July 2017

The Loss Of ATRX Creates Susceptibility To KRAS-Mediated Pancreatic Damage

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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

Pancreatic ductal adenocarcinoma (PDAC) is a lethal disease with a five-year survival rate of 8%. Oncogenic KRAS mutation is found in greater than 95% of PDAC cases, but additional mutations or injury are required for disease initiation and progression. Chromatin remodeling protein ATRX has been previously implicated in DNA repair, replication and maintaining genomic stability. I hypothesized that loss of ATRX could increase the susceptibility of pancreatic tissue to pancreatic injury or KRAS-mediated damage. In this study, combination of inducible ATRX loss in adult mice with recurrent pancreatic injury or oncogenic KRAS activation resulted in increased pancreatic damage, including fibrosis, inflammation and acinar-to-ductal metaplasia. Interestingly, this effect was gender-specific, causing pancreatic damage exclusively in female mice with ATRX loss/KRAS activation. This study defines a novel role for ATRX within the exocrine pancreas, and provides insight into the epigenetic factors that can influence susceptibility to pancreatic disease.

Keywords

Exocrine pancreas, acinar cell, pancreatic ductal adenocarcinoma, ATRX, chromatin remodeler, pancreatitis, acinar-to-ductal metaplasia, KRAS
Co-Authorship Statement

I completed all experimental work and performed data analysis, with the exception of Figure 3.2, in which all experiments and analysis were performed by Ryan Baker.

Charis Johnson, Kurt Berger & other members of the Pin lab assisted with mouse work and dissection of pancreatic tissue.
Acknowledgments

First, I would like to thank my supervisor, Dr. Chris Pin, for his mentorship and guidance throughout my time in the lab. Thank you for giving me a chance as an undergraduate student, and allowing me to develop and gain confidence as a graduate student. You have always encouraged me to think critically and take the lead in my own research, and I would not be where I am today without your support and guidance. I can’t thank you enough for being a great mentor.

To the Department of Physiology & Pharmacology, as well as the Children’s Health Research Institute, you have provided me with a wonderful atmosphere to learn and develop as a graduate student. I would like to thank my committee members Drs. Tom Drysdale & Nathalie Bérubé for their guidance and advice throughout my thesis.

To all past and present members of the Pin lab, you made the lab an enjoyable place to work every day. To Ryan Baker, thank you for all your help on this ATRX project, and to Kurt Berger, thank you for your support and guidance in troubleshooting any protocol. A special thanks to Charis Johnson, for training me when I first started in the Pin lab. I will always be grateful for your patience, expertise and guidance throughout this process.

To Melissa Fenech & Jelena Toma, my fellow graduate students, I’m so thankful for your friendship over the past couple years. Thank you for always being there to support me, keep me sane and make me laugh. My time in the Pin lab would not have been the same without you both.

To all my wonderful friends and my sister Sarah, thank you for being there when I needed you, and for being understanding when I was busy in the lab. Throughout this process, you were always there to keep me balanced and provide a fresh perspective, and I’m so grateful for that.

Lastly, Mom & Dad, thank you for being there for me every step of the way. Everything I have achieved would not have been possible without your support and encouragement. I’m incredibly lucky to have parents like you.
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<td>ADD</td>
<td>ATRX-DNMT3-DNMT3L</td>
</tr>
<tr>
<td>ADM</td>
<td>acinar-to-ductal metaplasia</td>
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<tr>
<td>ALT</td>
<td>alternative lengthening of telomeres</td>
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<tr>
<td>AREG</td>
<td>amphiregulin</td>
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<td>ARID1A</td>
<td>AT-rich interaction domain 1A</td>
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<td>ATP</td>
<td>adenosine trisphosphate</td>
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<td>ATRXt</td>
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<td>BMI1</td>
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<td>breast cancer type 2 susceptibility protein</td>
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<td>brahma homologue</td>
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<td>bovine serum albumin</td>
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<td>CHD</td>
<td>chromodomain, helicase, DNA binding</td>
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<td>CP</td>
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<td>C-X-C chemokine receptor 2</td>
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<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
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<td>death domain associated protein</td>
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<td>ECM</td>
<td>extra-cellular matrix</td>
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<td>epidermal growth factor</td>
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<td>ERK</td>
<td>extracellular signal-related kinase</td>
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<td>GAP</td>
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<td>Harvey rat sarcoma viral oncogene homolog</td>
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<td>IL-6</td>
<td>interleukin-6</td>
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<td>INO80</td>
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<td>IPMN</td>
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<td>ISWI</td>
<td>imitation switch</td>
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<td>MAPK</td>
<td>mitogen activated protein kinase</td>
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<td>MAPKK</td>
<td>mitogen activated protein kinase kinase</td>
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<td>MAPKKKK</td>
<td>mitogen activated protein kinase kinase kinase</td>
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<td>MCN</td>
<td>mucinous cystic neoplasm</td>
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<tr>
<td>MECP2</td>
<td>methyl-CpG binding protein</td>
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<td>MEK</td>
<td>MAPK/ERK kinase</td>
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<td>MRN</td>
<td>MRE11-RAD50-NBS1</td>
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<td>NFATc1</td>
<td>nuclear factor of activated T cells 1</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor κB</td>
</tr>
<tr>
<td>NRAS</td>
<td>neuroblastoma rat sarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>OIS</td>
<td>oncogene-induced senescence</td>
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<tr>
<td>PanIN</td>
<td>pancreatic intraepithelial neoplasia</td>
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<td>PBRM1</td>
<td>polybromo 1</td>
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<td>PDAC</td>
<td>pancreatic ductal adenocarcinoma</td>
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<td>plant homeodomain</td>
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<td>pancreatic neuroendocrine tumour</td>
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<td>PP</td>
<td>pancreatic polypeptide</td>
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<td>PRC2</td>
<td>polycomb repressive complex 2</td>
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<td>PSC</td>
<td>pancreatic stellate cell</td>
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<td>RAC</td>
<td>RAS-related C3 botulinum toxin substrate</td>
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<td>RAL</td>
<td>RAS-like proto-oncogene</td>
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<td>Acronym</td>
<td>Description</td>
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<tr>
<td>RAL-GDS</td>
<td>RAL-guanine nucleotide dissociation stimulator</td>
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<td>rat sarcoma</td>
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<td>qRT-PCR</td>
<td>quantitative reverse transcriptase polymerase chain reaction</td>
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<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>STAT3</td>
<td>signal transducer and activator of transcription 3</td>
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<tr>
<td>SWI/SNF</td>
<td>switching defective/sucrose non-fermenting</td>
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<tr>
<td>SWRI</td>
<td>SWI/SNF-related adenosine triphosphate complex</td>
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<td>TGF-β</td>
<td>transforming growth factor β</td>
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<td>TNF-α</td>
<td>tumour necrosis factor α</td>
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<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>XCI</td>
<td>X chromosome inactivation</td>
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</tbody>
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Chapter 1

1 Introduction

The pancreas is an organ of endodermal origin, which develops from the embryonic foregut (Jørgensen et al., 2007). The pancreas is divided into two functional compartments; the endocrine portion and the exocrine portion. The endocrine portion comprises approximately 1-2% of total pancreatic mass, acts to regulate glucose homeostasis and consists of cell clusters called islets of Langerhans (Murtaugh and Melton et al., 2003). There are five types of endocrine cells present with pancreatic islets - β-cells, α-cells, δ cells, PP cell and ε cells, which produce the hormones insulin, glucagon, somatostatin, pancreatic polypeptide and ghrelin respectively (Murtaugh and Melton, 2003; Gittes, 2009).

The exocrine portion of the pancreas, comprising the other 98-99% of total pancreatic mass, is responsible for the production and secretion of digestive enzymes to aid in the digestion and absorption of food (Shih et al., 2013). Cell types within the exocrine pancreas include pancreatic acinar cells, stellate cells, centroacinar cells and ductal cells (Cleveland et al., 2012). The functional unit of the exocrine pancreas are the pancreatic acinar cells, which are grouped into acini and have well-established basal-luminal polarity (Shih et al., 2013; Figure 1.1). Centroacinar cells are located in the transitional region between acinar cells and the ductal epithelium, which forms increasingly larger ductal structures before joining with the common bile duct (Cleveland et al., 2012).
Acinar cells produce and store zymogen granules containing inactive digestive enzymes. Following food ingestion, acinar cells are stimulated by cholecystokinin to exocytose their zymogen granules into adjacent pancreatic ducts. These granules move through the pancreatic ductal system and eventually drain into the duodenum of the small intestine to become active and assist in digestion (Shih et al., 2013). This thesis will focus on factors within the pancreatic acinar cell that increase susceptibility to diseases of the exocrine pancreas, including pancreatitis and pancreatic ductal adenocarcinoma (PDAC).
**Figure 1.1. Schematic of major cell types within the exocrine pancreas.** Acinar cells are organized into acini, surrounding a central duct composed of pancreatic duct cells. Pancreatic stellate cells are located in the peri-acinar space, while centroacinar cells are located in the transitional region between acinar and duct cells. Each acinar cell produces and stores zymogen granules containing inactive digestive enzymes.
1.1 Pancreatitis

Pancreatitis is an inflammatory disease predominantly thought to be caused by premature activation of digestive enzymes, particularly trypsin, within the pancreatic acinar cell leading to auto-digestion and tissue necrosis (Grady et al., 1998; Halangk et al., 2000; Murtaugh and Keefe, 2015). However, studies have suggested a more complex etiology for pancreatitis, as deletion of the trypsinogen gene in mice did not reduce pancreatitis-associated inflammation (Dawra et al., 2011), and activation of NF-κB signaling alone was shown to be sufficient to induce acute pancreatitis (Baumann et al., 2007). This suggests multiple pathways could be working in parallel to cause pancreatitis. The two main types of pancreatitis are acute and chronic pancreatitis. While most cases of acute pancreatitis are mild, there is significant morbidity and mortality associated with a subset of severe acute pancreatitis cases (Koutroumpakis et al., 2017; Hazra et al., 2014).

Approximately 70-80% of acute cases have an attributable cause, which include alcohol abuse, gall-stones, smoking, obesity and drug-hypersensitivity (Yadav et al., 2009; Badalov et al., 2007; Chen et al., 2012; Lerch and Aghdassi, 2010; Guda et al., 2011). Genetic factors play a role in pancreatitis; hereditary pancreatitis can be caused by mutations in the human cationic trypsinogen (PRSS1) and serine protease inhibitor, Kazal type 1 (SPINK1) genes (Teich et al., 2006; Witt et al., 2000), leading to premature enzyme activation within the pancreatic acinar cell. Additionally, mutations in chymotrypsin C (CTRC; Rosendahl et al., 2008) can lead to increased levels of active trypsin enzyme, while mutations in carboxypeptidase A1 (CPAI; Witt et al., 2013) are theorized to cause ER stress and are linked to pancreatitis. Lastly, mutations in the cystic
fibrosis transmembrane conductance regulator (CFTR) gene (Bishop et al., 2005; Sharer et al., 1998) can impair bicarbonate conductance and increase susceptibility to pancreatitis. Hereditary forms of pancreatitis have an earlier onset and present initially as acute episodes, before progressing to chronic pancreatitis (Howes et al., 2004; Girard et al., 1981). Further, multiple episodes of acute pancreatitis (recurrent acute pancreatitis) can be a risk factor for progression into chronic pancreatitis, especially in cases related to alcohol or smoking (Lankisch et al., 2009; Sankaran et al., 2015).

Acute episodes of pancreatitis result in reversible damage to the pancreas which may be resolved within approximately one week (Koutroumpakis et al., 2017). Conversely, chronic pancreatitis (CP) is a progressive, long-term disease which can lead to irreversible damage to the pancreas (Kloppel et al., 1993; Amman et al., 1996). CP is characterized by an extensive inflammatory response, fibrosis and eventual endocrine and exocrine insufficiency as the disease progresses (Kloppel et al., 1993; Amman et al., 1996; Malka et al., 2000). The leading cause for CP includes alcoholism (Nikkola et al., 2017; Irving et al., 2009) and biliary-related etiologies (Bertilsson et al., 2015; van Baal et al., 2012), while smoking (Andriulli et al., 2010) can also play a role. There are approximately 14,400 Canadians currently living with CP, and it is expected that more than 2,000 new cases will be diagnosed each year (Teshima et al., 2012). Patients with CP have a greatly reduced quality of life, with many patients becoming unemployed or retiring earlier than planned due to decreased physical, social and/or emotional wellbeing (Wehler et al., 2003). Diabetes mellitus is a common co-morbidity with CP; 20 years after onset approximately 46% of chronic pancreatitis patients have been diagnosed with
secondary diabetes (Type 3c diabetes mellitus) (Pan et al., 2016). Further, significant mortality is associated with CP, with one long-term study identifying 44% mortality within 10 years post-diagnosis (Yadav et al., 2011).

1.1.1 Animal models of pancreatitis

Animal models utilized to replicate acute human pancreatic injury includes hormonally-induced pancreatitis (e.g. cerulein), administration of basic amino acids (L-arginine), diet-induced pancreatitis (choline-deficient diet enriched with ethionine) and pancreatic duct ligation (Lampel et al., 1977; Tani et al., 1987; Tani et al., 1990; Gilliland et al., 1980; Mooren et al., 2003). The most commonly-used model is cerulein-induced pancreatitis (CIP), in which supraphysiological doses of cerulein, a cholecystokinin analogue, blocks enzyme secretion and leads to intracellular enzyme activation (Saluja et al., 1999). Treatment with cerulein can be administered over a short time period (4-7 injections in one day), to mimic acute pancreatitis, or extended over a few weeks to replicate the phenotype of recurrent pancreatitis (Elsasser et al., 1992). However, unlike CP in humans, mouse pancreas regenerates following recurrent pancreatitis, reversing the pancreatic damage (Elsasser et al., 1992). Long-term cerulein injections are still utilized as a model of CP, as it recapitulates characteristics of the disease including extensive fibrosis (Neuschwander-Tetri et al., 2000). This damage can be further amplified by combination of cerulein with an additional sensitizer, such as ethanol (Deng et al., 2005) or pancreatic duct ligation (Miyauchi et al., 2007).
As a result of studies using animal pancreatic injury models, the progression of pancreatitis has been characterized. Induction of pancreatic injury results in dysfunctional calcium signaling with premature zymogen activation in acinar cells and activation of inflammatory responses through release of TNF-α and the NF-κB pathway that can further amplify enzyme activation (Krüger et al., 2000; Mooren et al., 2003; Sendler et al., 2013; Dawra et al., 2011; Abdulla et al., 2011). Widespread apoptosis, necrosis and edema is observed (Dawra et al., 2011). Acinar cell damage leads to the release of chemokines and cytokines (including monocyte chemotactic protein, cytokine-induced neutrophil chemoattractant, interleukin-6 (IL-6)) which can enhance pancreatic inflammation and attract infiltrating immune cells, including neutrophils and macrophages (Brady et al., 2002; Shimada et al., 2002). Accordingly, blocking interleukin-6 (IL-6) or chemokine receptor CXCR2 attenuates inflammation in pancreatitis models (Chao et al., 2006; Steele et al., 2015).

In CP, recurrent injury and inflammation promotes fibrosis, which is mediated through activation of quiescent pancreatic stellate cells (PSCs) (Schneider et al., 2001; Mews et al., 2002). Macrophages present in CP have been shown to secrete factors that promote PSC-mediated fibrosis (Treiber et al., 2011). Further, PSCs can be activated by cytokines that are shown to be upregulated in acute pancreatitis (including TNF-α and IL-6), and TGF-β signaling can promote this process (Mews et al., 2002; van Laethem et al., 1996). Once activated, PSCs can secrete extracellular matrix components, primarily collagen, that contribute to fibrosis (Neushwander-Tetri et al., 2000).
1.1.2 Regeneration & Acinar to ductal metaplasia (ADM)

Pancreatic damage from acute pancreatitis appears to subside within 1-2 weeks following an episode of acute pancreatitis, indicating the regenerative capability of acinar tissue (Siveke et al., 2008; Fendrich et al., 2009). A major event in the pancreatic response to injury is acinar to ductal metaplasia (ADM), in which mature acinar cell markers (Mist1, Amylase) are downregulated in acinar cells, while markers typically expressed in pancreatic progenitor cells (sex-determining region Y box 9 (Sox9), pancreatic and duodenal homeobox 1 (Pdx1), hepatocyte nuclear factor 6 (Hnf6)) or duct cells (keratin 19 (Ck19)) are upregulated (Karki et al., 2015; Pinho et al., 2011; Prevot et al., 2012; Figure 1.2). This event could indicate trans-differentiation from acinar to duct cell, or rather dedifferentiation of acinar cells to a progenitor-like state. ADM can be important for regeneration, but the prolonged presence of ADM may sustain a loss of acinar identity and provide susceptibility for oncogenic transformation (Halbrook et al., 2017; Shi et al., 2013; Kopp et al., 2012). Several key factors have been identified that establish or maintain acinar cell identity, including pancreas specific transcription factor, 1a (Ptf1a), GATA-binding factor 6 (Gata6), nuclear receptor subfamily 5a2 (Nr5a2) and Mist1 (Hoang et al., 2016; Martinelli et al., 2013; von Figura et al., 2014b; Johnson et al., 2004). Accordingly, loss of Nr5a2 or Mist1 increases susceptibility to pancreatic damage and decreases the regenerative capability of tissue following injury (von Figura et al., 2014b; Kowalik et al., 2007). Factors involved in mature acinar cell organization (connexin 32 (Cx32), heat shock protein 27 (Hsp27)) can also influence response to
pancreatic injury by altering acinar cell-cell communication and the acinar cell cytoskeleton (Frossard et al., 2003; Kubisch et al., 2004).

Many signaling pathways required for ADM and subsequent pancreatic regeneration have been identified, including epidermal growth factor receptor (EGFR) and downstream KRAS activation of MEK/ERK signaling (Shi et al., 2013; Halbrook et al., 2017). Overexpression of EGF is sufficient to drive ADM in a mouse model (Means et al., 2003), and high levels of EGF ligands, including transforming growth factor α (TGF-α) and amphiregulin (AREG), or EGFR have been observed in CP (Ardito et al., 2012). Downstream of EGFR, inhibition of MEK signaling in a mouse model of CP was sufficient to block ADM (Halbrook et al., 2017), supporting the requirement for EGFR and MEK/ERK signaling in the occurrence of ADM.

Notch, Wnt and Hedgehog signaling all play a role in acinar regeneration following injury, by regulating the differentiation status of acinar cells post-injury (Morris et al., 2010a; Morris et al., 2010b; Siveke et al., 2008; Fendrich et al., 2009). Chromatin remodeling proteins such as Enhancer of Zeste 2 (EZH2; Mallen St. Clair et al., 2012) and B-cell specific Moloney murine leukemia virus insertion site 1 (BMI1; Fukuda et al., 2012) are important in pancreatic regeneration, promoting a proliferative state. Many of the pathways involved in pancreatic regeneration are also upregulated in pancreatic cancer (including Kras, Wnt and Notch signaling) (Bailey et al., 2016). Accordingly, CP is a risk factor for pancreatic cancer (Lowenfels et al., 1993; Raimondi et al., 2010). The
relative risk for the development of pancreatic cancer in chronic pancreatitis is 13.3-fold, with increased risk (69-fold) for those with hereditary pancreatitis (Raimondi et al., 2010). Identification of pathways or events that link CP to pancreatic cancer would be beneficial in limiting the progression of pancreatic disease.
Figure 1.2. Schematic of acinar-to-ductal metaplasia (ADM). In response to pancreatic injury, acinar cells can undergo a process involving upregulation of pancreatic progenitor (PDX1, SOX9, HNF6) and ductal markers (CK19) and decreased in mature acinar cell markers (MIST1, Amylase, Carboxypeptidase), progressing towards a duct cell phenotype.
1.2 Pancreatic Ductal Adenocarcinoma (PDAC)

Pancreatic cancer is a relatively rare but lethal disease, accounting for approximately 2.5% of new cancer cases in Canada last year, while causing 6% of cancer-related deaths (Canadian Cancer Statistics, 2016). The most common type of pancreatic cancer is pancreatic ductal adenocarcinoma (PDAC), which accounts for more than 85% of total pancreatic cancer cases (Ryan et al., 2014). Currently, the five-year survival rate for PDAC is approximately 8% (Pancreatic Cancer Facts, 2016) because PDAC is typically diagnosed in the late stages due to lack of early symptoms and biomarkers, and current treatments are limited. Less than 20% of cases are treated surgically, as this treatment option is typically reserved for cases that have not yet metastasized (Pancreatic Cancer Facts, 2016). Available chemotherapeutic agents include a standard first-line treatment gemcitabine (median survival 6.8 months), along with FOLFIRINOX, a combinatorial treatment which leads to slight improvement in survival (median survival 11.1 months; Conroy et al., 2011). Both treatments have limited efficacy long-term (Conroy et al., 2011). One of the difficulties in treating PDAC is the presence of a dense stroma that may support tumour progression and block effective treatments, consisting of a heterogeneous population of cells including fibroblasts, pancreatic stellate cells and inflammatory cells, and extra-cellular matrix (ECM) components (Neesse et al., 2015; Hwang et al., 2009; Mahadevan et al., 2007).
The etiology behind the majority of PDAC cases is unknown, with hereditary factors accounting for approximately 10% of PDAC cases (Ryan et al., 2014). Families with a history of pancreatic cancer are at higher risk, although the onset of familial pancreatic cancer occurs only 5 years earlier than the average pancreatic cancer age of diagnosis (Peterson et al., 2015). Genetic causes to account for increased familial risk have only been identified in a subset of cases, including hereditary syndromes such as familial breast cancer (BRCA2), Lynch syndrome and Peutz Jeghers syndrome (Klein et al., 2004; Shi et al., 2009; Peterson et al., 2015; Matsubayashi et al., 2017). Patients with mutation in the p16 gene (linked to familial atypical mole melanoma) have 17% increased risk of developing pancreatic cancer, and mutations in the palladin gene, encoding a protein involved in cytoskeletal organization, have been shown to play a role in familial pancreatic cancer (Pogue-Geile et al., 2006). Aside from genetic factors, lifestyle has been implicated in the etiology of pancreatic cancer, with proposed links to cigarette smoking, high alcohol intake and increased body mass (Bosetti et al., 2012; Wang et al., 2016; Aune et al., 2012).

1.2.1 Origin & acquired mutations in PDAC

One of the major controversies regarding pancreatic cancer is the cell of origin from which PDAC arises. Precursor lesions for PDAC include pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasms (IPMN) and mucinous cystic neoplasms (MCNs; Hruban et al., 2007). Although PDAC demonstrates ductal
characteristics, and these three precursor lesion types express ductal markers, lineage tracing experiments in mouse models strongly support acinar cells as the cell of origin for most PDAC cases (Kopp et al., 2012; Zhu et al., 2007; Ji et al., 2009; De La O et al., 2008; Morris et al., 2010b). These experiments demonstrated the capability of acinar cells to transdifferentiate into duct cells, in both mouse models and cultured human acinar cells (Strobel et al., 2007; Houbracken et al., 2011), and acinar cells can contribute to PanIN lesions (Kopp et al., 2012). PanINs are non-invasive, neoplastic precursor lesions which progress from low-grade PanIN1 to PanIN3, before reaching full-blown PDAC (Hruban et al., 2004). PanIN1 lesions can accumulate over time and can be found in non-malignant pancreata, while PanIN3 lesions are more typically associated with the occurrence of invasive PDAC (Andea et al., 2003; Hruban et al., 2004).

A series of acquired mutations occurs as PDAC develops from its precursor lesions. The earliest mutation is believed to occur in the KRAS gene, due to its presence in over 95% of PDAC cases (Morris et al., 2010b), and activating KRAS mutation are observed in over 90% of early-grade PanIN1 lesions (Kanda et al., 2012). Other common early mutations which increase in frequency throughout PDAC progression include CDKN2A (encoding p16^{INK4A}/p19^{ARF}), TP53, and SMAD4, which are linked to cell cycle regulation, DNA damage control and TGF-β signaling (Makohan-Moore et al., 2016; Waddell et al., 2015; Jones et al., 2008). However, PDAC is a genetically heterogeneous disease, and genomic sequencing has revealed additional mutations that occur at a lower frequency in subsets of PDAC (Jones et al., 2008; Bailey et al., 2016). An extensive study by Bailey et al., 2016 used these mutations to identify 10 common signaling pathways that may be
mutated in PDAC, and classified PDAC into four subtypes accordingly. These pathways included mutation in Notch signaling, SWI/SNF complexes, chromatin modifications and DNA repair. PDAC is also characterized by large variations in chromosomal structure. Waddell et al., (2015) demonstrated the presence of significant chromosomal rearrangements and alterations which could disrupt gene expression in PDAC. The wide range of acquired mutations and chromosomal instability in PDAC indicates the importance of maintaining genomic stability to prevent PDAC initiation and progression.

1.3 KRAS

1.3.1 KRAS gene & protein

It has been estimated that approximately 30% of all cancers contain an activating RAS mutation (Fernandez-Medarde et al., 2011). Within the specific RAS family, there are three different classes of GTPases: HRAS, NRAS and KRAS. The members of the RAS protein superfamily contain GTPase activity, and share a common catalytic G domain with variability in their cellular processes and function (Paduch et al., 2001). Each RAS protein (HRAS, NRAS, KRAS) is implicated in different cancer types, with KRAS mutations found most frequently in epithelial tumours including lung, colon and pancreas, as well as biliary tract, endometrial and ovarian cancers among others (Jančík et al., 2010; Sohal et al., 2016; Schubbert et al., 2007). Pancreatic adenocarcinomas have
the highest rate of oncogenic KRAS mutation, with over 95% of cases carrying mutations (Morris et al., 2010b).

The Kirsten rat sarcoma viral oncogene homolog (\textit{KRAS}) gene is located on the short arm of chromosome 12, and has two different isoforms; \textit{KRAS4A} and \textit{KRAS4B} due to alternative splicing of exon 4. \textit{KRAS4B} is more predominantly expressed in most human cells (Jančík et al., 2010), and the majority of studies involving the KRAS protein focus on the protein encoded by this isoform. The KRAS protein is a small 21kDa GTPase, activated via GTP-binding and inactive when bound to GDP (Jančík et al., 2010). The switch between active and inactive KRAS is mediated by two proteins, a guanine nucleotide exchange factor (GEF) which promotes GTP binding, and a GTPase activating protein (GAP) which promotes GTP hydrolysis (Jančík et al., 2010). Activating KRAS mutations impede the ability of GAPs to induce GTP hydrolysis, maintaining KRAS in an active state (Trahey et al., 1987). The most common amino acids for mutation include residues G12, G13 and Q61 (Buhrman et al., 2010; Scheffzek et al., 1997). Stimulation of KRAS activity by various growth factors or cytokines activates downstream signaling pathways, regulating cellular processes including cell survival, proliferation and differentiation (Crespo et al., 2000; Matallanas et al., 2006). External stimuli of KRAS activity include inflammatory signals, as well as GPCR-mediated activation, including EGFR signaling, through binding of EGFR ligands such as EGF and TGF\(\alpha\) (Daniluk et al., 2012; Harris et al., 2003; Logsdon et al., 2016).
1.3.2 Downstream pathways of KRAS

Activation of oncogenic KRAS, and subsequent downstream signaling pathways, is an important driving factor in PDAC initiation and progression (Collisson et al., 2012). The canonical downstream target of KRAS is mitogen-activated protein kinase (MAPK) signaling (Figure 1.3), in which the KRAS protein binds to serine/threonine kinase RAF (MAPKKK), inducing translocation of the protein to the plasma membrane where it is phosphorylated (Votjek et al., 1993; Marais et al., 1995). Active RAF then phosphorylates MEK (MAPKK), which can then phosphorylate and activate downstream proteins ERK1/2 (MAPK1/2; Crespo et al., 2000). Once phosphorylated, ERK proteins can translocate to the nucleus and target a variety of downstream effectors, including transcription factors such as JUN and ELK1 to regulate gene expression and cellular proliferation levels (Crespo et al., 2000; Schubbert et al., 2007).

However, KRAS signaling is diverse and context-dependent, and influences many downstream signaling pathways apart from MAPK signaling. Two major pathways activated by KRAS are the phosphatidylinositol 3-kinase (PI3K)-3-phosphoinositide-dependent protein kinase 1 (PDK1)-Akt pathway and the RalGDS/p38 MAPK pathway. The PI3K pathway is a well-characterized Ras-effector pathway known to mediate cell survival (Castellano and Downward, 2011). Activation of PI3K leads to production of the substrate phosphatidylinositol (3,4,5)-trisphosphate (PIP3), that binds and activates PDK1 and Akt/PKB and lead to a series of downstream phosphorylation events (Castellano and
Downward, 2011; Eser et al., 2013). In addition, KRAS activates exchange factors for Ral GTPases (including Ral-GDS), leading to subsequent activation of Ral and downstream phospholipase D (PLD; Lim et al., 2005). Other KRAS-mediated pathways include RAC and RHO, and PLCε pathways (Schubbert et al., 2007; Eser et al., 2014; Pylayeva-Gupta et al., 2011). Ras-regulation of Rho-GTPases has been demonstrated to influence cytoskeleton regulation (Chen et al., 2003; Sahai et al., 2001), while Ras-mediated PLCε signaling can activate protein kinase C (PKC) and regulate calcium signaling (Song et al., 2001).
Figure 1.3. MAPK signaling pathway downstream of active KRAS. Once activated (by various stimuli including EGFR signaling), the KRAS GTPase induces a series of phosphorylation events. Active KRAS phosphorylates RAF (mitogen activated protein kinase kinase kinase; MAPKKK), which phosphorylates MEK (mitogen activated protein kinase kinase; MAPKK) with subsequent phosphorylation of ERK (mitogen activated protein kinase; MAPK). Phosphorylated ERK can translocate into the nucleus and target specific transcription factors to alter gene expression and influence cellular proliferation.
1.3.3 KRAS mutations in PDAC

The MAPK pathway activated by KRAS is vital in driving oncogenic PDAC (Collisson et al., 2012; Collins et al., 2014), and studies demonstrate activation of endogenous or transgenic oncogenic KRAS (KRAS\textsuperscript{G12V} or KRAS\textsuperscript{G12D}) in early pancreas leads to the formation of PanINs and PDAC (Tuveson et al., 2004; Guerra et al., 2007; Grippo et al., 2003; Hingorani et al., 2003). Furthermore, the use of models in which KRAS activity can be turned on/off demonstrate the requirement of KRAS for both the initiation and maintenance of PDAC (Collins et al., 2012). However, adult acinar tissue is less susceptible than the developing pancreas to the induction of oncogenic KRAS (Huang et al., 2014; Ji et al., 2009; Morris et al., 2010b), and additional factors such as injury or acquired mutation are required to promote PanIN formation.

Daniluk et al., (2012) demonstrated that inflammatory stimuli in mice with oncogenic KRAS\textsuperscript{G12D} lead to PanINs and a chronic inflammatory response, as opposed to minimal effects from the same stimuli in wild-type mice. Furthermore, activation of inflammatory-associated factor NFATc1 and STAT3 co-operates with KRAS\textsuperscript{G12D} to promote pancreatic cancer (Baumgart et al., 2014). This provides evidence to suggest oncogenic KRAS makes acinar tissue more susceptible to the activation of inflammatory pathways. Accordingly, the combination of KRAS mutation with pancreatic injury greatly increases the amount of pancreatic damage. As opposed to wild-type mouse models, mice with oncogenic KRAS activation that undergo acute pancreatic injury do
not recover to the same extent, and form PanIN lesions (Collins et al., 2012; Morris et al., 2010a). Acute pancreatitis accelerated the onset of PDAC in mice with KRAS\textsuperscript{G12D} (Carriere et al., 2011), and induction of CP in KRAS\textsuperscript{G12D} mice was sufficient to promote PDAC formation (Guerra et al., 2007). These studies demonstrate a co-operative role for oncogenic KRAS activation and pancreatic injury in the occurrence of PDAC.

Oncogenic KRAS activation also promotes PDAC with loss of function in known tumour suppressor genes, including \textit{p16}\textsubscript{Ink4a}/\textit{p19}\textsubscript{Arf}, \textit{Trp53} and \textit{Dpc}/\textit{Smad4} (Bardeesy et al., 2006; Hingorani et al., 2005; Kojima et al., 2007; Izeradjene et al., 2007). Furthermore, combination of KRAS\textsuperscript{G12D} with loss of mature acinar cell factors such as \textit{Mist1}, \textit{Nr5a2} and \textit{Gata6} promotes PanIN formation (Shi et al., 2013; von Figura et al., 2014b; Martinelli et al., 2015). KRAS\textsuperscript{G12D} combined with upregulation of pathways associated with a developmental or progenitor pancreatic phenotype, including Notch and Hippo signaling, also promote PanIN formation (De La O et al., 2008; Zhang et al., 2014), suggesting maintenance of acinar cell differentiation constrains KRAS-mediated pancreatic disease. Lastly, oncogenic KRAS signaling can cooperate with the presence or loss of chromatin remodeling proteins to promote PDAC, including members of the polycomb protein family BMI1 and EZH2 (Bednar et al., 2015; Mallen-St. Clair et al., 2012), as well as SWI/SNF chromatin remodeling protein BRG1 (von Figura et al., 2014a).
1.4 Chromatin Modifications & SWI/SNF Chromatin Remodelers

1.4.1 Chromatin structure & modification

Modifications to chromatin can alter genomic stability and gene expression patterns and, thus, there is increasing evidence for a role of chromatin remodelers in PDAC suppression (Bailey et al., 2016; Wadell et al., 2015). The packaging of histones and DNA into chromatin is an essential organization of genome, which regulates various DNA processes including transcription, replication and repair. Chromatin consists of 147 DNA base pairs wrapped around an octamer of histone proteins (sets of histone variants H2A, H2B, H3 and H4) to form a nucleosome core, which are connected by sections of linker DNA (Luger et al., 1997; Richmond and Davey, 2003). Histone structure involves basic N-terminal tails that protrude from the nucleosome unit, which undergo histone modifications including acetylation, methylation and phosphorylation (Bannister and Kouzarides, 2011; Luger at el., 1997). Histone tail acetylation is almost always associated with increased chromatin accessibility and transcription, and conversely, deacetylation is considered to be a mechanism of gene silencing (Kouzarides, 2007). Methylation modifications are more specific to a particular arginine or lysine on a histone tail, can occur as mono, di or trimethylation and, depending upon location, can be activating or repressive marks (Kouzarides, 2007). For example, trimethylation of lysine 4 on histone 3 (H3K4me3) is shown to be an activating mark, while trimethylation of lysine 9 on
Histone 3 (H3K9me3) is repressive (Schuettengruber et al., 2007; Margueron et al., 2011; Zhao et al., 2007).

Histone modifications help regulate nucleosome structure and recruit chromatin remodeling enzymes dependent on the type of modification (Kouzarides, 2007). Once recruited, chromatin remodelers utilize their ATPase activity to incorporate specific histone variants, changing the accessibility and structure of the chromatin region (Bushbeck et al., 2017). Non-canonical histone variants (such as histone 3.3) are viewed as epigenetic marks that regulate genomic function, are synthesized throughout the cell cycle and are not dependent on replication for deposition (Bushbeck et al., 2017).

### 1.4.2 SWI/SNF family of chromatin remodelers

Chromatin remodelers that contain an ATPase subunit are divided into subgroups, including switching defective/sucrose non-fermenting (SWI/SNF), imitation switch (ISWI), chromodomain, helicase, DNA binding (CHD/Mi2), SWI/SNF-related adenosine triphosphate complex (SWRI) and inositol requiring 80 (INO80) ATPases, each with a distinct structure and ATPase (Wilson et al., 2011; Mohrmann et al., 2005). These groups utilize energy from ATP hydrolysis to rearrange chromatin structure by altering histone-DNA interaction and sliding nucleosomes along DNA (Mohrmann et al., 2005; Vignali et al., 2000). The first SWI/SNF complex was characterized in yeast (Stern et al., 1984;
Neigeborn and Carlson, 1984), with homologous complexes identified in *Drosophila* and mammals (Papoulos et al., 1998; Wang et al., 1996). The SWI/SNF complexes are large and consist of 9-12 subunits (Smith et al., 2003; Wang et al., 1996), and the composition of proteins can vary. In humans, there are two ATPase subunits which can be incorporated: BRM(SMARCA2) or BRG1(SMARCA4) (Bultman et al., 2000; Wang et al., 1996).

SWI/SNF complexes have many diverse regulatory roles in the genome, and mutations in SWI/SNF complexes have been found in a number of cancer types. A survey of 44 exome/genome sequencing studies identified SWI/SNF subunits mutations in 19.6% of all human tumours (Kadoch et al., 2013). *BRG1* mutations have been detected in lung cancer and multiple human cancer cell lines, including those lines derived from prostate, lung, breast and pancreas (Marquez-Vilendrer et al., 2016; Wong et al., 2000). Mutations in other subunits of SWI/SNF complexes have been correlated to cancer, including ARID1A in breast and clear cell ovarian cancers (Jones et al., 2011; Takao et al., 2017), and PBRM1, mutated in 41% of clear cell renal cancers (Varela et al., 2011). A study of esophageal squamous cell carcinomas demonstrated cases with inactivating mutation in *ARID1A, BRG1* and *ATRX* among others, suggesting SWI/SNF mutation may be an early occurrence in this cancer type (Nakazato et al., 2010). This thesis will focus specifically on SWI/SNF chromatin remodelling protein ATRX, and investigate the potential role of ATRX loss in promoting pancreatic disease.
1.4.3 \( \alpha \)-thalassemia/mental-retardation, X-linked protein (ATRX) syndrome

The ATRX syndrome, which includes the unusual co-presentation of intellectual disability and \( \alpha \)-thalassemia in male children from three unrelated families, was first defined in 1981 (Weatherall et al., 1981). Patients with ATRX syndrome present with a spectrum of clinical symptoms, including severe cognitive defects, facial abnormalities and \( \alpha \)-thalassemia (Gibbons et al., 1995). Additional symptoms may include urogenital and skeletal abnormalities, generalized hypotonia from birth, short stature and gastrointestinal issues (Gibbons et al., 2000a). As an X-linked disorder, ATRX syndrome occurs predominantly in males. Phenotypically normal carrier females who are heterozygous for ATRX mutation show skewed X chromosome inactivation (XCI) for the X-chromosome carrying the mutation (Gibbons et al., 1992). Mutations in the \( ATRY \) gene causing ATRX syndrome are typically missense and lead to hypomorphic function of the ATRX protein (Gibbons et al., 2008). It appears that complete loss of ATRX function is lethal, as mouse models carrying germ-line null \( Atrx \) mutations/deletions do not produce viable embryos (Garrick et al., 2006). It was shown that \( Atrx \) deletion in the 8-16 cell stage of the mouse embryo caused defects in the formation of extraembryonic trophoblast, causing lethality within 9 days of \( Atrx \) ablation (Garrick et al., 2006).
1.4.4 ATRX gene & protein structure

The *ATRX* gene is 36 exons long, and spans approximately 300 kb on the X-chromosome (Picketts et al., 1996). There are two alternatively spliced transcripts of approximately 10.5 kb, which are different at their 5' ends due to alternative splicing of exon 6 (Villard et al., 1997). *ATRX* is ubiquitously expressed in all tissue types, with high levels of expression in the brain (Gecz et al., 1994). The two isoforms of *ATRX* give rise to proteins of 265 kDa or 280 kDa. The ATRX protein belongs to the SNF2 subgroup of the SWI/SNF protein family (Picketts et al., 1996). It contains two functionally conserved domains; an N-terminus ADD (ATRX-DNMT3-DNMT3L) domain and a C-terminus SWI/SNF ATPase domain (Figure 1.4; Argentaro et al., 2007). The ADD domain is named based on its sequence homology with a family of *de novo* DNA methyltransferases, and is highly cysteine-rich with two different types of zinc finger motifs (Argentaro et al., 2007). The first motif is a zinc finger, and the second is an atypical plant homeodomain (PHD) zinc finger (Argentario et al., 2007; Gibbons et al., 1997). The C-terminus of the ATRX protein contains a SWI/SNF ATPase domain with helicase activity involved in chromatin remodeling (Mitson et al., 2011). The presence of mutations leading to ATRX syndrome are most often found in one of these two conserved domains, with approximately 49% of ATRX syndrome mutations in the ADD domain, and 30% in the SWI/SNF ATPase domain, indicating the functional importance of each domain (Gibbons et al., 2008). There is also a conserved, truncated isoform of the ATRX protein called ATRXt, caused by a failure to splice exon 11 of the *ATRX* gene (Garrick et al., 2004). The resultant protein lacks the C-terminal SWI/SNF domain,
suggesting it has reduced functionality compared to the full-length ATRX protein (Garrick et al., 2004).
**Figure 1.4. Schematic of ATRX protein.** The ATRX protein contains two function domains; a N-terminal ADD (ATRX-DNMT3-DNMT3L) domain and a C-terminal SWI/SNF ATPase domain. Within the ADD domain, there are two zinc finger motifs; GATA-1-like C2C2 and atypical plant homeodomain (PHD). Interaction sites between ATRX and death-domain associated protein (DAXX) or heterochromatin protein-1 (HP1) are indicated in pink.
1.4.5 Binding Partners & ATRX cellular localization

ATRX is a nuclear protein, associated with the nuclear matrix during interphase (Berube et al., 2000). Nuclear localization of ATRX can be altered with ATRX mutation, leading to a more diffuse nuclear pattern of staining (Cardoso et al., 2000). During the metaphase stage of mitosis, ATRX localizes to condensed chromosomes following a phosphorylation event in association with heterochromatin protein-1 (HP1) (Berube et al., 2000).

The ability of ATRX to bind to DNA through its N-terminal domain was demonstrated in vitro (Cardoso et al., 2000), and it can interact with and bind RNA (Sarma et al., 2014). ATRX is often localized to areas of heterochromatin and largely repetitive regions of DNA, including telomeres and centromeres (McDowell et al., 1999; Law et al., 2010; Lewis et al., 2010). The localization of ATRX to heterochromatin is believed to be mediated through its N-terminal ADD domain (McDowell et al., 1999;), as well as the interaction between ATRX and HP1. The ADD domain binds to histone 3 (H3) tails with the specific histone trimethylation mark H3K9me3, a marker of heterochromatin, especially in the absence of H3K4 methylation (Dhayalan et al., 2011). HP1 is thought to stabilize this ATRX recruitment event, as mutation in the HP1 binding site on the ATRX protein reduces its heterochromatic localization (Figure 1.4; Eustermann et al., 2011). An additional interaction occurs specifically in neurons, where methyl-CpG binding protein (MECP2) associates with ATRX to recruit it to heterochromatin (Nan et al., 2007).
Importantly, the localization of ATRX is not solely confined to heterochromatic regions; ATRX can bind to intergenic regions and gene bodies, especially those containing tandem repeat sequences (Law et al., 2010).

In addition to HP1, a major protein partner of ATRX is death domain-associated protein (DAXX). The DAXX protein was demonstrated using immunoprecipitation assays to be in complex with ATRX, and responsible for targeting ATRX to promyelocytic leukemia PML nuclear bodies (Xue et al., 2003; Tang et al., 2004). DAXX works in complex with ATRX to mediate incorporation of histone variant 3.3 (H3.3) at pericentric heterochromatin and telomeres in a replication-independent manner (Drane et al., 2010; Lewis et al., 2010). Once H3.3 is incorporated, it can be targeted for K9 trimethylation to produce a heterochromatic state and maintain stability of the telomere (Udugama et al., 2015).

1.4.6 Regulation of gene expression and DNA methylation

Loss of ATRX leads to decreased expression of the α-globin gene cluster located on chromosome 16 (Wilkie et al., 1990), suggesting it may act as a transcriptional regulator. The interaction of ATRX with the Su(var)3-9, enhancer of zeste, and trithorax (SET) domain of the EZH2 protein, part of the Polycomb Repressive Complex 2 (PRC2), provides further evidence of a regulatory role for ATRX in gene expression (Cardoso et
al., 1998). PRC2 is a histone methylase complex, associated with gene silencing and repressive chromatin (Margueron and Reinberg, 2011). ATRX can recruit PRC2 to Xist RNA during X chromosome inactivation, as well as other PRC2 targets throughout genome (Sarma et al., 2014). ATRX also associates with the inactivated X-chromosome (Baumann and De La Fuente, 2009), and is required to maintain gene silencing at interstitial heterochromatin and imprinted regions (Voon and Wong, 2015; Kernohan et al., 2010). Finally, ATRX influences DNA methylation throughout the genome, as patients with ATRX syndrome show patterns of altered DNA methylation (Gibbons et al., 2000b). DNA methylation analysis performed on blood samples of ATRX patients demonstrated differentially DNA methylated regions, including pericentromeric and telomeric regions in patients with ATRX mutation (Schenkel et al., 2017). Taken together, these studies strongly suggest a role for ATRX in altering gene expression. Further, ATRX regulates additional processes aside from gene regulation, including DNA replication, repair and cell cycle processes.

1.4.7 Cell cycle regulation

The effects of ATRX loss on mitosis in proliferating cells are evident in several different tissue types, including neuroprogenitor cells, Sertoli cells of the testes, skeletal muscle and forelimb mesenchyme (Bagheri-Fam et al., 2011; Bérubé et al., 2005; Huh et al., 2012; Ritchie et al., 2008). ATRX knockdown in HeLa cells led to prolonged prometaphase to metaphase transition, due to impaired chromosome congression to the
spindle equator (Ritchie et al., 2008). ATRX knockdown reduced cohesion between sister chromatids, and caused chromosome segregation defects during mitosis (Ritchie et al., 2008). Increased apoptosis was observed in Atrx-null mouse neuroprogenitor cells, cultured from E12.5 embryos containing a conditional neuroprogenitor-specific Atrx deletion, suggesting mitotic defects from ATRX loss may contribute to cell death in the developing forebrain (Berube et al., 2005; Ritchie et al., 2008; Seah et al., 2008). Cell cycle impairment was observed in skeletal muscle development, where ATRX loss led to delayed S phase progression and mitotic spindle defects in myoblasts (Huh et al., 2012), and in Atrx-null Sertoli cells of the testes, causing prolonged G2 to M phase transition and increased apoptosis (Bagheri-Fam et al., 2011). These studies demonstrate a role for ATRX in maintaining genomic stability in actively dividing cells. Interestingly, although Atrx deletion in the forelimb mesenchyme lead to brachydactyly and increased cell death (Soloman et al., 2013b), loss of ATRX in chondrocytes or osteoblasts had minimal effects on cartilage and skeletal development (Soloman et al., 2013a; Soloman et al., 2013b). Within the exocrine pancreas, the role of ATRX in pancreatic development has not yet been characterized. ATRX has also been implicated in regulating meiosis, as loss of ATRX led to defects in chromosome alignment along the meiotic spindle during metaphase II (De La Fuente et al., 2004). These authors proposed a role for ATRX in binding to centromeric heterochromatin, to mediate proper chromosome alignment during meiosis.
1.4.8 DNA replication & repair

The previous studies cited support a clear link between ATRX loss and replicative stress in proliferating cells, suggesting ATRX is required to maintain genomic stability during replication. More recently, studies have defined a role for ATRX in preventing replication fork stalling, which lead to collapse and double-stranded DNA breaks (DSBs) if unresolved by DNA repair responses (Branzei and Foiani, 2010; Clynes et al., 2014; Huh et al., 2016). It has been proposed that ATRX prevents the formation of DNA secondary structures during replication that lead to these stalled replication forks (Gibbons et al., 2010). ATRX binds to tandem-repeat sequences (Law et al., 2010), which are often guanine-rich (G-rich) sequences and have the propensity to form secondary DNA structures called G-quadruplexes (G4; Duquette et al., 2004). G-quadruplexes are abnormal and form potential obstacles for normal DNA processes including DNA replication and transcription (Rizzo et al., 2009; Levy et al., 2014) and stabilization of these G4 structures induces replicative stress (Rizzo et al., 2009; Rodriguez et al., 2012). Approximately 50% of ATRX binding sites indicated by ChIP were predicted to form G4 structures, and electrophoretic mobility shift assays indicated ATRX interacted with G4 DNA in vitro (Law et al., 2010). ATRX is believed to incorporate H3.3 into G-rich sequences to prevent G4 DNA formation and allow the proper passage of transcriptional machinery (Gibbons et al., 2010; Levy et al., 2014). In support of this mechanism, Atrx-null neuroprogenitor cells had increased susceptibility to treatment with a G4-stabilizing ligand (Watson et al., 2013).
In addition to the prevention of replication fork stalling, ATRX may also play a role in recruiting DNA repair complexes to resolve stalled forks. ATRX and the MRE11-RAD50-NBS1 (MRN) complex interact to facilitate replication fork restart and repair double stranded breaks (Clynes et al., 2014; Leung et al., 2013), indicating a role for ATRX in DNA repair. Loss of ATRX increases DNA damage in mouse ES cells (Clynes et al., 2014), and enhance sensitivity to a variety of DNA damaging agents (Conte et al., 2012). An apparent link exists between loss of ATRX and activation of p53, with the occurrence of p53-mediated apoptosis in $Atrx$-null neuroprogenitors and myoblasts (Conte et al., 2012; Watson et al., 2013; Huh et al., 2012).

### 1.4.9 Telomere maintenance & Alternative Lengthening of Telomeres (ALT)

In mouse embryonic stem cells, ATRX localized to telomeres with H3.3 during replication in the S-phase, and RNAi knockdown of $Atrx$ led to telomere dysfunction (Wong et al., 2010). Deletion of $Atrx$ in mouse neuroprogenitor cells lead to increased telomere fusion, and other telomeric defects including deletions and duplications (Watson et al., 2013). These studies indicate a role for ATRX-mediated telomere maintenance, which is supported by the upregulation of the alternative lengthening of telomeres (ALT) pathway that occurs with ATRX loss (Lovejoy et al., 2012; Clynes et al., 2015; Bower et al., 2012).
ALT is a mechanism through which telomeres maintain their length independent of telomerase enzyme by using homologous recombination (HR) (Bryan et al., 1997; Dunham et al., 2000). ALT is believed to be utilized in 10-15% of cancers but varies based on cancer type (Heaphy et al., 2011b). A role for ATRX in suppression of the ALT pathway has been well-established, although loss of ATRX alone is not sufficient to drive ALT (Clynes et al., 2014). A study of 22 ALT-positive cell lines demonstrated 90% were negative for ATRX (Lovejoy et al., 2012), while use of somatic cell hybrids demonstrated a correlation between ATRX loss and presence of the ALT pathway (Bower et al., 2012). Ectopic ATRX expression reversed the ALT phenotype, and ATRX-mediated suppression of the ALT pathway was dependent on DAXX (Clynes et al., 2015). These findings suggest a mechanism in which ATRX stabilized telomeric regions using H3.3 incorporation, preventing the formation of secondary DNA structures leading to double-stranded DNA breaks and subsequent HR.

1.4.10 Malignancies involving ATRX mutation

Given the previously described roles of ATRX in cell cycle processes, DNA replication and repair and telomere maintenance, it is not unexpected that ATRX mutations have been implicated in several types of cancer. One of the first reported cases of acquired, somatic ATRX mutations occurred in patients with α-thalassemia myelodysplasia syndrome (ATMDS; Gibbons et al., 2003). Additional studies identified ATRX mutations in several types of diffuse gliomas, including astrocytomas and glioblastomas (Jiao et al., 2012;
Kannan et al. 2012; Wiestler et al., 2013). Mutation frequency in adult gliomas is distributed evenly across the ATRX gene, and typically result in frameshifts or nonsense mutations (Jiao et al., 2012). These mutations often co-occur with TP53 or IDH1 mutations, and are correlated to the presence of ALT (Jiao et al., 2012; Kannan et al., 2012; Schwartzentruber et al., 2012). Assessment of ATRX in glioma can be prognostic (Pekmezci et al., 2017; Karsy et al., 2017), and ATRX loss has been indicated as a favourable prognostic factor in a subset of astrocytic tumours (Wiestler et al., 2013). In cases of paediatric glioblastoma, in which ATRX is mutated in approximately 30% of patients, mutations are typically found near the carboxy-terminal helicase domain (Schwartzentruber et al., 2012). ATRX mutations have also been implicated in paediatric osteosarcomas (Ellison et al., 2014; Lovejoy et al., 2012), and melanomas (Qadeer et al., 2014). ATRX has been suggested to act as a negative regulator of histone variant macro H2A1.2, which is linked to melanoma progression (Ratnakumar et al., 2012). In contrast to many other cancer types, ATRX is over-expressed in colorectal cancer cell lines (Athwal et al., 2015), and may cooperate with DAXX to incorporate the histone variant CENP-A into chromatin, altering the normal chromatin state.

1.4.11 ATRX mutation in pancreatic neuroendocrine tumours (PNETs)

ATRX mutations have been implicated in pancreatic neuroendocrine tumours (PNETs), a rare type of tumour with demonstrated chromosomal instability (Hu et al., 2010). Genomic analysis on 68 cases of PanNETs identified mutations for ATRX or DAXX in
43% of samples, while 44% contained mutations in the MEN1 tumour suppressor gene (Jiao et al., 2011). In the majority of ATRX-mutated cases, deletions or insertions and nonsense mutations in the ATRX gene lead to loss of the ATRX protein (Jiao et al., 2011). Mutations in ATRX and DAXX are almost always mutually exclusive, indicating they perform similar functions in a tumour-suppressive role (Jiao et al., 2011; Yachida et al., 2012). Loss of DAXX or ATRX was strongly correlated to the ALT phenotype, and the presence of chromosomal instability in PNETs (Heaphy et al., 2011a; Marinoni et al., 2014). Conflicting reports exist on the prognosis of DAXX/ATRX mutations in PNETs, and their effect on long-term survival is unclear. However, a recent study by Singhi et al (2017) demonstrated that DAXX/ATRX loss in PanNETs resulted in higher rates of metastasis and a poor survival rate. Within the exocrine pancreas, there is currently limited information regarding ATRX loss and PDAC.

1.5 Rationale, Hypothesis & Objectives

Pancreatic ductal adenocarcinoma (PDAC) is a rare but lethal disease of the exocrine pancreas, with a five-year survival rate of less than 6%. Constitutively activating KRAS mutation (KRAS\textsuperscript{G12D}) is present in greater than 95% of PDAC cases, but KRAS mutation alone is insufficient to drive PDAC progression. Additional factors, such as acquired somatic mutation or pancreatic injury, are required to promote disease progression. Previous studies have implicated a role for chromatin remodelers, including BMI1 (von Figura et al., 2014a), in constraining KRAS\textsuperscript{G12D}-mediated PDAC. However, the role of
chromatin remodeling protein ATRX has not yet been characterized in the context of the exocrine pancreas. In other tissues, ATRX is shown to play a role in many cellular processes including gene regulation, cell cycle progression, DNA replication and telomere maintenance. In the exocrine pancreas, ATRX loss could alter these processes, influencing acinar cell function or response to pancreatic insult, including pancreatitis or oncogenic KRAS activation.

**It is hypothesized that inducible deletion of Atrx will increase the susceptibility of acinar cells to pancreatic injury or KRAS\(^{G12D}\) oncogenic transformation.** Two main objectives were completed to address this hypothesis. First, mice with acinar cell-specific inducible Atrx deletion underwent recurrent pancreatic injury to determine the role of ATRX in the acinar cell response to injury. Second, acinar cell-specific Atrx deletion was combined with oncogenic KRAS\(^{G12D}\) activation in mice to determine the role of ATRX in constraining KRAS-mediated oncogenic transformation. This work defines a novel role for ATRX in disease progression in the exocrine pancreas, and provides further insight into acinar cell factors that influence susceptibility to pancreatic disease.
Chapter 2

2 Methods

2.1 Animal Care and Cre Induction

All mouse experiments were approved by the Animal Care and Use Committee at the University of Western Ontario (Protocol #2017-001). Male and female C57Bl6 mice expressing creERT from the Mist1 locus (Mist1^creERT/+; Shi et al., 2013) were crossed with mice harboring an Atrx allele with the 18th exon flanked by loxP sites (Berube et al., 2005), producing male (Mist1^creERT/+ Atrx^Δ18/y) and female (Mist1^creERT/+ Atrx^Δ18/Δ18) mice, collectively referred to as Mist1^creERT/+. Control mice were considered mice heterozygous for the creERT allele (Mist1^creERT/+) or wildtype for Mist1 (Mist1^+/+), and were collectively referred to as Mist1^creERT/+. Control mice were considered mice heterozygous for the creERT allele (Mist1^creERT/+) or wildtype for Mist1 (Mist1^+/+), and were collectively referred to as Mist1^creERT/+. Control mice were considered mice heterozygous for the creERT allele (Mist1^creERT/+) or wildtype for Mist1 (Mist1^+/+), and were collectively referred to as Mist1^creERT/+, as previous literature and extensive work in our laboratory has established no difference in phenotype between mice wild-type or heterozygous for Mist1 expression (Karki et al., 2015; unpublished data).

Mist1^creERT/+ Atrx^Δ18 mice were also crossed to mice containing an inducible form of oncogenic KRAS (loxP-STOP-loxP (LSL)-KRAS^G12D), to produce Mist1^creERT/+ LSL-KRAS^G12D Atrx^Δ18 mice (referred to as MKA). In 2-4 month old mice, tamoxifen (Sigma-Aldrich, St. Louis, MO, USA) was administered through oral gavage (2 mg/mouse), three
times over five days. Mice were monitored for a period of 60 days from first tamoxifen gavage, and body weight was measured weekly.

2.2 Cerulein Induced Pancreatitis (CIP)

To induce recurrent pancreatic injury, 60 days following tamoxifen gavage, control ($\text{Mist}^{creERT/+}$) and $\text{Mist}^{creERT/+}Atrx^{fl^{18}}$ mice (see Table 2.1 for n values) were given intraperitoneal injections of cerulein (Sigma-Aldrich, St. Louis, MO, USA, 75 $\mu$g/kg body weight), twice daily for 11 days, followed by a three-day recovery period. Saline injections were used as a control. Mice were weighed daily throughout the pancreatitis protocol. Three days following cessation of cerulein treatment, mice were sacrificed and pancreatic tissue was collected for analysis.

2.3 Serum Amylase Assay

Immediately following euthanasia, blood was collected from mice through intra-cardiac puncture and placed on ice for 30 minutes. Blood samples were centrifuged at 3,000 rpm at 4°C for 15 minutes, and supernatant (blood serum) collected. Levels of serum amylase were determined using the Phadebas amylase test (Magle Life Sciences, Cambridge, MA, USA), according to kit instructions. Briefly, serum samples were diluted in buffer (0.9% NaCl, 0.2% bovine-serum albumin [BSA], 20 mM CaCl$_2$), placed in 4 mL distilled
water and incubated at 37°C for 5 min. A Phadebas tablet was placed in each tube, incubated at 37°C for 15 min, and then 1 mL of 0.5M NaOH was added to each sample. Samples were centrifuged at 1500 rpm for 5 min, and absorbance of the supernatant was measured at 620 nm, using the Ultrospec 2100 pro UV/Visible spectrophotometer (Thermo Fisher Scientific, Fremont, CA, USA).

2.4 Histological Quantification and Analysis

Mouse pancreata (taken from the head-mid region of the pancreas) were fixed overnight in 4% formalin, washed with phosphate-buffered saline (PBS) for 24 hours, and placed in 70% ethanol prior to embedding in paraffin. Paraffin tissue sections (5 µm thickness) were stained using standard H&E, Alcian Blue or Masson’s Trichrome stain (ab150686; Abcam Inc.) protocols. The Alcian Blue kit used was specific to pH 2.5 to target all acidic mucins (Mucin Stain; ab150662; Abcam Inc.). Sections were imaged using the Aperio CS2 Digital Scanner and Aperio ImageScope software (Leica Biosystems Imaging Inc, San Diego, CA, USA). Levels of pancreatic damage in recurrent pancreatic injury and KRAS\textsuperscript{G12D} models were assessed using a grading scale based on three factors - fibrosis, inflammation and presence of acinar to ductal metaplasia. Tissue sections were scored on a scale from 0 – 4. Representative images and descriptions of each score can be found in Supplementary Figure S1; Supplemental Table S1.
Total tissue area was quantified using the Fiji software (Schindelin et al., 2012), and area of damage was quantified as a percentage of total area. Total number of pancreatic lesions (between 0-800, ranging from ADM to PanIN grade 3) were quantified and classified into the following categories; ADM, PanIN grade 1, PanIN grade 2, PanIN grade 3, based on morphological characteristics including cell shape (cuboidal or columnar), presence of mucin accumulation, nuclear atypia, pseudostratification and papillary or cribriform structure (see Supplemental Figure S5 for examples).

2.5 Immunohistochemistry (IHC) and Immunofluorescence (IF)

For immunohistochemical analysis (IHC), paraffin tissue sections (5 µm thickness) were stained using the ABC staining system (Santa Cruz Biotechnology Inc., Dallas, TX, sc-2018/2017) or the VectaStain ABC HRP kit with ImmPACT DAB Peroxidase (HRP) Substrate (Vector Laboratories, PK-4001/SK-4105, Brockville, ON, CA) according to kit instructions, counterstained with hematoxylin and coverslipped using Permount Mounting Medium (Thermo Fisher Scientific). Cleaved-caspase 3 staining was completed using the Ventana Discovery Ultra XT autostainer (Ventana Medical Systems Inc, Tuscon, AZ). Slides were imaged at 20x and 40x magnification using light microscopy (Leica DFC450 microscope camera on the Leica DM5500B microscope; Leica Microsystems Ltd., Wetzlar, Germany) with Leica LAS V4.4 software. Primary antibodies used are specific to ATRX (diluted in 1.5% mouse blocking serum in PBS,
1:100; Santa Cruz Biotechnology Inc.), carboxypeptidase (CPA; 1:1000; Abcam), SOX9 (1:500; Abcam Inc., Cambridge MA), PDX1 (1:1000; Abcam Inc.), Ki67 (1:500; Abcam Inc.) and cleaved caspase 3 (1:100; Cell Signaling Technology, Danvers, MA).

For immunofluorescence (IF), mouse pancreata were frozen in cryomatrix and sectioned into 6 µm sections. Tissue sections were kept at -20°C until processing, then warmed to room temperature and fixed in 4% formalin for 10 min, followed by 0.1% Triton in PBS for 10 min. Slides were placed in blocking solution (0.1% Triton X-100 in 5% BSA in PBS) for 30 min, and incubated in primary antibody overnight at 4°C. Following three 5 min PBS washes, slides were incubated in secondary antibody for 1 h at room temperature (RT), washed with PBS, stained with 4',6-diamidino-2-phenylindole (DAPI) and mounted with Permafluor mountant (Thermo Fisher Scientific). Slides were imaged at 40x magnification using fluorescence microscopy (Leica DFC365 FX camera on the Leica DM5500B microscope; Leica Microsystems Ltd.), and Leica LAS V4.4 software. Primary antibodies used are specific to ATRX (diluted in blocking solution, 1:100; Santa Cruz Biotechnology Inc.), MIST1 (1:500; Pin et al., 2000), β-catenin (1:500; Sigma), Insulin (1:500; Sigma), or γH2AX (1:200; Santa Cruz Biotechnology Inc.). Secondary antibodies used include anti-rabbit FITC and anti-mouse FITC (1:250; Jackson ImmunoResearch, West Grove, PA).
2.6 Protein Isolation & Western Blot Analyses

Mouse pancreata were flash frozen in liquid nitrogen immediately following dissection, then homogenized in a protein isolation buffer consisting of: 50 mM Tris pH 7.2, 5 mM MgCl₂, 1 mM CaCl₂, 10 mM DTT, 1% Nonidet P-40, DNAse (100 units/ml), RNAses (50 µg/ml), 1 mM PMSF, 30 mM NaF, 2 mM Na₃VO₄ and protease inhibitors leupeptin, aprotinin and pepstatin (5 µg/ml each). Once homogenized, samples were sonicated for 20 seconds, centrifuged at 14,000 rcf for 5 min at 4°C, and supernatant was retained. Protein concentrations were quantified using the Bradford protein assay (Bio-Rad, Hercules, CA). Isolated protein was resolved by SDS-PAGE in 10% acrylamide gels and transferred to a PVDF membrane (Bio-Rad) at 200 mA for 90 minutes. Blots were blocked with 5% non-fat dried milk (NFDM) and incubated in primary antibody overnight at 4°C in 5% NFDM (total ERK1/2 and SOX9 antibodies were incubated in 5% BSA-0.1% Tween20). Following incubation, blots were washed in PBS-0.1% Tween20, 4x for 5 min, then incubated in secondary antibody (anti-rabbit HRP, 1:10,000; Jackson Labs, Bar Harbor, ME) in 5% NFDM for 1 h at room temperature. Blots were washed 4x for 5 min in PBS-0.1% Tween20, and developed using Western Lightening chemiluminescence substrate (Perkin Elmer, Waltham, MA). Blots were visualized using the VersaDoc system with Quantity One 1-D analysis software (Bio-Rad). Primary antibodies used were specific for SOX9 (1:500; Abcam), CPA (1:1000; Abcam), amylase (1:1000; Abcam) and p44/42 MAPK (ERK1/2) (1:2500, Cell Signaling Technology).
2.7 TUNEL Assay

To assess apoptosis, 6 µm pancreatic sections were fixed in 4% formalin for 10 min at room temperature, washed with PBS for 3x for 5 min and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate in PBS, for 2 min on ice. Using the In Situ Cell Death detection kit (Roche, Laval, QC, CA), slides were labelled with the TUNEL reaction mixture for 1 h at 37°C. Following three PBS washes, slides were stained with DAPI and mounted with Permafluor mountant (Thermo Fisher Scientific). The number TUNEL-positive cells was quantified using 7 random fields of view from each mouse, and calculated as percentage of TUNEL positive cells compared to DAPI counts.

2.8 Quantitative real-time PCR

RNA was isolated from splenic pancreatic tissue using TRIZOL (Invitrogen, Carlsbad, CA, USA), and the SV Total RNA Isolation system (Promega, Madison, WI). Briefly, a small piece of pancreatic tissue (>2 cm wide) was immediately placed in 5 mL TRIZOL and homogenized for 30 sec. The solution was mixed after the addition of 1 mL chloroform, incubated at 4°C for 3 min and centrifuged (4°C) for 15 min. The supernatant was removed and washed 4x with 70% ethanol. DNase was added to the RNA for 15 min at RT, followed by DNase stop solution, 2 washes, and elution of RNA in RNase-free water. 1 µg of RNA was reverse-transcribed using ImPromII Reverse
transcriptase (Sigma). Quantitative RT-PCR (qRT-PCR) was carried out using primers specific to the *Mist1* gene:

Fwd: 5' GTGGTGCTAAAGCTACGTG 3'

Rev: 5' GACTGGGTCTGTCAGGTGT 3'

and the GoTaq PCR Mastermix system (Promega, Madison, WI, USA) using an ABI Prism 7900HT Sequence Detection System and SDS 2.2.1 software (Applied Biosystems, Foster City, CA, USA).

### 2.9 Statistical Analysis

Data was analyzed for significance using an unpaired, two-tailed T-test or two-way ANOVA with Tukey’s post-hoc test with GraphPad Prism 6 software. Values are depicted as means ± standard error of the mean (SEM). Significance is considered p<0.05.
Chapter 3

3 Results

To determine if ATRX affected the phenotype of mature acinar cells, mice with exon 18 in the Atrx gene flanked by loxP sites (Berube et al., 2005) were mated to mice expressing an inducible cre recombinase from the Mist1 locus (Shi et al., 2013). Since ATRX is an X-linked gene, male mice are referred to as Mist1<sup>creERT</sup>+/Atrx<sup>Δ18/y</sup> and female mice are Mist1<sup>creERT</sup>+/Atrx<sup>Δ18/Δ18</sup>. These mice will collectively be referred to as Mist1<sup>creERT</sup>/Atrx<sup>Δ18</sup> hereafter (Figure 3.1). The Mist1 gene (driving creERT) is expressed in serous exocrine cells of the pancreas but not duct or centroacinar cells of the pancreas (Pin et al., 2000). Tamoxifen induced cre recombination was induced in 2-4 month-old mice and ATRX accumulation assessed 7, 35 or 60 days after dosing (Figure 3.2A, B). Immunofluorescent (IF) analysis indicated 98% of the acinar cells were ATRX-negative 7 days following tamoxifen gavage, demonstrating efficient Atrx deletion. Two months post-gavage, 98% of cells remained negative for ATRX, indicating mature acinar cells can survive without ATRX expression (Figure 3.2B). IF co-staining for ATRX with insulin, a marker of pancreatic islets, demonstrated ATRX expression in islets, while surrounding acinar tissue was largely devoid of ATRX expression (Figure 3.2C). ATRX expression remained visible in other non-acinar cell types, including ductal, centroacinar and interstitial cells (Figure 3.2A, C; white arrows) based on nuclear morphology and tissue location.
Since ATRX can affect mitosis and DNA repair (Ritchie et al., 2008; Clynes et al., 2014; Conte et al., 2012; Leung et al., 2013), the levels of DNA damage and acinar cell death were examined 60 days following tamoxifen treatment. Assessment of γH2AX accumulation showed an increased presence in DNA double stranded breaks in \textit{Mist1}^{\text{creERT/+}} \textit{Atrx}^{\text{flA18}} mice, suggesting increased DNA damage with loss of ATRX (Supplemental Figure S2; Figure 3.2D). Furthermore, TUNEL analysis demonstrated increased acinar cell apoptosis (Supplemental Figure S2; Figure 3.2E), suggesting ATRX loss caused mild injury or cell stress in a subset of acini. Assessment of MIST1 expression levels, a marker of the mature acinar cell which is down-regulated during injury (Fazio et al., submitted) indicated decreased MIST1 accumulation by IF, and a trend towards a reduction in Mist1 mRNA in \textit{Mist1}^{\text{creERT/+}} \textit{Atrx}^{\text{flA18}} pancreatic tissue (Figure 3.2F). Combined these results suggest the loss of \textit{Atrx} results in a mild pancreatic injury, which may make the pancreas susceptible to additional stress.
Figure 3.1. Schematic of *Atrx* deficient mouse model. Cre-recombination events driven by expression from the *Mist1* promoter produces deletion of exon 18 of the *Atrx* gene, in combination with expression of oncogenic *KRAS*G12D in 2-4 month old mice.
Figure 3.2. Mature acinar cells do not require ATRX for survival, but ATRX loss induces low levels of pancreatic damage. (A) Immunofluorescence (IF) for ATRX expression in pancreatic tissue 7 (n=8), 35 (n=3), and 60 (n=4) days following tamoxifen gavage. White arrows indicate ATRX expression in non-acinar cell types, based on nuclei morphology. (B) Efficiency of Atrx deletion was quantified as percentage of acinar cells lacking ATRX expression. (C) IF for ATRX and insulin, a marker of pancreatic islets, demonstrates acinar-specific knockout of ATRX expression in Mist1^creERT/+ Atrx^flΔ18 mice. White arrows indicate ATRX expression in non-acinar cell types. (D) Quantification of γH2AX-positive acinar cells in control (Mist1^creERT/+ ) and Mist1^creERT/+ Atrx^flΔ18 mice shows increased presence of double-stranded DNA breaks in cells lacking ATRX (Mist1^creERT/+ , n=7; Mist1^creERT/+ Atrx^flΔ18 , n=11; * denotes p=0.004). (E) Quantification of TUNEL-positive apoptotic cells in control (Mist1^creERT/+ ) and Mist1^creERT/+ Atrx^flΔ18 mice demonstrate increased acinar cell death with loss of ATRX (Mist1^creERT/+ , n=9; Mist1^creERT/+ Atrx^flΔ18 , n=10; * denotes p=0.0006). (F) IF staining for MIST1 protein expression indicates decreased MIST1 in Mist1^creERT/+ Atrx^flΔ18 mice, as supported by qRT-PCR (Mist1^creERT/+ , n=4; Mist1^creERT/+ Atrx^flΔ18 , n=6; p=0.058 indicated by t-test). In all cases, bars represent means ± standard error.
**A**

\[ \text{Mist1}^{\text{creERT}+/+} \]

[Images showing ATRX expression levels at Day 7, Day 35, and Day 60.]

\[ \text{ATRX + DAPI} \]

\[ \text{ATRX} \]

\[ 25 \mu m \]

**B**

\[ \% \text{ ATRX Knockout} \]

\[ 0 \quad 20 \quad 40 \quad 60 \quad 80 \quad 100 \]

\[ \text{Day 7} \quad \text{Day 35} \quad \text{Day 60} \]

**C**

\[ \text{Mist1}^{\text{creERT}+/+} \]

\[ \text{Mist1}^{\text{creERT}+/+} \text{Atrx}^{\Delta18} \]

[Images showing insulin and ATRX expression.]

**D**

\[ \gamma-H2AX Positive Cells (\%) \]

\[ 0 \quad 1 \quad 2 \quad 3 \quad 4 \]

\[ \text{Mist1}^{\text{creERT}+/+} \quad \text{Atrx}^{\Delta18} \]

**E**

\[ \text{TUNEL positive cells (\%)} \]

\[ 0.0 \quad 0.2 \quad 0.4 \quad 0.6 \quad 0.8 \]

\[ \text{Mist1}^{\text{creERT}+/+} \quad \text{Atrx}^{\Delta18} \]

\[ * \]

**F**

\[ \beta-\text{Catenin + MIST1 + DAPI} \]

\[ \text{Mist1}^{\text{creERT}+/+} \text{Atrx}^{\Delta18} \]

[Images showing β-Catenin and MIST1 expression.]

**G**

\[ \text{Mist1} \text{ gene expression relative to Mrpl} \]

\[ 0.0 \quad 0.5 \quad 1.0 \quad 1.5 \]

\[ p=0.058 \]

\[ * \]
3.1 Loss of ATRX increases susceptibility to recurrent pancreatic injury

To determine if the mild injury observed in Mist1\textsuperscript{creERT/+} Atrx\textsuperscript{flΔ18} mice affects the pancreatic response to recurrent injury, Mist1\textsuperscript{creERT/+} and Mist1\textsuperscript{creERT/+} Atrx\textsuperscript{flΔ18} mice were subjected to recurrent pancreatic injury for 11 days (Figure 3.3A) and then allowed to recover for three days. Upon dissection, no gross morphological differences were observed between Mist1\textsuperscript{creERT/+} and Mist1\textsuperscript{creERT/+} Atrx\textsuperscript{flΔ18} mice (data not shown), and no significant difference in body weight was observed between groups, either prior to or throughout pancreatic injury (Figure 3.3B). Assessment of serum amylase levels, a marker of pancreatitis severity, revealed no differences between groups (Figure 3.3C). However, histological analysis showed marked differences between these two groups. Cerulein-treated Mist1\textsuperscript{creERT/+} mice show limited differences when compared to saline-treated controls, with the presence of intra-acinar edema (Figure 3.4A). This was unexpected but suggests that the cerulein dosing regime was suboptimal to produce levels of damage typically observed in recurrent pancreatic injury protocols, and resulted in minimal damage to the tissue. Conversely, cerulein-treated Mist1\textsuperscript{creERT/+} Atrx\textsuperscript{flΔ18} mice show increased inflammation, potential acinar-to-ductal metaplasia (ADM; Figure 3.4A; Table 3.1), and increased levels of fibrosis, as indicated by Trichrome stain analysis (Figure 3.4B; Table 3.1). Surprisingly, this damage appears to be more extensive in Mist1\textsuperscript{creERT/+} Atrx\textsuperscript{flΔ18} female mice (Table 3.1).
Figure 3.3. No alteration in body weight and serum amylase levels between cerulein-treated \textit{Mist1}^{creERT/+} and \textit{Mist1}^{creERT/+} \textit{Atrx}^{flΔ18} mice. (A) Experimental timeline for recurrent pancreatic injury model in 2-4 month old congenic \textit{Mist1}^{creERT/+} or \textit{Mist1}^{creERT/+} \textit{Atrx}^{flΔ18} mice. (B) Percent change in body weight of mice during prior to and during recurrent CIP (red arrow indicates cessation of treatment). (C) Serum amylase levels in mice (n=4-5 for each group) three days following cessation of recurrent CIP treatment. Bars represent mean ± standard error. No significant difference in body weight or amylase levels was observed between genotypes.
**Figure A**

A box labeled with Mist1creERT/+, Mist1creERT/-Atrx^{flΔ18} is shown with arrows indicating Tamoxifen Gavage and Cerulein Injection (2x daily, 11 days). A timeline shows Tamoxifen Gavage 60 days and Day 3 Sacrifice.

**Figure B**

A graph showing % change in body weight over weeks with data for Mist1creERT/+, Mist1creERT/-CIP, Atrx^{flΔ18} Saline, and Mist1creERT/-Saline.

**Figure C**

A bar graph showing Amylase (U/L) for Male and Female with data for Mist1creERT/-Saline, Atrx^{flΔ18} Saline, Mist1creERT/-CIP, and Atrx^{flΔ18} CIP.
Figure 3.4. Loss of ATRX increases susceptibility to recurrent pancreatic injury. (A) Representative H&E staining of saline or cerulein (CIP) treated Mist1creERT/+ and Mist1creERT/+ AtrxflΔ18 mice three days following cessation of pancreatic injury. Mist1creERT/+ CIP-treated mice show intra-acinar edema relative to saline-treated control mice. CIP-treated Mist1creERT/+ AtrxflΔ18 mice demonstrate increased pancreatic damage, fibrosis and putative ADM relative to CIP-treated Mist1creERT/+ mice (black arrows indicate the presence of inflammatory cells in CIP-treated Mist1creERT/+ AtrxflΔ18 mice). Similar analysis suggests that Mist1creERT/+ AtrxflΔ18 female mice show more extensive injury. Representative scale bar is indicated for each panel. (B) Representative Trichrome stain of Mist1creERT/+ and Mist1creERT/+ AtrxflΔ18 pancreatic tissue, three days following cessation of CIP treatment, indicating the level of fibrosis present throughout the tissue. Mist1creERT/+ AtrxflΔ18 mice exhibit increased fibrosis based on Trichrome staining (presence of blue indicates fibrotic areas, indicated by black arrow), to a greater extent in female Mist1creERT/+ AtrxflΔ18 mice.
A

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50 μm

B

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100 μm
Table 3.1. Morphometric analysis of pancreatic tissue following recurrent pancreatic damage

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(#{}) – indicates n value; see methodology for scoring. Histopathological assessment of pancreatic damage, as indicated by three factors; fibrosis, inflammation and the presence of ADM. Scores are represented on a grading scale from 0 – 4. There are increased levels of pancreatic damage in cerulein-treated Mist1creERT/+ AtrxflΔ18 mice compared to other groups.
To determine if increased damage observed in Mist1\textsuperscript{creERT/+} Atrx\textsuperscript{flΔ18} mice following injury correlates with increased levels of apoptosis, cleaved caspase-3 levels in acinar tissue were quantified using IHC (Figure 3.5). Results demonstrated no significant differences in levels of apoptosis between genotypes or genders. Similarly, assessment of proliferation in cerulein-treated Mist1\textsuperscript{creERT/+} and Mist1\textsuperscript{creERT/+} Atrx\textsuperscript{flΔ18} mice revealed no significant differences (Figure 3.6), indicating a change in apoptosis or proliferation was likely not a primary factor through which loss of ATRX altered the response to pancreatic injury (Figure 3.6).

ADM are identified by a reduction in differentiated acinar cell markers (including amylase, carboxypeptidase (CPA)), and a concomitant increase in progenitor/ductal markers (including SOX9) that are not typically expressed in acinar tissue (Karki et al., 2015; Pinho et al., 2011; Prevot et al., 2012). In wild-type pancreatic tissue, the transcription factor SOX9 is expressed only in ductal and centroacinar cells (Prevot et al., 2012). Previous studies showed SOX9 upregulation in both ADM and advanced pancreatic lesions (Kopp et al., 2012; Prevot et al., 2012). Three days following cessation of pancreatic injury, histological analysis suggested increased instances of ADM in Mist1\textsuperscript{creERT/+} Atrx\textsuperscript{flΔ18} mice relative to Mist1\textsuperscript{creERT/+} mice. To confirm the increased ADM within Mist1\textsuperscript{creERT/+} Atrx\textsuperscript{flΔ18} mice following injury, acinar tissue was examined for the presence of amylase, carboxypeptidase or SOX9. In corroboration with the histological analysis, IHC showed a consistent and significant decrease in CPA staining in female Mist1\textsuperscript{creERT/+} Atrx\textsuperscript{flΔ18} mice, in response to chronic injury (Figure 3.7A), which was not observed in Mist1\textsuperscript{creERT/+} mice or Mist1\textsuperscript{creERT/+} Atrx\textsuperscript{flΔ18} male mice. Western blot analyses
confirmed decreased CPA in Mist1creERT/+ AtrxflΔA18 cerulein-treated mice of both genders, compared to cerulein-treated control mice (Figure 3.7B). Conversely, Mist1creERT/+ cerulein-treated mice exhibited an upregulation of CPA compared to saline-treated controls. Amylase levels are also upregulated in cerulein-treated Mist1creERT/+ mice but not in cerulein-treated Mist1creERT/+ AtrxflΔA18 mice (Figure 3.7B).

IHC analysis for SOX9 showed increased nuclear staining in Mist1creERT/+ AtrxflΔA18 pancreatic tissue following CIP treatment (Figure 3.7A), although no increase in total Sox9 levels was observed by Western blot analyses (Figure 3.7B). Taken together, this data suggests loss of ATRX increases the sensitivity of acinar cells to recurrent pancreatic injury, leading to upregulation of progenitor markers, consistent with increased tissue damage and ADM.
Figure 3.5. Acinar-specific apoptosis is not significantly altered between Mist1^{creERT/+} and Mist1^{creERT/+} Atrx^{flA18} mice during recurrent CIP. (A) IHC analysis reveals variation in cleaved caspase 3 staining in Mist1^{creERT/+} (n=4 for both saline and cerulein-treated groups) and Mist1^{creERT/+} Atrx^{flA18} mice (male Mist1^{creERT/+} Atrx^{flA18} saline n=4, remaining groups n=5) of both genders (black arrows indicate acinar cells positive for caspase-3). Images demonstrate areas of high staining in cerulein-treated Mist1^{creERT/+} Atrx^{flA18} mice, although there is variability present within the tissue. (B) Quantification of percentage of acinar cells positive for cleaved caspase 3 expression. Bars represent mean ± standard error.
A

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50 μm

B

% acinar cells positive for cleaved caspase-3

- Male
- Female

Mist1creERT+/+  
AtrxflΔ18
Figure 3.6. Acinar cell proliferation is not significantly altered between Mist1\textsuperscript{creERT/+} and Mist1\textsuperscript{creERT/+} Atrx\textsuperscript{flΔ18} mice following CIP. (A) Representative IHC for Ki67 accumulation, indicating variation in the number of acinar cells undergoing proliferation in Mist1\textsuperscript{creERT/+} (n=3) and Mist1\textsuperscript{creERT/+} Atrx\textsuperscript{flΔ18} mice (n=3), following recurrent pancreatic injury. Black arrows indicate Ki67-positive acinar cells. Images demonstrate areas of high Ki67 staining present within tissue, although there is variability throughout the tissue. (B) Quantification of percentage of acinar cells positive for Ki67 expression. Bars represent mean ± standard error.
B

% acinar cells positive for Ki67

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<tr>
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Male

Female
Figure 3.7. *Mist1*<sup>creERT/+</sup> *Atrx<sup>flA18</sup> pancreatic tissue shows decreased digestive enzyme accumulation, and increased SOX9 staining. (A) IHC analysis for carboxypeptidase (CPA) and SOX9 in *Mist1*<sup>creERT/+</sup> or *Mist1*<sup>creERT/+</sup> *Atrx<sup>flA18</sup> mice, three days following cessation of recurrent CIP treatment. CPA staining was decreased in female *Mist1*<sup>creERT/+</sup> *Atrx<sup>flA18</sup> mice (acinus is indicated by a dotted line). Conversely, SOX9 staining was increased in both male *Mist1*<sup>creERT/+</sup> *Atrx<sup>flA18</sup> and female *Mist1*<sup>creERT/+</sup> *Atrx<sup>flA18</sup> tissue (black arrows). Higher magnification images show specific SOX9 staining. (B) Representative Western blot analysis for CPA and amylase indicated increased digestive enzymes specifically in *Mist1*<sup>creERT/+</sup> mice, while *Mist1*<sup>creERT/+</sup> *Atrx<sup>flA18</sup> mice demonstrated decreased enzymes. Total ERK1/2 (tERK1/2) was used as a loading control.
3.2 The loss of ATRX enhances oncogenic KRAS-induced pancreatic injury in female mice

If loss of Atrx increases the potential for ADM, this would suggest that

\( \text{Mist1}^{\text{creERT/}} \text{Atrx}^{\Delta18} \) mice would be more susceptible to oncogenic KRAS activation, as maintenance of the acinar phenotype is shown to be important in constraining KRAS-induced transformation (Shi et al., 2013; von Figura et al., 2014b; Martinelli et al., 2015). Further, chronic forms of pancreatic injury, which demonstrate sustained ADM, are a significant risk factor for the development of PDAC (Malka et al., 2002; Lowenfels et al., 1993). To assess the potential for increased damage with oncogenic KRAS,

\( \text{Mist1}^{\text{creERT/}} \text{Atrx}^{\Delta18} \) mice were mated to mice containing an inducible form of oncogenic KRAS (\( \text{LSL-KRAS}^{G12D} \)) to generate \( \text{Mist1}^{\text{creERT/}} \text{LSL-KRAS}^{G12D} \text{Atrx}^{\Delta18} \) mice (hereafter referred to as \( \text{MKA} \) mice) (Figure 3.8A). Control female mice included mice that are heterozygous (\( \text{Mist1}^{\text{creERT/}} \text{Atrx}^{\Delta18/x} \)) or wild-type (\( \text{Mist1}^{\text{creERT/}} \text{Atrx}^{x/x} \)) for the Atrx allele, and will collectively be referred to as \( \text{Mist1}^{\text{creERT/}} \) mice. Similarly, female mice with KRAS activation that were heterozygous for Atrx expression (\( \text{Mist1}^{\text{creERT/}} \text{LSL-KRAS}^{G12D} \text{Atrx}^{\Delta18/x} \)), or wild-type for Atrx expression (\( \text{Mist1}^{\text{creERT/}} \text{LSL-KRAS}^{G12D} \text{Atrx}^{x/x} \)) are collectively referred to as female \( \text{Mist1}^{\text{creERT/}} \text{LSL-KRAS}^{G12D} \) mice.

Cre-recombinase-mediated induction of \( \text{KRAS}^{G12D} \) and/or deletion of Atrx was performed in 2-4 month old congenic \( \text{Mist1}^{\text{creERT/}} \), \( \text{Mist1}^{\text{creERT/}} \text{Atrx}^{\Delta18} \), \( \text{Mist1}^{\text{creERT/}} \text{LSL-KRAS}^{G12D} \) and \( \text{MKA} \) mice, which were followed for two months prior to sacrifice (Figure
3.8A). Again, efficient Atrx deletion was confirmed using IHC (Figure 3.8B). There was no significant difference observed in body weight between groups (Figure 3.8C), and assessment of serum amylase levels revealed no differences (Figure 3.8D). Initial gross examination of the pancreas in situ demonstrated variability in the appearance of pancreatic tissue between groups, with the presence of enlarged spleens in both female Mist1creERT+/LSL-KRASG12D and MKA mice, and evidence of edema in female MKA mice (Supplemental Figure S3).
Figure 3.8. Combined Atrx deletion with oncogenic KRAS activation does not alter body weight of mice. (A) Experimental timeline for tamoxifen gavage in 2-4 month old congeneric Mist1<sup>creERT</sup>/+, Mist1<sup>creERT</sup>/+ Atrx<sup>flA18</sup>, Mist1<sup>creERT</sup>/+ LSL-KRAS<sup>G12D</sup> (denoted as LSL-KRAS<sup>G12D</sup>) and MKA mice. (B) IHC revealed ATRX expression in acinar (black arrow) and duct (red arrow) cells of Mist1<sup>creERT</sup>/+ mice. In Mist1<sup>creERT</sup>/+ Atrx<sup>flA18</sup> mice, ATRX expression was limited to islets (open arrow) and duct cells (red arrow). (C) Change in body weight (%) of mice (both genders) following Atrx deletion +/- oncogenic KRAS activation showed no significant differences (Mist1<sup>creERT</sup>/+ Atrx<sup>flA18</sup> denoted as Atrx<sup>flA18</sup>, Mist1<sup>creERT</sup>/+ LSL-KRAS<sup>G12D</sup> denoted as LSL-KRAS<sup>G12D</sup>). Points are represented as mean weight ± standard error. (D) Serum amylase levels in mice (n=3-8 in each group), two months following Atrx deletion +/- oncogenic KRAS activation. Bars represent mean ± standard error.
A

Mist1\textsuperscript{creERT/\+)
Mist1\textsuperscript{creERT/\+} Atrx\textsuperscript{flΔ18}
Mist1\textsuperscript{creERT/\+} LSL-KRAS\textsuperscript{G12D}
Mist1\textsuperscript{creERT/\+} LSL-KRAS\textsuperscript{G12D} Atrx\textsuperscript{flΔ18}

60 day Monitoring Period

Tamoxifen Gavage

B

Mist1\textsuperscript{creERT/\+}
Mist1\textsuperscript{creERT/\+} Atrx\textsuperscript{flΔ18}

C

\begin{center}
\begin{tabular}{c}
\textbf{% change in body weight} \\
\textbf{Week}
\end{tabular}
\end{center}

\begin{center}
\textbf{Amylase (U/L)}
\end{center}

D

\begin{center}
\textbf{Amylase (U/L)}
\end{center}

\begin{center}
\textbf{Male} \quad \textbf{Female}
\end{center}

\begin{center}
\textbf{Mist1\textsuperscript{creERT/\+} Atrx\textsuperscript{flΔ18}}
\textbf{LSL-KRAS\textsuperscript{G12D}}
\textbf{MK1}
\end{center}
Previous reports suggest that on its own, activation of oncogenic $\text{KRAS}^{G12D}$ in mature acinar cells is insufficient to cause widespread pancreatic damage (Morris et al., 2010b; Ji et al., 2009). Accordingly, $\text{Mist1}^{\text{creERT/+}}\text{LSL-KRAS}^{G12D}$ mice demonstrated normal pancreatic morphology, with few instances of ADM or more progressive PanIN lesions (Figure 3.9). However, $\text{MKA}$ mice demonstrated increased in levels of pancreatic damage, but exclusively in females (Figure 3.9). Female $\text{MKA}$ mice also exhibited increased levels of inflammation and fibrosis, along with disruption to the normal acinar cell organization (Figure 3.9; Table 3.2), relative to all other groups. Interestingly, male $\text{MKA}$ mice exhibited little to no pancreatic damage, even to a lesser extent than male $\text{Mist1}^{\text{creERT/+}}\text{LSL-KRAS}^{G12D}$ mice.
Figure 3.9. Female Mist1\textsuperscript{creERT\textasciitilde}+ Atrx\textsuperscript{flΔ18} mice show increased sensitivity to oncogenic KRAS activation. Representative H&E images of pancreatic tissue from male and female Mist1\textsuperscript{creERT\textasciitilde}, Mist1\textsuperscript{creERT\textasciitilde} Atrx\textsuperscript{flΔ18}, Mist1\textsuperscript{creERT\textasciitilde} LSL-KRAS\textsuperscript{G12D} and MKA mice 60 days after tamoxifen treatment. Increased tissue damage is observed in MKA females. Insets show higher magnification.
Male

Female

Mist1<sup>creERT/+</sup>

Mist1<sup>creERT/+</sup> Atrx<sup>Δ</sup>18

Mist1<sup>creERT/+</sup> LSL-KRAS<sup>G12D</sup>

Mist1<sup>creERT/+</sup> LSL-KRAS<sup>G12D</sup> Atrx<sup>Δ</sup>18
Table 3.2. Morphometric analysis of pancreatic tissue 60 days after activation of KRAS$^{G12D}$ and loss of Atrx

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<td>7±1.57</td>
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( #) – indicates n value; see methodology for scoring. Histopathological assessment of pancreatic damage, as indicated by three factors; fibrosis, inflammation and the presence of ADM. Scores are represented on a grading scale from 0 – 4. There are increased levels of pancreatic damage in female Mist1$^{creERT/ +}$ LSL-KRAS$^{G12D}$ Atrx$^{fl\Delta18}$ mice compared to other groups.
Trichrome staining for fibrosis indicated the increased fibrotic areas in female \textit{MKA} pancreatic tissue (Figure 3.10A; Supplementary Figure S3), while Alcian blue staining, which is characteristic of metaplastic lesions in this tissue, confirmed the presence of PanIN lesions (Figure 3.10A). The total number of lesions (ranging from ADM to PanIN2) was quantified within each group, and demonstrated a significant increase in the number of lesions within female \textit{MKA} mice (Figure 3.10C), although quantification of total area of damage (expressed as a percentage of total tissue area) was not significant (Figure 3.10B).

To determine the progression of PanIN formation within acinar tissue, \textit{Mist1}\textsuperscript{creERT/+ \textit{LSL-KRAS}}\textsuperscript{G12D} and \textit{MKA} mice were assessed for the presence of each lesion type (ranging from ADM to PanIN2); the percentage of pancreatic lobules containing at least one instance of each lesion type was quantified (Representative images of each in Supplemental Figure S5). \textit{Mist1}\textsuperscript{creERT/+ \textit{LSL-KRAS}}\textsuperscript{G12D} and \textit{MKA} mice both exhibited ADM, however the incidence of PanIN1 was increased 2.5-fold in female \textit{MKA} mice (Figure 3.10D). Furthermore, increased instances of PanIN2 were observed within female \textit{MKA} mice (Figure 3.10D). These results demonstrated that \textit{Atrx} loss increased sensitivity to \textit{KRAS}\textsuperscript{G12D} and promoted increased damage, characteristic of chronic pancreatitis, and PanIN lesion formation, within a relatively short two-month time period. Furthermore, it appeared that damage was exclusive to the female \textit{MKA} mice.
Figure 3.10. Increased pancreatic damage and progression in PanIN lesions were evident in female *MKA* mice. (A) Representative images of Alcian blue stain or Trichrome stain (indicator of fibrosis) confirms duct metaplasia (open arrow) and fibrosis (closed arrow) in areas of damage within female *MKA* mice. (B) Quantification of total lesion area (relative to total area of acinar tissue), and (C) total number of lesions within each group (* denotes p<0.05). Bars represent mean % of damage ± standard error. 

*Mist1*\(^{\text{creERT/+}}\) *Atrx*\(^{\text{fl}A18}\) are denoted as *Atrx*\(^{\text{fl}A18}\), and *Mist1*\(^{\text{creERT/+}}\) *LSL-KRAS*\(^{G12D}\) mice are denoted as *LSL-KRAS*\(^{G12D}\). Female *MKA* mice demonstrate increased number of lesions, as well as a trend towards increased area of pancreatic damage. (D) Average percentage of lobules containing at least one instance of ADM to PanIN 2. *Mist1*\(^{\text{creERT/+}}\) *LSL-KRAS*\(^{G12D}\) mice are denoted as *LSL-KRAS*\(^{G12D}\). Results demonstrate female *MKA* mice show increased levels of PanIN 1 and 2 lesions.
Similar to the recurrent pancreatic injury model, examination of SOX9 staining revealed no significant differences between $\textit{Mist}^1\text{CreERT}^+/+$ and $\textit{Mist}^1\text{CreERT}^+/+\text{Atrx}^{\text{fl}18}$ mice, nor increased staining in $\textit{Mist}^1\text{CreERT}^+/+\text{LSL-KRAS}^{G12D}$ and male $\textit{MKA}$ mice (Figure 3.11A). However, female $\textit{MKA}$ mice showed increased staining for SOX9 in the nucleus (Figure 3.12A, Figure 3.12A) that was widespread in both pancreatic lesions and acinar tissue adjacent to areas of damage, suggesting acinar cells with increased SOX9 may be primed to undergo the ADM process. Again, Western blot analyses revealed no differences in global SOX9 levels (Figure 3.12C). To confirm ADM, transcription factor PDX1 was assessed. PDX1 is a factor essential for pancreatic development with low levels of expression in mature acinar tissue, but upregulated in ADM and PanIN lesions (Roy et al., 2016). Increased PDX1 staining was observed in female $\textit{MKA}$ acinar tissue adjacent to areas of damage and in pancreatic lesions (Figure 3.12B, Figure 3.12B) compared to all other groups, suggesting upregulation of ADM factors occurs in acinar tissue prior to ADM.
Figure 3.11. Loss of ATRX alone does not alter SOX9 and PDX1 staining in acinar cells. IHC for SOX9 (A) and PDX1 (B) in control (\textit{Mist1}^{\textit{creERT}/+}) and mice lacking ATRX (\textit{Mist1}^{\textit{creERT}/+}\textit{Atrx}^{\textit{flΔ18}}) revealed no differences in staining in either gender.
Figure 3.12. Loss of ATRX combined with oncogenic KRAS lead to increased SOX9 and PDX1 staining in acinar tissue only in female *MKA* mice. IHC for SOX9 (A) and PDX1 (B) in *Mist1creERT2/LSL-KRASG12D* (denoted as *LSL-KRASG12D*) and *MKA* mice of both genders revealed increased acinar cell staining specifically in female *MKA* mice (black arrows), and expression in putative ADM (open arrows). (C) Representative western blot analyses for SOX9 reveals no significant differences between groups. Total ERK1/2 (tERK1/2) was used as a loading control.
3.3 Acinar cells with loss of ATRX contribute to KRAS-mediated pancreatic lesion formation

To determine if loss of ATRX alters proliferation in acinar cells, a largely post-mitotic tissue, IHC for Ki67 was performed (Figure 3.13A). Compared to mice with ATRX expression, \( \text{Mist1}^{\text{creERT/}+} \cdot \text{Atrx}^{\text{fl}18} \) mice demonstrated increased numbers of Ki67+ acinar cells, indicating an increased number of cells are undergoing active cell division (Figure 3.13). These results suggest that loss of ATRX results in increased acinar cell proliferation.

To further examine the manner in which ATRX loss may influence the development of pancreatic damage, ATRX staining was assessed within pancreatic lesions of \( \text{Mist1}^{\text{creERT/}+} \cdot \text{LSL-KRAS}^{G12D} \) and \( \text{MKA} \) mice of both genders. Lesions present within \( \text{Mist1}^{\text{creERT/}+} \cdot \text{LSL-KRAS}^{G12D} \) mice were mostly ATRX positive (Figure 3.14A, B). Female \( \text{Mist1}^{\text{creERT/}+} \cdot \text{LSL-KRAS}^{G12D} \) mice heterozygous for ATRX expression (\( \text{Mist1}^{\text{creERT/}+} \cdot \text{LSL-KRAS}^{G12D} \cdot \text{Atrx}^{\text{fl}18/x} \)) demonstrated 58±17% ATRX-positive lesions. However, lesions within male and female \( \text{MKA} \) mice exhibited 82±7% and 73±10% ATRX-negative lesions, respectively, with 17.5±7% and 27±10% ATRX-positive lesions (Figure 3.14A, B). From these results, it appears that while PanIN formation can occur independently of ATRX expression, ADM and PanINs are forming mostly from ATRX-negative cells. Interestingly, a pattern similar to the variable ATRX staining in female \( \text{MKA} \) mice is observed with Ki67 staining (Figure 3.14A).
Figure 3.13. Acinar cell proliferation is increased following the loss of ATRX. (A) Representative IHC indicated an increase in acinar-cell Ki67 staining in mice lacking ATRX, compared to control (Mist1<sup>creERT/+</sup>) mice (black arrow). (B) Quantification of Ki67+ acinar cells indicated a trend towards increased acinar cell proliferation with loss of ATRX in both genders, which did not increase in the presence of oncogenic KRAS. Mist1<sup>creERT/+</sup> Atrx<sup>flΔ18</sup> are denoted as Atrx<sup>flΔ18</sup>, and Mist1<sup>creERT/+</sup> LSL-KRAS<sup>G12D</sup> mice are denoted as LSL-KRAS<sup>G12D</sup>. 
A

Male

Female

Mist1 creERT/+

Mist1 creERT/+ Atrx flox

B

% of acinar cells positive for Ki67

Male

Female

Mist1 creERT/+ Atrx flox

LSL-KRAS G12D

MKα
Figure 3.14. Acinar cells with loss of ATRX contribute to KRAS-mediated pancreatic lesion formation. (A) IHC for Ki67 in Mist1<sup>creERT<sup>+/</sup> LSL-KRAS<sup>G12D</sup> (denoted as LSL-KRAS<sup>G12D</sup>) and MKA mice indicated positive Ki67 cells in male and female MKA mice (open arrows). ATRX expression was evident in PanINs in female Mist1<sup>creERT<sup>+/</sup> LSL-KRAS<sup>G12D</sup> mice (black arrow), while female MKA revealed both ATRX+ and ATRX- cells within the PanINs (* indicates lesion with some ATRX+ cells; ** indicates lesion with no ATRX+ cells), confirming cells with ATRX loss can contribute to ADM and PanIN lesions. (B) Quantification of lesions with ATRX+ or ATRX- cells in Mist1<sup>creERT<sup>+/</sup> Mist1<sup>creERT<sup>+/</sup> Mist1<sup>creERT<sup>+/</sup> Atrx<sup>flΔ18</sup>, Mist1<sup>creERT<sup>+/</sup> LSL-KRAS<sup>G12D</sup> and MKA mice. Bars represent mean % ± standard error.
Chapter 4

4 Discussion

4.1 Overall Findings

This work defines a novel role for chromatin remodeler ATRX within the adult exocrine pancreas, utilizing a cre-loxP mouse model to inducibly ablate ATRX expression in pancreatic acinar cells. Loss of ATRX within acinar tissue does not produce morphological changes or impact short-term survival, indicating ATRX is not required for mature acinar cell viability. However, ATRX loss caused modest increases in DNA damage, proliferation and apoptosis, indicating mild injury may be induced in acinar tissue with loss of ATRX.

When exposed to recurrent pancreatic injury, the loss of ATRX resulted in greater extent of pancreatic damage, demonstrating increased levels of fibrosis, inflammation and ADM. Furthermore, combination of ATRX loss with oncogenic KRAS activation produced extensive pancreatic damage indicative of chronic pancreatitis. Increased fibrosis and inflammation was observed, as well as the presence of PanIN lesions up to PanIN grade 2. Interestingly, this damage was exclusive to the female population of mice, suggesting a gender-specific effect of ATRX. Taken together, this work demonstrates that ATRX loss enhances the susceptibility of acinar tissue to pancreatic injury, either by
experimentally induced pancreatitis or oncogenic KRAS activation, through gender specific mechanisms. This pancreatic injury has the potential to progress into PDAC in females, and thus ATRX loss in humans may act in co-operation with KRAS to promote PDAC.

4.2 General Discussion

4.2.1 Role of ATRX in pancreatic injury

Three days following recurrent cerulein-induced pancreatitis, mice lacking ATRX had increased pancreatic damage, with a fibrotic response, inflammatory cell infiltration and instances of ADM, which are not observed in the control mice. Differences in levels of circulating serum amylase were not observed between control and Mist1<sup>creERT</sup>/+ Atrx<sup>flΔ18</sup> mice, however this is not unexpected as mice were allowed to recover for three days prior to sacrifice. There are at least two possible ways in which ATRX loss can lead to increased damage; either the loss of ATRX causes more extensive initial injury, or ATRX loss interferes with the regenerative process.

If the former option is true, this suggests that the initial damage in Mist1<sup>creERT</sup>/+ Atrx<sup>flΔ18</sup> mice requires a longer recovery time for complete regeneration to occur, explaining the difference in levels of damage between control and Mist1<sup>creERT</sup>/+ Atrx<sup>flΔ18</sup> mice, 3 days
post-injury. Previous work in our lab using an acute pancreatitis protocol indicated relatively similar amounts of damage in control and \( Mistl^{\text{creERT/}} \ Atrx^{\Delta18} \) mice, immediately following injury. However, repeated episodes of acute pancreatitis (recurrent pancreatitis) in this study could amplify the amount of initial damage observed with ATRX loss. In support of this, the downregulation of \( Mistl \) gene expression observed in mice lacking ATRX suggests an initial susceptibility to pancreatic injury, as loss of \( Mistl \) prior to injury has been shown to increase necrosis and tissue damage in acute cerulein-induced pancreatitis (Kowalik et al., 2007). To determine if ATRX loss does result in worse initial damage after recurrent injury, assessment of pancreatic tissue immediately following injury would allow for comparison between initial damage in control and \( Mistl^{\text{creERT/}} \ Atrx^{\Delta18} \) mice.

Conversely, it is possible that the loss of ATRX delays or impairs the regenerative process following injury. Previous studies have identified roles for chromatin remodeling proteins in regeneration after pancreatitis, including EZH2 and BMI1, members of the polycomb repressive complex family of proteins (Fukuda et al., 2012; Mallen-St. Clair et al., 2012). In both studies, acinar regeneration was compromised with loss of the chromatin remodeling protein, due to decreased proliferation. In this study, ATRX loss in non-injured acinar tissue increased DNA damage (indicated by positive \( \gamma \)H2AX staining) and acinar cell apoptosis in a small subset of acinar cells, potentially inducing these effects in the relatively few acinar cells that are actively undergoing cell division. This would be consistent with previous roles of ATRX in mitotically active tissue, where ATRX has been shown to maintain genomic stability and regulate cell cycle processes
(Ritchie et al., 2008; Watson et al., 2013; Clynes et al., 2014; Huh et al., 2012). It is possible that these defects become more wide-spread once injury is induced, and increased DNA damage and/or replicative defects may provide a barrier to acinar tissue regeneration. Interestingly, assessment of apoptosis and proliferation markers in cerulein-treated mice revealed variable results, suggesting that this is not the primary mechanism through which ATRX loss promotes damage.

Another possible mechanism of pancreatic damage could include increased inflammation with loss of ATRX, preventing proper regeneration from occurring. A typical inflammatory response in pancreatitis includes the infiltration of leukocytes and macrophages, causing secretion of inflammatory cytokine tumour necrosis factor \(\alpha\) (TNF\(\alpha\)) and interleukin-6 (IL-6), as well as other factors that promote pancreatic stellate cell activation and subsequent fibrosis (Sendler et al., 2013; Shimada et al., 2002; Treiber et al., 2011; Mews et al., 2002). Assessment and/or inhibition of these cytokines and their downstream pathways (NF-\(\kappa\)B, JAK/STAT signaling) in this pancreatitis model could further characterize a relationship between ATRX loss and inflammation. Future experiments could also include assessment of additional long term time points, 1-2 weeks following injury, to determine if ATRX loss has delayed or fully blocked regeneration from occurring.
4.2.2 SOX9 Regulation

Following pancreatic injury or KRAS activation, mice lacking ATRX exhibit increased instances of ADM, which contain obvious nuclear staining for SOX9, a progenitor cell marker expressed in ADM and early PanIN lesions. This is expected, as SOX9 expression as a driving factor in ADM has been well-established previously (Kopp et al., 2012; Prevot et al., 2013). Further, SOX9 staining was also observed in acinar tissue surrounding ADM, suggesting SOX9 precedes the morphological change from acinar cell to ADM. Interestingly, Western blot analyses of SOX9 did not demonstrate differential SOX9 levels, contradicting the immunohistochemical analyses of SOX9. This finding is aligned with work from our collaborating lab of Dr. Arthur Brown, in which they observed no changes in SOX9 mRNA levels, but altered IHC staining of SOX9. There are a couple potential reasons for this difference: post-translational modification of SOX9 or factors influencing antibody binding and/or specificity. Transcriptional regulation of SOX9 expression is downstream of several signaling pathways, including NF-kB (Sun et al., 2013), Wnt/β-catenin (Larsimont et al., 2015) and MAPK signaling (Ling et al., 2011). However, modifications of the SOX9 protein have also been demonstrated, including phosphorylation (Huang et al., 2000) and acetylation which prevented nuclear translocation of the SOX9 (Bar Oz et al., 2016). It is possible that post-translational modification of SOX9 alters the nuclear localization of this protein, leading to changes in staining intensity in IHC. Alternatively, these modifications or other alterations to SOX9 protein conformation could prevent antibody binding in immunohistochemistry, which would not be an obstacle in the denatured environment of Western blot analyses.
4.2.3 Role of ATRX in combination with oncogenic KRAS activation

My results indicate that Atrx deletion or oncogenic KRAS mutation alone in adult acinar tissue does not lead increased pancreatic damage. In accordance with previous literature (Grippo et al., 2003; Guerra et al., 2007), activating KRAS mutations required an additional acquired mutation, in this case ATRX loss, to promote pancreatic damage. Interestingly, this study demonstrates a gender specific pancreatic phenotype, in which ATRX loss/KRAS activation leads to extensive damage within the female cohort. Similar to the recurrent pancreatic injury model, increased infiltration of inflammatory cells and extensive fibrosis are present, consistent with a chronic pancreatitis phenotype. Furthermore, instances of ADM and more progressive PanIN lesions were present in the tissue. The presence of PanIN lesions indicates the potential for progression to PDAC, although full-blown pancreatic cancer was not observed in this study.

However, a study by Guerra et al., 2007 demonstrated that inducing KRAS activation in adult mice, when combined with chronic pancreatitis, produced invasive PDA within 5 months of oncogenic KRAS activation. The pancreatic injury observed in the female MKA mice indicates the presence of chronic pancreatitis. Due the presence of active KRAS in this mouse model, it is likely that extending the length of this experiment past two months could reveal the presence of PDAC in MKA female mice.
Studies of ATRX mutation in other cancer types (including pancreatic neuroendocrine tumours) demonstrate a strong correlation between loss of ATRX or its binding partner DAXX and upregulation of the alternative lengthening of telomeres (ALT) pathway (Lovejoy et al., 2010; Heaphy et al., 2011a). However, a study examining PDAC tumours (n=448) confirmed the absence of ALT in every case (Heaphy et al., 2011b). Further, PDAC is typically characterized by telomere attrition, with telomere shortening evident in all PanIN grades (van Heek et al., 2002). This strongly suggests that unlike other cancer types, loss of ATRX does not drive damage through an ALT phenotype. However, to rule out this possibility, assessment of telomere length in lesions of the female MKA mice could be performed.

The exact mechanism through which ATRX loss can promote activity of oncogenic KRAS is unclear. A well characterized function of ATRX is mediating deposition of histone variant H3.3, in conjunction with binding partner DAXX (Drane et al., 2010; Lewis et al., 2010). Loss of ATRX, and subsequent alteration of H3.3 deposition, could influence chromatin structure and modify gene expression, leading to activation of oncogenic KRAS and/or downstream pathways which further potentiate KRAS activity. Pathways downstream of KRAS are diverse and regulate many different cellular processes, including proliferation and cell survival, which could promote pancreatic damage (Castellano and Downward, 2011; Crespo et al., 2000). Performing similar experiments with an inducible DAXX deletion could reveal if ATRX activity in this model is DAXX-dependent. Alternatively, ATRX could be functioning in a DAXX-independent manner to influence gene expression, working in complex with other
proteins or complexes, including PRC2. Previous literature has confirmed interaction between ATRX and EZH2, a member of PRC2, leading to altered gene expression (Cardoso et al., 1998; Sarma et al., 2014). Whole genome analysis of mRNA expression, using RNA-sequencing of pancreatic tissue in mice, at a time point prior to the development of damage, could demonstrate changes in gene expression and help to pinpoint specific genes of interest.

### 4.2.4 Gender Specificity of Pancreatic Damage in MKA mice

The incidence of human PDAC between genders is relatively equal, with approximately the same number of cases occurring in males and females (Canadian Cancer Statistics, 2016). However, it is possible that ATRX loss defines a unique subtype of PDAC, in which the female population is more susceptible. The gender-specific mechanism producing extensive pancreatic damage specifically in female MKA mice remains unclear. In this study, female mice heterozygous (\(Atrx^{flΔ18/x}\)) or homozygous (\(Atrx^{flΔ18/flΔ18}\)) for the mutant \(Atrx\) allele showed different results when combined with KRAS activation. In mice that are heterozygous for the mutant \(Atrx\) allele (\(Atrx^{flΔ18/x}\)), it is expected that approximately half of acinar cells will lose ATRX expression completely due to X-inactivation of the wild-type \(Atrx\) allele. However, these mice did not demonstrate increased damage with KRAS mutation, and were subsequently grouped together with mice containing two wild-type \(Atrx\) alleles (\(Atrx^{x/x}\)) during analysis. These results suggest that complete loss of ATRX in acinar tissue is required to produce
pancreatic damage, possibly due to skewed X-inactivation of the chromosome containing the mutant Atrx allele. Interestingly, the male MKA mice appear to have the opposite effect from their female counterparts, showing a protective effect and decreased damage with ATRX loss/KRAS activation.

There are three potential mechanisms through which MKA mice may have gender-specific differences in damage. First, hormonal factors could be considered to play a role. Sex hormone receptors, including estrogen receptors (ER) have been known to play a role in the progression of other cancers such as breast and colon (Nussler et al., 2008; Perou et al., 2000). Further, specific rare types of pancreatic neoplasms, including papillary and mucinous cystic neoplasms, have a higher incidence in females and have been linked to ER upregulation (Kosumi et al., 2015; Satake et al., 2006). Estrogen receptors (specifically ERα) can be activated by MAPK signaling, and can mediate regulation of cellular growth and survival (Kato et al., 1995; Thomas and Gustafsson, 2011). It is possible that increased KRAS activity, due to ATRX loss, in female MKA mice could induce ER upregulation and growth factor signaling in acinar tissue. The level of expression of estrogen receptors, or other sex hormone receptors (progesterone) within pancreatic tissue of MKA mice could be assessed using immunohistochemical staining to test this theory.

Second, it is possible that aberrant gene expression from the silenced X-chromosome may occur with loss of ATRX. ATRX co-operates with polycomb repressive complex 2
(PRC2) to mediate gene silencing, including inactivation of the X chromosome (XCI) (Cardoso et al., 1998; Sarma et al., 2014). Loss of XCI has been demonstrated in other cancer types including ovarian and breast cancer (Chaligne et al., 2015), and genes located on the X chromosome have been implicated in cancer, including cancer/testis antigen and the \( Wtx \) gene (Kang et al., 2015; Rivera et al., 2007). It is possible that reactivation of the X-chromosome with loss of ATRX could greatly increase the expression of X-linked genes, an event which would be specific to female cohort of \( MKA \) mice. To confirm this theory, RNA-sequencing of acinar tissue would allow for complete assessment of changes in X-linked gene expression.

Finally, gender-specific mechanisms could be explained by a difference in inflammatory response between male and female mice. It is possible that female mice with ATRX loss are more susceptible to factors promoting inflammation, such as pancreatic injury. In support of this theory, female \( Mist1^{creERT^+/} \ Atrx^{flA18} \) mice that underwent recurrent pancreatic injury showed increased inflammation in comparison to their male counterparts, resulting in higher levels of damage. Accordingly, increased inflammation in female MKA mice could lead to increased damage, promoting widespread lesion formation. High levels of KRAS activity can induce senescence as a tumour suppressive mechanism, termed oncogene-induced senescence (OIS) (Collado et al., 2005; Serrano et al., 1997). Interestingly, Guerra et al., 2011 demonstrated that the presence of pancreatic inflammation could overcome this senescent mechanism, contributing to the progression of PDAC. In this model, an increased inflammatory response in female \( MKA \) mice could amplify oncogenic KRAS and activation of downstream pathways, including MAPK and
PI3K-PDK1-Akt signaling, leading to increased cell survival and proliferation.

Accordingly, increased $KRAS^{G12D}$ activity by inflammatory cytokines, including NF-κB and IL-6, has been demonstrated previously (Daniluk et al., 2012; Baumgart et al., 2014; Lesina et al., 2011). The presence of an inflammatory response in the female $MKA$ mice, which is not observed in male $MKA$ mice, could overcome OIS specifically within the female cohort of mice, leading to increased damage. Assessment of senescent markers within these mice could confirm this theory.

In summary, based on the results shown in this study, it is clear that ATRX loss can enhance pancreatic injury or susceptibility to KRAS-mediated pancreatic damage. Oncogenic $KRAS^{G12D}$ mutation places the KRAS protein in a primed state, leading to high levels of KRAS activity upon stimulation. It is proposed that ATRX loss is able to promote high levels of KRAS activity, which may be attenuated in male $MKA$ mice through oncogene-induced senescence (OIS) leading to decreased damage (Figure 4.1). Potential gender specific factors within female $MKA$ mice (including hormonal factors, X chromosome re-activation, or increased inflammation) may provide an additional driving factor for KRAS activity and pancreatic damage, leading to a female-specific phenotype.
Figure 4.1. Proposed model for the loss of ATRX contributing to KRAS$^{G12D}$-mediated PanIN formation. In this model, KRAS$^{G12D}$ makes acinar cells susceptible to further stimulation (including injury or further mutation). Loss of ATRX is sufficient to stimulate high levels of KRAS activity, triggering oncogene-induced senescence (OIS) in male mice and protecting acinar tissue from oncogenic transformation. In female mice, additional gender-specific factors (possibly inflammatory or hormonal differences) allow acinar cells to overcome OIS, leading to acinar-to-ductal metaplasia (ADM) and PanIN formation.
4.3 Limitations & Future Directions

This study defines a novel role for ATRX within the pancreas. However, there are limitations to the experimental design which must be considered. The Mist1 gene, used to drive cre-mediated recombination in this model, is specific to serous exocrine cells, and thus provided an acinar-specific deletion of Atrx/activation of KRAS within the pancreas. However, since MIST1 is expressed in other tissue types (including stomach and salivary gland), Atrx deletion and expression of oncogenic KRAS also occurred in these tissues. Therefore, there were non-pancreatic effects including the formation of tumours in the stomach and mouth of these mice. This effectively limited the timeframe of this study, and prevented the observation of pancreatic phenotype at potential later time points, where PDAC is most likely to develop. Use of a different acinar-specific cre-driver (e.g. Ptf1a) in future experiments would allow for longer-term studies. Furthermore, the dose of pancreatitis used in the recurrent pancreatitis experiments was suboptimal (75 µg/ml), compared to other studies which used similar cerulein administration protocols (Halbrook et al., 2017) and showed more extensive damage in their mice. Use of a higher cerulein dose in this experiment may have revealed greater differences between control mice and mice lacking ATRX.

Moving forward, studies to further investigate the cooperation between ATRX loss and KRAS activation will help to elucidate the exact mechanism through which this phenotype occurs. Both cell-autonomous (dependent on ATRX loss within the cell) and
non-cell autonomous (e.g. inflammation) mechanisms should be considered. Using cell culture techniques, acinar cells from male and female mice lacking ATRX could be isolated and plated in collagen matrix. The propensity of these cells to undergo conversion to a duct-like state in culture can be assessed, to determine if there is an inherent susceptibility to ADM in cells lacking ATRX (cell-autonomous event). Analysis of gene expression using RNA-sequencing could identify the influence of ATRX loss on gene expression, and potential pathways through which it may amplify KRAS activity. To investigate possible non-cell autonomous factors, including inflammation, anti-inflammatory treatments could be administered in these models of pancreatic injury and KRAS-induced pancreatic damage. This would indicate the potential importance of inflammation in mediating the phenotypes observed in this study.
Chapter 5

5 References


Chapter 6

6 Appendices

Supplemental Table S1. Histology grading criteria

<table>
<thead>
<tr>
<th>Acinar-to-ductal metaplasia (ADM)</th>
<th>Fibrosis</th>
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<tbody>
<tr>
<td><em>(based on worst pancreatic lobule)</em></td>
<td><em>(based on Trichrome Stain)</em></td>
</tr>
<tr>
<td>0 None present</td>
<td>0 None present</td>
</tr>
<tr>
<td>1 &gt;10% of lobule</td>
<td>1 &lt;5% of tissue area</td>
</tr>
<tr>
<td>2 10-30% of lobule</td>
<td>2 5-10% of tissue area</td>
</tr>
<tr>
<td>3 30-50% of lobule</td>
<td>3 10-20% of tissue area</td>
</tr>
<tr>
<td>4 &gt;50% of lobule</td>
<td>4 &gt;20% of tissue area</td>
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</tbody>
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**Inflammation**

| 0 None present                                      |
| 1 Focal – small, contained areas                    |
| 2 Mild – small, slightly diffuse areas              |
| 3 Moderate – diffuse areas                          |
| 4 Severe – diffuse areas, significant presence throughout the tissue |
Supplemental Figure S1. Representative images of histological grading scale.

Representative histological images for each possible score (0-4) on the histological grading scale utilized for recurrent pancreatitis and oncogenic KRAS models (see Table 3.1, 3.2). Three factors were assessed; inflammation, fibrosis (based on Trichrome stain), and acinar-to-ductal metaplasia (ADM).
Supplemental Figure S2. Increased γH2AX and TUNEL staining, 60 days following loss of ATRX. Representative immunofluorescence indicates an increased number of acinar cells positive for γH2AX and TUNEL staining (white arrows) in Mist1<sup>creERT</sup>+/Atrx<sup>flΔ18</sup>, compared to control mice.
Supplemental Figure S3. Minor morphological differences visible in pancreatic tissue, 60 days following $Atrx$ deletion and/or oncogenic KRAS activation. (A) Representative images of pancreas *in situ* in control, $Mist1^{creERT/+} Atrx^{flΔ18}$, $Mist1^{creERT/+} LSL$-$KRAS^{G12D}$ and $MKA$ mice (outlined by the dotted line). Edema is visible in female $MKA$ mice, as well as an enlarged spleen (indicated by white arrow).
Supplemental Figure S4. Increased fibrosis in MKA mice compared to Mist1creERT/+. A) Masson’s Trichrome stain in control and Mist1creERT/+, AtrxβΔ18 saline or cerulein-treated mice, three days following cessation of CIP treatment. (B) Trichrome stain in control, Mist1creERT/+, AtrxβΔ18, Mist1creERT/+ LSL-KRASG12D mice, 60 days following tamoxifen gavage (blue indicates fibrosis, indicated by black arrow).
Supplemental Figure S5. PanIN lesions progress to PanIN2 in MKA female mice.
Representative H&E images of each grade of pancreatic lesion, ranging from ADM – PanIN 2, evident in the acinar tissue of MKA female mice (black arrows).
# Curriculum Vitae

**Name:** Claire Young

**Post-secondary Education and Degrees:**

<table>
<thead>
<tr>
<th>Year</th>
<th>Degree</th>
<th>Institution</th>
<th>Specialization/Award</th>
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</thead>
</table>

**Honours and Awards:**

- Paediatric Summer Studentship 2014
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**Conferences:**

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