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The Role of p62/SQSTM1 in TGFβ-dependent EMT and Autophagy

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Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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Abstract

Transforming growth factor beta (TGFβ) is a cytokine that regulates cellular adhesion, proliferation and apoptosis. In the context of cancer, TGFβ induces processes such as epithelial-to-mesenchymal transition (EMT). More recently, TGFβ has been discovered to also induce autophagy, and the relationship between TGFβ-induced EMT and autophagy remains unknown. Due to its involvement in autophagy and its established interactions with key TGFβ signaling proteins, this thesis focuses on the sequestosome 1 (p62/SQSTM1) protein. Here, I have shown that p62/SQSTM1 co-localizes with TGFβ receptors at the same time point that the receptors localize to Rab7-positive late endosomes. siRNA-mediated silencing of p62/SQSTM1 was also observed to prime non-small cell lung cancer cells to undergo an E-cadherin to N-cadherin shift, but this is not due to alterations of canonical TGFβ signaling. Furthermore, TGFβ induces a loss in nuclear p62 protein levels and sustained TGFβ incubation reduces total p62 levels, while converting light chain 3 beta (LC3B) I to LC3BII (a process that is a marker of autophagy). Finally, TGFβ-induced EMT and autophagy was shown to be dependent on the presence of the p62/SQSTM1-interacting proteins, TRAF6 and aPKC. Taken together, these findings provide new insight into the role of p62 in TGFβ signaling and autophagy, as well as provide possible relationships between EMT and autophagic processes.

Keywords

Autophagy, transforming growth factor beta (TGFβ), p62/SQSTM1, epithelial-to-mesenchymal transition (EMT), cancer, atypical protein kinase C (aPKC), Tumor necrosis factor-associating factor 6 (TRAF6), Smad signalling
Co-Authorship Statement

The co-immunoprecipitation experiment presented in the Appendix was conducted by Dr. Adrian Gunaratne. The microarray analysis was conducted by Mr. David Carter from the London Regional Genomics Centre (London, ON). The data from the microarray studies were analyzed and compiled by E. Ng. All other experiments were designed by E. Ng and J. Di Guglielmo, and completed by E. Ng.
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Being part of the Di Guglielmo lab gave me the opportunity to do research alongside some pretty stellar people, and I’m lucky to be able to call my colleagues friends. To Craig Campbell, Colleen Ryan, Eddie Chan, Sam (Sanghyun) Lee and Anthony Ziccarelli, thank you for experiencing science, life, and randomness with me, and thank you for all the good memories: playing “who can copy me” in New Orleans and eating crawfish out of a plastic bag, talking about our future dreams and ambitions, learning to play golf, looking at pictures
of cute animals, and solving riddles at the Grad Club—and this is just naming a few! I’m very thankful to have had you guys as my lab family.

Outside of the lab, I am also very fortunate to have the most caring and understanding friends. Thank you to Tana Saiyin and Jessica Blom for helping me stay motivated and for being a shoulder to lean on. You two are always there for me, and I don’t know what I would’ve done without you. To my friends back at home, Polly Lau, Claudia Tsang and Crystal Wong, thank you for thinking of me even when we’re apart. Your messages and our hangouts definitely kept me going. And to Charles Han, thank you for holding my hand and sticking by me, even when things got tough. I’m blessed to be with someone who is as patient and compassionate as you.

Finally, to my family— to my mom, dad, and Jeh— I’m incredibly thankful for you. I am so grateful to have such a loving home that I can come back to, and I know I can always rely on you, regardless of what it is that I need. Thank you for your unwavering confidence in me, and thank you for your loud cheers of encouragement.
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<tbody>
<tr>
<td>A</td>
<td>Alanine</td>
</tr>
<tr>
<td>ActR-IIA</td>
<td>Activin receptor type IIA</td>
</tr>
<tr>
<td>ActR-IIB</td>
<td>Activin A receptor type IIB</td>
</tr>
<tr>
<td>Akt</td>
<td>Also known as PKB (protein kinase B)</td>
</tr>
<tr>
<td>ALK</td>
<td>Activin receptor-like kinase</td>
</tr>
<tr>
<td>AMH/MIS</td>
<td>Anti müllerian hormone/ müllerian inhibiting substance</td>
</tr>
<tr>
<td>AMHR-II</td>
<td>Anti müllerian hormone receptor type II</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>aPKC</td>
<td>Atypical Protein Kinase C</td>
</tr>
<tr>
<td>Atg</td>
<td>Autophagy-related</td>
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<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BMPR-IA</td>
<td>Bone morphogenetic protein receptor type IA</td>
</tr>
<tr>
<td>BMPR-IB</td>
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</tr>
<tr>
<td>BMPR-II</td>
<td>Bone morphogenetic protein receptor type II</td>
</tr>
<tr>
<td>C</td>
<td>Cysteine</td>
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<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
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<tr>
<td>Co-Smad</td>
<td>Common-mediator Smad (Smad4)</td>
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<tr>
<td>Dab2</td>
<td>Disabled-2</td>
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<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DAPK</td>
<td>Death associated protein kinase</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<td>-------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>E-cad</td>
<td>Epithelial cadherin (CDH1)</td>
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<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>EEA1</td>
<td>Early endosomal antigen-1</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FIP200</td>
<td>FAK family kinase interacting protein of 200 kDa</td>
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<tr>
<td>FYVE</td>
<td>Fab1, YOTB, Vac1, EEA1</td>
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<td>G</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>GDF</td>
<td>Growth and differentiation factor</td>
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<td>GFP</td>
<td>Green fluorescence protein</td>
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<td>GSK3β</td>
<td>Glycogen synthase kinase 3 beta</td>
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<td>HA</td>
<td>Hemagglutinin</td>
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<td>HCCs</td>
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<td>HEK</td>
<td>Human embryonic kidney</td>
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<td>HRP</td>
<td>Horse radish peroxidase</td>
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<td>I-Smad</td>
<td>Inhibitory smad</td>
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<td>IL-3</td>
<td>Interleukin 3</td>
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<td>IL-6</td>
<td>Interleukin 6</td>
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<tr>
<td>IP</td>
<td>Immunoprecipitate</td>
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<td>Acronym</td>
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</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal kinase</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>Keap1</td>
<td>Kelch-like ECH-associated protein 1</td>
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<tr>
<td>KIR</td>
<td>Keap1 interacting region</td>
</tr>
<tr>
<td>Kras</td>
<td>V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>LC3B</td>
<td>Microtubule-associated protein light chain 3</td>
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<tr>
<td>LIR</td>
<td>LC3-interacting region</td>
</tr>
<tr>
<td>LTBP</td>
<td>Latent TGFβ binding protein</td>
</tr>
<tr>
<td>MAD</td>
<td>Mothers against decapentaplegic</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEM</td>
<td>Modified eagle medium</td>
</tr>
<tr>
<td>MH1</td>
<td>Mad homology 1</td>
</tr>
<tr>
<td>MH2</td>
<td>Mad homology 2</td>
</tr>
<tr>
<td>miR</td>
<td>Micro ribonucleic acid</td>
</tr>
<tr>
<td>MIS</td>
<td>Müellerian inhibiting substance</td>
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<tr>
<td>MISRII</td>
<td>Müellerian inhibiting substance receptor II</td>
</tr>
<tr>
<td>MKK</td>
<td>Mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>mL</td>
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<td>mM</td>
<td>Millimolar</td>
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<tr>
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<td>Matrix metalloproteinase-2</td>
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<td>MMP9</td>
<td>Matrix metalloproteinase-9</td>
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<tr>
<td>mTORC1</td>
<td>Mammalian target of rapamycin complex 1</td>
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<tr>
<td>Mv1Lu</td>
<td>Mink lung cells</td>
</tr>
<tr>
<td>N-Cad</td>
<td>Neural cadherin</td>
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NBR1 Neighbour of BRCA1 gene 1
Nedd4 Neural precursor cell expressed developmentally down-regulated protein 4
NES Nuclear export sequence
NFκB Nuclear factor κ B
ng Nanograms
NLS Nuclear localization sequence
nm Nanometre
nM Nanomolar
Nrf2 Nuclear factor (erythroid-derived 2)-like 2
NSCLC Non-small cell lung cancer
P-Smad Phosphorylated Smad
PAI-1 Plasminogen activator inhibitor 1
Par6 Partitioning defective 6
PB1 Phox1 and Bem1p
PBS Phosphate buffered saline
PCR Polymerase chain reaction
PI3K Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKC Protein kinase C
PKCι Protein kinase C iota
PKCζ Protein kinase C zeta
pM Picomolar
PMSF Phenylmethane sulfonyl fluoride
R-Smad Receptor regulated Smad
<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
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<tbody>
<tr>
<td>Raptor</td>
<td>Regulatory-associated protein of mTOR</td>
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<tr>
<td>RhoA</td>
<td>Ras homolog gene family member A</td>
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<td>RIP</td>
<td>Receptor interacting protein</td>
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<td>Ribonucleic acid</td>
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<td>RNF168</td>
<td>Ring finger protein 168</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SARA</td>
<td>Smad anchor for receptor activation</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<td>SEM</td>
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<td>Small interfering RNA</td>
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<td>Small phenotype mothers against decapentaplegic homolog</td>
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<td>Sequestosome 1</td>
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<td>Thymine</td>
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<td>TGFβ-activated kinase 1</td>
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<tr>
<td>TB</td>
<td>TRAF6 binding domain</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline with tween</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
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<td>-------------</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
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<td>TRAF6</td>
<td>Tumor necrosis factor receptor associated factor 6</td>
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<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>µm</td>
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Chapter 1: Introduction

1.1. Lung Cancer

Cancer is the leading cause of death in Canada, claiming more lives than heart disease, accidents, and suicide combined (Canadian Cancer Society, 2017). Lung cancer in particular is responsible for the highest number of cancer deaths in the country, causing an estimated 21,000 deaths annually (Canadian Cancer Society, 2017).

There are two major types of lung cancer: small cell lung cancer and non-small cell lung cancer (NSCLC). Of the two types, NSCLC is slower growing than its counterpart, however, it is less responsive to treatment and also more prevalent— it constitutes 80-85% of lung cancer diagnoses (CancerCare, 2017). The NSCLC group consists of adenocarcinomas, squamous cell carcinomas, large cell carcinomas, and other subtypes. Adenocarcinoma is the most common of the NSCLCs and it usually originates from glandular cells found on the lung periphery. It is also the most common type of lung cancer in smokers and non-smokers alike (American Cancer Society Inc., 2017).

As with many epithelial tumors, malignancies in the lung may also possess the potential to migrate to other parts of the body and establish secondary tumors. This occurrence, called metastasis, involves several stages including invasion and intravasation into the lymph or bloodstream, extravasation, and colonization (as reviewed in Nguyen et al., 2009). It is of great importance to define this process, as 90% of cancer-related deaths are attributed to the establishment of metastatic lesions, as opposed to the primary tumor (Beck et al., 2014).
1.2. The TGFβ signaling pathway

1.2.1. TGFβ as both a tumor suppressor and a tumor promoter

The transforming growth factor beta (TGFβ) pathway is a critical regulator of many physiological processes including cell growth, morphogenesis, proliferation and apoptosis (Horbelt et al., 2012; Massague, 2012). Normally, TGFβ signaling acts to suppress tumor growth and cell proliferation. These processes are controlled by canonical TGFβ signaling through regulation of cytostatic genes. For example, TGFβ induces the expression of cyclin dependent kinase inhibitors such as p21\textsuperscript{CIP1} and p15\textsuperscript{INK4B} in neuronal and epithelial cells (Elliott & Blobe, 2005; Padua & Massagué, 2009), which inhibits the progression of the cell cycle from the G1 to S phase. Similarly, p57 is induced by TGFβ in haematopoietic cells to attenuate cell growth (Meulmeester & Ten Dijke, 2011). In addition to cell dependent kinases, TGFβ also suppresses the transcription factor c-Myc, which is a known oncogene that promotes cell growth (Zhang, 2009; Meulmeester & Ten Dijke, 2011). Thus, TGFβ is important in preventing uncontrolled cell proliferation.

Aberrant TGFβ signaling is implicated in the development of cardiovascular diseases, fibrodysplasia, and several types of cancer (Kang et al., 2009; Horbelt et al., 2012). Indeed, dysregulation of the TGFβ pathway has been found to be involved in the development of breast, colorectal, pancreatic and lung tumors (Elliott & Blobe, 2005; Horbelt et al., 2012).

In order for cells to become malignant, they must evade the tumor suppressive effects of TGFβ. This can occur in one of two ways: silencing TGFβ signaling altogether by
disrupting key components in the pathway, or exclusively repressing the branch of TGFβ signaling that suppresses tumor progression. The second of these mechanisms allows the tumor cells to then exploit the residual functional TGFβ signaling towards growth and invasion processes (Meulmeester & Ten Dijke, 2011; Massague, 2012). Thus, whether TGFβ acts as a tumor suppressor versus a tumor promoter is heavily dependent on context.

1.2.2. TGFβ and lung cancer

In the majority of pancreatic and colon cancers, the first of the aforementioned mechanisms is used to evade the tumor suppressive effects of TGFβ (Massagué, 2008). This type of TGFβ resistance is well defined. However, in other cancers such as those found in the lung, mechanisms used to select for and bypass tumor suppressive TGFβ pathways are poorly understood. In addition, the method by which these tumors shift to benefit from remaining TGFβ signaling has yet to be elucidated (Elliott & Blobe, 2005).

It is clear that lung cancer cells take advantage of TGFβ signaling: lung tumors overexpress TGFβ ligand, and a high serum level of TGFβ in adenocarcinoma patients has been shown to be correlated with poor prognosis (Elliott & Blobe, 2005; Xue et al., 2016). Furthermore, these studies have also suggested that higher levels of TGFβ in these patients were involved in the progression of tumor angiogenesis and metastasis (Jeon & Jen, 2010).
1.2.3. **Ligands and receptors of the TGFβ superfamily**

The ligands of the TGFβ superfamily can be divided into a TGFβ branch and a BMP branch (Fig 1.1A). Though structurally similar to the TGFβ subfamily, BMP ligands regulate osteogenesis, neurogenesis and developmental processes (Drabsch & Dijke, 2012). In contrast, ligands of the TGFβ subfamily are more involved in proliferation and apoptosis. Six isoforms of TGFβ ligand exist, however, mammals only express three: TGFβ1, TGFβ2, and TGFβ3. The first of these isoforms is most abundant in the body, and was the first to be discovered in 1983, having been observed to promote rat fibroblast growth (Drabsch & Dijke, 2012).

As a cytokine, TGFβ1 is produced by a variety of cells and tissues in the body. Its precursor polypeptide is 390 amino acids in length and it is glycosylated after transcription (Katz et al., 2013). Thereafter, two precursors dimerize via disulfide linkage to form an inactive homodimer, which is then secreted out of the cell (Annes et al., 2003; Kang et al., 2009). This latent form of TGFβ1 binds extracellular proteins until proteolytic cleavage and separation from the propeptide converts the ligand into its mature and active form (Annes et al., 2003). Normal serum levels of TGFβ1 in a healthy individual are found to be in the lower picomolar range (Kong et al., 1995; Tas et al., 2014), and the largest source of ligand production is from blood platelets. Upon injury, platelets release more TGFβ in order to control the inflammation, regeneration, and proliferation of cells (Massague, 2012). Tumors are also an abundant source of TGFβ. Although initially released to impede the progression of potentially malignant cells, TGFβ is also released by tumors to benefit their growth (Katz et al., 2013).
A.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Receptor II</th>
<th>Receptor I</th>
<th>Smad</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β</td>
<td>TβR-II</td>
<td>TβR-I</td>
<td>Smad 2, 3 + Smad4</td>
</tr>
<tr>
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<td>ActRII A,B</td>
<td>ALK4</td>
<td>Smad 2, 3 + Smad4</td>
</tr>
<tr>
<td>Nodal</td>
<td>ActRII A,B</td>
<td>ALK4, 7</td>
<td>Smad 2, 3 + Smad4</td>
</tr>
<tr>
<td>GDF- 1, 3</td>
<td>ActRII A,B</td>
<td>ALK4, 7</td>
<td>Smad 2, 3 + Smad4</td>
</tr>
<tr>
<td>BMP- 5, 6, 7</td>
<td>BMPRII, ActRII</td>
<td>BMPRIA, ALK2</td>
<td>Smad 1, 5, 8 + Smad4</td>
</tr>
<tr>
<td>GDF- 5, 6, 7</td>
<td>BMPRII, ActRII</td>
<td>BMPRIA, B</td>
<td>Smad 1, 5, 8 + Smad4</td>
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<tr>
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<td>AMHR-II</td>
<td>BMPRIA, ALK2</td>
<td>Smad 1, 5, 8 + Smad4</td>
</tr>
</tbody>
</table>

B.

- **Canonical Signaling**
  - Activated receptor complex
  - Gene transcription: E-cad, c-myc etc.
  - DNA

- **Non-canonical Signaling**
  - aPKC
  - TAK1
  - aPKC
  - p38
  - EMT
  - Apoptosis

C.

- **Clathrin-mediated endocytosis**
- **Caveolin-mediated endocytosis**
- No ligand: recycling via Rab11 positive endosomes

**Figure 1.1.**
**Figure 1.1. Overview of TGFβ signaling**

(A) Different ligands of the TGFβ family, their respective receptors, and associated downstream signaling proteins, termed Smads. (B) Signaling by TGFβ1 occurs when dimerized ligand binds the type II receptor, which then phosphorylates and binds the type I receptor to form an activated heterocomplex. Canonical signaling involves the phosphorylation and nuclear translocation of Smads, which associate with co-factors to modulate gene transcription. Non-canonical TGFβ pathways include signaling through the TRAF6-TAK1-MKK-p38 and Par6/aPKC pathways. (C) Receptor trafficking via clathrin and caveolin coated pits promotes signaling and degradation, respectively.

**Acronyms:** transforming growth factor beta (TGFβ), transforming growth factor beta receptor (TβR), bone morphogenic protein (BMP), bone morphogenic protein receptor (BMPR), activin receptor (ActR), anti-mullerian hormone/mullerian inhibiting substance (AMH/MIS), anti-mullerian hormone receptor (AMHR), growth differentiation factor (GDF), activin-like receptor kinase (ALK), Small phenotype mothers against decapentaplegic homolog (Smad), Smad anchor for receptor activation (SARA), atypical protein kinase C (aPKC), TNF receptor-associated factor 6 (TRAF6), transforming growth factor beta-activated kinase 1 (TAK1), mitogen-activated protein kinase kinase (MKK) partitioning defective 6 (Par6), Smad ubiquitin regulatory factor (Smurf).
Much like the ligands of the TGFβ superfamily, the type I and type II receptors can also be grouped into either the TGFβ or BMP subfamily. In humans, there are seven type I and five type II receptors, which assemble in different combinations as determined by the ligand (Fig 1.1A). TGFβ isoforms only bind one type of type II receptor, the constitutively active TGFβ receptor II (TβRII), which then also exclusively binds one type of type I receptor, TGFβ receptor I (TβRI, also called activin-like kinase 5; ALK5) (Drabsch & Dijke, 2012; Cantelli et al., 2016). A third type of receptor is also known to interact with TGFβ—the co-receptor known as TGFβ receptor III (TβRIII). This co-receptor can modulate the access of the type II receptor to different TGFβ isoforms by interacting with them, but TβRIII is not directly involved in signaling (Katz et al., 2013).

1.2.4. **TGFβ signaling**

Activation of classical TGFβ signaling occurs when ligand binds to the extracellular portion of a transmembrane type II receptor (Fig. 1.1B). The type II receptor then binds a type I receptor to form an activated heterotetrameric complex (composed of dimerized ligand, type II and type I receptors) that can activate several downstream pathways due to its serine/threonine kinase activity (Wrana et al., 1992). The canonical downstream effectors of the type I receptor are intracellular receptor-regulated Smads (R-Smads). The receptor complexes initiated by TGFβ and BMP cytokines activate Smads 2/3 and Smads 1/5/8, respectively, through phosphorylation. For TGFβ ligands, this phosphorylation occurs at two serine residues near the C-terminus of Smads 2/3. These activated R-Smads then leave the receptors to associate with Smad4, and the heteromeric Smad complex is then translocated to the nucleus. The accumulation of these nuclear Smads stimulate the
recruitment of various transcriptional cofactors, as well as alterations in gene transcription (Padua & Massagué, 2009). Some examples of transcription factors regulated by Smads are p300, p107, CBP, Swift, Snail and Twist, and downstream gene regulation includes that of c-Myc, osteocalcin, and E-Cadherin (Massagué et al., 2005; Heldin et al., 2012). There also exists a cell-dependent factor that is important for determining which transcription factors are recruited, and how gene expression is regulated (for example, the amounts of certain available transcription factors to be recruited) (Massagué et al., 2005).

There are also a number of atypical, non-Smad pathways through which TGFβ can signal (Fig. 1.1B). These include the activation of Par6-RhoA, TRAF6-TAK1-p38/JNK, and PI3K-Akt-mTOR pathways (Zhang, 2009; Meulmeester & Ten Dijke, 2011; Pickup et al., 2013). Interestingly, recent findings have shown that atypical protein kinase C (aPKC) is involved in both the canonical Smad-dependent pathway and atypical Par6 pathways of TGFβ (Gunaratne et al., 2015).

1.2.5. **SMAD proteins**

TGFβ signaling mobilizes Smad proteins, which are transcription factors capable of regulating hundreds of genes at once. Eight Smads exist in humans, and they can be grouped into three categories: receptor-regulated Smads (R-Smads), Co-operating Smads (Co-Smads) and Inhibitory Smads (I-Smads). Five Smads fall into the R-Smad category, Smads 1/2/3/5/8, and they are direct substrates of the TGFβ receptor superfamily (Drabsch & Dijke, 2012). Both the C-terminus and the N-terminus consist of Mad-homology (MH) domains that are connected by a variable linker region (Massagué et al.,
The ability of R-Smads to bind to DNA is attributed to its N-terminal MH1 domain, and the C-terminal MH2 domain allows R-Smads to bind other proteins (Horbelt et al., 2012). Prior to activation, R-Smads associate with proteins using its MH2 domain, and this sequesters and retains them to the cytoplasm until they are activated (Heldin & Moustakas, 2012). In the case of Smads 2/3, an example of such a protein is Smad Anchor for Receptor Activation (SARA). Activation of Smads 2/3 occurs when SARA facilitates its phosphorylation on two carboxy-terminal serine residues by type I TGFβ receptors, which subsequently interrupts the SARA-Smad interaction. This separation allows Smads 2/3 to bind to the Co-Smad (Smad4) and also exposes a nuclear import signal to allow the translocation of the Smad complex (Padua & Massagué, 2009). Smad nuclear import involves the nuclear localization sequence of Smads 2/3 as well as the importin nuclear pore protein (Hill, 2009). Once inside the nucleus, the Smad complex binds a 5’-CAGAC-3’ DNA motif in partnership with other transcription factors to regulate the expression of hundreds of genes (Massague, 2012; Macias et al., 2016). This includes the upregulation in the expression of the I-Smads, which establishes a negative feedback loop (Drabsch & Dijke, 2012).

Inhibitory I-Smads 6/7 bind to R-Smads in competition with TGFβ receptors (reviewed in Itoh et al., 2000). They down-regulate TGFβ signaling by recruiting E3 ubiquitin ligases, such as Smad ubiquitination regulatory factors (Smurfs) 1 and 2, that tag activated receptors to target them for proteasome-dependent degradation (Massagué et al., 2005). I-Smads promote the degradation of R-Smads in a similar manner by first recruiting phosphatases to deactivate them, and then recruiting ubiquitin ligases like Nedd4 (in the case of Smads 2/3) (Itoh et al., 2000; Heldin & Moustakas, 2012). Aside
from degradation, modification of the R-Smad linker region via phosphorylation can also regulate its activity (Schilling et al., 2006).

In addition to propagating TGFβ signaling, Smads can also act to integrate TGFβ with other signaling pathways. Smad-enabled crosstalk with TGFβ includes the incorporation of the Wnt, Akt, and p38/MAPK pathways (Massagué et al., 2005; Leivonen & Kahari, 2007).

1.2.6. *Receptor trafficking and internalization*

The method by which TGFβ receptors (TGFβRs) are trafficked and internalized can also regulate downstream signaling. TGFβR endocytosis can occur in two ways—through a clathrin-mediated pathway or a caveolin/lipid-raft pathway (Fig. 1.1C; Balogh et al., 2013).

The clathrin-mediated endocytosis of TβRII is constitutively active, and occurs when TβRII binds clathrin-coated pits. This process is mediated by the di-leucine motif found in the cytoplasmic domain of TβRII and is also dependent on the kinase activity of the type II—but not the type I—receptor (Anders et al., 1998; Kang et al., 2009). After internalization, the receptors are then found in early endosomes that are positive for markers Rab5 and early endosome antigen-1 (EEA1). In the absence of ligand, the receptors are then recycled back to the cell surface via Rab11 positive endosomes (Di Guglielmo et al., 2003; Chen, 2009). If, however, there is ligand present, downstream signaling occurs at the early endosome for an extended period of time (Le Roy & Wrana, 2005). Thereafter, the receptors are then sorted into either late endosomes (which destines
receptors to lysosomal degradation) or recycling Rab11 endosomes. The mechanism by which sorting occurs remains unknown (Chen, 2009).

Clathrin-mediated endocytosis promotes robust canonical TGFβ signaling through Smads (Runyan et al., 2005). In contrast, caveolin-mediated endocytosis facilitates the degradation of receptors which attenuates downstream signaling (Di Guglielmo et al., 2003; Kang et al., 2009). Thus, there is a balance in the proportion of receptors existing between the clathrin and caveolin compartments of the cell membrane, and it is an important regulator of signal transduction (Le Roy & Wrana, 2005). If receptors are shifted into lipid rafts, TβRI associates with caveolin-1, which inhibits downstream Smad signaling (Razani et al., 2001). Furthermore, the localization of Smad7 and Smurf 1/2 proteins to caveolin vesicles is also proposed to promote the turnover of TGFβRs.

1.3. Epithelial-to-mesenchymal transition

1.3.1. An introduction to EMT processes

Epithelial-to-mesenchymal transition (EMT) occurs when epithelial cells begin to undergo biochemical changes that initiate the loss of epithelial traits in exchange for the adoption of a mesenchymal phenotype (Fig. 1.2). It is characterized by a loss in apical-basal cell polarity and in the adhesion to the basement membrane, as well as the dissolution of cell-cell junctional complexes (Heldin et al., 2012). This is accompanied by an increase in matrix protease production, the remodeling of structural cell components (i.e. microtubules and microfilaments) and an enhancement in the cell’s
Figure 1.2.

Epithelial-to-mesenchymal transition (EMT)

**Protein Markers**
- N-cadherin
- Vimentin
- Fibronectin

**Physical Features**
- Actin stress fibres
- Anterior-posterior polarity
- Cell-ECM adhesion

Induced by factors such as TGFβ
Figure 1.2. Epithelial-to-mesenchymal transition

During EMT, cells lose their epithelial phenotype and then adopt a mesenchymal one. Features such as junctional complexes and cell-to-cell adhesion are lost, and the cell gains migratory capacity through this process. Cells that have undergone EMT have also been shown to be more resistant to apoptosis, and express markers such as N-cadherin, vimentin, and fibronectin.
resistance to apoptosis. At a molecular level, the transition of a cell from an epithelial to mesenchymal state can be followed with the use of protein markers. Epithelial cells express E-cadherin, occludins, claudins and cortical actin. These proteins maintain the adherens and tight junctions that are required for a tightly formed and organized layer of cells. This is in contrast to mesenchymal cell markers, which include N-cadherin, vimentin, fibronectin, and actin stress fibres (Lamouille et al., 2014). This expression profile supports the adhesion of mesenchymal cells to the extracellular matrix, as opposed to adhesion to other cells, and increases their migratory capacity (Miyazono, 2009).

There are three subtypes of EMT, each of which is defined by a different context and function (Miyazono et al., 2012). Type 1 EMT occurs during early development, which promotes the diversification of cells and supports gastrulation and thus, embryogenesis. Type 2 EMT is necessary for wound healing and tissue regeneration. In this context, EMT facilitates the formation of fibroblasts from secondary epithelial cells in response to inflammation and organ fibrosis. Type 3 EMT is a precursor process of metastasis and involves the development of migratory cells from tumorigenic epithelium (Kalluri & Weinberg, 2009).

1.3.2. EMT and cancer

Many hallmarks of the EMT process are associated with more aggressive and resistant tumors (reviewed in Ye & Weinberg, 2015). When epithelial cancer cells become mesenchymal, it allows them to undergo intravasation, which drives systemic dissemination (Kalluri & Weinberg, 2009). Similar to type 1 developmental EMT, the
upkeep of type 3 EMT programs relies on contextual signals from various locations in the body. Stromal cells are able to release a vast array of molecules that regulate EMT; these include Wnt, Notch, VEGF, and TGFβ (Ye & Weinberg, 2015).

1.3.3. **EMT and TGFβ**

Of the TGFβ isoforms, TGFβ2 and 3 play major roles in the progression of type 1 developmental EMT. TGFβ2 is involved in the formation of atrioventricular cushions, and palate fusion requires an EMT program that is driven by TGFβ3 (Thiery, 2002). In comparison, TGFβ1 is involved in post-natal fibrosis, wound healing and cancer-related type 2 and 3 EMT. Its expression is elevated during kidney, hepatic, and pulmonary fibroses, which all have a preceding EMT event (Lamouille et al., 2014). The overexpression of TGFβ1 also activates EMT programs that develop invasive tumor cells (Miyazono, 2009).

The activation of EMT in cancer cells by TGFβ is regulated at multiple levels. Both canonical and atypical TGFβ signaling not only modify transcriptional regulation and gene expression, but they also involve alternative splicing, epigenetic changes, and miRNA mechanisms of control, which all contribute to the induction of EMT (reviewed in Katsuno et al., 2013).

Within canonical TGFβ signaling, Smads directly enhance the expression of proteins, microRNAs and transcription factors. This includes the promotion of mesenchymal protein markers like vimentin and fibronectin (Lamouille et al., 2014) as well as the enhanced expression of microRNA miR-155, which aids in the dissolution of tight junctions (Kong et al., 2008). Transcription factors such as Snail, Slug, Twist, and Zeb 1
and 2, are also upregulated by Smad signaling. These factors are important for repressing E-cadherin expression, which is a critical step in the progression of EMT (Thiery, 2002). The expression of one factor can promote the expression of another, and partnerships also exist between them. For example, Snail can augment the expression of Twist, and together, Snail and Twist induce Zeb1 (Heldin et al., 2012). These interrelated transcription factors are also capable of indirectly supporting a mesenchymal phenotype. Alternative splicing of epithelial proteins occurs when Zeb 1 and 2 suppress the expression of certain RNA-binding proteins. Another example includes Snail-mediated epigenetic changes to epithelial genes, such as the methylation of the E-cadherin promoter, via interactions with methyl transferases (Katsuno et al., 2013).

Non-canonical TGFβ pathways are also important regulators of EMT processes. The tight junction protein Par6 undergoes aPKC-dependent phosphorylation by TβRII (Gunaratne et al., 2013). This causes Par6 to then recruit Smurf1 to tag RhoA for degradation through ubiquitination (Ozdamar et al., 2005). Since it is a critical regulator of the cytoskeleton, degradation of RhoA elicits structural changes such as the disruption of tight junctions and loss of cell polarity (Ozdamar et al., 2005; Miyazono, 2009). Another atypical TGFβ pathway that contributes to EMT starts with the activation of TNF receptor-associated factor 6 (TRAF6), whose downstream effector is p38/MAPK. This signaling stabilizes Snail by inhibiting GSK3β, which leads to further repression of E-cadherin (Lamouille et al., 2014).
1.4. Autophagy

Autophagy, Greek for “self-eating”, is a regulated process by which the cell can degrade, recycle, and repurpose its components. There are three types of autophagy, defined by the process by which cargo is delivered to the lysosome: macro-autophagy, micro-autophagy, and chaperone-mediated autophagy (Mizushima, 2007). Macro-autophagy involves the formation of a double membrane vesicle called an autophagosome, which sequesters cargo and then merges with the lysosome (Tanida, 2011). In microautophagy, cytoplasmic structures are engulfed directly by the lysosomal membrane without the use of an intermediate vesicle (Eskelin & Saftig, 2009). In chaperone-mediated autophagy, protein cargo forms a complex with chaperone proteins that is recognized by a receptor on the lysosomal membrane and then degraded after its translocation (Kaur & Debnath, 2015). In contrast with the other two types of autophagy, chaperone-mediated autophagy is very selective and is not capable of initiating the degradation of larger cargoes, whereas both macro and micro autophagy are capable of being selective and non-selective processes (Glick et al., 2010).

Of all the types of autophagy, macro-autophagy (which will henceforth be referred to as “autophagy”) has been of most interest due to its involvement in metabolism, cell death, and immune response, which characterizes it as an significant process in maintaining homeostasis (Eskelinen & Saftig, 2009). Dysfunctional autophagic processes can lead to infectious, neurodegenerative, pulmonary, and vascular diseases, and it has also been shown to have an important role in the initiation, progression, and therapeutic resistance of cancer (Choi & Ryter, 2013).
1.4.1. The basics of autophagy induction

Autophagy can be described to undergo six stages: initiation, nucleation, elongation, maturation, fusion, and degradation (Fig. 1.3A). In the initiation step, autophagic machinery found in the cytoplasm assembles into a scaffold complex that is essential for autophagy induction. These proteins include autophagy-related (Atg) proteins, such as Atg 13 and 101, which associate with UNC51-like kinase (ULK) 1 and FAK family kinase interacting protein of 200 kDa (FIP200). Together, these proteins form the ULK complex (Kaur & Debnath, 2015).

During nucleation, an additional complex known as class III PI3K, (which includes Beclin 1, vacuolar protein sorting 15 [VPS15], VPS34 and ATG14) is recruited to the ULK complex (Tanida, 2011). This initiates the production of a phospholipid that is specific for the extension of a phagophore, which is an isolated lipid bilayer. In mammalian cells the origin of this membrane is still unclear, although it is suggested that trans-Golgi endosomes or the endoplasmic reticulum are probable sources (He & Klionsky, 2009). The elongation of this phagophore also requires the maturation of the lipidation of microtubule-associated protein light chain 3 (LC3B)-II (reviewed in Schaaf et al., 2017). Its original precursor, LC3B, is found in the cytosol and is cleaved by the cysteine protease, Atg4, upon autophagy induction. This then produces LC3B-I, which is activated by Atg7, then conjugated to phosphatidylethanolamine by Atg 3 to form LC3B-II (Glick et al., 2010). Together with an Atg16-Atg12-Atg5 complex, LC3B-II facilitates the extension of the phagophore, such that the cytoplasmic cargo is engulfed (Farré & Subramani, 2016). The closure of the phagophore forms a double membrane organelle.
Figure 1.3.

A.

1. INITIATION

ULK complex: ULK1 - ATG13 - FIP200 - ATG101

2. NUCLEATION

Cargo (proteins, organelles)

Beclin1 - VPS15 - VPS34 - ATG14

3. ELONGATION

LC3B

4. MATURATION

LC3BI

ATG16 - ATG12 - ATG5

ATG complex dissociation

5. FUSION

Hydrolase

Autophagosome + lysosome

6. DEGRADATION

Autolysosome

B.

Normal epithelium

Autophagy deficiency = accumulation of cytotoxins

Formation of tumorigenic cells

Autophagy intact tumor cells have high survival and proliferation
Figure 1.3. Autophagy induction and its role in cancer

(A) Autophagy induction occurs in six phases: initiation, nucleation, elongation, maturation, degradation, and fusion. Initiation involves the formation of the ULK complex, which then localizes to the phagophore. More proteins are recruited during nucleation, and an Atg complex as well as the formation of LC3BII are required for the elongation of the phagophore around cargo that is targeted for autophagic degradation. Once the cargo is enclosed and the Atg complex dissociates, the autophagosome is considered mature. It then goes on to fuse with the lysosome, which contains hydrolases that degrade the engulfed autophagosome and its contents. (B) In early tumorigenesis, autophagy acts as a tumor suppressor by preventing the accumulation of cytotoxins. However, during tumor progression, autophagy can benefit cancer cells by supporting their survival and proliferation, especially in low-nutrient conditions.
called an autophagosome, and this maturation step also involves the dissociation of the Atg16-Atg12-Atg5 complex. LC3B-II, however, is bound to the autophagosome membrane and serves as a good marker for the induction of autophagy (Tanida, 2011). The outer membrane of the mature autophagosome then fuses to the lysosome, forming an autolysosome. The autophagic cargo is then degraded by lysosomal hydrolases (Levine et al., 2011).

1.4.2. Autophagy and cancer

Autophagy has several functions that allow it to act as both a tumor suppressor and a tumor promoter (Fig. 1.3B). First, autophagy acts to remove toxic waste products by degrading protein aggregates, and it also plays a major role in managing oxidative stress. In this manner, autophagy prevents tumorigenesis since it reduces the accumulation of potential mutagens (Choi & Ryter, 2013). Furthermore, autophagy has been shown to reduce inflammation and necrosis, both of which produce an environment conducive to cancer (Choi, 2012). There have also been studies showing more novel mechanisms of tumor suppression by autophagy. Young et al. (2009) observed that autophagy-deficient cells are able to avoid oncogene-induced senescence, a phenomenon in which potentially malignant cells stop dividing.

It has not yet been shown that genetic silencing of core autophagy proteins promotes the formation of human cancers, however, this has been seen in mouse models (White, 2015). For instance, a monoallelic deletion in the autophagy-dependent Beclin-1 gene promotes spontaneous tumorigenesis in the lungs and liver of mice (Eskelinen, 2011).
Once tumors are established, autophagy can also aid their survival, growth, and even therapeutic resistance (Kimmelman, 2011; White, 2015). Autophagy can also act as a tumor promoter since it provides an intracellular nutrient source. Due to the heterogeneity of tumors, some regions may not be as well vascularized and can even be hypoxic. In these areas, autophagy has been found to be upregulated in order to promote cell survival (Degenhardt et al., 2006).

In cancer cell lines, autophagy has been shown to promote a tumorigenic process, as many cell lines express relatively high levels of autophagy at rest even in the absence of metabolic stress (White et al., 2015). Furthermore, the survival of these cell lines can be reduced by the knockdown of core autophagic genes. This also holds true in genetically modified mouse models—Atg7-/- Kras-induced lung tumors were less proliferative when compared to control. They were also more sensitive to starvation due to the accumulation of lipids and defective mitochondria (Amaravadi et al., 2016).

1.4.3. **TGFβ-induced autophagy**

In a study done by Kiyono et al. (2009), TGFβ1 ligand was shown to be an inducer of autophagy in hepatocarcinoma and breast cancer cells. This activation was observed to be dependent on both canonical and atypical TGFβ signaling pathways, which then upregulate the expression of autophagic genes such as Beclin-1, Atg5 and Atg7 (Suzuki et al., 2010). Recent studies also suggest the involvement of the Disabled-2 (Dab2) endocytic adaptor protein in the regulation of autophagy by TGFβ (Jiang et al., 2016).

Due to its recent discovery, much is still unknown about TGFβ-induced autophagy and its relation to other TGFβ-regulated processes such as EMT.
1.5. p62: a multifunctional protein

Selective autophagy occurs when specific cargo is targeted for degradation by the autolysosome. In order for this to occur, adaptor proteins like p62 (also known as sequestosome-1, SQSTM1) are required to sequester the cargo to the autophagosome (Farré & Subramani, 2016). Though many scaffolding proteins exist, p62 was the first autophagy adaptor to be identified. It is unique since, unlike the other adaptor proteins, p62 is able to regulate signaling by interacting with key pathway components (Moscat et al., 2016).

1.5.1. Structure of p62

The SQSTM1 gene is on chromosome 5g35 in humans, and it encodes the p62 protein that is 440 amino acids in length. It is highly conserved and homologues of SQSTM1 exist in a variety of vertebrates (Bitto et al., 2014). Its transcription is upregulated by factors such as Nrf2, IL-3, and calcium (Puissant et al., 2012), and once translated and modified, p62 is distributed in numerous compartments of the cell, including the cytoplasm, nucleus, late endosomes, autophagosomes, and lysosomes (Sanchez et al., 1998; Katsuragi et al., 2015).

The different domains of p62 are what enable it to act as a multifunctional protein (Fig. 1.4A). From the N-terminus to the C-terminus, p62 contains a Phox1 and Bem1p (PB1) domain, a zinc finger domain (ZZ), a TRAF6 binding domain (TB), an LC3-interacting region (LIR), a Keap1 interacting region (KIR), and a ubiquitin-associated domain (UBA). It also has 2 nuclear localization sequences (NLS) that flank the TB
Figure 1.4.
Figure 1.4. p62: a multifunctional protein in autophagy

(A) The structural domains of p62 include the Phox-BEM1 (PB1), zinc finger (ZZ), TRAF6 binding (TB), LC3 interacting region (LIR), Keap1 interacting region (KIR) and ubiquitin associated (UBA) region. p62 also has two nuclear localization signals that flank the TB domain and one nuclear export signal. (B) The various domains of p62 enable it to function in numerous cell processes, such as autophagy, and signaling pathways, such as mTORC1, Nrf2, and NFκB. In autophagy, p62 selectively targets cargo for degradation and scaffolds it to the autophagosome via interaction with LC3BII. In signaling, p62 scaffolds and sequesters key protein players, which changes the outcome of certain pathways.
domain and a nuclear export signal just downstream of the second NLS (Katsuragi et al., 2015).

1.5.2. p62 and its role in autophagy

As mentioned above, p62 facilitates selective degradation of mitochondria, microbes, and protein aggregates by autophagy (Fig. 1.4B). p62 is first phosphorylated by ULK1 at Ser407 in its UBA domain (Lim et al., 2015). Then, the selected cargo associates with p62 by either the UBA domain or PB1 domain for ubiquitinated or non-ubiquitinated cargo, respectively. In the case of ubiquitinated cargo, binding with p62 can be enhanced by phosphorylation on Ser403 in the UBA domain of p62 (Matsumoto et al., 2011). Next, p62 then undergoes self-oligomerization using its Phox-Bem1 (PB1) domain in order to effectively package and deliver the cargo to the phagophore (Puissant et al., 2012). The oligomerization step is crucial for mediating successful degradation by autophagosomes (Komatsu & Ichimura, 2010).

The p62 oligomeric complexes then interact with the phagophore using its LIR domain. This region of p62 contains acidic and hydrophobic clusters, which can associate with several sites of LC3B-II, including two hydrophobic pockets and N-terminal basic residues (Katsuragi et al., 2015). Hydrolases in the autolysosome degrade both the cargo as well as p62, as both proteins are engulfed and localized to the autophagosome. Thus, the accumulation of p62 can serve as a marker for defective autophagy (Puissant et al., 2012).
1.5.3. *p62 and its role in signalling*

Alongside autophagy, p62 has been shown to regulate signaling, despite having no catalytic abilities. Although it does not participate in any signaling cascades such as phosphorylation, p62 is able to regulate signaling by scaffolding important signaling components. It has been shown to act as a signaling hub in the mTORC1, NFκB, and Nrf2 pathways (*Fig. 1.4B*; Bitto *et al.*, 2014; Katsu*ragi et al.*, 2015).

When the level of amino acids is elevated, the mTORC1 pathway is activated in order to manage protein synthesis. The amino acid sensing component, Rag GTPase, is able to associate with a subunit of the mTORC1 complex, Raptor, in a p62-dependent manner. Furthermore, p62 is necessary to initiate the auto-ubiquitination of TRAF6 (an E3 ubiquitin ligase), which allows it to then ubiquitinate mTOR and enhance its activation of mTORC1 (Katsu*ragi et al.*, 2015). This results in the down regulation of autophagic processes, and the upregulation of cell growth (Levine *et al.*, 2011; Puissant *et al.*, 2012).

The NFκB transcription factors regulate cell processes such as survival and differentiation once they are translocated to the nucleus (Moscat *et al.*, 2007). Its nuclear import is regulated by a protein complex, consisting of aPKC (PKC iota and zeta), TRAF6, and receptor interacting protein (RIP) (Puissant *et al.*, 2012). p62 is able to bind to each of these components, and scaffold them together to induce NFκB translocation. Using its PB1, TB, and ZZ domains, p62 associates with aPKC, TRAF6, and RIP, respectively (Katsu*ragi et al.*, 2015). Downstream effects of the NFκB pathway include the production of reactive oxygen species (ROS) scavengers and the induction of inflammatory response genes (Moscat *et al.*, 2016). A positive feedback loop is also
established since p62 is also up regulated in response to NFκB, and this sustains NFκB signaling (Moscat & Diaz-Meco, 2012).

Another pathway that helps to control oxidative stress is the Nrf2 pathway. Nrf2 is a transcription factor that is sequestered to the cytoplasm by a protein called Keap1. The non-covalent interaction between Nrf2 and Keap1 inhibits the activity of Nrf2 (Puissant et al., 2012). This is abrogated when p62 competitively binds Keap1 and targets it for degradation via selective autophagy using its KIR domain (Katsuragi et al., 2015). Nrf2 is then activated and able to induce the transcription of genes such as those involved in detoxification, repair, and ROS scavenging (Bitto et al., 2014). Similarly to NFκB, Nrf2 also sustains its signaling when establishing positive feedback by up regulating p62 expression (Puissant et al., 2012).

1.5.4. p62 and cancer

Due to its role in autophagy and various signaling pathways, p62 is undoubtedly an important regulator of cancer-relevant processes. Its involvement in both tumor promoting and tumor suppressive mechanisms necessitates that the effects of p62 are heavily context-dependent. For example, studies done by and reviewed in Moscat et al., 2016 reveal that p62 promotes tumorigenesis in hepatocarcinoma cells, but prevents cancer progression when expressed in the hepatostellate cells (tumor stromal fibroblasts). Thus, current studies are focused on discovering the consequences of p62 expression in a variety of contexts.
1.6. Rationale and hypothesis

In recent studies, it has been shown that sustained TGFβ signaling can induce autophagy (Kiyono et al., 2009; Jiang et al., 2016). Though much is known about starvation-induced autophagy, many aspects of TGFβ-induced autophagy have not yet been elucidated. It is of interest to assess whether knockdown of p62 or p62-associated proteins would alter autophagy, and to determine whether TGFβ-dependent autophagy may have a relationship with TGFβ-dependent EMT.

Alongside autophagy, p62 has been shown to act as a signaling hub in the mTORC1, NFκB, and Nrf2 pathways (Moscat et al., 2016). It remains unknown whether p62 has a role in TGFβ signaling, however, p62 does associate with key TGFβ signaling proteins: aPKC via its PB1 domain and TRAF6 via its TRAF6-binding domain (Katsuragi et al., 2015). Because of these interactions, it is plausible that p62 affects TGFβ signaling. Previous studies from our lab have shown that aPKC alters canonical TGFβ signaling (Gunaratne et al., 2015), but it is unknown whether p62 may regulate aPKC activity. Since both TRAF6 and aPKC independently form complexes with TGFβRs, p62 may also co-localize with TGFβ receptors.

Based on the aforementioned rationale, I hypothesize that p62 regulates TGFβ-dependent EMT and autophagy. To test this, aims of this thesis were (Fig. 1.5):

1. Assess effect of p62 silencing on canonical TGFβ signaling and EMT.
Figure 1.5.

Assess effect of siRNA-mediated p62 silencing on TGFβ-dependent Smad phosphorylation and epithelial-to-mesenchymal transition

Assess whether p62 or its associated proteins (aPKC and TRAF6) are required for the induction of TGFβ-dependent autophagy via siRNA knockdown
Figure 1.5. Thesis Aims

These aims are based on both aspects of p62: its role in autophagy and its role in signaling. The first aim will assess if p62 is required for TGFβ-dependent Smad phosphorylation and EMT, and the second will assess if p62 and p62-associated proteins are required for TGFβ-induced autophagy.
Chapter 2: Materials and Methods

2.1. Antibodies and reagents

All antibodies, sources, applications and working dilutions are provided in Table 2.1.

Table 2.1. Antibodies

<table>
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All reagents, sources, applications and working dilutions are provided in **Table 2.2**.

**Table 2.2. Reagents**

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2.2. Cell Culture and transfections

2.2.1. Cell culture

A549 non-small cell lung cancer (NSCLC) cells were cultured in Nutrient Mixture F-12 Ham Kaighn’s Modification (F12K; Sigma: N3520) supplemented with 10% fetal bovine serum (FBS). Mv1Lu mink lung cells stably transfected with HA-tagged TβRII were cultured in Minimal Essential Medium (MEM) supplemented with 1% non-essential amino acids, 0.3 mg/mL hygromycin and 10% FBS. All cell lines were kept in a cell culture incubator that was maintained at 37°C and had a high-humidity atmosphere composed of 5% CO₂.

2.2.2. Transfection

The following transfection protocol describes RNA silencing in A549 cells. Twenty-four hours prior to transfection, 175 000 A549 cells were plated per well of a 6-well dish. Knockdown was carried out using Lipofectamine RNAiMAX Reagent (Table 2.2) as per manufacturer’s protocol. Briefly, appropriate amount of RNAiMAX was diluted in opti-MEM and left to incubate for 5 minutes. In a separate tube, appropriate amount of siRNA was diluted in opti-MEM medium. RNAiMAX solution was then added to each siRNA solution (the volume of both solutions were equal). After gentle mixture by inversion, the mixture was left to incubate for 15 minutes. The solution was then added drop-wise onto the cells. Concentrations of siRNA towards their respective proteins were used as follows: 5 nM for siRNA directed to p62, 37.5 nM for siRNA directed to PKCε, 75 nM
for siRNA directed to PKCζ, and 15 nM for siRNA directed toward TRAF6. Cells were processed 48 hours post-transfection.

2.3. **Cell lysis and protein isolation**

2.3.1. *Cell lysis, protein isolation and measurement for immunoblotting*

To isolate protein, cells were first washed with cold phosphate buffered saline (PBS). Lysis buffer (50 mM Tris pH 7.5, 1 mM EDTA, 0.5% TX-100, 150 mM sodium chloride, 1 mg/mL pepstatin, 50 µM PMSF, 2.5 mM sodium fluoride, and 10 mM sodium pyrophosphate phosphatase inhibitor) was then put on the cells and left to incubate on a rocker at 4°C for 20 minutes. Cells were then scraped off the plate using cell scrapers and the lysate was then transferred to a 1.5 mL tube. Samples were centrifuged at 21 000 x g for 10 minutes at 4°C. After transferring the supernatant to a new tube and discarding the cell pellet, the lysates were stored at -20°C until use.

2.3.2. *Protein assay*

Cell lysate protein concentrations were measured according to the Lowry method (Fisher) prior to the addition of sample preparation buffer. Protein assays were carried out using the DC™ Protein Assay (Table 2.2) as per manufacturer’s protocol. Absorbance was measured using a Beckman Coulter DU730 spectrometer or a PerkinElmer Victor 3V Multi-Detection Microplate Reader.
2.4. **Nuclear fractionation**

Nuclear fractionation was done using NE-PER\textsuperscript{TM} Nuclear and Cytoplasmic Extraction Reagents (Table 2.2) as per the manufacturer’s protocol, and as described in Gunaratne et al., 2015. Briefly, cells were dissociated using trypsin, washed with PBS and pelleted. Cytoplasmic extraction reagents I and II were then added and samples were centrifuged (21 000 x g at 4°C) to separate nuclear protein (cell pellet) from the cytoplasmic protein (supernatant). Nuclear extraction reagent was then added to the pellet, vortexed, and centrifuged again to isolate nuclear protein.

2.5. **TGFβ treatment**

One day prior to TGFβ treatment, cells were washed once with PBS, then serum starved overnight using medium supplemented with 0.2% FBS. The following day, cells were treated with 250 pM of TGFβ unless otherwise stated (incubation time varies per experiment).

For the 5-day TGFβ experiments, cells were serum starved for 4 hours one day post-transfection, then treated with 250 pM TGFβ. For each day of the experiment, cells were thoroughly washed with PBS to remove cellular debris and then replaced with fresh medium supplemented with 0.2% FBS and 250 pM TGFβ.

2.6. **SDS-PAGE and immunoblotting**

In preparation for immunoblotting, sample preparation buffer (30% glycerol, 10% 1.5 M Tris (pH 6.8), 1.2% SDS, 0.018% bromophenol blue, and 15% β-mercaptoethanol) was
added to lysates, and then the samples were boiled at 100°C for 5 minutes before being subjected to SDS-PAGE. Cell lysates were subjected to gel electrophoresis (SDS-PAGE) using a stacking gel composed of 5% acrylamide and a separating gel composed of 10% acrylamide. For the 5-day TGFβ experiment, samples were run on a 15% acrylamide gel to ensure proper separation of LC3BI from LC3BII protein. A range of 50-100 µg of protein per sample was loaded into the gel, which was run at a constant 200 V. Proteins were then transferred onto a nitrocellulose membrane via wet transfer (Biorad), which was maintained at a cold temperature by using ice packs. After protein transfer, staining was done using Ponceau S (15% acetic acid; 4 mg/mL Ponceau S). Ponceau S was washed off the membranes using tris-buffered saline-tween 20 (TBST; 50 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.5). Membranes were then blocked with 5% skim milk diluted in TBST for 1 hour, rocking at room temperature. Primary antibodies diluted in TBST (dilutions for each antibody are shown in Table 2.2) were then left on the membranes to incubate overnight, rocking at 4°C. The following day, the membranes were washed three times with TBST, each wash being 10 minutes in duration rocking at room temperature. Membranes were then incubated with the appropriate HRP-conjugated secondary antibody diluted in TBST for 1 hour at room temperature. Following secondary antibody incubation, membranes were washed three more times (10 minutes each). Excess TBST was then drained from the membranes and, using a pipette, membranes were washed several times with enhanced chemiluminescent substrate (Table 2.2). Visualization was carried out using a Bio-Rad Versa-doc Imager and QuantityOne® 1-D Analysis software. Quantitation/densitometry was done using the volume tools within the QuantityOne® 1-D Analysis software.
2.7. Antibody feeding/TGFβ receptor internalization analysis

Colocalization of TβRII with p62 and rab7 was done using Mv1Lu mink lung cells that were stably transfected to over express pMEP4 with cDNA encoding HA-tagged TβRII under a zine-inducible promoter (Di Guglielmo et al., 2003; Gunaratne et al., 2012). One day after being plated on coverslips, cells were serum starved overnight using medium supplemented with 0.2% FBS and 50 µM zinc chloride. The following day, cells were placed on ice for 5 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 PBS, and then appropriate AlexaFluor™-conjugated secondary antibody was added and left to incubate on ice for 1 hour in the dark. Coverslips were washed another 3 times with PBS before being put back to 37°C at various times to induce trafficking. At the indicated times, coverslips were removed, fixed, and permeabilized (please refer to section 2.8 Immunofluorescence microscopy).

2.8. Immunofluorescence microscopy

Coverslips were first washed one time with PBS, then fixed using 4% PFA (left to incubate for 10 minutes at room temperature). After another 3 PBS washes, cells were permeabilized by incubating coverslips in 0.25% Triton X-100 for 5 minutes. After 3 PBS washes, coverslips were blocked using a 10% FBS solution diluted with PBS and left to rock at room temperature for 1 hour. The FBS solution was then aspirated and 300 µL of appropriate primary antibody (diluted in 10% FBS solution as described in Table 2.1) was immediately added to the coverslips. Primary antibody was left to incubate
overnight at 4°C on a rocker. The following day, coverslips were washed 3 times with PBS and appropriately diluted Alexafluor™-conjugated antibody (please see Table 2.1) was left to incubate on coverslips for 1 hour at room temperature in the dark. After another 3 PBS washes, coverslips were incubated in DAPI solution (1:1000 dilution in PBS) for 5 minutes. After 1 PBS rinse, coverslips were mounted onto glass slides using liquid Immunomount (Fisher Scientific). Samples were left to dry in the dark overnight before visualization. If imaging was not done immediately, slides were stored in the dark at -20°C until use.

Imaging of coverslips was done using an Olympus IX81 motorized inverted microscope and InVivo software.

2.9. Microarray: RNA quality assessment, probe preparation and GeneChip hybridization

All GeneChips were processed at the London Regional Genomics Centre (Robarts Research Institute, London, Ontario, Canada; http://www.lrgc.ca). RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA) and the RNA 6000 Nano kit (Caliper Life Sciences, Mountain View, CA). Single stranded complimentary DNA (sscDNA) was prepared from 200 ng of total RNA as per the Ambion WT Expression Kit (Applied Biosystems, Carlsbad, CA) and the Affymetrix GeneChip WT Terminal Labeling kit and Hybridization User Manual (Affymetrix, Santa Clara, CA). Total RNA was first converted to cDNA, followed by in vitro transcription to make cRNA. Five micrograms of single stranded cDNA were synthesized, end labeled
and hybridized, for 16 hours at 45°C, to Human Gene 1.0 ST arrays. All liquid handling steps were performed by a GeneChip Fluidics Station 450 and GeneChips were scanned with the GeneChip Scanner 3000 7G (Affymetrix, Santa Clara, CA) using Command Console v1.1. Probe level (.CEL file) data was generated using Affymetrix Command Console v1.1. Probes were summarized to gene level data in Partek Genomics Suite v6.5 using the RMA algorithm adjusted for GC content (Irizarry et al., 2003). Using Partek, any batch affect due to scan date was removed and an ANOVA (Yijk = \( \mu + \text{Condition} * \text{Timeij} + \epsilon_{ijk} \)) using Method of Moments was run to determine gene level p-values. Fold change comparisons are expressed relative to untreated siControl cells, and represent the average of two separate experiments (2 separate gene chips per condition). A fold change of ± 1.7 was considered as the cutoff for induction.

2.10. Statistical Analysis

All quantitation is representative of at least 3 replicates. Evaluation of results was done using a one-way or two-way ANOVA followed by a Bonferonni post-hoc test. These tests were carried out by using GraphPad Prism® Version 6 software. Values of p<0.05 were considered statistically significant.
Chapter 3: Results

3.1. P62 co-localizes with TβRII at the late endosome

Previous studies have shown that aPKC has interactions with both p62 (Sanchez et al., 1998) and TβRII (Gunaratne et al., 2013). Furthermore, Dr. Gunaratne also observed that p62 interacts with TGFβ receptors (unpublished data; Appendix Fig. A1). However, the subcellular location where this interaction may occur remained unknown. Thus, I first investigated the temporal subcellular localization of p62 and TGFβ receptors using immunofluorescence microscopy (Fig. 3.1).

Briefly, Mv1Lu cells stably transfected with extracellular HA-tagged TβRII were used to follow the internalization of cell surface labelled receptors in order to investigate whether they localize with p62/SQSTM1 during their trafficking from the cell surface to intracellular vesicles (Fig. 3.1). Cell surface TβRII were labeled using anti-HA antibody and fluorescent secondary antibody at 4°C (a temperature that does not permit vesicular trafficking) and the cells were then incubated at 37°C (to reinitiate vesicular trafficking) for various amounts of time before fixing and counterstaining with either markers for the early (EEA1) or late (Rab7) endosomal compartments (please see Methods Section 2.7 & 2.8). As incubation time at 37°C increases, TβRII signal gradually become more punctate, which is consistent with the internalization of the receptor. Indeed, I observed that after 1 hour of incubation at 37°C, TGFβ receptors co-localized with EEA1, as previously reported (Fig 3.1A; Di Guglielmo et al., 2003). When counterstained with anti-Rab7, moderate co-localization with TβRII was observed at the 3-hour time point.
(seen as a yellow signal; **Fig. 3.1B**). This indicates that the later trafficking of TβRII involves Rab7, and this agrees with previous studies (Balogh et al., 2015). Counterstaining with anti-p62 also shows moderate co-localization at the 3-hour time point (**Fig. 3.1C**). Taken together, my results suggest that there is a co-localization between p62 and TβRII at a time point when the TGFβ receptors arrive at the late endosome.

### 3.2. Receptor internalization is necessary for maximum Smad phosphorylation

Since p62 associates with TGFβ receptors (Appendix; **Fig. A1**) and co-localizes with TβRII (**Fig. 3.1**), it has potential to affect the canonical TGFβ signaling pathway. In order to assess this, I investigated whether silencing p62 would alter the extent and time course of Smad2 phosphorylation and Smad2 nuclear translocation in response to TGFβ stimulation. Briefly, A549 cells were transfected with control siRNA or siRNA targeting p62 (**Fig. 3.2A**) and incubated with increasing concentrations (0-10 pM) of TGFβ. Following western blotting using phopho-specific Smad2 antibodies, I observed that p62 silencing had no effect in the magnitude or duration of Smad2 phosphorylation at the various TGFβ concentrations (**Fig. 3.2B**). Furthermore, the presence or absence of p62 did not affect the ability of TGFβ to stimulate Smad2 nuclear localization, as assessed by immunofluorescence microscopy (**Fig. 3.2C**). In the absence of TGFβ, the Smad2 immunofluorescence signal had the characteristic hazy cellular pattern, indicating that it is present in the entirety of the cell. After TGFβ incubation, the Smad2 signal became
Figure 3.1A
Figure 3.1B
Figure 3.1C
Figure 3.1. Colocalization exists between TβRII and EEA1, between TβRII and Rab7, and between TβRII and p62

Immunofluorescence microscopy was carried out using Mv1Lu mink lung epithelial cells stably transfected with HA-tagged TβRII. After serum starving overnight, cell surface receptors were labelled at 4°C with anti-HA. Cells were then incubated at 37°C for various times before fixing and counter staining with anti-EEA1 (A), anti-Rab7 (B), or anti-p62 (C) antibodies. Bar = 10 µm. Images represent n=3.
Figure 3.2
Figure 3.2. p62 knockdown does not affect Smad phosphorylation and translocation

(A) 5 nM of siRNA towards p62 is sufficient to knockdown p62 protein levels. (B) A549 NSCLC cells were transfected with the indicated siRNA and serum starved the next day overnight. 48 hours post-transfection, cells were then incubated with increasing amounts of TGFβ for 1 h. Total lysates were subjected to SDS-PAGE and immunoblotting. (C) A549 NSCLC cells were transfected with the indicated siRNA and serum-starved the next day overnight. 48 hours post-transfection, cells were incubated with 250 pM TGFβ for 1 h, then fixed, permeablized and stained for Smad2. Images represent n=3.
crisp and co-localized with the DAPI-stained nucleus, indicating nuclear translocation (Fig. 3.2C). The immunofluorescence micrographs containing the p62 knockdown condition appeared very similar to the control, indicating that silencing p62 did not affect Smad2 nuclear translocation (Fig. 3.2C).

A potential reason for the lack of effect of p62 silencing on TGFβ dependent signal transduction could be that the interaction between p62 and TGFβ receptors occurs in the late endosome. Indeed, TGFβ signaling has been shown to occur in the early endosomal compartment and therefore the signal transduction pathway is fully engaged by the time that the receptors access the p62-positive compartment. In fact, previous studies have used Dynamin mutants to show that receptor internalization is necessary for the maximum phosphorylation of Smad (Di Guglielmo et al., 2003; Runyan et al., 2005). In order to test this in my cell system, I pharmacologically inhibited Dynamin activity, and by extension receptor endocytosis, using Dyngo-4a. Using antibody feeding and immunofluorescence microscopy, I first established that Dyngo-4a was indeed inhibiting internalization of cell surface TGFβ receptors (Fig 3.3A). In control cells, the internalization of receptors was observed within 1 hour to result in a punctate, perinuclear TβRII stain that co-localized with EEA1 (a marker for the early endosome). Using these parameters, I observed that Dyngo4a retained immunofluorescent receptor signal at the cell surface, indicating that inhibition of dynamin blocked receptor internalization.

I then assessed the effect of dynamin inhibition on Smad phosphorylation using western blotting (Fig 3.3B). A549 NSCLC cells were treated with increasing concentrations of Dyngo-4a in the presence or absence of TGFβ ligand for one hour and the cell lysates were then analyzed by immunoblotting. I observed that TGFβ-dependent Smad
Figure 3.3A
Figure 3.3B and 3.3C
Figure 3.3. Inhibition of receptor internalization by Dyngo-4a attenuates TGFβ-dependent Smad phosphorylation

(A) Immunofluorescence microscopy was carried out using Mv1Lu mink lung epithelial cells stably transfected with HA-tagged TβRII. After serum starving overnight, cell surface receptors were labelled at 4°C with anti-HA. Cells were then incubated at 37°C for various times before fixing and counter staining with anti-EEA1 antibody. Bar = 10 µm. (B) A549 NSCLC cells were serum starved overnight and incubated with the increasing concentrations of Dyngo-4a for one hour. The cells were then incubated in the presence or absence of 250 pM TGFβ for one hour before lysing. Cell lysates were subjected to SDS-PAGE and immunoblotting using anti-Phospho-Smad2 and anti-Smad2 antibodies. (C) Quantitation of P-Smad2 levels in conditions with TGFβ incubation. Bars labelled with uppercase letters are significantly different (p<0.05) from bars labelled with the same lowercase letter. Images represent n=3.
phosphorylation was significantly attenuated after incubation with 40 \( \mu \)M and 50 \( \mu \)M of Dyngo-4a (Fig 3.3C), which supports the notion that receptor signaling occurs during early events in receptor endocytosis and that access to the late endosomal compartment, where p62 resides, occurs after signal transduction is initiated.

3.3. TGFβ reduces levels of nuclear p62

Although I observed that p62 silencing did not appear to alter Smad2 nuclear translocation (Fig. 3.2C), I further investigated this using a quantitative method. Briefly, A549 cells were transfected with control siRNA or siRNA targeted towards p62, incubated with TGFβ for 30 minutes, washed, then further incubated for either 2.5 or 5.5 hours before lysis and subcellular fractionation. Cytoplasmic and nuclear fractions were isolated and subjected to immunoblotting (Fig. 3.4A). Consistent with my immunofluorescence microscopy analysis, there were no significant differences observed in the time course of nuclear Smad2 localization (Fig. 3.4B).

As a parallel study, I also investigated the levels of nuclear p62, as the nucleocytoplasmic shuttling of p62 has been reported to occur in response to autophagy (Pankiv et al., 2010). Since TGFβ has been shown to also induce autophagy (Kiyono et al., 2009; Ding & Choi, 2014; Jiang et al., 2016), I assessed if the amount of nuclear p62 would change in response to TGFβ treatment (Fig. 3.4A). Interestingly, nuclear p62 appeared as a doublet in the sip62 condition, which may be indicative of post-translational modifications. Furthermore, the amount of p62 observed in the nucleus of control cells decreased significantly in response to TGFβ treatment (Fig. 3.4C).
Figure 3.4
Figure 3.4. 

p62 knockdown does not affect Smad2 translocation. TGFβ reduces the amount of nuclear p62

(A) A549 NSCLC cells were transfected with the indicated siRNA and then serum starved the next day. 48 hours post-transfection, cells were incubated with 250 pM TGFβ for 30 minutes, washed, and then further incubated for 2.5 or 5.5 h before lysis and fractionation. Cytoplasmic and nuclear fractions were then subjected to SDS-PAGE and immunoblotting. P62 knockdown did not affect nuclear Smad2 levels (B), however, nuclear p62 levels decreased in SiControl cells, 3 hours after the start of the TGFβ treatment (C). Images represent n=3.
3.4. P62 knockdown reduces basal E-cadherin and induces preliminary stress fibres in NSCLC cells

Nucleocytoplasmic shuttling of p62 has been proposed to affect the ubiquitination and degradation of transcription factors, some of which are involved in EMT (Bertrand et al., 2015; Grassi et al., 2015). Since I observed that TGFβ reduced the levels of nuclear p62, it was of interest to explore whether p62 knockdown would have an effect on TGFβ-dependent EMT. TGFβ-induced EMT involves a reduction in epithelial cell markers (for e.g., E-cadherin) and an induction of mesenchymal cell markers (for e.g., N-cadherin). I therefore assessed E- and N-cadherin proteins levels after incubation with TGFβ ligand. Briefly, A549 cells were transfected with control siRNA or siRNA targeted towards p62, incubated with TGFβ for 24 or 48 hours and subjected to immunoblotting (Fig. 3.5A). I observed that TGFβ induced a robust loss of E-cadherin and an increase in N-cadherin levels as previously described by our laboratory (Fig. 3.5A; Gunaratne et al., 2013). Interestingly, this cadherin switch was very similar in control and p62-silenced cells; however, it was observed that basal levels of E-cadherin in p62-silenced cells were significantly lower compared to control (Fig. 3.5B). This suggests that knockdown of p62 may prime cells to undergo EMT. Furthermore, I observed that p62 levels were also significantly attenuated by incubation with TGFβ for 24 or 48 hours (Fig. 3.5D). In order to further assess if p62 silencing would prime A549 cells to undergo EMT, I carried out microarray studies.

Microarray analysis was used as a method to identify possible alterations in gene expression after silencing p62. Results could potentially explain how p62 silencing
Figure 3.5

A)

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>siCtrl</th>
<th>sip62</th>
</tr>
</thead>
<tbody>
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<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>24</td>
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<tr>
<td>48</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
</tbody>
</table>

- **E-Cadherin**
- **N-Cadherin**
- **p62**
- **GAPDH**

B)

Relative E-Cad Levels

- si Control
- si p62

Relative N-Cad Levels

- si Control
- si p62

Relative p62 Levels

- si Control
- si p62
Figure 3.5. p62 knockdown reduces basal E-cadherin levels in a TGFβ-independent manner

(A) A549 NSCLC cells were transfected with the indicated siRNA and serum starved the next day. 48 hours after transfection, cells were then incubated with 250 pM TGFβ for 0, 24, or 48 hours. Total cell lysates were subjected to SDS-PAGE and immunoblotting. (B) TGFβ induces a reduction in E-cadherin levels at the same rate between the control and p62 knockdown conditions, however, p62 knockdown reduced the basal level of E-cadherin. No differences were observed in N-cadherin induction between control and p62-silenced cells. Levels of p62 were also significantly attenuated after incubation with TGFβ for 24 or 48 hours. Images represent n=3.
attenuates levels of basal E-cadherin expression (Fig. 3.5). To conduct this experiment, A549 cells were transfected with either control siRNA or siRNA towards p62. Forty-eight hours post-transfection, total RNA was extracted and submitted to the London Regional Genomics Centre for microarray analysis. After normalization (please refer to Methods section 2.9), results were processed and summarized (Table A1). In support of my immunoblotting findings, p62 silencing attenuated the transcription of E-cadherin (CDH1) but did not affect the relative levels of N-cadherin (CHD2) transcript levels.

To further my analyses, I next assessed the structure of the actin cytoskeleton, as it also realigns from the cell cortex into stress fibers in cells undergoing EMT (Thiery, 2002; Lamouille et al., 2014). Using phalloidin, which stains for filamentous actin, I analyzed the effects of p62 knockdown on TGFβ-dependent EMT by immunofluorescence microscopy (Fig. 3.6). The control cells contained cortical actin organization in the absence of TGFβ, but robust stress fibers in cells that were incubated with TGFβ for 48 hours (Fig. 3.6A). Interestingly, the silencing of p62 induced the formation of preliminary stress fibres, even in the absence of any TGFβ induction (Fig. 3.6B). However, after the addition of TGFβ ligand, the stress fibre formation did not appear to be different between the siControl and sip62 cells, consistent with my findings that once EMT is initiated, the TGFβ effect supercedes the effects of p62. Taken together, these results suggest that the presence of p62 may maintain cells in a more epithelial state and that removal of p62, either by siRNA or TGFβ, supports EMT.
Figure 3.6
Figure 3.6. p62 knockdown induces preliminary formation of actin stress fibres

(A) A549 cells were transfected with either control siRNA or siRNA towards p62. After serum starving overnight, cells were then incubated in the presence or absence of TGFβ for 48 h. Cells were then fixed, permeabilized, and stained for p62 and filamentous actin. Bar=10 µm. (B) In each replicate, 10 images were taken and the longest stress fibre and shortest cell axis from three cells per image were measured blindly. The values presented represent the average length of the longest stress fibres relative to the shortest axis within the same cell. Bars labelled with uppercase letters are significantly different (p<0.05) from bars labelled with the same lowercase letter. Images represent n=3.
3.5. P62 in TGFβ-induced autophagy

Recent findings show that sustained TGFβ treatment induces autophagy (Jiang et al., 2016). Since proteins that are involved in TGFβ signaling, namely aPKC and TRAF6, also associate with p62, I next investigated if the presence or absence of these proteins would affect TGFβ-dependent autophagy. I therefore silenced p62, aPKC or TRAF6 and assessed LC3BII protein levels, as the conversion of LC3BI to LC3BII is a marker of autophagy. Briefly, A549 cells were transfected with control siRNA or siRNA targeting p62, aPKC or TRAF6 and incubated in the presence (Fig. 3.7) or absence (Fig. 3.8) of TGFβ for 0 to 5 days. Total cell lysates were subjected to immunoblotting for EMT (E-cadherin, N-cadherin) or autophagic (LC3BII) markers. I observed that cells expressing control siRNA contained elevated LC3BII levels in response to TGFβ over the time course of the experiment (Fig. 3.8B). Interestingly, TRAF6 levels were also observed to be significantly higher after incubation with TGFβ (Fig. 3.8C).

Knockdown of aPKC appeared to moderately hinder TGFβ-dependent induction of autophagy, whereas TRAF6 silencing was observed to have a slightly more dramatic effect (Fig. 3.8B). Indeed, the levels of TRAF6 protein appear to parallel those of LC3BII, indicating that it has potential to serve as a novel marker for TGFβ-dependent autophagy.

With respect to EMT, aPKC knockdown was also seen to inhibit this process — specifically the cadherin switch (Fig 3.7A). This effect was previously observed by our lab (Gunaratne et al., 2013). Additionally, in the absence of TGFβ, p62 knockdown
**Figure 3.7**

A) Western blot analysis showing the expression levels of E-Cadherin, N-Cadherin, aPKC, p62, TRAF6, LC3B, LC3B II, and GAPDH over time (days 0 to 5) for siControl, sip62, siPKCγ, and siTRAF6. The graphs indicate the relative expression levels for each protein.

B) Bar graph illustrating the relative LC3B II levels for different treatments over time (days 0 to 5). The treatments include siControl, sip62, siaPKC, and siTRAF6. The data shows statistically significant differences (A, B, C, D) at specific time points.

C) Bar graph depicting the relative TRAF6 levels for the same treatments over time. The graph highlights the significance of the results (a, b, c, d) at different time points.
Figure 3.7. Autophagy is induced by sustained TGFβ treatment

(A) A549 NSCLC cells were transfected with the indicated siRNA. 24 hours post-transfection, cells were incubated with 0.2% serum medium and treated with 250 pM TGFβ for 0 to 5 days. Cells were washed and medium/TGFβ ligand was replaced every day they were in culture. Total cell lysates were then subjected to SDS-PAGE and immunoblotting. An increase in LC3BII protein levels is indicative of autophagy induction. (B) Quantitation shows a significant reduction in TGFβ-dependent LC3BII induction after silencing aPKC or TRAF6. (C) In the presence of TGFβ, quantitation of TRAF6 levels shows a similar trend of induction relative to LC3BII levels. Bars labelled with uppercase letters are significantly different (p<0.05) from bars labelled with the same lowercase letter. Images represent n=3.
Figure 3.8
Figure 3.8. Effect of different knockdowns and sustained incubation in serum-starved medium

(A) A549 NSCLC cells were transfected with the indicated siRNA. 24 hours post-transfection, cells were incubated with 0.2% serum medium for 0 to 5 days. Cells were washed and medium was replaced every day they were in culture. Total cell lysates were then subjected to SDS-PAGE and immunoblotting. (B) LC3BII induction is dependent on the presence of TGFβ ligand. (C) TRAF6 induction is dependent on the presence of TGFβ ligand. (D) Silencing p62 attenuates the rise in E-cadherin levels after 2 and 3 days in culture. Bars labelled with uppercase letters are significantly different (p<0.05) from bars labelled with the same lowercase letter. Images represent n=3.
appears to attenuate the rise in E-cadherin levels, thus further supporting my findings above (Fig. 3.8D).

3.6. Knockdown of aPKC (but not p62 or TRAF6) attenuates Smad phosphorylation

Since TRAF6 levels were seen to parallel levels of inducted LC3BII, it was important to investigate whether TRAF6 would have an effect on canonical TGFβ signaling. I therefore investigated the extent and duration of Smad phosphorylation in cells with TRAF6 knockdown, and compared this to the effects of aPKC and p62 knockdown (Fig 3.9). Briefly, A549 cells were transfected with the indicated siRNAs (Fig. 3.9C), and incubated with TGFβ for 30 minutes, washed, then further incubated for either 1 or 4 hours (Fig. 3.9A). Total lysates were then subjected to immunoblotting. In control cells, I observed that the time course of Smad2 phosphorylation was highest after 30 min of incubation and decreased thereafter (Fig. 3.9) I also observed that aPKC knockdown (but not p62 or TRAF6 knockdown) attenuated the amount of Smad phosphorylation at all time points post-incubation with TGFβ (Fig. 3.9B). This supports our previous findings that show an attenuation of Smad2 nuclear translocation, as well as attenuation of the EMT cadherin switch after aPKC knockdown (Gunaratne et al., 2015). Interestingly however, since TRAF6 silencing did not affect TGFβ-dependent Smad2 phosphorylation but did inhibit TGFβ-dependent autophagy, this suggests that TRAF6 is downstream of (or parallel to) the TGFβ signaling pathway during autophagy.
Figure 3.9
Figure 3.9. aPKC knockdown attenuates Smad phosphorylation

(A) A549 NSCLC cells were transfected with the indicated siRNA and serum starved the next day. 48 hours post-transfection, cells were then incubated with 250 pM of TGFβ for 30 minutes, washed, and then further incubated for 1 or 4 h before lysis. Lysates were then subjected to SDS-PAGE and immunoblotting. aPKC knockdown attenuated Smad phosphorylation (B). Bars labelled with uppercase letters are significantly different (p<0.05) from bars labelled with the same lowercase letter. (C) Knockdown efficiency of p62, aPKC, and TRAF6 at 0 h. Images represent n=3.
Taken together, my results suggest that p62 may prime cells to undergo TGFβ-dependent EMT and efficient autophagy and future studies analyzing the mechanism(s) will be of great interest.
Chapter 4 : Discussion

4.1. Summary and general discussion

Several cancer types (including non-small cell lung cancer) are able to evade the tumor suppressive components of TGFβ while keeping its signaling intact. Thus, it is important to characterize TGFβ regulated processes within this context. The present study is the first to explore the role of the p62 protein in canonical TGFβ signaling as well as in TGFβ-dependent autophagy. Here we suggest that silencing p62 does not affect canonical TGFβ signaling through Smads, however, nuclear p62 levels appear to be responsive to TGFβ. In addition, knockdown of p62 appears to prime NSCLC cells for TGFβ-induced E-cadherin loss but also hinders the progression of TGFβ-induced autophagic processes. Furthermore, knockdown of the p62 and TGFβ receptor associated proteins aPKC and TRAF6 also hinder TGFβ-induced autophagy, as assessed by the conversion of LC3BI to LC3BII.

4.2. p62 localizes with TβRII at the late endosome

Previous studies from our lab have shown that aPKC is an important regulator of TGFβ signaling (Gunaratne et al., 2012, 2013, 2015). As a binding partner to aPKC, p62 has potential in modulating the activity of aPKC. Although previous studies suggest that p62 is not capable of altering the kinase activity of aPKC (Moscat et al., 2007), it is still capable of regulating activity via scaffolding or degradation. Since aPKC has been shown to associate with the TGFβ receptors (Gunaratne et al., 2013), I first decided to test
Figure 4.1

A) TGFβ

- aPKC
- EMT
- Autophagy

Conclusion #1
p62 attenuates EMT processes in a Smad-independent manner; aPKC promotes TGFβ-dependent EMT

Conclusion #2
p62 and its related proteins (aPKC and TRAF6) promote the induction of TGFβ-dependent autophagic processes

B) Model #1
- EMT
- Autophagy

Model #2
- EMT
- Autophagy
Figure 4.1. Thesis general summary

(A) These conclusions are based on both aspects of p62: its role in autophagy and its role in signaling. The first conclusion is that p62 is required to attenuate EMT processes in a Smad-independent manner, and the second is that p62 is promotes TGFβ-induced autophagic processes. Additional observations include the involvement of aPKC in promoting both EMT and autophagic processes, and the promotion of TGFβ-dependent autophagic LC3BI induction by TRAF6. (B) Two models describing the EMT-autophagy relationship that are supported by the data. The first model shows EMT induction leading to autophagy, and the second portrays EMT and autophagy as independent processes.
whether p62 would also co-localize to TβRII. Using stably transfected cells overexpressing HA-tagged TβRII, we observed that after 3 hours of internalization, TβRII localizes to Rab7 positive endosomes (Fig. 3.1B). Since TβRII co-localizes to p62 at the same time point (Fig. 3.1C), this suggests that TβRII and p62 are found together at the late endosome. This notion is further supported by previous studies that show co-localization between p62 and Rab7 (Sanchez et al., 1998). Further, preliminary co-immunoprecipitation studies show a direct association between p62 and TβRII (Appendix; Fig. A1). This finding implicates possible roles of p62 in the regulation of TGFβ signaling, possibly through the autophagic degradation of the type II receptor. Indeed, previous studies suggest that p62 facilitates lysosomal degradation of aPKC (Sanchez et al., 1998), so it remains plausible that the type II receptor is also anchored to lysosome-targeted endosomes via p62.

4.3. p62 and TRAF6 do not affect canonical TGFβ signaling

Due to its localization with TβRII and its potential in modulating aPKC activity, I investigated whether p62 affects canonical Smad signaling through siRNA-mediated silencing. In my studies I found that knockdown of p62 does not affect the magnitude of Smad2 phosphorylation, the nuclear translocation of Smad2, as well as the length of time Smad2 spends in the nucleus. This can be explained by temporal differences between the transduction of the canonical signaling pathway and the association of p62 with the type II receptor (as explained above). Since Smad phosphorylation maximally occurs at the early endosome (Fig. 3.3; Runyan et al., 2005) and p62 does not co-localize with the receptors until they reach the late endosome, the silencing of p62 expression may only
have effects on signaling events downstream of Smad phosphorylation. Indeed, other proteins that are known to regulate Smad phosphorylation such as SARA and endofin are found in early endosomes (Chen, 2009).

This finding also suggests that p62 is not critical for the scaffolding of aPKC to proteins relevant to the canonical signaling pathway. Although previous studies have found that p62 mediates the lysosomal degradation of aPKC (Sanchez et al., 1998), it is possible that aPKC can be degraded by other means, such as proteasomal degradation. It is also possible that p62 may regulate atypical TGFβ signaling that is relevant to EMT, such as the TRAF6 or Par6 pathways. Since aPKC has been shown to regulate both of these pathways (Gunaratne et al., 2013, 2015) and p62 binds both aPKC and TRAF6 (Puissant et al., 2012; Katsuragi et al., 2015), there is potential for p62 to be involved in non-canonical TGFβ signaling pathways.

4.4. The status of p62 as a tumor promoter vs. tumor suppressor

It is clear that p62 is involved in tumorigenesis, as several studies show that its expression is abnormal in various cancer types, including lung (Inoue et al., 2012), ovarian (Iwadate et al., 2014), breast (Yuan & Xi, 2013), and prostate (Kitamura et al., 2006). In my investigations involving the silencing of p62 expression, I have found that p62 initiates E-cadherin loss (Fig. 3.5), as well as the preliminary formation of stress fibers (Fig. 3.6), suggesting that p62 has a tumor suppressor status in A549 lung adenocarcinoma cells. This, however, appears to be independent of canonical TGFβ signaling (Fig. 3.2 & 3.4; Discussion section 4.2). The literature suggests that this
classification is heavily context-dependent, and many factors can influence how p62 acts in tumorigenesis (Linares et al., 2011; Moscat & Diaz-Meco, 2012; Puissant et al., 2012). Some of these factors include cell phenotype (i.e. epithelial vs. stromal cells), the stage of the tumor, p62 cellular localization, and possibly even the phosphorylation of p62. p62 is also involved in both tumor suppressing and tumor promoting pathways, which also indicates dependence on signaling crosstalk (reviewed in Puissant et al., 2012).

4.4.1. The effect of cell phenotype on p62 status

The first factor that may affect the tumor suppressor/promoter status of p62 is cell phenotype and signaling. In an investigation done in hepatocarcinoma cells (HCCs) by Umemura et al., 2016, p62 was identified as a tumor promoter since ectopic expression of p62 in neoplastic livers was able to initiate tumorigenesis. This effect was attributed to the activation of mTORC1 and Nrf2, which support the survival of hepatocarcinoma progenitor cells by preventing the accumulation of reactive oxygen species (ROS). In contrast, an earlier study done by Valencia et al., 2014 showed that p62 expression has tumor suppressive effects in the stromal cells that support HCC. This characterization was also attributed to p62’s regulation of mTORC1—lower p62 levels would cause less mTORC1 activation and the accumulation of ROS, which then initiates the release of IL-6, and subsequently, TGFβ. In more recent studies, Duran et al., 2016 identified p62 as a mediator of vitamin D tumor suppression which occurs through the inhibition of Smad3-DNA binding. Taken together, these studies suggest a tumor-promoting role of p62 in hepatocarcinoma cells, and a tumor-suppressing role in hepatic stromal cells. In relation to my investigations, A549 NSCLC cells appear to be more similar to the stromal
phenotype. In support of this, previous studies have identified a mutation in the A549 cell line of the Nrf2-inhibitor, Keap1 (Singh et al., 2006), rendering it dysfunctional. This then suggests that p62 would not affect the already over-active Nrf2, since p62 was shown to act by inhibiting and sequestering Keap1. It would be interesting to further investigate TGFβ ligand secretion from p62-silenced A549 cells, as well as the effects of vitamin D release as a possible mechanism to explain the loss of basal E-cadherin expression (Fig. 3.5) and preliminary stress fiber formation (Fig. 3.6).

4.4.2. Cell signaling pathways and p62 status

As previously mentioned, the attenuation of E-cadherin expression by p62 knockdown is independent of canonical TGFβ signaling (Fig. 3.2 & 3.4), and could possibly be explained by regulation of the atypical TGFβ pathways. There are, however, other signaling pathways that control EMT processes and the expression of E-cadherin, such as the Wnt pathway. p62 is capable of regulating Wnt signaling by degrading the protein Dishevelled (Dvl; Gao et al., 2010). After the activation of Wnt signaling, Dsh acts to promote the nuclear translocation of the β-catenin, which binds other transcription factors and recruits other co-factors in a complex that has been shown to up-regulate the expression of Snail and Twist (Howe et al., 2003; Derk ten et al., 2008). Thus, the silencing of p62 expression would attenuate Dvl degradation and promote downstream Wnt signaling and EMT processes. A recent study done in A549 cells shows that activation of Wnt down-regulates the expression of E-cadherin in a β-catenin-dependent manner (Song et al., 2015), therefore, regulation of Wnt signaling remains a possible explanation for my observations after p62 knockdown. Since there is also crosstalk
between the Wnt and TGFβ signaling pathways, a third possibility remains in which p62 regulates both pathways such that the end result is a down regulation of basal E-cadherin expression.

Another major tumorigenic signaling pathway in which p62 is involved is the NFκB pathway (Diaz-Meco & Moscat, 2012). Although previously described in a context related to Paget’s disease and the formation of the ternary complex with aPKC and TRAF6 (Introduction Section 1.5.3), p62 has also been shown to regulate Ras-induced NFκB signaling. In a study done by Duran et al., 2008, it was shown that p62 expression was up-regulated and required for Ras-induced NFκB signaling and subsequent tumorigenesis. Ras is also interrelated to TGFβ signaling, as well as the conduction of EMT processes and the expression of Snail (Horiguchi et al., 2009; Kim et al., 2014; Saitoh et al., 2016). Since p62 was characterized as a tumor promoter in the study done by Duran et al., this aspect of Ras cell signaling deserves further exploration in the A549 cell model to elucidate possible discrepancies in signaling. Indeed, the role of p62 in Ras signalling appears to be complex, as our microarray data (Table A1) show that silencing p62 down-regulates the expression of Ras-related proteins that both inhibit (Rab1b; Jiang et al., 2015) and promote (Rab22a; Su et al., 2016) tumorigenesis. Furthermore, crosstalk between TGFβ and Ras could also be investigated.

4.4.3. Tumor stage and p62 status

Numerous studies agree that the accumulation of p62 is conducive to tumorigenesis in preneoplastic tissues (Duran et al., 2008; Inoue et al., 2012; Iwadate et al., 2014). Previous studies have shown that silencing p62 expression in A549 cells appears to
inhibit cell proliferation and growth of the primary tumor (Nihira et al., 2014), but in my studies, I have observed that it may be conducive to migratory processes. This can be explained by the autophagy paradox. At early stages in carcinogenesis, dysfunctional autophagic processes lead to the accumulation of p62 and potential mutagens. Conversely, autophagy (and the degradation of p62) is able to sustain tumor cell survival and promote metastasis once tumors are established. Thus, it is important to consider the stage of the tumor in the deliberation of whether p62 supports or opposes cancer progression. Ellis et al., 2014 showed that localized melanoma development correlated with higher levels of p62, however, this expression significantly declined when observing metastatic stage III/IV tumors. Furthermore, patients with “low p62” tumors had higher incidences of metastatic lesions with reduced disease free survival over a period of 7 years. Therefore, although p62 accumulation can support initial tumor establishment and localized growth, it may inhibit the metastasis of more advanced cancers.

4.4.4. *Phosphorylation status and nuclear localization of p62*

Phosphorylation status is another aspect of p62 that can regulate its behaviour in tumor progression vs. suppression. p62 phosphorylation has been shown to be important in autophagy induction (Matsumoto et al., 2011) as well as p62-mediated regulation of cell signaling (Katsuragi et al., 2015). Further, phosphorylation of p62 has also been implicated in the management of appropriate mitotic transitions. Expression of unphosphorylatable p62 promoted faster proliferation and greater tumor establishment of Ras-transformed cells (Linares et al., 2011).
Phosphorylation also modulates the nucleocytoplasmic shuttling of p62, specifically at a site near its second (more downstream) nuclear localization signal (Pankiv et al., 2010). This is also meaningful in the context of tumorigenesis: several studies suggest that cytoplasmic p62 levels serves as a prognostic marker for more aggressive ovarian (Iwadate et al., 2014), oral squamous cell (Liu et al., 2014), and prostate (Kitamura et al., 2006) tumors. The function of nuclear p62 is still not well understood, however, a recent study shows that p62 regulates histone ubiquitination and response to DNA damage by directly associating with RNF168 (Wang et al., 2016). Autophagy deficiency-induced accumulation of p62 in the nucleus inhibits RNF168 and impedes its function towards DNA damage repair.

Interestingly, I observed a decrease in nuclear p62 levels in response to TGFβ ligand (Fig. 3.4), which could potentially be accredited to a change in a phosphorylation status, although I did not assess this. This result agrees with the tumorigenic profile of TGFβ signaling in cancer cells, since lower nuclear p62 correlates cancer aggressiveness and metastasis (Iwadate et al., 2014). It would be interesting to explore whether DNA damage repair by RNF168 is activated by lower nuclear p62 induced by TGFβ.

### 4.5. TRAF6 is important in TGFβ-dependent autophagy

Prolonged incubation of A549 cells induced autophagy induction (Fig. 3.7 & 3.8), as measured by the induction of LC3BII levels. Knockdown of either aPKC or TRAF6, but not p62, appeared to attenuate LC3BII induction (Fig. 3.7). A possible explanation for why p62-associated proteins (as opposed to p62) are required for TGFβ-dependent autophagy is because of functionally redundant proteins of p62, such as neighbour of
BRCA1 gene 1 (NBR1). Due to its structural similarity, NBR1 is also capable of selectively targeting ubiquitinated cargo to autophagosomes via LC3B association (Komatsu & Ichimura, 2010). Thus, even after silencing p62 expression, the induction of LC3BII by TGFβ may occur since NBR1 is still being expressed. Outside the context of autophagy, however, NBR1 lacks many functional domains that p62 uses to regulate signaling (Bitto et al., 2014).

This study is the first to identify potential involvement of aPKC and TRAF6 in autophagy, and it would be of great interest to elucidate how these proteins might regulate this process. I observed that TRAF6 levels appear to follow that of LC3BII (Fig. 3.7), indicating that it may serve as a novel marker for autophagy.

4.6. Relationship between EMT and autophagy

Due to its apparent complexity, several models have arisen to explain the relationship between EMT and autophagy. It has been proposed that EMT is inhibited by autophagy, that autophagy induces EMT, that EMT induces autophagy, and that EMT and autophagy are independent processes (reviewed in Gugnoni et al., 2016). By observing both EMT and autophagy, the present study provides temporal evidence that supports 2 models: EMT processes (i.e. the cadherin switch) induce autophagic processes (i.e. induction of LC3BII protein), and EMT processes and autophagic processes are independent of each other (Fig. 4.1B). However, it is important to note that there is much left unknown about TGFβ-induced autophagy. Nutrient-stress-induced autophagy may share a different relationship with EMT than that of TGFβ-induced autophagy, and further investigations
should be directed towards identifying differences in how autophagy is induced in these two contexts.

Additionally, the EMT-autophagy relationship may also be dependent on cell type, tumor stage, and other contextual factors.

4.7. Limitations and future directions

All of these studies were conducted in established cell lines, and this is the major limitation of my investigation. It would be interesting to repeat these experiments in an in vivo model, or perhaps using human tumor samples. Further, stable protein overexpression in mink lung cells was used to observe co-localization due to the inability to detect such low endogenous levels of TβRII in other cell lines through immunofluorescence microscopy. A caveat of overexpression studies includes the possibility of falsely positive associations, since some proteins form aggregates and inorganic structures once transfected.

In these studies, several cellular processes were determined using protein markers. Firstly, TGFβ signalling was assessed by measuring Smad2 phosphorylation. This, however, neglects other downstream TGFβ signalling events, which include the production of PAI-1 and the secretion of fibronectin. A similar limitation exists in the assessment of EMT, which was done using E-cadherin and N-cadherin protein markers. Use of additional mesenchymal protein markers, such as vimentin, would strengthen my current observations. Lastly, autophagy was assessed by observing the conversion of LC3BI to LC3BII. This is correlative with autophagosome formation, and visual methods
of assessment (such as electron microscopy and immunofluorescence microscopy) would bolster my findings.

It would be very interesting to further investigate the mechanism and consequence of TGFβ-attenuated nuclear p62 levels. Recent studies have shown that p62 associates with transcription factors SNAIL and TWIST (Qiang et al., 2014; Grassi et al., 2015), which are both regulated under TGFβ, and may provide a link between p62 silencing and EMT processes. Additional studies using nuclear export mutants or Leptomycin B could be done to shed light on the effects of nuclear p62. Since p62 was observed to co-localize with TβRII (Fig 3.1C), p62 may also potentially affect the half-life of TGFβ receptors. Furthermore, p62 may also have effects on non-canonical TGFβ pathways (and subsequent EMT), but this remains unexplored. Lastly, in order to further explore the TGFβ-dependent EMT-autophagy relationship, it would be interesting to observe the effects of autophagy inhibition on EMT. This could be done either by the use of 3-methyladenine or by the knockdown of autophagy-dependent proteins like Atg5, 7, or Beclin1.

4.8. Significance

TGFβ regulates EMT and autophagy, both of which are relevant to metastasis and tumor progression. In the present study, I have investigated both TGFβ-dependent EMT and autophagy in lung adenocarcinoma through a study of the p62 protein. This research proposes that p62 attenuates EMT processes but is necessary for autophagy induction. These effects, however, appear to be Smad-independent. Interestingly, both nuclear and total p62 protein levels are reduced in response to TGFβ ligand. Taken together, we have
found that p62 acts to suppress EMT processes in NSCLC. Although its involvement in carcinogenesis is apparent, p62 is a multifunctional protein and is also involved in other processes like autophagy. As such, the consideration of p62 as a potential therapeutic target is complex. Additional studies on the consequences TGFβ-dependent autophagy will be necessary to fully characterize the tumor promoting vs. suppressive status of p62.
References


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Horbelt D, Denkis A & Knaus P (2012). A portrait of Transforming Growth Factor β


Komatsu M & Ichimura Y (2010). Physiological significance of selective degradation of


Appendix

<table>
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<tr>
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<th>HA-TβRII</th>
<th>Flag-TβRI</th>
<th>HA-p62</th>
<th>Flag-Par6</th>
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<td>+</td>
<td>+</td>
</tr>
<tr>
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<td>+</td>
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**Figure A1**

**IP: TβRII**

**Cell Lysates**
Figure A1. TβRII associates with p62

HEK 293T cells were transfected with cDNA encoding HA-tagged TGFβ type II receptor (HA-TβRII), Flag-tagged type I TGFβ receptor (Flag-TβRI), HA-tagged p62 and/or Flag-tagged Par6 as indicated. Cells were then lysed and immunoprecipitated (IP) using anti (α)-TβRII antibodies. The immunoprecipitates were immunoblotted with anti-Flag and anti-HA antibodies to visualize immunoprecipitated Flag-tagged TβRI and Par6, HA-tagged TβRII and p62 (top panel). Cell lysates were also immunoblotted with anti-Flag and anti-HA antibodies to visualize relative protein expression. This unpublished figure was used with permission from Dr. Adrian Gunaratne.
## Table A1

<table>
<thead>
<tr>
<th>Gene Assignment</th>
<th>Gene Symbol</th>
<th>Fold Change (sip62 vs. siControl)</th>
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<tbody>
<tr>
<td>NM_001122665 // DEAD (Asp-Glu-Ala-Asp) box helicase 3, Y-linked</td>
<td>DDX3Y</td>
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<td>CLDN1</td>
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<tr>
<td>NM_006931 // solute carrier family 2 (facilitated glucose transporter), member</td>
<td>SLC2A3</td>
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<td>NM_001281435 // mitogen-activated protein kinase kinase 4</td>
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<td>NM_001311197 // relaxin 3</td>
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Table A1. Knockdown of p62 alters gene expression by microarray analysis

A549 NSCLC cells were transfected with either control siRNA or siRNA directed toward p62. Forty-eight hours post-transfection, total RNA was extracted and subjected to microarray analysis. Fold change comparisons are shown as relative differences of the sip62 condition when compared to siControl. Highlighted genes are relevant to characterize the role of p62 in carcinogenesis. Table represents n=2.
Curriculum Vitae

EVELYN NG

EDUCATION

MASTER OF SCIENCE (MSc) IN PHYSIOLOGY & PHARMACOLOGY 2015-present
University of Western Ontario, London, Ontario
- “The role of p62/SQSTM1 in TGFβ-dependent EMT and autophagy” (anticipated completion: June 2017)

BACHELOR OF MEDICAL SCIENCE (BMSc) HONORS SPECIALIZATION IN PHYSIOLOGY 2011-2015
University of Western Ontario, London, Ontario
- Graduated with distinction in academic achievement (overall average over 80% each year with no grade lower than 70% in the entire program with no failed courses)

RELATED WORK EXPERIENCE

TEACHING ASSISTANT 2015-present
University of Western Ontario, London, Ontario
- Assisting in a third year physiology lab course which emphasizes experimental design and execution
- Prepared tutorials to teach students physiological concepts behind experiments

HONORS

GRADUATE STUDENT POSTER PRESENTATIONS- 1ST PLACE 2015
University of Western Ontario, London, Ontario
- Placed first in the new student category within the Department of Physiology & Pharmacology

WESTERN GRADUATE RESEARCH SCHOLARSHIP (WGRS) 2015
University of Western Ontario, London, Ontario

GORDON J. SPYLO AWARD 2015
University of Western Ontario, London, Ontario
- Awarded to a graduating student from an Honors Physiology Program who, in the opinion of classmates and faculty, demonstrated enthusiasm and who promoted camaraderie amongst the students

DEAN'S HONOR LIST 2011-2015
WESTERN SCHOLARSHIP OF DISTINCTION 2011

PRESENTATIONS & PUBLICATIONS

A poster was presented and an abstract was published for the following events

AMERICAN ASSOCIATION OF CANCER RESEARCH (AACR) ANNUAL MEETING 2016/2017
LONDON HEALTH RESEARCH DAY 2016/2017
ANNUAL PHYS/PHARM RESEARCH DAY 2015/2016