The Effects Of Autophagy And Proteasomes On TGFβ Signalling And EMT

Charles Brandon Trelford, The University of Western Ontario

Supervisor: Di Guglielmo, Gianni, M, The University of Western Ontario
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Abstract

Transforming growth factor-β (TGFβ) signalling regulates growth, proliferation, immunity, and development. Although TGFβ typically antagonizes tumour formation, tumour cells often acquire mutations within the TGFβ signalling pathway that activate epithelial-mesenchymal transition (EMT). During EMT, epithelial tumour cells lose epithelial-like properties and acquire mesenchymal-like characteristics, which allows tumour cells to detach from the primary tumour and establish metastatic colonies. In addition to EMT, TGFβ augments tumourigenesis by increasing the degradation of damaged macromolecules and organelles via autophagy. Autophagy contributes to radiotherapy and chemotherapy resistance by mitigating the damages inflicted on tumour cells. Currently, there is a growing interest in the relationship between TGFβ signalling and protein degradative pathways as inhibitors of both autophagy and proteasomes block TGFβ-dependent EMT. Therefore, understanding pro-tumourigenic TGFβ signalling and its relationship with degradative processes has become a topic of interest for identifying novel therapeutic targets.

Since the mechanism of TGFβ-dependent autophagy activation was unknown, this work utilized immunoblotting and fluorescence microscopy of non-small cell lung cancer (NSCLC) cells that stably expressed a green fluorescent protein and red fluorescent protein conjugated to microtubule-associated protein light chain 3B (LC3B) and LC3B with a glycine deletion, respectively, to elucidate TGFβ signalling branches that activate autophagy. Indeed, both Smad-dependent and -independent TGFβ signalling, activate autophagy by increasing the proportion of active uncoordinated 51-like autophagy activating kinase 1 (ULK1). This work also demonstrated that impeding autophagy using small interfering RNA targeting autophagy-related genes as well as pharmacological inhibitors including chloroquine, spautin-1, and ULK-101 blocked TGFβ receptor endocytosis, Smad phosphorylation, Smad nuclear translocation, EMT, and cell migration. Likewise, proteasome inhibitors, such as MG132 and lactacystin, promoted lysosomal-targeting of TGFβRII and dampened TGFβ-induced R-Smad phosphorylation, R-Smad nuclear translocation, and EMT. Since protein 62/sequestosome 1 (p62/SQSTM1) delivers cargo to both degradative pathways, its expression was silenced using small interfering RNA. Silencing p62/SQSTM1 disrupted TGFβ signalling and promoted EMT; however, there were no changes to autophagy.
In conclusion, this work discovered that altering autophagy or proteasome activity attenuated TGFβ signalling and blocked the pro-tumourigenic properties of TGFβ in NSCLC.

**Keywords**

Transforming growth factor-β (TGFβ)

Receptor trafficking

Smad signalling

Smad4

Non-Smad signalling

TGFβ-activated kinase 1 (TAK1)

Tumour necrosis factor receptor-associated factor 6 (TRAF6)

P38 mitogen-activated protein kinase (p38 MAPK)

Epithelial-mesenchymal transition (EMT)

Autophagy

Lysosome

Uncoordinated 51-like autophagy activating kinase 1 (ULK1)

Mechanistic target of rapamycin (mTOR)

Proteasome

Protein 62/Sequestosome 1 (p62/SQSTM1)
Summary for Lay Audience

Lung cancer is the leading cause of cancer mortality worldwide and mutations in transforming growth factor beta (TGFβ) signalling have been implicated in cancer development. TGFβ promotes epithelial-mesenchymal transition (EMT), which allows cancer cells to become invasive and spread throughout the body. Furthermore, TGFβ activates autophagy, a mechanism for cells to degrade damaged or non-essential cellular components. In cancer, TGFβ-dependent autophagy protects tumour cells from chemotherapeutic drugs and primes cells for invasion. The bulk of protein degradation is facilitated by autophagy and several degradative organelles called proteasomes. Given that recent reports suggest that inhibiting autophagy and proteasome activity blocks EMT, there is a need to explore the role of degradative pathways in TGFβ-dependent tumourigenesis.

In this work, using a model to measure autophagic degradation in combination with known inhibitors and activators of autophagy, I verified that TGFβ activates autophagy and outlined the mechanism of TGFβ-dependent autophagy in lung cancer cell lines. I observed that blocking autophagy disrupts TGFβ-dependent tumourigenesis by decreasing TGFβ signalling, EMT, and cell migration. More specifically, autophagy inhibition blocks the internalization of TGFβ receptors and therefore prevents the activation of downstream signalling molecules that TGFβ relies on to propagate its signalling. Proteasome inhibitors also blocked TGFβ signalling, EMT, and cell migration. However, because proteasomes and autophagy compensate for one another, proteasome inhibitors activated autophagy, which lead to rapid degradation of TGFβ receptors.

Since both autophagy and proteasome inhibitors disrupted pro-tumourigenic TGFβ signalling, I next targeted protein 62/sequestosome 1 (p62/SQSTM1) because it delivers cellular components to both degradative pathways. I observed that decreasing p62/SQSTM1 activity had no effect on autophagy, blocked TGFβ signalling, and activated EMT. Therefore, using this methodology more work is needed to identify a target protein or pathway that regulates autophagy, proteasomes, EMT, and TGFβ signalling.

In conclusion, this work identified that inhibiting proteasomes and autophagy impeded pro-tumourigenic TGFβ signalling. This study has the potential to define the relationship between TGFβ, autophagy, proteasomes, and cancer invasion, which is essential to understanding how
tumours that can selectively eliminate damaged proteins and organelles can evade chemotherapeutic toxicities.

Co-Authorship Statement

Chapter 1 of this thesis was published in Biochemical Journal in Sept. 2021 (Biochem. J. 2021 Sept; 478(18):3395-3421.)

This peer reviewed review paper is entitled “Molecular mechanism of mammalian autophagy”. Charles B. Trelford reviewed the literature, wrote the paper, and designed the figures. Dr. Gianni M. Di Guglielmo edited the paper and prepared it for submission.

Chapter 1 of this thesis was submitted to Frontiers in Molecular Biosciences.

This submitted review paper is entitled “New insights into the tumour-promoting properties of Transforming Growth Factor-β”. Charles B. Trelford reviewed the literature, wrote the paper, and designed the figures. Dr. Lina Dagnino edited the paper. Dr. Gianni M. Di Guglielmo edited the paper and prepared it for submission.

Chapter 2 of this thesis was published in Biology Open in Nov. 2020 (Biol Open. 2020 Nov; 9 (11)).

This peer reviewed research paper is entitled “Assessing methods to quantitatively validate TGFβ-dependent autophagy”. The microarray was performed by Adrian Gunaratne and Charles B. Trelford performed the analysis. All other experiments and figures were completed by Charles B. Trelford. Dr. Gianni M. Di Guglielmo edited the paper and prepared it for submission.


This peer reviewed research paper is entitled “Canonical and non-canonical TGFβ signalling activate autophagy in an ULK1-dependent manner”. Figure 3.1C was originally composed by Dr. Gianni M. Di Guglielmo. This experiment was repeated by Charles B. Trelford, and all other experiments and figures were completed by Charles B. Trelford.
Chapter 4 of this thesis was published in Biochimica et Biophysica Acta in May. 2022 (BBA. 2022 Sept; 1869 (9)).

This peer reviewed research paper is entitled “Autophagy regulates transforming growth factor β signalling and receptor trafficking”. Figure 4.15A was originally composed by Dr. Gianni M. Di Guglielmo. These experiments were repeated by Charles B. Trelford, and all other experiments and figures were completed by Charles B. Trelford.

Chapter 5 of this thesis was published in Cellular Signalling in Cellular Signalling in July. 2022 (Cell Signal. 2022 July).

This peer reviewed research paper is entitled “Prolonged proteasome inhibition antagonizes TGFβ1-dependent signalling by promoting the lysosomal-targeting of TGFβRII”. All experiments and figures were completed by Charles B. Trelford. Dr. Gianni M. Di Guglielmo edited the paper and prepared it for submission.

Chapter 6 of this thesis was published in Cellular Signalling in May. 2021 (Cell Signal. 2021 May; 85).

This peer reviewed research paper is entitled “p62/SQSTM1 regulated transforming growth factor beta signalling and epithelial to mesenchymal transition”. Figures 6.1A, 6.8A, and 6.9B were originally generated by Dr. Craig Campbell and Figures 6.1C-E and the microarray were generated by Eveln Ng. These experiments were repeated by Charles B. Trelford, and all other experiments including the analysis of the microarray in the paper were completed by Charles B. Trelford. Dr. Gianni M. Di Guglielmo edited the paper and prepared it for submission.
Acknowledgments

At first, the idea of completing a Ph.D seemed like a daunting task. However, with the help of several wonderful people it become manageable.

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<th>Abbreviation</th>
<th>Full Name</th>
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<tbody>
<tr>
<td>ACVR</td>
<td>Activin Receptor</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>AMBRA</td>
<td>Autophagy and Beclin 1 Regulator</td>
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<tr>
<td>AMH</td>
<td>Anti-Muellerian Hormone</td>
</tr>
<tr>
<td>AMHR</td>
<td>Anti-Muellerian Hormone Receptor</td>
</tr>
<tr>
<td>AMPK</td>
<td>5’ Adenosine Monophosphate-Activated Protein Kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AP1</td>
<td>Activator Protein 1</td>
</tr>
<tr>
<td>AP2</td>
<td>Clathrin Coat Adaptor Complex 2</td>
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<td>ATF</td>
<td>Activating Transcription Factor</td>
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<td>ATG</td>
<td>Autophagy-Related Gene</td>
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<td>BCL</td>
<td>B-cell Lymphoma</td>
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<td>Bone Morphogenetic Protein</td>
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<td>BMPR</td>
<td>Bone Morphogenetic Protein Receptor</td>
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<tr>
<td>C</td>
<td>Carboxyl</td>
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<td>CDC</td>
<td>Cell Division Control Protein</td>
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<td>CDH</td>
<td>Cadherin</td>
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<td>CDK</td>
<td>Cyclin-Dependent Kinase</td>
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<td>Carboxyl Terminus of Heat Shock Cognate-Interacting Protein</td>
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<td>CHMP</td>
<td>Charged Multivesicular Body Protein</td>
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<tr>
<td>CMA</td>
<td>Chaperone-Mediated Autophagy</td>
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<td>Co-IP</td>
<td>Co-immunoprecipitation</td>
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<td>Co-Smad</td>
<td>Common Sma and mother against decapentaplegic</td>
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<td>DAPI</td>
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<td>DUB</td>
<td>Deubiquitinating Enzyme</td>
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<td>Term</td>
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<tr>
<td>EB1</td>
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<td>EEA1</td>
<td>Early Endosome Antigen 1</td>
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<td>ELK1</td>
<td>E-twenty-six Like-1 Protein</td>
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<td>EMT</td>
<td>Epithelial-Mesenchymal Transition</td>
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<td>Epithelial-Mesenchymal Transition-Transcription Factors</td>
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<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<td>ERK</td>
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</tr>
<tr>
<td>ESCRT</td>
<td>Endosomal Sorting Complex Required for Transport</td>
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<td>FBS</td>
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<td>GDF</td>
<td>Growth Differentiation Factors</td>
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<td>Green Fluorescent Protein</td>
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<td>GABA Type A Receptor-Associated Protein Interaction Motif</td>
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<td>Grb</td>
<td>Growth Factor Receptor Bound</td>
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<td>Hemagglutinin</td>
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<td>Human Bronchiole Epithelial</td>
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<td>HEY</td>
<td>Hairy/Enhancer-of-Split Related with YRPW Motif Protein</td>
</tr>
<tr>
<td>HOPS</td>
<td>Homotypic Fusion and Vacuole Protein Sorting</td>
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<tr>
<td>HMGA</td>
<td>High Mobility Group AT-hook</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>I-Smad</td>
<td>Inhibitory-Sma and mother against decapentaplectic</td>
</tr>
<tr>
<td>IAM</td>
<td>Inner Autophagosome Membrane</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>JNK</td>
<td>cJun amino-terminal kinase</td>
</tr>
<tr>
<td>K</td>
<td>Lysine</td>
</tr>
<tr>
<td>LAMP</td>
<td>Lysosomal-Associated Membrane Protein</td>
</tr>
<tr>
<td>LAP</td>
<td>Latency-Associated Peptide</td>
</tr>
<tr>
<td>LC3</td>
<td>Microtubule-Associated Protein Light Chain 3</td>
</tr>
<tr>
<td>LIR</td>
<td>Microtubule-Associated Protein Light Chain 3 Interacting Region</td>
</tr>
<tr>
<td>LTBP</td>
<td>Latent Transforming Growth Factor-β Binding Protein</td>
</tr>
<tr>
<td>MA</td>
<td>Macroautophagy</td>
</tr>
<tr>
<td>mA</td>
<td>microautophagy</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse Embryonic Fibroblast</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-Activated Protein Kinase Kinase</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
</tr>
<tr>
<td>MET-TF</td>
<td>Mesenchymal-Epithelial Transition-Transcription Factor</td>
</tr>
<tr>
<td>MH</td>
<td>Mad Homology</td>
</tr>
<tr>
<td>miR</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mechanistic Target of Rapamycin</td>
</tr>
<tr>
<td>MVB</td>
<td>Multivesicular Body</td>
</tr>
<tr>
<td>Mv1Lu</td>
<td>Mink Lung Cell Line</td>
</tr>
<tr>
<td>N</td>
<td>Amine</td>
</tr>
<tr>
<td>NES</td>
<td>Nuclear Export Signal</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear Localization Signal</td>
</tr>
<tr>
<td>NPS</td>
<td>Nuclear Pore Signal</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-Small Cell Lung Cancer</td>
</tr>
<tr>
<td>OAM</td>
<td>Outer Autophagosome Membrane</td>
</tr>
<tr>
<td>P-mTOR</td>
<td>Phosphorylated Mechanistic Target of Rapamycin</td>
</tr>
<tr>
<td>P-p38 MAPK</td>
<td>Phosphorylated p38 Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>P-Smad</td>
<td>Phosphorylated Sma and mother against decapentaplegic</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>P-TAK1</td>
<td>Phosphorylated Transforming Growth Factor-β-Activated Kinase</td>
</tr>
<tr>
<td>P-ULK</td>
<td>Phosphorylated Uncoordinated-51-like Autophagy Activating Kinase</td>
</tr>
<tr>
<td>PAI</td>
<td>Plasminogen Activator Inhibitor</td>
</tr>
<tr>
<td>Par6</td>
<td>Partitioning Defective 6 Homolog</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PB1</td>
<td>Phox Bem1</td>
</tr>
<tr>
<td>PCBP</td>
<td>Poly(RC) Binding Protein</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-Kinase</td>
</tr>
<tr>
<td>PI3P</td>
<td>Phosphatidylinositol 3-Phosphate</td>
</tr>
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<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl Fluoride</td>
</tr>
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<td>PPXY</td>
<td>Proline-Proline-X-Tyrosine</td>
</tr>
<tr>
<td>Pre-Pro-TGFβ</td>
<td>Precursor-pro-TGFβ</td>
</tr>
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<td>PRX1</td>
<td>Paired-related Homeobox 1</td>
</tr>
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<td>PS</td>
<td>Phosphatidylinerine</td>
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<td>P38 MAPK</td>
<td>Protein 38 Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>P62</td>
<td>Protein 62</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
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<td>R-Smad</td>
<td>Receptor-Sma and mother against decapentaplegic</td>
</tr>
<tr>
<td>Raf</td>
<td>Mitogen-Activated Protein Kinase Kinase Kinase</td>
</tr>
<tr>
<td>RFP</td>
<td>Red Fluorescent Protein</td>
</tr>
<tr>
<td>RhoA</td>
<td>Ras homolog family member A</td>
</tr>
<tr>
<td>RILP</td>
<td>Rab-Interacting Lysosomal Protein</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RUBCN</td>
<td>Rubicon Autophagy Regulator</td>
</tr>
<tr>
<td>S</td>
<td>Serine</td>
</tr>
<tr>
<td>SAD</td>
<td>Smad Activation Domain</td>
</tr>
<tr>
<td>SAPK</td>
<td>Stress-Activated Protein Kinase</td>
</tr>
<tr>
<td>SARA</td>
<td>Smad Anchor for Receptor Activation</td>
</tr>
<tr>
<td>SCLC</td>
<td>Small Cell Lung Cancer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>ShcA</td>
<td>Src Homology Domain Containing Protein A</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small Interfering RNA</td>
</tr>
<tr>
<td>SLUG</td>
<td>Snail Family Transcriptional Repressor 2</td>
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<td>Smad</td>
<td>Sma and mother against decapentaplegic</td>
</tr>
<tr>
<td>SMA</td>
<td>Smooth Muscle Actin</td>
</tr>
<tr>
<td>Smurf</td>
<td>Smad ubiquitination regulatory factor</td>
</tr>
<tr>
<td>SNAIL</td>
<td>Snail Family Transcriptional Repressor 1</td>
</tr>
<tr>
<td>SNAP</td>
<td>Synaptosome-Associated Protein</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble N-Ethylmaleimide-Sensitive-Factor Attachment Receptors</td>
</tr>
<tr>
<td>SnoN</td>
<td>Ski-Related Protein N</td>
</tr>
<tr>
<td>Sos</td>
<td>Son of Sevenless</td>
</tr>
<tr>
<td>Spautin</td>
<td>Specific and Potent Autophagy Inhibitor</td>
</tr>
<tr>
<td>SQSTM1</td>
<td>Sequestosome 1</td>
</tr>
<tr>
<td>SS</td>
<td>Disulfide bond</td>
</tr>
<tr>
<td>SSXS</td>
<td>Serine-Serine-X-Serine</td>
</tr>
<tr>
<td>STX</td>
<td>Syntaxin</td>
</tr>
<tr>
<td>T-cell</td>
<td>T-lymphocyte</td>
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<tr>
<td>TAB</td>
<td>Transforming Growth Factor-β-Activated Kinase 1 Binding Protein</td>
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<tr>
<td>TAK1</td>
<td>Transforming Growth Factor-β-Activated Kinase 1</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumour-Associated Macrophage</td>
</tr>
<tr>
<td>TAN</td>
<td>Tumour-Associated Neutrophil</td>
</tr>
<tr>
<td>TB</td>
<td>Tumour Necrosis Factor Receptor-Associated Factor Binding</td>
</tr>
<tr>
<td>TECPR</td>
<td>Tectonin β-Propeller Repeat Containing</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming Growth Factor-β</td>
</tr>
<tr>
<td>TGFβR</td>
<td>Transforming Growth Factor-β Receptor</td>
</tr>
<tr>
<td>TNTE</td>
<td>Tris, sodium chloride, Triton X-100, and Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>TRAF</td>
<td>Tumour Necrosis Factor Receptor-Associated Factor</td>
</tr>
<tr>
<td>TSG101</td>
<td>Tumour Susceptibility Gene 101</td>
</tr>
<tr>
<td>TWIST</td>
<td>Twist-Related Protein</td>
</tr>
<tr>
<td>UBA</td>
<td>Ubiquitin-Associated Domain</td>
</tr>
<tr>
<td>ULK</td>
<td>Uncoordinated-51-like Autophagy Activating Kinase</td>
</tr>
<tr>
<td>UPP</td>
<td>Ubiquitin-Proteasome Pathway</td>
</tr>
<tr>
<td>USP</td>
<td>Ubiquitin-Specific Peptidase</td>
</tr>
<tr>
<td>VAMP</td>
<td>Vesicle-Associated Membrane Protein</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular Endothelial Growth Factor Receptor</td>
</tr>
<tr>
<td>VPS</td>
<td>Vacuolar Protein Sorting</td>
</tr>
<tr>
<td>WIPI</td>
<td>WD-repeat Protein Interacting with Phosphoinositides</td>
</tr>
<tr>
<td>ZEB</td>
<td>Zinc Finger E-box Binding Homeobox</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>Zinc</td>
</tr>
<tr>
<td>ZZ</td>
<td>ZZ-Type Zinc Finger</td>
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Chapter 1

INTRODUCTION

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Components of this chapter have been submitted to Front. Mol. Biosci.
1 Introduction

1.1 Introduction to Transforming Growth Factor-β

Transforming growth factor-β (TGFβ) cytokines are central modulators of development, growth, proliferation, immune function, apoptosis, and homeostasis that play key roles in cellular communication. For instance, TGFβ modulates protein synthesis and function by regulating transcription, translation, and post-translational modifications. Alterations in TGFβ signalling pathways have been implicated in numerous pathologies, including congenital diseases, fibrotic disorders, immune dysfunction, and tumourigenesis. The regulation of TGFβ signalling in cancer is complex, as it generally plays a tumour suppressive role in normal tissues. In contrast, mutations or abnormalities in the tumour suppressive arms of TGFβ signalling are common in many cancer types. In tumours, this cytokine becomes a factor that induces epithelial-mesenchymal transition (EMT), metastasis, angiogenesis, autophagy, and disrupts immune cell recognition and destruction. Analyzing the signalling pathways activated by TGFβ is necessary to understand the effects that they have in the microenvironment of tumours, and the potential mechanisms involved in their dysregulation.

1.2 TGFβ pathways

The TGFβ superfamily consists of 33-members of secreted cytokines that are ubiquitously expressed in vertebrates and invertebrates. This superfamily includes the TGFβ-like and the bone morphogenetic protein (BMP)-like subfamilies, whose members differ from each other structurally and functionally. The TGFβ subfamily consists of TGFβ1, TGFβ2, TGFβ3, activin A, activin B, nodal, myostatin, inhibin, and growth differentiation factors (GDFs) that include GDF1, GDF3, GDF5, GDF11, and GDF15. The BMP subfamily includes 12 BMP proteins, lefty1, lefty2, anti-muellerian hormone (AMH), GDF6, GDF7, and GDF10.

As homodimers or heterodimers, TGFβ superfamily members signal through heteromeric TGFβ receptor complexes. Seven different type I receptors, five different type II receptors, and betaglycan and endoglin type III receptors have been described in
morphogenetic protein
transforming growth factor
vertebrates and invertebrates. Receptor activation leads to signalling cascades modulated by several classes of Sma and mother against decapentaplegic (Smad) proteins, such as common Smads (co-Smads), receptor regulated Smads (R-Smads), and inhibitory Smads (I-Smads) as well as non-Smad proteins. An extensive number of TGFβ superfamily members activate a specific subset of receptors and signalling molecules (Table 1.1), but this thesis will focus on TGFβ ligands.

**Table 1.1: The ligands, receptors, and Smad proteins for each TGFβ superfamily.**

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>TGFβ-like</th>
<th>BMP-like</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ligands</strong></td>
<td>TGFβ1, TGFβ2, TGFβ3, Activin A, Activin B, Nodal, Myostatin, Inhibin, GDF1, GDF3, GDF5, GDF11, and GDF15</td>
<td>BMP2, BMP2, BMP3, BMP4, BMP5, BMP6, BMP7, BMP8, BMP9, BMP10, BMP14, BMP15, GDF6, GDF7, GDF10, AMH, Lefty1, and Lefty2</td>
</tr>
<tr>
<td><strong>Type II receptors</strong></td>
<td>TGFβRII, ACVRII, and ACVRIB</td>
<td>BMPRII, ACVRII, AMHRII and ACVRIB</td>
</tr>
<tr>
<td><strong>Type I receptors</strong></td>
<td>TGFβRI, ACVRIB, and ACVRIC</td>
<td>ACVRL1, ACVRIA, BMPRIA, and BMPRIB</td>
</tr>
<tr>
<td><strong>R-Smads</strong></td>
<td>Smad2 and Smad3</td>
<td>Smad1, Smad5, and Smad8</td>
</tr>
<tr>
<td><strong>Co-Smads</strong></td>
<td>Smad4</td>
<td>Smad4</td>
</tr>
<tr>
<td><strong>I-Smads</strong></td>
<td>Smad7</td>
<td>Smad6 and Smad7</td>
</tr>
</tbody>
</table>

transforming growth factor-β (TGFβ), growth and differentiation factor (GDF), bone morphogenetic protein (BMP), anti-muellerian hormone (AMH), transforming growth
factor-β receptor (TGFβR), activin receptor (ACVR), bone morphogenetic protein receptor (BMPR), anti-muellerian hormone receptor (AMHR), and Sma and mother against decapentaplegic (Smad).

1.3 Synthesis and post-translational modification of TGFβ

In most metazoans, three genes encoding TGFβ isoforms have been described, and in humans the TGFβ1, TGFβ2, and TGFβ3 genes are located on chromosomes 19, 1, and 14, respectively. Although TGFβ1, TGFβ2, and TGFβ3 genes are highly conserved across species, there are some exceptions. For instance, TGFβ4 has been identified in avian species; however, genetic mapping of chicken TGFβ4 suggested that it is orthologous to human TGFβ1. Moreover, some South African frogs (Xenopus laevis) express a TGFβ5 gene. Translation of the TGFβ1, TGFβ2, and TGFβ3 mRNA generates precursor polypeptides termed pre-pro-TGFβ, which are composed, respectively, of 390, 412, and 412 amino acid residues. The pre-pro-TGFβ species are composed of a signal peptide, a large amino-terminal latency-associated peptide (LAP), which ensures proper folding and transportation through the Golgi complex, and the residues of the mature ligand. Following signal peptide removal, disulfide isomerase catalyzes the formation of three disulfide bonds between two pre-pro-TGFβ monomers, linking cysteine residues at two positions in the LAP and one position in what will become the mature ligand. For instance, two pre-pro-TGFβ1 monomers form three disulfide bonds at positions 223, 225, and 356 from each monomer. These modification gives rise to pro-TGFβ. Within the Golgi complex membrane, furin and other convertases cleave LAP to generate small latent TGFβ complexes. Non-covalent bonds tether LAP to TGFβ, rendering the latter inactive. Small latent TGFβ complexes, composed of a mature 25 kDa TGFβ dimer and 2 LAP moieties, are subsequently packaged into secretory vesicles in the Golgi complex. Once secreted from the cell, the small latent TGFβ complexes are retained in the extracellular matrix (ECM), bound to latent TGFβ binding proteins (LTBPs) to form large latent TGFβ complexes. TGFβ dimers can subsequently be released from the large latent TGFβ complexes through various enzymatic reactions or allosteric mechanisms (Fig. 1.1).
The enzymatic activation of TGFβ through proteolysis requires matrix metalloproteinases (MMPs), plasmin, and other proteases\textsuperscript{22,23}. MMP2 and MMP9 are Ca\textsuperscript{2+}-dependent Zn\textsuperscript{2+}-containing endopeptidases that target the LAP-binding domains of LTBPs, releasing TGFβ from the large latent TGFβ complexes. Plasmin generated at the cell surface, following plasminogen cleavage by urokinase plasminogen, also contributes to TGFβ release from LAPS\textsuperscript{24,25}. Alternatively, allosteric activation of TGFβ is dependent on several LAP-binding cell surface proteins, such as thrombospondin-1, mannose 6-phosphate receptors, and integrins, which induce conformational rearrangements of LAP\textsuperscript{26–29}. Modifications of LAP are also induced by reactive oxygen species\textsuperscript{30} as well as acidic (pH<2) or basic (pH>12) environments\textsuperscript{31}. Since these diverse LAP conformers no longer favour binding to TGFβ, the latter is released from the large latent TGFβ complexes.
Figure 1.1: TGFβ ligand maturation.

Following Transforming Growth Factor-β (TGFB) gene (red) transcription and TGFB mRNA translation in the nucleus and endoplasmic reticulum, respectively, TGFβ is synthesized as a precursor pro-TGFβ (pre-pro-TGFβ). Pre-pro-TGFβ contains an amine (N) terminal signal peptide, latency-associated peptide, and mature ligand. The N terminal signal peptide ensures transportation to the Golgi complex. In the Golgi complex, the signal peptide is cleaved, and disulfide isomerases catalyze disulfide bonds (SS) between two pre-pro-TGFβ monomers to generate pro-TGFβ. Furin convertases modify the latency-
associated peptides, which are bonded with mature ligands non-covalently to generate a small latent TGFβ complex. The small latent TGFβ complex is secreted from the cell and attaches to latent TGFβ binding proteins in the extracellular matrix to form a large latent TGFβ complex. Mature ligands are released from the large latent TGFβ complexes via allosteric interactions or proteolysis mediated by enzymes.

1.4 Smad-dependent TGFβ signalling

After TGFβ ligands are released from large latent TGFβ complexes, they bind to cognate cell surface receptors. The Type I and II TGFβ receptors (TGFβRI and TGFβRII) exhibit serine-threonine kinase activity, and initiate signalling cascades upon ligand stimulation\textsuperscript{32}. Type III TGFβ receptors (TGFβRIIs) do not exhibit catalytic activity, but may facilitate the interaction between TGFβ ligands and TGFβRII\textsuperscript{33,34}. TGFβ signalling is initiated when TGFβ binds to TGFβRII, triggering the association and phosphorylation of the glycine/serine domain in the cytoplasmic tails of TGFβRI\textsuperscript{1}. TGFβRI in turn phosphorylates downstream intracellular signalling molecules to induce canonical Smad-dependent and non-canonical Smad-independent TGFβ signalling\textsuperscript{35–38}.

All three classes of Smad proteins, R-Smads (Smad2/3), co-Smad (Smad4), and I-Smads (Smad6/7), temporally regulate TGFβ signalling\textsuperscript{39}. Signal initiation begins when TGFβRI phosphorylates Smad2 or Smad3 on the carboxyl (C) terminus serine-serine-x-serine (SSXS) motif. Phosphorylated Smad2/3 is then released from the Smad anchor for receptor activation (SARA) protein into the cytoplasm, where it can form heterodimeric or heterotrimeric complexes with Smad4\textsuperscript{37,40}. These complexes subsequently translocate into the nucleus, where they regulate gene expression directly, by activating transcription, or indirectly by modulating the activity of other transcription factors\textsuperscript{41}. Smad targeted genes include I-Smads\textsuperscript{42}, cyclin-dependent kinase 4 (CDK4)\textsuperscript{43}, and EMT-transcription factors, including Snail Family Transcriptional Repressor 1 and 2 (SNAIL and SLUG), Zinc Finger E-box Binding Homeobox 1 and 2 (ZEB1 and ZEB2), Twist-related Protein 1 (TWIST1), Forkhead box C2 (FOXC2), Forkhead box A1 (FOXA1), Forkhead box A2 (FOXA2), Paired-related Homeobox 1 (PRX1), and High Mobility Group AT-hook 2 (HMGA2; Fig. 1.2)\textsuperscript{44,45}. 
Through negative feedback mechanisms, Smad6 and Smad7 terminate TGFβ pathway activation (Fig. 1.2). I-Smads block R-Smad access to TGFβRI or recruit phosphatases\(^\text{46,47}\), leading to dephosphorylation of active receptors\(^\text{48}\). I-Smads also form complexes with E3 ubiquitin ligases, such as Smad ubiquitination regulatory factor 1 or 2 (Smurf1 or Smurf2), resulting in the degradation of TGFβ receptors\(^\text{47,49}\).

Figure 1.2: Canonical (Smad-dependent) TGFβ signalling.
Transforming growth factor-β (TGFβ) receptor type III (TGFβRIII) presents TGFβ to the type II receptor (TGFβRII). The TGFβ-TGFβRII complex phosphorylates TGFβ receptor type I (TGFβRI), which in turn phosphorylates Smad2 or Smad3. Phosphorylated Smad2/3 are released from the Smad anchor for receptor activation (SARA) protein. Smad2 and Smad3 may translocate into the nucleus or form heterodimers/heterotrimers with Smad4 prior to nuclear translocation. Once in the nucleus, Smads function as transcription factors or interact with other transcription factors to regulate gene expression. Examples of genes regulated by Smads include, cyclin-dependent kinases (CDKs), Snail Family Transcriptional Repressor 1 and 2 (SNAIL/SLUG), Zinc Finger E-box Binding Homeobox 1 and 2 (ZEB1/ZEB2), Twist-related Protein 1 (TWIST1), Forkhead box C2 (FOXC2), Forkhead box A1 (FOXA1), Forkhead box A2 (FOXA2), Paired-related Homeobox 1 (PRX1), High Mobility Group AT-hook 2 (HMG2), and Smad7—which in turn dampens TGFβ signal transduction.

1.5 Structure of Smad proteins

Smad structure accounts for differences in Smad function. Structurally, Smad proteins have a Mad Homology 1 (MH1) domain, separated by a flexible linker region from a MH2 domain. MH1 domains contain a nuclear localization signal and β-hairpin loop that mediates interactions with glycine cysteine-rich Smad-binding elements on DNA, whereas MH2 domains interact with TGFβ receptors and mediate binding to other Smad proteins, transcription factors, and co-activators or co-repressors of transcription. Among the three regions, the greatest variability is observed within the linker region. The linker region of R-Smads contain phosphorylation sites for multiple kinases, such as CDKs and mitogen-activated protein kinases (MAPKs). Furthermore, within the linker region, both R-Smads and I-Smads, but not Smad4, have a proline-proline-x-tyrosine (PPXY) motif to bind to E3 ubiquitin ligases. Although MH1 and MH2 domains are highly conserved, there are some notable differences. I-Smads are missing the MH1 domain, therefore, cannot bind to DNA. The MH2 domains of R-Smads have a β1-strand, L3 loop, and α-helix 5 structure that together mediates binding to TGFβRI or SARA. Although the structure of Smad2 and Smad3 are similar, there are notable differences. For instance, Smad2 has two inserts in its MH1 domain.
these inserts, known as the E3 insert, was once believed to disrupt the β-hairpin loop, preventing Smad2 from binding DNA\textsuperscript{56,57}. Further analysis indicated that different conformations of the E3 insert regulate MH1 domain structure, which explains why in some instances Smad2 has been shown to bind to DNA (Fig. 1.3)\textsuperscript{58}.

Although Smad4 is essential to many TGFβ-dependent changes in gene expression, Smad4 is not essential for R-Smad nuclear translocation nor is it necessary for some TGFβ-dependent transcriptional functions\textsuperscript{59}. Smad4 also performs TGFβ-independent functions that include silencing the expression of TGFβ target genes in T-lymphocytes (T-cells)\textsuperscript{60}, upregulating genes that promote natural killer (NK) cell maturation\textsuperscript{61}, and tumour suppression by mediating Aurora A kinase degradation\textsuperscript{62}. Although the roles of Smad4 remain incompletely understood, Smad4 is the only Smad with a nuclear export signal and a Smad activation domain (SAD) within its linker region (Fig. 1.3). The SAD region is recognized by the chromatin modifiers p300 and CREB-binding protein co-activators\textsuperscript{63}. Although Smad4 SAD deletion cells are still able to bind p300 and CREB co-activators, these Smad4-p300 and Smad4-CREB complexes are unable to activate transcription\textsuperscript{64}. In this manner, Smad4 contributes to the regulation of gene expression through p300 and CREB-binding protein co-activator complexes.
Smads are comprised of Mad Homology 1 (MH1) and MH2 domains separated by a variable linker region. MH1 domains are responsible for DNA binding, whereas MH2 domains facilitate protein-protein interactions. The MH1 domain is located toward the amine (N) terminus while the MH2 domain flanks the carboxyl (C) terminus. The MH1 domains contain a nuclear localization signal (NLS) and β-hairpin loop, which mediates DNA binding. Smad2 contains an E3 insert within its β-hairpin loop, whereas I-Smads are missing the MH1 domain altogether. R-Smads contain kinase sites that are phosphorylated by a variety of kinases and a proline-proline-x-tyrosine (PPXY) motif. Common Smad (Co-Smad) contains a nuclear export signal (NES) and Smad activation domain (SAD).
within the linker region. I-Smads contain a PPXY motif in the linker region. In the MH2 domain, R-Smads contain a NES, whereas all Smads contain an L3 loop and nuclear pore signal (NPS). Finally, transforming growth factor-β receptor type I (TGFβRI) phosphorylates R-Smads on the serine-serine-x-serine (SSXS) motif located in the MH2 domain.

1.6 Smad-independent TGFβ signalling

Smad-independent TGFβ signalling occurs through various pathways (Fig. 1.4). One involves the MAPK cascade via tumour necrosis factor receptor-associated factor 6 (TRAF6). Upon stimulation by TGFβ, TGFβRI associates with TRAF6, leading to lysine (K)63 polyubiquitination of this protein. K63-linked polyubiquitination provides a scaffold that subsequently recruits TGFβ-activated kinase 1 (TAK1), as well as TAK1-binding proteins. After TAK1-dependent phosphorylation, MAPK kinase 3/6 phosphorylates c-Jun amino-terminal kinase (JNK) and p38 MAPK. JNK and p38 MAPK translocate into the nucleus, where they phosphorylate several targets, including p53, activator protein 1 (AP1), E-twenty-six like-1 protein (ELK1), activating transcription factor 2 (ATF2), and cJun. These transcription factors regulate the expression of genes involved in apoptosis, inflammation, motility, development, cell-cell attachments, cell-ECM attachments, and proliferation.

The protein kinase B (AKT) pathway is activated when tyrosine residues on TGFβRI are phosphorylated. TGFβRI may then function as a tyrosine kinase and phosphorylate phosphoinositide 3-kinase (PI3K), which in turn activates AKT. Downstream targets of AKT include mechanistic target of rapamycin (mTOR), a regulator of cell growth, proliferation, motility, survival, autophagy, transcription, and protein synthesis. Additionally, AKT inhibits Forkhead box O (FoxO) transcription factors, which are important regulators of CDKs, survival, DNA repair, and T-cell activity.

Tyrosine residues on the src homology domain containing protein A (ShcA) was also reported to be phosphorylated by TGFβRI. ShcA forms a complex containing growth factor receptor bound 2 (Grb2) and son of sevenless (Sos) to activate Ras. The latter initiates downstream MAPK cascades that ultimately phosphorylates extracellular signal-regulated kinase (ERK). ERK phosphorylates transcription factors, such as AP1 and
ELK1, that regulate the expression of genes essential for cell-cell attachments, cell-ECM attachments, motility, differentiation, proliferation, and apoptosis\textsuperscript{10,65}.

In addition to Ras, Smad-independent TGFβ signalling increases the activity of other small GTPases\textsuperscript{74}. Specifically, TGFβRII can phosphorylate partitioning defective 6 homolog (Par6), whereas Ras homolog family member A (RhoA), and cell division control protein 42 (CDC42) activation relies on TGFβRI activity\textsuperscript{75,76}. These proteins modulate cell-cell and cell-ECM attachments by regulating the function, stability, and organization of proteins essential to adherens and tight junctions. RhoA also promotes cell migration by inducing stress fiber formation\textsuperscript{77,78}. Stress fibers are contractile actomyosin bundles found in non-muscle cells composed of filamentous actin, $\alpha$-actinin, and non-muscle myosin II filaments that may aid in cell movement\textsuperscript{79,80}. 
phosphoinositide activated kinase 1 (TAK1), src homology domain containing protein A (ShcA), (Figure 1.4).

Non-canonical TGFβ signalling pathways.

In non-canonical transforming growth factor-β (TGFβ) signalling, TGFβ receptor type I (TGFβRI) phosphorylates numerous downstream signalling molecules including TGFβ-activated kinase 1 (TAK1), src homology domain containing protein A (ShcA), phosphoinosotide 3-kinase (PI3K), partitioning defective 6 homolog (Par6), Ras homolog
family member A (RhoA), and cell division control protein 42 (CDC42). The Par6/CDC42/RhoA pathway regulates adherens junctions, tight junctions, and stress fiber formation. PI3K phosphorylates protein kinase B (AKT), which inhibits Forkhead box O (FoxO) transcription factors that regulate genes responsible for DNA repair, cell cycle arrest, survival, and T-lymphocyte function. AKT also regulates cell growth, proliferation, motility, and survival by activating mechanistic target of rapamycin (mTOR). After ShcA is phosphorylated, it forms a complex with growth factor receptor bound 2 (Grb2) and son of sevenless (Sos) to phosphorylate membrane bound Ras. This initiates a signalling cascade involving mitogen-activated protein kinase kinase kinase (Raf), mitogen-activated protein kinase kinase (MEK), and extracellular signal-regulated kinase 1 (ERK1). ERK1 upregulates activator protein 1 (AP1) and E-twenty-six like-1 protein (ELK1) transcription factors. AP1 upregulates genes that regulate differentiation, proliferation, and apoptosis, whereas ELK1 upregulates genes involved with cell-cell attachments, cell-extracellular matrix (ECM) attachments, and motility. TGFβRI phosphorylation promotes lysine (K)63-linked polyubiquitination of tumour necrosis factor receptor-associated factor 6 (TRAF6). TRAF6 forms a complex with TAK1 binding protein 2 and 3 (TAB2 and TAB3) to recruit TAK1. TGFβRI phosphorylates TAK1, which initiates signalling cascades that phosphorylate mitogen-activated protein kinase kinase 3/6 (MKK3/6). MKK3/6 phosphorylates c-Jun amino-terminal kinase (JNK) and p38 MAPK. JNK regulates c-Jun and AP1 transcription factors, whereas p38 MAPK regulates activating transcription factor 2 (ATF2), p53, and ELK1 transcription factors. cJun upregulates genes involved with proliferation and survival, whereas ATF2 upregulates genes that modulate development, motility, apoptosis, and inflammation.

1.7 TGFβ receptor endocytosis regulates signalling strength and duration

Endocytosis of TGFβRI, TGFβRII, and TGFβ-TGFβRII complexes are mediated via clathrin- or caveolae-dependent mechanisms (Fig. 1.5). Clathrin-dependent endocytosis allows TGFβ signalling to continue following receptor internalization and is associated with signal amplification. Clathrin-coated pits sequester TGFβ receptors via
the clathrin coat adaptor complex 2 (AP2)\textsuperscript{83}. AP2 is a heterotetramer that binds to clathrin and consists of four adaptins (β2, µ2, α, and σ2)\textsuperscript{84}. Unlike many receptors within the plasma membrane that bind to µ2-adaptin, TGFβ receptors directly bind to β2-adaptin\textsuperscript{83}. Next, several proteins facilitate budding and fission of clathrin-coated pits that are internalized as clathrin-coated vesicles. Clathrin-coated vesicles subsequently shed AP2 and fuse with the early endosome membrane compartment in a Rab5-dependent manner\textsuperscript{85}. Early endosome membrane compartments are enriched in phosphatidylinositol 3-phosphate (PI3P), which serve as recruitment sites for FYVE domain-containing proteins, such as early endosome antigen 1 (EEA1), endofin, and SARA\textsuperscript{86}. By associating with SARA on early-endosomal membranes, the R-Smads, Smad2/3, are poised to interact with TGFβ receptors\textsuperscript{87}. Since regions involved in clathrin-dependent internalization are enriched in SARA, these routes of subcellular trafficking promote TGFβRI-dependent R-Smad phosphorylation\textsuperscript{50}. SARA also amplifies TGFβ signalling because SARA overexpression leads to endosomal swelling, which delays receptor recycling/degradation\textsuperscript{88}. In support of this, when the localization of SARA and EEA1-positive early endosomes was disrupted, there was a decrease in both TGFβ-induced Smad2 phosphorylation and Smad2 nuclear translocation\textsuperscript{89}. Finally, endofin facilitates TGFβ signalling because it binds to TGFβRI and Smad4, which brings Smad4 in close proximity to phosphorylated R-Smads. Indeed, endofin knockdown reduced transcriptional responses to TGFβ and impaired TGFβ-dependent apoptosis\textsuperscript{90}. Therefore, clathrin-dependent trafficking of TGFβ receptors enables R-Smad phosphorylation in the early endosome and prolongs the duration in which ligands, receptors, and downstream signalling molecules are in close proximity. The early endosome is primarily responsible for sorting endocytosed TGFβ receptors, which may either recycle back to the plasma membrane in Rab11-positive vesicles\textsuperscript{91} or be degraded in Rab7-positive late endosomes and lysosomes\textsuperscript{92} (Fig. 1.5A).

Caveolae are plasma membrane invaginations enriched with caveolin-1 that are localized in membrane rafts, plasma membrane subdomains rich in cholesterol and glycosphingolipids\textsuperscript{93}. Caveolin-positive vesicles may mature into or fuse with pre-existing caveosomes or early endosomes in a Rab5-independent or -dependent manner, respectively\textsuperscript{94}. Caveolin-dependent endocytosis is associated with dampening and
disrupting TGFβ signalling. Unlike clathrin-coated vesicles, SARA localizes away from membrane rafts and Smad7-Smurf2 complexes are commonly associated with caveolin-positive vesicles. Due to the association with Smad7-Smurf2, TGFβRII/TGFβRI complexes within caveolin-positive vesicles are targeted for proteasomal degradation. Caveolin-1 also has been shown to directly bind to TGFβRI following stimulation, which suppresses Smad2 phosphorylation possibly by antagonizing TGFβRI kinase activity (Fig. 1.5B). Caveolin-1 also disrupts TGFβ signalling through association with CD109, a TGFβ co-receptor. In the presence of ligands, CD109 promotes the localization of TGFβ receptors in caveolae and increases receptor degradation. Indeed, after the TGFβRII/TGFβRI complexes are endocytosed in caveolin-positive vesicles, TGFβ signalling is inhibited. However, the activation of some non-Smad signalling pathways, such as p38 MAPK, rely on the localization of TGFβ receptors in caveolae.

In summary, the route of TGFβ receptor subcellular trafficking regulates signalling duration, strength, and receptor fate. Although some TGFβ signalling occurs in the absence of receptor internalization, clathrin- or caveolae-dependent endocytosis can enhance or dampen TGFβ signal transduction pathways.
Figure 1.5: Clathrin- and caveolae-dependent endocytosis regulate the duration and strength of TGFβ signalling.

(A) Clathrin-dependent receptor trafficking is mediated by triskelion shaped clathrin proteins (green). Clathrin tethers transforming growth factor-β (TGFβ) receptors to clathrin-coated pits via the β2 adaptin of the clathrin coat adaptor complex 2 (AP2).
Clathrin-coated pits pinch off the plasma membrane to form clathrin-coated vesicles that fuse with early endosome membrane compartments by a Rab5-dependent process. In the presence of TGFβ, TGFβ receptors within clathrin-coated vesicles are active and phosphorylate downstream signalling molecules, such as Smads. Clathrin-coated pits and vesicles are enriched in Smad anchor for receptor activation (SARA) proteins that bind to receptor regulated Smads (R-Smads), which augments TGFβ signalling. Early endosomes bind to FYVE domain-containing proteins, such as endofin and SARA. Endofin enhances TGFβ signalling in early endosomes by tethering common Smad (co-Smad; Smad4) to early endosomes. Clathrin-dependent receptor trafficking promotes R-Smad phosphorylation, which subsequently enters the nucleus with and without Smad4 to regulate transcription. The fates of the TGFβ receptors subjected to clathrin-dependent receptor trafficking involve recycling back to the plasma membrane in Rab11-positive vesicles or lysosomal degradation. Lysosomal degradation occurs after early endosomes mature into Rab7-positive late endosomes, which eventually fuse with lysosomes.

(B) Caveolae-dependent receptor trafficking is facilitated by caveolin-1 proteins (red). Caveolae-coated vesicles are associated with dampening TGFβ signalling; however, non-canonical p38 MAPK signalling requires caveolae-coated vesicles. Caveolin-1 may bind to TGFβ receptor type I (TGFβRI) directly and attenuate its kinase activity. Caveolae-coated vesicles are enriched with Smad7-Smurf2 complexes that target TGFβ receptors to proteasome-dependent degradation. Prior to degradation, caveolae-coated vesicles may fuse with early endosomes in a Rab5-dependent manner or mature into caveolin-1-positive endosomes known as caveosomes.

1.8 The role of the ubiquitin-proteasome pathway in TGFβ signalling

The ubiquitin-proteasome pathway (UPP) also regulates the strength and duration of TGFβ signalling\(^\text{100}\). The polyubiquitination of TGFβ receptors, R-Smads, and downstream effectors is dependent on E1 (activating), E2 (conjugating), and E3 (ubiquitin ligase) enzymes\(^\text{47}\). E1 enzymes hydrolyze ATP to activate the C terminus of ubiquitin. Activated ubiquitin is then transferred to an E2 enzyme. E3 enzymes subsequently bind to
E2-ubiquitin conjugates and transfers ubiquitin to K residues on TGFβ receptors, R-Smads or downstream effectors. K48-linked polyubiquitin chains target TGFβ receptors, R-Smads, and downstream effectors to 26S proteasomes, which are multi-subunit proteases. Deubiquitinating enzymes decrease proteasome-dependent degradation by removing ubiquitin (Fig. 1.6). Although ubiquitination is important for proteasome-dependent degradation, it is also necessary to facilitate signalling. For instance, K63-linked polyubiquitination functions as a scaffold to recruit and activate protein kinase complexes. As previously discussed, ubiquitin ligases catalyze K63-linked polyubiquitin chains on TRAF6 to recruit TAK1 to facilitate Smad-independent TGFβ signalling.

Given that TGFβ signalling regulates a diverse set of cellular processes, modulating TGFβ signalling through a balance of ubiquitin ligase and deubiquitinating enzyme activity is important. By degrading TGFβ receptors, R-Smads, and downstream effectors, E3 ubiquitin ligases, protect cells from excessive TGFβ signalling. However, there are numerous examples where ubiquitin ligases prolong TGFβ signalling. For instance, Smad2-Smurf2 complexes lead to the destruction of Ski-related protein N (SnoN) and Ski, which are protooncogenes that impede TGFβ signalling. Arkadia, an E3 ubiquitin ligase, amplifies TGFβ signalling by ubiquitinating I-Smads. Paradoxically, if deubiquitinating enzymes remove K48-linked polyubiquitin chains on SnoN, Ski or Smad7, TGFβ signalling is dampened. Therefore, ubiquitin ligases and deubiquitinating enzymes may both antagonize or promote TGFβ signalling depending on the function of the ubiquitinated protein.
Figure 1.6: The effect of the ubiquitin-proteasome pathway on TGFβ signalling.

Transforming growth factor-β (TGFβ) signalling is tightly regulated by the ubiquitin-proteasome pathway. After TGFβ binds to the TGFβ-receptors, the ubiquitin-proteasome pathway is activated to prevent uncontrolled TGFβ signalling. E1 activating enzymes hydrolyse ATP to bind to ubiquitin. Ubiquitin is then transferred to an E2 conjugating enzyme. Smad7 binds to E3 ubiquitin ligases, which conjugates ubiquitin to TGFβ receptors, receptor regulated Smads (R-Smads), Smad4, and R-Smad-Smad4 complexes. This process is repeated until TGFβ receptors, R-Smads, Smad4 or R-Smad-Smad4 complexes are polyubiquitinated. Polyubiquitinated components of the TGFβ pathway are then subject to (1) proteasome-dependent degradation or (2) the removal of the ubiquitin-linked chains mediated by deubiquitinating enzymes (DUBs).

1.9 Mutations in genes involved in TGFβ signalling

Alterations in the TGFβ signalling pathway due to genetic mutations are the underlying cause of various hereditary congenital malformations, as well as diseases that arise later in life\textsuperscript{109,110}. Germline mutations impair embryonic development, whereas increased susceptibility to develop cancer is associated with somatic mutations\textsuperscript{5}. The clinical consequences of mutations in the TGFβ signalling pathway are complex, because
the tumour microenvironment and TGFβ signalling vary among patients and among different tissues within the same individual.\(^2\)

Genetically engineered mouse models with targeted inactivation of various TGFβ ligands have been generated to investigate the importance of TGFβ on development and viability.\(^{111}\) \(Tgfβ1^{-/-}\) mice can either succumb during mid-gestation as a result of vascular and hematopoiesis defects, or a few weeks after as a consequence of systemic inflammation.\(^{112-114}\) Death occurs shortly before, during or within minutes of birth in \(Tgfβ2^{-/-}\) mice, due to impaired cardiovascular function. These animals exhibit cardiac, craniofacial, limb, eye, inner ear, and urogenital defects.\(^{115,116}\) \(Tgfβ3^{-/-}\) mice exhibit cleft palates that interfere with feeding, eventually resulting in death.\(^{116,117}\) The majority of \(Smad\)-null mice die in utero, indicating that Smad proteins are required for proper embryonic development.\(^{118}\) Specifically, \(Smad2^{-/-}\) and \(Smad4^{-/-}\) mice die early in embryogenesis, due to defects in the organization of the primitive germ layers and extensive mesodermal defects.\(^{119,120}\) \(Smad3^{-/-}\) mice are viable, but exhibit impaired local inflammatory responses and accelerated wound healing.\(^{121,122}\)

In patients, familial juvenile polyposis, which increases the risk of gastrointestinal cancer, correlated with \(SMAD4\) mutants that produced truncated proteins with a loss or partial loss of function.\(^{123,124}\) Although juvenile polyposis patients have been screened for \(SMAD2\) and \(SMAD3\) mutations, only \(SMAD4\) germline mutants are identified as an underlying cause of juvenile polyposis.\(^{125}\) However, screening colorectal adenoma patients revealed that mutations to the \(SMAD4\) loci are rare.\(^{126}\) \(SMAD4\) mutations in patients with juvenile polyposis syndrome may also develop hereditary hemorrhagic telangiectasia, which results in abnormal vascular structures.\(^{127}\)

Frameshift and missense mutations in \(TGFBR1\) are common in several tumour types.\(^{128}\) For example, the \(TGFBR1*6A\) mutation in exon 1 is a loss of three Alanine residues in a 9-Alanine repeat region that increases cancer susceptibility associated with impaired anti-proliferative TGFβ signalling.\(^{129}\) Inactivating mutations in \(TGFBR2\) are frequently present in tumours that exhibit microsatellite instability, such as those found in subsets of colon carcinomas, which express truncated mutant forms of TGFβR2.\(^{131}\) \(SMAD4\) is the most common Smad family gene mutated in malignant tumours.\(^{132}\) Inactivating \(SMAD4\) mutations have been found in approximately 50% of pancreatic
adenocarcinomas\textsuperscript{123}, 20\% of colorectal carcinomas\textsuperscript{120}, and 5\% of head and neck squamous cell carcinomas\textsuperscript{133}. \textit{SMAD4} mutations also correlate with tumour formation\textsuperscript{134} and may predict poor prognosis and aggressive tumour phenotypes\textsuperscript{135}. For instance, mice with conditional targeted inactivation of \textit{Smad4} in the oral epithelium developed spontaneous squamous cell carcinomas\textsuperscript{136}. Although somatic mutations of the TGF\(\beta\) pathway may promote tumour formation, similar mutations in cancerous cells that rely on TGF\(\beta\) can decrease tumour growth\textsuperscript{137}. Since somatic mutations of the TGF\(\beta\) pathway may promote or block tumourigenesis depending on the stage of the disease, this is important to bear in mind when assessing the benefits and risks of using TGF\(\beta\) signalling inhibitors in cancer treatment\textsuperscript{138}.

\subsection*{1.10 Introduction to pro-tumourigenic TGF\(\beta\) signalling}

Cells escape the tumour suppressing arms of TGF\(\beta\) signalling through mutations that impede specific TGF\(\beta\) pathways or abnormalities in processes that dampen TGF\(\beta\) signalling\textsuperscript{7}. Functional inactivation of the tumour suppressing arms of TGF\(\beta\) signalling can contribute to carcinogenesis through various mechanisms\textsuperscript{2,7}. Major mechanisms that contribute to the pro-tumourigenic effects of TGF\(\beta\) include inhibition of immune function, activation of angiogenesis/lymphangiogenesis, and the initiation of EMT\textsuperscript{139–141}.

The ability of the immune system to distinguish external pathogens, damaged tissue, and tumour cells from healthy cells requires careful coordination of components that regulate immune processes\textsuperscript{142}. The components of the immune system are categorized as being a part of the innate or adaptive immune system\textsuperscript{143}. The leukocytes of the innate immune system may destroy tumour cell directly or activate cells of the adaptive immune system by either presenting antigens to cell surface receptors or releasing humoral signals\textsuperscript{142,143}. To evade the immune system, cells in the tumour and its microenvironment produce excessive amounts of immunosuppressive cytokines, such as TGF\(\beta\)\textsuperscript{139,140}. TGF\(\beta\) inhibits many components of both the innate and adaptive immune systems, which creates an environment favourable for tumour growth\textsuperscript{144}.

As tumours grow, blood carrying oxygen and nutrients is blocked from reaching interior tumour cells\textsuperscript{145}. To bypass this, TGF\(\beta\) alters cellular processes within endothelial
cells to mediate angiogenesis\textsuperscript{141}. Angiogenesis, the physiological process that forms capillaries from pre-existing blood vessels, is essential to tumour growth and invasion\textsuperscript{146}.

Epithelial-mesenchymal transition (EMT), a biological process whereby cells of epithelial origin acquire characteristics of mesenchymal cells, is essential for embryogenesis and wound healing\textsuperscript{147,148}. EMT is also involved in the ability of carcinoma cells to develop motile and invasive phenotypes, thus contributing to tumour progression and metastasis\textsuperscript{149}. During EMT, there is a loss of epithelial properties, such as apical/basolateral polarity, cytoskeleton polarization, cell-cell adhesions (adherens junctions, tight junctions, and gap junctions), and attachment to the basal lamina. Subsequently, the cells acquire mesenchymal characteristics including spindle-shaped morphology, transient focal point cell-cell attachments, lamellipodia/filopodia formation, front-back polarity, stress fibers, and increased motility (\textbf{Fig. 1.7})\textsuperscript{148,150}.

\textbf{Figure 1.7: Epithelial-mesenchymal transition.}

Epithelial-mesenchymal transition (EMT) is the biological process of an epithelial cell losing its epithelial properties, such as apical/basolateral polarity, tight junctions, gap junctions, adherens junctions, and hemidesmosome, and develop mesenchymal properties, which includes the capacity to breakdown the basal lamina, back/front polarity, spindle-shaped morphology, stress fiber formation, and N-Cadherin-dependent cell-cell attachments.

The profound phenotypical and morphological characteristics observed during EMT are amplified by signals that tumour cells receive from the tumour microenvironment,
such as TGFβ. TGFβ contributes to the initiation of the EMT program, via transcription-dependent and -independent mechanisms. TGFβ upregulates the expression of various EMT-transcription factors (SNAIL, SLUG, TWIST, ZEB1, ZEB2, FOXC2, FOXA1, FOXA2, PRX1, and HMGA2), which decrease the expression of epithelial genes, whilst increasing that of mesenchymal genes (Fig. 1.8). For example, SNAIL, SLUG, and ZEB1 downregulate the expression of E-Cadherin, a protein required for strong adherens junctions observed in epithelial cells, whereas TWIST upregulates the expression of N-Cadherin, a mesenchymal protein that forms weak transient cell-cell interactions.

TGFβ can promote EMT through non-canonical, Smad3-dependent regulation of RNA splicing. Phosphorylation of Smad3 on Threonine, subsequent to TGFβ receptor stimulation, impairs binding to Smad4 and to DNA, but induces Smad3 association with the RNA-binding protein poly(RC) binding protein 1 (PCBP1) in the nucleus. The Smad3-PCBP1 species catalyzes alternative splicing of myriad transcripts involved in EMT, including RNAs encoding the CD44 glycoprotein, which modulates cell-cell adhesion. Multiple CD44 splice variants exist. CD44E is preferentially expressed in normal epithelial cells, whereas the mesenchymal isoform CD44s is ubiquitous. In epithelial carcinoma cells, Smad3-PCBP1 complexes induce a splicing switch from CD44E to CD44s, resulting in activation of EMT and invasion. Similarly, complex formation between Smad3, PCBP1, and the RNA-binding protein Rbfox2 mediates expression of the alternative TAK1 splice variant TAK1Δglutamic acid 12 (TAK1ΔE12). TAK1ΔE12 is constitutively active, which means downstream signalling kinases, such as p38 MAPK and JNK, are constitutively phosphorylated. Transcription factors regulated by p38 MAPK and JNK are involved with upregulating genes that promote proliferation and EMT (Fig. 1.8).

TGFβ can also promote EMT by upregulating DNA methyltransferases, which hypermethylate promoters of various genes involved in the regulation of the cell cycle, apoptosis, cell-cell attachments, ECM production, and cell movement. For example, in ovarian carcinoma cells, reduced transcription of CDH1, which encodes E-Cadherin, is associated with hypermethylation in the presence of TGFβ (Fig. 1.8).
Figure 1.8: TGFβ induces epithelial-mesenchymal transition (EMT) by altering EMT-transcription factor activity.

As the concentration of transforming growth factor-β (TGFβ) increases, the epithelial-mesenchymal transition (EMT) program becomes more advanced. After TGFβ binds to the TGFβ receptors, it upregulates EMT-transcription factors (EMT-TFs), such as Snail Family Transcriptional Repressor 1 and 2 (SNAI1/SNAI2), Twist-related Protein 1 (TWIST1), Forkhead box C2 (FOXC2), Forkhead box A1 (FOXA1), Forkhead box A2 (FOXA2), Paired-related Homeobox 1 (PRX1), and High Mobility Group AT-hook 2 (HMGA2). EMT-TFs downregulate epithelial markers ((E-Cadherin, claudins, occludins, cytokeratins, integrins, microRNA(miR)-34, and miR-200)) and upregulate mesenchymal markers ((N-Cadherin, vimentin, matrix metalloproteinases (MMPs), fibronectin, α-smooth muscle actin (α-SMA), and miR-21)). TGFβ induces EMT by increasing DNA methyltransferase activity.

In the presence of TGFβ, DNA methyltransferase methylates (M) the promoters of epithelial genes, such as Cadherin 1 (CDH1). Also, when TGFβ receptor type 1 phosphorylates Smad3 at threonine 179 (T179-Smad3), it may associate with the RNA-binding protein poly (RC) binding protein 1 (PCBP1). Smad3-PCBP1 complexes alter CD44 splicing from CD44E, which is found in epithelial cells, to CD44s. CD44s splice variants modulate cell-cell adhesion to promote EMT. The Smad3-PCBP1 complex associated with Rbfox2 that mediates alternative splicing of TGFβ-activated kinase 1
(TAK1) to favour TAK1Δglutamic acid 12 (TAK1ΔE12) variants. TAK1ΔE12 is constitutively active, which leads to the constitutive phosphorylation of p38 MAPK (p38) and cJun amino-terminal Kinase (JNK). P38 and JNK upregulate genes that promote EMT.

There are several factors involved with TGFβ-dependent EMT regulation. First, a cells chromatin structure and epigenetics dictate if SNAIL and other EMT-transcription factors can access genes subject to their regulation\textsuperscript{162,179}. Second, miRNAs block the expression of EMT-transcription factors. For instance, microRNA-34 and microRNA-200 prevent the translation of SNAIL and ZEB1, respectively\textsuperscript{148,180,181}. Finally, each cell type has different intracellular signalling configurations. Therefore, the rate in which different cell types conduct Smad-dependent or -independent signalling is not the same\textsuperscript{182}. In conclusion, cells that upregulate microRNAs that block EMT-transcription factor translation, contain DNA methylation in the promoters of genes regulated by EMT-transcription factors, and favour tumour suppressive TGFβ pathways are less likely to undergo TGFβ-dependent EMT.

1.11 Introduction to autophagy

Autophagy, Greek for self-devouring, is a catabolic process where cells degrade and recycle their own macromolecules and organelles primarily via lysosomes\textsuperscript{183}. Autophagy is essential for recycling the building blocks of lipids, carbohydrates, and proteins as well as eliminating invading pathogens, protein aggregates, and damaged organelles\textsuperscript{184}. Although autophagy is primarily facilitated by lysosomes, which are acidic organelles that contain luminal degradative hydrolases, other acidic vesicles, such as late endosomes, contribute to autophagic degradation\textsuperscript{185}. Due to distinct molecular processes, protein complexes, and biological structures that target cellular materials to lysosomes, autophagy is categorized into three subtypes called microautophagy (mA), chaperone-mediated autophagy (CMA), and macroautophagy (MA). Briefly, mA is the direct uptake and degradation of cellular material via endosomes or lysosomes, CMA is facilitated by chaperone proteins that target specific proteins to lysosomes, and MA is a multi-step process involving several autophagy-related genes (ATGs) to form autophagosomes, which are double membrane vesicles that engulf macromolecules and organelles prior to fusing
with lysosomes\textsuperscript{186,187}. However, non-canonical MA can also occur when autophagosome formation does not rely on all \textit{ATGs} or the canonical hierarchal steps of autophagosome formation\textsuperscript{188}.

Since the 1950s, electron microscopy has been used to visualize lysosomes and autophagosomes\textsuperscript{189}. However, the term autophagy was not coined until 1963 when Christian de Duve discovered the cellular functions of lysosomes and described that “Its enzymes dissolve the substances ingested by the cell and under certain circumstances can dissolve the cell itself”\textsuperscript{190}. In the 1990s, another major autophagy revelation was achieved when Yoshinori Ohsumi identified several \textit{ATGs} and many of their respective functions in budding yeast\textsuperscript{191–193}. Over the years, the field of autophagy has grown to explore the role of autophagy in disease, homeostasis, metabolism, wound healing, immunity, and cell death. This led to the discovery of several distinct autophagy pathways that rely on different molecular mechanisms, biological structures, and protein complexes to facilitate degradation\textsuperscript{194}.

1.12 Macroautophagy (MA)

In general, mA and MA coordinate selective and bulk degradation of proteins and organelles whereas CMA mediates the degradation of select proteins\textsuperscript{195}. Each subtype of autophagy is important for homeostasis and thus is highly coordinated to remove/recycle unnecessary or damaged cellular material. However, this thesis focused on MA for several reasons. First, MA is the only autophagy subtype that produces large structures that are easily visible via microscopy. Second, its impact on autophagic flux far exceeds that of the other autophagy subtypes. Third, a broad scope of cell stressors and diseases significantly upregulate MA whereas their effects on CMA and mA are unknown. Lastly, MA can be measured using several molecular techniques, including western blotting, immunofluorescence microscopy, confocal microscopy, pH sensitive stains, electron microscopy, and RNA analysis\textsuperscript{196}.

Since the discovery of \textit{ATGs} in yeast and their respective mammalian homologs, studies investigating autophagy have shifted from structural/morphological based investigations to observing the molecular pathways involved in the formation of these structures\textsuperscript{197,198}. This led to the discovery of several proteins essential for the assembly of
MA structures, sequestering cellular materials for degradation, and trafficking to lysosomes. Knockout experiments made identifying proteins necessary for MA relatively straightforward, but characterizing specific protein functions proved challenging due to the fact that a single cell is continuously producing multiple MA structures that vary in size and assembly time. Although the processes that facilitate autophagosome formation proceed concurrently, MA is separated into six hierarchal stages distinguished by the activity of large protein complexes and morphological changes of MA structures. The six stages of MA include the activation of autophagy initiation complexes, nucleation of phagophores, phagophore extension, autophagosome maturation, autophagosome-lysosome fusion, and lysosomal-mediated degradation (Fig. 1.9).
During the initiation phase, cell stress activates 5’ adenosine monophosphate-activated protein kinase (AMPK). AMPK inhibits mechanistic target of rapamycin (mTOR) and...
activates uncoordinated-51-like autophagy activating kinase 1 (ULK1). mTOR is an inhibitor of autophagy because it adds an inhibitory phosphate group to ULK1. ULK1 phosphorylates autophagy-related protein (Atg)13 and focal adhesion kinase family kinase interacting protein of 200 kDa (FIP200). Together, Atg13, Atg101, FIP200, and ULK1 comprise the ULK1 complex. The ULK1 complex phosphorylates beclin 1 and autophagy and beclin 1 regulator 1 (AMBRA1), which initiates the nucleation stage. During nucleation, a class III phosphoinositide 3-kinase (PI3K) complex consisting of Atg14L, vacuolar protein sorting (VPS)34, VPS15, and beclin 1 bind to omegasomes on the endoplasmic reticulum. The PI3K complex creates phosphatidylinositol 3-phosphate (PI3P) lipids in the omegasome membrane to recruit downstream Atgs such as WD-repeat protein interacting with phosphoinositides 2 (WIPI2). B-cell lymphoma 2 (BCL2), AMBRA1, and endophilin B1 (EB1) are beclin 1 binding proteins. BCL2 antagonizes the formation of the PI3K complex whereas AMBRA1 and EB1 promote autophagy. The phagophore elongation phase of MA is characterized by two ubiquitin-like protein conjugation reactions. The first begins by Atg7 forming Atg12-Atg5 complexes. Atg10 then mediates the development of non-covalent bonds between Atg5 and Atg16L1 to form Atg12-Atg5-Atg16L1. In the second conjugation pathway, Atg4 cleaves the microtubule-associated protein light chain 3 (LC3) to expose a carboxyl terminal glycine (G)120, which produces LC3-I. LC3-I is activated by Atg7 and conjugated to phosphatidylethanolamine (PE) by Atg3 to form LC3-II. Atg12-Atg5-Atg16L1 complexes facilitate phagophore elongation by inserting LC3-II and lipids obtained from Atg9A vesicles into the phagophore. Phagophores engulf cytosolic materials non-selectively or by autophagy cargo receptors tethering the cytosolic materials to LC3-II. Autophagosome maturation is mediated by charged multivesicular body protein 2a (CHMP2A), VPS4, VPS37A, VPS28, and tumour susceptibility gene 101 (TSG101) cleaving mature autophagosomes off the endoplasmic reticulum membrane. The maturation stage is also associated with shedding Atgs and PI3P from the outer autophagosome membrane. For autophagosomes to fuse with lysosomes, they must migrate to perinuclear cell regions. Migration is facilitated along microtubules in which LC3-II attaches to Rab-interacting lysosomal protein (RILP), which binds to dynein, a microtubule motor protein. Once autophagosome membranes are in close contact to lysosome membranes, numerous proteins involved in the soluble N-
ethylmaleimide-sensitive-factor attachment receptors (SNARE) complex, homotypic fusion and VPS (HOPS) complex, and Rab7 facilitate fusion. Once the autophagosome fuses with the lysosome, autolysosomes are formed, which is the location of hydrolase-dependent degradation.

The initiation of autophagy is controlled by two serine-threonine kinases called uncoordinated-51-like autophagy activating kinase 1 (ULK1) and mTOR\textsuperscript{200}. The mTOR kinase is a component of mTORC1, which is a known autophagy inhibitor. In nutrient rich cellular environments, mTOR phosphorylates ULK1 to attenuate the activation of protein complexes that initiate MA\textsuperscript{200}. Alternatively, cell stressors and nutrient poor conditions upregulate 5’ adenosine monophosphate-activated protein kinase (AMPK). AMPK-dependent phosphorylation activates ULK1 and other downstream autophagy-related proteins (Atgs) important to autophagosome formation\textsuperscript{201}. Upon AMPK-dependent phosphorylation, ULK1 assembles the ULK1 protein complex by phosphorylating Atg13 and focal adhesion kinase family kinase interacting protein of 200 kDa (FIP200). The entirety of the ULK1 complex consists of ULK1, Atg13, Atg101, and FIP200 that are each responsible for specific functions\textsuperscript{202}. FIP200 is a scaffolding protein that tethers the complex together\textsuperscript{203,204}, Atg13 binds to Atg14L, a component of a class III PI3K complex\textsuperscript{205}, Atg101 stabilizes Atg13 by protecting it from proteasome-mediated degradation\textsuperscript{206}, and ULK1 phosphorylates two components of the class III PI3K complex called beclin 1 and autophagy and beclin 1 regulator 1 (AMBRA1) to initiate phagophore nucleation\textsuperscript{201,207}.

Beclin 1 recruits Atg14L, VPS34, VPS15, AMBRA1, B-cell lymphoma 2 (BCL2), and endophilin B1 to form a large PI3K complex responsible for nucleating phagophore assembly sites on omegasomes\textsuperscript{207,208}. Omegasomes are omega shaped outgrowths of the rough endoplasmic reticulum (ER) membrane enriched with PI3P\textsuperscript{209}. BCL2, AMBRA1, and Atg14L are beclin 1 binding proteins that regulate its ability to initiate the formation of the PI3K complex. Endophilin B1 is responsible for recruiting Atgs to the ER and creating curvature of the phagophore during assembly\textsuperscript{210,211}. VPS15 binds and activates VPS34, a class III PI3K that increases the formation of PI3P lipids\textsuperscript{208}. Enriching the membrane with PI3P sites recruits WD-repeat protein interacting with phosphoinositides
(WIPI)-1α/2 that functions as a scaffolding protein for downstream Atgs, such as microtubule-associated protein light chain 3 (LC3)\textsuperscript{212,213}.

Phagophore elongation is facilitated by two distinct molecular mechanisms that function similarly to ubiquitin-like conjugation systems. In the first ubiquitin-like conjugation system, Atg12 is activated by Atg7 and conjugated to Atg5 via Atg10. Meanwhile, FIP200 targets Atg16L1 to the phagophore assembly site where it forms non-covalent bonds with Atg5\textsuperscript{214,215}. The Atg12-Atg5-Atg16L1 complex tethers to phagophore membranes and participates in recruiting lipids to elongate phagophore membranes\textsuperscript{215–217}. The second ubiquitin-like conjugation system is responsible for the post-translation modification of LC3 proteins. In the cytosol, Atg4(A-D), a cysteine protease, cleaves the C terminus of LC3 to expose a conserved glycine (G)\textsubscript{120} residue, which produces LC3-I. LC3-I is activated by Atg7 and conjugated to phosphatidylethanolamine (PE) within the phagophore membrane by Atg3 to form LC3-II\textsuperscript{218}. LC3-II is incorporated into the inner and outer phagophore membranes via Atg12-Atg5-Atg16L1\textsuperscript{215}, which is tethered to PI3P sites within phagophore membranes by WIPI2\textsuperscript{219}. Finally, phagophore elongation relies on Atg9A, the only multi-spanning transmembrane Atg, to incorporate lipids into growing phagophores\textsuperscript{205,220,221} and deform growing phagophore membranes to create membrane curvature\textsuperscript{222}.

Temporally, as more lipids are incorporated to extend phagophore membranes, the phagophore closes and pinches off the ER to become a mature autophagosome. Recently, it was discovered that phagophore closure is similar to MVB formation and closure due to its dependency on endosomal sorting complex required for transport (ESCRT) proteins\textsuperscript{223}. Phagophore closure is driven by VPS37A, an ESCRT I protein, binding to phagophores and recruiting the other two ESCRT I proteins (VPS28 and tumour susceptibility gene 101) and charged multivesicular body protein 2a (CHMP2A), an ESCRT III protein. CHMP2A, in coordination with the VPS4, is responsible for phagophore membrane fission to generate an inner and outer autophagosome membrane (IAM and OAM)\textsuperscript{224,225}. In addition to membrane closure, autophagosome maturation is dependent on shedding PI3P and LC3-II from the OAM. One possible explanation for this is that cells recycle these proteins to spare them from degradation. Alternatively, since several Atgs bind to PI3P and use a LC3 interacting region (LIR) motif to bind LC3-II, both PI3P and LC3-II may be removed from
the OAM to ensure that these Atgs are recycled. Therefore, during autophagosome maturation, most of the PI3P and Atgs on the OAM are removed by myotubularin phosphatase 3 and Atg4, respectively.\textsuperscript{226,227}

Mature autophagosomes fuse directly with lysosomes or late endosomes to facilitate degradation of the enclosed proteins and organelles.\textsuperscript{228} Autophagosome-late endosome fusion generates an intermediate vesicle, referred to as an amphisome, prior to fusing with lysosomes.\textsuperscript{229} For fusion to occur, autophagosomes must traffic toward perinuclear cellular regions, which is the cellular location containing the majority of lysosomes, via microtubules. LC3 proteins and the related family, GABA type A receptor-associated protein (GABARAP), are essential for microtubule-mediated autophagosome trafficking.\textsuperscript{230} For instance, the C terminal glycine-PE tethers LC3 and GABARAP family members to the OAM. However, the N terminal-\(\alpha\)-helices of LC3 and GABARAP binds tubulin and proteins containing LIRs or GABARAP interaction motifs (GIMs), respectively.\textsuperscript{231,232} LIR/GIM containing proteins that contribute to autophagosome trafficking include FYVE and coiled-coil domain containing 1 (FYCO1) and JNK-interacting protein 1. FYCO1 binds to PI3P within autophagosome membranes and kinesin, a microtubule motor protein, that migrates toward the positive end of the microtubule (away from the perinuclear region) whereas JNK-interacting protein 1 attaches to dynein, a microtubule motor protein, that migrates toward the negative end of the microtubule (towards perinuclear regions).\textsuperscript{235} Additionally, autophagosome trafficking is modulated by the Rab7 GTPase. Rab7 attaches to the OAM and binds to FYCO1 and Rab-interacting lysosomal protein (RILP), which attaches to dynein.\textsuperscript{236}

Once the autophagosome and lysosomal membranes are in close contact, Rab7, homotypic fusion and vacuole protein sorting (HOPS) complex, and soluble N-ethylmaleimide-sensitive-factor attachment receptors (SNARE) complexes work together to facilitate autophagosome-lysosome fusion. Rab7 binds to VPS39/VPS41 of the HOPS complex, which is a heterohexameric complex comprised of VPS11, VPS16, VPS18, VPS33A, VPS39, and VPS4.\textsuperscript{237,238} Within autophagosome membranes, syntaxin 17 (STX17) and synaptosome-associated protein 29 (SNAP29) form a SNARE complex stabilized by Atg14L\textsuperscript{239} and VPS16/VPS33A.\textsuperscript{240,241} As a result, the HOPS complex assembles trans-SNARE complexes between the autophagosome SNARE proteins (STX17
and SNAP29) and the lysosomal SNARE protein called vesicle-associated membrane protein 7 (VAMP7)\(^{241}\). Trans-SNARE complexes are stabilized by ectopic P-granules autophagy protein 5 homolog, which is recruited to autophagosomes by its two LIR motifs and Rab7\(^{242}\). Finally, tectonin β-propeller repeat containing 1 (TECPR1) binds to PI3P sites and competes with Atg16L1 for Atg5 to form the Atg12-Atg5-TECPR1 complex that tethers autophagosomes to lysosomes\(^{243}\).

After autophagosome-lysosome fusion, a short-lived vesicle called the autolysosome is formed\(^{244}\). Degradation is initiated by lysosomal phospholipases breaking down the IAM\(^{245}\). Once the IAM is compromised, the cytosolic material that was transported to the lysosomes is degraded by more than 60 different lysosomal hydrolases\(^{246}\). Since the lysosomes are acidic vesicles, most of these degradative enzymes optimally function in low pH. Finally, after proteins, lipids, and carbohydrates are broken down into amino acids, fatty acids, and monosaccharides, respectively, they are exported to the cytoplasm. These degradative products are exported via transporters within the lysosomal membrane, lysosomal exocytosis or vesicular trafficking\(^{247}\).

### 1.13 The activation of autophagy by TGFβ

Similar to TGFβ, the tumour regulatory consequences of autophagy are context dependent, as autophagy can result in either tumour suppression or promotion, depending on the stage of tumour development\(^{199,248}\). In non-cancerous tissues, autophagy functions as a homeostatic safeguard by removing protein aggregates, damaged organelles, and other metabolic stressors, all of which protects the tissues against neoplastic transformation\(^{249,250}\). On the other hand, autophagy participates in the survival of established tumour cells under conditions of hypoxia, oxidative damage, metabolic stress, and starvation\(^{248,251,252}\). Autophagy has been linked to EMT, MMP secretion, angiogenesis, evasion of immune surveillance, promigratory cytokine secretion, anoikis resistance, and stemness in tumour cells\(^{253}\). Autophagy has also been implicated in resistance to chemotherapeutic agents that target rapidly dividing cells, because it promotes tumour cell dormancy\(^{254}\). Accordingly, silencing of autophagic proteins can increase the efficacy of chemotherapeutic agents\(^{255}\). Autophagy can also improve survival of circulating tumour
cells and establishment of the pre-metastatic niche\textsuperscript{253}, as well as increase tumour cell survival after metastasis (Table \textbf{1.2})\textsuperscript{252,256}.

\textbf{Table 1.2: The tumour promoting properties of autophagy.}

<table>
<thead>
<tr>
<th>Primary tumors</th>
<th>Secondary tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increases EMT</td>
<td>Tumor cell dormancy</td>
</tr>
<tr>
<td>Increases motility</td>
<td>Drug resistance</td>
</tr>
<tr>
<td>Increases cytokine secretion</td>
<td>Survival</td>
</tr>
<tr>
<td>Increases cell adhesion turnover</td>
<td>Establishes metastatic colonies</td>
</tr>
<tr>
<td>Anoikis resistance</td>
<td></td>
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<tr>
<td>Immunosuppression</td>
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<td>Drug resistance</td>
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Epithelial-mesenchymal transition (EMT)

Immunosuppression, increased angiogenesis, and EMT are the most widely studied mechanisms whereby TGFβ promotes tumourigenesis. However, the pro-tumourigenic activity of TGFβ likely includes additional biological processes, such as autophagy\textsuperscript{257}. TGFβ signalling can influence autophagosome formation, \textit{ATG} expression, and steady-state Atg levels. For instance, TGFβ signalling activates transcription of genes essential to autophagy, such as \textit{ATG5}, \textit{ATG7}, \textit{BECNIL1}, and \textit{DAPK1}\textsuperscript{257,258} and increases steady-state levels of beclin 1, Atg7, Atg5, and LC3-II\textsuperscript{259}. TGFβ treatment has also been shown to increase the number of autophagosomes and autolysosomes as well as LC3-lysosome co-localization (Fig. \textbf{1.10A, B})\textsuperscript{251,260}. However, specific branches of the TGFβ pathway that activate autophagy have yet to be elucidated.
Figure 1.10: TGFβ signalling upregulates autophagy.
(A) Diagram illustrating how TGFβ signalling induces macroautophagy. After TGFβ binds to the TGFβ receptors, it increases steady-state levels of beclin 1, which is a component of the PI3K complex. The PI3K complex then nucleates phagophore assembly. As the phagophore membranes are elongated with lipids and LC3B, cargo proteins, and organelles are sequestered within autophagosomes by autophagy cargo receptors. Once phagophore assembly is complete, it forms a mature double membrane vesicle called an autophagosome. Autophagosomes fuse with lysosomes to generate autolysosomes. The autophagosomes and cargo are degraded by lysosomal degradative enzymes.

(B) Schematic illustrating that in the absence of TGFβ there are few autolysosomes and autophagosomes; however, after TGFβ activates autophagy, autolysosome and autophagosome formation is increased.

Recently, TGFβ-induced EMT was found to be attenuated following autophagy inhibition251. The fact that TGFβ-dependent EMT relies on autophagy is not surprising due to the extensive roles that autophagy has in tumour development, maintenance, and metastasis (Fig. 1.11A)261. Although research of the effect of TGFβ-induced autophagy in tumourigenesis is scarce, we know that as TGFβ signalling and autophagy are upregulated, angiogenesis and EMT are increased whereas the immune response is dampened (Fig. 1.11B)262. For instance, in pancreatic ductal adenocarcinoma cells, autophagy is required for TGFβ-induced migration, proliferation, and invasion263,264. TGFβ-induced autophagy also decreases the expression of proinflammatory cytokines in macrophages265. Furthermore, genomic analysis of colon cancer revealed that autophagy upregulates immune checkpoint molecules that dampen the immune response, whereas EMT, TGFβ, and angiogenic pathways were enhanced266. Finally, in vivo xenograft models of breast cancer demonstrated that TGFβ-induced autophagy protected fibroblasts from cell death-mediated by nutrient starvation and increased CAF phenotypes267.
Figure 1.11: The interplay between autophagy and TGFβ signalling in tumourigenesis.

(A) A schematic summarizing the impact of TGFβ-induced autophagy on EMT, immune surveillance, angiogenesis, and metastasis. Epithelial cells acquire mutations to the TGFβ pathway until they become cancerous and proliferate rapidly to form the primary tumour. TGFβ-induced autophagy protects tumour cells from the innate immune system (macrophages, dendritic cells, neutrophils, and Natural Killer cells) and cells of the adaptive immune system (Naive T-cell, Cytotoxic T-cells, Helper T-cells, B-cells, and plasma cells). Furthermore, TGFβ and autophagy can prevent activation of immune cells that reside in lymph nodes. TGFβ-induced autophagy promotes the release of vascular endothelial growth factor (VEGF) that stimulate angiogenesis. Overtime, cells acquire a mesenchymal-like phenotype and release matrix metalloproteinases (MMPs) to breakdown the basal lamina and intravasate into the bloodstream. TGFβ-induced autophagy promotes intravasation because it protects cells that detach from the basal lamina against anoikis...
dependent cell death. The mesenchymal-like tumour cells extravasate from the blood vessel at a distant site from the primary tumour. Autophagy is critical for promoting phenotypes to help tumour cells adapt to new environments and establish secondary tumour sites.

(B) As the concentration of transforming growth factor-β (TGFβ) increases, the immune response is inhibited, whereas angiogenesis, epithelial-mesenchymal transition (EMT), and autophagy are activated.

1.14 The link between protein degradation pathways and TGFβ

Although there are several catabolic processes that regulate protein quality control in mammalian cells, the UPP and autophagy/lysosome pathway are the two central processes\(^{268}\). Due to difference in substrate selectivity, preparation for degradation, and degradative organelles, the UPP and autophagy do not necessarily compete with one another. Instead, their relationship may be described as compensatory. For instance, when autophagy or the UPP are disrupted, the other major route of protein degradation increases protein turnover to compensate for the disruption\(^{268}\). One explanation is that both lysosome and proteosome-dependent degradation rely on ubiquitination to identify proteins destined for degradation\(^{269–271}\). Also, both autophagy and the UPP depend on adaptor proteins such as protein 62/sequestosome 1 (p62/SQSTM1) to deliver substrate proteins\(^{272}\). Currently, the mechanism of how p62/SQSTM1 decides which pathway receives the ubiquitinated protein remains unknown. What is known is that p62/SQSTM1 is an autophagy cargo receptor protein that functions in autophagic degradation, regulates EMT, binds to ubiquitin, and is important for TGFβ signalling\(^{273–275}\).

P62/SQSTM1 is composed of several domains including a phox bem1 (PB1) domain, ZZ-type zinc finger (ZZ) domain, TRAF binding (TB) domain, LC3-interacting region (LIR), and ubiquitin-associated (UBA) domain. The UBA domain allows p62/SQSTM1 to functions as a ubiquitin receptor protein that targets ubiquitinated proteins to proteasomes\(^{272,276}\). In addition to regulating autophagy and the proteasome, p62/SQSTM1 can sequester several downstream TGFβ signalling molecules, including
p38 MAPK, TRAF6, and aPKC using the ZZ, TB, and PB1 domains, respectively. These proteins have been implicated in modulating autophagy induction and TGFβ receptor trafficking\(^\text{277}\). Furthermore, using the PB1 domain, p62/SQSTM1 self-oligomerizes to sequester intracellular cargo during cell stress or disruption of the protein turnover pathways\(^\text{278}\). Also, between the ZZ and TB domains, there is a region of p62/SQSTM1 that interacts with Raptor, a component of mTORC1, which is an additional link between p62/SQSTM1 and autophagy (Fig. 1.12).

An image based genome wide small interfering RNA screen in mammalian cells identified Smurf1 as a mediator of selective autophagy\(^\text{279}\). Since we know that Smurf1 also mediates the UPP, this suggests that TGFβ-specific signalling modulators also have the potential to regulate protein degradation pathways. Therefore, there is crosstalk between TGFβ signalling, autophagy, and the UPP. Given that autophagy, proteasomes, and p62/SQSTM1 regulate TGFβ-dependent EMT\(^\text{251,280,281}\) and are altered by TGFβ treatment\(^\text{282,283}\), proteins such as p62/SQSTM1 may be important to understanding the crosstalk between protein degradation pathways and TGFβ signalling.

**Figure 1.12: Domains and structure of p62/SQSTM1.**

From the amnio (N) terminal to carboxyl (C) terminal, p62/SQSTM1 is comprised of the phox bem1 (PB1), ZZ-type zinc finger (ZZ), tumour necrosis factor receptor-associated factor (TRAF) binding (TB), microtubule-associated protein light chain 3 (LC3)-interacting region (LIR), and ubiquitin-associated (UBA) domains. The PB1 domain allows protein 62/sequestosome 1 (p62/SQSTM1) to interact with atypical protein kinase C (aPKC) and self-oligomerize. The ZZ and TB domain have been shown to interact with
downstream transforming growth factor-β (TGFβ) signalling molecules, such as p38 mitogen-activated protein kinase (MAPK) and TRAF6, respectively. Between the ZZ and TB domains, p62/SQSTM1 associates with Raptor, which is a component of mechanistic target of rapamycin complex 1 (mTORC1). The LIR binds to LC3 and is necessary to facilitate selective autophagy. The UBA domain recognizes ubiquitin prior to delivering ubiquitin-conjugated proteins to proteasomes or lysosomes.

1.15 Overview of cell models

Although the signalling pathways activated by TGFβ have previously been characterized, the regulation of these pathways determines a cell’s response to TGFβ signals. For instance, TGFβ signalling is regulated at multiple points through a network of proteins and biological processes whose expression and prevalence is cell type-dependent. Therefore, the model selected for my investigation is pivotal to understanding the effects of TGFβ signalling on the microenvironment of tumours, and the mechanisms involved in TGFβ signalling dysregulation.

This work primarily used A549 and H1299 lung adenocarcinoma cell lines, which are established models of non-small cell lung cancer (NSCLC). These cell lines have been used to investigate TGFβ signalling\textsuperscript{37}, EMT\textsuperscript{284}, autophagy\textsuperscript{285}, and p62/SQSTM1\textsuperscript{286}. Although H1299 cells do not express p53\textsuperscript{287}, the TGFβ signalling pathways for both cell lines are functional and intact\textsuperscript{288,289}. A549 and H1299 cell lines were transfected to stably express a green fluorescent protein-LC3B-red fluorescent protein-LC3B with a C terminal glycine deletion (GFP-LC3B-RFP-LC3BΔG) vector that quantified autophagic flux. Finally, mink lung (Mv1Lu) cells stably expressing hemagglutinin (HA)-tagged TGFβRII under a zinc inducible promoter were used to assess TGFβ receptor endocytosis. Previously, my lab demonstrated that the antibodies against the HA-tag detected TGFβRII trafficking to early endosome and late endosome membrane compartments\textsuperscript{99}. These experiments were repeated using A549 cells and H1299 cells; however, due to poor expression and the lack of quality of human TGFβ receptor antibodies, immunofluorescence microscopy using NSCLC cell lines was limited.
1.16 Purpose of study, hypothesis, and aims

Cancer, a group of diseases characterized by abnormal cell proliferation and invasion, is responsible for more than 30% of Canadian mortalities\textsuperscript{290}. Lung cancer in particular is a major concern for oncologists and patients alike since it is the leading cause of cancer mortality for Canadians\textsuperscript{291}. The 16\% five-year survival rate associated with lung cancer can be attributed to the fact that early stages of the disease are relatively asymptomatic\textsuperscript{292}. As such, by the time patients seek therapeutic interventions; they have developed advanced forms of the disease, which are associated with poor clinical outcomes\textsuperscript{293}. When comparing the two major subtypes of lung cancer referred to as small cell lung cancer (SCLC) and NSCLC, NSCLCs are responsible for more than 85\% of lung cancer cases and are less responsive to chemotherapeutic treatments\textsuperscript{294,295}. This combination of high incidence and chemotherapeutic resistance has fueled the demand for novel NSCLC interventional candidates\textsuperscript{296}.

For decades, NSCLC has commonly been treated with surgery, radiation therapy, chemotherapy, and palliative care\textsuperscript{297}. For early stages of NSCLC, surgical resection was, and still is, the gold standard treatment; however, cardiac and pulmonary comorbidities such as emphysema and heart disease deem patients surgically inoperable\textsuperscript{298}. For those who are surgically operable, about 30-50\% of patients relapse and develop more advanced forms of the disease\textsuperscript{292,299}. The standard of care for advanced NSCLC is platinum-based double-agent chemotherapeutic regimens such as carboplatin and paclitaxel, cisplatin and paclitaxel, and cisplatin and docetaxel\textsuperscript{300}. However, previous studies investigating chemotherapeutic efficacy report that 40\% of patients who present with metastatic disease are beyond the therapeutic scope that modern medicine can provide\textsuperscript{301}. This need to improve patient prognosis has fueled the research for novel markers for targeted therapy\textsuperscript{295,302}.

Given that TGFβ, UPP, and autophagy control a diverse set of cellular processes, I investigated how these three pathways may be co-ordinately implicated in tumourigenesis. My hypothesis is that TGFβ signalling and the protein degradation pathways of UPP/autophagy are reciprocally regulated in NSCLC. This hypothesis was tested through five aims:
1. Identify molecular techniques and assays appropriate to monitor TGFβ-dependent autophagy in NSCLC.

2. Elucidate the TGFβ signal transduction pathways that facilitate autophagy and how TGFβ upregulates autophagy.

3. Examine how inhibitors of autophagy and siRNA against ATGs impact TGFβ-dependent signalling, TGFβ receptor endocytosis, and TGFβ receptor trafficking.

4. Characterize how proteasome inhibitors affect autophagy, TGFβ signalling, TGFβ receptor endocytosis, and TGFβ receptor trafficking.

5. Determine how p62/SQSTM1 regulates autophagy, TGFβ signalling, and EMT.
Chapter 2

Assessing methods to quantitatively validate TGFβ-dependent autophagy

Components of this chapter have been published in Biol Open. (2020) 9(11).
2 Summary

Here, I assessed how transforming growth factor-β1 (TGFβ1) modulates autophagy-related gene (ATG) expression and autophagy-related protein (Atg) levels. I also examined the impact of TGFβ1 on microtubule-associated protein light chain 3B (LC3B) lipidation, LC3B puncta formation, and autophagosome-lysosome co-localization in non-small cell lung cancer (NSCLC) cell lines. These experimental approaches were validated using pharmacological autophagy inhibitors (chloroquine and spautin-1) and an autophagy activator (MG132). I found that TGFβ1, chloroquine, and MG132 had little effect on Atg protein levels but increased LC3B lipidation, LC3B puncta formation, and autophagosome-lysosome co-localization. Since similar outcomes were observed using chloroquine and MG132, I concluded that several techniques employed to assess TGFβ-dependent autophagy may not differentiate between the activation of autophagy vs. lysosomal inhibition. Thus, NSCLC cell lines stably expressing a GFP-LC3B-RFP-LC3BΔG autophagic flux probe were used to assess TGFβ1-mediated autophagy. Using this approach, I observed that TGFβ1, MG132, and serum starvation increased autophagic flux, whereas chloroquine and spautin-1 decreased autophagic flux. Finally, this supported that Atg5 and Atg7 are critical for TGFβ1-dependent autophagy in NSCLC cells. The application of this model will fuel future experiments to characterize TGFβ1-dependent autophagy, which is necessary to understand the molecular processes that link TGFβ1, autophagy, and tumourigenesis.

2.1 Introduction

Using lysosomal enzymes to recycle unwanted, superfluous, and aggregated proteins and eliminate damaged organelles, autophagy regulates iron, carbohydrate, fatty acid, amino acid, and cholesterol homeostasis. As such, autophagy protects cells from tumourigenesis, which was verified by spontaneous tumour formation in ATG knockout models. Paradoxically, in cancer cells, autophagy has been linked to epithelial-mesenchymal transition (EMT), anoikis resistance, stem cell phenotypes, quiescent phenotypes, cell migration, and resistance to cancer therapies, which promote tumourigenesis. As previously discussed, TGFβ may augment tumourigenesis by
upregulating autophagy\textsuperscript{248,306}. Therefore, autophagy inhibitors may impede the tumour promoting properties of TGFβ\textsuperscript{252}.

Although impairing autophagy disrupts TGFβ-dependent EMT, there is still much unknown about the relationship between TGFβ and autophagy\textsuperscript{251}. What is known is that TGFβ upregulates the expression of \textit{ATG}s\textsuperscript{259}, increases the steady-state levels of Atgs\textsuperscript{307}, induces LC3 puncta formation\textsuperscript{306}, promotes LC3-lysosome co-localization, and increases the number of autophagosomes\textsuperscript{251}. However, there is no evidence to suggest that TGFβ increases autophagic flux\textsuperscript{260}. This is important because autophagic flux indicates changes to the rate of degradation, which means evidence that TGFβ alters degradation is lacking. For this reason, this thesis first aimed to highlight potential technical pitfalls in the investigations of TGFβ1-dependent autophagy and develop strategies designed to interpret the impact of TGFβ1 on autophagy more accurately.

### 2.2 Materials and Methods

#### Antibodies and reagents

Primary antibodies were purchased from the following vendors: anti-beclin 1 (BECN1; Cell Signalling Technology, 3738S), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Cell Signalling Technology, 2118S), anti-tubulin (Cell Signalling Technology, 2144S), anti-LC3B (Cell Signalling Technology, 9236S), anti-uncoordinated-51-like autophagy activating kinase (ULK1; Cell Signalling Technology, 8054S), anti-phospho-S465/467-Smad2 (P-Smad2; Cell Signalling Technology, 3108L), anti-Smad2/3 (BD Transduction laboratories, 562586), anti-autophagy related protein 3 (Atg3; Cell Signalling Technology, 3415S), anti-Atg5 (Cell Signalling Technology, 12994S), anti-Atg7 (Cell Signalling Technology, 8558S), anti-Atg9A (Novus Biologicals, NB110-56893), anti-Atg16L1 (Cell Signalling Technology, 8089S), and anti-Atg12 (Cell Signalling Technology, 4180S). Secondary antibodies used for western blot analysis were as follows: Horseradish peroxidase (HRP)-conjugated goat anti-rabbit-IgG (Thermo Fisher Scientific, 31460) and goat anti-mouse-IgG (Thermo Fisher Scientific, 31430). Human Ambion small interfering RNA (siRNA) constructs were purchased from Thermo Fisher Scientific (si-ATG7, si-ATG5 and si-Control with catalog numbers 4392420, 4392420 and 4457289, respectively). For fluorescence microscopy, LysoTracker Deep Red (Invitrogen,
and Hoechst stain (Invitrogen, H3569) labelled lysosomes and nuclei, respectively. The pharmacological agents that modulate autophagy were specific and potent autophagy inhibitor-1 (spautin-1; Sigma, SML0440), chloroquine (Acquired from the Shepherd lab, London, Canada), and MG132 (Sigma, M7449).

**Cell culture and transfections**

H1299 cells and A549 cells are NSCLC cell lines that were cultured in Roswell Park Memorial Institute (RPMI; Corning, 10-043-CVR) and Kaelihn’s Modification of Hams F-12 (F-12K; Corning, 10-025-CV) media, respectively. Both cell lines were passaged using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA; Sigma, T2605), centrifuged at 1000x g for 2 minutes, and resuspended in fresh media supplemented with 10% fetal bovine serum (FBS). A humidified tissue incubator cultured the cells at 37°C under 5% CO₂. Cells were treated with 250 pM TGFβ1, 50 μM chloroquine, 10 μM spautin-1, 10 μM MG132 in media supplemented with 10% FBS. Transient siRNA knockdowns in H1299 cells and A549 cells were performed using optimem media (Thermo Fisher Scientific, 22600134) and Lipofectamine RNAiMAX (Thermo Fisher Scientific, 13778150) as per manufacture’s protocol. Stable GFP-LC3B-RFP-LC3BΔG expressing cells were generated using a cDNA pMRX-IP-GFP-LC3B-RFP-LC3BΔG vector (Addgene, 84573) and PolyJet transfection reagent (Froggabio, Toronto, ON). Transfected cells were isolated using 1 μg/mL puromycin (Thermo Fisher Scientific, A1113802) in growth media supplemented with 10% FBS.

**Microarray data analysis**

Expression of autophagy specific genes were analyzed from my labs previously published microarray dataset of untreated and TGFβ-treated A549 cells\(^{308}\) (NCBI Gene Expression Omnibus website, GEO; GSE26241).

**Immunoblotting**

Protein isolation was achieved using a lysis buffer containing 50 mM Tris pH 7.5, 150 mM sodium chloride, 1 mM EDTA, 0.5% Triton X-100, 1 mg/mL pepstatin, 50 μM phenylmethylsulfonyl fluoride, 2.5 mM sodium fluoride, and 10 mM sodium pyrophosphate phosphatase inhibitor (TNTE). Cell lysates were then centrifuged at 21000x
g at 4°C for 12 minutes. Protein concentration was determined using the DC™ protein assay (Bio-Rad, Hercules, California, U.S.A) and a Victor 3V Multi-Detection Microplate Reader (PerkinElmer, Waltham Massachusetts, U.S.A). Prior to immunoblotting, 8x loading buffer (30% glycerol, 10% 1.5 M Tris (pH 6.8), 1.2% sodium dodecyl sulfate (SDS), 0.018% bromophenol blue, and 15% β-mercaptoethanol) was added to the protein lysates. Protein lysates mixed with loading buffer were utilized for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Each well of the polyacrylamide gel received approximately 50 µg of protein, which was run at a constant 120 volts for 100 minutes. Following a standard wet transfer protocol, proteins were transferred onto a nitrocellulose membrane using a constant 100 volts for 80 minutes. Nitrocellulose membranes were blocked with 5% skim milk for 1-hour, rocking at room temperature. Primary antibodies were incubated overnight with the nitrocellulose membranes, rocking at 4°C. On the following day, nitrocellulose membranes were incubated with the appropriate HRP-conjugated secondary antibody for 1-hour at room temperature. Enhanced chemiluminescent substrate (Bio-Rad, 1705060) was added 5 minutes prior to visualizing using a Versa-doc Imager (Bio-Rad). Finally, QuantityOne® 1-D Analysis software (Bio-Rad) was used to analyze the relative intensity of protein bands.

**MTT assay**

A549 cells and H1299 cells were treated with increasing concentrations of chloroquine, spautin-1 or MG132 for 24 and 48 hours in a 96 well plate. After the incubation period, the cells were subject to an MTT assay (Sigma, 11465007001) as per manufacturer’s protocol. Briefly, I added 10 µL of the MTT labelling reagent to each well and left it in a humidified tissue incubator at 37°C under 5% CO₂ for 4 hours. Next, I added 100 µL of the solubilization solution to each well and placed it in a humidified tissue incubator overnight. The next day a Victor 3V Multi-Detection Microplate Reader measured the absorbance of the 550 nm and 690 nm wavelengths. The values for each wavelength were subtracted by the blank (cell null) treatment wavelength values. The 550 nm-690 nm absorbances for the no treatment cells were standardized to 100% viability and all treatments were relative to the no treatment control.

**Assessing autophagosome and lysosome co-localization**
A549 cells stably expressing GFP-LC3B were treated with pharmacological modulators of autophagy in the presence and absence of TGFβ1 for 24 hours. LysoTracker Deep Red labelled lysosomes and Hoechst stain labelled the nucleus 2 hours and 10 minutes prior to imaging, respectively. Using a 63x objective of an Olympus IX 81 inverted fluorescence microscope (Olympus, Canada), I imaged the Hoechst stain, GFP-LC3B, and LysoTracker Deep Red. Co-localization was observed by the appearance of yellow puncta, which suggested that the GFP-LC3B and lysosomes were in close proximity. The co-localization plug-in of ImageJ version 2.0 quantified each image. Each data point represents quantitation from ≥ 100 cells from each condition.

**Autophagic flux assay**

Autophagic flux was measured using A549 cells and H1299 cells that were transfected with a cDNA pMRX-IP-GFP-LC3B-RFP-LC3BΔG vector developed by the Mizushima laboratory (Addgene)\(^{309}\). After the transfected cells express this vector, they produce two forms of LC3B: LC3B conjugated to green fluorescent protein (GFP-LC3B) and a mutant LC3B with a C-terminal glycine deletion conjugated to red fluorescent protein (RFP-LC3BΔG). The LC3BΔG cannot be incorporated into the autophagosome membrane, and thus as autophagy occurs the GFP-LC3B is degraded whereas the RFP-LC3BΔG remains immune to autophagic degradation\(^{309}\). Immunoblotting using LC3B specific antibodies could distinguish the RFP-LC3BΔG, GFP-LC3B-I and GFP-LC3B-II bands, which are quantified using QuantityOne® 1-D Analysis software to determine the GFP/RFP ratio. Furthermore, using a 63x objective of an Olympus IX 81 inverted fluorescence microscope, I imaged the green and red channels. The GFP/RFP ratio was determined by ImageJ version 2.0, which quantified the average pixel intensity for green and red channels.

**Statistical Analysis**

One-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparisons test, a two-way/three-way ANOVA followed by either Tukey’s or Sidak’s multiple comparison tests and Student’s t-tests were used to evaluate the significance of the results. Statistical analyses were performed using GraphPad Prism Software 8.1 and P-values <0.05 were statistically significant.
2.3 Results

*TGFβ1 has little effect on the expression of ATGs in A549 NSCLC cell lines*

The purpose of this work was to explore different techniques to provide quantitative evidence that TGFβ1 induces autophagy in NSCLC cells. To examine how TGFβ1 regulated autophagy, I first utilized microarray analysis to determine the impact of TGFβ1 on the expression of *ATGs* in A549 cells (Table 2.1). A549 cells were treated with 250 pM TGFβ1 for 0 hours (control) or 1-hour, which was followed by an 8-hour or 24-hour washout. I observed that TGFβ1 elicited only a modest change in the expression of *ATGs*. Indeed, there was a small increase in *ATG4D, ATG9A, ATG16L1, GABA Type A receptor-associated protein L1 (GABARAPL1), GABA Type A receptor-associated protein L3 (GABARAPL3), and microtubule-associated protein light chain 3A (LC3A)*; and a minor decrease in the expression of *ATG3*. The presence and activity of TGFβ1 was verified by the increase in the expression of *CDH2*, a mesenchymal marker, and a decrease in the expression of *CDH1*, an epithelial marker, which are known targets of TGFβ1 signalling (Table 2.1)\(^{150,153}\). Thus, although TGFβ1 had a modest effect on the expression of some *ATGs*, it had little influence on the expression of the majority of *ATGs* in A549 cells.
Table 2.1: The effect of TGFβ1 on autophagic marker expression.

<table>
<thead>
<tr>
<th>Genes</th>
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<tr>
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Autophagy and beclin 1 regulator 1 (AMBRA1), Autophagy-related gene (ATG), beclin 1 (BECN1), GABA type A receptor-associated protein (GABARAP), Microtubule-associated protein light chain 3 (MAP1LC3), protein 62/sequestosome 1 (p62/SQSTM1), Rubicon autophagy regulator (RUBCN), uncoordinated-51-like autophagy activating kinase (ULK), Cadherin 1 (CDH1), and Cadherin 2 (CDH2). Bold font indicates significant difference with respect to the 0 hour control.

*TGFβ1 induces LC3B lipidation but does not increase Atg levels in NSCLC cell lines*

Next, I assessed the impact of TGFβ1 on the steady-state levels of several Atgs that facilitate or regulate autophagy. A549 cells and H1299 cells were treated with 250 pM TGFβ1 for 24 hours prior to lysis and immunoblotted for Atgs whose genes were found to be induced (Atg9A, Atg16L1, and ULK1), reduced (Atg3) or unchanged (Atg7, Atg12 and Atg12-Atg5 complex, BECN1, and LC3B) in Table 2.1. Furthermore, I immunoblotted for P-Smad2, Smad2, and GAPDH (loading control). P-Smad2 verified the presence and activity of TGFβ1 in both cell lines. In A549 cells, TGFβ1 had no significant impact on the protein levels of Atg7, BECN1, Atg12 or Atg12-Atg5 complex formation. Interestingly, TGFβ1 decreased the protein levels of Atg3 and Atg9A, whereas it increased ULK1 and LC3B-II protein levels (Fig. 2.1A). In H1299 cells, TGFβ1 had no significant impact on the protein levels of BECN1, Atg3, Atg12 or Atg12-Atg5 complex formation. However, in this cell line, TGFβ1 significantly decreased Atg7 and Atg9A protein levels and increased ULK1 and LC3B-II protein levels (Fig. 2.1B). Therefore, after assessing the impact that TGFβ1 had on steady-state Atgs, I found that the levels of ULK1 and LC3B were consistent indicators of TGFβ1-induced autophagy in both NSCLC cell lines.
Figure 2.1: The effect of TGFβ1 on Atg levels and LC3B lipidation.
A549 cells (A) and H1299 cells (B) were treated with 250 pM TGFβ1 for 24 hours. Cells were lysed and subjected to SDS-PAGE and immunoblotting for anti-Atg3, anti-Atg5, anti-Atg7, anti-Atg9A, anti-Atg12, anti-Atg16L1, anti-BECN1, anti-ULK1, anti-LC3B, anti-P-Smad2, anti-Smad2, and anti-GAPDH (loading control) antibodies. The steady-state levels of Atg3, Atg7, Atg9A, Atg12, Atg12-Atg5, Atg16L1, BECN1,ULK1, and LC3B were quantitated using QuantityOne software and graphed (n=3 ± SD). Significance is indicated as *=P<0.05, **P<0.01, and ****=P<0.0001.

The caveat of using LC3B lipidation and Atg levels as readouts for TGFβ1-induced autophagy

Although LC3-II protein levels are considered to be proportional to the amount of autophagosomes, their utility as an indicator for autophagy remains unresolved. For this reason, I investigated if using LC3 lipidation would be useful to draw conclusions regarding TGFβ1-induced autophagy. First, I analyzed how known inhibitors and activators of autophagy impacted LC3 lipidation. The pharmacological inhibitors of autophagy selected for the study were chloroquine and spautin-1 whereas MG132, a proteasomal inhibitor, functioned as an activator of autophagy. For each compound, I determined the optimal dose and treatment duration that would have a significant effect on LC3B-II protein levels but would not affect cell viability, as assessed by MTT assays in A549 cells (Fig. 2.2). The doses and treatment durations that had low cell mortality (50 µM chloroquine, 10 µM spautin-1, and 10 µM MG132 for 24 hours) were selected as the treatment regimen for each pharmacological autophagy modulator. I found that chloroquine increased steady-state LC3B-II protein levels (Fig. 2.2A), whereas spautin-1 decreased steady-state LC3B-II protein levels (Fig. 2.2B). Furthermore, MG132 increased steady-state LC3B-II protein levels (Fig. 2.2C). Based on my observations that both chloroquine (an inhibitor of autophagy) and MG132 (an activator of autophagy) increased LC3B-II protein levels, the TGFβ1-dependent increase in LC3B lipidation was insufficient to conclude that TGFβ1 activated autophagy.
Figure 2.2: The effects of chloroquine, spautin-1, and MG132 on LC3 lipidation and viability.
(A) A549 cells were treated with 0-50 μM chloroquine for 24 hours (left panel) or with 50 μM chloroquine for 0-24 hours (middle panel). Cells were lysed and subjected to SDS-PAGE and immunoblotting using anti-GAPDH and anti-LC3B antibodies. Phosphoimaging analysis of steady-state LC3B-II levels are shown graphically below representative immunoblots. A549 cells were treated with 0-50 μM chloroquine for 24 or 48 hours and subjected to an MTT assay (right panel). The data were graphed from 4 independent experiments (mean ± SD). Significance is indicated as **P<0.01, ***=P<0.001, and ****=P<0.0001.

(B) A549 cells were treated with 0-10 μM spautin-1 for 24 hours (left panel) or with 10 μM spautin-1 for 0-24 hours (middle panel). Cells were lysed and subjected to SDS-PAGE and immunoblotting using anti-GAPDH and anti-LC3B antibodies. Phosphoimaging analysis of steady-state LC3B-II levels are shown graphically below representative immunoblots. A549 cells were treated with 0-10 μM spautin-1 for 24 or 48 hours and subjected to an MTT assay (right panel). The data were graphed from 4 independent experiments (mean ± SD). Significance is indicated as *=P<0.05.

(C) A549 cells were treated with 0-10 μM MG132 for 24 hours (left panel) or with 10 μM MG132 for 0-24 hours (middle panel). Cells were lysed and subjected to SDS-PAGE and immunoblotting using anti-GAPDH and anti-LC3B antibodies. Phosphoimaging analysis of steady-state LC3B-II levels are shown graphically below representative immunoblots. A549 cells were treated with 0-10 μM MG132 for 24 or 48 hours and subjected to an MTT assay (right panel). The data were graphed from 4 independent experiments (mean ± SD). Significance is indicated as *=P<0.05, **P<0.01, ***=P<0.001, and ****=P<0.0001.

As shown above, TGFβ1 had consistent effects on 3 of the 10 Atgs (Atg9A, ULK1, and LC3B-II) in A549 cells and H1299 cells. To study this further, I treated A549 cells and H1299 cells with 50 μM chloroquine, 10 μM spautin-1 or 10 μM MG132 in the presence and absence of 250 pM TGFβ1 for 24 hours. The cells were lysed, and immunoblotted for Atg3, Atg5, Atg7, Atg9A, Atg12, Atg12-Atg5 complex formation, BECN1, ULK1, and LC3B-II (Fig. 2.3). Once again, P-Smad2 and Smad2 levels were assessed to confirm TGFβ1 activity. In both cell lines, there were no significant changes in the protein levels of Atg3, Atg7, Atg9A, BECN1, Atg16L1 or Atg12-Atg5 complex formation; MG132
increased the steady-state of Atg12 and LC3B-II protein levels and chloroquine significantly increased steady-state ULK1 and LC3B-II protein levels (Fig. 2.3A, B). These results suggested that the TGFβ1-dependent increase of ULK1 and LC3B-II was insufficient to verify TGFβ1-induced autophagy. Also using steady-state Atg levels to assess TGFβ1-induced autophagy is unreliable because most Atg levels were relatively stable despite chloroquine, spautin-1, and MG132 treatment.
Figure 2.3: The effect of chloroquine, spautin-1, and MG132 on autophagy-related protein (Atg) levels.

A549 cells (A) and H1299 cells (B) were treated with 50 μM chloroquine, 10 μM spautin-1 or 10 μM MG132 in the presence or absence of 250 pM TGFβ1 for 24 hours. Cells were
lysed and subjected to SDS-PAGE and immunoblotting using anti-Atg3, anti-Atg5, anti-Atg7, anti-Atg9A, anti-Atg12, anti-Atg16L1, anti-BECN1, anti-ULK1, anti-LC3B, anti-P-Smad2, anti-Smad2, and anti-GAPDH (loading control) antibodies. The steady-state levels of Atg3, Atg7, Atg9A, Atg12, Atg12-Atg5, Atg16L1, BECN1, ULK1, and LC3B were quantitated using QuantityOne software and graphed (n=3, mean ± SD). Significance is indicated as *P<0.05, **P<0.01, and ****P<0.0001.

Assessing autophagy using LC3B puncta formation and LC3B-lysosome co-localization in A549 cells

Next, I utilized A549 cells stably expressing GFP-labelled LC3B protein to determine if TGFβ1 increased GFP-LC3B puncta formation or GFP-LC3B-lysosome co-localization. Although some GFP is quenched within the lysosomal lumen196, GFP-LC3B-lysosome co-localization was detected and fluctuated in response to treatment. A549 cells were treated with 50 µM chloroquine, 10 µM spautin-1 or 10 µM MG132 in the presence and absence of 250 pM TGFβ1 for 24 hours prior to LysoTracker Deep Red incubation to identify lysosomes (Fig. 2.4). After examining the images obtained from untreated or chloroquine-treated cells (Fig. 2.4A), I observed that 24 hours of TGFβ1 increased GFP-LC3B puncta formation (Fig. 2.4B) and GFP-LC3B-lysosome co-localization relative to untreated cells (Fig. 2.4C). Next, I examined the images containing cells that were treated with vehicle dimethyl sulfoxide (DMSO), spautin-1 or MG132 (Fig. 2.4D) and observed that MG132 increased GFP-LC3B puncta formation and spautin-1+TGFβ1 decreased GFP-LC3B puncta formation compared to the DMSO and TGFβ1 treatment, respectively (Fig. 2.4E). Finally, MG132 increased GFP-LC3B-lysosome co-localization relative to DMSO (control) treatment, whereas the combination of spautin-1 and TGFβ1 decreased GFP-LC3B-lysosome co-localization compared to TGFβ1 treatment alone (Fig. 2.4F). Since these results suggested that both chloroquine and MG132 increased GFP-LC3B puncta formation and GFP-LC3B-lysosome co-localization, I was unable to conclude that the TGFβ1-dependent increase of GFP-LC3B puncta formation or GFP-LC3B-lysosome co-localization represented an induction of autophagy.
Figure 2.4: The effect of pharmacological modulation of autophagy and TGFβ1 on LC3 puncta formation and autophagosome-lysosome co-localization.
(A) A549 cells transfected with a cDNA pMRX-IP-GFP-LC3B-RFP-LC3BΔG vector were treated with 50 μM chloroquine in the presence or absence of 250 pM TGFβ1. LysoTracker Deep Red (red) and Hoechst stain (blue) were added 2 hours and 10 minutes, respectively, prior to imaging. Images were obtained with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Scale bars = 10 μm.

(B) Cells and number of puncta/cell were counted using ImageJ version 2.0 software. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as *=P<0.05, **=P<0.01, and ****=P<0.0001.

(C) ImageJ version 2.0 was used to quantify the number of yellow pixels per cell area for chloroquine and no treatment. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as *=P<0.05 and ****=P<0.0001.

(D) A549 cells transfected with a cDNA pMRX-IP-GFP-LC3B-RFP-LC3BΔG vector were treated with 10 μM spautin-1, 10 μM MG132, or DMSO (vehicle control) in the presence or absence of 250 pM TGFβ1. LysoTracker Deep Red (red) and Hoechst stain (blue) were added 2 hours and 10 minutes, respectively, prior to imaging. Images were obtained with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Scale bars = 10 μm.

(E) ImageJ version 2.0 was used to count the number of cells and puncta per image for all treatments. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as *=P<0.05, **=P<0.01, ***P<0.001, and ****=P<0.0001.

(F) ImageJ version 2.0 was used to quantify the number of yellow pixels per cell area for all treatments. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as *=P<0.05, **=P<0.01, ***P<0.001, and ****=P<0.0001.

TGFβ1 increases autophagic flux

Although I investigated the impact that TGFβ1 had on ATG expression, Atg levels, GFP-LC3B puncta formation, and LC3B-lysosome co-localization, the data did not consistently support the notion that TGFβ1 induced autophagy in NSCLC cell lines. Therefore, I next assessed the ability of TGFβ1 to alter autophagic flux in A549 cells and H1299 cells, using the pMRX-IP-GFP-LC3B-RFP-LC3BΔG autophagic flux vector developed by the Mizushima laboratory. Briefly, A549 cells and H1299 cells were
generated to stably express the GFP-LC3B-RFP-LC3BΔG reporter and the resulting GFP/RFP ratio was used to monitor autophagic flux, which was assessed via both immunoblotting and fluorescence microscopy (Fig. 2.5). In A549 cells, quantitation of western blots indicated that 6-48 hours of TGFβ1 incubation significantly increased steady-state LC3B-II protein levels and 24 and 48 hours of TGFβ1 significantly decreased the GFP/RFP ratio (Fig. 2.5A). Assessing autophagic flux in A549 cells using fluorescence microscopy revealed that 6 hours of TGFβ1 did not impact the GFP/RFP ratio whereas 24 hours of TGFβ1 significantly decreased the GFP/RFP ratio (Fig. 2.5B). In H1299 cells, quantitative analysis of the western blots indicated that steady-state LC3B-II protein levels were significantly increased after 3 and 24 hours of TGFβ1 incubation. Like A549 cells, 24 and 48 hours of TGFβ1 significantly decreased the GFP/RFP ratio in H1299 cells (Fig. 2.5C). Furthermore, 24 hours of TGFβ1 significantly decreased the GFP/RFP ratio in H1299 cells when it was assessed via fluorescence microscopy (Fig. 2.5D). Therefore, assessing the GFP/RFP ratio in GFP-LC3B-RFP-LC3BΔG stably transfected cells suggested that TGFβ1 activated autophagy in both NSCLC cell lines.
Figure 2.5: Using a GFP-LC3B-RFP-LC3BΔG probe to assess TGFβ1-dependent autophagy.
(A) A549 cells stably expressing a cDNA pMRX-IP-GFP-LC3B-RFP-LC3BΔG construct were treated with 250 pM TGFβ1 for 0-48 hours, lysed, subjected to SDS-PAGE, and immunoblotted with anti-P-Smad2, anti-Smad2, anti-LC3B, anti-GAPDH, and anti-tubulin antibodies. Quantitative analysis of steady-state LC3B-II levels and the GFP/RFP ratio are shown graphically to the right of representative immunoblots. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as *P<0.05, **P<0.01, and ****P<0.0001.

(B) A549 cells stably expressing a cDNA pMRX-IP-GFP-LC3B-RFP-LC3BΔG construct were treated with 250 pM TGFβ1 for 6 or 24 hours. Hoechst stain (blue) was added 10 minutes prior to imaging. Images were obtained with a 63x objective using an Olympus IX 81 inverted fluorescence microscope and ImageJ quantified the green and red pixel intensity. The GFP/RFP ratio is shown below representative images. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as *=P<0.05. Scale bars = 10 μm.

(C) H1299 cells stably expressing a cDNA pMRX-IP-GFP-LC3B-RFP-LC3BΔG construct were treated with 250 pM TGFβ1 for 0-48 hours, lysed, subjected to SDS-PAGE, and immunoblotted with anti-P-Smad2, anti-Smad2, anti-LC3B, anti-GAPDH, and anti-tubulin antibodies. Phosphoimaging analysis of steady-state LC3B-II levels and the GFP/RFP ratio are shown graphically to the right of representative immunoblots. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as *P<0.05 and **P<0.01.

(D) H1299 cells stably expressing a cDNA pMRX-IP-GFP-LC3B-RFP-LC3BΔG construct were treated with 250 pM TGFβ1 for 6 or 24 hours. Hoechst stain (blue) was added 10 minutes prior to imaging. Images were obtained with a 63x objective using an Olympus IX 81 inverted fluorescence microscope and ImageJ quantified the green and red pixel intensity. The GFP/RFP ratio is shown below representative images. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as **P<0.01. Scale bars = 10 μm.

Verifying the GFP-LC3B-RFP-LC3BΔG probe as an appropriate tool to assess autophagic flux.
Next, I assessed the accuracy of the GFP/RFP ratio obtained from cells stably transfected with a GFP-LC3B-RFP-LC3ΔG vector at predicting autophagic flux. A549 cells and H1299 cells were treated with 50 \( \mu \)M chloroquine, 10 \( \mu \)M spautin-1 or 10 \( \mu \)M MG132 in the presence and absence of 250 pM TGF\( \beta \)1 for 24 hours. Cells were either lysed and immunoblotted for P-Smad2, Smad2, and LC3B or subjected to fluorescence microscopy. In both cell lines, the quantitation of GFP- and RFP-labelled LC3B in the western blots showed that TGF\( \beta \)1 significantly decreased the GFP/RFP ratio but increased steady-state LC3B-II protein levels (Fig. 2.6 & 2.7). Quantitation of the western blots containing chloroquine treatments indicated that chloroquine significantly increased steady-state LC3B-II protein levels and the GFP/RFP ratio compared to untreated A549 cells (Fig. 2.6A) and H1299 cells (Fig. 2.7A). Additionally, the combination of chloroquine and TGF\( \beta \)1 significantly increased the GFP/RFP ratio with respect to the TGF\( \beta \)1 treatment in both cell lines (Fig. 2.6A & 2.7A). Consistent with the western blotting results, quantifying the GFP/RFP ratio via fluorescence microscopy indicated that compared to untreated cells, TGF\( \beta \)1 decreased the GFP/RFP ratio and chloroquine increased the GFP/RFP ratio in A549 cells and H1299 cells. Furthermore, the combination of chloroquine and TGF\( \beta \)1 had a significantly greater GFP/RFP ratio compared to the TGF\( \beta \)1 treatment in both cell lines (Fig. 2.6B & 2.7B). Having observed consistent results using chloroquine, an inhibitor of late-stage autophagy events, I next assessed spautin-1, which inhibits earlier autophagic processes.

Quantitation of the western blots containing spautin-1 treated cells suggested that the combination of spautin-1 and TGF\( \beta \)1 significantly decreased steady-state LC3B-II protein levels and increased the GFP/RFP ratio with respect to the TGF\( \beta \)1 treatment in both cell lines (Fig. 2.6C & 2.7C). Quantifying the GFP/RFP ratio via fluorescence microscopy indicated that TGF\( \beta \)1 decreased the GFP/RFP ratio, whereas spautin-1 increased the GFP/RFP ratio. Finally, the combination of spautin-1 and TGF\( \beta \)1 had a significantly greater GFP/RFP ratio compared to the TGF\( \beta \)1 treatment (Fig. 2.6D & 2.7D). These results were consistent in showing that the inhibition of early or late autophagic events produced similar GFP/RFP ratios. Finally, quantitation of the western blots containing MG132 treatments revealed that MG132 significantly decreased the GFP/RFP ratio, whereas it
increased LC3B-II protein levels with respect to the DMSO control in both cell lines (Fig. 2.6E & 2.7E). Additionally, compared to TGFβ1 treatment, the combination of MG132 and TGFβ1 significantly decreased the GFP/RFP ratio in H1299 cells, but increased steady-state LC3B-II protein levels in both cell lines (Fig. 2.6E & 2.7E). Quantifying the GFP/RFP ratio via fluorescence microscopy indicated that TGFβ1 and MG132 decreased the GFP/RFP ratio in both cell lines (Fig. 2.6F & 2.7F). Taken together, this data suggested that chloroquine and spautin-1 decreased autophagic flux, whereas MG132 and TGFβ1 increased autophagic flux in the NSCLC cell lines.
Figure 2.6: The effect of chloroquine, spautin-1, and MG132 on autophagic flux in A549 cells.
(A) A549 cells stably expressing a cDNA pMRX-IP-GFP-LC3B-RFP-LC3BΔG construct were treated with 50 μM chloroquine in the presence and absence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted with anti-P-Smad2, anti-Smad2, anti-LC3B, and anti-tubulin antibodies. Quantitative analysis of steady-state LC3B-II levels and the GFP/RFP ratio are shown graphically to the right of representative immunoblots. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as *=P<0.05, **=P<0.01, and ****=P<0.0001.

(B) A549 cells stably expressing a cDNA pMRX-IP-GFP-LC3B-RFP-LC3BΔG construct were treated with 50 μM chloroquine in the presence and absence of 250 pM TGFβ1 for 24 hours. Hoechst stain (blue) was added 10 minutes prior to imaging. Images were obtained with a 63x objective using an Olympus IX 81 inverted fluorescence microscope and ImageJ quantified the green and red pixel intensity. The GFP/RFP ratio is shown graphically to the right of representative images. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as **P<0.01, ***=P<0.001, and ****=P<0.0001. Scale bars = 10 μm.

(C) A549 cells stably expressing a cDNA pMRX-IP-GFP-LC3B-RFP-LC3BΔG construct were treated with 10 μM spautin-1 in the presence and absence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted with anti-P-Smad2, anti-Smad2, anti-LC3B, and anti-tubulin antibodies. Quantitative analysis of steady-state LC3B-II levels and the GFP/RFP ratio are shown graphically to the right of representative immunoblots. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as *=P<0.05, **=P<0.01, and ***=P<0.001.

(D) A549 cells stably expressing a cDNA pMRX-IP-GFP-LC3B-RFP-LC3BΔG construct were treated with 10 μM spautin-1 in the presence and absence of 250 pM TGFβ1 for 24 hours. Hoechst stain (blue) was added 10 minutes prior to imaging. Images were obtained with a 63x objective using an Olympus IX 81 inverted fluorescence microscope and ImageJ quantified the green and red pixel intensity. The GFP/RFP ratio is shown graphically to the right of representative images. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as *=P<0.05, **P<0.01, and ****=P<0.0001. Scale bars = 10 μm.
(E) A549 cells stably expressing a cDNA pMRX-IP-GFP-LC3B-RFP-LC3BΔG construct were treated with 10 μM MG132 in the presence and absence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted with anti-P-Smad2, anti-Smad2, anti-LC3B, anti-GAPDH, and anti-tubulin antibodies. Quantitative analysis of steady-state LC3B-II levels and the GFP/RFP ratio are shown graphically to the right of representative immunoblots. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as *=P<0.05, **=P<0.01, and ***P<0.001.

(F) A549 cells stably expressing a cDNA pMRX-IP-GFP-LC3B-RFP-LC3BΔG construct were treated with 10 μM MG132 in the presence and absence of 250 pM TGFβ1 for 24 hours. Hoechst stain (blue) was added 10 minutes prior to imaging. Images were obtained with a 63x objective using an Olympus IX 81 inverted fluorescence microscope and ImageJ quantified the green and red pixel intensity. The GFP/RFP ratio is shown graphically to the right of representative images. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as ****=P<0.0001. Scale bars = 10 μm.
Figure 2.7: The effect of chloroquine, spautin-1, and MG132 on autophagic flux in H1299 cells.
(A) H1299 cells stably expressing a cDNA pMRX-IP-GFP-LC3B-RFP-LC3BΔG construct were treated with 50 μM chloroquine in the presence and absence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE and immunoblotted with anti-P-Smad2, anti-Smad2, anti-LC3B, anti-GAPDH and anti-tubulin antibodies. Phosphoimaging analysis of steady-state LC3B-II levels and the GFP/RFP ratio are shown graphically to the right of representative immunoblots. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as *=P<0.05 and ***P<0.001.

(B) H1299 cells stably expressing a cDNA pMRX-IP-GFP-LC3B-RFP-LC3BΔG construct were treated with 50 μM chloroquine in the presence and absence of 250 pM TGFβ1 for 24 hours. Hoechst stain (blue) was added 10 minutes prior to imaging. Images were obtained with a 63x objective using an Olympus IX 81 inverted fluorescence microscope and ImageJ quantified the green and red pixel intensity. The GFP/RFP ratio is shown graphically to the right of representative images. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as **P<0.01, ***P<0.001, and ****P<0.0001. Scale bars = 10 μm.

(C) H1299 cells stably expressing a cDNA pMRX-IP-GFP-LC3B-RFP-LC3BΔG construct were treated with 10 μM spautin-1 in the presence and absence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE and immunoblotted with anti-P-Smad2, anti-Smad2, anti-LC3B, anti-GAPDH and anti-tubulin antibodies. Phosphoimaging analysis of steady-state LC3B-II levels and the GFP/RFP ratio are shown graphically to the right of representative immunoblots. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as *=P<0.05, **=P<0.01, and ****=P<0.0001.

(D) H1299 cells stably expressing a cDNA pMRX-IP-GFP-LC3B-RFP-LC3BΔG construct were treated with 10 μM spautin-1 in the presence and absence of 250 pM TGFβ1 for 24 hours. Hoechst stain (blue) was added 10 minutes prior to imaging. Images were obtained with a 63x objective using an Olympus IX 81 inverted fluorescence microscope and ImageJ quantified the green and red pixel intensity. The GFP/RFP ratio is shown graphically to the right of representative images. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as *=P<0.05 and **P<0.01, ***P<0.001, and ****P<0.0001. Scale bars = 10 μm.
(E) H1299 cells stably expressing a cDNA pMRX-IP-GFP-LC3B-RFP-LC3BΔG construct were treated with 5 μM MG132 in the presence and absence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE and immunoblotted with anti-P-Smad2, anti-Smad2, anti-LC3B, anti-GAPDH and anti-tubulin antibodies. Phosphoimaging analysis of steady-state LC3B-II levels and the GFP/RFP ratio are shown graphically to the right of representative immunoblots. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as *=P<0.05 and **P<0.01.

(F) H1299 cells stably expressing a cDNA pMRX-IP-GFP-LC3B-RFP-LC3BΔG construct were treated with 5 μM MG132 in the presence and absence of 250 pM TGFβ1 for 24 hours. Hoechst stain (blue) was added 10 minutes prior to imaging. Images were obtained with a 63x objective using an Olympus IX 81 inverted fluorescence microscope and ImageJ quantified the green and red pixel intensity. The GFP/RFP ratio is shown graphically to the right of representative images. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as **P<0.01 and ***P<0.001. Scale bars = 10 μm.

Since this autophagic flux probe suggested that chloroquine and spautin-1 are inhibitors of autophagy, whereas TGFβ1 and MG132 were observed to activate autophagy, I next confirmed these results by assessing this autophagic flux model using starvation-induced autophagy. A549 cells or H1299 cells stably expressing GFP-LC3B-RFP-LC3BΔG were serum starved for 0-24 hours prior to being lysed and immunoblotted for LC3B and tubulin or subjected to fluorescence microscopy. In A549 cells, 24 hours of serum starvation significantly increased and decreased LC3B-II/LC3B-I and GFP/RFP ratios, respectively (Fig. 2.8A). Quantifying the GFP/RFP fluorescence microscopy autophagic flux ratio revealed that 24 hours of serum starvation significantly increased autophagy with respect to the control (Fig. 2.8B). In H1299 cells, 4 and 6 hours of serum starvation significantly increased the LC3B-II/LC3B-I ratio whereas 4, 6, and 24 hours of serum starvation significantly decreased the GFP/RFP ratio (Fig. 2.8C). Quantifying the GFP/RFP ratio via fluorescence microscopy revealed that 6 and 24 hours of serum starvation significantly decreased the GFP/RFP ratio with respect to the control (Fig. 2.8D). Therefore, this data suggested that serum starvation increased autophagic flux in my NSCLC cell lines.
Figure 2.8: Assessing the effect of serum starvation on autophagic flux.
(A) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were serum starved for 0, 4, 6 or 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted with anti-LC3B and anti-tubulin antibodies. Quantitative analysis of LC3B-II/LC3B-I and GFP/RFP ratios are shown graphically to the right of representative immunoblots. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as **=P<0.01 and ****P<0.0001.

(B) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were serum starved for 0, 6 or 24 hours. Hoechst stain (blue) was added 10 minutes prior to imaging. Images were obtained with a 63x objective using an Olympus IX 81 inverted fluorescence microscope and ImageJ was used to quantify the green and red pixel intensity. The GFP/RFP ratio is shown graphically to the right of representative images. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as ***=P<0.001. Scale bars = 10 μm.

(C) H1299 cells stably expressing GFP-LC3B-RFP-LC3BΔG were serum starved for 0, 4, 6 or 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted with anti-LC3B and anti-tubulin antibodies. Phosphoimaging analysis of LC3B-II/LC3B-I and GFP/RFP ratios are shown graphically to the right of representative immunoblots. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as *=P<0.05 and **=P<0.01.

(D) H1299 cells stably expressing GFP-LC3B-RFP-LC3BΔG were serum starved for 0, 6 or 24 hours. Hoechst stain (blue) was added 10 minutes prior to imaging. Images were obtained with a 63x objective using an Olympus IX 81 inverted fluorescence microscope and ImageJ quantified the green and red pixel intensity. The GFP/RFP ratio is shown graphically to the right of representative images. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as **=P<0.01 and ***=P<0.001. Scale bars = 10 μm.

**Utilizing GFP-LC3B-RFP-LC3BΔG transfected cells to assess if Atg5 and Atg7 are essential to TGFβ1-dependent autophagy**

Finally, I tested the application of GFP-LC3B-RFP-LC3BΔG transfected NSCLC cells to characterize the influence of silencing ATG5 and ATG7 (ATG5/7) expression on
TGFβ1-induced autophagy. A549 cells and H1299 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with ATG5 and ATG7 siRNA (si-ATG5/7) or control siRNA (si-Control) for 24 hours. The cells were then treated with 250 pM TGFβ1 for 24 hours and immunoblotted for Atg7, Atg5, P-Smad2, Smad2, LC3B, and tubulin. I observed that silencing ATG5 and ATG7 decreased TGFβ1-dependent Smad2 phosphorylation and the LC3B-II/LC3B-I ratio, while increasing the GFP/RFP ratio in A549 cells (Fig. 2.9A, B) and H1299 cells (Fig. 2.9C, D). Furthermore, fluorescence microscopy indicated that the combination of si-ATG5/7 and TGFβ1 significantly increased the GFP/RFP autophagic ratio compared to TGFβ1-treated control cells, indicating that ATG5/7 silencing decreased TGFβ1-dependent autophagy (Fig. 2.9E, F). Taken together, these data show that Atg5 and Atg7 are essential for TGFβ1-dependent autophagy in both A549 and H1299 NSCLC cells.
The effect of ATG5 and ATG7 silencing on TGFβ1-dependent autophagy.
(A) A549 cells stably expressing GFP-LC3B-RFP-LC3ΔG were transfected with 10 nM control siRNA (si-Control) or 5 nM siRNA targeting ATG5 and 5 nM siRNA targeting ATG7 (si-ATG5/7) were incubated for 24 hours in the presence or absence of 250 pM TGFβ1. The cells were lysed, subjected to SDS-PAGE, and immunoblotted with anti-Atg7, anti-Atg5, anti-P-Smad2, anti-Smad2, anti-LC3B, and anti-tubulin antibodies. Quantitative analysis of LC3B-II/LC3B-I and GFP/RFP ratios are shown graphically to the right of representative immunoblots. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as *=P<0.05, **=P<0.01, and ***P<0.001.

(B) A549 cells were transfected with 10 nM si-Control or 10 nM si-ATG5/7 and incubated with 250 pM TGFβ1 for 0, 24 or 48h. Cells were then lysed and immunoblotted with anti-Atg7, anti-Atg5, anti-P-Smad2, anti-Smad2, and anti-GAPDH antibodies.

(C) H1299 cells stably expressing GFP-LC3B-RFP-LC3ΔG were transfected with 10 nM si-Control or 10 nM si-ATG5/7 and incubated for 24 hours in the presence or absence of 250 pM TGFβ1. The cells were lysed, subjected to SDS-PAGE, and immunoblotted with anti-Atg7, anti-Atg5, anti-P-Smad2, anti-Smad2, anti-LC3B, and anti-tubulin antibodies. Phosphoimaging analysis of LC3B-II/LC3B-I and GFP/RFP ratios are shown graphically to the right of representative immunoblots. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as *=P<0.05, **=P<0.01, and ****P<0.0001.

(D) H1299 cells were transfected with 10 nM si-Control or 10 nM si-ATG5/7 and incubated with 250 pM TGFβ1 for 0, 24 or 48h. Cells were then lysed and immunoblotted with anti-Atg7, anti-Atg5, anti-P-Smad2, anti-Smad2, and anti-GAPDH antibodies.

(E) A549 cells stably expressing GFP-LC3B-RFP-LC3ΔG were transfected with 10 nM si-Control or 10 nM si-ATG5/7 and incubated for 24 hours in the presence or absence of 250 pM TGFβ1. Hoechst stain (blue) was added 10 minutes prior to imaging. Images were obtained with a 63x objective using an Olympus IX 81 inverted fluorescence microscope and ImageJ quantified the green and red pixel intensity. The GFP/RFP ratio is shown graphically to the right of representative images. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as ***P<0.001 and ****P<0.0001. Scale bars = 10 μm.
(F) H1299 cells stably expressing GFP-LC3B-RFP-LC3ΔG were transfected with 10 nM si-Control or 10 nM si-ATG5/7 and incubated for 24 hours in the presence or absence of 250 pM TGFβ1. Hoechst stain (blue) was added 10 minutes prior to imaging. Images were obtained with a 63x objective using an Olympus IX 81 inverted fluorescence microscope and ImageJ quantified the green and red pixel intensity. The GFP/RFP ratio is shown graphically to the right of representative images. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as **=P<0.01. Scale bars = 10 μm.

2.4 Discussion

Here, I examined the utility of monitoring autophagy via ATG expression, Atg levels, LC3B lipidation, LC3B puncta formation, and autophagosome-lysosome co-localization. Using NSCLC cells, I found that TGFβ1 had limited effects on ATG expression and altered the protein levels of a subset of Atgs. However, it increased LC3B lipidation, LC3B puncta formation, and autophagosome-lysosome co-localization. Another experimental technique that I considered for this study was transmission electron microscopy (TEM). Although TEM is considered the gold standard to identify double-membrane vesicles observed during cellular autophagy, I excluded it from this work because 1) TGFβ1 had already been suggested to increase the number of autophagosomes in NSCLC cells, and 2) the use of this technique is limited for cell population based quantitation.

I utilized autophagy inhibitors (spautin-1 and chloroquine) and an autophagy activator (MG132) to expose the limitations of the previously mentioned techniques. However, to understand this, one must first recognize how these pharmacological agents modulate autophagy. For instance, spautin-1 antagonizes the activity of ubiquitin specific peptidase (USP)10 and USP13 that are responsible for removing ubiquitin from the BECN1 complex. As such, spautin-1 increases the proteasome-mediated degradation of the BECN1 complex, which ultimately decreases autophagosome formation and inhibits relatively early events during autophagy. Chloroquine is an acidotropic agent that accumulates in lysosomes and increases lysosomal pH to prevent lysosomal-mediated
degradation. Therefore, autophagosomes accumulate because lysosomal-mediated degradation is reduced, and chloroquine is considered an inhibitor of later events in autophagy\textsuperscript{228}. Finally, MG132 antagonizes the catalytic subunits within the 20S core particle of the 26S proteasome, which increases the protein load on the cell. Since proteasome and lysosome-mediated degradation are compensatory, as proteasomal activity decreases, autophagic activity increases to alleviate the excess protein load\textsuperscript{268,312}.

Although many publications have used similar experimental techniques to justify that particular experimental conditions induced autophagy, repeating my experiments with known inhibitors and activators of autophagy suggested that this may not be the case. For instance, chloroquine and MG132 had little affect on Atg levels and increased LC3B lipidation, GFP-LC3B puncta formation, and autophagosome-lysosome co-localization. The explanation for this is that many of these techniques rely on autophagosome structures to make interpretations of autophagy\textsuperscript{189,250}. However, all of these techniques do not monitor degradation, which is the final, and most important, stage of autophagy\textsuperscript{314,315}. Since chloroquine prevents autophagosome degradation, autophagosomes accumulate and may be incorrectly interpreted by several techniques as increased autophagy. Other autophagy inhibitors that do not impede lysosomal degradation, such as spautin-1, are not at risk of this limitation. Furthermore, if TEM experiments were performed, based on my results and what is reported in the literature\textsuperscript{191,236,316}, I would expect chloroquine, MG132, serum starvation, and TGF\(\beta\)1 to increase the number of autophagosomes whereas spautin-1 would decrease the amount of autophagosomes. Ultimately, since a known inhibitor and activator of autophagy resulted in similar outcomes, it would be inaccurate to conclude that TGF\(\beta\)1 activated autophagy based on these parameters alone.

I next reviewed the literature for an experimental technique to measure autophagy that was not confounded by previously mentioned limitations. I found that assessing autophagic flux measures autophagic degradation, and as such may distinguish between autophagy activators and lysosomal inhibitors\textsuperscript{317}. Currently, there are several autophagic flux probes available for experimentation; however, I selected to stably express the GFP-LC3B-RFP-LC3B\(\Delta\)G construct in NSCLC cells. Using these cell lines, I determined that TGF\(\beta\)1 elicited a time-dependent decrease of the GFP/RFP ratio, that suggests increased autophagy. Indeed, since GFP-LC3B undergoes autophagic degradation whereas the RFP-
LC3BΔG does not, a decrease in the GFP/RFP ratio suggested that autophagic flux increased. I next validated my model using chloroquine, spautin-1, MG132, and serum starvation. As expected, the autophagy inhibitors increased the GFP/RFP ratio whereas autophagy activators decreased the GFP/RFP ratio. Based on these data, I concluded that TGFβ1 activated autophagy in NSCLC cells.

After I verified that TGFβ1 activated autophagy, I provided an example as to how one may use GFP-LC3B-RFP-LC3BΔG transfected cells to characterize the mechanism of TGFβ1-induced autophagy. I have shown that TGFβ1-induced autophagy is dependent on the presence of Atg5 and Atg7. Although this may seem straightforward because Atg5 and Atg7 are key components for phagophore elongation, Atg5/7-independent autophagy is possible. As such, the application of this model may identify specific targets of the TGFβ pathway that are directly responsible for autophagy activation so that one may specifically hinder them in cancer to limit off-target and non-specific effects.
Chapter 3

Canonical and non-canonical TGFβ signalling activate autophagy in a ULK1-dependent manner

Components of this chapter have been published in Front Cell Dev Biol. (2021) 9.
3 Summary

The mechanism(s) in which transforming growth factor-β (TGFβ) modulates autophagy in cancer remain unclear. Here, I characterized the TGFβ signalling pathways that induce autophagy in non-small cell lung cancer (NSCLC) cells that stably express GFP-LC3B-RFP-LC3BΔG constructs to measure autophagic flux. I demonstrated that TGFβ1 increases uncoordinated 51-like autophagy activating kinase 1 (ULK1) protein levels, 5’ adenosine monophosphate-activated protein kinase (AMPK)-dependent ULK1 phosphorylation at serine (S) 555, and ULK1 complex formation but decreases mechanistic target of rapamycin (mTOR) activity on ULK1. Further analysis revealed that the TGFβ-activated kinase 1/tumour necrosis factor receptor-associated factor 6/p38 mitogen-activated protein kinase (TAK1-TRAF6-p38 MAPK) pathway and Smad4 were important for TGFβ1-induced autophagy. The TAK1-TRAF6-p38 MAPK pathway was essential for downregulating mTOR S2448 phosphorylation and increasing ULK1 S555 phosphorylation and autophagosome formation. SiRNA-mediated Smad4 silencing did not alter mTOR-dependent ULK1 S757 phosphorylation, but it reduced AMPK-dependent ULK1 S555 phosphorylation and autophagosome formation. Additionally, both Smad4 silencing and inhibiting the TAK1-TRAF6-p38 MAPK pathway decreased autophagosome-lysosome co-localization in the presence of TGFβ. Therefore, my results suggest that the Smad4 and TAK1-TRAF6-p38 MAPK signalling pathways are essential for TGFβ1-induced autophagy and provide specific targets for the inhibition of TGFβ signalling in tumour cells that utilize autophagy in their EMT program.

3.1 Introduction

Virtually all cells increase autophagic flux to eliminate the influx of damaged cellular materials mediated by cell stress to survive^{319}. However, cells have mechanisms to dampen autophagic flux because excessive degradation may initiate cell death^{320}. For example, cells modulate autophagic flux through post-translational modifications of autophagy-related protein 1 (Atg1)^{321}. The phosphorylation status and activation of Atg1—ULK1 in mammals—is determined by a balance between mTOR and AMPK activity^{204,322}. When the rate of autophagy is detrimental to cells, mTOR phosphorylates ULK1 at S757
to disrupt ULK1-AMPK interactions$^{323}$. Alternatively, cell stressors impede mTOR and activate AMPK to directly phosphorylate ULK1 at S317, S555, and S778$^{324}$. AMPK-dependent phosphorylation of ULK1 results in the formation of the ULK1 complex$^{325}$.

Autophagic degradation requires multiple Atgs downstream of the ULK1 complex to generate double membrane vesicles known as autophagosomes that engulf cellular materials prior to fusing with lysosomes or late endosomes$^{184}$. Briefly, the ULK1 complex initiates autophagy by phosphorylating beclin 1 at S30 to assemble a phosphoinositide-3 kinase (PI3K) complex$^{207,326}$, which inserts phosphatidylinositol lipids into membranes to recruit Atgs responsible for autophagosome formation$^{327}$. Autophagosome growth is facilitated via Atg12-Atg5-Atg16L1 complexes incorporating lipids and Atg8—microtubule-associated protein light chain 3 (LC3) in mammals—into autophagosome membranes$^{328}$. Prior to membrane incorporation, LC3 is post-translationally modified into LC3-I and LC3-II, which involves an Atg4-dependent cleavage to expose a C-terminal glycine residue (LC3-I) that is conjugated to phosphatidylethanolamine (LC3-II) by Atg7 and Atg3$^{331}$. As autophagosomes develop, autophagy cargo receptors tether cellular materials destined for degradation to LC3-II$^{213}$. Once autophagosomes fully form, they migrate via microtubules and dynein toward lysosomes in perinuclear regions of cells$^{234}$. Autophagosomes fuse with lysosomes to generate autophagolysosomes$^{329}$ that contain lysosomal enzymes responsible for degrading autophagosomes and their cellular cargo$^{244}$.

Although autophagy is important for cellular homeostasis and survival, the protective functions of autophagy act as a double-edged sword in tumourigenesis$^{275,305}$. For example, autophagy has been linked to drug resistance$^{330}$, epithelial-mesenchymal transition (EMT)$^{331}$, cell migration$^{332}$, metastasis$^{333}$, anoikis resistance$^{334}$, and aggressive tumour phenotypes$^{261}$. As such, there is a need to understand the signalling pathways that may activate autophagy to promote tumourigenesis.

In the past decade, several reports have suggested that TGFβ activates autophagy$^{248,257,259,260,307,331}$. My previous chapter verified that TGFβ increased autophagic flux using cells stably expressing GFP-LC3B-RFP-LC3BΔG$^{260}$. After Atg4 cleaves GFP-LC3B-RFP-LC3BΔG to generate GFP-LC3B and RFP-LC3BΔG, RFP-LC3BΔG cannot be conjugated to a phosphatidylethanolamine nor be incorporated into the autophagosome membrane. Therefore, during autophagy, the GFP-LC3B is degraded whereas the RFP-
LC3BΔG is resistant to autophagic degradation\textsuperscript{309}. However, the specific TGFβ signalling pathway(s) responsible for autophagy remain(s) unclear.

Here, using NSCLC cell lines expressing GFP-LC3B-RFP-LC3BΔG, I evaluated the role of specific components of canonical and non-canonical TGFβ1 signalling pathways and assessed the impact on autophagic flux. The purpose of this work was to identify specific branches of the TGFβ signalling pathway responsible for activating autophagy in NSCLC cell lines to highlight molecular targets for cancer therapy. Targeting specific branches rather than the entire pathway is advantageous because TGFβ1 is essential to cell survival and plays an anti-tumourigenic role in most cells.

### 3.2 Materials and Methods

**Antibodies and reagents**

Primary antibodies were purchased from the following vendors: anti-GAPDH (Cell Signalling Technology, 2118S), anti-phospho-S465/467-Smad2 (P-Smad2; Cell Signalling Technology, 3108L), anti-Smad2/3 (BD Transduction laboratories, 562586), anti-LC3B (Cell Signalling Technology, 9236S), anti-ULK1 (Cell Signalling Technology, 8054S), anti-phospho-S555-ULK1 (Cell Signalling Technology, 5869S), anti-phospho-S757-ULK1 (Cell Signalling Technology, 6888S), anti-ULK2 (Santa Cruz, sc-293453), anti-Smad anchor for receptor activation (SARA; Cell Signalling Technology, 13285S), anti-Smad4 (Cell Signalling Technology, 38454S), anti-mTOR (Cell Signalling Technology, 2972S), anti-phospho-S2448-mTOR (P-mTOR; Cell Signalling Technology, 2971S), anti-5′ adenosine monophosphate-activated protein kinase-α (AMPKα; Cell Signalling Technology, 2532S), anti-phospho-T172-AMPKα (P-AMPK; Cell Signalling Technology, 50081S), anti-atypical protein kinase C-ζ (aPKCζ; Santa Cruz, sc-17781), anti-atypical protein kinase C-τ (aPKCτ; Santa Cruz, sc-17837), anti-TAK1 (Cell Signalling Technology, 5206S), anti-TRAF6 (Cell Signalling Technology, 8028S), anti-cleaved PARP (Cell Signalling Technology, 5625S), and anti-TGFβ receptor type III (TGFβRIII; Santa Cruz, sc-74511). Secondary antibodies used for western blot analysis were as follows: Horseradish peroxidase (HRP)-conjugated goat anti-rabbit-IgG (Thermo Fisher Scientific, 31460) and goat anti-mouse-IgG (Thermo Fisher Scientific, 31430). Fluorescently-
conjugated donkey anti-rabbit-IgG (Life Technologies, A31572) was used for immunofluorescence studies. Two different Human Ambion siRNA constructs were purchased from Thermo Fisher Scientific for each knockdown experiment. The siRNA targets included si-SARA, si-Smad4, si-TGFβRIII, si-TAK1, si-TRAFl6, si-PKCζ, si-PKCθ, si-ULK1, and si-ULK2 with the catalog number 4392420 and si-Control (4457289). For fluorescence microscopy, Hoechst stain (Invitrogen, H3569) labelled nuclei prior to live cell imaging. The pharmacological agents that modulated TGFβ signalling were SB431542 (Selleckchem, S1067), LY294002 (Sigma Aldrich, L9908-1MG), p38 MAPK Inhibitor (Calbiochem, 506126), Compound C (Calbiochem, 171260), and ULK-101 (Selleckchem, S8793).

**Cell culture and transfections**

A549 cells and H1299 cells are NSCLC cell lines that were cultured in a humidified tissue incubator at 37°C under 5% CO₂. A549 cells and H1299 cells were incubated with Kaighn’s Modification of Hams F-12 (F-12K; Corning, 10-025-CV) and Roswell Park Memorial Institute (RPMI; Corning, 10-043-CVR) media, respectively. Cells were passaged using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA; Sigma, T2605), centrifuged at 1000x g for 2 minutes and resuspended in fresh media supplemented with 10% fetal bovine serum (FBS). Cells were treated with 250 pM TGFβ1, 10 µM ULK-101, 10 µM Compound C, 20 µM SB431542, 40 µM LY294002, and 10 µM p38 MAPK inhibitor in media supplemented with 10% FBS. Transient siRNA knockdowns were performed using Lipofectamine RNAiMAX (Thermo Fisher Scientific, 13778150) and optimem media (Thermo Fisher Scientific, 22600134) as per manufacture’s protocol. Stable GFP-LC3B-RFP-LC3BΔG expressing cells were generated using PolyJet transfection reagent (Froggabio, Toronto, ON) and a cDNA pMRX-IP-GFP-LC3B-RFP-LC3BΔG vector (Addgene, 84573). Transfected cells were isolated using growth media supplemented with 10% FBS and 1 µg/mL puromycin (Thermo Fisher Scientific, A1113802).

**Immunoblotting**

Please see Chapter 2.
**Confocal Microscopy**

A549 cells cultured on glass coverslips were treated with 0 or 250 pM TGFβ for 24 hours. Following treatment, the cells were washed with 1x phosphate buffered saline (PBS), fixed using 4% paraformaldehyde for 10 minutes, permeabilized after 5 minutes of 0.1% Triton X-100, and blocked with 10% FBS diluted in 1x PBS for 1-hour. Antibodies against ULK1 were diluted in a 10% FBS 1x PBS solution to a final concentration of 1:100. The cells were left in 4°C rocking with the antibody solution overnight. The following day, the cells were washed with 1x PBS, and an anti-rabbit secondary antibody diluted in a 10% FBS 1x PBS solution to a final concentration of 1:250 was added. The cells and secondary antibody were left rocking in the dark at room temperature for 1-hour. The cells were washed with 1x PBS and were subject to a 1:1000 dilution of DAPI dissolved in a 1x PBS solution for 10 minutes. Coverslips were then mounted onto microscope slides using Immu-mount (Thermo Fisher Scientific, 9990402) and were left in the dark overnight. A 63x objective of a Nikon Eclipse Ti2 (Nikon Instruments) confocal microscope imaged the coverslips and ImageJ (version 2.0) quantified relative nuclear ULK1 intensity/Total ULK1 intensity. This experiment was repeated in A549 cells treated with siRNA against Smad4 and A549 cells treated with siRNA against TAK1 and TRAF6 in combination with a p38 MAPK inhibitor. Each data point represents quantitation from ≥ 100 cells from each condition.

**Autophagic flux assay**

A549 cells and H1299 cells were transfected with a cDNA pMRX-IP-GFP-LC3B-RFP-LC3ΔAΔG vector developed by the Mizushima laboratory as described in Chapter 2.

**Assessing autophagosome and lysosome co-localization**

A549 cells stably expressing GFP-LC3B were treated with siRNA against Smad4 or siRNA targeting TRAF6 and TAK1 in combination with a p38 MAPK inhibitor for 24 hours. Each experiment was conducted in the presence and absence of TGFβ1 for 24 hours. LysoTracker Deep Red labelled lysosomes and Hoechst stain labelled the nucleus 2 hours and 10 minutes prior to imaging, respectively. Imaging and quantitation was performed as previously described.
Quantifying LC3B Puncta using ImageJ

A549 cells expressing GFP-LC3B that were subjected to live imaging using a 63x objective of an Olympus IX 81 inverted fluorescence microscope were utilized to determine the relative LC3B puncta per cell. ImageJ version 2.0 quantified the number of puncta/cell utilizing puncta size, pixel count, and circularity plug-ins.

Statistical Analysis

Statistical significance was evaluated using a Student’s t-test, one-way ANOVA followed by Dunnett’s multiple comparisons test, or a two-way/three-way ANOVA followed by either Tukey’s or Sidak’s multiple comparison tests. Statistical analyses were performed using GraphPad Prism (Software 8.0) and P-values <0.05 were statistically significant.

3.3 Results

TGFβ1 activates autophagy by regulating the mTOR-ULK1 pathway

In the previous chapter, I reported that TGFβ1 induced ULK1 protein levels and stimulated autophagy in NSCLC cells260; however, the mechanism of how this was achieved remained unknown. To this end, I first investigated if TGFβ1 alters AMPK and mTOR activity by following site-specific ULK1 phosphorylation. I measured ULK1 S555 phosphorylation to assess AMPK-dependent activity, ULK1 S757 to measure mTOR-dependent phosphorylation of ULK1, and mTOR S2448 phosphorylation to assess active mTOR335. A549 and H1299 NSCLC cells were treated with TGFβ1 for 24 hours prior to lysis and immunoblotting. I observed that in response to TGFβ1, ULK1 phosphorylation of S555 tripled in A549 cells (Fig. 3.1A) and doubled in H1299 cells (Fig. 3.1B). Although there was at least a twofold increase in ULK1 protein levels in both cell lines, the ratio of phospho-S555-ULK1/ULK1 rose significantly. Furthermore, I observed that TGFβ1 had little influence on mTOR protein levels but produced a slight, yet significant, decrease in P-mTOR in both A549 cells and H1299 cells (Fig. 3.1A, B). Since a low P-mTOR/mTOR ratio increases the amount of ULK1 available for AMPK-dependent S555 phosphorylation and a high phospho-S555-ULK1/ULK1 ratio indicates an increase of active ULK1, I
postulated that TGFβ1 increases the amount of post-translationally modified ULK1 to initiate autophagy.

One hallmark of autophagy is the cellular redistribution of autophagy initiating ULK complexes to omegasomes\textsuperscript{336}. Therefore, to investigate if TGFβ1 alters the subcellular localization of ULK1 and ULK2, I carried out immunofluorescence microscopy. I observed that TGFβ1 treatment induces a co-localization of both ULK1 and ULK2 in cytoplasmic puncta (Fig. 3.1C). Interestingly, in response to TGFβ1, I also observed a small, but reproducible decrease in the nuclear signal for both ULK1 and ULK2. To confirm this observation, I carried out confocal microscopy and observed an approximate 20% decrease in nuclear ULK1 and ULK2 in response to TGFβ1 (Fig. 3.2).
Figure 3.1: The effect of TGFβ1 on mTOR and ULK1 activity in NSCLC cells.

A549 cells (A) or H1299 cells (B) were treated with 250 pM TGFβ1 for 24 hours. Cells were lysed, subjected to SDS-PAGE, and immunoblotting for anti-mTOR, anti-phospho-
S2448-mTOR, anti-ULK1, anti-phospho-S555-ULK, anti-phospho-S757-ULK1, and anti-GAPDH (loading control) antibodies. The steady-state levels of phospho-S555-ULK1, ULK1, phospho-S2448-mTOR, and mTOR were quantified using QuantityOne software and the phospho-mTOR/mTOR and phospho-ULK1/ULK1 ratios were graphed (n=3 ± SD) to the right of representative immunoblots. Significance is indicated as *=P<0.05.

(C) A549 cells were treated with 250 pM TGFβ1 for 24 hours. Cells were fixed and stained with DAPI (blue) and antibodies against ULK1 (green) and ULK2 (red). A 63x objective of an Olympus IX 81 inverted fluorescence microscope imaged the cells. Bar = 10 µm.

Figure 3.2: The effect of TGFβ1 on the nuclear intensity of ULK1 and ULK2.

A549 cells were treated with 250 pM TGFβ1 for 24 hours. The cells were fixed and stained with DAPI (blue), anti-ULK1 (green), and anti-ULK2 (red). A 63x objective of a Nikon Eclipse Ti2 confocal microscope imaged the coverslips and ImageJ (version 2.0) quantified relative nuclear ULK intensity/Total ULK intensity, which are graphed below representative images (n=3 ± SD). Significance is indicated as *=P<0.05 and ****=P<0.0001. Bar = 10 µm.

To assess the role of ULK1 and/or ULK2 on TGFβ1-dependent autophagy, I used ULK-101, a pharmacological inhibitor of both ULK1 and ULK2. For this analysis, I
utilized A549 cells and H1299 cells stably expressing GFP-LC3B-RFP-LC3BΔG to measure autophagic flux as previously described\textsuperscript{260}. These cells were treated with ULK-101 in the presence and absence of TGFβ1, and in both cell lines, I observed that TGFβ1 increased ULK1 and LC3B-II protein levels, whereas it decreased ULK2 protein levels and the GFP/RFP ratio. Interestingly, in the presence of TGFβ1, ULK-101 decreased ULK1, ULK2, and LC3B-II protein levels and decreased autophagic flux (as measured by an increased GFP/RFP ratio; \textbf{Fig. 3.3A, B}). Next, I assessed autophagic flux in control or ULK-101-treated A549 cells in the presence or absence of TGFβ1 using fluorescence microscopy (\textbf{Fig. 3.3C}). I observed that TGFβ1 significantly decreased the GFP/RFP ratio by 50±10\% and that ULK-101 restored the GFP/RFP ratio to control levels (\textbf{Fig. 3.3D}). Furthermore, I quantified GFP-LC3B puncta/cell and observed that although TGFβ1 increased the number of GFP-LC3B puncta/cell, ULK-101 decreased the ratio of GFP-LC3B puncta/cell in the presence and absence of TGFβ1 (\textbf{Fig. 3.3E}).
Figure 3.3: The effect of inhibiting ULK activity on TGFβ1-dependent autophagy in NSCLC cell lines.
A549 cells (A) or H1299 cells (B) stably expressing GFP-LC3B-RFP-LC3BΔG were treated with 10 µM of the ULK1/2 inhibitor, ULK-101, or DMSO (vehicle control) in the presence and absence of 250 pM TGFβ1 for 24 hours. Cells were lysed, subjected to SDS-PAGE, and immunoblotting for anti-ULK1, anti-ULK2, anti-LC3B, and anti-GAPDH antibodies. Quantitative analysis of steady-state ULK1, ULK2, and LC3B-II protein levels as well as the GFP/RFP ratio are shown graphically below representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05, **=P<0.01, and ***=P<0.001.

(C) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were treated as described above. Hoechst stain (blue) was added 10 minutes prior to imaging with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Bar = 10 µm.

(D) ImageJ was used to quantify the green and red pixel intensity, and the GFP/RFP ratio is shown graphically below representative images (n=3 ± SD). Significance is indicated as **=P<0.01.

(E) Cells and number of GFP-LC3B puncta/cell were counted using ImageJ version 2.0 software. The data were graphed and shown below representative images (n=3 ± SD). Significance is indicated as ***=P<0.001 and ****=P<0.0001.

TGFβ1 induced autophagy is dependent on ULK1 activity

Since ULK-101 inhibits the kinase activity of both ULK1 and ULK2, I next specifically targeted ULK1 or ULK2 using siRNA. A549 cells or H1299 cells were treated with control siRNA (si-Control), siRNA targeting ULK-1 (si-ULK1) or ULK-2 (si-ULK2), or a combination of both si-ULK1 and si-ULK2 followed by TGFβ1 stimulation. Western blotting indicated that in A549 cells, two different siRNAs targeting ULK1 significantly decreased ULK1 protein levels by >80% and increased ULK2 and LC3B-II protein levels by 150±18% and 140±9%, respectively. Furthermore, in the presence of TGFβ1, the two ULK1 siRNAs increased the GFP/RFP ratio compared to the TGFβ1 treatment alone, suggesting that ULK1 activity is necessary for TGFβ1-induced autophagy in A549 cells (Fig. 3.4A, B). In H1299 cells, the ULK1 siRNAs also reduced ULK1 protein levels by >80% and increased ULK2 protein levels by 150±21%. Additionally, the ULK1 siRNAs had no affect on LC3B-II protein levels but consistent with A549 cells, increased the GFP/RFP ratio, suggesting that ULK1 activity is important for TGFβ1-induced autophagy
in H1299 cells as well (Fig. 3.4C, D). In both cell lines, the ULK2 siRNAs decreased ULK2 protein levels, increased LC3B-II and ULK1 protein levels but had no affect on the GFP/RFP ratio (Fig. 3.4A-D). Taken together, these results suggest that ULK1 but not ULK2 is involved with TGFβ1-induced autophagy.

As a parallel approach, I carried out fluorescence microscopy on GFP-LC3B-RFP-LC3BΔG expressing cell lines (Fig. 3.4E). A549 cells transfected with two different ULK1 siRNAs had significantly increased GFP/RFP ratios by 35±9%, whereas si-ULK2 had little effect (Fig. 3.4F & data not shown). Finally, quantifying GFP-LC3B puncta/cell revealed that in the presence of TGFβ1, all treatments with siRNAs targeting ULK1 had fewer GFP-LC3B puncta/cell (Fig. 3.4G & data not shown). Therefore, my results suggest that TGFβ1 activates autophagy by increasing AMPK-dependent ULK1 S555 phosphorylation.
Figure 3.4: Assessing ULK1 and ULK2 silencing on TGFβ1-dependent autophagy in NSCLC cell lines.
(A) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with 10 nM control siRNA (si-Control), 5 nM siRNA targeting ULK1 (si-ULK1) or 5 nM siRNA targeting ULK2 (si-ULK2) for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-ULK1, anti-ULK2, anti-LC3B, and anti-GAPDH (loading control) antibodies. Quantitative analysis of steady-state ULK1, ULK2, and LC3B-II protein levels as well as the GFP/RFP ratio are shown graphically to the right of representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05, **=P<0.01, ***=P<0.001, and ****=P<0.0001.

(B) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with 10 nM si-Control and a second 5 nM si-ULK1 and/or a second 5 nM si-ULK2 for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-ULK1, anti-ULK2, anti-LC3B, and anti-GAPDH antibodies. Quantitative analysis of the GFP/RFP ratios are shown graphically to the right of representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05.

(C) H1299 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with 10 nM si-Control, 5 nM si-ULK1 and/or 5 nM si-ULK2 for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-ULK1, anti-ULK2, anti-LC3B, and anti-GAPDH antibodies. Quantitative analysis of steady-state ULK1, ULK2, and LC3B-II protein levels as well as the GFP/RFP ratio are shown graphically to the right of representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05, **=P<0.01, ***=P<0.001, and ****=P<0.0001.

(D) H1299 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with 10 nM si-Control and a second 5 nM si-ULK1 and/or a second 5 nM si-ULK2 for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-ULK1, anti-ULK2, anti-LC3B, and anti-GAPDH antibodies. Quantitative analysis of steady-state ULK1, ULK2, and the GFP/RFP ratios are shown graphically to the right of representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05.
(E) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were treated as described above. Hoechst stain (blue) was added 10 minutes prior to imaging with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Bar = 10 µm.

(F) ImageJ was used to quantify the green and red pixel intensity, and the GFP/RFP ratio is shown graphically to the right of representative images (n=3 ± SD). Significance is indicated as *=P<0.05, **=P<0.01, and ***=P<0.001.

(G) Cells and number of GFP-LC3B puncta/cell were counted using ImageJ version 2.0 software. The data were graphed and shown graphically to the right of representative images (n=3 ± SD). Significance is indicated as **=P<0.01 and ****=P<0.0001.

**TGFβ1-induced autophagy relies on TGFβ receptor type I kinase activity**

Although I determined that TGFβ1-dependent autophagy is facilitated by ULK1, the signalling pathway(s) that impact ULK1 protein levels and phosphorylation were unknown. Therefore, I assessed which TGFβ receptors are essential to TGFβ1-dependent autophagy. First, I inhibited the TGFβ receptor type II/ TGFβ receptor type I (TGFβRII/TGFβRI) complex using SB431542, which blocks the kinase activity of TGFβRI138. A549 cells and H1299 cells were treated with SB431542 in the presence and absence of TGFβ1 and immunoblotted for ULK1, P-Smad2, Smad2, and LC3B. I observed that SB431542 inhibited TGFβ1-dependent Smad2 phosphorylation in both cell lines (Fig. 3.5). In A549 cells, SB431542 blocked the TGFβ1-dependent decrease of the GFP/RFP ratio and increase of ULK1 and LC3B-II protein levels (Fig. 3.5A). In H1299 cells, SB431542 disrupted the TGFβ1-dependent decrease of the GFP/RFP ratio and increase of ULK1 protein levels. However, SB431542 treatments significantly increased LC3B-II protein levels by 210±29% compared to control (Fig. 3.5B). To confirm that TGFβRI kinase activity is necessary for TGFβ1-induced autophagy, I next utilized fluorescence microscopy to visualize cells expressing GFP-LC3B-RFP-LC3BΔG as described above (Fig. 3.5C). Quantifying the GFP and RFP channels revealed that SB431542 increased the TGFβ1-dependent GFP/RFP ratio by 30±5%, indicating that SB431542 inhibited TGFβ1-dependent autophagic flux (Fig. 3.5D). After analyzing the GFP-LC3B puncta/cell using the fluorescence images, I determined that SB431542, in the presence of TGFβ1, decreased the amount of LC3B puncta/cell with respect to the TGFβ1 treatment (Fig. 3.5E).
Figure 3.5: The effect of SB431542 on TGFβ1-induced autophagy in NSCLC cell lines.

A549 cells (A) or H1299 cells (B) stably expressing GFP-LC3B-RFP-LC3BΔG were treated with 20 μM SB431542 or DMSO (vehicle control) in the presence and absence of
250 pM TGFβ1 for 24 hours. Cells were lysed, subjected to SDS-PAGE, and immunoblotted for anti-ULK1, anti-LC3B, and anti-GAPDH (loading control) antibodies. Quantitative analysis of steady-state ULK1 and LC3B-II protein levels and the GFP/RFP ratio are shown graphically below representative immunoblots (n=3 ± SD). Significance is indicated as **=P<0.01, ***=P<0.001, and ****=P<0.0001.

(C) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were treated as described above. Hoechst stain (blue) was added 10 minutes prior to imaging with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Bar = 10 µm.

(D) ImageJ was used to quantify the green and red pixel intensity, and the GFP/RFP ratio is shown graphically below representative images (n=3 ± SD). Significance is indicated as **=P<0.01 and ****=P<0.0001.

(E) Cells and number of GFP-LC3B puncta/cell were counted using ImageJ version 2.0 software. The data were graphed and shown below representative images (n=3 ± SD). Significance is indicated as ****=P<0.0001.

TGFβ receptor type III silencing had no affect on TGFβ1-dependent autophagy

Next, I assessed any involvement of the TGFβ receptor type III (TGFβRIII) in TGFβ1-dependent autophagy. I used an siRNA approach because, unlike TGFβRII and TGFβRI, this receptor does not have any intrinsic enzymatic activity. Interestingly, A549 cells expressing two different siRNAs targeting TGFβRIII exhibited a slightly higher basal level of autophagic flux, but TGFβ1-dependent autophagy remained unperturbed by TGFβRIII silencing (Fig. 3.6A, B). Next, I repeated this experiment in A549 cells but prepared the cells for live cell fluorescence microscopy. Indeed, TGFβRIII silencing had no affect on TGFβ1-dependent autophagy but increased autophagic flux (Fig. 3.6C). Finally, as observed in A549 cells, H1299 cells expressing two different siRNAs targeting TGFβRIII had no change to TGFβ1-induced autophagy, and it increased autophagic flux compared to si-Control (Fig. 3.6D). Taken together, these results confirm that only the activity of the TGFβRII/TGFβRI complex is necessary for the TGFβ1-dependent increase of autophagic flux in both NSCLC cell lines.
Figure 3.6: The effect of TGFβRIII silencing on TGFβ1-dependent autophagy in NSCLC cell lines.

(A) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with a 10 nM control siRNA (si-Control) or 10 nM siRNA targeting TGFβRIII (si-TGFβRIII) for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours,
lysed, subjected to SDS-PAGE, and immunoblotted for anti-TGFβRIII, anti-P-Smad2, anti-Smad2, anti-LC3B, and anti-GAPDH (loading control) antibodies. Quantitative analysis of steady-state LC3B-II protein levels and the GFP/RFP ratio are shown graphically to the right of representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05, **=P<0.01, and ***=P<0.001.

(B) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with 10 nM si-Control or a second 10 nM si-TGFβRIII for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-TGFβRIII, anti-LC3B, and anti-GAPDH antibodies. Quantitative analysis of the GFP/RFP ratios are shown graphically to the right of representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05 and **=P<0.01.

(C) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were treated with 10 nM si-Control or two different siRNAs targeting TGFβRIII for 48 hours. Hoechst stain (blue) was added 10 minutes prior to imaging with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Bar = 10 µm. Significance is indicated as ****=P<0.0001.

(D) H1299 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with 10 nM si-Control or two different 10 nM siRNAs targeting TGFβRIII for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-TGFβRIII, anti-LC3B, and anti-GAPDH antibodies. Quantitative analysis of the GFP/RFP ratios are shown graphically to the right of representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05, **=P<0.01, and ***=P<0.001.

**Smad4-dependent TGFβ1 signalling activates autophagy**

After TGFβ1 binds to TGFβRII/TGFβRII complexes, it initiates canonical and non-canonical signalling339. Since I observed that inhibiting TGFβ receptor kinase activity and Smad2 phosphorylation resulted in inhibition of autophagy (Fig. 3.5), I assessed if reducing the accessibility of Smad2 to the TGFβ receptor complex would affect TGFβ1-dependent autophagy. This was carried out by two different siRNAs targeting Smad anchor for receptor activation (SARA). Interestingly, reducing SARA levels in both A549 cells or H1299 cells did not inhibit TGFβ1-dependent induction of LC3B-II protein levels, or inhibit autophagy (Fig. 3.7A-D). Furthermore, after fluorescence microscopy (Fig. 3.7E),
I verified that SARA silencing had no influence on autophagic flux (Fig. 3.7F) or alter relative GFP-LC3B puncta/cell (Fig. 3.7G). These results suggest that if the canonical TGFβ signalling pathway results in autophagy, removing a major member of the pathway, such as Smad4, may be necessary to alter TGFβ1-dependent autophagy. Therefore, I next evaluated if Smad4 silencing via siRNA targeting (si-Smad4) influenced TGFβ1-dependent autophagy.
Figure 3.7: The effect of SARA silencing on TGFβ1-dependent autophagy in NSCLC cell lines.
(A) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with a 10 nM control siRNA (si-Control) or 10 nM siRNA targeting SARA (si-SARA) for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-SARA, anti-P-Smad2, anti-Smad2, anti-LC3B, and anti-GAPDH (loading control) antibodies. Quantitative analysis of steady-state LC3B-II protein levels and the GFP/RFP ratio are shown graphically to the right of representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05, **=P<0.01, and ****=P<0.0001.

(B) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with 10 nM si-Control or a 10 nM second si-SARA for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-SARA, anti-LC3B, and anti-GAPDH antibodies. Quantitative analysis of the GFP/RFP ratios are shown graphically to the right of representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05 and **=P<0.01.

(C) H1299 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with 10 nM si-Control or 10 nM si-SARA for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-SARA, anti-LC3B, and anti-GAPDH antibodies. Quantitative analysis of steady-state LC3B-II protein levels and the GFP/RFP ratio are shown graphically to the right of representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05 and **=P<0.01.

(D) H1299 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with 10 nM si-Control or two different 10 nM siRNAs targeting SARA for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-SARA, anti-LC3B, and anti-GAPDH antibodies. Quantitative analysis of the GFP/RFP ratios are shown graphically to the right of representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05.

(E) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were treated as described above. Hoechst stain (blue) was added 10 minutes prior to imaging with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Bar = 10 µm.
(F) ImageJ was used to quantify the green and red pixel intensities, and the GFP/RFP ratio is shown to the right of representative images (n=3 ± SD). Significance is indicated as *=P<0.05 and **=P<0.01.

(G) Cells and number of GFP-LC3B puncta/cell were counted using ImageJ version 2.0 software. The data were graphed and shown to the right of representative images (n=3 ± SD). Significance is indicated as *=P<0.05.

A549 cells and H1299 cells were transfected with si-Control or two different siRNAs targeting Smad4 for 24 hours. The NSCLC cells were then incubated for an additional 24 hours in the presence or absence of TGFβ1, lysed, and immunoblotted for Smad4, P-Smad2, Smad2, and LC3B. In both cell lines, I observed that TGFβ1 increased LC3B-II protein levels and decreased the GFP/RFP ratio (Fig. 3.8A-D). Interestingly, Smad4 silencing increased the proportion of P-Smad2, which suggested that TGFβ1-dependent autophagy does not rely on P-Smad2 (Fig. 3.8A, C). Although Smad4 silencing had differing affects on LC3B-II protein levels in A549 cells vs. H1299 cells, both cell lines showed attenuated TGFβ1-dependent GFP/RFP ratio in the absence of Smad4, suggesting that Smad4 is necessary to induce TGFβ1-dependent autophagic flux (Fig. 3.8A-D). To investigate this further, I used fluorescence microscopy to image the GFP/RFP ratio in A549 cells (Fig. 3.8E). Quantifying the GFP/RFP ratios indicated that TGFβ1 decreased the GFP/RFP ratio compared to the si-Control treatment by 60±5%. Alternatively, siRNAs targeting Smad4 in the presence of TGFβ1 had elevated GFP/RFP ratios that were no longer significantly different compared to the control (Fig. 3.8F & data not shown). Lastly, I examined the influence that Smad4 had on relative GFP-LC3B puncta/cell. Although the TGFβ1 treatment significantly increased the relative number of GFP-LC3B puncta/cell, I observed that TGFβ1 treated Smad4-silenced cells did not have significantly increased GFP-LC3B puncta/cell ratios (Fig. 3.8G & data not shown). These results support the conclusion that TGFβ1 induces autophagy via Smad4. Having ascertained that the canonical pathway was important for promoting TGFβ1-dependent autophagy, I next assessed the contribution of non-canonical TGFβ pathways.
Figure 3.8: The effect of Smad4 silencing on TGFβ1-dependent autophagy in NSCLC cell lines.
(A) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with a 10 nM control siRNA (si-Control) or 10 nM siRNA targeting Smad4 (si-Smad4) for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-Smad4, anti-P-Smad2, anti-Smad2, anti-LC3B, and anti-GAPDH (loading control) antibodies. Quantitative analysis of steady-state LC3B-II protein levels and the GFP/RFP ratio are shown graphically to the right of representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05 and **=P<0.01.

(B) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with 10 nM si-Control or a second 10 nM si-Smad4 for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-Smad4, anti-LC3B, and anti-GAPDH antibodies. Quantitative analysis of the GFP/RFP ratios are shown graphically to the right of representative immunoblots (n=3 ± SD). Significance is indicated as **=P<0.01.

(C) H1299 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with 10 nM si-Control or 10 nM si-Smad4 for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-Smad4, anti-P-Smad2, anti-Smad2, anti-LC3B, and anti-GAPDH antibodies. Quantitative analysis of the GFP/RFP ratio are shown graphically to the right of representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05, **=P<0.01, and ***=P<0.001.

(D) H1299 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with 10 nM si-Control or two different 10 nM siRNAs targeting Smad4 for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-Smad4, anti-LC3B, and anti-GAPDH antibodies. Quantitative analysis of the GFP/RFP ratios are shown graphically to the right of representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05.

(E) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were treated as described above. Hoechst stain (blue) was added 10 minutes prior to imaging with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Bar = 10 µm.
(F) ImageJ was used to quantify the green and red pixel intensities, and the GFP/RFP ratio is shown to the right of representative images (n=3 ± SD). Significance is indicated as *=P<0.05.

(G) Cells and number of GFP-LC3B puncta/cell were counted using ImageJ version 2.0 software. The data were graphed and shown to the right of representative images (n=3 ± SD). Significance is indicated as *=P<0.05 and **=P<0.01.

Non-canonical TGFβ1 signalling upregulates autophagy

I first investigated the role of the PI3K non-canonical TGFβ signalling pathway on TGFβ1-induced autophagy using LY294002, an inhibitor of the PI3K-mTOR pathway.70,306 A549 cells and H1299 cells were treated with LY294002 in the presence and absence of TGFβ1, lysed, and immunoblotted for mTOR, P-mTOR, LC3B, and GAPDH. I observed that LY294002 treatment increased LC3B-II protein levels and reduced the GFP/RFP ratio to a greater extent than the TGFβ1 treatment alone (Fig. 3.9A, B). These results suggest that the PI3K pathway and autophagic flux are inversely proportional to one another. I verified that the PI3K pathway does not facilitate TGFβ1-dependent autophagy by treating A549 cells with LY294002, with and without TGFβ1, prior to fluorescence microscopy imaging (Fig. 3.9C). In all cases where the cells were treated with LY294002, I observed a marked decrease in the GFP-LC3B signal, and the quantitation of the GFP/RFP ratios suggested that LY294002 decreased the GFP/RFP ratio in the presence and absence of TGFβ1 (Fig. 3.9D). Finally, I observed that both TGFβ1 and LY294002 increased the amount of GFP-LC3B puncta/cell; however, LY294002 significantly increased (>50%) the number of GFP-LC3B puncta/cell compared to the TGFβ1 treatment alone (Fig. 3.9E). Since these results suggest that any PI3K activity that is stimulated by TGFβ would impede autophagy, I next directed my attention to another non-canonical TGFβ pathway, the aPKC pathway.
Figure 3.9: The effect of LY294002 on TGFβ1-induced autophagy in NSCLC cell lines.

A549 cells (A) or H1299 cells (B) stably expressing GFP-LC3B-RFP-LC3BΔG were treated with 40 μM LY294002 or DMSO (vehicle control) in the presence and absence of...
250 pM TGFβ1 for 24 hours. Cells were lysed, subjected to SDS-PAGE, and immunoblotted for anti-mTOR, anti-P-mTOR, anti-LC3B, and anti-GAPDH (loading control) antibodies. Quantitative analysis of steady-state LC3B-II protein levels as well as the GFP/RFP ratio are shown below representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05, **=P<0.01, ***=P<0.001, and ****=P<0.0001.

(C) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were treated as described above. Hoechst stain (blue) was added 10 minutes prior to imaging with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Bar = 10 µm.

(D) ImageJ was used to quantify the green and red pixel intensities, and the GFP/RFP ratio is shown below representative images (n=3 ± SD). Significance is indicated as ****=P<0.0001.

(E) Cells and number of GFP-LC3B puncta/cell were counted using ImageJ version 2.0 software. The data were graphed and shown below representative images (n=3 ± SD). Significance is indicated as *=P<0.05, **=P<0.01, and ****=P<0.0001.

Through their association with par6, both aPKCζ and aPKCι have been shown to be involved with TGFβ-dependent processes, such as EMT and apoptosis. To investigate if this pathway is involved with autophagy, I utilized siRNAs selective for aPKCζ (si-aPKCζ) or aPKCι (si-aPKCι). A549 cells and H1299 cells were treated with si-Control, si-aPKCζ or si-aPKCι in the presence or absence of TGFβ1, lysed, and immunoblotted for aPKCζ, aPKCι, LC3B, and GAPDH. In both cell lines, although no effects were observed for si-aPKCι treatments, si-aPKCζ increased LC3B-II protein levels. Significantly, silencing aPKCζ decreased autophagic flux in A549 cells only (Fig. 3.10A, B). To verify this, I treated A549 cells with si-Control, si-aPKCζ or si-aPKCι and used fluorescence microscopy to image GFP-LC3B-RFP-LC3BΔG (Fig. 3.10C). I observed that si-aPKCι had no effect on autophagic flux or relative LC3 puncta/cell, whereas si-aPKCζ, in the presence of TGFβ1, increased the GFP/RFP ratio compared to the TGFβ1 treatment (Fig. 3.10D, E). Finally, although silencing aPKCζ had no significant effect on the number of GFP-LC3B puncta/cell, in the presence of TGFβ1, si-aPKCζ was the only treatment that was not increased with respect to the control (Fig. 3.10E).
Figure 3.10: The influence of silencing aPKCζ or aPKCt on TGFβ1-dependent autophagy in NSCLC cell lines.

A549 cells (A) and H1299 cells (B) stably expressing a cDNA pMRX-IP-GFP-LC3B-RFP-LC3BΔG construct were transfected with 10 nM si-Control, 10 nM siRNA targeting aPKCζ (si-aPKCζ) or 10 nM siRNA targeting aPKCt (si-aPKCt) for 48 hours. The cells were also incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed,
subjected to SDS-PAGE, and immunoblotted for anti-aPKCζ, anti-aPKCι, anti-LC3B, and anti-GAPDH antibodies. Quantitative analysis of steady-state LC3B-II protein levels and the GFP/RFP ratio are shown graphically below representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05, **=P<0.01, and ***=P<0.001.

(C) A549 cells stably expressing a cDNA pMRX-IP-GFP-LC3B-RFP-LC3BΔG construct were treated as described above. Hoechst stain (blue) was added 10 minutes prior to imaging with a 63x objective using an Olympus IX 81 inverted fluorescence microscope.

(D) ImageJ quantified the green and red pixel intensity, and the GFP/RFP ratio is shown graphically to the right of representative images (n=3 ± SD). Significance is indicated as *=P<0.05 and ***=P<0.001.

(E) Cells and number of GFP-LC3B puncta/cell were counted using ImageJ version 2.0 software. The data were graphed and shown graphically to the right of representative images (n=3 ± SD). Significance is indicated as *=P<0.05.

Since my lab had previously observed that aPKCι silencing increases aPKCζ protein levels37, I utilized a double si-aPKCι and si-aPKCζ knockdown approach. A549 cells and H1299 cells were treated with si-Control or two different combinations of si-aPKCζ/si-aPKCι in the presence or absence of TGFβ1, lysed, subjected to SDS-PAGE, and immunoblotted for aPKCζ, aPKCι, LC3B, and GAPDH. In A549 cells, I observed that TGFβ1 increased LC3B-II protein levels and decreased the GFP/RFP ratio in the presence of si-Control and si-aPKCζ/si-aPKCι treatments (Fig. 3.11A, B). In H1299 cells, I found that si-aPKCζ/si-aPKCι and TGFβ1 increased LC3B-II protein levels compared to the si-Control treatment, however, in the presence of TGFβ1, si-aPKCζ/si-aPKCι significantly reduced LC3B-II protein levels by 25±5%. Additionally, in the presence of TGFβ1, the GFP/RFP ratio of the si-aPKCζ/si-aPKCι treatment was not statistically different compared to the si-Control treatment (Fig. 3.11C, D). Based on these results, aPKCs may not be involved with TGFβ1-dependent autophagy. To confirm this, I treated A549 cells with si-Control or si-aPKCζ/si-aPKCι and used fluorescence microscopy to image GFP-LC3B and RFP-LC3BΔG (Fig. 3.11E). Quantitation of the GFP/RFP ratios indicated that all TGFβ1 treatments had reduced GFP/RFP ratios with respect to the si-Control treatment (Fig. 3.11F), and that TGFβ1 increased the number of GFP-LC3B puncta/cell regardless of
aPKC knockdown (Fig. 3.11G). Having observed that TGFβ1 does not require aPKCζ or aPKCι to activate autophagy, I next directed my attention to the TAK1-TRAF6-p38 MAPK pathway.
Figure 3.11: The influence of aPKC knockdown on TGFβ1-dependent autophagy in NSCLC cell lines.
(A) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with a 10 nM control siRNA (si-Control) or 5 nM siRNA targeting aPKCζ and 5 nM siRNA targeting aPKCζ+si-aPKCζ for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-aPKCζ, anti-aPKCζ, anti-LC3B, and anti-GAPDH (loading control) antibodies. Quantitative analysis of steady-state LC3B-II protein levels and the GFP/RFP ratio are shown to the right of representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05 and **=P<0.01.

(B) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with 10 nM si-Control or a second combination of 10 nM si-aPKCζ+si-aPKCζ for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-aPKCζ, anti-aPKCζ, anti-LC3B, and anti-GAPDH antibodies. Quantitative analysis of the GFP/RFP ratio are shown to the right of representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05.

(C) H1299 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with 10 nM si-Control or 10 nM si-aPKCζ+si-aPKCζ for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-aPKCζ, anti-aPKCζ, anti-LC3B, and anti-GAPDH antibodies. Quantitative analysis of steady-state LC3B-II protein levels and the GFP/RFP ratio are shown to the right of representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05, **=P<0.01, and ****=P<0.0001.

(D) H1299 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with 10 nM si-Control or a second combination of 10 nM si-aPKCζ+si-aPKCζ for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-aPKCζ, anti-aPKCζ, anti-LC3B, and anti-GAPDH antibodies. Quantitative analysis of the GFP/RFP ratio are shown to the right of representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05.

(E) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were treated as described above. Hoechst stain (blue) was added 10 minutes prior to imaging with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Bar = 10 µm.
(F) ImageJ was used to quantify the green and red pixel intensities, and the GFP/RFP ratio is shown graphically to the right of representative images (n=3 ± SD). Significance is indicated as **=P<0.01.

(G) Cells and number of GFP-LC3B puncta/cell were counted using ImageJ version 2.0 software. The data were graphed and shown graphically to the right of representative images (n=3 ± SD). Significance is indicated as **=P<0.01.

To assess if the TAK1-TRAF6-p38 MAPK pathway was involved with TGFβ1-dependent autophagy, I first inhibited each component of the pathway separately. The effects of pharmacologically inhibiting p38 MAPK in A549 cells and H1299 cells was assessed by immunoblotting for cleaved PARP, as TGFβ1 increases PARP cleavage via p38 MAPK308. In both cell lines, I observed that the p38 MAPK inhibitor blocked TGFβ1-dependent PARP cleavage, however, it did not alter TGFβ1-dependent autophagy, as assessed by western blotting or fluorescence microscopy, nor did it alter relative GFP-LC3B puncta/cell (Fig. 3.12A-E). I next assessed the involvement of TRAF6 in TGFβ1-dependent autophagy using two different siRNAs specific for TRAF6 (si-TRAF6). A549 cells and H1299 cells treated with si-Control or si-TRAF6 in the presence or absence of TGFβ1 showed that TRAF6 silencing did not affect TGFβ1 mediated changes to LC3B-II protein levels. Although TRAF6 silencing may decrease the GFP/RFP ratio in A549 cells compared to the si-Control, it does not impact the effect of TGFβ1 on the GFP/RFP ratio in both cell lines (Fig. 3.13A-D). To verify that TRAF6 silencing had no effect on TGFβ1-induced autophagy, I used fluorescence microscopy on A549 cells treated as described above (Fig. 3.13E). In the presence and absence of TGFβ1, si-TRAF6 did not alter the GFP/RFP ratios or impact the number of GFP-LC3B puncta/cell (Fig. 3.13F-G). Finally, I used two different siRNAs specific for TAK1 (si-TAK1) to silence TAK1 in A549 cells and H1299 cells. In both cell lines, the first si-TAK1 decreased LC3B-II protein levels and partially reversed TGFβ1-dependent autophagic flux, as assessed by western blotting. However, the second si-TAK1 had no impact on the GFP/RFP ratio (Fig. 3.14A-D). When I assessed the influence of TAK1 silencing on autophagic flux and relative GFP-LC3B puncta/cell via fluorescence microscopy (Fig. 3.14E), I observed no significant differences (Fig. 3.14F, G). However, the promising results from the western blot analysis obtained using the first si-TAK1 prompted me to try a combination of inhibitors of this pathway.
Figure 3.12: The effect of p38 MAPK on TGFβ1-induced autophagy in NSCLC cell lines.

A549 cells (A) or H1299 cells (B) stably expressing GFP-LC3B-RFP-LC3BΔG were treated with 10 μM p38 MAPK or equivalent volumes of DMSO in the presence and absence of 250 pM TGFβ1 for 24 hours. Cells were lysed, subjected to SDS-PAGE, and
immunoblotted for anti-cleaved PARP, anti-LC3B, and anti-GAPDH (loading control) antibodies. Quantitative analysis of steady-state LC3B-II protein levels and the GFP/RFP ratio are shown graphically below representative immunoblots (n=3 ± SD). Significance is indicated as **=P<0.01 and ***=P<0.001.

(C) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were treated as described above. Hoechst stain (blue) was added 10 minutes prior to imaging with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Bar = 10 µm.

(D) ImageJ quantified the green and red pixel intensity, and the GFP/RFP ratio is shown graphically below representative images (n=3 ± SD). Significance is indicated as *=P<0.05.

(E) Cells and number of GFP-LC3B puncta/cell were counted using ImageJ version 2.0 software. The data were graphed and shown graphically below representative images (n=3 ± SD). Significance is indicated as **=P<0.01.
Figure 3.13: The effect of TRAF6 silencing on TGFβ1-dependent autophagy in NSCLC cell lines.
(A) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with a 10 nM control siRNA (si-Control) or 10 nM si-TRAF6 for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-TRAF6, anti-LC3B, and anti-GAPDH (loading control) antibodies. Quantitative analysis of steady-state LC3B-II protein levels and the GFP/RFP ratio are shown graphically to the right of representative immunoblots (n=3 ± SD). Significance is indicated as **=P<0.01 and ****=P<0.0001.

(B) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with 10 nM si-Control or a second 10 nM si-TRAF6 for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-TRAF6, anti-LC3B, and anti-GAPDH antibodies. Quantitative analysis of the GFP/RFP ratios are shown graphically to the right of representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05 and ***=P<0.001.

(C) H1299 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with 10 nM si-Control or 10 nM si-TRAF6 for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-TRAF6, anti-LC3B, and anti-GAPDH antibodies. Quantitative analysis of steady-state LC3B-II protein levels and the GFP/RFP ratio are shown graphically to the right of representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05 and **=P<0.01.

(D) H1299 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with 10 nM si-Control or a second 10 nM si-TRAF6 for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-TRAF6, anti-LC3B, and anti-GAPDH antibodies. Quantitative analysis of the GFP/RFP ratios are shown graphically to the right of representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05.

(E) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG construct were treated as described above. Hoechst stain (blue) was added 10 minutes prior to imaging with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Bar = 10 µm.
(F) ImageJ quantified the green and red pixel intensity, and the GFP/RFP ratio is shown graphically to the right of representative images (n=3 ± SD). Significance is indicated as * = P<0.05.

(G) Cells and number of GFP-LC3B puncta/cell were counted using ImageJ version 2.0 software. The data were graphed and shown graphically to the right of representative images (n=3 ± SD). Significance is indicated as ***/=P<0.001.
Figure 3.14: The effect of TAK1 silencing on TGFβ1-dependent autophagy in NSCLC cell lines.

(A) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with 10 nM si-Control or a 10 nM siRNA targeting TAK1 (si-TAK1) for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-TAK1, anti-LC3B, and anti-GAPDH (loading control) antibodies. Quantitative analysis of steady-state LC3B-II protein levels and the GFP/RFP ratio are shown graphically to the right of representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05, **=P<0.01, and ***=P<0.001.

(B) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with 10 nM si-Control or a second 10 nM si-TAK1 for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-TAK1, anti-LC3B, and anti-GAPDH antibodies. Quantitative analysis of steady-state LC3B-II protein levels and the GFP/RFP ratio are shown graphically to the right of representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05.

(C) H1299 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with 10 nM si-Control or 10 nM si-TAK1 for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-TAK1, anti-LC3B, and anti-GAPDH antibodies. Quantitative analysis of steady-state LC3B-II protein levels and the GFP/RFP ratio are shown graphically to the right of representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05.

(D) H1299 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with 10 nM si-Control or a second 10 nM si-TAK1 for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-TAK1, anti-LC3B, and anti-GAPDH antibodies. Quantitative analysis of steady-state LC3B-II protein levels and the GFP/RFP ratio are shown graphically to the right of representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05.

(E) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were treated as described above. Hoechst stain (blue) was added 10 minutes prior to imaging with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Bar = 10 µm.
(F) ImageJ quantified the green and red pixel intensity, and the GFP/RFP ratio is shown graphically to the right of representative images (n=3 ± SD). Significance is indicated as *=P<0.05.

(G) Cells and number of GFP-LC3B puncta/cell were counted using ImageJ version 2.0 software. The data were graphed and shown graphically to the right of representative images (n=3 ± SD). Significance is indicated as ***=P<0.001 and ****=P<0.0001.

Next, I inhibited TAK1, TRAF6, and p38 MAPK activity simultaneously to achieve maximal blockade of this non-canonical TGFβ signalling pathway (Fig. 3.15). A549 cells and H1299 cells were treated with two different siRNAs against TRAF6 and TAK1 (si-TRAF6 and si-TAK1) as well as a p38 MAPK inhibitor in the presence and absence of TGFβ1. The cells were then lysed, subjected to SDS-PAGE, and immunoblotted for TAK1, TRAF6, cleaved PARP, and LC3B. In both cell lines, I observed that using two sets of siRNA to TAK1 and TRAF6, in combination with a p38 MAPK inhibitor decreased LC3B-II protein levels and increased the GFP/RFP ratio (Fig. 3.15A-D). To verify the role of this pathway in TGFβ1-induced autophagy, I used fluorescence microscopy to image A549 cells treated as previously described (Fig. 3.15E). Quantitation revealed that inhibiting the TAK1-TRAF6-p38 MAPK pathway, in the presence of TGFβ1, significantly increased the GFP/RFP ratio by 20±5% compared to the TGFβ1 treatment (Fig. 3.15F & data not shown). Additionally, in the presence and absence of TGFβ1, inhibiting the TAK1-TRAF6-p38 MAPK pathway reduced the relative number of GFP-LC3B puncta/cell (Fig. 3.15G & data not shown). In conclusion, TGFβ1 relies on the TAK1-TRAF6-p38 MAPK to upregulate autophagy.
Figure 3.15: The effect of TAK1-TRAF6-p38 MAPK pathway on TGFβ1-dependent autophagy in NSCLC cell lines.
(A) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with a 10 nM control siRNA (si-Control) or 5 nM siRNA targeting TAK1 (si-TAK1) and 5 nM siRNA targeting TRAF6 (si-TRAF6) for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 and 10 μM p38 MAPK inhibitor (p38i) for 24 hours. The cells were then lysed, subjected to SDS-PAGE, and immunoblotted for anti-TAK1, anti-TRAF6, anti-cleaved PARP, anti-LC3B, and anti-GAPDH (loading control) antibodies. Quantitative analysis of steady-state LC3B-II protein levels and the GFP/RFP ratio are shown to the right of representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05, **=P<0.01, and ****=P<0.0001.

(B) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with 10 nM si-Control or a second 5 nM si-TAK1 and a second 5 nM si-TRAF6 for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 and 10 μM p38i for 24 hours. The cells were then lysed, subjected to SDS-PAGE, and immunoblotted for anti-TAK1, anti-TRAF6, anti-cleaved PARP, anti-LC3B, and anti-GAPDH antibodies. Quantitative analysis of the GFP/RFP ratios are shown to the right of representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05 and **=P<0.01.

(C) H1299 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with 10 nM si-Control or 5 nM si-TAK1 and 5 nM si-TRAF6 for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 and 10 μM p38i for 24 hours. The cells were then lysed, subjected to SDS-PAGE, and immunoblotted for anti-TAK1, anti-TRAF6, anti-cleaved PARP, anti-LC3B, and anti-GAPDH antibodies. Quantitative analysis of steady-state LC3B-II protein levels and the GFP/RFP ratio are shown to the right of representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05 and **=P<0.01.

(D) H1299 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with 10 nM si-Control or a second 5 nM si-TAK1 and a second 5 nM si-TRAF6 for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 and 10 μM p38i for 24 hours. The cells were then lysed, subjected to SDS-PAGE, and immunoblotted for anti-TAK1, anti-TRAF6, anti-cleaved PARP, anti-LC3B, and anti-GAPDH antibodies. Quantitative analysis of the GFP/RFP ratios are shown to the right of representative immunoblots (n=3 ± SD).
A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were treated as described above. Hoechst stain (blue) was added 10 minutes prior to imaging with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Bar = 10 µm.

ImageJ was used to quantify the green and red pixel intensities, and the GFP/RFP ratio is shown to the right of representative images (n=3 ± SD). Significance is indicated as *P<0.05 and ***P<0.001.

Cells and number of GFP-LC3B puncta/cell were counted using ImageJ version 2.0 software. The data were graphed and shown to the right of representative images (n=3 ± SD). Significance is indicated as ***P<0.001 and ****P<0.0001.

* TGFβ1-induced autophagosome-lysosome co-localization is regulated by Smad4 and TAK1-TRAF6-p38 MAPK signalling pathways

Above I observed that the Smad4 and TAK1-TRAF6-p38 MAPK branches of the canonical and non-canonical TGFβ signalling pathways influence TGFβ1-dependent autophagy. To gain more mechanistic insight, I next utilized A549 cells stably expressing GFP-labelled LC3B protein to determine if either Smad4 silencing or inhibiting the TAK1-TRAF6-p38 MAPK pathway disrupted the TGFβ1-dependent increase of GFP-LC3B-lysosome co-localization. Briefly, A549 cells expressing GFP-LC3B were transfected with si-Control or si-Smad4, in the presence and absence of 250 pM TGFβ1 for 24 hours and labelled with LysoTracker Deep Red to identify lysosomes. I observed that in the absence of TGFβ there was little GFP-LC3B co-localizing with lysosomes; however, TGFβ1 induced the accumulation of GFP-LC3B into LysoTracker-positive puncta. Interestingly, Smad4 silencing reduced both GFP-LC3B accumulation within cells and the co-localization with lysosomes (Fig. 3.16A). Inhibiting the TAK1-TRAF6-p38 MAPK pathway using a combination of si-TAK1, si-TRAF6, and p38 MAPK inhibitor yielded similar results, as inhibiting the TAK1-TRAF6-p38 MAPK pathway blocked the TGFβ1-dependent increase in GFP-LC3B-lysosome co-localization (Fig. 3.16B). In summary, these results verified that both Smad4 and the TAK1-TRAF6-p38 MAPK signalling pathways are necessary for TGFβ1 to induce autophagosome and lysosome co-localization, which temporally occurs immediately prior to lysosomal-dependent degradation.
Figure 3.16: The effect of canonical and non-canonical TGFβ signalling on autophagosome/lysosome co-localization.
(A) A549 cells expressing GFP-LC3B-RFP-LC3BΔG were transfected with 10 nM si-Control or 10 nM si-Smad4 for 48 hours. The cells were then incubated in the absence or presence of 250 pM TGFβ1 for 24 hours. LysoTracker Deep Red (red) and Hoechst stain (blue) were added 2 hours and 10 minutes, respectively, prior to imaging. Images were obtained with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Scale bars = 10 μm. ImageJ version 2.0 was used to quantify the number of yellow pixels per cell area for each treatment. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as **=P<0.01 and ****=P<0.0001.

(B) A549 cells expressing GFP-LC3B-RFP-LC3BΔG were transfected with 10 nM si-Control or 5 nM si-TAK1 and 5 nM si-TRAF6 for 48 hours. The cells were then incubated in the absence or presence of 250 pM TGFβ1 and 10 μM p38 MAPK inhibitor for 24 hours. LysoTracker Deep Red (red) and Hoechst stain (blue) were added 2 hours and 10 minutes, respectively, prior to imaging. Images were obtained with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Scale bars = 10 μm. ImageJ version 2.0 was used to quantify the number of yellow pixels per cell area for each treatment. The data were graphed from 3 independent experiments (mean ± SD). Significance is indicated as *=P<0.05 and **=P<0.001.

Smad4 regulates ULK1 phosphorylation and TAK1-TRAF6-p38 MAPK activation inhibits the mTOR-ULK1 pathway

Since TGFβ1 activates autophagy using Smad4 and TAK1-TRAF6-p38 MAPK signalling pathways, I next investigated if these pathways influenced mTOR and ULK1 phosphorylation. A549 cells and H1299 cells were treated with si-Control or two siRNAs targeting Smad4 in the presence or absence of TGFβ1, lysed, subjected to SDS-PAGE, and immunoblotted using both antibodies and phospho-specific antibodies for mTOR, ULK1, and AMPKα as well as GAPDH. In both cell lines, the Smad4 knockdowns had no effect on the P-mTOR/mTOR or P-S757-ULK1/ULK1 ratios. However, in the presence of TGFβ1, Smad4 silencing decreased the P-S555-ULK1/ULK1 in A549 cells by 50±15% and 45±18% and in H1299 cells by 50±19% and 55±20%. To assess if this could be due to increased AMPKα activity, I analyzed AMPKα-T172 phosphorylation status and observed
that P-AMPKα levels remained constant in the presence or absence of TGFβ1 and/or Smad4 (Fig. 3.17A-D).

Next, A549 cells and H1299 cells were treated with two different siRNAs against TRAF6 and TAK1 as well as a p38 MAPK inhibitor in the presence and absence of TGFβ1. The cells were then lysed, subjected to SDS-PAGE, and immunoblotted for P-mTOR, mTOR, P-S555-ULK1, P-S757-ULK1, ULK1, AMPKα, P-T172-AMPKα, and GAPDH. In both cell lines, inhibiting the TAK1-TRAF6-p38 MAPK pathway had no impact on the P-S757-ULK1/ULK1 ratios (Fig. 3.17E-H). In A549 cells treated with TGFβ1, inhibiting the TAK1-TRAF6-p38 MAPK pathway increased the P-mTOR/mTOR ratio by 25±12% and 35±15% and decreased the P-S555-ULK1/ULK1 ratio by 40±15% and 30±5% (Fig. 3.17E, F). In H1299 cells treated with TGFβ1, inhibiting the TAK1-TRAF6-p38 MAPK pathway increased the P-mTOR/mTOR ratio by 20±5% and 25±10% and decreased the P-S555-ULK1/ULK1 ratio by 30±10% and 35±15% (Fig. 3.17G, H). Interestingly, inhibiting the TAK1-TRAF6-p38 MAPK pathway increased the basal level of AMPKα-T172 phosphorylation; however, the P-AMPKα/AMPKα ratio was unchanged in response to TGFβ1 (Fig. 3.17E, G).
Figure 3.17: The effect of TAK1-TRAF6-p38 MAPK pathway and Smad4 on mTOR and ULK1 activity in NSCLC cells.

(A) A549 cells were transfected with a 10 nM control siRNA (si-Control) or 10 nM siRNA against Smad4 (si-Smad4) for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-mTOR, anti-phospho-S2448-mTOR, anti-phospho-S757-ULK1, anti-ULK1, anti-phospho-S555-ULK1, anti-AMPKα, anti-phospho-T172-AMPKα, and
anti-GAPDH (loading control) antibodies. The phospho-S2448-mTOR/mTOR (P-mTOR/mTOR), phospho-S555-ULK1/ULK1, anti-phospho-S757-ULK1/ULK1, and phospho-T172-AMPKα/AMPKα (P-AMPK/AMPK) ratios were quantified using QuantityOne software and the graphs (n=3 ± SD) are located to the right of representative immunoblots. Significance is indicated as *=P<0.05 and **=P<0.01.

(B) A549 cells were transfected with 10 nM si-Control or a second 10 nM si-Smad4 for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-mTOR, anti-phospho-S2448-mTOR, anti-ULK1, anti-phospho-S555-ULK1, and anti-GAPDH antibodies. The P-mTOR/mTOR and phospho-S555-ULK1/ULK1 ratios were quantified using QuantityOne software and the graphs (n=3 ± SD) are located to the right of representative immunoblots. Significance is indicated as *=P<0.05 and **=P<0.01.

(C) H1299 cells were transfected with 10 nM si-Control or 10 nM si-Smad4 for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-mTOR, anti-phospho-S2448-mTOR, anti-phospho-S757-ULK1, anti-ULK1, anti-phospho-S555-ULK1, anti-AMPKα, anti-phospho-T172-AMPKα, and anti-GAPDH antibodies. The P-mTOR/mTOR, phospho-S555-ULK1/ULK1, anti-phospho-S757-ULK1/ULK1, and P-AMPK/AMPK ratios were quantified using QuantityOne software and the graphs (n=3 ± SD) are located to the right of representative immunoblots. Significance is indicated as *=P<0.05 and ****=P<0.0001.

(D) H1299 cells were transfected with 10 nM si-Control or a second 10 nM si-Smad4 for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-mTOR, anti-phospho-S2448-mTOR, anti-ULK1, anti-phospho-S555-ULK1, and anti-GAPDH antibodies. The P-mTOR/mTOR and phospho-S555-ULK1/ULK1 ratios were quantified using QuantityOne software and the graphs (n=3 ± SD) are located to the right of representative immunoblots. Significance is indicated as *=P<0.05 and **=P<0.01.

(E) A549 cells were transfected with 10 nM si-Control or 5 nM siRNAs targeting TAK1 or 5 nM siRNAs targeting TRAF6 (si-TAK1 and si-TRAF6) for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 and 10 µM p38 MAPK inhibitor (p38i) for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-mTOR,
anti-phospho-S2448-mTOR, anti-phospho-S757-ULK1, anti-ULK1, anti-phospho-S555-ULK1, anti-AMPKα, anti-phospho-T172-AMPKα, and anti-GAPDH antibodies. The P-mTOR/mTOR, phospho-S555-ULK1/ULK1, anti-phospho-S757-ULK1/ULK1, and P-AMPK/AMPK ratios were quantified using QuantityOne software and the graphs (n=3 ± SD) are located to the right of representative immunoblots. Significance is indicated as *=P<0.05 and **=P<0.01.

(F) A549 cells were transfected with 10 nM si-Control or a second 5 nM si-TAK1 and a second 5 nM si-TRAF6 for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 and 10 μM p38i for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-mTOR, anti-phospho-S2448-mTOR, anti-ULK1, anti-phospho-S555-ULK1, and anti-GAPDH antibodies. The P-mTOR/mTOR and phospho-S555-ULK1/ULK1 ratios were quantified using QuantityOne software and the graphs (n=3 ± SD) are located to the right of representative immunoblots. Significance is indicated as *=P<0.05 and **=P<0.01.

(G) H1299 cells were transfected with 10 nM si-Control or 5 nM si-TAK1 and 5 nM si-TRAF6 for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 and 10 μM p38i for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-mTOR, anti-phospho-S2448-mTOR, anti-phospho-S757-ULK1, anti-ULK1, anti-phospho-S555-ULK1, anti-AMPKα, anti-phospho-T172-AMPKα, and anti-GAPDH antibodies. The P-mTOR/mTOR and phospho-S555-ULK1/ULK1, and P-AMPK/AMPK ratios were quantified using QuantityOne software and the graphs (n=3 ± SD) are located to the right of representative immunoblots. Significance is indicated as *=P<0.05, **=P<0.01, and ***=P<0.001.

(H) H1299 cells were transfected with 10 nM si-Control or a second 5 nM si-TAK1 and a second 5 nM si-TRAF6 for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 and 10 μM p38i for 24 hours, lysed, subjected to SDS-PAGE, and immunoblotted for anti-mTOR, anti-phospho-S2448-mTOR, anti-ULK1, anti-phospho-S555-ULK1, and anti-GAPDH antibodies. The P-mTOR/mTOR and phospho-S555-ULK1/ULK1 ratios were quantified using QuantityOne software and the graphs (n=3 ± SD) are located to the right of representative immunoblots. Significance is indicated as *=P<0.05.
Since these results suggested that AMPKα activity may not be necessary for TGFβ1-dependent autophagy, I inhibited AMPKα activity in A549 cells using Compound C, which was verified when Compound C decreased the P-AMPKα/AMPKα ratios. I also observed that Compound C increased steady-state LC3B-II levels and P-ULK1/ULK1 ratios but increased the GFP/RFP ratio (Fig. 3.18A). To confirm, the importance of AMPKα activity on autophagic flux in A549 cells, I used fluorescence microscopy and observed that Compound C increased basal autophagy but had no impact on TGFβ1-dependent autophagy, which opposed the western blot results (Fig. 3.18B). Therefore, AMPKα activity may not be necessary for TGFβ1-dependent autophagy in these NSCLC cell lines.
Figure 3.18: The effect of Compound C on TGFβ1-induced autophagy in NSCLC cell lines.

(A) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were treated with 10 μM Compound C or DMSO (vehicle control) in the presence and absence of 250 pM TGFβ1 for 24 hours. Cells were lysed, subjected to SDS-PAGE, and immunoblotted for anti-ULK1, anti-phospho-S555-ULK1, anti-AMPKα, anti-phospho-T172-AMPKα, anti-LC3B, and anti-GAPDH (loading control) antibodies. Quantitative analysis of steady-state LC3B-II protein levels and the GFP/RFP, P-AMPKα/AMPKα, and P-ULK1/ULK1 ratios are
shown below representative immunoblots (n=3 ± SD). Significance is indicated as *=P<0.05, **=P<0.01, ***=P<0.001, and ****=P<0.0001.

(B) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were treated as described above. Hoechst stain (blue) was added 10 minutes prior to imaging with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Bar = 10 µm. ImageJ was used to quantify the green and red pixel intensities, and the GFP/RFP ratio is shown below representative images (n=3 ± SD). Significance is indicated as **=P<0.01, ***=P<0.001, and ****=P<0.0001.

Finally, to assess if canonical and/or non-canonical pathways would induce the nuclear export of ULK1 in response to TGFβ1, I carried out confocal microscopy in cells treated with siRNAs targeting Smad4 or TAK1+TRAF6, in combination with a p38 MAPK inhibitor. I observed that perturbing either pathway inhibits TGFβ1-dependent ULK1 cellular re-localization from the nucleus (Fig. 3.19A, B).
Figure 3.19: The effect of TAK1-TRAF6-p38 MAPK pathway and Smad4 on the cellular distribution of ULK1.
(A) A549 cells were transfected with 10 nM si-Control or two different 10 nM siRNAs targeting Smad4 (si-Smad4) for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 for 24 hours, fixed, and stained with DAPI (blue) or anti-ULK1 (green). A 63x objective of a Nikon Eclipse Ti2 confocal microscope imaged the coverslips and ImageJ (version 2.0) quantified relative nuclear ULK1 intensity/Total ULK1 intensity, which are graphed below representative images (n=3 ± SD). Significance is indicated as **=P<0.01, ***=P<0.001, and ****=P<0.0001. Bar = 10 µm.

(B) A549 cells were transfected with 10 nM si-Control or two different 5 nM siRNAs against TAK1 (si-TAK1) or 5 nM siRNAs against TRAF6 (si-TRAF6) for 48 hours. The cells were incubated in the absence or presence of 250 pM TGFβ1 and 10 µM p38 MAPK inhibitor for 24 hours. The cells were then fixed and stained with DAPI (blue) or anti-ULK1 (green). A 63x objective of a Nikon Eclipse Ti2 confocal microscope imaged the coverslips and ImageJ (version 2.0) quantified relative nuclear ULK1 intensity/Total ULK1 intensity, which are graphed below representative images (n=3 ± SD). Significance is indicated as *=P<0.05 and **=P<0.01. Bar = 10 µm.

Taken together, my results show that TGFβ1 induces autophagy by increasing ULK1 activity, which is dependent on TGFβRI kinase activity, the canonical Smad4 signalling pathway, and the non-canonical TAK1-TRAF6-p38 MAPK signalling pathway (Fig. 3.20).
Based on the inhibitory strategies used in this study (indicated in blue), specific canonical and non-canonical TGFβ pathways were observed to regulate TGFβ1-dependent autophagy. Both pathways were observed to converge on ULK1 activity and were necessary for lysosomal-targeting of LC3B.
3.4 Discussion

In Chapter 2, I uncovered several aspects of TGFβ1-dependent autophagy in NSCLC cells, and observed that TGFβ1 increased ULK1, ATG9A, ATG16L1, and LC3 gene expression, but only the protein levels of LC3B-II and ULK1 were increased by TGFβ1. I also observed that LC3B-II protein levels are limited in measuring autophagy and therefore methods that investigate autophagic flux should be used to measure TGFβ1-dependent autophagy. Finally, I reported that siRNA-mediated ATG5/7 knockdown decreased TGFβ1-dependent autophagic flux in NSCLC cells. Therefore, although macroautophagy can be activated independently of ATG5/7 or ULK1 activity, my results suggest that the majority of autophagic degradation initiated by TGFβ1 is mediated by canonical macroautophagy.

Here, using pharmacological inhibitors and siRNA to target specific TGFβ1 signalling pathways, I mechanistically characterized TGFβ1-dependent autophagy in two NSCLC cell lines. I observed that TGFβ1-dependent autophagy was diminished in the absence of Smad4 protein or the disruption of the TAK1-TRAF6-p38 MAPK pathway. Further analysis revealed that Smad4 knockdown did not alter P-mTOR/mTOR ratios, suggesting that it affects autophagy downstream of mTOR. Consistent with this hypothesis, I found that Smad4 upregulated AMPK-dependent ULK1 S555 phosphorylation. However, since Smad4 knockdown did not disrupt the increase of ULK1 protein levels, TGFβ1 may alter ULK1 expression or degradation via a Smad4-independent mechanism. Alternatively, the TAK1-TRAF6-p38 MAPK pathway may influence autophagy by impeding mTOR S2448 phosphorylation. This would explain why inhibiting the TAK1-TRAF6-p38 MAPK pathway increased the P-mTOR/mTOR ratio, decreased autophagic flux, and reduced the P-S555-ULK1/ULK1 ratio.

The link between Smad4 and TGFβ1-dependent autophagy that I observed in NSCLC cells was consistent with studies investigating TGFβ-dependent autophagy in pancreatic ductal adenocarcinoma cell lines and breast cancer cell lines. However, the role of Smad4 in TGFβ1-dependent autophagy is complex and remains an area that needs to be further investigated. This is because Smad4 was observed to not be essential for TGFβ1-dependent autophagy in Smad4 negative cell lines. Also, the presence of Smad4 may not...
be sufficient to drive TGFβ-dependent autophagy. For example, when I inhibited the TAK1-TRAF6-p38 MAPK pathway, Smad4 did not sustain TGFβ1-dependent autophagy in the NSCLC cell lines. Finally, there is some evidence to suggest that Smad4 impedes autophagy. For instance, in orthotopic pancreatic tissue samples, SMAD4 expression was inversely correlated to autophagy. Therefore, the role of Smad4 in TGFβ-dependent autophagy is likely cell type-dependent. In support of this, miRNA targeting of Smad4 in breast cancer cells attenuated autophagy whereas Smad4 depletion protected pancreatic cancer cells from radiotherapy by inducing autophagy. Less unclear is the importance of Smad4 in tumourigenesis. To date, SMAD4 is known as the most common Smad family gene mutated in cancer. Currently, more research is needed to characterize the relationship between Smad4, autophagy, and cancer to determine if Smad4 genetic targeting in cancer cells could impede tumourigenesis by hindering both TGFβ and autophagy-dependent drivers of cancer.

Since members of the TAK1-TRAF6-p38 MAPK pathway have been shown to affect autophagy, I decided to study this pathway in TGFβ1-dependent autophagy. A possible explanation for the lack of knowledge with respect to how it is involved in TGFβ1-dependent autophagy, is that this pathway is accessed by numerous stimuli. For instance, TAK1 is activated by tumour necrosis factors, toll-like receptors, interleukins, and TGFβ ligands prior to activating p38 MAPK and cJun amino-terminal kinase, which regulate metabolism, growth, survival, and tumourigenesis. Polyubiquitination and activation of TRAF6 is initiated by interleukins and toll-like receptors during innate proinflammatory responses; nucleotide-binding and oligomerization domain containing protein 2 receptors recognizing bacteria; recognition of viral RNAs; TGFβ receptors; receptor activator of nuclear factor kappa-B ligands during osteoclast differentiation; and several cell surface receptors on B-lymphocytes and T-lymphocytes. Therefore, due to the broad spectrum of stimuli that induce TAK1, TRAF6 or p38 MAPK activation, I knew little about their respective roles in autophagy and even less with regards to TGFβ1-dependent autophagy.

TAK1 functions as an upstream AMPK kinase by phosphorylating AMPKα at T172. For this reason, the increase in P-AMPKα/AMPKα ratio in cells subject to TAK1-TRAF6-p38 MAPK pathway inhibition was surprising. Since AMPK stimulates autophagy by phosphorylating ULK1 to form the ULK1 complex and by suppressing mTOR
activity\textsuperscript{344}, TAK1 has become a target of interest to suppress autophagy. For instance, TAK1 inactivation in mice has resulted in the accumulation of dysfunctional mitochondria in skeletal muscle\textsuperscript{345}. Furthermore, compared to their wild-type counterparts, mice with hepatocyte depletion of TAK1 developed hepatosteatosis due to autophagy suppression in which further analysis indicated that TAK1 depletion suppressed AMPK activity and increased mTOR activity. However, mTOR inhibition restored autophagy, therefore, consistent with my findings, TAK1 may influence autophagy at the level of or upstream of mTOR\textsuperscript{346}.

Experiments investigating TAK1 have highlighted a relationship between TGFβ signalling, autophagy, and cancer. For example, the genetic deletion of TAK1 blocked growth and migration of hepatocellular carcinoma\textsuperscript{346}. Likewise, TAK1 knockdown experiments attenuated tumour growth in xenograft models\textsuperscript{345,346}. One possible explanation for this is that TAK1 expression is positively correlated with mTOR expression and phosphorylation. Therefore, as the activity of TAK1 decreases, autophagic flux decreases and disrupts the tumour promoting properties of autophagy in cancer cells\textsuperscript{347}. In support of this, inhibition of TAK1 in Kras-dependent NSCLC cell lines induced apoptosis by inhibiting protective autophagy\textsuperscript{348}.

TRAF6 is an E3 ubiquitin ligase proven to be essential for toll-like receptor 4-dependent autophagy. TRAF6 stabilizes beclin 1 by conjugating it to lysine(K)63-linked polyubiquitin chains\textsuperscript{349}. Furthermore, TRAF6 in partnership with autophagy and beclin 1 regulator 1 tethers ULK1 to K63-linked polyubiquitin chains to promote its stability, self-association, and kinase activity\textsuperscript{350,351}. Interestingly, TRAF6 may be a suitable therapeutic target for the pro-tumourigenic properties of autophagy. For instance, peroxiredoxin 1, an antioxidant enzyme, was observed to inhibit TRAF6 ubiquitin-ligase activity, autophagy, and cancer cell migration\textsuperscript{352}. Additionally, blocking TRAF6 in mice models of cancer cachexia attenuated autophagy-dependent muscle wasting\textsuperscript{353}. For this reason, future work is needed to explore how silencing TRAF6 influences TGFβ-dependent autophagy and the pro-tumourigenic properties of TGFβ.

To date, p38 MAPK has been implicated in augmenting cancer cachexia by upregulating autophagy. For example, stimulating toll-like receptors in mice upregulated \textit{ATG6}, \textit{ATG7}, and \textit{ATG12} expression in a p38 MAPK-dependent manner to promote
muscle wasting. When p38 MAPK activity was blocked with SB202190, ATG genes were downregulated and mice were rescued from muscle wasting phenotypes\textsuperscript{354}. However, the role of p38 MAPK in autophagy is cell type-dependent. For instance, in microglial cells, after lipopolysaccharide stimulate toll-like receptors, p38 MAPK is activated and phosphorylates ULK1, which disrupts ULK1 from recruiting Atg13 and other components of the ULK1 complex\textsuperscript{355}. Furthermore, another study identified that when SB202190 blocked p38 MAPK activity, the p53-dependent apoptotic response is interrupted and autophagy was upregulated, which promoted cancer cell resistance to 5-fluorouracil\textsuperscript{356}. Recently, evidence has emerged that flavopereirine, a chemotherapeutic agent that decreases the proliferation and viability of cancer cells largely through unknown mechanisms, inhibited autophagy by upregulating the p38 MAPK pathway\textsuperscript{357}. Although these forms of autophagy are independent of TGFβ, they are still important to understanding a potential relationship between TGFβ, cancer, and autophagy.

In summary, TGFβ1 regulates autophagy using Smad4 and TAK1–TRAF6–p38 MAPK pathways to influence AMPK-dependent ULK1 S555 phosphorylation. Future work will evaluate how inhibiting autophagy and silencing Smad4 and the TAK1–TRAF6–p38 MAPK pathway impacts pro-tumourigenic properties of TGFβ.
Chapter 4

Autophagy regulates transforming growth factor-β signalling and receptor trafficking

Components of this chapter have been published in BBA. (2022) 1869 (9).
4 Summary

Transforming growth factor-β (TGFβ) signalling is regulated by the endocytosis of cell surface receptors and their subcellular trafficking into the endo-lysosomal system. Here, I investigated how autophagy, a cellular quality control network that delivers material to lysosomes, regulates TGFβ signalling pathways that induce epithelial-mesenchymal transition (EMT) and cell migration. I impaired autophagy in non-small cell lung cancer (NSCLC) cells using chloroquine, spautin-1, ULK-101 or small interfering RNA (siRNA) targeting autophagy-related gene (ATG)5 and ATG7 and observed that inhibiting autophagy results in a decrease in the expression of TGFβ1-dependent EMT-transcription factors as well as attenuated stress fiber formation and cell migration. This correlated with decreased internalization of cell surface TGFβ receptors and their trafficking to early/late endosomal and lysosomal compartments. The effects of autophagy inhibition on TGFβ signalling were investigated by Smad2/Smad3 phosphorylation and cellular localization using western blotting, subcellular fractionation, and immunofluorescence microscopy. I observed that inhibiting autophagy decreased the amount and timeframe of Smad2/Smad3 signalling. Taken together, my results suggest that inhibiting autophagy attenuates pro-tumourigenic TGFβ signalling by regulating receptor trafficking, resulting in impaired Smad2/Smad3 phosphorylation and nuclear accumulation.

4.1 Introduction

Aberrant TGFβ signalling leads to abnormal communication pathways that contribute to human diseases, such as cancer. Complications arise in multiple stages of TGFβ signalling, such as receptor internalization, trafficking, and degradation that can promote tumourigenesis. One of the mechanisms of tumourigenesis driven by aberrant TGFβ signalling is through the induction of EMT, where epithelial cells acquire mesenchymal properties that may lead to metastasis. Additionally, TGFβ signalling may augment tumourigenesis by upregulating macroautophagy, hereafter referred to as
autophagy, which promotes cancer cell survival in the presence of stressful stimuli, fulfils extensive metabolic needs, and removes damaged organelles and macromolecules\textsuperscript{248,253,260}.

TGFβ signalling is initiated with ligand binding to the extracellular domain of the constitutively activated TGFβ receptor type II (TGFβRII). This binding changes TGFβRII conformation to promote association with and phosphorylation of TGFβ receptor type I (TGFβRI). Once phosphorylated, TGFβRI phosphorylates receptor regulated Smads (R-Smads), such as Smad2 or Smad3, that are then released from the Smad anchor for receptor activation protein. The R-Smads form heterodimers with Smad4 that enter the nucleus to function as transcription factors or interact with other transcriptional machinery to modulate transcription\textsuperscript{7,32,95}. Furthermore, TGFβ signal transduction is dependent on subcellular trafficking of TGFβ receptors\textsuperscript{89,95}. The two internalization pathways for TGFβ receptors are clathrin-dependent and membrane raft-dependent, which promote TGFβ receptor signalling or receptor degradation, respectively\textsuperscript{81}. Clathrin-mediated endocytosis targets the TGFβRII/TGFβRI complexes to the early endosome, which may either recycle back to the cell membrane or develop into late endosomes that are degraded by lysosomes\textsuperscript{99}. Alternatively, in membrane rafts, Smad7 competes with R-Smads for TGFβRII binding and recruits Smad ubiquitin regulation factor 2 (Smurf2), an E3 ubiquitin ligase, that conjugates TGFβ receptors to ubiquitin chains to promote rapid receptor turnover via proteasomes\textsuperscript{359}.

In Chapter 3, I demonstrated that TGFβ1 induced autophagy through Smad4 and the non-canonical TGFβ-activated kinase 1-tumour necrosis factor receptor-associated factor 6-p38 mitogen-activated protein kinase (TAK1-TRAF6-p38 MAPK) pathways in NSCLC cells\textsuperscript{360}. Since the impact of TGFβ on autophagy has been characterized, I next assessed how autophagy impacts TGFβ signalling. Although a recent report suggested that disrupting autophagy impedes TGFβ-dependent processes in NSCLC\textsuperscript{251}, much is still unknown about the relationship between autophagy and TGFβ signal transduction. Here, I verified that inhibiting autophagy affected TGFβ1-induced E- to N-Cadherin shift, stress fiber formation, and cell migration. Furthermore, I investigated how autophagy regulated TGFβ1 signalling through the Smad pathway, TGFβ receptor internalization, and trafficking. Therefore, the purpose of this work was to characterize how inhibiting autophagy disrupts pro-tumourigenic TGFβ signalling.
4.2 Materials and Methods

Antibodies and reagents

Primary antibodies were purchased from the following commercial sources: anti-E-Cadherin (BD Transduction laboratories, 610182), anti-N-Cadherin (BD Transduction laboratories, 610921), anti-protein 62/sequestosome 1 (p62/SQSTM1; Cell Signalling Technology, 5114S), anti-microtubule-associated protein light chain 3B (LC3B; Cell Signalling Technology, 9236S), anti-ki67 (Santa Cruz, sc-23900), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Cell Signalling Technology, 2118S), anti-lamin A/C (Santa Cruz, sc-6125), anti-Phospho-Ser465/467-Smad2 (P-Smad2; Cell Signalling Technology, 3108L), anti-Smad2/3 (BD Transduction laboratories, 562586), anti-Smad3 (Cell Signalling Technology, 9513S), anti-Phospho-Ser243/245-Smad3 (P-Smad3; Cell Signalling Technology, 9520S), anti-Smad4 (Cell Signalling Technology, 38454S), anti-TAK1 (Cell Signalling Technology, 5206S), anti-TRAF6 (Cell Signalling Technology, 8028S), anti-Cleaved PARP (Cell Signalling Technology, 5625S), anti-TGFβRII (Santa Cruz, sc-17799), anti-TGFβRI (Santa Cruz, sc-518018), anti-human influenza hemagglutinin (HA; Cell Signalling Technology, 3724S), anti-autophagy-related protein 5 (Atg5; Cell Signalling Technology, 12994S), anti-autophagy-related protein 7 (Atg7; Cell Signalling Technology, 8558S), anti-Snail (Cell Signalling Technology, 3879S), anti-Slug (Cell Signalling Technology, 9585S) anti-lysosomal-associated membrane protein 1 (LAMP1; Cell Signalling Technology, 3243S), anti-early endosome antigen 1 (EEA1; BD Biosciences, 612007), anti-Caveolin-1 (Cell Signalling Technology, 3267S), and anti-Rab7 (Santa Cruz, sc-376362). Horseradish-peroxidase (HRP)-conjugated secondary goat anti-rabbit-IgG (Thermo Fisher Scientific, 31460), goat anti-mouse-IgG (Thermo Fisher Scientific, 31430), and donkey anti-goat-IgG (Thermo Fisher Scientific, PA1-28664) were used for western blot analysis. Fluorescently-conjugated donkey anti-mouse-IgG (Life Technologies, A21206) and donkey anti-rabbit-IgG (Life Technologies, A31572) were used for immunofluorescence studies. Two Human Ambion siRNA constructs were purchased from Thermo Fisher Scientific for si-ATG7 (s20652 and s20650), si-ATG5 (s18160 and s18159), si-Smad4 (s8403 and s8405), si-TAK1 (s13766 and s13767), and si-TRAF6 (s14389 and s14390) and these constructs were compared to a control siRNA (si-
All the siRNA mediated experiments in this study were carried out using both series of siRNAs. A p38 MAPK inhibitor (Calbiochem, 506126) impeded p38 MAPK activity whereas Dyngo-4a (Cayman Chemical, 1256493-34-1) inhibited dynamin. The pharmacological agents that decreased autophagic flux were specific potent autophagy inhibitor 1 (spautin-1; Sigma, SML0440), uncoordinated 51-like autophagy activating kinase-101 (ULK-101; Selleckchem, S8793), and chloroquine (acquired from the Shepherd lab, London, Canada). Rapamycin (Sigma Aldrich, 553210), a specific and potent mechanistic target of rapamycin (mTOR) inhibitor, was used to increase autophagy\textsuperscript{196}.

Cell culture and transfections

NSCLC cell lines, H1299 cells and A549 cells, were grown in Roswell Park Memorial Institute (RPMI; Corning, 10-043-CV) and Kaighn’s Modification of Ham’s F-12 (F-12K; Corning, 10-025-CV) media, respectively, supplemented with 10% fetal bovine serum (FBS). Mink lung (Mv1Lu) cells were grown in Minimum Essential Media (MEM; Corning, 10-009-CVR) supplemented with 10% FBS, non-essential amino acids, and 0.3 mg/mL hygromycin. Cells were cultured in a humidified tissue incubator at 37°C under 5% CO\textsubscript{2}. The cells were passaged by detaching them from the plates using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA), centrifuged at 1000x g for 2 minutes and resuspended in fresh media supplemented with 10% FBS. SiRNA knockdowns were mediated using opti-MEM media (Thermo Fisher Scientific, 51985091) and Lipofectamine RNAiMAX (Thermo Fisher Scientific, 13778150) as per the manufacturer’s protocol. Cells were treated with a receptor saturating dose of TGFβ1 (250 pM), 5 nM si-ATG5, 5 nM si-ATG7, 10 nM si-Smad4, 10 nM si-TAK1, 10 nM si-TRAF6, 10 nM p38 MAPK inhibitor, 25 µM Dyngo-4a, 50 µM chloroquine, 10 µM spautin-1, or 10 µM ULK-101.

Nuclear and cytoplasmic fractionation

The nuclear and cytoplasmic compartments of the cell were isolated using a NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Fisher Scientific, 78835) as per manufacturer’s protocol. Briefly, cells were detached using trypsin-EDTA and centrifuged at 1000x g for 2 minutes. The supernatant was aspirated, and the cell pellet was washed with 1x PBS and centrifuged at 1000x g for 2 minutes. Cytoplasmic extraction
reagents I and II were then added and samples were centrifuged (21 000x g at 4°C) for 5 minutes to separate nuclear protein (pellet) from the cytoplasmic protein (supernatant). Nuclear extraction reagent was then added to the pellet, vortexed, and centrifuged (21 000x g at 4°C) for 10 minutes to isolate nuclear proteins. The lysates from both the nuclear and cytoplasmic fraction were processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

**Immunoblotting**

Protein was isolated from cells using 50 mM Tris pH 7.5, 150 mM sodium chloride, 1 mM EDTA, 0.5% Triton X-100, 1 mg/mL pepstatin, 50 μM phenylmethylsulfonyl fluoride (PMSF), 2.5 mM sodium fluoride, and 10 mM sodium pyrophosphate phosphatase inhibitor (TNTE) lysis buffer for 20 minutes. The lysates were centrifuged at 21000x g at 4°C for 12 minutes. The protein concentrations were determined using the DC protein assay (Bio-Rad, 500-0116) and a Victor 3V Multi-Detection Microplate Reader (PerkinElmer, Waltham, MA, U.S.A). Cell lysates were processed for SDS-PAGE and approximately 50 μg of protein per sample was analyzed. Proteins were transferred onto a nitrocellulose membrane and membranes were blocked with 5% skim milk for 1-hour at room temperature. Primary antibodies were left on the membranes to incubate overnight, rocking at 4°C. On the following day, nitrocellulose membranes were incubated with the appropriate HRP-conjugated secondary antibody for 1-hour at room temperature, washed, and visualized using enhanced chemiluminescent substrate (Bio-Rad, 1705060) and a Versa-doc Imager (Bio-Rad, Hercules, CA, USA). Quantitation was carried out using QuantityOne 1-D analysis software.

**Antibody feeding/TGFβ receptor internalization analysis**

Co-localization of HA-TGFβRII with EEA1, Rab7, and LAMP1 was done using Mv1Lu cells that were stably transfected to over express pMEP4 with cDNA encoding HA-tagged TGFβRII under a zinc-inducible promoter. Cells were serum starved overnight using MEM supplemented with 0.2% FBS, 50 μM zinc chloride, chloroquine, spautin-1, ULK-101, and TGFβ1. The following day, cells were placed on ice for 10 minutes to interrupt receptor trafficking. Primary anti-HA antibody (1:250) was diluted in 0.2% FBS MEM and left to incubate on the cells for 2 hours on ice. The coverslips were
washed 3 times with 1x PBS, and then appropriate Alexa Fluor-conjugated secondary antibodies (1:250) were added and left to incubate on ice for 1-hour in the dark. Coverslips were washed another 3 times with 1x PBS before being put back to 37°C for 0-3 hours to induce trafficking. At the indicated times, coverslips were removed, fixed, permeabilized, and processed for confocal microscopy. A 63x objective of a Nikon Eclipse Ti2 (Nikon Instruments) confocal microscope imaged the coverslips and the co-localization plug-in of ImageJ (version 2.0) quantified relative percent HA-TGFβRII co-localization with EEA1, Rab7, and LAMP1.

**Immunofluorescence microscopy/stress fiber analysis**

Cells were washed with 1x PBS, fixed with 4% paraformaldehyde for 10 minutes, permeabilized with 0.25% Triton X-100 for 5 minutes, blocked with 10% FBS in 1x PBS for 1-hour, and incubated with primary (ki67 and Smad2/3) antibodies at 4°C overnight. The primary antibodies (1:100) were diluted using 10% FBS in 1x PBS. The appropriate fluorescent-probe-conjugated secondary antibodies (1:250) were incubated with the coverslips for 1-hour at room temperature. 4’,6-diamidino-2-phenylindole (DAPI) stained nucleic acids and Alexa Fluor-555 phalloidin (Invitrogen, A34055) was used to label filamentous actin. The coverslips were mounted on microscope slides using Immuno-Mount (Thermo Fisher Scientific, 9990402) and they were left at room temperature in the dark overnight. A 63x objective of an Olympus IX 81 inverted fluorescence microscope (Olympus, Canada) was used to image the cells. For quantitation of DAPI-Smad2 co-localization and ki67 staining, the images were analyzed using the co-localization and cell counter plug-in of ImageJ (version 2.0), respectively. Each data point represents quantitation from ≥ 100 cells from each condition.

**Isolating membrane raft lipid compartments**

H1299 cells were grown to 90% confluency in 10 cm dishes prior to treatment with pharmacological autophagy inhibitors or si-ATG5/7 for 24 hours. Cells were lysed in 0.5 M Na2CO3, pH 11.0 containing 1 mg/mL pepstatin, and 50 μM PMSF. The cell lysates were homogenized with two 15s bursts using a Polytron tissue homogenizer (Brinkmann Instruments) and sonicated twice for 15s using a Vibra Cell Sonicator (Sonics and Materials). The cell lysates were adjusted to 40% (w/v) sucrose, subjected to
ultracentrifugation, isolated into 12 fractions, and prepared for SDS-PAGE as previously described\textsuperscript{33}. Caveolin-1-positive fractions contained membrane rafts whereas EEA1-positive fractions represent non-raft membrane compartments. Fractions were pooled so that we could compare TGFβ receptor levels between EEA1 and Caveolin-1-rich membrane compartments using SDS-PAGE followed by immunoblotting.

\textit{Scratch migration assay}

Cells were grown to achieve a confluent monolayer, after which, a pipette tip was used to scrape a trench of cells. The cells were then treated with pharmacological autophagy inhibitors in the presence and absence of 250 pM TGFβ1 for 24 hours. Alternatively, cells were treated with siRNA targeting ATG5, ATG7, Smad4, TAK1 or TRAF6 for 24 hours prior to the scratch and TGFβ1 treatment. An Olympus IX 81 inverted fluorescence microscope imaged the cells using a 10x objective. Each treatment was imaged 3 times after the scratch and then they were imaged in the same locations 24 hours post-scratch. ImageJ (version 2.0) measured the proportion of the scratch width that diminished from cells migrating into the scratch area.

\textit{Transwell migration assay}

Cells were subjected to 24 hours of chloroquine or spautin-1 treatment with or without 250 pM TGFβ1. The cells were detached from the plates using 0.25% trypsin-EDTA, resuspended in serum free RPMI or F-12K media, and counted using a hemocytometer. Each well of a transwell plate with a pore size of 8 µm (Corning, CLS3464) received 25 000 cells suspended in 200 µL of serum free media. RPMI or F-12K media containing 10% FBS was added to the bottom chamber. After 24 hours, the transwells upper chambers were washed with 1x PBS, fixed with 4% paraformaldehyde for 10 minutes and stained with DAPI for 5 minutes. The membranes were cut and mounted onto microscope slides using Immu-Mount and a glass coverslip. An Olympus IX 81 inverted fluorescence microscope was used to image nuclei on the transwell membranes that were counted using ImageJ (version 2.0).

\textit{Statistical Analysis}

Statistical significance was evaluated using a Student’s t-test, one-way ANOVA
followed by Dunnett’s multiple comparisons test, or a two-way/three-way ANOVA followed by either Tukey’s or Sidak’s multiple comparison tests. Statistical analyses were performed using GraphPad Prism (Software 9.0) and P-values <0.05 were statistically significant.

4.3 Results

*Pharmacological modulators of autophagy disrupt TGFβRII internalization and trafficking*

The relationship between TGFβ receptor trafficking to the early-endosomal compartment and maximal induction of R-Smad phosphorylation has been well established[^9[^5[^6][^1][^2]]]. To assess the timeframe of TGFβ receptor trafficking, I followed the endocytosis of surface TGFβRII into the early- and late-endosomal compartments of Mv1Lu lung cells ([Fig. 4.1](#fig4)). To carry this out, I used a series of small molecule inhibitors of ‘early’ or ‘late’ autophagy that interestingly also affect steps within the endocytic pathway. ULK-101 inhibits the kinase activity of ULK1, which has been shown to alter the internalization of cell surface proteins[^3][^6][^3][^7][^3][^8]. Spautin-1 inhibits VPS34-dependent trafficking of cargo from the early endosome[^3][^5] and inhibits the formation of the beclin 1 complex in the nucleating phase of autophagy[^3][^4]. Chloroquine is an acidotropic agent that inhibits the acidification of the lumen of several vesicles/compartments within the endosomal/lysosomal system[^2][^3][^1][^0] ([Fig. 4.1A](#fig4)).

I followed the internalization of cell surface TGFβRII and observed that the maximum localization with the EEA1-positive early endosomal compartment occurred after 1-hour of endocytosis and decreased after 3 hours. Interestingly, in cells treated with chloroquine, spautin-1, or ULK-101 the majority of TGFβ receptors were visibly close to the cell periphery, consistent with an inhibition of the early events in the endocytic process. Indeed, although TGFβ receptors measurably co-localized with the EEA1 marker after 1-hour of internalization (26 ± 3% in the control cells), it was significantly reduced in the presence of all three inhibitors ([Fig. 4.1B](#fig4)). To follow TGFβRII trafficking to the late-endosomal membrane compartment, I investigated TGFβRII co-localization with the late-
endosomal marker, Rab7. In untreated control cells, TGFβRII internalized and formed partial yellow puncta with Rab7, suggesting co-localization of receptors in the late-endosomal compartment. I observed that the trafficking of TGFβRII into the Rab7-positive late endosomes, occurred predominately after 3 hours of endocytosis, and this was decreased in cells treated with chloroquine, spautin-1, and ULK-101. Additionally, the late endosomes in the chloroquine treatment groups appeared enlarged, which is consistent with a chloroquine dependent perturbation of late endosome-lysosome fusion (Fig. 4.1C).

Since trafficking of TGFβ receptors to the lysosome results in receptor degradation\textsuperscript{95}, I next assessed if TGFβ1, chloroquine, spautin-1 or ULK-101 affected the delivery of TGFβRII to LAMP1-positive lysosomes. After the cell surface receptors were labelled, I induced endocytosis by incubating the cells at 37°C for 3 hours and counterstained using anti-LAMP1 antibodies. TGFβ1 increased the amount of TGFβRII co-localizing with LAMP1; however, chloroquine, spautin-1, and ULK-101 decreased co-localization, which suggested that TGFβ1 increased lysosomal-mediated degradation of TGFβRII whereas the pharmacological modulators of autophagy decreased it (Fig. 4.1D). Given that pharmacological autophagy inhibitors impeded TGFβRII trafficking, I next examined how autophagy inhibition dampened TGFβRII endocytosis by investigating the role of autophagy on the localization of TGFβRII to membrane rafts.
C

0  1h  3h
DAPI  DAPI  DAPI
Rab7  Rab7  Rab7
TGFβRII  TGFβRII  TGFβRII
Overlay  Overlay  Overlay

Time (h)
0  1  3  1  3  1  3  1  3

TGFβRII-Rab7 co-localization (%)

0  10  20

Control  Chloroquine  Spautin-1  ULK-101

Chloroquine
1h  3h
DAPI  DAPI  DAPI  DAPI
Rab7  Rab7  Rab7  Rab7
TGFβRII  TGFβRII  TGFβRII  TGFβRII
Overlay  Overlay  Overlay  Overlay

Spautin-1
1h  3h
DAPI  DAPI  DAPI  DAPI
Rab7  Rab7  Rab7  Rab7
TGFβRII  TGFβRII  TGFβRII  TGFβRII
Overlay  Overlay  Overlay  Overlay

ULK-101
1h  3h
DAPI  DAPI  DAPI  DAPI
Rab7  Rab7  Rab7  Rab7
TGFβRII  TGFβRII  TGFβRII  TGFβRII
Overlay  Overlay  Overlay  Overlay
Figure 4.1: TGFβRII internalization and trafficking is regulated by autophagy.
(A) Schematic of how autophagy inhibitors (red) inhibit specific steps in endocytosis.

(B) Mv1Lu cells expressing HA-tagged TGFβRII were treated with 50 µM chloroquine, 10 µM spautin-1 or 10 µM ULK-101 for 12 hours. The cells were then placed at 4°C and incubated with an anti-HA antibody followed by Alexa Fluor-555-secondary antibody to fluorescently label cell surface TGFβ receptors (red). To stimulate receptor internalization and trafficking, the cells were incubated at 37°C for 0-3 hours in the absence or presence of chloroquine, spautin-1 or ULK-101. The cells were then fixed, permeabilized, and labelled with anti-EEA1 antibodies (green). After staining cells with DAPI to visualize nuclei (blue), micrographs were acquired using a Nikon Eclipse Ti2 confocal microscope (63x objective). ImageJ software (version 2.0) was used to quantify the area of co-localization between TGFβRII and EEA1 and graphed as % co-localization.

(C) Mv1Lu cells overexpressing HA-tagged TGFβRII were treated with 50 µM chloroquine, 10 µM spautin-1 or 10 µM ULK-101 and processed as described in Panel A, with the exception that the cells were counterstained using anti-Rab7 antibodies (green). Quantitation of TGFβRII co-localization with Rab7 graphed as % co-localization.

(D) Mv1Lu cells overexpressing HA-tagged TGFβRII were treated with 50 µM chloroquine, 10 µM spautin-1 or 10 µM ULK-101 for 12 hours. The cells were then placed at 4°C and incubated with an anti-HA antibody followed by Alexa Fluor-555-secondary antibody to fluorescently label cell surface TGFβ receptors (red). To stimulate receptor internalization and trafficking to degradative compartments, the cells were incubated at 37°C in the absence or presence of 250 pM TGFβ1 for 0-3 hours in the absence or presence of chloroquine, spautin-1 or ULK-101. The cells were then fixed, permeabilized, and labelled with anti-LAMP1 antibodies (green). After staining cells with DAPI to visualize nuclei (blue), micrographs were acquired using a Nikon Eclipse Ti2 confocal microscope (63x objective). ImageJ software (version 2.0) was used to quantify the area of co-localization between TGFβRII and LAMP1 from three independent experiment (mean ± SD) and graphed as % co-localization. In all panels, the scale bar = 10 µm. Significance was defined as *=P<0.05, **=P<0.01, ***=P<0.001, and ****=P<0.0001 with respect to no treatment controls.

Autophagy inhibition increases the proportion of TGFβ receptors within membrane rafts
Surface TGFβRII are localized in membrane raft or non-raft lipid domains. Membrane raft domains are enriched with Caveolae that facilitate endocytosis whereas non-raft membrane compartments rely on clathrin-dependent trafficking. Compared to TGFβRII internalized via clathrin-mediated endocytosis, TGFβRII trafficked via Caveolae are less active in TGFβ signal transduction. Therefore, the abundance of TGFβRII within membrane rafts may dictate the duration and strength of TGFβ signalling. For this reason, I next assessed if autophagy inhibition impacted the proportion of TGFβRII that resides in membrane rafts and non-raft cellular compartments. Briefly, H1299 cells were treated with chloroquine, ULK-101, spautin-1, and siRNA against ATG5 and ATG7 (si-ATG5/7) for 24 hours. Cells were then processed for sucrose density gradient ultracentrifugation and separated fractions were subjected to SDS-PAGE and immunoblotting for anti-EEA1 (non-raft marker) and anti-Caveolin-1 (membrane raft marker). The fractions containing EEA1 were pooled to represent non-raft lysates whereas the two fractions containing the strongest Caveolin-1 signal were pooled to represent membrane raft lysates (Fig. 4.2A-C). In response to chloroquine, ULK-101, spautin-1, or si-ATG5/7 treatment, I either observed a decrease or increase in the steady-state TGFβRII and TGFβRI protein levels in non-raft membrane compartments and membrane raft compartments, respectively. Overall, there was a decrease in the non-raft:raft ratios for both receptors (Fig. 4.3A-D). Since autophagy regulates endocytosis and the lipid domain localization of TGFβ receptors, I next assessed how autophagy inhibition effects TGFβ signal transduction compared to Dyngo-4a, a dynamin inhibitor that blocks clathrin-mediated endocytosis.
Figure 4.2: Separating membrane raft and non-raft lysate fractions.

A549 cells were treated with 50 µM chloroquine (A), 10 µM spautin-1, 10 µM ULK-101 (B), and 10 nM si-ATG5/7 (C) for 12 hours. Chloroquine treatments were compared to the
negative control, ULK-101, and spautin-1 treatments were compared to the dimethyl sulfoxide (DMSO) vehicle control whereas si-ATG5/7 was compared to si-Control. The cells were lysed, homogenized, and sonicated prior to ultracentrifugation in a sucrose density gradient. The cell lysates were separated into 12 fractions prior to being subjected to SDS-PAGE and immunoblotting using anti-EEA1 and anti-Caveolin-1 antibodies. Fractions 10 and 11 containing EEA1 and fractions 4 and 5 containing Caveolin-1 were pooled.

Figure 4.3: The effect of autophagy inhibition on TGFβRII and TGFβRI membrane raft localization.
A549 cells and H1299 cells were treated with 50 µM chloroquine (A), 10 µM ULK-101 (B), 10 µM spautin-1 (C), and 10 nM si-ATG5/7 (D) for 12 hours. The cells were lysed, homogenized, and sonicated prior to ultracentrifugation in a sucrose density gradient. The cell lysates were subjected to SDS-PAGE and immunoblotting using anti-Atg7, anti-Atg5, anti-EEA1, anti-caveolin-1, anti-TGFβRI, and anti-TGFβRII antibodies. Relative TGFβRII levels, non-raft:lipid raft TGFβRII ratios, TGFβRI levels, and non-raft:lipid raft TGFβRI ratios were graphed from 3 independent experiments (mean ± SD) and significance was defined as *=P<0.05, **=P<0.001, ***=P<0.001, and ****=P<0.0001.

Blocking autophagy damps maximal induction of R-Smad phosphorylation

Smad2 and Smad3 regulation is coordinated at the level of TGFβ receptors and within the cytoplasm and nucleus. Indeed, the nucleus contains phosphatases, kinases, E3 ubiquitin ligases, and other proteins that lead to the nuclear export and/or degradation of phosphorylated R-Smads. Since maximal induction of R-Smad phosphorylation occurs early in the TGFβ signal transduction pathway, Dyngo-4a-, spautin-1-, ULK-101-, chloroquine-, or si-ATG5/7-treated A549 cells or H1299 cells were incubated with TGFβ1 for 1-hour. Cells were lysed, subjected to SDS-PAGE, and immunoblotted for R-Smad phosphorylation. In A549 cells, Dyngo-4a and ULK-101 were observed to reduce TGFβ-dependent Smad2 and Smad3 phosphorylation and chloroquine dampened Smad3 phosphorylation (Fig. 4.4A). Alternatively, in H1299 cells, Dyngo-4a, spautin-1, and ULK-101 decreased Smad2 phosphorylation, whereas only Dyngo-4a and ULK-101 decreased Smad3 phosphorylation (Fig. 4.4B). Next, I assessed siRNA targeting of ATG5/7 on maximal induction of R-Smad phosphorylation and observed that Dyngo-4a and both combinations of si-ATG5/7 dampened Smad2 and Smad3 phosphorylation in both cell lines (Fig. 4.4C, D). ULK-101 and si-ATG5/7 treated cells provided evidence for the importance of autophagy during maximal R-Smad phosphorylation. However, due to neither spautin-1 nor chloroquine impacting maximal R-Smad phosphorylation, I next assessed if these pharmacological autophagy inhibitors influence the duration of TGFβ1 signalling.
Figure 4.4: The effect of autophagy inhibition on R-Smad phosphorylation.

A549 cells (A) and H1299 cells (B) were treated with 25 μM Dyngo-4a, 10 μM spautin-1, 50 μM chloroquine or 10 μM ULK-101 for 24 hours in the presence and absence of 250
pM TGFβ1 for 1-hour. Cells were lysed and subjected to SDS-PAGE and immunoblotting using anti-P-Smad2, anti-Smad2, anti-P-Smad3, anti-Smad3, and anti-GAPDH (loading control) antibodies. Steady-state P-Smad2, Smad2, P-Smad3, and Smad3 levels were quantified, and the ratios are shown graphically beside the representative immunoblots. The data were graphed from 4 independent experiments (mean ± SD). Significance is indicated as *=P<0.05, **=P<0.01, ***=P<0.001, and ****=P<0.0001.

A549 cells (C) and H1299 cells (D) were treated with 25 µM Dyngo-4a, 10 nM si-Control, and 5 nM of two different siRNAs against ATG5 or ATG7 for 24 hours with and without 250 pM TGFβ1 for 1-hour. Cells were lysed and subjected to SDS-PAGE and immunoblotting using anti-P-Smad2, anti-Smad2, anti-P-Smad3, anti-Smad3, anti-Atg7, anti-Atg5, and anti-GAPDH antibodies. Steady-state P-Smad2, Smad2, P-Smad3, and Smad3 levels were quantified, and the ratios are shown graphically beside the representative immunoblots. The data were graphed from 4 independent experiments (mean ± SD). Significance is indicated as ****=P<0.0001.

Disrupting autophagy impedes TGFβ1-dependent R-Smad phosphorylation and increases Phospho-Smad turnover

Based on the observation that the maximal P-Smad2/Smad2 and P-Smad3/Smad3 ratios were reduced by autophagy inhibitors, I next investigated if autophagy regulates the time course of R-Smad phosphorylation. For this investigation, I incubated cells for 1-hour with TGFβ1, which induced robust Smad2 and Smad3 phosphorylation, followed by a washout, where cells were then lysed after a time course of 0-8 hours. In A549 cells, I observed that Dyngo-4a, ULK-101, and si-ATG5/7 dampened Smad2 and Smad3 phosphorylation as all experimental time points had lower P-Smad2/Smad2 and P-Smad3/Smad3 ratios with respect to the control cells. Likewise, in H1299 cells, Dyngo-4a, ULK-101, and si-ATG5/7 decreased Smad2 phosphorylation at all experimental time points. Although Dyngo-4a and si-ATG5/7 decreased Smad3 phosphorylation at all experimental time points, ULK-101 decreased the P-Smad3/Smad3 ratio 2 hours post-TGFβ1 treatment in H1299 cells (Fig. 4.5A-C). Chloroquine decreased the P-Smad2/Smad2 ratio compared to control cells 6 hours and 8 hours post-TGFβ1 treatment in A549 cells and 8 hours post-TGFβ1 treatment in H1299 cells. Furthermore, chloroquine
decreased the P-Smad3/Smad3 ratio compared to control cells 8 hours post-TGFβ1 treatment in A549 cells and 6 hours and 8 hours post-TGFβ1 treatment in H1299 cells (Fig. 4.5D). Similarly, spautin-1 reduced the P-Smad2/Smad2 and P-Smad3/Smad3 ratios compared to control cells 6 hours and 8 hours post-TGFβ1 treatment in A549 cells and 8 hours post-TGFβ1 treatment in H1299 cells (Fig. 4.5E). To summarize, inhibitors of autophagy decreased relative P-Smad2/Smad2 and P-Smad3/Smad3 ratios post-TGFβ1 treatment by either impeding maximal R-Smad phosphorylation and/or increasing the rate of P-Smad2 or P-Smad3 loss.
Figure 4.5: The effect of autophagy inhibition on P-Smad turnover.
A549 cells and H1299 cells were pre-treated with 25 μM Dyngo-4a (A), 10 μM ULK-101 (B), 10 nM si-ATG5/7 (C), 50 μM chloroquine (D) or 10 μM spautin-1 (E) followed by a 1-hour treatment of 250 pM TGFβ1. The cells were then washed and chased with chloroquine, spautin-1, ULK-101 or si-ATG5/7 for an additional 0-8 hours. Cell lysates were subjected to SDS-PAGE and immunoblotting using anti-P-Smad2, anti-Smad2, anti-P-Smad3, anti-Smad3, anti-Asg7, anti-Asg5, and anti-GAPDH (loading control) antibodies. Steady-state P-Smad2, Smad2, P-Smad3, and Smad3 levels were quantified, and the ratio is shown graphically below the representative immunoblots. The data represents 3 independent experiments (mean ± SD). Significance was defined as *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001.

Unlike autophagy inhibitors, blocking TGFβ1-dependent autophagy had no effect on long term TGFβ1 signalling

Given that decreasing autophagy disrupts maximal R-Smad phosphorylation or increases P-Smad2 or P-Smad3 turnover, I next investigated if autophagy inhibitors affect the extent of TGFβ1 signalling, which was assessed by R-Smad phosphorylation after 24 hours of TGFβ1 stimulation. A549 cells and H1299 cells were treated with chloroquine, spautin-1, and ULK-101 for 24 hours in the presence and absence of TGFβ1 prior to lysis and immunoblotting for R-Smad phosphorylation (Fig. 4.6). In A549 cells, I observed that chloroquine, spautin-1, and ULK-101, decreased the proportion of Smad2 and Smad3 phosphorylation after 24 hours of TGFβ1 treatment (Fig. 4.6A). However, these pharmacological autophagy inhibitors only decreased the proportion of Smad2 phosphorylation in H1299 cells (Fig. 4.6B). Similarly, siRNA targeting of ATG5/7 decreased Smad2 and Smad3 phosphorylation in A549 cells (Fig. 4.6A) but only dampened Smad2 phosphorylation in H1299 cells (Fig. 4.6B).

Given that Smad4 and TAK1-TRAF6-p38 MAPK facilitate TGFβ1-dependent autophagy, I next investigated if disrupting TGFβ1-dependent autophagy impacted R-Smad phosphorylation. Interestingly, I found that si-Smad4 increased the proportion of Smad2 phosphorylated in both cell lines and Smad3 phosphorylated in A549 cells, whereas blocking the TAK1-TRAF6-p38 MAPK pathway with si-TAK1, si-TRAF6, and a p38 MAPK inhibitor had no impact on R-Smad phosphorylation (Fig. 4.6A, B). To assess the
inhibition of the p38 MAPK branch, I investigated PARP cleavage, as p38 MAPK is essential during TGFβ-dependent apoptosis. I observed lower cleaved PARP steady-state levels, which indicated that the p38 MAPK inhibitor was indeed functional. Taken together, these results suggest that disrupting autophagy suppresses extended R-Smad phosphorylation; however, this may be independent of TGFβ1 signalling pathways that induce autophagy. Given that the nuclear translocation of R-Smads occurs downstream of phosphorylation, I next assessed the effect of autophagy inhibitors on R-Smad nuclear translocation.
Figure 4.6: Pharmacological inhibition of autophagy decreases the P-Smad/Smad ratios in NSCLC cells.
A549 cells (A) and H1299 cells (B) were treated with 50 μM chloroquine, 10 μM spautin-1, 10 μM ULK-101, 10 nM si-ATG5/7, 10 nM si-Smad4, 10 nM si-TAK1, 10 nM si-TRAF6, and 10 μM p38 MAPK inhibitor (p38 MAPK i) for 24 hours in the presence or absence of 250 pM TGFβ1. Cells were lysed, subjected to SDS-PAGE, and immunoblotted using anti-P-Smad2, anti-Smad2, anti-P-Smad3, anti-Smad3, anti-Atg7, anti-Atg5, and anti-GAPDH (loading control) antibodies. Steady-state P-Smad2, Smad2, P-Smad3, and Smad3 levels were quantified, and the ratios are shown graphically beside the representative immunoblots. The data were graphed from 4 independent experiments (mean ± SD). Significance is indicated as *=P<0.05, **=P<0.01, ***=P<0.001, and ****=P<0.0001.

**Autophagy inhibition decreases nuclear protein levels of R-Smads**

Since R-Smad phosphorylation precedes translocation into the nucleus to affect gene transcription\(^5^0\), I used subcellular fractionation to investigate the impact of modulating the strength vs. duration of R-Smad phosphorylation on its nuclear accumulation. A549 cells and H1299 cells were treated with spautin-1, chloroquine or ULK-101 for 24 hours in the presence of TGFβ1 for 0-24 hours, and the cytoplasmic and nuclear fractions were isolated and subjected to SDS-PAGE and immunoblotting. I first assessed the autophagy inhibitors that only decreased the duration of TGFβ1-dependent R-Smad phosphorylation, chloroquine, and spautin-1. As expected, both chloroquine and spautin-1 inhibited TGFβ1-dependent nuclear Smad2 accumulation 24 hours after ligand administration (Fig. 4.7A-D). Surprisingly, I observed that spautin-1 reduced Smad2 protein accumulation even after 1-hour of TGFβ1 treatment in both NSCLC cell lines (Fig. 4.7A, B), despite this inhibitor not affecting Smad2 phosphorylation at this time point (Fig. 4.5E). Chloroquine decreased nuclear Smad2 levels 24 hours after TGFβ1 treatment in A549 cells (Fig. 4.7C) and 1 and 24 hours after TGFβ1 treatment in H1299 cells (Fig. 4.7D). Next, I investigated the modes of autophagy inhibition that dampened maximum R-Smad phosphorylation, ULK-101 and si-ATG5/7. Indeed, ULK-101 decreased nuclear Smad2 levels 1 and 24 hours after TGFβ1 treatment in A549 cells (Fig. 4.7E) and 24 hours after TGFβ1 treatment in H1299 cells (Fig. 4.7F). Finally, si-ATG5/7 reduced nuclear...
Smad2 protein levels after 1 and 24 hours of TGFβ1 treatment in both cell lines (Fig. 4.7G, H).

Figure 4.7: The effect that inhibiting autophagy has on the cellular distribution of Smad2.
A549 cells or H1299 cells were treated with 10 μM spautin-1 (A & B), 50 μM chloroquine (C & D), 10 μM ULK-101 (E & F), or 10 nM si-ATG5/7 (G & H) for 24 hours in the presence and absence of 250 pM TGFβ1 for 0, 1 or 24 hours. The cells were then lysed, fractionated into cytoplasmic and nuclear cell fractions, and subjected to SDS-PAGE and immunoblotted with anti-Smad2, anti-GAPDH (cytoplasmic marker), anti-Atg7, anti-Atg5, and anti-lamin A/C (nuclear marker) antibodies. The data were graphed from 3 independent experiments (mean ± SD) and significance was defined as *=P<0.05, **=P<0.01, ***=P<0.001, and ****=P<0.0001.

To visually assess the influence of autophagy inhibition on the nuclear levels of Smad2 and Smad3, I treated A549 cells and H1299 cells with chloroquine, spautin-1, ULK-101, and si-ATG5/7 for 24 hours, which was followed by 0, 1, and 24 hours of TGFβ1 treatment and carried out immunofluorescence microscopy. In A549 cells, I observed that spautin-1 and ULK-101 decreased the nuclear/cytoplasmic R-Smad ratio after 1 and 24 hours of TGFβ1 treatment with respect to TGFβ1-treated cells. Additionally, si-ATG5/7 reduced the nuclear/cytoplasmic R-Smad ratio after 1 and 24 hours of TGFβ1 treatment with respect to the control cells treated with TGFβ1. Chloroquine, on the other hand, decreased the nuclear/cytoplasmic R-Smad ratio after 24 hours of TGFβ1 treatment (Fig. 4.8A). In H1299 cells, I found that chloroquine and spautin-1 reduced the nuclear/cytoplasmic R-Smad ratio after 1 and 24 hours of TGFβ1 treatment with respect to the control. As observed with A549 cells, si-ATG5/7 reduced the nuclear/cytoplasmic R-Smad ratio after 1 and 24 hours of TGFβ1 treatment with respect to the si-Control. Lastly, ULK-101 decreased the nuclear/cytoplasmic R-Smad ratio after 24 hours of TGFβ1 treatment with respect to the control (Fig. 4.8B). In summary, after 1 and 24 hours or only 24 hours of TGFβ1 treatment, all autophagy inhibitors decreased the amount of fluorescently labelled Smad2 and Smad3 in the nucleus (Fig. 4.8), which suggests that autophagy inhibition impedes R-Smad nuclear translocation.
Figure 4.8: Examining if autophagy inhibition impacts nuclear R-Smad levels.

A549 cells (A) or H1299 cells (B) were treated with 10 μM spautin-1, 50 μM chloroquine, 10 μM ULK-101, or 10 nM si-ATG5/7 for 24 hours in the presence or absence of 250 pM TGFβ1. The cells were processed for immunofluorescence microscopy using anti-Smad2/3 (green) and DAPI (blue) to label Smad2 and Smad3 proteins and nuclei, respectively. Images were obtained with a 63x objective using an Olympus IX 81 inverted fluorescence
microscope. ImageJ (version 2.0) was utilized to calculate the relative nuclear:cytoplasmic Smad2 ratio using pixel intensity. This data was derived from 3 independent experiments (mean ± SD). Significance is indicated as *=P<0.05, **=P<0.01, ***=P<0.001, and ****=P<0.0001 with respect to the controls. Scale bars = 10 μm

*Decreasing autophagy suppresses TGFβ1-dependent EMT cell markers*

Having observed that autophagy regulates TGFβRII trafficking, R-Smad phosphorylation, and nuclear translocation, I next assessed how impeding autophagy and TGFβ1-dependent autophagy influenced pro-tumourigenic TGFβ1 signalling. For this reason, I examined the relationship between autophagy, E- to N-Cadherin shift, and EMT-transcription factors. Briefly, cells were treated with chloroquine, spautin-1 or ULK-101 in the presence and absence of TGFβ1 for 24 hours prior to lysis and immunoblotting (Fig. 4.9 & 4.10). I observed that Slug and Snail, EMT-transcription factors that modulate CDH1 and CDH2 expression, increased after TGFβ1 induction in both cell lines, and were inhibited by chloroquine (Fig. 4.9A & 4.10A). The gene products of CDH1 (E-Cadherin) and CDH2 (N-Cadherin) were also analyzed as E-Cadherin and N-Cadherin protein levels serve as markers for epithelial and mesenchymal cells, respectively. I observed that the TGFβ1-dependent loss of E-Cadherin in A549 cells was unaffected by chloroquine (Fig. 4.9A), and interestingly, H1299 cells did not contain detectable E-Cadherin levels by immunoblotting (Fig. 4.10). However, consistent with the A549 cells, chloroquine inhibited the TGFβ1-dependent increase in N-Cadherin protein levels in H1299 cells (Fig. 4.10A). In support of these findings, after analysis of spautin-1 and ULK-101 treated cells, I observed an inhibition of TGFβ1-dependent transcription factor production (Slug and Snail) as well as the E- to N-Cadherin shift in both cell lines (Fig. 4.9B, C & 4.10B, C).

To support my observations that pharmacological inhibition of autophagy impedes TGFβ1-dependent E- to N-Cadherin shift, I next assessed the influence of targeting ATG5 and ATG7 via siRNA. I investigated the influence of si-ATG5/7 on TGFβ1-dependent EMT via immunoblotting for E- or N-Cadherin, Slug, and Snail. As observed with the pharmacological inhibitors, ATG5 and ATG7 silencing had no affect on E-Cadherin protein levels in A549 cells, however, targeting ATG5/7 inhibited TGFβ1-dependent N-Cadherin, Slug, and Snail protein induction in both cell lines (Fig. 4.9D & 4.10D). These
results suggest that impeding autophagy interferes with TGFβ1-dependent expression of mesenchymal markers.

Next, I investigated the impact of silencing Smad4 and TAK1-TRAF6-p38 MAPK autophagy activating TGFβ1 signalling pathways on TGFβ1-induced E- to N-Cadherin shift and EMT markers. Here, the results were more nuanced, as silencing of Smad4 did not inhibit the TGFβ1-mediated loss of E-Cadherin or TGFβ1-dependent increase of N-Cadherin in A549 cell, but it did inhibit Slug and Snail expression (Fig. 4.9E). Interestingly, all three markers were inhibited in H1299 cells (Fig. 4.10E). Lastly, disrupting the TAK1-TRAF6-p38 MAPK pathway increased the basal levels of E-Cadherin expression in A549 cells but did not inhibit the TGFβ1-dependent loss of E-Cadherin in A549 cells or gain of N-Cadherin in both cell lines. Furthermore, disrupting the TAK1-TRAF6-p38 MAPK pathway blocked the TGFβ1-dependent induction of Slug and Snail in A549 cells but only affected Snail expression in H1299 cells (Fig. 4.9F & 4.10F). Taken together, these results suggest that impeding autophagy does not influence TGFβ1-mediated changes to E-Cadherin protein levels but blocks the increase of N-Cadherin, Slug, and Snail, which suggests that autophagy is required for the TGFβ1-dependent E- to N-Cadherin shift.
Figure 4.9: Decreasing autophagy impedes TGFβ1-mediated E- to N-Cadherin shift in A549 cells.
A549 cells were treated with 50 μM chloroquine (A), 10 μM spautin-1 (B), 10 μM ULK-101 (C), or 5 nM siRNA targeting ATG5 and ATG7 (si-ATG5/7) (D), 10 nM siRNA targeting Smad4 (si-Smad4) (E), and 5 nM siRNA targeting TAK1 (si-TAK1), 5 nM siRNA targeting TRAF6 (si-TRAF6), and 10 μM p38 MAPK inhibitor (p38 MAPK i) (F) in the presence or absence of 250 pM TGFβ1 for 24 hours. The cells were then lysed, subjected to SDS-PAGE, and immunoblotted using anti-E-Cadherin, anti-N-Cadherin, anti-Slug, anti-Snail, anti-Atg5, anti-Atg7, anti-Smad4, anti-TAK1, anti-TRAF6, anti-cleaved PARP or anti-GAPDH (loading control) antibodies. Quantitation of steady-state E-Cadherin, N-Cadherin, Snail, and Slug levels is shown graphically beside the representative immunoblots. The data were graphed from 4 independent experiments (mean ± SD). Significance is indicated as *=P<0.05, **=P<0.01, ***=P<0.001, and ****=P<0.0001.
Figure 4.10: Decreasing autophagy impedes TGFβ1-mediated E- to N-Cadherin shift in H1299 cells.
H1299 cells were treated with 50 μM chloroquine (A), 10 μM spautin-1 (B), 10 μM ULK-101 (C), or 5 nM siRNA targeting ATG5 and ATG7 (si-ATG5/7) (D), 10 nM siRNA targeting Smad4 (si-Smad4) (E), and 5 nM siRNA targeting TAK1 (si-TAK1), 5 nM siRNA targeting TRAF6 (si-TRAF6), and 10 μM p38 MAPK inhibitor (p38 MAPK i) (F) in the presence or absence of 250 pM TGFβ1 for 24 hours. The cells were then lysed and subjected to SDS-PAGE and immunoblotting using anti-E-Cadherin, anti-N-Cadherin, anti-Slug, anti-Snail, anti-Atg5, anti-Atg7, anti-Smad4, anti-TAK1, anti-TRAF6, anti-cleaved PARP or anti-GAPDH (loading control) antibodies. Quantitation of steady-state E-Cadherin, N-Cadherin, Snail, and Slug levels is shown graphically beside the representative immunoblots. The data were graphed from 4 independent experiments (mean ± SD). Significance is indicated as *=P<0.05, **=P<0.01, ***=P<0.001, and ****=P<0.0001.

**Dampening autophagy blocks TGFβ1-mediated stress fiber formation**

Since stress fiber formation is a characteristic of the TGFβ1-dependent EMT program, I next assessed how pharmacological inhibitors of autophagy affected stress fiber formation in A549 cells and H1299 cells. Using fluorescence microscopy, I determined the proportion of cells containing stress fibers by labelling filamentous actin with phalloidin and observed that chloroquine, spautin-1, and ULK-101 significantly decreased TGFβ1-dependent stress fiber formation in A549 cells (Fig. 4.11A) and H1299 cells (Fig. 4.11B). Next, I examined the influence of si-ATG5/7, si-Smad4, and blocking the TAK1-TRAF6-p38 MAPK pathway on TGFβ1-dependent stress fiber formation and observed that in both cell lines si-ATG5/7, si-Smad4, and TAK1-TRAF6-p38 MAPK pathway attenuation disrupted the formation of stress fibers in the presence of TGFβ1 (Fig. 4.11A, B). Therefore, TGFβ signalling pathways that activate autophagy are also necessary for TGFβ-dependent stress fiber formation. The results thus far suggested that autophagy inhibition may also affect TGFβ1-dependent cell migration, as cell migration is correlated to N-Cadherin protein levels and stress fiber formation.
Figure 4.11: Disrupting autophagy impairs TGFβ1-induced stress fiber formation.
A549 cells (A) and H1299 cells (B) were treated with 10 μM spautin-1, 50 μM chloroquine, 10 μM ULK-101, 10 nM si-Smad4, 10 nM si-ATG5/7, or 5 nM si-TAK1, 5 nM si-TRAF6, and 10 μM p38 MAPK inhibitor (p38 MAPK i) for 24 hours in the presence or absence of 250 pM TGFβ1. The cells were processed for fluorescence microscopy using Alexa Fluor-555 phalloidin (red) and DAPI (blue) to label filamentous actin and nuclei, respectively. Images were obtained with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. Cells containing stress fibers were counted and graphed. This data was derived from 3 independent experiments (mean ± SD). Significance is indicated as *=P<0.05, **=P<0.01, ***=P<0.001, and ****=P<0.0001 with respect to the controls. Scale bars = 10 μm

Decreasing autophagy blocks TGFβ1-dependent cell migration

To assess the effects of chloroquine, spautin-1, and ULK-101 on TGFβ1-dependent cell migration, I first employed a monolayer scratch wound healing assay using A549 cells and H1299 cells (Fig. 4.12). I observed that the TGFβ1-mediated increase in cell migration was significantly decreased by chloroquine, spautin-1, and ULK-101 treatments in A549 cells (Fig. 4.12A) and H1299 cells (Fig. 4.12B). Similarly, si-ATG5/7, si-Smad4, and TAK1-TRAF6-p38 MAPK pathway attenuation disrupted TGFβ1-dependent cell migration in A549 cells (Fig. 4.12A) and H1299 cells (Fig. 4.12B). In support of these findings, I assessed the influence of chloroquine and spautin-1 on TGFβ1-dependent cell migration in A549 cells and H1299 cells using transwell assays. Indeed, TGFβ1 increased the percentage of migrated cells, which was significantly reduced by chloroquine and spautin-1 treatments in A549 cells (Fig. 4.13A) and H1299 cells (Fig. 4.13B).
Figure 4.12: Inhibiting autophagy impairs TGFβ1-dependent cell migration.

Confluent monolayers of A549 cells (A) and H1299 cells (B) were scratched and treated with 10 μM spautin-1, 50 μM chloroquine, 10 μM ULK-101, 10 nM si-Smad4, 10 nM si-
ATG5/7, or 5 nM si-TAK1, 5 nM si-TRAF6, and 10 μM p38 MAPK inhibitor (p38 MAPK i) for 24 hours in the presence or absence of 250 pM TGFβ1. Images were acquired immediately after the scratch was made (0 h) and 24 hours (24h) post-scratch using a 10x objective of an Olympus IX 81 microscope and quantitated using ImageJ software. The data graphed represents 3 independent experiments (mean ± SD). Significance is indicated as *=P<0.05, **=P<0.01, ***=P<0.001, and ****=P<0.0001. Scale bars = 40 μm.
Figure 4.13: The effect of autophagy inhibition on cell migration.
A549 cells (A) or H1299 cells (B) were transferred into transwell inserts suspended in low serum (0.2% FBS) F-12K or RPMI media, respectively, with high serum (10% FBS) media placed in the bottom chamber. The cells were treated with 50 μM chloroquine or 10 μM spautin-1 in the presence or absence of 250 pM TGFβ1 for 24 hours. The transwell membranes were fixed, stained with DAPI, and mounted on microscope slides. Images were acquired with an Olympus IX 81 microscope using a 10x objective and nuclei were counted using ImageJ. Percent migration was determined for each treatment group and compared to Control (untreated cells). The quantitation represents 3 independent experiments (mean ± SD). Significance is indicated as *=P<0.05, **=P<0.01, ***=P<0.001, and ****=P<0.0001. Scale bars = 40 μm.

Next, I verified that these observations were not due to alterations in cell proliferation using ki67, a nuclear marker for proliferating cells. A549 cells were treated with TGFβ1, chloroquine, DMSO, spautin-1, ULK-101, si-Control, and si-ATG5/7 for 24 hours prior to fixation, permeabilization, and ki67 labelling. I observed that only chloroquine and TGFβ1 decreased the percentage of ki67-labelled cells, which suggested that chloroquine and TGFβ1 decreased cell proliferation (Fig. 4.14A). Given that spautin-1, ULK-101, and si-ATG5/7 had no effect on cell proliferation (Fig. 4.14B, C) in combination with TGFβ1 increasing cell migration, I concluded that autophagy inhibition decreased cell migration. Therefore, the results suggest that TGFβ1-dependent EMT, stress fiber formation, and cell migration of A549 cells and H1299 cells relies on autophagy. Furthermore, TGFβ1 signalling pathways that upregulate autophagy also induce E- to N-Cadherin shift, EMT-transcription factors, stress fibers, and cell migration.
Figure 4.14: The effect of autophagy inhibition on A549 cell proliferation.

A549 cells were treated with 250 pM TGFβ1 or 50 μM chloroquine (A); control siRNA (si-Control) or siRNA targeting ATG5/7 (si-ATG5/7; B); 10 μM spautin-1, 10 μM ULK-101 or equivalent volumes of dimethyl sulfoxide (DMSO) (C). The cells were then fixed, permeabilized, and labelled with anti-ki67 antibodies (green). After staining cells with DAPI to visualize nuclei (blue), micrographs were acquired using an Olympus IX 81 microscope (63x objective). The proportion of cells positive for ki67 was determined using imageJ (version 2.0) and graphed (n=3 ± SD). Significance is defined as ***P<0.001 compared to controls. Scale bars = 10 μm

*Increasing autophagy had no effect on TGFβ1 signalling*
Given that impeding autophagy affected TGFβRII trafficking, R-Smad phosphorylation, E- to N-Cadherin shift, stress fiber formation, and cell migration, I next assessed how increasing autophagy may influence these processes. Rapamycin, an inhibitor of mTOR, was utilized as an autophagy activator\textsuperscript{196}. Using Mv1Lu cells to assess TGFβRII trafficking, I observed that rapamycin had no significant effect on receptor internalization or trafficking (Fig. 4.15A). Next, I investigated the effect of rapamycin on R-Smad phosphorylation, E- to N-Cadherin shift, and autophagy markers. Although rapamycin decreased steady-state p62/SQSTM1 levels in the presence or absence of TGFβ, the mTOR activator had no affect on steady-state LC3B-II, E-Cadherin, and N-Cadherin or the P-Smad2/Smad2 ratio (Fig. 4.15B). Given that E-Cadherin to N-Cadherin shift was not impacted by rapamycin treatment, I assessed the impact of rapamycin on stress fiber formation and cell migration. Although rapamycin decreased basal stress fiber formation, it did not impede TGFβ1-dependent stress fiber formation (Fig. 4.15C). Furthermore, rapamycin had no influence on cell migration (Fig. 4.15D). Therefore, activating autophagy with rapamycin does not affect TGFβ1 signalling or TGFβ1-dependent outcomes.
Figure 4.15: The effect of rapamycin on TGFβ1 signalling.

(A) Mv1Lu cells expressing HA-tagged TGFβ receptor type II (TGFβRII) were treated with 1 μM rapamycin for 12 hours. The cells were then placed at 4°C and incubated with an anti-HA antibody followed by Alexa Fluor-555-secondary antibody to fluorescently label cell surface TGFβRII (red). To stimulate receptor internalization and trafficking, the
cells were incubated at 37°C for 1-hour in the absence or presence of rapamycin. The cells were then fixed, permeabilized, and labelled with anti-EEA1 antibodies (green). After staining cells with DAPI to visualize nuclei (blue), micrographs were acquired using an Olympus IX 81 inverted fluorescence microscope (63x objective). ImageJ software (version 2.0) was used to quantify the area of co-localization between TGFβRII and EEA1 and graphed as % co-localization. Scale bars = 10 μm

(B) A549 cells were treated with 1 μM rapamycin in the presence or absence of 250 pM TGFβ1 for 24 hours. The cells were then lysed and subjected to SDS-PAGE and immunoblotting using anti-E-Cadherin, anti-N-Cadherin, anti-p62, anti-P-Smad2, anti-Smad2, anti-LC3B or anti-GAPDH (loading control) antibodies. Quantitation of steady-state E-Cadherin, N-Cadherin, p62, LC3B-II, and P-Smad2/Smad2 are shown graphically beside the representative immunoblots. The data were graphed from 4 independent experiments (mean ± SD). Significance is indicated as *=P<0.05, **=P<0.01, and ****=P<0.0001.

(C) A549 cells were treated with 1 μM rapamycin for 24 hours in the presence or absence of 250 pM TGFβ1. The cells were processed for fluorescence microscopy using Alexa Fluor-555 phalloidin (red) and DAPI (blue) to label filamentous actin and nuclei, respectively. Images were obtained with a 63x objective using an Olympus IX 81 microscope. Cells containing stress fibers were counted and graphed. This data was derived from 3 independent experiments (mean ± SD). Significance is indicated as *=P<0.05 and ****=P<0.0001 with respect to the controls. Scale bars = 10 μm

(D) Confluent monolayers of A549 cells were scratched and treated with 1 μM rapamycin for 24 hours in the presence or absence of 250 pM TGFβ1. Images were acquired immediately after the scratch was made (0 h) and 24 hours (24h) post-scratch using a 10x objective of an Olympus IX 81 microscope and quantitated using ImageJ software. The data graphed represents 3 independent experiments (mean ± SD). Significance is indicated as *=P<0.05 and **=P<0.01. Scale bars = 40 μm.

Taken together, my results suggest that the inhibition of autophagy delays TGFβ receptor internalization and transit through the early- and late-endosomal system. This altered receptor trafficking may be the mechanism whereby the inhibition of autophagy
would dampen TGFβ1-dependent Smad2/3 phosphorylation and nuclear translocation, and result in attenuated EMT, stress fiber formation, and cell migration (Fig. 4.16).
Figure 4.16: Summary of the effects of autophagy inhibition on TGFβ1 signalling and outcomes.
TGFβ signalling is initiated when TGFβ ligands bind to TGFβ receptor type II (TGFβRII) that in turn phosphorylates TGFβ receptor type I (TGFβRI). The TGFβ-TGFβ receptor complexes are internalized and develop into early endosomes. Early endosomes fuse into late endosomes, which eventually are degraded via lysosomes. In the early endosomes, TGFβ-TGFβ receptor complexes phosphorylate Smad2/3 (S2/3). Phosphorylated S2/3 may then bind to Smad4 (S4) and translocate into the nucleus where they regulate transcription of genes that promote an E-Cadherin to N-Cadherin shift and stress fiber formation. Increasing N-Cadherin and stress fibers may then lead to an increase in cell migration.

Autophagy inhibition (red) impedes TGFβ-TGFβ receptor internalization and trafficking to early endosomes, late endosomes, and lysosomes. Autophagy inhibition also decreases S2/3 phosphorylation and nuclear translocation. As a result, autophagy inhibition blocks TGFβ-dependent outcomes including E-Cadherin to N-Cadherin shift, stress fiber formation, and cell migration.

4.4 Discussion

Since many tumours of epithelial origin rely on TGFβ signalling and autophagy\textsuperscript{151,152,251}, I examined the effect of blocking early vs. late events during the autophagic process on TGFβ receptor trafficking, TGFβ1-dependent R-Smad phosphorylation, modulation of EMT-transcription factors, EMT markers, and cell migration. Although previous work has shown a connection between autophagy and EMT\textsuperscript{251}, I sought to characterize how autophagy regulates early vs. late TGFβ signalling and outcomes.

As the majority of autophagic flux is mediated by macroautophagy\textsuperscript{375}, targeting autophagosome assembly and lysosomal fusion are the most common techniques utilized to decrease autophagic flux\textsuperscript{196}. Several pharmacological agents have been designed to impede protein complexes responsible for coordinating autophagosome lipid recruitment, Atg incorporation within autophagosome membranes, and autophagosome-lysosome fusion\textsuperscript{196,253,376}. Cells were treated with a late autophagic inhibitor, chloroquine, which is an acidotropic agent that impedes autophagosome-lysosome fusion and lysosomal degradation\textsuperscript{228,310}. To corroborate the effect of chloroquine on TGFβ1-dependent autophagy, I used two other autophagy inhibitors that target processes earlier on in the
pathway. The ULK1 and ULK2 kinase inhibitor, ULK-101, inhibits in the initiation step of macroautophagy, whereas spautin-1 inhibits the Beclin complex in the nucleation step of macroautophagy. Furthermore, I assessed the impact of siRNA mediated knockdown of genes essential to autophagy. The double knockdown of Atg5 and Atg7 is necessary for autophagy inhibition because together they regulate phagophore elongation and conjugate LC3B-I to a phosphatidylethanolamine to form LC3B-II.335

Although chloroquine, spautin-1, and ULK-101 are effective at disrupting autophagy, there are some limitations regarding their use. For instance, chloroquine impedes not just autophagy but non-degradative lysosomal functions, such as the regulation of energy metabolism and protein secretion.377 Furthermore, spautin-1 inhibits ubiquitin specific peptidase (USP) 10 and USP 13, which may increase the proteasome-dependent degradation of all proteins that rely on them to remove ubiquitin.313 Finally, when ULK-101 suppresses ULK1 and ULK2 activity, all ULK1 and ULK2 autophagy independent processes, such as lipid metabolism, cytokine secretion, and endoplasmic reticulum to Golgi apparatus anterograde transport may be impacted. To address this, I corroborated my findings with all three pharmacological autophagy inhibitors and ATG5/7 knockdown. Although ATG5/7-independent macroautophagy has been reported, in Chapter 2, I demonstrated that ATG5/7 knockdown significantly decreased autophagic flux in the presence and absence of TGFB1 using the same cell line systems.260

In this study, I have identified that autophagy plays a role in TGFB signal transduction. I first verified the previously reported relationship between TGFB and autophagy, in which disrupting autophagy blocks TGFB-induced EMT. Briefly, in TGFB1-treated A549 cells or H1299 cells, I observed an increase in N-Cadherin protein levels, EMT-transcription factor protein levels, stress fiber formation, and cell migration. These are all characteristics of mesenchymal cells, which indicated that TGFB1-dependent EMT was in process.36,44,152 Interestingly, autophagy inhibitors did not disrupt the TGFB1 mediated loss of E-Cadherin, which suggests that, in the presence of TGFB1, cells with compromised autophagy remain in a transition state between epithelial and mesenchymal phenotypes. Additionally, given that TGFB1 driven E-Cadherin was not impacted, future work is needed to differentiate the impact of autophagy on Cadherin trafficking versus Cadherin transcription.
Since R-Smads, such as Smad2 and Smad3, are activated upon phosphorylation and associate with other transcription factors\textsuperscript{40}, autophagy inhibition decreased the amount of active Smad-dependent transcription. Surprisingly, disrupting TGFβ1-dependent autophagy using si-Smad4 or TAK1-TRAF6-p38 MAPK pathway inhibition did not dampen R-Smad phosphorylation. In fact, si-Smad4 increased R-Smad phosphorylation. One potential explanation is that Smad4 binding to R-Smads triggers nuclear translocation, where they encounter several phosphatases that have been shown to dephosphorylate R-Smads\textsuperscript{382}.

Impeding autophagy dampened cellular processes downstream of phosphorylated R-Smads such as \textit{SNAI1} (Snail) and \textit{SNAI2} (Slug) transcription\textsuperscript{162,170,383}. This was evident when I observed that TGFβ1 increased the protein levels of Snail and Slug, which were reduced via autophagy inhibition. With lower levels of Snail and Slug, EMT would be unfavourable and thus cells would remain epithelial\textsuperscript{170,383}. These results led to further investigation using a time-course to assess Smad phosphorylation and turnover. Since chloroquine and spautin-1 increased P-Smad2/P-Smad3 turnover and ULK-101 and si-ATG5/7 decreased the extent of R-Smad phosphorylation, I concluded that the observed changes were likely due to alterations in Smad function. Indeed, cellular fractionation assays and immunofluorescence microscopy indicated that disrupting autophagy decreases the amount of nuclear R-Smad. As a result, autophagy may have been essential to the nuclear translocation of R-Smads. Given that Smad trafficking may be regulated by autophagy, I hypothesized that autophagy also regulates TGFβ receptor trafficking. If TGFβ receptor trafficking was altered, this would explain why autophagy inhibitors decrease P-Smad2 and P-Smad3 levels as Smad phosphorylation relies on receptor trafficking\textsuperscript{33,49,82,95}. Indeed, autophagy inhibitors increasing the proportion of TGFβ receptors within membrane rafts supported these findings.

One outstanding question that remains is how autophagy regulates upstream membrane raft retention of TGFβRII to reduce TGFβRII internalization and R-Smad phosphorylation. Autophagy inhibition either activates other protein degradation processes, such as proteasomes, to facilitate the selective degradation of TGFβ receptors that reside in non-raft membrane compartments or encourages the transfer of TGFβ receptors to membrane raft compartments. Additionally, future work needs to examine if
the effect of autophagy inhibition on membrane raft retention is specific to TGFβRII. This is important because membrane compartments impact receptor-ligand binding, receptor-internalization, receptor trafficking, and receptor fate.

Recently, it was reported that TGFβ-dependent EMT, migration, and proliferation can be blunted by proteasome inhibitors. Therefore, the proteasome, may influence TGFβ signalling similar to what has already been observed by autophagy. Indeed, the relationship between autophagy and proteasome activity is one of compensation. For instance, when proteasomes are old or damaged, aggregated proteins accumulate, which results in cell stress. To compensate for this, cells increase autophagic flux. As a result, tumour cells may respond to TGFβ by altering the balance between autophagic and proteasomal activities. Regardless, TGFβ signalling is dependent on autophagy and the proteasome, which are the two primary protein degradation pathways. Additionally, it has been reported that knockdown of endosomal sorting complex required for transport (ESCRT) machinery prolongs TGFβ signalling by extending the duration in which active TGFβ receptors remain in endosomes. Therefore, if ESCRT machinery is degraded via autophagy, altering autophagy could increase ESCRT machinery, which has been identified as a route for degrading TGFβ receptors.

It is not surprising that autophagy is involved with TGFβ-mediated cell communication, as both processes maintain cellular homeostasis and can affect tumourigenesis. For example, the protective functions of autophagy act as a double-edged sword in cancer because autophagy assists with resistance to metabolic, oxidative, and chemotherapeutic damage, which allows tumour cells to survive in unfavourable environments. Furthermore, in tumours, autophagy has been associated with EMT, increased cell motility, and metastasis, all of which are induced by TGFβ. For these reasons, autophagy and TGFβ are important for maintaining tumour cell survival and progressing benign tumours to advanced forms of the disease. Future studies aimed at understanding the relationship between TGFβ and autophagy will provide insight as to how tumours that selectively eliminate damaged proteins and organelles can evade chemotherapeutic toxicities and promote tumour cell invasion.
Chapter 5

Prolonged proteasome inhibition antagonizes TGFβ1-dependent signalling by promoting the lysosomal-targeting of TGFβRII

Components of this chapter have been published in Cell. Signal. (2022).
5 Summary

Impairing autophagy disrupts transforming growth factor-β1 (TGFβ1) signalling and epithelial-mesenchymal transition (EMT) in non-small cell lung cancer (NSCLC). Since autophagy and proteasome-mediated degradation are interdependent, I investigated how downregulating the catalytic activity of proteasomes influenced TGFβ1-dependent signalling and EMT. Proteasome-dependent degradation was inhibited in A549 and H1299 NSCLC cells using MG132 and lactacystin, which are reversible and irreversible proteasome inhibitors, respectively. After 1-hour of TGFβ1 treatment, proteasome inhibition decreased nuclear Smad2 and Smad3 levels. In the presence of TGFβ1, short-term proteasome inhibition increased receptor regulated Smad (R-Smad) phosphorylation and steady-state TGFβ receptor type II (TGFβRII) levels. However, prolonged proteasome inhibition decreased TGFβ1-dependent R-Smad phosphorylation and steady-state TGFβRII and TGFβ receptor type I (TGFβRI) levels. Prolonged proteasome inhibition also blunted E-Cadherin to N-Cadherin shift, stress fiber formation, and increased apoptotic signalling. Interestingly, TGFβ-activated kinase 1, tumour necrosis factor receptor-associated factor 6, and p38 mitogen-activated protein kinase mediated apoptosis during prolonged proteasome inhibition. In cells transfected with a pMRX-IP-GFP-LC3B-RFP-LC3BΔG construct, proteasome inhibition increased autophagic flux, steady-state microtubule-associated protein light chain 3B-II (LC3B-II) and active uncoordinated 51-like autophagy activating kinase 1 (ULK1) levels, and co-localization of lysosomes with autophagy cargo proteins and autophagy-related proteins (Atgs). Given that proteasome inhibition increased TGFβRII endocytosis to early/late endosomes and increased trafficking to lysosomes, I concluded that proteasome inhibition disrupted TGFβ signalling through the lysosomal-targeting of TGFβRII.

5.1 Introduction

The ubiquitin-proteasome pathway (UPP) and autophagy facilitate the bulk of protein degradation\textsuperscript{268}. In mammals, the most dominant proteasome utilized for the removal of misfolded, damaged, and superfluous proteins is the 26S proteasome. The 26S proteasome is predominately comprised of one 20S core particle and two 19S regulatory
Each 20S core particle is comprised of four stacked heptameric ring structures in which the outer and inner rings consist of α and β subunits, respectively. The α1-7 subunits of the two outer rings form gates to block access to unfolded proteins whereas the β1, β2, and β5 subunits of the two inner rings contain proteases that catalyze peptidylglutamyl-peptide hydrolysing (caspase)-like, trypsin-like, and chymotrypsin-like reactions, respectively, to break peptide bonds. Alternatively, the 19S regulatory particles bind to the α subunits of the core particle and contain an ATPase to facilitate protein unfolding, ubiquitin receptors to recognize ubiquitinated proteins, and a deubiquitinating enzyme to recycle ubiquitin.

Macroautophagy, hereafter referred to as autophagy, relies on Atgs to form double membrane vesicles called autophagosomes that sequester macromolecules and organelles prior to fusing with lysosomes. Autophagy functions as a protein quality control process that directs aggregated or damaged proteins toward lysosomes. Canonical autophagy activation depends on post-translational modifications to ULK1 that are mediated by mechanistic target of rapamycin (mTOR) and anti-5' adenosine monophosphate-activated protein kinase (AMPK), which downregulate and upregulate autophagy, respectively.

Protein degradation pathways play an intricate role in cell signalling. For example, TGFβ signalling is regulated by both autophagy and the UPP. Upon TGFβ binding to cell surface TGFβRII, TGFβRI is transphosphorylated and the TGFβ-TGFβ receptor complexes are endocytosed through two distinct pathways. Endocytosis mediated via caveolae antagonizes TGFβ signalling by targeting receptors for degradation. Alternatively, clathrin-mediated endocytosis targets TGFβ-TGFβ receptor complexes to early endosomes where TGFβRI phosphorylates R-Smads and non-Smad proteins to facilitate canonical and non-canonical TGFβ signalling, respectively. Cells then recycle TGFβ receptors or degrade them via proteasomes or lysosomes. Likewise, Smad proteins and downstream signalling molecules are subject to ubiquitin conjugation followed by proteasome-dependent degradation.

The interplay between cell signalling and proteasomal/lysosomal activity make protein degradation pathways a therapeutic target for tumourigenesis. Indeed, clinical studies report that proteasome inhibitors and autophagy inhibitors increase tumour cell susceptibility to radiotherapy, chemotherapy, and cell death. In NSCLC, TGFβ1 has
been shown to promote tumourigenesis by both upregulating autophagy and increasing mesenchymal/invasive properties through the activation of EMT\textsuperscript{260,394}. Although autophagy inhibition has been shown to attenuate TGF\(\beta\)I-dependent EMT in NSCLC\textsuperscript{331}, the influence of downregulating proteasomal activity on EMT is complex. In some instances, proteasome inhibitors have activated EMT\textsuperscript{395,396}; however, depending on the cell line, proteasome inhibitors may antagonize EMT\textsuperscript{280,397}. Since a relationship between autophagy, EMT, and TGF\(\beta\) signalling has been established, this work characterized how proteasomes regulate these processes in NSCLC cells.

5.2 Materials and Methods

Antibodies and reagents

Primary antibodies were as follows: anti-E-Cadherin (BD Transduction laboratories, 610,182), anti-N-Cadherin (BD Transduction laboratories, 610,921), anti-protein 62/sequestosome 1 (p62/SQSTM1; Cell Signalling Technology, 5114S), anti-p62/SQSTM1 (Abnova, H00008878-M01), anti-lysosomal-associated membrane protein 1 (LAMP1; Cell Signalling Technology, 3243S), anti-lamin A/C (Santa Cruz, sc-6125), anti-ki67 (Santa Cruz, sc-23900), anti-LC3B (Cell Signalling Technology, 9236S), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Cell Signalling Technology, 2118S), anti-TGF\(\beta\) receptor type III (TGF\(\beta\)RIII; Santa Cruz, sc-74511), anti-TGF\(\beta\)RII (Santa Cruz, sc-17799), anti-TGF\(\beta\)RI (Invitrogen, PA5-78198), anti-TGF\(\beta\)-activated kinase 1 (TAK1; Cell Signalling Technology, 5206S), anti-phospho-T181/184-TAK1 (P-TAK1; Cell Signalling Technology, 4531S), anti-stress-activated protein kinase/c-jun amino-terminal kinase (SAPK/JNK; Cell Signalling Technology, 9252), anti-phospho-T183/185-SAPK/JNK (P-SAPK/P-JNK; Cell Signalling Technology, 9251S), anti-P38 mitogen-activated protein kinase (P38 MAPK; Cell Signalling Technology, 9212S), Phospho-T180/Y182-P38 MAPK (P-P38 MAPK; Cell Signalling Technology, 9211S), anti-Cleaved PARP (Cell Signalling Technology, 5625S), anti-caspase 3 (Santa Cruz, sc-7272), anti-phospho-S465/467-Smad2 (P-Smad2; Cell Signalling Technology, 3108L), anti-Smad2/3 (BD Transduction laboratories, 562586), anti-Smad3 (Cell Signalling Technology, 9513S), anti-phospho-S243/245-Smad3 (P-Smad3; Cell Signalling Technology, 9520S), anti-early endosome antigen 1 (EEA1; BD Transduction laboratories,
610,182), anti-hemagglutinin (HA; Y-11; Santa Cruz, sc-805), anti-Rab7 (Santa Cruz, sc-376,362), anti-Slug (Cell Signalling Technology, 9585S), anti-Snail (Cell Signalling Technology, 3879S), anti-mTOR (Cell Signalling Technology, 2972S), anti-phospho-S2448-mTOR (P-mTOR; Cell Signalling Technology, 2971S), anti-AMPKα (Cell Signalling Technology, 2532S), anti-phospho-T172-AMPKα (P-AMPKα; Cell Signalling Technology, 50081S), anti-ULK2 (Santa Cruz, sc-292353), anti-ULK1 (Cell Signalling Technology, 8054S), and anti-phospho-S555-ULK1 (P-ULK1; Cell Signalling Technology, 5869S). Horseradish-peroxidase (HRP)-conjugated secondary goat anti-rabbit-IgG (Thermo Fisher Scientific, 31,460) and goat anti-mouse-IgG (Thermo Fisher Scientific, 31,430) were used for western blot analysis. Alexa Fluor-conjugated donkey anti-mouse-IgG (Life Technologies, A21206) and donkey anti-rabbit-IgG (Life Technologies, A31572) were used for immunofluorescence studies. Two different Human Ambion siRNA constructs were purchased from Thermo Fisher Scientific for each knockdown experiment. The siRNA targets included si-TAK1 and si-TRAF6 with the catalog number 4392420 and si-Control (4457289). MG132 (Sigma Aldrich, M7449) and lactacystin (Cayman Chemicals, 70,980) were used to inhibit the proteasome whereas chloroquine (acquired from the Shepherd lab, London, Canada) and specific potent autophagy inhibitor 1 (spautin-1; Sigma Aldrich, SML0440) inhibited autophagy. Rapamycin (Sigma Aldrich, R0395) inhibited mTOR to activate autophagy. A p38 MAPK Inhibitor (Calbiochem, 506126) disrupted p38 MAPK activity.

Cell culture

A549 cells and H1299 cells are NSCLC cell lines purchased from ATCC and cultured in Kaighn’s modification of F-12 (F-12K; Corning, 10-025-CV) and Roswell Park Memorial Institute (RPMI; Corning, 10-043-CVR), respectively, supplemented with 10% fetal bovine serum (FBS). Mink lung (Mv1Lu) cells were grown in Minimum Essential Medium (MEM; Corning) supplemented with 10% FBS, 0.3 mg/mL Hygromycin B (Invitrogen) and MEM non-essential amino acids (Gibco). Cells were cultured in a humidified tissue incubator at 37°C under 5% CO2. The cell lines were passaged using 0.25% of trypsin-ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, T2605), centrifuged at 1000× g for 3 min, and resuspended in fresh media supplemented with 10% FBS. TGFβ1-dependent experiments were serum starved overnight using media
supplemented with 0.2% FBS. Unless specified, cells were treated with 250 pM TGFβ1, 50 μM chloroquine, 10 μM spautin-1, 5 μM MG132, 5 μM lactacystin or 1 μM rapamycin.

**Immunoblotting**

Cell lysis, protein quantitation, sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting were performed as previously described\(^2\).\(^{20}\)

**Nuclear and cytoplasmic fractionation**

Nuclear and cytoplasmic compartments of A549 cells were isolated using a NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Fisher Scientific, 78835). A549 cells were detached using trypsin-EDTA and centrifuged at 1000x g for 3 minutes. Next, 200 μL of cytoplasmic extraction reagents I and 11 μL of cytoplasmic extraction reagent II were added to lyse the cell pellet. Cell lysates were vortexed for 15 seconds and samples were centrifuged (21 000x g at 4°C) for 5 minutes to separate nuclear protein (pellet) from the cytoplasmic protein (supernatant). The cytoplasmic fraction was transferred to a separate pre-chilled tube and the pellet was washed with 1x PBS. Next, 100 μL of the nuclear extraction reagent was added to the pellet, which incubated on ice for 40 minutes. Every 10 minutes the nuclear lysates were vortexed and after 40 minutes, centrifuged (21 000x g at 4°C) for 10 minutes to isolate nuclear proteins (supernatant). The lysates from both the nuclear and cytoplasmic fraction were processed for SDS-PAGE and immunoblotting.

**Coomassie brilliant blue staining**

A549 cells and H1299 cells were lysed using a lysis buffer containing 50 mM Tris pH 7.5, 150 mM sodium chloride, 1 mM EDTA, 0.5% Triton X-100, 1 mg/mL, pepstatin, 50 μM phenylmethylsulfonyl fluoride, 2.5 mM sodium fluoride, and 10 mM sodium pyrophosphate phosphatase inhibitor (TNTE). Cell lysates were prepared for SDS-PAGE using an 8x loading buffer (30% glycerol, 10% 1.5 M Tris (pH 6.8), 1.2% sodium dodecyl sulfate (SDS), 0.018% bromophenol blue, and 15% β-mercaptoethanol). Cell lysates were loading into a 10% polyacrylamide gel and run at 120 V for 1-hour. The gels were rocked with Brilliant Blue R-250 (BioShop, CBB250.25) for 1-hour and de-stained overnight at
room temperature using polyacrylamide gel de-staining solution (isopropanol and acetic acid). Gels were visualized using a Versa-doc Imager (Bio-Rad) and QuantityOne® 1-D Analysis software (Bio-Rad) was used to analyze the relative intensity of protein bands.

**Immunofluorescence, confocal, and phase contrast microscopy**

A549 cells and H1299 cells were rinsed with 1× phosphate buffer saline (PBS), fixed with 4% paraformaldehyde for 10 minutes, permeabilized with 0.25% Triton X-100 for 5 minutes, blocked with 10% FBS diluted in 1× PBS for 1-hour, and incubated with primary antibodies at 4°C overnight. The primary anti-p62/SQSTM1, anti-LAMP1, anti-Rab7, anti-ki67, anti-ULK1, anti-ULK2, and anti-EEA1 antibodies were used at a 1:100 dilution that were diluted using 10% FBS in 1× PBS. The appropriate fluorescent-probe-conjugated secondary antibodies at a 1:250 dilution were incubated with the coverslips for 1-hour at room temperature. A 1:1000 dilution of 10 mg/mL 4′,6-diamidino-2-phenylindole (DAPI) stained nucleic acids and a 1:250 dilution of Alexa Fluor-555 phalloidin stained filamentous actin after incubating with the sample for 5 and 20 minutes, respectively. The coverslips were mounted on pre-cleaned frosted microscope slides (Thermo Fisher Scientific, 9990402) using Immuno-mount (Thermo Fisher Scientific) and they were left at room temperature in the dark overnight. For live cell fluorescence microscopy, LysoTracker Deep Red (Invitrogen, L12492) and Hoechst stain (Invitrogen, H3569) labelled lysosomes and nuclei, respectively.

An Olympus IX 81 inverted fluorescence microscope (Olympus, Canada) or Nikon Eclipse Ti2 confocal microscope (Nikon Instruments) imaged the fluorescent probes. ImageJ (version 2.0) measured protein co-localization and performed cell counting functions. Each data point represents quantitation from ≥ 100 cells from each condition. Cell morphology was assessed using phase contrast microscopy. A Leica DM6000 B (Leica Microsystems, Germany) phase contrast microscope imaged A549 cells treated with MG132 or lactacystin in the presence and absence of TGFβ1 for 24 hours.

**Antibody feeding/TGFβ receptor internalization analysis**

Co-localization of HA-tagged TGFβRII (HA-TGFβRII) with EEA1, Rab7, and LAMP1 using Mv1Lu cells stably transfected to over express pMEP4 with cDNA encoding
HA-TGFβRII (HAT cells) under a zinc-inducible promoter was achieved as previously described\textsuperscript{394}.

**Autophagic flux assay**

As previously described\textsuperscript{260}, A549 cells and H1299 cells were transfected with a cDNA pMRX-IP-GFP-LC3B-RFP-LC3BΔG vector. After DMSO, MG132 or lactacystin treatment in the presence and absence of TGFβ1 for 24 hours, the GFP/RFP ratio was assessed via immunoblotting. The RFP-LC3BΔG, GFP-LC3B-I, and GFP-LC3B-II bands were labelled with LC3B specific antibodies, imaged using a Versa-doc, and quantified using QuantityOne® 1-D Analysis software.

**Statistical analysis**

An unpaired two-tailed Student’s T-test, one-way ANOVA followed by Dunnett’s multiple comparisons test, and two-way ANOVA followed by a Tukey’s multiple comparison test evaluated the statistical significance of the results. Statistical analysis was performed using GraphPad Prism (Software 9.0) and P-values <0.05 were statistically significant.

### 5.3 Results

**Inhibition of proteasome activity decreased nuclear R-Smad levels**

In Chapter 4, I found that autophagy inhibitors disrupted TGFβ1 signalling outcomes partly through decreasing the nuclear steady-state levels of R-Smad transcription factors\textsuperscript{399}. Since R-Smad nuclear translocation was regulated by autophagy, and autophagy and the UPP represent two primary protein degradation pathways\textsuperscript{272}, I assessed if R-Smad nuclear translocation is dependent on proteasome activity. A549 cells were treated with MG132 or lactacystin, which are reversible and irreversible antagonists of the 20S proteasome’s catalytic subunits, respectively\textsuperscript{312,400}, for 24 hours in the presence or absence of TGFβ1 for 0, 1 or 24 hours. The cytoplasmic and nuclear fractions were isolated, subjected to SDS-PAGE, and immunoblotted for anti-Smad2, anti-Smad3, anti-GAPDH (cytoplasmic marker), and anti-lamin A/C (nuclear marker). As previously reported, I observed that 1-hour TGFβ1 maximally increased nuclear R-Smad levels\textsuperscript{401}. Interestingly,
compared to dimethyl sulfoxide (DMSO) controls, neither MG132 nor lactacystin altered nuclear R-Smad levels after 0 and 24 hours of TGFβ1 treatment. However, in the presence of 1-hour TGFβ1, MG132 decreased the proportion of nuclear Smad2 and Smad3 and increased the proportion of Smad3 in the cytoplasmic fractions. Although lactacystin decreased nuclear Smad2 and Smad3 levels in the presence of 1-hour TGFβ1, only the effect on Smad2 was significant (Figure 5.1A). Next, I assessed the influence of proteasome inhibition on the nuclear levels of Smad2/3 using immunofluorescence microscopy. A549 cells were treated with MG132 or lactacystin for 24 hours in combination with 0, 1, and 24 hours of TGFβ1. The cells were fixed, permeabilized, blocked, and labelled with an anti-Smad2/3 antibody (green) and DAPI (blue) prior to imaging. Indeed, both MG132 and lactacystin decreased the maximal nuclear/cytoplasmic Smad2/3 ratio induced by 1-hour TGFβ1 treatment but had no effect after 0 or 24 hours of TGFβ1 (Figure 5.1B). These results suggest that proteasome inhibition may decrease maximal nuclear R-Smad levels by interfering with R-Smad nuclear translocation.
Figure 5.1: Examining if proteasome inhibition impacts nuclear R-Smad levels.

(A) A549 cells were treated with 5 μM MG132, 5 μM lactacystin or equivalent volumes of dimethyl sulfoxide (DMSO) for 24 hours. Cells were treated with 250 pM TGFβ1 for 0, 1 or 24 hours. The cells were then lysed, fractionated into cytoplasmic and nuclear cell fractions, and subjected to SDS-PAGE and immunoblotted with anti-Smad2, anti-Smad3, anti-GAPDH (cytoplasmic marker) and anti-lamin A/C (nuclear marker) antibodies. The
data were graphed from 3 independent experiments (mean ± SD) and significance was defined as * = P<0.05, ** = P<0.01, and **** = P<0.0001.

(B) A549 cells were treated with 5 μM MG132, 5 μM lactacystin or equivalent volumes of DMSO for 24 hours in the presence or absence of 250 pM TGFβ1. The cells were processed for immunofluorescence microscopy using anti-Smad2/3 (green) and DAPI (blue) to label Smad2/3 protein and nuclei, respectively. Images were obtained with a 63x objective using an Olympus IX 81 inverted fluorescence microscope. ImageJ (version 2.0) was utilized to calculate the relative nuclear: cytoplasmic Smad2 ratio using pixel intensity. This data was derived from 3 independent experiments (mean ± SD). Significance is indicated as * = P<0.05, ** = P<0.01, and **** = P<0.0001 with respect to the controls. Scale bars = 10 μm.

Temporal inhibition of proteasome activity alters TGFβ-dependent R-Smad phosphorylation

Given that R-Smad phosphorylation precedes and enhances nuclear translocation50,53, I next assessed if proteasome inhibitors regulate R-Smad phosphorylation. Previously, short-term inhibition of proteasome activity (< 6 hours) was shown to reduce TGFβ receptor degradation359 and should therefore enhance phospho-R-Smad levels402,403. However, I demonstrated that prolonged (>16 hours) proteasome inhibitor treatment may decrease the P-Smad2/Smad2 ratio260. I assessed the mechanism for these differences using a 0-24-hour time course of MG132 or lactacystin in combination with a 1-hour ± TGFβ1 incubation during the final hour (Figure 5.2A). Cells were then lysed and analyzed for R-Smad phosphorylation by western blotting (Figure 5.2B, C). I observed that both proteasome inhibitors had no significant effect on P-Smad2 or P-Smad3 levels in the absence of TGFβ1. However, TGFβ-dependent R-Smad phosphorylation was enhanced if cells were pre-incubated for 2-4 hours with either MG132 or lactacystin. Consistent with my previous findings260, 16 and 24 hours of MG132 or lactacystin incubation significantly decreased P-Smad2/Smad2 and P-Smad3/Smad3 ratios in both cell lines (Figure 5.2B, C). Next, I assessed if the impact of prolonged proteasome inhibitor treatment on R-Smad phosphorylation was concentration dependent. A549 cells and H1299 cells were treated with 0-10 μM MG132 or lactacystin for 24 hours and TGFβ1 during the final hour. Cells were lysed, subjected to SDS-PAGE, and immunoblotted for
anti-P-Smad2, anti-Smad2, anti-P-Smad3, anti-Smad3, and anti-GAPDH antibodies. Interestingly, in both cell lines, I observed that 1-10 μM of MG132 or lactacystin decreased maximum P-Smad2/Smad2 and P-Smad3/Smad3 ratios induced by TGFβ1 (Figure 5.3). These results suggest that independent of concentration, prolonged proteasome inhibitor treatment decreases R-Smad phosphorylation.

**Figure 5.2:** The effect of proteasome inhibitors on R-Smad phosphorylation is dependent on treatment duration.
(A) Schematic of experimental design.

(B) A549 cells and (C) H1299 cells were treated with 5 μM MG132, 5 μM lactacystin or vehicle control (DMSO) for 0-24 hours ± 250 pM TGFβ1 for the last hour of incubation. Cells were lysed and subjected to SDS-PAGE and immunoblotting for anti-P-Smad2, anti-Smad2, anti-P-Smad3, anti-Smad3, and anti-GAPDH (loading control) antibodies. The steady-state levels of P-Smad2/Smad2 and P-Smad3/Smad3 were quantified using QuantityOne software and graphed (n=3 ± SD). Significance is defined as *=P<0.05, **=P<0.01, ***=P<0.001, and ****=P<0.0001 compared to control (-).

Figure 5.3: The effect of the concentration of MG132 or lactacystin on R-Smad phosphorylation.

A549 cells and H1299 cells were treated with 1, 2.5, 5, and 10 μM MG132/lactacystin or equivalent volumes of dimethyl sulfoxide (DMSO) for 24 hours prior to a 1-hour 250 pM TGFβ1 treatment. Cells were lysed, subjected to SDS-PAGE, and immunoblotted for anti-P-Smad2, anti-Smad2, anti-P-Smad3, anti-Smad3, and anti-GAPDH (loading control) antibodies. The steady-state levels of P-Smad2/Smad2 and P-Smad3/Smad3 were quantified using QuantityOne software and graphed (n=4 ± SD). Significance is defined as *=P<0.05, **=P<0.01, ***=P<0.001, and ****=P<0.0001 compared to DMSO.

Prolonged inhibition of proteasomal activity promotes TGFβ receptor turnover
Given that the interactions between TGFβ1 and TGFβ receptors are necessary for R-Smad phosphorylation, decreasing TGFβ receptors could in turn reduce R-Smad phosphorylation\(^7\). I examined the possibility that proteasome inhibitors reduced steady-state TGFβ receptor levels by treating A549 cells and H1299 cells with MG132 or lactacystin in the presence and absence of TGFβ1 for 24 hours prior to lysis and immunoblotting for anti-TGFβRIII, anti-TGFβRII, anti-TGFβRI, anti-P-Smad2, anti-Smad2, anti-P-Smad3, anti-Smad3, and anti-GAPDH antibodies (Figure 5.4). In both cell lines, incubation with TGFβ1 decreased TGFβRII protein levels, increased the phospho-R-Smad/R-Smad ratios, and had no effect on TGFβRIII protein levels. Interestingly, TGFβ1 treatment increased TGFβRI protein levels in A549 cells, but not in H1299 cells (Figure 5.4A, B). When I incubated A549 cells with MG132 or lactacystin, I observed no effect on steady-state TGFβRIII protein levels, but they decreased TGFβRII and TGFβRI protein levels. Consistent with having fewer receptors in the presence of proteasome inhibitors, TGFβ-dependent P-Smad2/Smad2 and the P-Smad3/Smad3 ratios were lower compared to the TGFβ1 treatment (Figure 5.4A). As observed with A549 cells, MG132 or lactacystin decreased TGFβRII and TGFβRI protein levels, as well as the P-Smad2/Smad2 and the P-Smad3/Smad3 ratios in H1299 cells. However, unlike A549 cells, incubation with lactacystin reduced TGFβRIII protein levels in H1299 cells (Figure 5.4B).
Figure 5.4: Prolonged proteasome inhibition decreases steady-state TGFβ receptor levels and R-Smad phosphorylation in NSCLC cell lines.

(A) A549 cells and (B) H1299 cells were treated with 5 μM MG132 or 5 μM lactacystin in the presence and absence of 250 pM TGFβ1 for 24 hours. Equivalent volumes of dimethyl sulfoxide (DMSO) were the control for MG132 and lactacystin treatments. Cells were lysed and subjected to SDS-PAGE and immunoblotting for anti-TGFβRII, anti-TGFβRI, anti-TGFβRII, anti-P-Smad2, anti-Smad2, anti-P-Smad3, anti-Smad3, and anti-GAPDH antibodies. The steady-state levels of TGFβRII, TGFβRII, TGFβRI, P-Smad2/Smad2, and P-Smad3/Smad3 were quantified using QuantityOne software and graphed (n=4 ± SD). Unless specified, significance is defined as *=P<0.05, **=P<0.01, ***P<0.001, and ****P<0.0001 compared to the DMSO treatment for TGFβRII, TGFβRII and TGFβRI.
graphs and *=P<0.05, **P<0.01, and ****=P<0.0001 compared to the TGFβ1 treatment for the P-Smad2/Smad2 and P-Smad3/Smad3 graphs.

Having observed that steady-state TGFβ receptor levels were consistently decreased by prolonged proteasome inhibitor treatment in both NSCLC cell lines, I repeated the time course of proteasome inhibition (Figure 5.2A) to examine TGFβRIII, TGFβRII, and TGFβRI levels during short-term and prolonged proteasome inhibitor treatment (Figure 5.5). Consistent with the R-Smad phosphorylation time course (Figure 1), I observed that 2-4 hours of MG132 or lactacystin treatment increased steady-state TGFβRII levels, whereas 16-24 hours of proteasome inhibition significantly decreased TGFβRII levels in both cell lines (Figure 5.5A, B). Neither MG132 nor lactacystin significantly affected TGFβRIII or TGFβRI levels in A549 cells (Figure 5.5A). However, in H1299 cells, 24 hours of MG132 decreased TGFβRI levels whereas 24 hours of lactacystin decreased TGFβRIII and TGFβRI levels (Figure 5.5B). In both cell lines and in two separate experiments, TGFβRII was decreased by MG132 and lactacystin (Figure 5.5A, B). Therefore, I next assessed how proteasome inhibition decreased steady-state TGFβRII levels.
Figure 5.5: The effect of proteasome inhibitors on TGFβRII levels is dependent on treatment duration.

(A) A549 cells and (B) H1299 cells were treated with 5 μM MG132, 5 μM lactacystin or equivalent volumes of dimethyl sulfoxide (DMSO) for 0-24 hours in the presence and absence of 250 pM TGFβ1 for 1-hour. Cells were lysed and subjected to SDS-PAGE and immunoblotting with anti-TGFβRIII, anti-TGFβRII, anti-TGFβRI, and anti-GAPDH (loading control) antibodies. The steady-state levels of TGFβRIII, TGFβRII, and TGFβRI were quantified using QuantityOne software and graphed (n=3 ± SD). Significance is defined as #=P<0.05, ##=P<0.01, and ####=P<0.0001 compared to 0-hour control or *=P<0.05, **=P<0.01, ***=P<0.001, and ****=P<0.0001 compared to 1-hour TGFβ1.
Proteasome inhibition increased TGFβRII endocytosis to early and late endosomes and promoted TGFβRII-targeting to lysosomes

Given that Smad phosphorylation relies on TGFβRII internalization and endocytosis, and prolonged proteasome inhibition rapidly decreased P-Smad2, P-Smad3, and TGFβRII levels, I next assessed if proteasome inhibition would alter TGFβRII trafficking. Previously, using a Mv1Lu cell model expressing extracellular HA-tagged TGFβRII, I highlighted that cell surface TGFβRII internalized into early endosomes and late endosomes within 1-hour and 3 hours, respectively. Here, I used this model to monitor the impact of downregulating proteasomal activity on TGFβRII endocytosis. Mv1Lu cells were treated overnight with Zn²⁺ as well as DMSO, MG132 or lactacystin. Endocytosis was disrupted by placing the cells on ice for 10 minutes, which was followed by HA-TGFβRII labelling. HA-TGFβRII internalization was induced by incubating the cells at 37°C for 1 and 3 hours. First, I investigated the endocytosis of Mv1Lu cell surface HA-TGFβRII into the early-endosomal membrane compartment, which was identified using an EEA1 antibody. I observed that both MG132 and lactacystin increased HA-TGFβRII and EEA1 co-localization after 1 and 3 hours of HA-TGFβRII endocytosis compared to DMSO treated cells (Figure 5.6A). Furthermore, I observed that both MG132 and lactacystin increased HA-TGFβRII co-localization with Rab7-positive late endosome membrane compartments after 1 and 3 hours of HA-TGFβRII endocytosis (Figure 5.6B). If proteasome inhibitors initially increased the proportion of receptors endocytosed, which regulates the extent of R-Smad phosphorylation, this could explain the mechanism of how short-term proteasome inhibitor treatment increased the P-Smad2/Smad2 and P-Smad3/Smad3 ratios. Therefore, I next examined the mechanism of how prolonged proteasome inhibition reduces TGFβRII, P-Smad2/Smad2, and P-Smad3/Smad3.

Since trafficking to lysosomes represents one route of TGFβRII degradation, I assessed if MG132 and lactacystin affected the delivery of HA-TGFβRII to LAMP1-positive lysosomes in Mv1Lu cells. After the Mv1Lu cell surface HA-TGFβRII was labelled, I induced endocytosis by incubating the cells at 37°C for 3 hours and counterstained using anti-LAMP1 antibodies. I observed that MG132 and lactacystin increased HA-TGFβRII co-localization with LAMP1-positive lysosomes after 3 hours of
HA-TGFβRII endocytosis (Figure 5.6C). These results suggested that downregulating proteasome activity increased TGFβRII-targeting to the lysosomal compartment.

Figure 5.6: The proteasome regulates TGFβ receptor trafficking.
(A) Mv1Lu cells overexpressing HA-tagged TGFβRII (HAT cells) were treated with 5 μM MG132, 5 μM lactacystin or equivalent volumes of dimethyl sulfoxide (DMSO) for 12 hours. The cells were then placed at 4°C and incubated with an anti-HA antibody followed by Alexa Fluor-555-secondary antibody to fluorescently label cell surface HA-TGFβRII (red). HA-TGFβRII internalization and trafficking was stimulated by incubating the cells at 37°C for 0-3 hours in the presence of MG132, lactacystin or DMSO. The cells were then fixed, permeabilized, and labelled with anti-EEA1 antibodies (green). After staining cells with DAPI to visualize nuclei (blue), micrographs were acquired using a Nikon Eclipse Ti2 confocal microscope (63x objective). ImageJ software (version 2.0) was used to quantify the area of co-localization between HA-TGFβRII and EEA1, which was graphed (n=4 ± SD) as % co-localization. Significance is indicated as *=P<0.05, **=P<0.01, and ****=P<0.0001. Scale bars = 10 μm

(B) HAT cells were treated with 5 μM MG132, 5 μM lactacystin or equivalent volumes of DMSO and processed as described in Panel A, with the exception that the cells were counterstained using anti-Rab7 antibodies (green). Quantitation of HA-TGFβRII co-localization with Rab7 was performed using ImageJ (version 2.0) and graphed (n=4 ± SD) as % co-localization. Significance is indicated as **=P<0.01 and ****=P<0.0001. Scale bars = 10 μm

(C) HAT cells were treated with 5 μM MG132, 5 μM lactacystin or equivalent volumes of DMSO and processed as described in Panel A, with the exception that the cells were counterstained using anti-LAMP1 antibodies (green). Quantitation of HA-TGFβRII co-localization with Rab7 was performed using ImageJ (version 2.0) and graphed (n=4 ± SD) as % co-localization. Significance is indicated as *=P<0.05 and ***=P<0.001. Scale bars = 10 μm

Proteasome inhibitors increased the co-localization of autophagy-related proteins (Atgs) and autophagy cargo receptors with lysosomes

Since TGFβ receptor signalling and trafficking was integrally linked with TGFβ-dependent autophagy in Chapter 4, I next assessed if the altered TGFβ receptor trafficking that is observed during prolonged proteasome inhibition also affects TGFβ-dependent autophagy. To carry this out, I first examined the cellular distribution and co-
localization of lysosomes, autophagy cargo receptors (p62/SQSTM1) and Atgs (ULK1 and LC3B)\textsuperscript{196}.

To assess if MG132 and lactacystin altered the cellular localization of lysosomes, I treated A549 cells with MG132 or lactacystin for 24 hours prior to fixing, permeabilization, and labelling using anti-LAMP1 antibodies. I observed that MG132 and lactacystin increased the perinuclear localization of LAMP1-positive lysosomes (Figure 5.7A), which is a feature of cells that exhibit upregulated autophagy\textsuperscript{406}. To corroborate this finding, A549 cells were treated with MG132 or lactacystin for 24 hours prior to incubating with LysoTracker Deep Red and subjected to fluorescent live cell imaging. As with the LAMP1 staining, the LysoTracker staining was also perinuclear in live A549 cells (Figure 5.7B).

I next assessed the subcellular localization of ULK proteins, as I previously observed that ULK activity and cellular localization are altered by TGFβ-dependent autophagy initiation in A549 cells\textsuperscript{360}. Briefly, A549 cells were treated with MG132 or lactacystin for 24 hours prior to processing and labelling with anti-ULK1 and anti-ULK2 antibodies and visualization by immunofluorescence microscopy. As a positive control, I also subjected A549 cells to low serum, a known method to activate autophagy\textsuperscript{196}. Although there were no noticeable changes with ULK2 cellular distribution, I observed that MG132, lactacystin, and serum starvation increased the intensity of ULK1 puncta as well as its perinuclear localization (Figure 5.7C).

I next investigated p62/SQSTM1 because it is an autophagy cargo receptor protein that sequesters autophagic cargo prior to being incorporated and degraded by lysosomes\textsuperscript{407}. Briefly, after A549 cells were treated with MG132, lactacystin or low serum media for 24 hours, the cells were fixed, permeabilized, and labelled with anti-LAMP1 and anti-p62/SQSTM1 antibodies. I observed that proteasome inhibition and serum starvation both increased p62/SQSTM1-LAMP1 co-localization and redistributed p62/SQSTM1 to the perinuclear cellular region of cells (Figure 5.7D). Finally, A549 cells were treated with MG132, lactacystin or low serum media for 24 hours, and processed for immunofluorescence microscopy using anti-ULK1 and anti-p62/SQSTM1 antibodies. I
observed that proteasome inhibitors and serum starvation increased ULK1-p62/SQSTM1 co-localization (Figure 5.7E).

Figure 5.7: The effect of proteasome inhibitors on the cellular localization of lysosomes, autophagy cargo receptors, and Atgs.
(A) A549 cells were treated with 5 μM MG132, 5 μM lactacystin or equivalent volumes of dimethyl sulfoxide (DMSO) for 24 hours. The cells were then fixed, permeabilized, and labelled with anti-LAMP1 (green) antibodies. After staining cells with DAPI to visualize nuclei (blue), micrographs were acquired using an Olympus IX 81 inverted fluorescence microscope (63x objective). Scale bars = 10 μm

(B) A549 cells were treated with 5 μM MG132, 5 μM lactacystin or equivalent volumes of DMSO for 24 hours. LysoTracker Deep Red (red) and Hoechst (blue) were added to the cells 2 hours and 10 minutes, respectively, prior to imaging. Images were acquired using an Olympus IX 81 inverted fluorescence microscope (63x objective). Scale bars = 10 μm

(C) A549 cells were treated with 5 μM MG132, 5 μM lactacystin, equivalent volumes of DMSO or serum starved for 24 hours. The cells were then fixed, permeabilized, and labelled with anti-ULK1 (green) and anti-ULK2 (red) antibodies. After staining cells with DAPI to visualize nuclei (blue), micrographs were acquired using an Olympus IX 81 inverted fluorescence microscope (63x objective). ImageJ (version 2.0) quantified the amount of ULK1 puncta/cell, which was graphed (n=3 ± SD) below representative images. Significance is indicated as ***=P<0.001. Scale bars = 10 μm

(D) A549 cells were treated with 5 μM MG132, 5 μM lactacystin, equivalent volumes of DMSO or serum starved for 24 hours. The cells were then fixed, permeabilized, and labelled with anti-LAMP1 (green) and anti-p62 (red) antibodies. After staining cells with DAPI to visualize nuclei (blue), micrographs were acquired using an Olympus IX 81 inverted fluorescence microscope (63x objective). Quantitation of p62 co-localization with LAMP1 was performed using ImageJ (version 2.0) and graphed (n=3 ± SD) as % co-localization below representative images. Significance is indicated as *=P<0.05. Scale bars = 10 μm

(E) A549 cells were treated with 5 μM MG132, 5 μM lactacystin, equivalent volumes of DMSO or serum starved for 24 hours. The cells were then fixed, permeabilized, and labelled with anti-ULK1 (green) and anti-p62 (red) antibodies. After staining cells with DAPI to visualize nuclei (blue), micrographs were acquired using an Olympus IX 81 inverted fluorescence microscope (63x objective). Quantitation of p62 co-localization with ULK1 was performed using ImageJ (version 2.0) and graphed (n=3 ± SD) as % co-
localization to the right of representative images. Significance is indicated as **=P<0.01, ***=P<0.001, and ****=P<0.0001. Scale bars = 10 μm

Thus far, utilizing immunofluorescence microscopy in A549 cells suggested that autophagy may be activated by proteasome inhibition. To test this hypothesis, I investigated the impact of MG132 and lactacystin on LC3, a standard marker of autophagy. LC3B-II proteins are incorporated into autophagosomes and are proportional to the number of autophagosomes in a cell. Therefore, when LC3B-co-localizes with lysosomes, it suggests that autophagic vesicles are subject to lysosomal-dependent degradation. For this reason, I treated A549 cells stably expressing GFP-LC3B with MG132, lactacystin or equivalent volumes of DMSO for 8 hours prior to LysoTracker Deep Red and Hoechst incubation. As I previously observed post-24 hours of MG132, 8 hours of MG132 or lactacystin increased GFP-LC3B-lysosome co-localization, which may indicate that autophagy is upregulated (Figure 5.8A). Finally, A549 cells and H1299 cells were treated with MG132 or lactacystin for 0-24 hours prior to lysis and immunoblotting for anti-p62/SQSTM1, anti-LC3B, and anti-GAPDH antibodies. In A549 cells, proteasomal inhibition had no influence on steady-state p62/SQSTM1 but increased LC3B-II protein levels after 24 hours. However, in H1299 cells, 24 hours of MG132 decreased steady-state p62/SQSTM1 whereas both proteasome inhibitors increased steady-state LC3B-II levels after 24 hours (Figure 5.8B). Therefore, my results strongly suggest that proteasome inhibition increased lysosomal activity and the co-localization of Atgs and p62/SQSTM1 with lysosomes.
Figure 5.8: The effect of MG132 and lactacystin on autophagosome-lysosome co-localization and steady-state LC3B levels.

(A) A549 cells transfected with a cDNA pMRX-IP-GFP-LC3B-RFP-LC3BΔG vector were treated with 5 μM MG132, 5 μM lactacystin or equivalent volumes of dimethyl sulfoxide (DMSO) for 8 hours. LysoTracker Deep Red (red) and Hoechst (blue) were added to the cells 2 hours and 10 minutes, respectively, prior to imaging. Images were acquired using...
an Olympus IX 81 inverted fluorescence microscope (63x objective). ImageJ (version 2.0) quantified the relative area of GFP-LC3B-LysoTracker co-localization. Scale bars = 10 μm.

(B) A549 cells and H1299 cells were treated with 5 μM MG132, 5 μM lactacystin or equivalent volumes of DMSO for 0-24 hours. Cells were lysed, subjected to SDS-PAGE, and immunoblotted for anti-p62/SQSTM1, anti-LC3B, and anti-GAPDH (loading control) antibodies. The steady-state levels of p62/SQSTM1 and LC3B-II were quantified using QuantityOne software and graphed (n=4 ± SD). Significance is defined as ****=P<0.0001 compared to 0-hour control (-).

**Proteasome inhibitors activate autophagy in NSCLC cells**

Although I observed that proteasome inhibitors increased the perinuclear localization of lysosomes, co-localization of lysosomes with Atgs and p62/SQSTM1, and steady-state LC3B-II levels, I next sought to accurately assess how inhibition of the proteasome would alter autophagic flux. For instance, previously I demonstrated that ATG gene expression, Atg protein levels, GFP-LC3B-lysosome co-localization, and GFP-LC3B puncta are unreliable at monitoring lysosomal-dependent degradation. Therefore, I measured autophagic flux using A549 cells stably expressing a pMRX-IP-GFP-LC3B-RFP-LC3BΔG vector. Indeed, 24 hours of MG132 or lactacystin decreased the GFP/RFP ratio, which suggests that prolonged proteasome inhibition increased autophagic flux (Figure 5.9A, B). Given that the mechanism of autophagy activation via proteasome inhibition remains unknown, I next assessed the activity of AMPKα and mTOR, as AMPKα and mTOR regulate autophagy initiation by activating and inhibiting ULK1 activity, respectively. Briefly, I treated A549 cells and H1299 cells with MG132 or lactacystin in the presence and absence of TGFβ1 for 24 hours prior to lysis and immunoblotting for anti-P-mTOR, anti-mTOR, anti-P-ULK1, anti-ULK1, anti-P-AMPKα, anti-AMPKα, anti-LC3B, and anti-GAPDH antibodies. As previously reported in Chapter 3, TGFβ1 decreased the P-mTOR/mTOR ratio, increased the P-ULK1/ULK1 ratio, and had no impact on the P-AMPKα/AMPKα ratio in NSCLC cells. Here, I observed that in both cell lines, MG132 and lactacystin decreased the P-mTOR/mTOR and P-AMPKα/AMPKα ratios and increased both steady-state LC3B-II and the P-ULK1/ULK1 ratio (Figure 5.9C). These results show that MG132 and lactacystin activate autophagy by
decreasing mTOR activity and increasing ULK1 phosphorylation, which are important to canonical autophagy initiation\(^{196}\).

**Figure 5.9:** The effect of proteasome inhibition on macroautophagy and TGFβ-dependent autophagy.
A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were treated with 5 μM MG132 (A), 5 μM lactacystin (B) or equivalent volumes of DMSO for 24 hours. Cells were lysed, subjected to SDS-PAGE, and immunoblotted for anti-LC3B and anti-GAPDH (loading control) antibodies. The green fluorescent protein/red fluorescent protein (GFP/RFP) ratio were quantified using QuantityOne software and graphed (n=4 ± SD). Significance is defined as ***=P<0.001 and ****=P<0.0001 compared to DMSO treatments.

(C) A549 cells and H1299 cells were treated with 5 μM MG132 or 5 μM lactacystin in the presence and absence of 250 pM TGFβ1 for 24 hours. Equivalent volumes of DMSO were the control for MG132 and lactacystin treatments. Cells were lysed, subjected to SDS-PAGE, and immunoblotted for anti-P-mTOR, anti-mTOR, anti-P-ULK1, anti-ULK1, P-AMPKα, anti-AMPKα, anti-LC3B, and anti-GAPDH antibodies. The steady-state levels of P-mTOR/mTOR, P-ULK1/ULK1, P-AMPKα/AMPKα and LC3B-II were quantified using QuantityOne software and graphed (n=4 ± SD). Unless specified, significance is defined as *=P<0.05, **=P<0.01, ***=P<0.001, and ****=P<0.0001 compared to DMSO.

Given that autophagy inhibition affects R-Smad phosphorylation after 24 hours, I next assessed if MG132 disrupted Smad phosphorylation by upregulating autophagy. To inhibit autophagy, A549 cells and H1299 cells were treated with chloroquine or spautin-1. To activate autophagy, cells were treated with MG132 or rapamycin. Cells were subjected to autophagy inhibitors or activators for 24 hours prior to a 1-hour TGFβ1 treatment. Cells were lysed, subjected to SDS-PAGE, and immunoblotted for anti-P-Smad2, anti-Smad2, and anti-GAPDH antibodies. I observed that chloroquine, spautin-1, and rapamycin had no effect on the P-Smad2/Smad2 ratio, whereas it was decreased by MG132 (Figure 5.10). Since the autophagy activating mTOR inhibitor, rapamycin, had no impact on the P-Smad2/Smad2 ratio, I concluded that the effect of MG132 on R-Smad phosphorylation was due to targeting TGFβRII to lysosomes.
Figure 5.10: Proteasome inhibitors decrease maximal R-Smad phosphorylation.

A549 cells and H1299 cells were treated with 50 μM chloroquine, 10 μM spautin-1, 10 μM MG132 or 1 μM rapamycin for 24 hours. No treatment was the control for chloroquine treated cells, but dimethyl sulfoxide (DMSO) served as the control for spautin-1, MG132 and rapamycin treated cells. During the last hour of treatment, 250 pM TGFβ1 was added. Cells were lysed, subjected to SDS-PAGE, and immunoblotted for anti-P-Smad2, anti-Smad2, and anti-GAPDH (loading control) antibodies. The steady-state levels of P-Smad2/Smad2 were quantified using QuantityOne software and graphed (n=3 ± SD). Significance is indicated as ***=P<0.001 compared to DMSO control.

*Downregulating proteasomal activity impedes TGFβ1-dependent EMT in NSCLC cells*

Given that TGFβRII is important for TGFβ-induced EMT,408,409 I assessed if increasing the lysosomal-targeting of TGFβRII impacted the acquisition of a mesenchymal-like cell morphology in the presence of TGFβ1. A549 cells were treated with
MG132, lactacystin or equivalent volumes of DMSO in the presence and absence of TGFβ1 for 24 hours. Cells were fixed and phase contrast microscopy assessed cell morphology. Indeed, I observed that DMSO treated cells were round whereas TGFβ1 increased spindle-shape morphology, which is consistent of an epithelial-mesenchymal phenotype transition. In the presence of TGFβ1, MG132 and lactacystin blocked the development of spindle-shape cell morphology (Figure 5.11A).

Since prolonged proteasome inhibition blocked the acquisition of a mesenchymal-like cell morphology, I next investigated the influence of prolonged proteasome inhibition on TGFβ1-dependent EMT. I treated A549 cells and H1299 cells with MG132 or lactacystin, in the presence and absence of TGFβ1, for 24 hours prior to lysis and immunoblotting for anti-N-Cadherin, anti-E-Cadherin, anti-Snail, anti-Slug, and anti-GAPDH antibodies. Snail and Slug are EMT-transcription factors that increase the transcription of mesenchymal markers, such as N-Cadherin, and decrease the transcription of epithelial markers, such as E-Cadherin. As expected, TGFβ1 induced N-Cadherin, Snail, and Slug protein levels in both cell lines and decreased E-Cadherin protein levels in A549 cells (Figure 5.11B). Furthermore, in A549 cells, MG132 and lactacystin decreased E-Cadherin protein levels in the absence of TGFβ1 but had no additive effects with TGFβ1. In both cell lines, MG132 and lactacystin decreased N-Cadherin protein levels and increased Snail protein levels regardless of the presence of TGFβ1. Both MG132 and lactacystin increased Slug protein levels in A549 cells; however, only MG132 increased Slug protein levels in H1299 cells (Figure 5.11B). Interestingly, in A549 cells and H1299 cells, proteasome inhibitors increased the steady-state levels of EMT-transcription factors, decreased steady-state E-Cadherin levels but blocked the induction of N-Cadherin, which is important in the EMT shift induced by TGFβ1.

Given that the proteasome inhibitors resulted in differential modulation of EMT transcription factor expression, I assessed an additional marker of TGFβ1-dependent EMT, actin stress fiber formation. I visualized and quantified the proportion of A549 cells and H1299 cells containing stress fibers after cells were treated with DMSO, MG132 or lactacystin in the presence and absence of TGFβ1 for 24 hours. Prior to confocal microscope imaging, cells were fixed, permeabilized, and stained with phalloidin and DAPI to label actin and the nucleus, respectively. Even though MG132 increased the
proportion of A549 cells containing stress fibers, the proteasome inhibitors attenuated TGFβ1-induced stress fiber formation in both cell lines (Figure 5.11C). Since stress fibers facilitate cell migration, I next investigated how proteasome inhibition impacted H1299 cell migration by treating a confluent monolayer of H1299 cells with MG132 for 24 hours. After treatment, a scratch was made in the H1299 cell monolayer, and this was imaged twice in the same position 8 hours apart. I observed that MG132 blocked H1299 cell migration (data not shown). Although 24 hours of proteasome inhibition increased the steady-state levels of EMT-transcription factors, they blocked the TGFβ1-dependent N-Cadherin induction, stress fiber formation, and NSCLC cell migration. In conclusion, proteasome inhibition may promote a partial-EMT phenotype, but blocks TGFβ1-dependent EMT.
Figure 5.11: The effect of proteasome inhibition on TGFβ-dependent EMT.
(A) A549 cells were treated with 5 μM MG132, 5 μM lactacystin or equivalent volumes of dimethyl sulfoxide (DMSO) in the presence and absence of 250 pM TGFβ1 for 24 hours. The cells were then fixed and processed for microscopy. A 40x objective of a Leica DM6000 B phase contrast microscope imaged the cells. Representative images depict the morphology of DMSO, MG132, lactacystin, TGFβ1, MG132+TGFβ1, and lactacystin+TGFβ1 treated cells. Plasma membrane perimeter was highlighted with a dotted white line. Scale bars = 10 μm

(B) A549 cells and H1299 cells were treated with 5 μM MG132 or 5 μM lactacystin in the presence and absence of 250 pM TGFβ1 for 24 hours. Equivalent volumes of DMSO were the control for MG132 and lactacystin treatments. Cells were lysed, subjected to SDS-PAGE, and immunoblotted for anti-N-Cadherin, anti-E-Cadherin, anti-Snail, anti-Slug and anti-GAPDH (loading control) antibodies. The steady state levels of N-Cadherin, E-Cadherin, Snail, and Slug were quantified using QuantityOne software and graphed (n=3 ± SD). Unless specified, significance is defined as *=P<0.05, **=P<0.01, ***P<0.001, and ****=P<0.0001 compared to DMSO.

(C) A549 cells and H1299 cells were treated with 5 μM MG132, 5 μM lactacystin or equivalent volumes of DMSO for 24 hours in the presence and absence of 250 pM TGFβ1. The cells were processed for fluorescence microscopy using Alexa Fluor-555 phalloidin (red) and DAPI (blue) to label filamentous actin and nuclei, respectively. Images were obtained with a 63x objective using a Nikon Eclipse Ti2 confocal microscope. Cells containing stress fibers were counted using imageJ (version 2.0) and graphed. This data was derived from 3 independent experiments (mean ± SD). Unless specified, significance is indicated as *=P<0.05 and ****=P<0.0001 with respect to the controls. Scale bars = 10 μm

Proteasome inhibitors induce apoptosis through the TAK1-SAPK/JNK-P38 pathway

To investigate if downregulating proteasomal activity decreased cell migration instead of cell proliferation, I treated A549 cells and H1299 cells with MG132 and lactacystin in the presence and absence of TGFβ1 for 24 hours. A549 cells and H1299 cells were fixed, permeabilized, and stained with DAPI and anti-ki67, which has affinity for an antigen present during proliferating phases of the cell cycle. Using confocal microscopy, I observed that TGFβ1 decreased the proportion of cells positive for ki67 in both cell lines;
however, in A549 cells, MG132 and lactacystin increased the proportion of cells positive for ki67 compared to the TGFβ1 treatment. Finally, in H1299 cells, MG132 and lactacystin treatments were not significantly different compared to the DMSO control in the presence of TGFβ1 (Figure 5.12A).

Although the proportion of proliferating cells were not decreased by proteasome inhibition, I observed that MG132 and lactacystin activate autophagy. Given that autophagy is upregulated to protect cells from damage and death\(^\text{410}\), I investigated if proteasome inhibition induced cellular apoptosis. A549 cells and H1299 cells were treated with MG132 or lactacystin for 24 hours prior to lysis and immunoblotting for anti-cleaved PARP and anti-GAPDH antibodies. In A549 cells, MG132 increased steady-state cleaved PARP after 8 hours whereas it was increased after 24 hours of lactacystin treatment. In H1299 cells, 24 hours of MG132 or lactacystin treatment increased steady-state cleaved PARP (Figure 5.12B). Next, I assessed the impact of proteasome inhibitor dosage on apoptosis by treating A549 cells and H1299 cells with 0-10 µM of MG132 or lactacystin for 24 hours. Cells were then lysed and immunoblotted for anti-cleaved PARP, anti-caspase 3, and anti-GAPDH antibodies (Figure 5.12C, D). I found that steady-state cleaved PARP and the cleaved caspase 3/caspase 3 ratio were increased by 5 µM and 10 µM MG132, respectively in A549 cells. However, in H1299 cells, 2.5 µM and 10 µM MG132 respectively increased steady-state cleaved PARP and the cleaved caspase 3/caspase 3 ratio (Figure 5.12C). Alternatively, 5 µM and 10 µM lactacystin increased steady-state cleaved PARP and the cleaved caspase 3/caspase 3 ratio in both cell lines (Figure 5.12D).
Figure 5.12: The effect of MG132 and lactacystin on proliferation and apoptosis.
(A) A549 cells and H1299 cells were treated with 5 μM MG132, 5 μM lactacystin or equivalent volumes of dimethyl sulfoxide (DMSO) for 24 hours in the presence and absence of 250 pM TGFβ1. The cells were then fixed, permeabilized, and labelled with anti-ki67 antibodies (green). After staining cells with DAPI to visualize nuclei (blue), micrographs were acquired using a Nikon Eclipse Ti2 confocal microscope (63x objective). The proportion of cells positive for ki67 was determined using imageJ (version 2.0) and graphed (n=3 ± SD). Unless specified, significance is defined as *=P<0.05, **=P<0.01, and ***P<0.001 compared to DMSO. Scale bars = 10 μm

(B) A549 cells and H1299 cells were treated with 10 μM MG132, 10 μM lactacystin or equivalent volumes of DMSO for 0-24 hours prior to lysis, SDS-PAGE, and immunoblotting for anti-cleaved PARP and anti-GAPDH (loading control) antibodies. The steady-state levels of cleaved PARP were quantified using QuantityOne software and graphed (n=4 ± SD). Significance is defined as *=P<0.05, **=P<0.01, and ****=P<0.0001 compared to 0 hours of treatment.

A549 cells and H1299 cells were treated with 2.5, 5, and 10 μM MG132 (C), lactacystin (D) or equivalent volumes of DMSO for 24 hours prior to lysis, SDS-PAGE, and immunoblotting for anti-cleaved PARP, anti-caspase 3, and anti-GAPDH antibodies. The steady-state levels of cleaved PARP and cleaved caspase 3/caspase 3 were quantified using QuantityOne software and graphed (n=4 ± SD). Significance is defined as **=P<0.01, ***P<0.001, and ****=P<0.0001 compared to DMSO.

Given that TGFβ1 activates autophagy and apoptosis through the TAK1-SAPK/JNK-P38 non-canonical signalling pathway, I next assessed if this pathway regulates apoptosis induced by proteasome inhibitors. Indeed, western blotting for these proteins in A549 cells and H1299 cells treated with MG132 or lactacystin revealed that proteasome inhibition increases P-TAK1/TAK1, P-SAPK/SAPK, P-JNK/JNK, and P-P38/P38 ratios in both cell lines (Figure 5.13A, B). Next, I disrupted the TAK1-TRAF6-P38 signalling branch in A549 cells and H1299 cells using siRNA against TAK1 and TRAF6 for 24 hours. Cells were then treated with DMSO or a P38 MAPK inhibitor in the presence and absence of MG132 or lactacystin for 24 hours. Cells were lysed, subjected to SDS-PAGE, and immunoblotted for anti-TAK1, anti-TRAF6, anti-cleaved PARP, anti-caspase 3, and anti-GAPDH antibodies. In A549 cells treated with MG132, although
disrupting the TAK1-TRAF6-P38 pathway had no influence on the cleaved caspase 3/caspase 3 ratio, it decreased steady-state cleaved PARP levels. Furthermore, disrupting the TAK1-TRAF6-P38 pathway blocked lactacystin induced PARP and Caspase 3 cleavage (Figure 5.13C). In H1299 cells treated with proteasome inhibitors, TAK1-TRAF6-P38 pathway disruption decreased cleaved PARP and cleaved caspase 3/caspase 3 ratios (Figure 5.13D). Therefore, both proteasome inhibitors may activate apoptosis through the TAK1-TRAF6-P38 signalling pathway.
Figure 5.13: Proteasome inhibitors induce apoptosis through the TAK1-SAPK/JNK-P38 pathway.
(A) A549 cells and (B) H1299 cells were treated with 5 μM MG132, 5 μM lactacystin or equivalent volumes of dimethyl sulfoxide (DMSO) for 24 hours prior to lysis, SDS-PAGE, and immunoblotting for anti-TAK1, anti-P-TAK1, anti-P-SAPK/P-JNK, anti-SAPK/JNK, anti-P-P38, anti-P38, and anti-GAPDH antibodies. The steady-state levels of P-TAK1/TAK1, P-SAPK/SAPK, P-JNK/JNK, and P-P38/P38 were quantified using QuantityOne software and graphed (n=4 ± SD). Significance is defined as *=P<0.05, **=P<0.01, ***=P<0.001, and ****=P<0.0001 compared to DMSO.

A549 cells (C) or H1299 cells (D) were transfected with 10 nM si-Control or 5 nM siRNA targeting TAK1 (si-TAK1) and 5 nM siRNA targeting TRAF6 (si-TRAF6) for 48 hours. The cells were incubated with DMSO control or 10 μM P38 MAPK inhibitor in the absence or presence of 5 μM MG132 or 5 μM lactacystin for 16 hours. The cells were then lysed, subjected to SDS-PAGE and immunoblotted for anti-TAK1, anti-TRAF6, anti-cleaved PARP, anti-caspase 3, and anti-GAPDH antibodies. Quantitative analysis of steady-state cleaved PARP protein levels and the cleaved caspase 3/caspase 3 ratio are shown to the right of representative immunoblots (n=3±SD). Significance is indicated as *=P<0.05, **=P<0.01, ***=P<0.001, and ****=P<0.0001.

Assessing the reversibility of proteasome inhibition on apoptosis, EMT, autophagy, and TGFβ signalling

Since NSCLC cells can be induced to undergo apoptosis in response to prolonged proteasome inhibition, I investigated if the impact of proteasome inhibition could be reversed. Briefly, A549 cells and H1299 cells were treated with MG132 and lactacystin in the presence and absence of TGFβ1 for 24 hours. The cells were then washed with PBS and only half of the cells were treated again with MG132 or lactacystin for 24 hours whereas all cells treated with TGFβ1 were retreated for an additional 24 hours. The cells were lysed, subjected to SDS-PAGE, and immunoblotted for anti-N-Cadherin, anti-E-Cadherin, anti-p62/SQSTM1, anti-TGFβRII, anti-cleaved PARP, anti-P-Smad2, anti-Smad2, anti-P-Smad3, anti-Smad3, anti-Snail, anti-Slug, anti-LC3B, and anti-GAPDH antibodies. As previously observed, 48 hours of TGFβ1 treatment increased N-Cadherin, Snail, Slug, P-Smad2/Smad2, and P-Smad3/Smad3 yet decreased TGFβRII protein levels in A549 cells and H1299 cells (Figure 5.14 & 5.15). In A549 cells, TGFβ1 also decreased
E-Cadherin and p62/SQSTM1 protein levels (Figure 5.14). In both cell lines, MG132 and lactacystin blocked the TGFβ1-dependent changes to N-Cadherin, p62/SQSTM1, Snail, and Slug protein levels and disrupted alterations to the P-Smad2/Smad2 and P-Smad3/Smad3 ratios. Additionally, MG132 and lactacystin increased cleaved PARP and LC3B-II protein levels in both cell lines. However, when cells were rescued from MG132 and lactacystin via PBS washout, cleaved PARP and LC3B-II protein levels were significantly decreased (Figure 5.14 & 5.15). Furthermore, in A549 cells treated with proteasome inhibitors for 24 hours, TGFβ1 decreased p62/SQSTM1 protein levels post-PBS washout, which verified that TGFβ1 facilitates p62/SQSTM1 degradation via proteasomes394 (Figure 5.14). In H1299 cells, the PBS washout, rescued p62/SQSTM1 levels in cells previously treated by MG132 (Figure 5.15). Interestingly, after the PBS washout, the P-Smad2/Smad2, P-Smad3/Smad3, Snail, and Slug were increased in cells previously treated with MG132; however, there were no changes in cells treated with lactacystin. These results verified that MG132 and lactacystin are reversible and irreversible proteasome antagonists, respectively312,400. Finally, in A549 cells, I also observed that in post-PBS washout, cells treated with MG132 and lactacystin had significantly higher TGFβRII levels, which suggests that in response to the proteasome inhibitors increasing autophagic degradation of TGFβRII, cells may upregulate TGFβRII synthesis (Figure 5.14).
Figure 5.14: Reversibility of the effect of proteasome inhibitors on apoptosis, EMT, autophagy, and TGFβ signalling in A549 cells.

A549 cells were treated with 5 μM MG132, 5 μM lactacystin or equivalent volumes of dimethyl sulfoxide (DMSO) in the presence and absence of 250 pM TGFβ1 for 48 hours. After 24 hours, half the cells were washed with PBS and re-incubated with 10% FBS with and without 250 pM TGFβ1 for 24 hours. Cells were lysed and subjected to SDS-PAGE and immunoblotting for anti-N-Cadherin, anti-E-Cadherin, anti-Snail, anti-Slug, anti-cleaved PARP, anti-TGFβRII, anti-p62, anti-P-Smad2, anti-Smad2, anti-P-Smad3, anti-
Smad3, anti-LC3B, and anti-GAPDH (loading control) antibodies. The steady-state levels of N-Cadherin, E-Cadherin, Snail, Slug, Cleaved PARP, TGFβRII, p62, P-Smad2/Smad2, P-Smad3/Smad3, and LC3B-II were quantified using QuantityOne software and graphed (n=3 ± SD). Unless specified, significance is defined as *=P<0.05, **=P<0.01, ***P<0.001, and ****=P<0.0001 compared to DMSO.

Figure 5.15: Reversibility of the effect of proteasome inhibitors on apoptosis, EMT, autophagy, and TGFβ signalling in H1299 cells.

H1299 cells were treated with 5 μM MG132, 5 μM lactacystin or equivalent volumes of dimethyl sulfoxide (DMSO) in the presence and absence of 250 pM TGFβ1 for 48 hours.
After 24 hours, half the cells were washed with PBS and re-incubated with 10% FBS with and without 250 pM TGFβ1 for 24 hours. Cells were lysed, subjected to SDS-PAGE, and immunoblotted for anti-N-Cadherin, anti-Snail, anti-Slug, anti-cleaved PARP, anti-TGFβRII, anti-p62, anti-P-Smad2, anti-Smad2, anti-P-Smad3, anti-Smad3, anti-LC3B, and anti-GAPDH (loading control) antibodies. The steady-state levels of N-Cadherin, Snail, Slug, Cleaved PARP, TGFβRII, p62, P-Smad2/Smad2, P-Smad3/Smad3, and LC3B-II were quantified using QuantityOne software and graphed (n=3 ± SD). Unless specified, significance is defined as *=P<0.05, **=P<0.01, ***P<0.001, and ****=P<0.0001 compared to DMSO.

I verified that MG132 and lactacystin were biologically active using lysates of A549 cells and H1299 cells treated with MG132 or lactacystin for 0-24 hours prior to SDS-PAGE and Coomassie blue staining. Although no significant differences were observed in A549 cells (Figure 5.16A), short-term proteasome inhibition typically had greater Coomassie blue stain intensity compared to DMSO controls, and prolonged proteasome inhibitor treatment decreased Coomassie blue stain intensity in H1299 cells (Figure 5.16B). Therefore, short-term disruption of proteasomes increased total protein levels, which suggested that proteasome-targeted proteins were spared. Whilst total protein levels were unchanged or decreased by prolonged proteasome inhibition, there was no reason to suspect that the biological activity of either proteasome inhibitor changed as there were no observable differences between MG132 (reversible) or lactacystin (irreversible) treatments. Instead, consistent with my findings, it may indicate that proteins spared from proteasome-dependent degradation are being re-routed to autophagy pathways or prolonged proteasome inhibition decreased cell viability.
Figure 5.16: The temporal effect of proteasome inhibitors on total protein levels.
(A) A549 cells and (B) H1299 cells were treated with 5 μM MG132, 5 μM lactacystin or equivalent volumes of dimethyl sulfoxide (DMSO) for 0-24 hours. Cells were lysed, subjected to SDS-PAGE, and stained with Brilliant Blue R-250 (Coomassie). The Coomassie stain intensity was quantified using QuantityOne software and graphed (n=3 ± SD). Significance is defined *=P<0.05 and **=P<0.01 compared to the control.

Taken together, my results support the model that proteasome activity, autophagy, apoptosis, EMT, and TGFβ1 signalling are linked (Figure 5.17). One potential mechanism for this is that proteasome inhibition upregulates autophagy, increases lysosomal activity, and promotes TGFβRII trafficking to early endosomes, late endosomes, and lysosomes. Therefore, fewer TGFβRII receptors may be available to bind to TGFβ1, which decreases R-Smad phosphorylation and TGFβ1 signalling. Interestingly, if 24 hours of proteasome inhibition decreased E-Cadherin levels and increased EMT-transcription factor levels, decreasing proteasomal degradation may prime cells to undergo EMT.

Figure 5.17: Schematic of the regulation of proteasome inhibition on TGFβ1 biology in NSCLC cells.

TGFβ1 binds to cell surface TGFβ receptors, which are internalized into the EEA1-positive early endosomal compartment to promote R-Smad phosphorylation. Receptors may then traffic to Rab7-positive late endosomes prior to trafficking to LAMP1-positive lysosomes. TGFβRII may be degraded by lysosomes or proteasomes. The inhibition of proteasomes using MG132 and lactacystin increases TGFβRII trafficking to EEA1, Rab7, and LAMP1-positive compartments. Proteasome inhibitors also decrease R-Smad phosphorylation.
Overall, proteasome inhibition increases apoptosis and autophagy, but reduces TGFβ-dependent EMT.

5.4 Discussion

Autophagy initiated by TGFβ1, MG132, and lactacystin rely on reducing mTOR activity and increasing the proportion of P-ULK1 that initiates the formation of the ULK1 complex. In some investigations, proteasome inhibitors decreased steady-state ULK1 levels and phosphorylation\textsuperscript{411}. One plausible mechanism could be due to differences in transcription factor expression, as proteasome inhibitors impact the expression of transcription factors in a cell type-dependent manner\textsuperscript{412}. For instance, proteasome inhibition leads to an accumulation of unfolded and misfolded proteins, which activates the unfolded protein response\textsuperscript{413}. The unfolded protein response and proteasome inhibitors have been shown to alter the expression of transcription factors, such as FoxM1\textsuperscript{414}. Furthermore, my results show differences in the protein levels of Snail and Slug transcription factors between A549 cells and H1299 cells treated with proteasome inhibitors. Interestingly, I found that 24 hours of proteasome inhibitors increased Snail and Slug whereas after 48 hours of the same proteasome inhibitors, Snail and Slug were decreased. Also, proteasome inhibition decreased the amount of nuclear R-Smads, post 1-hour of TGFβ1 treatment, which may have limited the activity of phospho-R-Smads. Taken together, this may explain why observed differences in the P-ULK1/ULK1, P-AMPKα/AMPKα, and P-mTOR/mTOR ratios exist in different cell lines subjected to the same treatments.

Since proteasome inhibition decreased steady-state TGFβRII levels, and previously in Chapter 3, I demonstrated that TGFβRIII knockdown increased autophagy\textsuperscript{360}, promoting the degradation of TGFβRII may explain the link between proteasome inhibition and elevated autophagic flux. Although it may seem counterintuitive that decreasing TGFβRII upregulates autophagic flux as TGFβ1 activates autophagy\textsuperscript{260}, TGFβ1 and proteasomes regulate several transcription factors that may impede or enhance the transcription of a specific subset of genes. For this reason, it is possible that autophagy can increase in the presence of TGFβ1 and increase upon TGFβRII degradation via proteasome inhibition if
proteasome inhibitors impact transcription factors with similar activity as the ones activated by TGFβ1.

Previously, in Chapter 3, I reported that TGFβ1-dependent autophagy relies on canonical and non-canonical TGFβ1 signalling pathways. More specifically, Smad4, a common Smad that binds to R-Smads prior to translocating into the nucleus to modulate transcription, represents the canonical component. Interestingly, when Smad4 protein levels were knocked down, TGFβ1-dependent autophagy was attenuated and the P-Smad2/Smad2 ratio was increased. Alternatively, TGFβ1-dependent autophagy was blocked when I inhibited the non-canonical TAK1-TRAF6-p38 MAPK signalling pathway, which had no effect on the P-Smad2/Smad2 ratio. Therefore, TGFβ1-dependent autophagy may not be dependent on P-Smad2 levels. Further support for this is that TGFβ1-dependent autophagy was observed when proteasome inhibitors decreased the P-Smad2/Smad2 and P-Smad3/Smad3 ratios but did not impede the TGFβ1-dependent increase in the P-ULK1/ULK1 ratio. Here, I illustrated that the TAK1-TRAF6-p38 MAPK signalling pathway is not only important for autophagy but also for apoptosis activation.

P62/SQSTM1 may be important for autophagy when the proteasomal activity is inhibited. For instance, it was previously reported that MG132 increased ULK1-dependent phosphorylation of p62/SQSTM1, which increased its binding affinity to ubiquitin. Here, I found that proteasome inhibitors increased p62/SQSTM1-LAMP1 co-localization. In other words, when the catalytic activity of proteasomes is suppressed, the increase in p62/SQSTM1-LAMP1 co-localization coincides with an increase in autophagic flux. Interestingly, steady-state p62/SQSTM1 levels were not reduced by proteasome inhibitors in A549 cells, which suggested that proteasome inhibitors not only promoted the lysosome-dependent degradation of p62/SQSTM1 but may upregulate its expression. Indeed, it has been reported that as a protective mechanism against damage mediated by misfolded proteins that accumulate upon proteasome inhibition, cells upregulate p62/SQSTM1 expression to sequester cell damaging proteins. Therefore, in NSCLC cells, p62/SQSTM1 may be important for macroautophagy initiated by compromised proteasomal activity.

Endocytosis and trafficking of receptors are important to ensure signalling strength and duration. Modulating endocytosis and trafficking is utilized in most cells to regulate
extracellular signalling\textsuperscript{95}. Since p62/SQSTM1 and TGFβRII have been found to associate in late endosome membrane compartments\textsuperscript{394}, it followed that proteasome inhibitors would increase their co-localization with LAMP1. Proteasome inhibition also promoted the co-localization between TGFβRII and EEA1/Rab7/LAMP1 membrane compartments. These results were surprising because UPP disruption has been proposed to impede the internalization and trafficking of several membrane proteins and enzymes. For instance, endocytosis of the epidermal growth factor receptor\textsuperscript{417}, growth hormone receptor\textsuperscript{418}, glutamate receptor\textsuperscript{419}, and interleukin receptor type 2\textsuperscript{420} are reduced immediately following proteasome inhibition. In some instances, there was a stark decrease in receptor degradation; however, internalization was not affected, which suggests that proteasomes may regulate protein trafficking. Furthermore, for some lectin receptors, proteasome inhibitors have no effect on internalization or trafficking\textsuperscript{421}. However, internalization of the nucleotide receptor P\textsubscript{2}Y\textsubscript{13}, which is responsible for high density lipoprotein endocytosis, was increased by proteasomal inhibition\textsuperscript{422}. Therefore, although it is less common, proteasome inhibition may enhance receptor endocytosis.

Although TGFβRI levels were decreased in H1299 cells, the results were not consistent and thus I only assessed endocytosis and trafficking of TGFβRII. Given the differences in steady-state levels of TGFβRII and TGFβRI in the presence of proteasome inhibitors, TGFβRI endocytosis and trafficking may differ from that of TGFβRII. Therefore, future studies will focus on examining differences between plasma membrane retention, endocytosis, and trafficking of TGFβRI, TGFβRII, and TGFβRIII receptors. These finding will reveal if cells preferentially spare or degrade specific receptors to modulate TGFβ signalling and outcomes.

Here, I downregulated the catalytic activity of proteasomes using MG132 and lactacystin. MG132 is a cell permeable peptide aldehyde that reversibly antagonizes chymotrypsin-like proteasome reactions\textsuperscript{423}. However, MG132 and other peptide aldehydes may have off-target effects, such as calpains and lysosomal cysteine proteases, like cathepsins\textsuperscript{424}. Furthermore, MG132 is rapidly metabolized by liver cytochrome P450 3A enzymes\textsuperscript{425}. Therefore, incomplete proteasome selectivity and low metabolic stability limits the therapeutic potential of MG132. Alternatively, lactacystin is a non-peptide proteasome inhibitor that converts to a β-lactone in a neutral pH\textsuperscript{426}. The lactacystin β-
lactone irreversibly binds to and reacts with threonine residues in the 20S proteasome core particle that acetylates and inhibits the catalytic activity of threonine hydroxyl groups\textsuperscript{400}. Although lactacystin is more selective than MG132, it still has a single off-target effect on cathepsin A\textsuperscript{427}. In comparison to MG132, it has a longer half-life with relatively fewer off-target effects, which make it a useful proteasome inhibitor\textsuperscript{400}.

The relationship between autophagy, UPP, EMT, and TGF\(\beta\)1 signalling is complex. In general, TGF\(\beta\)1 activated autophagy in a ULK-1-dependent manner, promoted EMT, degraded p62/SQSTM1 via proteasomes, and induced TGF\(\beta\) receptor endocytosis and degradation via proteasomes/lysosomes. Previously, I reported that autophagy inhibitors suppressed TGF\(\beta\)1-dependent autophagy\textsuperscript{260}, attenuated TGF\(\beta\)1-induced EMT, decreased R-Smad phosphorylation, blocked p62/SQSTM1 protein degradation in the absence of TGF\(\beta\)1, and delayed TGF\(\beta\) receptor internalization and trafficking\textsuperscript{399}. Here, I observed that proteasome inhibitors activated autophagy, attenuated TGF\(\beta\)1-induced EMT, decreased R-Smad phosphorylation, increased TGF\(\beta\) receptor internalization and endocytosis, and promoted lysosomal degradation of p62/SQSTM1 and TGF\(\beta\) receptors.

In summary, this work aimed to investigate the relationship between the proteasome, autophagy, and TGF\(\beta\) signal transduction to improve my understanding of their interdependence. For instance, in tumourigenesis, autophagy augments cancer. Therefore, inhibiting the proteasome in some tumour cells may be counterintuitive because they upregulate autophagic flux. Furthermore, autophagy and proteasome inhibitors suppress TGF\(\beta\) signalling, which may activate abnormal cellular communication pathways. Additionally, decreasing p62/SQSTM1 expression may also blunt the therapeutic effects of proteasome inhibitors. Future studies investigating therapeutic cocktails that may simultaneously target proteasome activity, autophagy, and TGF\(\beta\) signalling would clarify how these processes influence tumourigenesis.
Chapter 6

p62/SQSTM1 regulates transforming growth factor-β signalling and epithelial-mesenchymal transition

Components of this chapter have been published in Cell. Signal. (2021) 85.
6 Summary

Here, I investigated the role of an autophagy cargo receptor, protein 62/sequestosome 1 (p62/SQSTM1), in regulating transforming growth factor-β (TGFβ) receptor trafficking as well as TGFβ1-dependent Smad2 phosphorylation, autophagy, and epithelial-mesenchymal transition (EMT) in A549 NSCLC cells. Using immunofluorescence microscopy, p62/SQSTM1 was observed to co-localize with TGFβ receptors in the late endosome. Small interfering RNA (siRNA)-mediated silencing of p62/SQSTM1 resulted in an attenuated time-course of Smad2 phosphorylation but did not affect Smad2 nuclear translocation. Furthermore, p62/SQSTM1 silencing increased the phosphorylation of TGFβ-activated kinase 1 (TAK1) and p38 mitogen-activated protein kinase (p38), which facilitate Smad-independent TGFβ signalling. Since p62/SQSTM1 may regulate the balance of Smad-dependent and -independent TGFβ signalling, I next assessed if p62/SQSTM1 was important to TGFβ-dependent EMT. Indeed, p62/SQSTM1 silencing promoted TGFβ1-dependent EMT marker expression, actin stress fiber formation, and migration in A549 cells. I further observed that Smad4-independent TGFβ1 signalling decreased p62/SQSTM1 protein levels via a proteasome-dependent mechanism. Although p62/SQSTM1 silencing did not impede TGFβ1-dependent autophagy, my results suggest that p62/SQSTM1 may aid in maintaining A549 cells in an epithelial state and TGFβ1 decreases p62/SQSTM1 prior to inducing EMT and autophagy.

6.1 Introduction

Given that both proteasome or autophagy inhibition altered TGFβ receptor trafficking and impeded R-Smad phosphorylation, EMT, and cell migration, I next investigated why targeting different degradative processes yielded similar results. Due to its involvement with both autophagy and proteasome activity, p62/SQSTM1 became the focus of this chapter. In autophagy, microtubule-associated protein light-chain 3B-II (LC3B-II) proteins are inserted into autophagosome membranes and bind to autophagy cargo receptors, such as p62/SQSTM1. Using a ubiquitin-associated (UBA) domain, phox-bem1 (PB1) domain, and LC3 interacting region (LIR), p62/SQSTM1 facilitates the selective degradation of lysosomal substrates. Briefly, p62/SQSTM1 recognizes ubiquitin-
conjugated lysosomal substrates using its UBA domain; the PB1 domain facilitates p62/SQSTM1 scaffolding, which is necessary for lysosomal-targeting; and the LIR tethers p62/SQSTM1 to LC3 within phagophores\textsuperscript{429,430}. In the ubiquitin-proteasome pathway (UPP), p62/SQSTM1 binds to ubiquitinated proteins using its UBA domain and delivers them to proteasomes by binding to the regulatory particle via the PB1 domain\textsuperscript{431}. Therefore, although autophagy and the UPP are two distinctive degradative pathways, they interact using shared cargo delivery proteins\textsuperscript{272}.

Although the role of p62/SQSTM1 on TGFβ signalling is unknown, it is known that p62/SQSTM1 regulates the selective degradation of components of the TGFβ pathway\textsuperscript{353,432}. For example, p62/SQSTM1 targets TGFβ signalling molecules, such as atypical protein kinase C (aPKC), to proteasomes and autophagosomes\textsuperscript{277}. In A549 non-small cell lung cancer (NSCLC) cells, aPKC is essential for TGFβ-dependent EMT, migration, and influences TGFβ receptor trafficking\textsuperscript{36,37,308,339}. Therefore, this introduces the possibility that p62/SQSTM1 may regulate TGFβ receptor trafficking, signalling, EMT, proteasomes, and autophagy.

6.2 Materials and Methods

\textit{Antibodies and reagents}

Primary antibodies were as follows: anti-E-Cadherin (BD Transduction laboratories, 610182), anti-N-Cadherin (BD Transduction laboratories, 610921), anti-p62/SQSTM1 (Cell Signalling Technology, 5114S), anti-p62/SQSTM1 (Abnova, H00008878-M01), anti-lysosomal-associated membrane protein 1 (LAMP1; Cell Signalling Technology, 3243S), anti-LC3B (Cell Signalling Technology, 9236S), anti-Smad4 (Cell Signalling Technology, 38454S), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Cell Signalling Technology, 2118S), anti-20S proteasome β1 (Santa Cruz, sc-374405), anti-tubulin (Cell Signalling Technology, 2144S), anti-phospho-Ser465/467-Smad2 (P-Smad2; Cell Signalling Technology, 3108L), anti-Smad2/3 (BD Transduction laboratories, 562586), anti-lamin A/C (Santa Cruz, sc-6125), anti-early endosome antigen 1 (EEA1; BD Transduction laboratories, 610182), anti-hemagglutinin (HA; Y-11; Santa Cruz, sc-805), anti-Rab7 (Santa Cruz, sc-376362), anti-plasminogen activator inhibitor-1 (PAI-1; Cell Signalling Technology, 11907S), anti-Slug (Cell
Signalling Technology, 9585S), anti-Snail (Cell Signalling Technology, 3879S), anti-Twist1 (Thermo Fisher Scientific, MA5-17195), anti-TAK1 (Cell Signalling Technology, 5206S), anti-phospho-T181/184-TAK1 (P-TAK1; Cell Signalling Technology, 4531S), anti-tumour necrosis factor receptor-associated factor 6 (TRAF6; Cell Signalling Technology, 8028S), anti-cJun-amino-terminal kinase/stress-activated protein kinase (JNK/SAPK; Cell Signalling Technology, 9252S), anti-phospho-T183/185-SAPK/JNK (P-SAPK/P-JNK; Cell Signalling Technology, 9251S), anti-p38 (Cell Signalling Technology, 9212S), anti-phospho-T180/Y182-p38 (P-p38; Cell Signalling Technology, 9211S), and anti-TGFβ receptor type II (TGFβRII; Santa Cruz, sc-220). Horseradish peroxidase (HRP)-conjugated secondary goat anti-rabbit-IgG (Thermo Fisher Scientific, 31460), goat anti-mouse-IgG (Thermo Fisher Scientific, 31430), and donkey anti-goat-IgG (Santa Cruz, sc-2020) were used for western blot analysis or immunofluorescence. Alexa Fluor-conjugated donkey anti-mouse-IgG (Life Technologies, A21206) and donkey anti-rabbit-IgG (Life Technologies, A31572) were used for immunofluorescence studies. Two different human ambion siRNA constructs were purchased from Thermo Fisher Scientific against autophagy-related 7 (ATG7), ATG5, p62/SQSTM1, Smad4, and a scrambled siRNA (si-Control) with catalog numbers 4392420, 4392420, s16960, AM16708, and 4390844, respectively. MG132 (Sigma Aldrich, M7449) and lactacystin (Cayman Chemicals, 70980) were used to inhibit the proteasome whereas chloroquine (acquired from the Shepherd lab, London, Canada), uncoordinated-51-like autophagy activating kinase-101 (ULK-101; Selleckchem, S8793), and specific potent autophagy inhibitor 1 (spautin-1; Sigma, SML0440) inhibited autophagy. Additionally, SB431542 was utilized to antagonize TGFβ receptor type I (TGFβRI) kinase activity.

**Cell culture and transfections**

A549 cells were grown in Kaighn’s Modification of Hams F-12 (F-12K) medium (Corning), respectively, supplemented with 10% fetal bovine serum (FBS). Cells were cultured in a humidified tissue incubator at 37°C under 5% CO₂. A549 cells were treated with 250 pM TGFβ1 using low serum media (0.2% FBS) after cells were serum starved overnight. Mink lung (Mv1Lu) cells were grown in Minimum Essential Medium (MEM; Corning) supplemented with 10% FBS, 0.3 mg/mL Hygromycin B (Invitrogen), and MEM non-essential amino acids (Gibco). p62/SQSTM1 knockdown was mediated using
Optimem medium and Lipofectamine RNAiMAX (Thermo Fisher Scientific) as per manufacturer’s protocol. pCMV5-p62/SQSTM1 over-expression was achieved using a PolyJet transfection reagent (SignaGen Laboratories). Cells were treated with 250 pM TGFβ1, 50 µM chloroquine, 10 µM lactacystin, 20 µM SB431542, and 10 µM MG132 in media supplemented with 10% FBS. A cDNA pMRX-IP-GFP-LC3B-RFP-LC3BΔG vector (Addgene, 84573) and PolyJet transfection reagent (Froggabio, Toronto, ON) generated GFP-LC3B-RFP-LC3BΔG expressing A549 cells. Transfected A549 cells were isolated using 1 µg/mL puromycin (Thermo Fisher Scientific, A1113802) in F-12K growth media supplemented with 10% FBS.

**Nuclear and cytoplasmic fractionation**

The nuclear and cytoplasmic compartments of the cell were isolated using a NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific) as per manufacturer’s protocol. Briefly, cells were detached from 10 cm plates using trypsin-ethylenediaminetetraacetic acid (EDTA) and centrifuged at 1000x g for 2 minutes. The supernatant was aspirated leaving a cell pellet that was washed with phosphate buffer saline (PBS) and centrifuged at 1000x g for 2 minutes. Cytoplasmic extraction reagents I and II were then added and samples were centrifuged (21 000x g at 4°C) for 5 minutes to separate nuclear protein (pellet) from the cytoplasmic protein (supernatant). Nuclear extraction reagent was then added to the pellet, vortexed, and centrifuged (21 000x g at 4°C) for 10 minutes to isolate nuclear proteins. The lysates from both the nuclear and cytoplasmic fraction were processed for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

**Immunoblotting**

Protein was isolated from cells using TNTE lysis buffer containing 50 mM Tris pH 7.5, 150 mM sodium chloride, 1 mM EDTA, 0.5% Triton X-100, 1 mg/mL pepstatin, 50 µM phenylmethylsulfonyl fluoride, 2.5 mM sodium fluoride, and 10 mM sodium pyrophosphate phosphatase inhibitor. The lysates were centrifuged at 21000x g at 4°C for 10 minutes. The protein concentrations were determined using the DC® protein assay (Bio-Rad) and a Victor 3V Multi-Detection Microplate Reader (PerkinElmer). Cell lysates were subjected to SDS-PAGE (approximately 50 µg of protein per sample) and transferred
onto a nitrocellulose membrane. Membranes were blocked with 5% skim milk and primary antibodies were left on the membranes to incubate overnight, rocking at 4°C. On the following day, nitrocellulose membranes were incubated with the appropriate HRP-conjugated secondary antibody for 1-hour at room temperature. The membranes were washed several times with enhanced chemiluminescent substrate (Bio-Rad) and visualized using a Versa-doc Imager (Bio-Rad) and QuantityOne® 1-D Analysis software.

Co-immunoprecipitation (Co-IP)

A549 cells were grown in 10 cm plates and lysed at 90% confluency using TNTE lysis buffer. After cells were lysed, 100 µL of total lysate for each sample was transferred to separate 1.5 mL tubes containing SDS-PAGE sample preparation buffer and stored at -20°C. The remaining lysates for each sample were divided into two equal volumes and incubated overnight at 4°C with one containing the primary antibody targeting the protein of interest and the other containing a control IgG antibody. The animal of origin for the control IgG antibody corresponds to the secondary antibody required by the primary antibody utilized for the pulldown. Protein G sepharose beads (GE Healthcare) were then added to the lysates for 4 hours. The beads were centrifuged at 1000x g for 2 minutes and the supernatant was aspirated. The beads were washed, and this cycle was repeated three times. Laemmlli sample preparation buffer was added to each sample, and the total lysates. Co-IPs and IgG controls were subjected to SDS-PAGE and immunoblotting.

Scratch migration assay

A549 cells were treated with si-Control or si-p62/SQSTM1 in the presence and absence of 250 pM TGFβ1 for 24 hours. Following treatment, a pipette tip scraped a trench in the cell monolayer. Each scratch was imaged 3 times with an Olympus IX 81 inverted fluorescence microscope using a 10x objective both immediately and 24 hours post-scratch. ImageJ (version 2.0) measured the proportion of the scratch width that diminished from cells migrating into the scratch area.

Immunofluorescence microscopy

A549 cells were rinsed with 1x PBS, fixed with 4% paraformaldehyde for 10 minutes, permeabilized with 0.25% Triton X-100 for 5 minutes, blocked with 10% FBS
diluted in 1x PBS for 1-hour, and incubated with primary antibodies at 4°C overnight. The primary anti-p62/SQSTM1, anti-LAMP1, anti-20S proteasome β1, anti-Rab7, and anti-EEA1 antibodies were used at a 1:100 dilution that were diluted using 10% FBS in 1x PBS. The appropriate fluorescent-probe-conjugated secondary antibodies at a 1:250 dilution was incubated with the coverslips for 1-hour at room temperature. 4′,6-diamidino-2-phenylindole (DAPI) stained nucleic acids and Alexa Fluor-555 phalloidin stained filamentous actin after incubating with the sample for 5 and 20 minutes, respectively. The coverslips were mounted on microscope slides using Immu-mount (Thermo Fisher Scientific) and they were left at room temperature in the dark overnight. An Olympus IX 81 inverted fluorescence microscope (Olympus, Canada) was used to image the fluorescent probes.

**Antibody feeding/TGFβ receptor internalization analysis**

Co-localization of TGFβRII with p62/SQSTM1, EEA1, and Rab7 was done using Mv1Lu cells that were stably transfected to overexpress pMEP4 with cDNA encoding HA-tagged TGFβRII under a zinc-inducible promoter. Cells were serum starved overnight using F-12K medium supplemented with 0.2% FBS and 50 μM zinc chloride 24 hours after being plated on coverslips. The following day, cells were placed on ice for 10 minutes to interrupt receptor trafficking. Primary anti-HA antibody (1:250) was diluted in 0.2% FBS medium and left to incubate on the cells for 2 hours on ice. The coverslips were washed 3 times with 1x PBS, and then appropriate Alexa Fluor-conjugated secondary antibodies were added and left to incubate on ice for 1-hour in the dark. Coverslips were washed another 3 times with 1x PBS before being put back to 37°C for 0, 1 or 3 hours to induce trafficking. At the indicated times, coverslips were removed, fixed, and permeabilized.

**Reverse transcription and qPCR**

A RNeasy mini kit isolated RNA, which was reverse transcribed to cDNA using a C1000 touch thermal cycler (Bio-Rad). A Real-Time CFX96 real-time quantitative polymerase chain reaction (qPCR) system assessed the mRNA expression using primers specific to CDH1, CDH2, SNAI1, SLUG, SERPINE1, and β-ACTIN. The primer sequences can be found in Table 6.1.
Table 6.1: Primer sequences used for qPCR analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward and reverse primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNAIL</td>
<td>AATCGGAAGCCTAACCTACAGCG</td>
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<tr>
<td></td>
<td>GTCGCCAGATGAGATTTGGCA</td>
</tr>
<tr>
<td>SLUG</td>
<td>ATACCAAAACCAGACATCTCTCA</td>
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<tr>
<td></td>
<td>GACTCAGTCGCCCTCCAAGATG</td>
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<td></td>
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<tr>
<td>β-ACTIN</td>
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<tr>
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<tr>
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</table>

Microarray data analysis

Using a previously published microarray dataset of untreated and TGFβ1-treated A549 cells, I measured the expression of autophagy specific genes (NCBI Gene Expression Omnibus website, GEO; GSE26241) and the expression of EMT markers. I also measured the expression of EMT markers and autophagy-related genes using a microarray dataset of si-Control and si-p62/SQSTM1 (NCBI Gene Expression Omnibus website, GEO; GSE208588).

Statistical Analysis

One-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparisons test, a two-way/three-way ANOVA followed by a Tukey’s multiple comparison tests and a student’s T test were used to evaluate the significance of the results. Statistical analysis was performed using GraphPad Prism Software 8.0 and P-values <0.05 were statistically significant.

6.3 Results

p62/SQSTM1 and TGFβRII co-localize in the late endosome compartment

My labs previous work showed that the interaction of partitioning defective 6 homolog (Par6)/aPKC isoforms with the TGFβ receptors were essential for TGFβ-dependent signalling, EMT, and cell migration. Furthermore, since aPKC associates with p62/SQSTM1, I assessed if there was a physical interaction between p62/SQSTM1
and TGFβ receptors in A549 cells. Using co-immunoprecipitation followed by immunoblotting, I observed that p62/SQSTM1 interacts with TGFβRII (Fig. 6.1A). Based on several studies that reported p62/SQSTM1 resides in the early- and late-endosomal membrane compartments434,435, I next assessed where the interaction of TGFβ receptors with p62/SQSTM1 may occur. To determine the timeframe and location of TGFβRII and p62/SQSTM1 association, I followed the endocytosis of cell surface TGFβ receptors into the early- and late-endosomal compartments. I observed that the trafficking of TGFβ receptors into the EEA1-positive early endosome and the Rab7-positive late endosome peaked after 1 and 3 hours of endocytosis, respectively (Fig. 6.1B, C). The co-localization between TGFβRII and p62/SQSTM1 was greatest 3 hours after receptor endocytosis, suggesting that p62/SQSTM1 and TGFβRII co-localize on the late endosome membrane compartment (Fig. 6.1D). Since trafficking to the late-endosomal/lysosomal system results in the degradation of cellular proteins185, I next investigated the effect of TGFβRII internalization via TGFβ1 treatment on p62/SQSTM1 steady-state levels in lung tumour cells.
Figure 6.1: p62/SQSTM1 and TGFβ receptors associate and co-localize in lung epithelial cells.

(A) A549 cell lysates were lysed and immunoprecipitated (IP) with non-specific IgG (Control IgG) or anti-transforming growth factor-β receptor type II (TGFβRII) antibodies.
The IP samples and total cell lysates were then processed for SDS-PAGE and immunoblotted using anti-TGFβRII or anti-p62/SQSTM1 antibodies.  

(B) Mv1Lu cells expressing HA-tagged TGFβRII were incubated at 4°C (0 hours of endocytosis) and then further incubated at 37°C for an additional 1 or 3 hours (h) to induce receptor endocytosis. The cells were fixed, permeabilized, and counterstained with anti-early endosome antigen 1 (EEA1) antibodies to identify the early endosomal compartment. TGFβRII (red) and EEA1-positive early endosomes (green) co-localization is indicated by yellow. ImageJ quantified the relative area of co-localization and the graph (Mean ± SD) is located on the right of representative images. Significance is defined as **=P<0.01 with respect to the control. Scale bar = 10 µm.  

(C) Mv1Lu cells expressing HA-tagged TGFβRII were processed as described in Panel B with the exception that they were incubated with anti-Rab7 antibodies post-fixation. TGFβRII (red) and Rab7-positive late endosomes (green) co-localization is indicated by yellow. ImageJ quantified the relative area of co-localization and the graph (Mean ± SD) is located on the right of representative images. Significance is defined as ****=P<0.0001 with respect to the control. Scale bar = 10 µm.  

(D) Mv1Lu cells expressing HA-tagged TGFβRII were processed as described in Panel B with the exception that they were incubated with anti-p62/SQSTM1 antibodies post-fixation. Co-localization of TGFβRII (red) with p62/SQSTM1 (green) is indicated by yellow. ImageJ quantified the relative area of co-localization and the graph (Mean ± SD) is located on the right of representative images. Significance is defined as ***=P<0.001 with respect to the control. Scale bar = 10 µm.  

*Smad4-independent TGFβ1 signalling facilitates a decrease in p62/SQSTM1 protein levels*  

I first assessed the levels of p62/SQSTM1 steady-state levels in cells incubated with TGFβ1 and observed a significant decrease in p62/SQSTM1 protein levels after 24 and 48 hours of ligand stimulation (35±9% and 42±12% compared to untreated cells, respectively; Fig. 6.2A). Next, I examined the time frame of p62/SQSTM1 protein loss in response to TGFβ1 and compared this to a time course of TGFβ1-dependent Smad2 phosphorylation. As the TGFβ1 treatment duration increased, p62/SQSTM1 protein levels decreased. Although TGFβ1-dependent Smad2 phosphorylation levels peaked at 1-hour, p62/SQSTM1 protein levels were significantly decreased after 6 hours of TGFβ1 treatment.
I next assessed which TGFβ1 signalling pathway(s) may be responsible for the loss of p62/SQSTM1 protein levels. First, I treated A549 cells with SB431542, a TGFβRI kinase inhibitor, in the presence and absence of TGFβ1 for 24 hours. I found that inhibiting TGFβRI attenuated the TGFβ1-dependent p62/SQSTM1 loss (Fig. 6.2C). Having ascertained that TGFβRI kinase activity is necessary for p62/SQSTM1 protein loss, I assessed if this was dependent on Smad signalling. To carry this out, I treated A549 cells with an siRNA that targeted Smad4 (si-Smad4) to assess if impeding Smad4-dependent TGFβ1 signalling would rescue p62/SQSTM1 protein levels. Although si-Smad4 significantly increased p62/SQSTM1 protein levels compared to si-Control, silencing Smad4 did not block the TGFβ1-dependent p62/SQSTM1 loss (Fig. 6.2D). Therefore, Smad4-independent TGFβ1 signalling facilitates p62/SQSTM1 protein loss. Since p62/SQSTM1 protein loss was not due to altering gene expression, as the p62/SQSTM1 mRNA levels were unchanged in response to TGFβ1 stimulation (Fig. 6.3), I therefore evaluated if the decrease in p62/SQSTM1 protein levels was dependent on protein degradation.
Figure 6.2: Smad4-independent TGFβ1 signalling decreases p62/SQSTM1 protein levels.

(A) A549 cells were treated with 250 pM TGFβ1 for 0, 24 or 48 hours (h) prior to lysis. Cell lysates were subjected to SDS-PAGE and immunoblotting using an anti-GAPDH (loading control) and anti-p62/SQSTM1 antibodies. The relative levels of p62/SQSTM1
were quantitated using QuantityOne Analysis software and graphed. The graphs represent 4 independent experiments (mean ± SD). Significance is represented as **=P<0.01 with respect to the control.

(B) A549 cells were treated with 250 pM TGFβ1 for 0-48 h, lysed, and processed for SDS-PAGE, and immunoblotted with anti-p62/SQSTM1, anti-phosphorylated Smad2 (P-Smad2), anti-Smad2, and anti-GAPDH antibodies. The relative levels of P-Smad2/Smad2 and p62/SQSTM1 were quantitated from 3 independent experiments and graphed as the mean ± SD. Significance was defined as ***=P<0.001 and ****=P<0.0001 with respect to 1-hour TGFβ1 for P-Smad2/Smad2 and 0-hour TGFβ1 for p62/SQSTM1.

(C) A549 cells were treated with 10 µM SB431542 and 250 pM TGFβ1 for 24 h, lysed, processed for SDS-PAGE, and immunoblotted with anti-p62/SQSTM1, anti-P-Smad2, anti-Smad2, and anti-GAPDH antibodies. Equivalent volumes of dimethyl sulfoxide (DMSO; vehicle control) were the control for SB431542 treated cells. The relative levels of p62/SQSTM1 were quantitated from 3 independent experiments and graphed as the mean ± SD. Significance defined as **=P<0.01 with respect to the control or between specified treatments.

(D) A549 cells transfected with 10 nM control siRNA (si-Control) or 10 nM siRNA targeting Smad4 (si-Smad4) were treated for 24 h with 250 pM TGFβ1, lysed, and immunoblotted for Smad4, p62/SQSTM1, and GAPDH as indicated. Three separate experiments were performed and p62/SQSTM1 levels were quantitated using QuantityOne Analysis software. The relative levels of p62/SQSTM1 were graphed as the Mean ± SD and significance was defined as **=P<0.01, ***=P<0.001, and ****=P<0.0001 with respect to the control or between specified treatments.
Microarray analysis was mined from the NCBI Gene Expression Omnibus website (GEO; GSE26241) from A549 cells treated with 250 pM TGFβ1 for 0 or 1 hours (h) followed by an 8- or 24-hour washout as I previously described in Chapter 2. The Transcript of interest was p62/SQSTM1.

**TGFβ1-dependent p62/SQSTM1 degradation is independent of TGFβ1-induced autophagy**

Due to the fact that autophagy and the UPP are major protein quality control pathways, I investigated if TGFβ1-induced p62/SQSTM1 degradation was dependent on activities of the lysosome and/or the proteasome. Based on my previous work in Chapter 2 that indicated that TGFβ1 induces autophagy in A549 cells, I assessed if TGFβ1 induces p62/SQSTM1 degradation via the lysosome. First, I examined the steady-state levels of p62/SQSTM1 after pharmacologically inhibiting the acidification of the lysosome using chloroquine. I observed that chloroquine did not affect steady-state p62/SQSTM1 levels on its own and did not attenuate the TGFβ1-mediated p62/SQSTM1 protein loss (Fig. 6.4A). Next, I assessed the effect of spautin-1 on TGFβ1-dependent p62/SQSTM1 loss. I observed that 24 hours of spautin-1 treatment significantly decreased p62/SQSTM1 protein levels by 32±6% with respect to control cells but did not impede TGFβ1-dependent p62/SQSTM1 degradation (Fig. 6.4B). To confirm these results, A549 cells were treated with ULK-101, an inhibitor of autophagy activating ULK1 and ULK2 proteins, and TGFβ1 for 24 hours. Inhibiting ULK1 and ULK2 did not alter TGFβ1-dependent p62/SQSTM1 degradation (Fig. 6.4C). Although I observed that A549 cells transfected with si-Control and siRNA targeting autophagy-related 5 (ATG5) and ATG7 (si-ATG5/7)
decreased Atg5 and Atg7 steady-state protein levels, ATG5/7 silencing showed no differences in p62/SQSTM1 protein levels and did not attenuate TGFβ1-dependent p62/SQSTM1 degradation (Fig. 6.4D).

Since inhibiting autophagy did not disrupt TGFβ1-mediated p62/SQSTM1 degradation, I investigated if activating autophagy would affect p62/SQSTM1 protein levels. Using rapamycin to activate autophagy\(^{398}\), I observed that p62/SQSTM1 protein levels were significantly reduced with respect to control cells and the TGFβ1-dependent p62/SQSTM1 protein loss was enhanced in the presence of rapamycin (Fig. 6.4E). Finally, I assessed if altering cellular p62/SQSTM1 levels would affect TGFβ1-dependent autophagy in NSCLC cells. I first assessed the impact that si-p62/SQSTM1 had on the expression of several ATGs via microarray. As indicated by the bold font, only the microtubule-associated protein light chain 3C (MAPLC3C) and ATG5 expressions were significantly increased by si-p62/SQSTM1 with respect to si-Control (Table 6.2). To investigate the role of p62/SQSTM1 on autophagy further, I treated A549 cells expressing GFP-LC3B-RFP-LC3BΔG with TGFβ1 and/or si-p62/SQSTM1. I previously showed in Chapter 2 that this cell line undergoes TGFβ1-dependent autophagy by measuring the GFP/RFP ratio that is a readout for the degradation of LC3B vs. the LC3BΔG internal control. I observed that TGFβ1 significantly increased LC3B-II steady-state levels to 218±15%, compared to untreated cells and decreased the GFP-LC3B/RFP-LC3BΔG ratio. However, silencing p62/SQSTM1 had no effect on LC3B-II protein levels or the GFP-LC3B/RFP-LC3BΔG ratio in the presence or absence of TGFβ1 (Fig. 6.4F). Given that siRNA targeting p62/SQSTM1 did not alter TGFβ1-dependent LC3B-II production nor the GFP-LC3B/RFP-LC3BΔG ratio, I concluded that p62/SQSTM1 protein levels are unrelated to TGFβ1-dependent autophagy in NSCLC cells.
Figure 6.4: Autophagy does not facilitate TGFβ1-mediated p62/SQSTM1 degradation.
(A) A549 cells were treated with 50 μM chloroquine and 250 pM TGFβ1 for 24 hours (h), lysed, processed for SDS-PAGE, and immunoblotted with anti-p62/SQSTM1 and anti-GAPDH (loading control) antibodies. The relative levels of p62/SQSTM1 were quantitated from 3 independent experiments and graphed as the mean ± SD. Significance is defined as ****=P<0.0001 with respect to the control or between specified treatments.

(B) A549 cells were treated with 10 μM spautin-1 and 250 pM TGFβ1 for 24 h, lysed, processed for SDS-PAGE, and immunoblotted with anti-p62/SQSTM1 and anti-GAPDH antibodies. Equivalent volumes of dimethyl sulfoxide (DMSO) were used as a vehicle control for spautin-1 treated cells. The relative levels of p62/SQSTM1 were quantitated from 3 independent experiments and graphed as the mean ± SD. Significance is defined as **=P<0.01, ***=P<0.001, and ****=P<0.0001 with respect to the control or between specified treatments.

(C) A549 cells were treated with 10 μM ULK-101 and 250 pM TGFβ1 for 24 h, lysed, processed for SDS-PAGE, and immunoblotted with anti-p62/SQSTM1 and anti-GAPDH antibodies. Equivalent volumes of DMSO were the vehicle control for ULK-101 treated cells. The relative levels of p62/SQSTM1 were quantitated from 3 independent experiments and graphed as the mean ± SD. Significance defined as **=P<0.01 with respect to the control.

(D) A549 cells transfected with 10 nM control siRNA (si-Control) or 5 nM siRNA targeting autophagy-related (ATG)5 and 5 nM siRNA targeting ATG7 (si-ATG5/7) were treated for 24 h and 48 h with 250 pM TGFβ1, lysed, processed for SDS-PAGE, and immunoblotted for p62/SQSTM1, Atg7, Atg5, and GAPDH as indicated. Three separate experiments were performed and p62/SQSTM1, Atg7, and Atg5 levels were quantitated using QuantityOne Analysis software. The relative levels were graphed as the mean ± SD and significance is defined as *=P<0.05, **=P<0.01, ***=P<0.001, and ****=P<0.0001 with respect to the control or between specified treatments.

(E) A549 cells were treated with 200 nM rapamycin and 250 pM TGFβ1 for 24 h, lysed, processed for SDS-PAGE, and immunoblotted with anti-p62/SQSTM1 and anti-GAPDH antibodies. Equivalent volumes of DMSO were used as the vehicle control for rapamycin treated cells. The relative levels of p62/SQSTM1 were quantitated from 3 independent
experiments and graphed as the mean ± SD. Significance defined as *=P<0.05, **=P<0.01, and ****=P<0.0001 with respect to the control or between specified treatments.

(F) A549 cells stably expressing GFP-LC3B-RFP-LC3BΔG were transfected with 10 nM si-Control or 10 nM siRNA targeting p62/SQSTM1 (si-p62/SQSTM1) were incubated for 24 h in the presence or absence of 250 pM TGFβ1. The cells were lysed, processed for SDS-PAGE, and immunoblotted using anti-p62/SQSTM1, anti-LC3B, and anti-GAPDH antibodies. The relative levels of LC3B-II and GFP/RFP ratio were quantitated using QuantityOne Analysis software and graphed. The graphs represent 3 independent experiments (mean ± SD). Significance represented as **=P<0.01 and ***=P<0.001 with respect to the control or between specified treatments.

Table 6.2: The effect of si-p62/SQSTM1 on ATG expression.

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Autophagy-related gene (ATG), GABA type A receptor-associated protein (GABARAP), and Microtubule-associated protein light chain 3 (MAP1LC3).
TGFβ1-induced p62/SQSTM1 degradation by means of a proteasome-dependent pathway

Since inhibiting lysosomal acidification or proteins essential for initiating autophagy did not modulate TGFβ1-dependent p62/SQSTM1 degradation, I next investigated the impact of pharmacologically inhibiting the proteasome. First, I treated A549 cells with the proteasome inhibitor, lactacystin\textsuperscript{400}, in the presence and absence of TGFβ1. Although lactacystin decreased p62/SQSTM1 protein levels by 23±4% compared to vehicle-treated cells, it did not increase TGFβ1-dependent degradation of p62/SQSTM1 compared to TGFβ1 treatment alone (Fig. 6.5A). Indeed, it was observed to partially attenuate the TGFβ1 affect on p62/SQSTM1 loss. To further assess if inhibiting proteasome activity would inhibit TGFβ1-dependent p62/SQSTM1 loss, cells were treated with a second proteasome inhibitor, MG132\textsuperscript{312}. I observed that MG132 did not reduce steady-state p62/SQSTM1 levels on its own and greatly inhibited the loss of p62/SQSTM1 in the presence of TGFβ1 (Fig. 6.5B). To assess the potential mechanism of how inhibiting the proteasome would decrease TGFβ1-dependent p62/SQSTM1 loss, I investigated the co-localization of p62/SQSTM1 with lysosomal and proteasomal compartments by immunofluorescence microscopy. Briefly, A549 cells were treated with TGFβ1 in the presence and absence of MG132 and the cells were then subjected to immunofluorescence microscopy to assess how MG132 and/or TGFβ1 impacted the co-localization of p62/SQSTM1 with the late-endosomal/lysosomal marker, LAMP1 or a proteasome marker, 20S proteasome β1. Although TGFβ1 reduced overall p62/SQSTM1 intensity in the cells, it also induced the co-localization of p62/SQSTM1 with LAMP1 by 46±9% (upper panels) but reduced co-localization with the 20S proteasome subunit by 52±1% (lower panels). Moreover, MG132 increased the co-localization of p62/SQSTM1 with both LAMP1 and the 20S proteasome subunit, and MG132 blocked TGFβ1 from decreasing p62/SQSTM1 intensity. Interestingly, MG132 also altered the subcellular localization of p62/SQSTM1, LAMP1-positive, and 20S proteasome-positive compartments to the perinuclear region of the cell (Fig. 6.5C). Taken together, these results suggest that the perturbation of the proteasome induces the co-localization of p62/SQSTM1 with the lysosome and proteasome and attenuates TGFβ1-dependent p62/SQSTM1 degradation.
Figure 6.5: TGFβ1-mediated p62/SQSTM1 degradation is dependent on the proteasome.
(A) A549 cells were treated with 250 pM TGFβ1 or 10 μM lactacystin for 24 hours (h). Equivalent volumes of dimethyl sulfoxide (DMSO) were used as the controls for lactacystin treatments. The cells were lysed, processed for SDS-PAGE, and immunoblotted using anti-p62/SQSTM1 and anti-GAPDH (loading control) antibodies. The relative levels of p62/SQSTM1 were quantitated using QuantityOne Analysis software and graphed. The graphs represent 3 independent experiments (mean ± SD). Significance represented as * = P<0.05 and ** = P<0.01 with respect to the control.

(B) A549 cells were treated with 10 μM MG132 and 250 pM TGFβ1 for 24 h, lysed, processed for SDS-PAGE, and immunoblotted with anti-p62/SQSTM1 and anti-GAPDH antibodies. Equivalent volumes of DMSO were used as the controls for MG132 treated cells. The relative levels of p62/SQSTM1 were quantitated from 3 independent experiments and graphed as the mean ± SD. Significance defined as ** = P<0.01 and *** = P<0.001 with respect to the control or between specified treatments.

(C) A549 cells were incubated with 10 μM MG132 in the presence or absence of 250 pM TGFβ1 for 24 h. The cells were fixed, permeabilized, and incubated with anti-p62/SQSTM1, anti-lysosomal-associated membrane protein 1 (LAMP1) and anti-20S proteasome antibodies prior to incubation with secondary fluorescently labelled antibodies. The co-localization of p62/SQSTM1 (red) with the lysosome (LAMP1; green) or proteasome (20S; green) is shown in yellow. ImageJ quantified the relative area of co-localization and the graph (Mean ± SD) is located on the right of representative images. Significance represented as ** = P<0.01, *** = P<0.001, and **** = P<0.0001 with respect to the control or between specified treatments. Scale bar = 10 μm.

*p62/SQSTM1 knockdown increased Smad-independent TGFβ signalling*

To assess why TGFβ1 facilitates p62/SQSTM1 degradation, I investigated if there is a reciprocal relationship between p62/SQSTM1 protein levels with Smad-dependent or Smad-independent TGFβ signalling pathways. First, I assessed how p62/SQSTM1 knockdown influenced Smad-independent TGFβ1 signalling and observed that p62/SQSTM1 silencing increased P-mTOR/mTOR and P-TAK1/TAK1 ratios in the absence of TGFβ1. In the presence of TGFβ1, although the P-mTOR/mTOR ratios were not significantly different, p62/SQSTM1 silencing increased the P-TAK1/TAK1 and P-
p38/p38 ratios compared to si-Control. However, p62/SQSTM1 silencing does not affect JNK/SAPK phosphorylation or steady-state TRAF6 levels in the presence or absence of TGFβ1 (Fig. 6.6). Overall, p62/SQSTM1 silencing promoted mTOR, TAK1, and p38 signalling pathways.

Figure 6.6: The effect of silencing p62/SQSTM1 on Smad-independent TGFβ1 signalling.

A549 cells transfected with 10 nM si-Control or 10 nM si-p62/SQSTM1 were treated with 250 pM TGFβ1 for 0, 1 or 24 hours (h). The cells were lysed, processed for SDS-PAGE, and immunoblotted for anti-mTOR, anti-P-mTOR, anti-TAK1, anti-P-TAK1, anti-TRAF6, anti-JNK/SAPK, anti-P-JNK/P-SAPK, anti-p38 MAPK, and anti-P-p38 MAPK antibodies. A loading control (GAPDH) was also included in the analysis. Five separate experiments were performed and TRAF6 levels as well as P-mTOR/mTOR, P-TAK1/TAK1, P54-JNK/JNK, P46-JNK/JNK, and P-p38 MAPK/p38 MAPK ratios were quantitated using QuantityOne Analysis software. For all experiments, this data represents three independent experiments (Mean ± SD). Significance is defined as * = P<0.05 and ** = P<0.01 with respect to the control or between specified treatments.

*p62/SQSTM1 knockdown decreases Smad-dependent TGFβ signalling*
After assessing the effect of p62/SQSTM1 silencing on the Smad-independent pathways that regulate TGFβ1-dependent autophagy, I examined the influence that p62/SQSTM1 silencing had on Smad2 phosphorylation and observed that in the presence of TGFβ1 for 24 hours, Smad2 was phosphorylated to a lesser degree in p62/SQSTM1 knockdown cells (67±9%) compared to control cells (Fig. 6.7A). Next, to determine if p62/SQSTM1 is important to the amplitude and/or duration of TGFβ1-dependent Smad2 phosphorylation, I carried out a TGFβ1 time course experiment in A549 cells transfected with si-Control or si-p62/SQSTM1. For the time course, Smad2 phosphorylation was measured after a 1-hour TGFβ1 ligand pulse, followed by a 1-8-hour chase. Immunoblotting revealed that when p62/SQSTM1 protein levels were significantly decreased, the proportion of P-Smad2/Smad2 was halved 8 hours post-ligand washout compared to the si-Control (Fig. 6.7B). Since Smad2 phosphorylation leads to its association with Smad4 and nuclear translocation, I next investigated the effect of silencing p62/SQSTM1 on Smad2 nuclear accumulation. After cells were treated with 250 pM TGFβ1 for 0-6 hours, nuclear and cytoplasmic cell fractions were isolated, and the fractions were subjected to immunoblotting. Interestingly, I observed that p62/SQSTM1 silencing had little effect on the protein levels of nuclear or cytoplasmic Smad2, or the ability of Smad2 to translocate to the nucleus (Fig. 6.7C). Taken together, p62/SQSTM1 silencing enhanced the activity of Smad-independent TGFβ1 signalling pathways but reduced Smad2 phosphorylation. Since p62/SQSTM1 silencing reduced Smad2 phosphorylation but not its nuclear accumulation, I next investigated if this would alter Smad-dependent gene transcription.
Figure 6.7: siRNA-mediated p62/SQSTM1 silencing shortens the duration of TGFβ1-dependent Smad2 phosphorylation.

(A) A549 cells transfected with 10 nM si-Control or 10 nM si-p62/SQSTM1 were treated for 24 h with 250 pM TGFβ1, lysed, processed for SDS-PAGE, and immunoblotted for phosphorylated Smad2 (P-Smad2), Smad2, and p62/SQSTM1 as indicated. A loading
control (GAPDH) was also included in the analysis. Three separate experiments were performed and p62/SQSTM1, P-Smad2, and Smad2 levels were quantitated using QuantityOne Analysis software. The relative levels of p62/SQSTM1 and the ratio of P-Smad2/Smad2 were graphed (Mean ± SD). Significance defined as *=P<0.05, **=P<0.01, and ****=P<0.0001 with respect to the control or between specified treatments.

(B) A549 cells transfected with 10 nM si-Control or 10 nM si-p62/SQSTM1 were treated for 1-h with 250 pM TGFβ1 and then incubated for an additional 0-8 h before lysis. Immunoblotting was then carried out as described in Panel A. Three separate experiments were performed and p62/SQSTM1, P-Smad2, and Smad2 levels were quantitated using QuantityOne Analysis software. The relative levels of p62/SQSTM1 and the ratio of P-Smad2/Smad2 were graphed (Mean ± SD). Significance defined as *=P<0.05, **=P<0.01, and ***=P<0.001 compared to si-Control or #=P<0.05 compared to 1-h TGFβ1.

(C) A549 cells transfected with 10 nM si-Control or 10 nM si-p62/SQSTM1 were incubated with 250 pM TGFβ1 for 0, 0.5, 3, and 6 h. The cells were then lysed, fractionated into cytoplasmic and nuclear cellfractions, subjected to SDS-PAGE, and immunoblotted with anti-Smad2, anti-p62/SQSTM1, anti-tubulin (cytoplasmic marker) or anti-lamin A/C (nuclear marker) antibodies.

*p62/SQSTM1 regulates Smad-dependent gene transcription*

Given that p62/SQSTM1 silencing alters the balance of Smad-dependent and Smad-independent TGFβ1 signalling, I assessed the effect that p62/SQSTM1 knockdown had on the expression of Smad-dependent genes, with an emphasis on epithelial and mesenchymal markers. I performed microarray analysis in A549 cells transfected with si-Control or si-p62/SQSTM1 for 48 hours. The microarray assessed the expression of p62/SQSTM1, epithelial markers, mesenchymal markers, EMT-transcription factors (EMT-TFs), mesenchymal-epithelial transition-transcription factors (MET-TFs), and microRNAs that target EMT-TFs and MET-TFs. Indeed, the expression of several transcription factors such as *Twist1, SNAIL, zinc finger E-box-binding homeobox 1 (ZEB1)*, and *Hairy/enhancer-of-split related with YRPW motif protein 1 (HEY1)* were significantly decreased by p62/SQSTM1 silencing (Table 6.3).
Table 6.3: The effect of si-p62/SQSTM1 on EMT-transcription factors.

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Cadherin (CDH), Zinc Finger E-box-binding Homebox (ZEB), Hairy/enhancer-of-split related with YRPW motif protein 1 (HEY1), Forkhead box A1 (FOXA1), MicroRNA (MIR), Plasminogen Activator Inhibitor-1 (PAI-1), and Smad ubiquitination regulatory factor (SMURF).

To follow up on the microarray analyses, using qPCR, with the help of Dr. Craig Campbell, we measured the relative mRNA levels of an epithelial marker (E-Cadherin; CDH1), a mesenchymal marker (N-Cadherin; CDH2), SERPINE1, which encodes PAI-1, SNAIL, and SLUG. Interestingly, we observed that p62/SQSTM1 silencing significantly decreased CDH1 mRNA levels in the presence or absence of TGFβ1, whereas the increase in TGFβ1-dependent CDH2 expression was enhanced in the absence of p62/SQSTM1. Additionally, siRNA-mediated silencing of p62/SQSTM1 decreased SERPINE1 expression in the presence of TGFβ1. Finally, although p62/SQSTM1 silencing did not alter SNAIL expression, the increase in TGFβ1-dependent SLUG expression was enhanced by the loss of p62/SQSTM1 (Fig. 6.8A).
Given that p62/SQSTM1 knockdown influenced the expression of Smad-dependent genes, I next investigated the influence of silencing p62/SQSTM1 on the corresponding steady-state protein levels. After I verified that p62/SQSTM1 knockdown and 24 hours of TGFβ1 significantly decreased p62/SQSTM1 protein levels by 70±5% and 30±5% compared to untreated cells, respectively, I assessed the protein levels of EMT-TFs and PAI-1. I observed that the TGFβ1-dependent increase of steady-state PAI-1 levels was decreased by p62/SQSTM1 knockdown. However, p62/SQSTM1 silencing did not alter the TGFβ1-dependent increase of steady-state Snail or Slug levels. Finally, neither TGFβ1 nor p62/SQSTM1 knockdown altered steady-state Twist1 levels (Fig. 6.8B). Taken together, these results suggest that p62/SQSTM1 regulates the expression of some TGFβ1-dependent genes.
Figure 6.8: p62/SQSTM1 silencing modulates the expression of Smad regulated genes.

(A) Dr. Craig Campbell transfected A549 cells with 10 nM si-Control or 10 nM si-p62/SQSTM1 that were treated with 250 pM TGFβ1 for 24 hours (h) and processed for quantitative polymerase chain reaction (qPCR) analysis to assess mRNA that encode E-Cadherin (CDH1), N-Cadherin (CDH2), PAI1 (SERPINE1), Snail (SNAI1), and Slug (SNAI2). For all experiments, this data represents three independent experiments (Mean ± SD). Significance is defined as *=P<0.05, **=P<0.001, and ***=P<0.0001 with respect to the control or between specified treatments.

(B) A549 cells transfected with 10 nM si-Control or 10 nM si-p62/SQSTM1 were treated with 250 pM TGFβ1 for 0, 1 or 24 h. The cells were lysed, subjected to SDS-PAGE, and immunoblotted for anti-PAI-1, anti-P-Smad2, anti-p62/SQSTM1, anti-Slug, anti-Snail,
and anti-Twist1 antibodies. A loading control (GAPDH) was also included in the analysis. Three separate experiments were performed and p62/SQSTM1, PAI-1, Slug, Snail, and Twist levels were quantitated using QuantityOne Analysis software. For all experiments, this data represents three independent experiments (Mean ± SD). Significance is defined as * = P<0.05, ** = P<0.01, *** = P<0.001, and **** = P<0.0001 with respect to the control or between specified treatments.

**p62/SQSTM1 silencing regulates E-Cadherin expression, stress fiber formation, and cell migration**

Since my observations suggested that TGFβ1 decreased p62/SQSTM1 steady-state protein levels and p62/SQSTM1 silencing altered TGFβ1-dependent gene regulation of epithelial and mesenchymal markers, I next evaluated if p62/SQSTM1 plays a role in TGFβ1-dependent EMT. I first assessed the ability of p62/SQSTM1 to influence the E- to N-Cadherin shift that occurs during EMT. Briefly, immunoblotting was used to investigate how p62/SQSTM1 knockdown affected basal and TGFβ1-dependent E-Cadherin and/or N-Cadherin protein levels. Both p62/SQSTM1 knockdown and TGFβ1 significantly decreased E-Cadherin protein levels. In fact, the lack of cellular p62/SQSTM1 increased the TGFβ1-dependent loss of E-Cadherin. Interestingly, p62/SQSTM1 silencing did not affect basal N-Cadherin protein levels and furthermore did not alter the TGFβ1-dependent increase in N-Cadherin levels after 24 or 48 hours of TGFβ1 treatment (Fig. 6.9A), suggesting that this may be specific to the expression of E-Cadherin. To investigate this further, together with Dr. Craig Campbell, we assessed the consequences of p62/SQSTM1 over-expression on E-Cadherin protein levels. We observed that basal E-Cadherin protein levels were significantly increased when p62/SQSTM1 was overexpressed. Furthermore, the overexpression of p62/SQSTM1 also interfered with TGFβ1-dependent E-Cadherin loss (Fig. 6.9B). These results suggest that p62/SQSTM1 may aid in the maintenance of E-Cadherin protein levels and impede EMT.

To assess if the interference of p62/SQSTM1 with EMT resulted in downstream consequences, I carried out fluorescence microscopy to assess how p62/SQSTM1 knockdown influenced TGFβ1-dependent stress fiber formation, which is a property of cells undergoing EMT. First, I quantified the pixel intensity of p62/SQSTM1, which
revealed that TGFβ1 and siRNA targeting p62/SQSTM1 decreased p62/SQSTM1 signal intensity. Next, I observed that, in the presence and absence of TGFβ1, the proportion of cells containing stress fibers was significantly increased by p62/SQSTM1 knockdown compared to control cells. Indeed, the combination of TGFβ1-treatment and p62/SQSTM1 silencing induced the most stress fiber formation (82±3% of cells) compared to TGFβ1-treated control cells (56±2% of cells; Fig. 6.9C). Finally, I assessed how p62/SQSTM1 knockdown influenced TGFβ1-dependent migration using a scratch assay. I observed that, in the presence and absence of TGFβ1, p62/SQSTM1 knockdown increased cell migration (Fig. 6.9D). Taken together my results suggest that p62/SQSTM1 levels in A549 cells regulate E-Cadherin levels and that TGFβ signalling reduces p62/SQSTM1 levels to prime these epithelial cells to undergo EMT.
Figure 6.9: p62/SQSTM1 silencing promotes stress fiber formation and migration.
(A) A549 cells transfected with 10 nM control siRNA (si-Control) or 10 nM siRNA targeting p62/SQSTM1 (si-p62/SQSTM1) were incubated in the absence or presence of 250 pM TGFβ1 for 0-48 hours (h). The cells were then lysed, subjected to SDS-PAGE, and immunoblotted for E-Cadherin, N-Cadherin, phosphorylated Smad2 (P-Smad2), Smad2, p62/SQSTM1, and GAPDH (loading control) as indicated. Four separate experiments were carried out as described and E-Cadherin and N-Cadherin levels were quantitated using QuantityOne Analysis software. The relative levels of E-Cadherin and N-Cadherin were graphed (Mean ± SD). Significance is defined as *=P<0.05, **=P<0.01, and ****=P<0.0001 with respect to the control or between specified treatments.

(B) Dr. Craig Campbell transfected A549 cells with pCMV5 empty vector (-) or a vector containing HA-tagged p62/SQSTM1 for 24 h. The cells were then treated with 250 pM TGFβ1 for 0-48 h and lysed. Cell lysates were processed for SDS-PAGE and immunoblotted with anti-E-Cadherin, anti-p62/SQSTM1, and anti-GAPDH antibodies. The relative levels of E-Cadherin were quantitated from 3 independent experiments and graphed as the mean ± SD. Significance defined as *=P<0.05, **=P<0.01, and ****=P<0.0001 with respect to the control.

(C) A549 cells transfected with 10 nM si-Control or 10 nM si-p62/SQSTM1 were incubated in the presence or absence of 250 pM TGFβ1 for 48 h. The cells were then fixed, permeabilized, and incubated with Alexa Fluor555-Phalloidin to label filamentous actin, anti-p62/SQSTM1, and DAPI (nuclear stain). Cortical actin or stress fibers were visualized using an Olympus IX 81 inverted fluorescence microscope. Scale bar = 10 µm. The proportion of cells containing stress fibers were compared to the total number of cells for each treatment and graphed (left). The intensity of p62/SQSTM1 staining was quantified by ImageJ and graphed (right). For all experiments, this data was derived from 4 independent replicates where each value represents a mean ± SD. Significance defined as **=P<0.01, ***=P<0.001, and ****=P<0.0001 with respect to the control or between specified treatments.

(D) Confluent monolayers of A549 cells treated with 10 nM si-Control or 10 nM si-p62/SQSTM1 were incubated in the presence or absence of 250 pM TGFβ1 for 24 h. Images were acquired immediately after scratching (0 h) and 24 h post-scratch using a 10x
objective of an Olympus IX 81 microscope. ImageJ quantified the width of the scratch from 3 independent experiments, and this was graphed (mean ± SD). Significance is indicated as *=P<0.05 and ****=P<0.0001 with respect to the control or between specified treatments. Scale bars = 40 μm.

6.4 Discussion

In general, NSCLCs are able to evade the tumour suppressive arms of TGFβ signalling while keeping tumour progressive signalling intact45. Numerous studies using A549 cells have highlighted that TGFβ is a potent inducer of EMT, autophagy, and promotes proliferation and survival of tumour cells44,151,152,306. For these reasons, I utilized A549 cells to characterize the relationship between TGFβ signalling, EMT, autophagy, and p62/SQSTM1.

It is known that p62/SQSTM1 interacts with TGFβ signalling molecules, such as aPKC249. Previous studies have shown that aPKC is an important regulator of TGFβ signalling, EMT, and associates with TGFβ receptors36,308,339. As a binding partner to aPKC, I believed that p62/SQSTM1 had potential to modulate TGFβ signalling and EMT. I first investigated the role of p62/SQSTM1 on TGFβ signalling by investigating if it regulated TGFβ receptor trafficking. Indeed, p62/SQSTM1 co-localized with TGFβ receptors in the late endosome compartment. In addition to p62/SQSTM1 silencing increasing p38 and TAK1 phosphorylation, which are components of Smad-independent TGFβ signalling, I found that p62/SQSTM1 knockdown significantly blunted the duration of Smad2 phosphorylation. Since phosphorylated R-Smads function as transcription factors and Smad2 phosphorylation may alter the timeframe for its nuclear translocation, I assessed if p62/SQSTM1 knockdown influenced Smad2 nuclear translocation and transcriptional activity. p62/SQSTM1 silencing did not impede Smad2 nuclear translocation, which suggests that p62/SQSTM1 is not essential to processes involved with Smad2 nuclear delivery. However, p62/SQSTM1 knockdown altered the expression of Smad-dependent genes. For instance, silencing p62/SQSTM1 resulted in a decrease in CDH1 and SERPINE1 expression, yet increased CDH2 and SLUG expression. Therefore, when p62/SQSTM1 is silenced in A549 cells, TGFβ signalling is altered to induce a pro-mesenchymal transcriptome that could augment an EMT program.
The fact that p62/SQSTM1 silencing altered Smad2 phosphorylation and the expression of Smad targeted genes is not surprising because p62/SQSTM1 influences transcription by regulating transcription factor stability\textsuperscript{281,387}. As determined by the microarray and qPCR, p62/SQSTM1 silencing altered the expression of several transcription factors that regulate genes that promote epithelial or mesenchymal properties. As previously mentioned, p62/SQSTM1 knockdown decreased and increased SNAI1 and SNAI2 expression, respectively. This is paradoxical because Snail and Slug function as EMT-TFs that repress the expression of epithelial genes such as CDH1.\textsuperscript{153,438} In order to investigate if reduced p62/SQSTM1 expression altered transcription factors to augment an EMT program, I assessed the relationship between p62/SQSTM1 and E-Cadherin protein levels. Indeed, p62/SQSTM1 knockdown significantly decreased E-Cadherin protein levels while p62/SQSTM1 over-expression significantly increased E-Cadherin protein levels. Furthermore, p62/SQSTM1 knockdown increased the proportion of cells containing stress fibers and A549 cell migration. Based on these data, p62/SQSTM1 protein levels are associated with maintaining A549 cells in an epithelial state.

Although p62/SQSTM1 impacts TGFβ1 signalling, it is important to note that TGFβ1 also regulates p62/SQSTM1 levels. However, the effects of TGFβ1 on p62/SQSTM1 expression and protein levels may be variable, as it has been reported that TGFβ can increase\textsuperscript{281} or decrease\textsuperscript{251} p62/SQSTM1 protein levels. In this study, TGFβ1 had no effect on p62/SQSTM1 expression but decreased p62/SQSTM1 protein levels. The literature suggests that the manner in which p62/SQSTM1 responds to TGFβ is context-dependent, and many factors can influence how p62/SQSTM1 acts in tumourigenesis\textsuperscript{275,439}. Some of these factors include cell phenotype (i.e. epithelial vs. stromal cells), the stage of the tumour, and/or cellular localization\textsuperscript{407}. p62/SQSTM1 is also involved in both tumour suppressing and tumour promoting pathways, which indicates that the function of p62/SQSTM1 in tumours is dependent on signalling crosstalk\textsuperscript{276}. For this reason, I investigated when TGFβ1-mediated p62/SQSTM1 degradation occurred with respect to TGFβ1-dependent EMT. A TGFβ1 time course revealed that p62/SQSTM1 protein levels decreased prior to significant changes in E-Cadherin or N-Cadherin protein levels (data not shown), which conveys that TGFβ may need to eliminate p62/SQSTM1 to induce EMT in A549 cells.
Since I observed that attenuating TGFβRI kinase activity with SB431542 rescued p62/SQSTM1 protein levels, I concluded that downstream signalling of TGFβRI is responsible for TGFβ1-dependent p62/SQSTM1 degradation. To determine if Smad-dependent or -independent TGFβ1 signalling was responsible for p62/SQSTM1 degradation, I first treated A549 cells with si-Smad4. Interestingly, si-Smad4 increased p62/SQSTM1 protein levels compared to si-Control, which suggests that Smad4 upregulates genes that degrade p62/SQSTM1. However, Smad4 silencing did not rescue p62/SQSTM1 from TGFβ1-dependent degradation. Finally, microarray analysis determined that TGFβ1 has no influence on p62/SQSTM1 expression. In conclusion, because TGFβ1 does not alter p62/SQSTM1 expression, the changes observed in p62/SQSTM1 protein levels is mediated by degradation. Moreover, since si-Smad4 does not attenuate TGFβ1-dependent p62/SQSTM1 degradation, this process is Smad4-independent.

In order to determine the mechanism of TGFβ1-mediated p62/SQSTM1 degradation, my focus shifted to primary protein quality control pathways, which include autophagy and the UPP. Autophagy is defined as a collection of catabolic processes characterized by lysosomal degradation of a cells own macromolecules and organelles. Alternatively, the UPP relies on ubiquitination, which is a process where ubiquitin is conjugated to proteins by E1, E2, and E3 enzymes. Ubiquitin receptor proteins, such as p62/SQSTM1, recognize the ubiquitin chains on the target protein and transport it to the 26S proteasome, a multi-subunit protease, that degrades ubiquitin-conjugated proteins. Using its ubiquitin-associated domain, p62/SQSTM1 binds to ubiquitinated proteins and facilitates their delivery to lysosomes or 26S proteasomes. As such, p62/SQSTM1 is known to be a cargo receptor and substrate for both lysosomal and proteasomal-dependent degradation.

I found that TGFβ1 increased p62/SQSTM1 and LAMP1 co-localization, which suggested that p62/SQSTM1 is degraded by lysosomes. One would expect an inverse relationship between autophagy and p62/SQSTM1 protein levels because p62/SQSTM1, p62/SQSTM1 target proteins, and autophagosomes are all degraded by lysosomes. However, disrupting autophagosome production with ULK-101 and spautin-1 and attenuating lysosomal degradation with chloroquine did not decrease p62/SQSTM1
protein loss in the presence of TGFβ1. In contrast, activating autophagy with rapamycin increased p62/SQSTM1 degradation in the presence and absence of TGFβ1. Therefore, although autophagy was observed to induce p62/SQSTM1 protein degradation, lysosomes were not observed to be responsible for TGFβ1-dependent p62/SQSTM1 degradation. Alternatively, proteasome inhibitors, such as lactacystin and MG132, blocked the TGFβ1-mediated decrease in p62/SQSTM1 protein levels, which suggested that TGFβ1 decreased p62/SQSTM1 protein levels via proteasomes. Interestingly, proteasome inhibitors activate autophagy because the UPP and autophagy are highly coordinated processes to control protein degradation. When proteasomes are inhibited, unfolded, and damaged proteins accumulate and form protein aggregates that disrupt cellular physiology and could initiate cell death responses. Therefore, in the presence of MG132 and lactacystin, autophagy is upregulated to sequester protein aggregates and preserve cellular homeostasis. As such, since these proteasomal inhibitors activate autophagy and impeded TGFβ1-dependent p62/SQSTM1 degradation, I am certain that lysosomes do not mediate the bulk of p62/SQSTM1 degradation in the presence of TGFβ1.

Since p62/SQSTM1 functions as an autophagy cargo receptor, it was surprising that silencing p62/SQSTM1 had no impact on autophagic flux or the expression of ATGs. Therefore, the impact that p62/SQSTM1 silencing had on Smad2 phosphorylation and EMT is autophagy-independent. Furthermore, this result highlighted the need for further investigation of the role of p62/SQSTM1 in TGFβ1-induced autophagy. Future experiments will focus on defining the proteasomes’ role in TGFβ1-dependent p62/SQSTM1 degradation and characterize the relationship between the UPP, autophagy, and TGFβ activity.

It is clear that p62/SQSTM1 is involved in tumourigenesis, as several studies show that its expression is abnormal in various cancer types, including lung, ovarian, breast, and prostate. In this study, I outlined that p62/SQSTM1 modulates TGFβ1 signalling, possibly through altering the time course of Smad2 phosphorylation and TGFβRII trafficking, upregulates Smad-independent TGFβ1 signalling pathways, and that TGFβ1 reduces p62/SQSTM1 protein levels to induce EMT. However, as described above, the function of p62/SQSTM1 in cancer may be cell type-dependent. As such, the consideration of p62/SQSTM1 as a potential therapeutic target is complex and additional
studies on other cell lines and models will characterize the role of p62/SQSTM1 in tumourigenesis.
Chapter 7

DISCUSSION
7 Chapter 7

7.1 Significant Contributions

Chapter 2

This work was the first to show that transforming growth factor-β (TGFβ) increased the rate of autophagic degradation in NSCLC cells. Although other work had previously indicated that TGFβ increases autophagy-related gene (ATG) transcription, steady-state autophagy-related protein (Atg) levels, autophagosomes, and green fluorescent protein (GFP)-microtubule-associated protein light chain 3 (LC3) puncta, these techniques did not fully differentiate autophagy activation from antagonizing lysosomal activity. This work was also the first to highlight that TGFβ-dependent autophagy is dependent on Atg5 and Atg7.

Chapter 3

Here, I elucidated the mechanism of TGFβ1-dependent autophagy. This work demonstrated that both Smad4 and the TGFβ-activated kinase 1/tumour necrosis factor receptor-associated factor 6/p38 mitogen-activated protein kinase (TAK1-TRAF6-p38 MAPK) pathway are two signalling branches that drive pro-autophagic TGFβ1 signalling. Further investigation revealed that TGFβ1 activated canonical macroautophagy by antagonizing mTOR activity and increasing the proportion of active ULK1, which mediates macroautophagy initiation.

Chapter 4

This work was the first to characterize the mechanism describing how autophagy inhibition regulates TGFβ-induced epithelial-mesenchymal transition (EMT) and Smad phosphorylation. Indeed, I observed that autophagy inhibitors disrupted TGFβ receptor type II (TGFβRII) internalization and trafficking. This ultimately decreased receptor regulated Smad (R-Smad) phosphorylation and nuclear translocation, which mitigated the impact that TGFβ had on cellular processes including transcription, EMT, and cell migration.
This work also linked the pro-autophagic TGFβ1 signalling pathways to protumourigenic signalling. For instance, when the Smad4 or TAK1-TRAF6-p38 MAPK pathways were silenced, I observed a reversal in the steady-state Snail and Slug levels, stress fiber formation, and cell migration compared to the TGFβ1 treatment.

Chapter 5

Although other reports have suggested that proteasome inhibitors affect EMT, apoptosis, and TGFβRII degradation, this work was the first to develop a working model to describe how these processes are linked. I observed that proteasome inhibitors activate apoptosis in part through the TAK1 and p38 MAPK pathway, which my data implicated in TGFβ-dependent autophagy. Furthermore, proteasome inhibitors increased canonical macroautophagy and TGFβRII-lysosome co-localization, which suggested that upon the attenuation of proteasome activity, TGFβRII is targeted to lysosomes. Therefore, fewer TGFβ receptors available to bind to TGFβ1 decreased R-Smad phosphorylation and TGFβ1-dependent outcomes, such as EMT, stress fiber formation, and cell migration.

Chapter 6

This work was the first to characterize the role of p62/SQSTM1 in autophagy, EMT, and TGFβ1 signalling in A549 cells. I observed that p62/SQSTM1 silencing had no effect on autophagy; however, it decreased R-Smad phosphorylation and primed cells to undergo EMT. Additionally, I found that TGFβ1 decreased steady-state p62/SQSTM1 levels via proteasomes prior to initiating EMT.

7.2 General Summary

The strength and duration of TGFβ signalling is linked to receptor endocytosis and trafficking. TGFβ receptors internalized via clathrin-coated pits into early endosomes propagate signalling whereas internalization via caveolae promotes receptor degradation and attenuates signalling. TGFβ signalling also depends on the availability of downstream Smad and non-Smad proteins. Smad proteins are transcription factors that alter cell behaviour through modulating transcription whereas non-Smad proteins can influence transcription, translation, post-translation modifications, and protein
interactions\textsuperscript{39,65}. Together, Smad-dependent and -independent TGF\(\beta\) signals regulate a wide array of biological processes including apoptosis, growth, differentiation, proliferation, migration, and autophagy\textsuperscript{35}. Given that many of these pleotropic signalling pathways modulate cellular homeostasis, TGF\(\beta\) has been implicated in both suppressing and augmenting tumourigenesis\textsuperscript{449}. Indeed, TGF\(\beta\) in non-cancerous cells prevents growth, differentiation, and migration but induces cell death\textsuperscript{450}. However, many tumour cells acquire mutations within TGF\(\beta\) signalling branches that block tumour suppressive signalling, which exacerbates pro-tumourigenic signals\textsuperscript{451}. As a result, TGF\(\beta\) signalling has been implicated in EMT\textsuperscript{164}, angiogenesis\textsuperscript{452}, immunosuppression\textsuperscript{140}, and autophagy upregulation\textsuperscript{260}.

Like TGF\(\beta\), the role of autophagy in tumourigenesis is complex. For instance, lysosomal degradation of damaged or superfluous organelles and macromolecules shield cells from accumulated damages that promote tumourigenesis\textsuperscript{185}. Alternatively, tumour cells upregulate autophagy to encourage EMT, anoikis resistance, evasion of drug therapies, and to endure hypoxic and metabolic stress\textsuperscript{253}. Indeed, it has been shown that impeding autophagy increases tumour cell death in response to therapies and minimizes the formation of metastatic colonies\textsuperscript{316,376}.

Recently, autophagy inhibitors were found to suppress TGF\(\beta\)-dependent EMT\textsuperscript{251}. Therefore, in this thesis, I examined the relationship between TGF\(\beta\), EMT, and autophagy using NSCLC cell lines. Although TGF\(\beta\)1 is known to activate autophagy\textsuperscript{248,306,307}, the mechanism of TGF\(\beta\)1-dependent autophagy was unknown. Furthermore, there are few experimental models that accurately measure autophagy because many experimental approaches currently employed have difficulty distinguishing between inhibiting autophagosome-lysosome fusion/autophagosome degradation from upregulating autophagic degradation\textsuperscript{196}. For this reason, I first needed to validate a model that could accurately measure autophagic flux. Eventually, using proven autophagy inhibitors (spautin-1, chloroquine, ULK-101, and si-ATG5/7) and autophagy inducers (MG132, lactacystin, rapamycin, and serum starvation), I discovered NSCLC cells stably transfected with a pMRX-IP-GFP-LC3B-RFP-LC3B\(\Delta\)G vector could accurately measure TGF\(\beta\)1-dependent autophagy. Next, I examined the mechanism of TGF\(\beta\)1-dependent autophagy.
by silencing specific components of Smad-dependent and -independent TGFβ signalling pathways. I observed that silencing Smad4 or the TAK1-TRAF6-p38 MAPK pathway disrupted autophagy in the presence of TGFβ1. Furthermore, I found that TGFβ1 activates canonical macroautophagy and silencing Smad4 or the TAK1-TRAF6-p38 MAPK decreased P-S555-ULK1 phosphorylation and/or increased P-S2448-mTOR phosphorylation. Therefore, Smad4 and TAK1-TRAF6-p38 MAPK TGFβ1 signalling branches increased the proportion of ULK1 primed to initiate autophagy and decreased the proportion of mTOR that functions to antagonize autophagy.187

Since autophagy has been linked to TGFβ signalling251, I next examined how autophagy regulates TGFβ signalling. I observed that autophagy inhibitors delayed TGFβRII internalization and trafficking to early endosomes, late endosomes, and lysosome membrane compartments. As a result, R-Smad phosphorylation was reduced by autophagy inhibitors and ATG5/7 siRNA targeting. Since Smad phosphorylation and nuclear translocation are linked, I found that autophagy inhibition decreased nuclear Smad levels. Therefore, fewer nuclear R-Smads would dampen signalling and explain how autophagy inhibitors impeded TGFβ-dependent outcomes, such as E- to N-Cadherin shift, stress fiber formation, and cell migration.

Recently, it was reported that TGFβ-dependent EMT, migration, and proliferation can be blunted by proteasome inhibitors46,280. Therefore, similar to autophagy, the proteasome may influence TGFβ signalling. Furthermore, proteasome and autophagic degradation have been reported to compensate for one another. Indeed, degradation mediated by lysosomes and proteasomes is carefully coordinated such that if one pathway is inhibited the other will compensate by increasing degradation.384 For example, when proteasomes are old or damaged, aggregated proteins accumulate, which results in cell stress. To compensate for this, proteins then clump into clusters called aggregates, which are targeted to autophagosomes via autophagy cargo receptors.268 For this reason, it is important to understand how altering the balance between autophagic and proteasomal activities may contribute to tumourigenesis.

During my work to investigate how proteasomes regulate TGFβ signalling and EMT, I noticed significant overlaps in how NSCLC cells respond to proteasome inhibitors compared to autophagy inhibition. For instance, in addition to inducing autophagy and
apoptosis, I determined that pharmacologically impeding proteasome degradation increased lysosomal-mediated degradation of TGFβRII that ultimately impeded R-Smad phosphorylation, R-Smad nuclear translocation, EMT, and cell migration. More specifically, this implies that the rapid TGFβRII turnover mediated by lysosomes during proteasome inhibition reduced R-Smad phosphorylation because fewer receptors available for TGFβ1 binding would decrease R-Smad phosphorylation, R-Smad nuclear translocation, and downstream outcomes, such as EMT and cell migration. One notable difference between targeting proteasomes versus autophagy was that proteasome inhibitors did not delay or decrease TGFβRII trafficking but instead increased it. Regardless of whether TGFβRII could not internalize to phosphorylate R-Smads (autophagy inhibition) or were degraded prior to phosphorylating R-Smads (proteasome inhibitors), evidence of pro-tumourigenic TGFβ signalling was attenuated by proteasome and lysosome inhibitors. Therefore, disrupting the balance of proteasomal and autophagic degradation is sufficient to disrupt TGFβ1 signalling.

Interestingly, I observed that proteasome inhibitors may have increased apoptosis and autophagy through the TAK1-cJun amino-terminal kinase/stress-activated protein kinase (JNK/SAPK)-p38 MAPK pathway, which I linked to autophagy activation\(^{\text{360}}\). This was surprising because proteasome inhibitors may disrupt and upregulate Smad-dependent and Smad-independent TGFβ signalling, respectively. I also found that proteasome inhibitors upregulated canonical macroautophagy by modulating mTOR and ULK1 activity. Given that TAK1 and p38 MAPK promote TGFβ-dependent autophagy, these proteins may be important mediators of autophagy upon proteasome inhibition.

Given that proteasome inhibitors activate autophagy and increase lysosomal-targeting of TGFβRII, whereas autophagy inhibitors delay TGFβRII trafficking, I next investigated the effect of targeting both proteasomes and autophagy on TGFβ signalling. p62/SQSTM1, an autophagy cargo receptor, delivers cargo to proteasomes and lysosomes and interacts with downstream TGFβ signalling molecules. Therefore, I was interested in how p62/SQSTM1 silencing influenced proteasome/lysosome-dependent degradation as well as TGFβ signalling. I observed that p62/SQSTM1 participates in TGFβRII trafficking and associates with TGFβRII in late endosomes. p62/SQSTM1 also regulated the balance of Smad-dependent and -independent TGFβ signalling. For instance, p62/SQSTM1
silencing upregulated Smad-independent pathways and dampened R-Smad phosphorylation. Although p62/SQSTM1 silencing did not impede autophagy, I found that it upregulated EMT markers, stress fiber formation, and cell migration. Using A549 NSCLC cells, I concluded that TGFβ1 promotes p62/SQSTM1 degradation via proteasomes prior to inducing EMT. In summary, the activity of lysosomes and proteasomes regulate homeostasis and altering the balance of their activities may drive tumourigenesis and aberrant TGFβ signalling.

The hypothesis tested in this thesis was that if proteasome activity, lysosome activity, and TGFβ signalling are dependent on one another then blocking protein degradative pathways will impede pro-tumourigenic TGFβ signalling and disrupting TGFβ signalling will alter the balance between autophagy and proteasome-dependent degradation. Overall, I observed that inhibiting either autophagy or proteasomes affected TGFβRII trafficking and impeded R-Smad phosphorylation, Smad nuclear translocation, E- to N-Cadherin shift, stress fiber formation, and cell migration. Additionally, silencing p62/SQSTM1, a protein that delivers cargo to both degradative pathways, may alter TGFβRII trafficking, R-Smad phosphorylation, Smad activity, and EMT (Fig. 7.1A-C). Therefore, my work highlights that using inhibitors of degradation pathways may be a useful strategy to impede pro-tumourigenic TGFβ signalling in NSCLC.
Figure 7.1: Summary of the impact of autophagy inhibition, 26S proteasome inhibition, and p62/SQSTM1 siRNA on TGFβ signalling in NSCLC cells.

TGFβ binds to TGFβ receptor type II (TGFβRII) that results in TGFβRI phosphorylation and activation. The TGFβ receptors are endocytosed into the early endosome membrane compartment where TGFβRI phosphorylates receptor regulated Smads (R-Smads) and non-Smad proteins, such as TRAF6 and TAK1, which leads to p38 MAPK
phosphorylation. R-Smads with or without Smad4 may translocate to the nucleus to regulate gene expression. In NSCLC, TGFβ signalling alters the transcriptome to promote epithelial-mesenchymal transition (EMT). Smad4 activates TGFβ-dependent autophagy by directly increasing ULK1 activity whereas the TAK1-TRAF6-p38 MAPK pathway increases autophagy by inhibiting mTOR, which is an inhibitor of ULK1. Together, autolysosomes and 26S proteasomes facilitate most of the degradation within cells.

(A) Autophagy inhibitors decrease (red line) TGFβRII internalization and trafficking to early endosomes, R-Smad phosphorylation, R-Smad nuclear translocation, and EMT. Autophagy inhibitors increase (green arrow) apoptosis and 26S proteasome activity. Also, si-Smad4 or si-TAK1+si-TRAF6+p38 MAPK inhibitor (p38 MAPK I) block TGFβ-dependent autophagy.

(B) Proteasome inhibitors decrease R-Smad phosphorylation, R-Smad nuclear translocation, and EMT whereas TGFβRII trafficking, apoptosis, and autophagy are increased.

(C) P62/SQSTM1 silencing may impede TGFβRII trafficking and R-Smad phosphorylation. However, p62/SQSTM1 silencing promotes EMT and had no observable affect (dotted line) on R-Smad nuclear translocation, autophagy or 26S proteasome activity.

7.3 Limitations

The majority of this work was completed using A549 and H1299 NSCLC cell lines. However, all experiments involving receptor trafficking required mink lung (Mv1Lu) cells that were stably transfected to over express pMEP4 with cDNA encoding HA-TGFβRII. Although I could not locate a TGFβRII overexpressing vector compatible with human NSCLC cells, I generated A549 cells and H1299 cells that express HA-TGFβ. I hoped that receptor trafficking could be monitored after HA-TGFβ binds to TGFβRII, which initiates receptor internalization. However, although the transfection was successful (Fig. 7.2A), HA-TGFβ could be visualized via immunofluorescence (Fig. 7.2B), and HA-TGFβ induced Smad2 phosphorylation (Fig. 7.2C), the number of receptors internalized were too few to emit a strong enough signal to be a reliable measurement for receptor endocytosis (Fig. 7.2D). Therefore, all finding regarding the influence of autophagy inhibition,
proteasome inhibition, and p62/SQSTM1 on TGFβRII endocytosis were completed using Mv1Lu cells.

**Figure 7.2:** Using HA-TGFβ transfected cells to monitor TGFβRII endocytosis.

**(A)** A549 cells that stably expressed HA-TGFβ were lysed and subjected to SDS-PAGE and immunoblotted for anti-HA antibodies. Western blots were imaged using a Versa Doc.

**(B)** Control cells and A549/H1299 cells stably expressing HA-TGFβ were fixed, permeabilized, and stained with DAPI (blue) or anti-HA (red) antibodies. Cells were imaged using an Olympus IX 81 inverted fluorescent microscope. Scale bar = 10 µm

**(C)** H1299 cells were treated with 0-250 pM TGFβ1 or H1299 cells that stably expressed HA-TGFβ were treated with 0-100% HA-TGFβ conditioned media for 24 hours. Cells were lysed, subjected to SDS-PAGE, and immunoblotted for anti-P-Smad2, anti-Smad2, and anti-GAPDH (loading control) antibodies. Western blots were imaged using a Versa Doc.
(D) A549 cells and H1299 cells stably expressed HA-TGFβ were treated with HA-TGFβ conditioned media for 24 hours. Endocytosis was then blocked by placing the cells in 4°C for 10 minutes. The cells were then incubated with anti-HA antibody for 3 hours. The cells were washed, fixed, permeabilized, and stained with DAPI (blue) or anti-HA (red) antibodies. Cells were imaged using an Olympus IX 81 inverted fluorescent microscope. Scale bar = 10 µm.

Another potential limitation with my experimental model for TGFβRII trafficking is that the siRNA against human ATG5 and ATG7 could not silence mink Atg5 and Atg7 protein levels. This was illustrated when I immunoblotted for Atg7 and Atg5 post-knockdown and observed no significant differences between si-Control and si-ATG5/7 in Mv1Lu cells (Fig. 7.3). For this reason, my conclusions describing the influence of autophagy on TGFβRII trafficking relies solely on pharmacological inhibitors. This needs to be taken into account because each of the pharmacological inhibitors used in my experiments contribute to receptor trafficking. Therefore, it is difficult to differentiate each pharmacological inhibitor’s impact on receptor trafficking compared to the affect of autophagy inhibition on TGFβRII trafficking.

![Western Blot](image)

**Figure 7.3:** Human si-ATG5/7 had no impact on steady-state Atg7 and Atg5 levels in Mv1Lu cells.

Mv1Lu cells were treated with 10 nM si-Control or 10 nM si-ATG5/7 for 48 hours prior to lysis. The cells were then subjected to SDS-PAGE and immunobblotted with anti-Atg7 and anti-Atg5 antibodies. Western blots were imaged using a Versa Doc (BioRad).

Both autophagy and TGFβ signalling were investigated using pharmacological agents and siRNA targeting experimental approaches. However, only pharmacological agents assessed the influence of proteasome inhibition on autophagy and TGFβ signalling. MG132 and lactacystin are potent inhibitors of the proteasome β5 subunit, which catalyzes
chymotrypsin-like reactions\textsuperscript{453}. As previously mentioned in Chapter 5, both of these agents have some off-target effects, such as Cathepsins\textsuperscript{424,427}. For this reason, I targeted the proteasome \( \beta \) subunit using siRNA and assessed markers for apoptosis EMT, TGF\( \beta \) signalling, and autophagy in both NSCLC cell lines (Figure 7.4). Although there was considerable overlap with the data obtained from the pharmacological inhibition of proteasome activity, there were some inconsistencies.

Figure 7.4: The effect of silencing the proteasome \( \beta \) subunit.
A549 cells and H1299 cells were treated with two different 10 nM siRNAs against the proteasome β5 subunit for 72 hours at 15 mM concentrations. During the final 24 hours of treatment, the cells were treated with 250 pM TGFβ1. Cells were lysed, subjected to SDS-PAGE, and immunoblotted for anti-P-mTOR, anti-mTOR, anti-N-Cadherin, anti-E-Cadherin, anti-Cleaved PARP, anti-TGFβRII, anti-TGFβRI, anti-p62, anti-P-Smad2, anti-P-Smad3, anti-Smad2/3, anti-Snail, anti-Slug, anti-proteasome β5, anti-LC3B, and anti-GAPDH antibodies. Three independent experiments were performed, and representative immunoblots are depicted above.

These inconsistencies may stem from proteasome activity being regulated by a balance of proteasome load and capacity. Proteasome inhibitors increase proteasome load but decrease proteasome capacity, which ultimately promotes the unfolded protein response. Given that pharmacological agents block the activity of proteasome β5 subunits, we were able to distinguish between short-term and prolonged proteasome inhibition. Alternatively, proteasome β5 subunit siRNA leads to the degradation of mRNA and decreases proteasome β5 protein levels, but functional proteasomes remain intact. Proteasome activity will be disrupted once older proteasome are eliminated and proteasome synthesis is altered due to the lack of proteasome β5 subunits. However, the rate of proteasome turnover varies in different cell types meaning that decreasing proteasome β5 protein levels does not necessarily indicate that proteasome activity is antagonized. Therefore, we were unable to determine short-term or prolonged proteasome inhibition using proteasome β5 subunit siRNA and future work is needed to derive an accurate protocol to achieve genetic ablation of proteasome activity.

Although A549 cells and H1299 cells are both NSCLC cells, differences between the cell types made interpreting my findings difficult. For example, using E- to N-Cadherin shift as one indicator of EMT was useful in A549 cells; however, E-Cadherin levels in H1299 cells were undetectable via immunoblotting. As such, the Cadherin shift solely relied on N-Cadherin protein levels. Furthermore, TGFβ1 slightly increased/had no affect and decreased steady-state p62/SQSTM1 levels in H1299 cells and A549 cells, respectively. This is an issue because p62/SQSTM1 plays an intimate role in TGFβ signalling, autophagy, and the ubiquitin-proteasome pathway. Due to this variation, I only explored the role of p62/SQSTM1 on EMT, TGFβ1 signalling, and TGFβ1-dependent
autophagy in A549 cells. Perhaps these experiments may have yielded different results in H1299 cells, and for this reason more NSCLC cell lines should be assessed to ensure the universality of my observations. Additionally, in response to TGFβ1, A549 cells produced larger and easily detectable stress fibers whereas H1299 cells migrated greater distances. Therefore, depending on the readout, it was beneficial to use one cell type over the other.

As previously discussed, I transfected A549 cells and H1299 cells with a pMRX-IP-GFP-LC3B-RFP-LC3BΔG vector to measure the rate of autophagic degradation known as autophagic flux. I decided to use the GFP-LC3B-RFP-LC3BΔG vector over the GFP-LC3B-RFP vector because, compared to RFP, the RFP-LC3BΔG internal control was more similar to GFP-LC3B. Furthermore, the Mizushima lab found that this vector was more stable and less susceptible to degradation. As such, I believed this to be a more accurate model. However, I found that TGFβ1 and other treatments decreased the RFP-LC3BΔG when I assessed my samples using immunoblotting and fluorescence microscopy experimental procedures. In support of this, proteasome inhibitors increased the RFP-LC3BΔG. Taken together, RFP-LC3BΔG can be degraded by proteasomes and may be subject to non-selective autophagic degradation. If different treatments alter RFP-LC3BΔG degradation, this should be taken into account when assessing GFP/RFP ratios as a representation of autophagic flux.

Another limitation of this work is that pharmacological agents such as chloroquine and MG132 have several off-target effects. For instance, chloroquine, once considered a lysosomotropic agent, is now considered acidotropic because it increases the pH of all acidic vesicles. Interestingly, MG132 is a proteasome inhibitor that induces autophagy yet is a known inhibitor of some proteases within the cytosol and lysosome. Therefore, it is difficult to conclude that some of my findings are entirely due to attenuating autophagy or proteasome function.

The work generated in my thesis was primarily conducted in human NSCLC cell lines to gain a mechanistic understanding of how autophagy and the ubiquitin-proteasome pathway regulate TGFβ signalling to promote tumourigenesis. The advantage of using cells lines is that they provide a simplified platform to conduct experiments, an endless supply of materials, and rapid experimental setups that bypass ethical concerns. However, cell lines may not capture the cell-cell and cell-matrix communication that occur in in vivo
models. Furthermore, 2D monolayers do not illicit the same mechanical stressors as what has been observed in 3D culture or in vivo. Therefore, 2D monolayer models are inappropriate to explore novel NSCLC therapies, which is the future direction of this work.

7.4 Future Directions

In the future, it would be interesting to examine autophagy inhibitors and proteasome inhibitors as potential NSCLC therapies that aim to disrupt metastasis and chemotherapeutic resistance. In order to achieve this, I will require a reliable in vivo pre-clinical model to test the potency, efficacy, and selectivity of autophagy inhibitors and/or proteasome inhibitors that can specifically impede pro-cancerous TGFβ pathways. Currently, several pre-clinical in vivo cancer models, such as genetically modified and orthotopic murine models fail to accurately reproduce pre-metastatic stages of tumour development, which is the developmental period that EMT plays a tumourigenic role. Additionally, outcomes obtained from many in vivo cancer models rarely translate to successful clinical trials due to the fact that they fail to reproduce the physiology, pharmacology, and progression of human carcinogenesis. However, my lab has previously shown that in ovo chick chorioallantoic membrane (CAM) models are efficacious at modeling primary tumour growth that may be excised for further analysis (Fig. 7.5A, B). The highly vascularized nature of chick CAM models provide a unique in vivo environment that is necessary to study tumour formation, autophagy, and metastasis free from several—but not all—restrictions that impact murine models.
Figure 7.5: NSCLC tumour growth on chick chorioallantoic (CAM) membranes.

(A) Visible NSCLC tumours were formed on the CAM of avian embryos 7 days after implantation (1 x 10^6 cells/embryo). Glass coverslips were placed over tumours for visualization.

(B) Four representative tumours formed on the CAMs of HBE, A549 or H1299 implanted embryos were excised and imaged. Scale bar = 1 cm. Courtesy of the Di Guglielmo lab.

Using the chick CAM model, we could identify an autophagic marker, such as p62/SQSTM1, that responds to TGFβ as a potential target to inhibit autophagy and EMT in a 3D biological system. Using p62/SQSTM1 as an example, we could investigate how $P62/SQSTM1^{+/+}$ A549 cells and $P62/SQSTM1^{-/-}$ H1299 cells differ from wild-type cells with respect to the expression profile of EMT and autophagic markers as well as autophagosome formation. Next, I would prepare chick CAMs for infection, experimentation, and imaging.
using protocols well established in the field. Briefly, the chick CAMs will incubate for 6 embryo developmental days (EDDs) prior to the introduction of wild-type cells, \( P62/SQSTM1^{-/-} \) A549 cells or \( P62/SQSTM1^{-/-} \) H1299 cells\(^{457} \). After EDD 12, growth media supplemented with TGF\( \beta \) will be added every 24 hours to the allantoic vesicle\(^{458} \). Previous work using this model suggests that A549 cells require a 7-day incubation period to allow primary tumours to develop and metastasize to secondary sites. Therefore, on EDD 19, tumours will be counted using an Olympus IX 81 fluorescence microscope and larger tumours will be excised. Prior to lysing the tumour cells to isolate the protein, tumour size will be estimated by calculating the tumour volume using the modified ellipsoid formula \( \frac{1}{2} ( \text{length} \times \text{width}^2 ) \). The proteins will be subject to immunoblotting techniques using antibodies targeting EMT and autophagic markers. Tumour metastasis in different chick organs will be assessed using PCR to detect human specific Alu-sequences and mRNA endogenously expressed by the chick CAM\(^{459} \). Finally, in order to assess autophagosome formation, the chick CAMs will be sectioned into 5 \( \mu \)m thick sections that are prepared for transmission electron microscopy\(^{460} \).

Using the chick CAM model system for this experiment has some limitations with respect to experimental duration and optimization. First of all, each cell line will respond differently to TGF\( \beta \) and gene knockout, which will vary how long it will take the cells to metastasize. Additionally, although chick CAM models lack immune systems, the cells added are still acceptable to apoptosis, necrosis, or could fail to adhere to the CAM, which will hinder primary tumour development and metastasis. To ensure that all chick CAM models have formed primary tumours and metastasized so that they are ready for experimentation around the same EDD, the number of cells from each cell line introduced into the model will vary. Although a sterilized laboratory is not required for experimentation, chick CAMs are sensitive to temperature and humidity. Finally, the primary and secondary tumours may not be visible at the time of excision, which will prevent protein collection or making any observations on tumour morphology.

After suitable targets for TGF\( \beta \)-induced autophagy and EMT are identified, the next step will be to determine if eliminating the target increases chick CAM tumours susceptibility to cytotoxic agents. If this technique proves to be successful in \textit{in ovo} chick CAM models, we could demonstrate its efficacy in an \textit{in vivo} pre-clinical non-obese
diabetic severely combined immunodeficient (NOD SCID) mouse model. Once again, using p62/SQSTM1 as an example, I would design a p62/SQSTM1 specific microRNA (miRNA) sequence and clone it into a pcDNA/miR expression vector that contains a CMV promoter and a luciferase gene and promoter. In the next step, I would recombine the miRNA sequence in an adenovirus-associated vector (AAV), which will be amplified and purified. I would then generate human P62/SQSTM1−/− A549 cells and P62/SQSTM1−/− H1299 cells as well as wild-type A549 cells and H1299 cells all of which express luciferase.

The NOD SCID mice experimental procedure will involve subcutaneous implantation of the P62/SQSTM1−/− A549 cells, P62/SQSTM1−/− H1299 cells, A549 cells or H1299 cells expressing luciferase. Cytotoxic agents will then be administered, and I will compare both primary tumour size and the number of metastatic colonies found in NOD SCID mice that received P62/SQSTM1 silenced cells to the NOD SCID mice implanted with wild-type cells. Bioluminescence imaging will be utilized to assess tumour size and metastasis.

However, as it is presented here, a novel cancer therapy identified using these experimental techniques have several limitations that need to be addressed before Health Canada would grant permission for human clinical trials. Firstly, inhibiting autophagy and EMT would have little impact in patients who have tumours cells that show no evidence of excessive autophagy or if the cells have already metastasized, respectively. Therefore, my therapy highlights the demand for personalized medicine in which the genome and proteome of patient tumours need to be screened to ensure that they are appropriate candidates for this therapy. Secondly, if the targeted autophagic marker only responds to elevated concentrations of TGFβ, inhibiting this marker will play no role in patients that have autophagy and EMT induced in a TGFβ-independent manner. Thirdly, I will need to ensure that my therapy is specific to the tumour cells and have few off-target effects. Precaution needs to be taken when inhibiting autophagy because autophagy serves an anti-tumourigenic function in healthy tissues. As such, if autophagy is inhibited in healthy tissues, this could make the individual susceptible to secondary carcinogenesis and inflammation from cellular necrosis.
7.5 Conclusion

In conclusion, I have demonstrated that autophagy and proteasome-dependent degradation are linked and together regulate TGFβ signalling. When either degradative pathway is disrupted, pro-tumourigenic TGFβ signalling such as E- to N-Cadherin shift, stress fiber formation, and cell migration are attenuated. I verified that TGFβ activates autophagy in NSCLC through the ULK1-dependent macroautophagy initiation stage and demonstrated that the Smad4 or TAK1-TRAF6-p38 MAPK pathways are responsible. I have provided mechanistic insight into the reliance of A549 cells on p62/SQSTM1 to maintain epithelial phenotypes. As such, TGFβ induces proteasome-mediated p62/SQSTM1 degradation prior to initiating EMT. Given the importance of TGFβ signalling in tumourigenesis, continued understanding of the mechanism of how degradative processes may drive pro-tumourigenic TGFβ signalling will further understanding of the complex cellular signals within the tumour microenvironment. Using this information, it may aid in the identification of important proteins and processes that can be targeted to attenuate tumour growth and metastasis and thus improve patient outcomes.
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Appendix

List of Pharmacological Agent Used

<table>
<thead>
<tr>
<th>Pharmacological Agent</th>
<th>Primary Target and IC$_{50}$ values (nM)</th>
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<tbody>
<tr>
<td>Chloroquine</td>
<td>Lysosomal enzymes (31.83-600)</td>
</tr>
<tr>
<td>Spautin-1</td>
<td>USP10 (580) and USP13 (690)</td>
</tr>
<tr>
<td>ULK-101</td>
<td>ULK1 (8.3) and ULK2 (30)</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>mTORC1 (0.1)</td>
</tr>
<tr>
<td>MG132</td>
<td>Protasome (100) and calpain (1200)</td>
</tr>
<tr>
<td>Lactacystin</td>
<td>Protasome (500)</td>
</tr>
<tr>
<td>Dyngo-4a</td>
<td>Dynamin (200-400)</td>
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<tr>
<td>P38 MAPK Inhibitor</td>
<td>P38 MAPK (35)</td>
</tr>
<tr>
<td>SB431542</td>
<td>TGFβRI (94)</td>
</tr>
<tr>
<td>LY294002</td>
<td>PI3K (1400)</td>
</tr>
</tbody>
</table>

Ubiquitin-specific peptidase (USP), Uncoordinated 51-like autophagy activating kinase (ULK), Mechanistic target of rapamycin complex (mTORC), Mitogen-activated protein kinase (MAPK), Transforming growth factor β receptor, (TGFβR), and Phosphoinositide 3-kinase (PI3K).
Curriculum Vitae

Name: Charles B. Trelford

Post-secondary Education and Degrees:
The University of Western Ontario
London, Ontario, Canada

The University of Western Ontario
London, Ontario, Canada
2017-2022 Ph.D.

Honours and Awards:
Mogenson Trust Physiology and Pharmacology Graduate Research Scholarship
2019

Canadian Glaucoma Society
Best Glaucoma Paper Award
2021

Hari and Gudrun Sharma Award
2021

Province of Ontario Graduate Scholarship Doctoral Fellowship
2020-2021, 2021-2022

Related Work Experience:
Teaching Assistant
The University of Western Ontario

Co-course Coordinator
The University of Western Ontario
2020-2022.

Publications:


Trelford, C.B and Di Guglielmo, G.M (2022) Prolonged proteasome inhibition antagonizes TGFβ1-dependent signalling by promoting the lysosomal-targeting of TGFβRII. Cellular Signalling.