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The ATP2C2 Gene as Transcribed from a Novel Transcriptional Start Site in Pancreatic Acinar Cells

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Graduate Program in Pharmacology and Toxicology

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

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ABSTRACT

Strict regulation of cytosolic Ca\textsuperscript{2+} is essential to regulated exocytosis and proper pancreatic acinar cell function, controlled in part by pumps that shuttle Ca\textsuperscript{2+} out of the cytosol. Our laboratory identified a novel isoform of Secretory Ca\textsuperscript{2+} ATPase 2 (SPCA2) containing only the carboxy terminus. Pancreatic SPCA2, is an approximately 17-20 kDa, protein encoded by the Atp2c2 gene and is completely absent in Mist1\textsuperscript{-/-} acini. The focus of this thesis was to understand the transcriptional regulation of Atp2c2 in the pancreas. Pancreatic Atp2c2 appears to be transcribed from an alternative transcriptional start site (TSS) and regulated by MIST1. Bioinformatic analyses identified a truncated Atp2c2 isoform initiated within the 23rd intron of the Atp2c2 gene. Comparative analysis suggested this isoform differs between humans and mice. ChIP-seq analysis identified enrichment for trimethylated (Me3) histone 3 (H3) at lysine 4 (K4) in intron 23 in pancreatic tissue, which was confirmed by ChIP-qPCR. ChIP-qPCR also identified enrichment of Acetylated Histone 3 (H3Ac), H3K36Me3, MIST1 and RNA Pol II within this region. Luciferase assays and immunofluorescence confirmed this region possessed promoter activity and produced a protein of the correct size. Our results identify a unique TSS for Atp2c2 within the pancreas that is regulated by MIST1.
ACKNOWLEDGMENTS

First and foremost I would like to thank my supervisor Dr. Christopher Pin. Thank you for being endlessly patient and optimistic when I was feeling frustrated and defeated. Your guidance and constructive criticism over the past two years have been instrumental in my growth as a scientist. You constantly encouraged me to think critically. I appreciate that you were always available to discuss things with me. The time I spent discussing and troubleshooting this project with you was invaluable to my progress. Being a part of your lab was a wonderful experience. The manner in which you are able to juggle so many responsibilities and still make time for all of the members of your lab while also maintaining a positive and encouraging attitude makes you an excellent supervisor. Thank you for everything, without your supervision and guidance I would never have grown so much as a scientist and as a person during this experience.

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To my mom and dad I would like to acknowledge how influential you have been in all that I have accomplished. I thank you for your endless support and encouragement. I am lucky to have such loving parents who always encouraged me to pursue my dreams and who have never doubted my ability to achieve anything I put my mind to. I love you both and I wouldn’t be the person I am today without you.
Rachel, thank you for always being there to listen to me and support me. I appreciate that you have listened to my concerns and have always been excited and curious to hear about what I am doing. You will be a fantastic nurse and are an even better sister. I love you.

To my best science friend Anusha, without you I don’t know what I would have done. Thank you for being an amazing friend and roommate. I am so lucky to have had you during this experience, because nobody quite gets how research can be both so frustrating and so exciting like someone who is also doing it. I am so grateful for all of your help and support over the past few years. You are so dedicated and intelligent and I cannot wait until I am able to celebrate your Ph. D defense with you.

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TABLE OF CONTENTS

CERTIFICATE OF EXAMINATION .............................................................. ERROR! BOOKMARK NOT DEFINED.

ABSTRACT ........................................................................................................ II

ACKNOWLEDGMENTS ...................................................................................... III

TABLE OF CONTENTS ....................................................................................... V

LIST OF FIGURES ............................................................................................. V

LIST OF ABBREVIATIONS ................................................................................ VII

1.0 INTRODUCTION ......................................................................................... 1

1.1 THE PANCREAS ......................................................................................... 2

1.2 ACINAR REGULATED EXOCYTOSIS ....................................................... 5
SECRETEGOGUE STIMULATED CA\textsuperscript{2+} RELEASE .................................. 6

1.3 CALCIUM AND THE CELL ......................................................................... 7
CALCIUM ATPASES ............................................................................................. 8
SARCOENDOPLASMIC RETICULAR CA\textsuperscript{2+} ATPASES (SERCA) ...................... 8
PLASMA MEMBRANE CA\textsuperscript{2+} ATPASES (PMCA) ..................................... 9
SECRETORY PATHWAY CA\textsuperscript{2+} ATPASES (SPCA) ................................... 10

1.4 SECRETORY PATHWAY CA\textsuperscript{2+} ATPASE 2 (SPCA2) ...................... 11

1.5 STORE OPERATED CA\textsuperscript{2+} ENTRY ............................................... 16

1.6 GENE REGULATION IN THE PANCREAS .............................................. 17
MIST1 and ATP2c2 TRANSCRIPTIONAL REGULATION .................................... 18
RNA POLYMERASE (RNA POL) ........................................................................ 18
RNA POLYMERASE II DRIVEN TRANSCRIPTION ....................................... 19

1.7 EPIGENETIC TRANSCRIPTIONAL REGULATION .................................... 20
DNA METHYLATION .......................................................................................... 21
HISTONE MODIFICATIONS .............................................................................. 22

1.8 HYPOTHESIS AND OBJECTIVES ......................................................... 23

2.0 METHODS ................................................................................................. 24

2.1 MICE ......................................................................................................... 25

2.2 CHROMATIN ISOLATION, AND CHIP .................................................... 25
ACINAR CELL CHROMATIN ISOLATION ....................................................... 25
CHROMATIN ISOLATION FROM WHOLE PANCREAS ................................. 26
CHROMATIN SONICATION AND SONICATION CHECK ............................. 27

2.3 PRIMER DESIGN, PCR, AND qPCR ....................................................... 29
PRIMER DESIGN ............................................................................................... 29
POLYMERASE CHAIN REACTION (PCR) ......................................................... 30
QUANTITATIVE CHIP POLYMERASE CHAIN REACTION .............................. 30

2.4 BIOINFORMATICS ...................................................................................... 31

2.5 CELL CULTURE ......................................................................................... 31

2.6 TRANSFECTIONS AND CONSTRUCT DESIGN ....................................... 32
CONSTRUCT DESIGN ....................................................................................... 32
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Intracellular organization of a pancreatic acinar cell.</td>
<td>3</td>
</tr>
<tr>
<td>1.2</td>
<td>Atp2c2 gene and Secretory Pathway Ca$^{2+}$ ATPase protein</td>
<td>14</td>
</tr>
<tr>
<td>3.1</td>
<td>Atp2c2 gene and Secretory Pathway Ca$^{2+}$ ATPase protein</td>
<td>40</td>
</tr>
<tr>
<td>3.2</td>
<td>H3K4Me3 and H3K27Me3 enrichment on Atp2c2 in pancreatic acinar cells</td>
<td>44</td>
</tr>
<tr>
<td>3.3</td>
<td>Truncated Atp2c2c appears to be pancreas specific.</td>
<td>46</td>
</tr>
<tr>
<td>3.4</td>
<td>Analyses of pancreatic Atp2c2 epigenetic modifications suggesting a novel TSS in region of Exon 24.</td>
<td>49</td>
</tr>
<tr>
<td>3.5</td>
<td>Enrichment of RNA Polymerase II and MIST1.</td>
<td>51</td>
</tr>
<tr>
<td>3.6</td>
<td>Expression of -2286/+1544 ATG FLAG SPCA2c.</td>
<td>55</td>
</tr>
<tr>
<td>3.7</td>
<td>Luciferase Expression of Atp2c2c promoter constructs.</td>
<td>60</td>
</tr>
<tr>
<td>4.1</td>
<td>Overview of Atp2c2-c Epigenetic Modifications, RNA Pol II, and MIST1 Enrichment.</td>
<td>69</td>
</tr>
</tbody>
</table>
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bHLH</td>
<td>Basic helix-loop-helix</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>c</td>
<td>carboxy</td>
</tr>
<tr>
<td>C</td>
<td>cytosine</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>calcium</td>
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<tr>
<td>cADPR</td>
<td>cyclic ADP ribose</td>
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<tr>
<td>CE</td>
<td>cytoplasmic extract</td>
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<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
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<td>ChIP-Seq</td>
<td>Chromatin Immunoprecipitation followed by Next Generation sequencing</td>
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<tr>
<td>DAPI</td>
<td>4,6 diamidino-2-phenylindole dihydrochloride hydrate</td>
</tr>
<tr>
<td>DCE</td>
<td>downstream core element</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxy-nucleotide triphosphate</td>
</tr>
<tr>
<td>DPE</td>
<td>downstream promoter element</td>
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<tr>
<td>DTT</td>
<td>dithioreitol</td>
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<tr>
<td>EBI</td>
<td>European Bioinformatics Institute</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>EMBL</td>
<td>European Molecular Biology Laboratory</td>
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<td>ENCODE</td>
<td>Encyclopedia of DNA Elements</td>
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<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>F12-K</td>
<td>F12 Kaighn’s modification</td>
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<tr>
<td>FITC</td>
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<td>G</td>
<td>guanine</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>GPCR</td>
<td>G-protein coupled receptor</td>
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<tr>
<td>H</td>
<td>histone protein</td>
</tr>
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<td>HEK293A</td>
<td>human embryonic kidney 293 adherent</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
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<tr>
<td>IF</td>
<td>immunofluorescence</td>
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<tr>
<td>Inr</td>
<td>initiator element</td>
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<tr>
<td>IP₃</td>
<td>inositol 1,4,5-triphosphate</td>
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<tr>
<td>K</td>
<td>lysine</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
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<tr>
<td>Mn²⁺</td>
<td>manganese</td>
</tr>
<tr>
<td>NAADP</td>
<td>nicotinic acid adenine dinucleotide</td>
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<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
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<tr>
<td>NFDM</td>
<td>non-fat dried milk</td>
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<td>PAGE</td>
<td>polyacrylamide gel</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PDX1</td>
<td>pancreatic and duodenal homeobox 1</td>
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<tr>
<td>PIC</td>
<td>preinitiation complex</td>
</tr>
<tr>
<td>PMCA</td>
<td>plasma membrane calcium ATPase</td>
</tr>
<tr>
<td>PP</td>
<td>Pancreatic polypeptide cells</td>
</tr>
<tr>
<td>PTF</td>
<td>pancreatic transcription factor</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RNA Pol</td>
<td>ribonucleic acid polymerase</td>
</tr>
<tr>
<td>RNA-Seq</td>
<td>RNA isolation followed by Next Generation Sequencing</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the means</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarcoendoplasmic reticular calcium ATPase</td>
</tr>
<tr>
<td>SICE</td>
<td>store independent calcium entry</td>
</tr>
<tr>
<td>SNARE</td>
<td>N-ethylmaleimide-sensitive fusion protein attachment protein receptors</td>
</tr>
<tr>
<td>SOCE</td>
<td>store operated calcium entry</td>
</tr>
<tr>
<td>SPCA</td>
<td>secretory pathway calcium ATPase</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>T</td>
<td>thymine</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
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<tr>
<td>TF</td>
<td>transcription factor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>TRITC</td>
<td>tetramethylrhodamine isothiocyanate</td>
</tr>
<tr>
<td>TRK</td>
<td>tyrosine receptor kinase</td>
</tr>
<tr>
<td>TSS</td>
<td>transcriptional start site</td>
</tr>
<tr>
<td>UCSC</td>
<td>University of California, Santa Cruz</td>
</tr>
<tr>
<td>ZG</td>
<td>zymogen granule</td>
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INTRODUCTION
1.1 The Pancreas

The pancreas is a branched, glandular organ located in the upper abdominal cavity, which consists largely of exocrine tissue in addition to portions of endocrine tissue termed the Islets of Langerhans (Arda, Benitez, & Kim, 2013). The Islets of Langerhans are made up of five cells types, which are identified by their production and secretion of different hormones. These cell types include β cells, which produce and secrete insulin to lower blood glucose, α cells, which make and secrete glucagon, δ cells, which produce and release somatostatin, ε cells, which produce and release ghrelin, and pancreatic polypeptide (PP) cells, which regulate pancreatic secretion (Hezel et al., 2008).

The exocrine portion of the pancreas, which is the focus of this thesis, is required for the digestion of fats, carbohydrates, and proteins. The exocrine pancreas is responsible for the secretion of more than 20 digestive enzymes and is composed of acinar and ductal cells. Acinar cells are serous exocrine epithelial cells that form grape-like clusters and secrete digestive enzymes and sodium chloride rich fluid into the branched tubes formed by ductal cells (Low, Shukla, & Thorn, 2010). Pancreatic ductal cells produce bicarbonate that aids in carrying zymogen granules to the duodenum through the common bile duct and counteracts the acidic secretions coming from the stomach (Arda et al., 2013). Acinar cells produce digestive enzymes such as proteases, amylase, lipase, secretin, and cholecystokinin, and package these in the form of zymogen granules (ZG). These cells are highly polarized with basally located nuclei and endoplasmic reticulum (ER) and apically directed zymogen secretion (Dingsdale, Voronina, Haynes, Tepikin, & Lur, 2012; Hezel et al., 2008) [Figure 1.1].
Figure 1.1 Intracellular organization of a pancreatic acinar cell.
1.2 Acinar Regulated Exocytosis

Acinar regulated exocytosis involves stimulus-secretion coupling in which parasympathetic or hormonal stimulation of acinar cells results in secretion. The primary stimulants for acinar cell secretion are parasympathetic cholinergic input and actions of the hormone cholecystokinin causing an increase in intracellular free calcium (Ca\(^{2+}\)). The increase in cytoplasmic Ca\(^{2+}\) acts as the primary signaling mechanism for secretion (Low et al., 2010; Williams, 2001, 2011). Local increases in Ca\(^{2+}\) at the apical border result in fusion of ZGs to the apical pole of the acinar cell where they can fuse with the membrane and release their contents into the duct (Low et al., 2010; O H Petersen, 2009). ZG fusion to the membrane is mediated through soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors (SNAREs). This initial Ca\(^{2+}\) release appears to come from intracellular stores and is followed by Ca\(^{2+}\) efflux and additional influx through store operated calcium channels located in the basolateral membrane (Williams, 2001; Yule, 2010).

The acinar cell response to heightened intracellular Ca\(^{2+}\) levels is multifaceted and involves Ca\(^{2+}\) oscillations, waves, and local spikes (Low et al., 2010; Wäsle & Edwardson, 2002). These changes in intracellular Ca\(^{2+}\) are the result of tightly regulated control of intracellular Ca\(^{2+}\) stores within the endoplasmic reticulum and mitochondria, extracellular Ca\(^{2+}\) accumulations, and channels involved in Ca\(^{2+}\) release such as inositol 1,4,5-triphosphate receptors (IP\(_3\)R), and ryanodine receptors (Low et al., 2010).
Secretogogue Stimulated Ca\textsuperscript{2+} Release

The main secretogogues in pancreatic acinar cells are the neurotransmitter acetylcholine (Ach), which activates the muscarinic cholinergic receptor M3, and the hormone cholecystokinin (CCK) (Ole H Petersen, 2012). The receptors for these secretogogues are all heterotrimeric G protein coupled receptors of the G\textsubscript{q} family. Binding of ligand induces an intracellular conformational change in the receptor, which results in activation of the G protein via the binding of GTP. This causes dissociation of the \(\alpha\)-subunit of the heterotrimeric G protein and activates phospholipase-c (PLC).

Phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}) is cleaved by PLC into inositol 1,4,5-triphosphate (IP\textsubscript{3}), which bind ligand gated Ca\textsuperscript{2+} channels (IP\textsubscript{3} receptors), leading to the release of Ca\textsuperscript{2+} from the ER. All three IP\textsubscript{3}Rs are expressed in acinar cells with IP\textsubscript{3}R2 and IP\textsubscript{3}R3 making up 90% of channels and IP\textsubscript{3}R1 making up only 10% (Yule, 2010). After release of ER Ca\textsuperscript{2+} stores, Ca\textsuperscript{2+} ATPases extrude Ca\textsuperscript{2+} from the cytoplasm by effluxing it out of the cell and moving Ca\textsuperscript{2+} back into intracellular stores. Some Ca\textsuperscript{2+} is pumped back into the ER stores but stores are not fully replenished because of Ca\textsuperscript{2+} lost through efflux. These stores are maintained via store operated Ca\textsuperscript{2+} channels allowing Ca\textsuperscript{2+} entry at the basolateral membrane (Hewavitharana, Deng, Soboloff, & Gill, 2007; Williams, 2001; Yule, 2010).

In response to secretogogues, Ca\textsuperscript{2+} oscillations appear to be mediated through IP\textsubscript{3}R. It is also suggested that independent regulators of Ca\textsuperscript{2+} such as nicotinic acid adenine dinucleotide (NAADP) and cyclic ADP ribose (cADPR), may be responsible for these oscillations via signaling through two pore channels (TPCs) and ryanodine receptors, respectively. TPCs are located on lysosomes and release endo-lysosomal Ca\textsuperscript{2+}.
stores in response to NAADP binding. cADPR activates ryanodine receptors resulting in the amplified release of ER Ca\(^{2+}\) stores (Zhao, Graeff, & Lee, 2012). Oscillations initially occur at the apical pole of the acinar cell where they are believed to stimulate regulated exocytosis (Williams, 2001). Abnormal accumulation of Ca\(^{2+}\) within the cytosol can have numerous effects on cell function, physiology and pathology.

1.3 Calcium and the Cell

Ca\(^{2+}\) plays a critical role in many cellular processes and has been identified as one of the most important and versatile second messengers, important in conveying extracellular signals into intracellular responses (Skupin & Thurley, 2012). Ca\(^{2+}\) signaling can result in both long term changes such as regulation of transcription, proliferation, and cell division, or transient responses such as exocytosis and contraction (Hewavitharana et al., 2007). The ability of Ca\(^{2+}\) to act as a vital second messenger is afforded by the maintenance of a calcium gradient across the cell membrane, with high levels of extracellular Ca\(^{2+}\) and high levels of Ca\(^{2+}\) in intracellular compartments such as the ER, sarcoplasmic reticulum (SR), mitochondria, Golgi, endosomes, lysosomes, and secretory granules and maintenance of low levels of cytoplasmic Ca\(^{2+}\). For cells to utilize the Ca\(^{2+}\) gradient, they must maintain intracellular levels by expelling, compartmentalizing, and chelating Ca\(^{2+}\) (Clapham, 2007). I have already described the process by which Ca\(^{2+}\) enters into the cytosol, and will now describe the mechanisms by which Ca\(^{2+}\) exits the cytosol.
Calcium ATPases

Ca\(^{2+}\) ATPases are crucial in maintaining the Ca\(^{2+}\) gradient between the intracellular and extracellular environments by pumping Ca\(^{2+}\) out of the cytosol. They help to restore cytoplasmic Ca\(^{2+}\) to basal levels after cell stimulation and help maintain Ca\(^{2+}\) stores in the ER, Golgi, and other compartments (Wuytack, Raeymaekers, & Missiaen, 2002). The three families of Ca\(^{2+}\) ATPases are the sarcoendoplasmic reticular Ca\(^{2+}\) ATPases (SERCA), plasma membrane Ca\(^{2+}\) ATPases (PMCA), and secretory pathway Ca\(^{2+}\) ATPases (SPCA). All three of these are P-type Ca\(^{2+}\) ATPases with PMCAs being the oldest phylogenetically, followed by the SPCAs and then the SERCAs (Vandecaetsbeek, Vangheluwe, Raeymaekers, Wuytack, & Vanoevelen, 2011). SERCA pumps are responsible for pumping Ca\(^{2+}\) from the cytosol into the ER and PMCAs are responsible for pumping Ca\(^{2+}\) from the cytosol out of the cell (Clapham, 2007). The less studied SPCAs are found within the Golgi membrane and pump Ca\(^{2+}\) from the cytosol into the Golgi (Vanoevelen et al., 2005). Ca\(^{2+}\) ATPases exist as different isoforms and splice variants allowing them to function in highly specialized ways in different cell types and allowing the strict regulation of Ca\(^{2+}\) levels required.

Sarcoendoplasmic Reticular Ca\(^{2+}\) ATPases (SERCA)

Sarcoendoplasmic Reticular Ca\(^{2+}\) ATPases contribute to maintaining ER stores of Ca\(^{2+}\) and lowering cytoplasmic Ca\(^{2+}\) levels to resting levels. These ATPases are transcribed from the ATP2A gene family and contain two Ca\(^{2+}\) binding sites, transporting two Ca\(^{2+}\) at the expense of one adenosine triphosphate (ATP). SERCA2 is the most widely expressed SERCA and is expressed in cardiac muscle, fast twitch skeletal muscle,
smooth muscle, and cerebellar neurons. SERCA2 exists as four different isoforms: SERCA2a, 2b, 2c, and 2d, the products of alternative splicing. SERCA2b is considered the housekeeping SERCA and SERCA2a is the muscle specific isoform found in cardiac, skeletal and smooth muscle. SERCA2c and SERCA2d were more recently discovered in the heart (Vandecaetsbeek et al., 2011). SERCA1 is the most specialized SERCA and is highly expressed in fast-twitch skeletal muscle. SERCA1 is expressed in two isoforms: SERCA1a is expressed in adults and SERCA1b is expressed in neonates and adult muscle undergoing regeneration (Vandecaetsbeek et al., 2011). SERCA3 seems to be a highly specialized SERCA that is always coexpressed with SERCA2b. SERCA3 has six splice variants in humans (SERCA3a-f) and three splice variants in mice (SERCA3a-c). It is expressed in secretory cells including the trachea, pancreas, colon, lymphocytes, platelets, mast cells and thyroid (Vandecaetsbeek et al., 2011; Wuytack et al., 2002).

**Plasma Membrane Ca$^{2+}$ ATPases (PMCA)**

Plasma membrane Ca$^{2+}$ ATPases lower intracellular Ca$^{2+}$ by pumping Ca$^{2+}$ out of the cytosol. These pumps are expressed from the ATP2B genes as four different isoforms: PMCA1, 2, 3, and 4. PMCA1 is ubiquitously expressed beginning in the earliest stages of the embryo. PMCA2 and PMCA3 have a more restricted expression profile with PMCA2 expressed in the lactating mammary gland, PMCA3 expressed in skeletal muscle and both expressed in the nervous system. PMCA4 has a more widespread tissue expression found in the heart, brain and at high concentrations in the spermatozoa (Giacomello, De Mario, Scarlatti, Primerano, & Carafoli, 2013). The PMCA1s are expressed as over 30 different isoforms resulting from alternative splicing of their primary transcripts. These
various splice variants result in isoforms, which have more tissue specific cellular localization, tissue specific Ca\(^{2+}\) regulating abilities, and differences in calmodulin affinity (Di Leva, Domi, Fedrizzi, Lim, & Carafoli, 2008).

**Secretory Pathway Ca\(^{2+}\) ATPases (SPCA)**

Secretory pathway Ca\(^{2+}\) ATPases (SPCAs) are found within the membrane of the Golgi and are responsible for pumping Ca\(^{2+}\) and manganese (Mn\(^{2+}\)) ions from the cytosol into the Golgi to be utilized for proper protein sorting, processing, and glycosylation. Similar to PMCAs the SPCAs contain only a single ion binding site (Vandecaetsbeek et al., 2011). Two SPCAs are found in higher vertebrates: SPCA1 and SPCA2. SPCA1 is transcribed from \(ATP2C1\), which is located on chromosome 3 in humans. This protein is expressed in all cell types and tissues that were studied and thus appears to be the housekeeping Ca\(^{2+}\)/Mn\(^{2+}\) pump. \(ATP2C1\) is alternatively spliced into four proteins SPCA1a-d, they differ in only their carboxyl termini and all are functional with the exception of SPCA1c. \(ATP2C1\) is the gene that is associated with the skin disorder Hailey-Hailey disease and the absence of this gene results in embryonic lethality by embryonic day 10.5 due to widespread cell apoptosis, caused by the inability of the Golgi to handle Mn\(^{2+}\) and Ca\(^{2+}\) (Okunade et al., 2007; Vandecaetsbeek et al., 2011; Vanoevelen et al., 2005). There is a second SPCA protein (SPCA2) that has much more limited information regarding its gene structure, expression and function. SPCA2 is the focus of this thesis.
1.4 Secretory Pathway Ca\(^{2+}\) ATPase 2 (SPCA2)

SPCA2 is a protein of approximately 103 kilo Daltons (kDa) and is transcribed from the *Atp2c2* gene, which consists of 27 exons. *Atp2c2* is located on chromosome 8 in mice and chromosome 16 in humans with a restricted tissue expression. While SPCA2 can transport both Ca\(^{2+}\) and Mn\(^{2+}\) ions, it has been shown to have a much higher affinity for Ca\(^{2+}\) (Dode et al., 2006). SPCA2 displays a restricted tissue distribution, which includes the brain, colon, prostate, lactating mammary gland, salivary glands, testes, and the pancreas (Vanoeveren et al., 2005). The expression of this protein is primarily restricted to cells with highly active exocytosis systems suggesting it may play a role in cellular secretion. SPCA2 is highly upregulated in the mammary gland during lactation, and is responsible for lowering prolactin induced increased intracellular Ca\(^{2+}\) levels by pumping Ca\(^{2+}\) into the Golgi (Anantamongkol, Takemura, Suthiphongchai, Krishnamra, & Horio, 2007; M. Feng et al., 2011).

Interestingly SPCA2 expression is also increased in human breast cancer derived cell lines and human breast tumors conferring an increase in intracellular Ca\(^{2+}\) levels and increased tumorigenicity, suggesting other potential roles for this protein in cellular Ca\(^{2+}\) maintenance. Importantly, the SPCA2-mediated Ca\(^{2+}\) signaling was independent of ER and Golgi stores and was found to interact with the store operated calcium channel Orai1 at the plasma membrane to activate downstream Ca\(^{2+}\) signaling. This Ca\(^{2+}\) signaling ability was dependent on the expression of positively charged lysines 927, 931, and 937 and arginine 938 and a putative PDZ domain found in the C-termini of the protein, which are conserved between human, rat, and mouse. This role of SPCA2 in increasing intracellular Ca\(^{2+}\) levels is in contrast to the usual role of Ca\(^{2+}\) ATPase pumps...
in lowering intracellular Ca\textsuperscript{2+} levels suggests it may play an important physiological role in secretory tissues (M. Feng et al., 2011).

Previous work from our lab showed that SPCA2 expression in the pancreas is restricted to the acinar cells and a subset of islet cells, and is not found in the ducts, intercalated ducts, or blood vessels. Surprisingly, SPCA2 is localized to the basolateral portion of the acinar cell, a pattern that is consistent with localization to the ER instead of the Golgi. In addition, the pancreatic Atp2c2 transcript and SPCA2 protein are both much smaller than expected. The protein detected in the pancreas is approximately 20 kDa in size and includes at least exons 24 to 27 [Figure 1.2]. Since this is a truncated form of SPCA2, we have termed it SPCA2c (Garside et al., 2010). SPCA2c can be recognized using an antibody generated to the carboxyl terminus of the full-length protein. All of these differences suggest that SPCA2c may be performing a different function than the full-length isoform.

SPCA2 was found to be highly upregulated in lactating mouse mammary gland in conjunction with elevated expression of the store operated calcium channel Orai1 channel. Orai1 and SPCA2 colocalize at the plasma membrane in the lactating mammary gland mediating Ca\textsuperscript{2+} influx into the basal membrane of these cells. It appears that this Ca\textsuperscript{2+} influx occurs by a mechanism termed store independent Ca\textsuperscript{2+} entry (SICE).

Knockdown of SPCA2 inhibited localization of Orai1 to the plasma membrane and instead, Orai1 localized to the perinuclear membrane. The converse was also true for SPCA2 when Orai1 was knocked down (Cross, Breitwieser, Reinhardt, & Rao, 2013). The full-length isoform of SPCA2 appeared to be more effective in localizing Orai1 at the plasma membrane and amino acids 67 to 106 in the protein effectively interacted with
Orai1. However, this interaction did not elicit Ca\(^{2+}\) influx, suggesting that the amino terminus plays a role in localization to the plasma membrane and the carboxyl terminus plays the crucial role in Ca\(^{2+}\) entry (M. Feng et al., 2011). This theory is further supported by the fact that tethering the carboxyl terminus of the protein (SPCA2c) allows interaction with Orai1 and Ca\(^{2+}\) influx, but free SPCA2c is unable to effectively localize with Orai1 at the plasma membrane. In culture, the loss of SPCA2 also inhibited the formation of mammospheres suggesting that it may also play a role in structural organization of secretory acini (Cross et al., 2013).

It appears that in lactating mammary gland SPCA2 and Orai1 interact in a manner to allow SICE, and if this type of Ca\(^{2+}\) entry becomes hyperactive, this results in cell dedifferentiation and tumorigenesis (M.-Y. Feng & Rao, 2013). In the pancreas however the loss of SPCA2c is associated with high intracellular Ca\(^{2+}\) levels (Garside et al., 2010). In the pancreas it appears that SPCA2c is unlikely to interact with Orai1 at the plasma membrane suggesting it may be involved in regulating intracellular Ca\(^{2+}\) levels via a different mechanism (Cross et al., 2013).
A
cation binding site

B
ATPase domain

C
Hydrolase domain

D
Transmembrane domain

N-terminus

C-terminus

Atp2c2c

Spca2c
Figure 0.1 Atp2c2 gene and Secretory Pathway Ca2+ ATPase protein

(A) Atp2c2 gene schematic, exons in black and introns in grey. (B) Atp2c2 truncated carboxy gene (Atp2c2c) (C) SPCA2 protein structure in mouse. Cation binding site in green, cation ATPase domain in purple, hydrolase domain in blue, and transmembrane domains in black. (D) Truncated SPCA2 carboxy protein (SPCA2c).
1.5 Store Operated Ca\(^{2+}\) Entry

Stromal interaction molecules (STIMs) and Orai proteins work together in mediating the SOC pathway and in replenishing diminished ER Ca\(^{2+}\) stores. STIM1 is a single membrane spanning protein that acts as a sensor to detect lowered levels of Ca\(^{2+}\) in ER stores and is expressed at the cell membrane and ER membrane. Orai1 is a four transmembrane domain SOC channel localized to the plasma membrane with both its carboxyl and amino termini found in the cytoplasm. STIM1 interacts with the c-terminus of Orai1 and together they allow Store operated Ca\(^{2+}\) entry (SOCE) into the cell to replenish ER stores (Collins, Zhu-Mauldin, Marchase, & Chatham, 2013; Hong et al., 2011). In pancreatic acinar cells STIM1 is located in the ER membrane and upon stimulation it translocates to the plasma membrane via ER puncta. Orai1 is located at the apical, basal, and lateral membranes of the pancreatic acinar cell, and basal and lateral Orai1 have been shown to interact with STIM1 to allow Ca\(^{2+}\) influx for replenishment of ER Ca\(^{2+}\) stores. The highest density of Orai1 channels are however found located at the apical membrane though there is not a clear consensus on what the role of these apical Orai1 channels is. Hong et. al. (2011) observed moderate co-localization, approximately 40%, of Orai1 at the apical pole with STIM1 suggesting it may be involved in SOCE. Dingsdale et. al. (2012) however did not observe any co-localization with STIM1 at the apical membrane, suggesting that Orai1 may function in a store operated Ca\(^{2+}\) independent manner at the apical membrane of pancreatic acinar cells. Due to SPCA2c’s structure and localization, it seems that its role in pancreatic acinar cells is more complex than other Ca\(^{2+}\) ATPases. However, before functional studies can be addressed, including targeted gene ablation, it is important to understand the complete coding region for
SPCA2c. The main question this thesis will address is whether SPCA2c is the result of alternative splicing or a novel transcriptional start site. Knowing this will also enable researchers to understand how it is regulated.

1.6 Gene Regulation in the Pancreas

Transcription begins with the binding of RNA Polymerase II (RNA Pol II). Regulation of transcription is controlled at two levels: at the level of transcription factors and transcriptional apparatus and secondly via chromatin and its regulators (Lee & Young, 2013). The development of the pancreas begins with dorsal budding of the embryonic posterior foregut endoderm and various transcriptional cascades drive the development of the endoderm dorsal bud into the pancreas. Pancreatic progenitor cells expressing TF pancreatic and duodenal homeobox (Pdx) 1 followed by expression of bHLH TF pancreatic transcription factor (Ptf) 1a, arise in the mouse on embryonic day 9.0 (Arda et al., 2013). By embryonic day 12.5 continued branching divides the populations into tip progenitor cells which become acinar cells and trunk cells which give rise to ductal and endocrine cell populations (Cleveland, Sawyer, Afelik, Jensen, & Leach, 2012). The Ptf1-J heterotrimeric complex consisting of the transcription factors Ptf1, Tcf12, and Rbpj forms, which drives acinar cells fate and maturation (Benitez, Goodyer, & Kim, 2012). The mature acinar cell phenotype is maintained by the Ptf1-L complex consisting of the TFs Ptf1a, and Rbpj1, which is required for the expression of genes encoding mitochondrial machinery, enzyme production, and exocytosis components (Cleveland et. al., 2012; Benitez, Goodyer, & Kim, 2012; Pin et. al., 2001).
MIST1 and *Atp2c2* Transcriptional Regulation

MIST1 is a basic helix loop helix (bHLH) transcription factor (TF) expressed in serous exocrine tissues. MIST1 induces expression of genes necessary for secretory function and regulated exocytosis (Capoccia et al., 2011; Pin, Rukstalis, Johnson, & Konieczny, 2001; Tian et al., 2010). MIST1 binds to E-box consensus sites (CANNTG) and recently ChIP-Seq revealed in pancreas MIST1 preferred binding to an internal TA or GC (Direnzo et al., 2012). It is required for the full maturation and function of pancreatic acinar cells. *Mist1*−/− mice display disrupted acinar cell polarity and regulated exocytosis (Pin et al., 2001). Aberrant Ca^{2+} signaling is common to most models of pancreatic injury. In *Mist1*−/− mice, all Ca^{2+} ATPases are normally expressed with the exception of SPCA2, which is absent suggesting that MIST regulates SPCA2 expression (Garside et al., 2010).

RNA Polymerase (RNA Pol)

Eukaryotic RNA Polymerases are large proteins made up of 12 subunits. There are three classes of transcription in eukaryotic cells: ribosomal RNA (rRNA) transcription, messenger RNA transcription (mRNA) and transfer RNA (tRNA). The enzyme RNA Pol I transcribes rRNA in the nucleolus, RNA Pol II transcribes mRNA in the nucleoplasm for eventual translation into protein, and RNA Pol III transcribes small RNAs and tRNAs in the nucleoplasm.
**RNA Polymerase II Driven Transcription**

Initiation of transcription begins at the promoter, which is found immediately upstream (5') of the transcriptional start site (TSS). The promoter region is a sequence larger than 200 bps, which contains binding elements for TFs required for transcription initiation and RNA Pol II recruitment (Lewin, 2008). About 60% of human promoters contain a high CpG content that are termed CpG islands. These regions remain unmethylated in active genes but may be methylated in mature and differentiated cells not expressing these genes (Jiang, Liu, Chen, Cao, & Tao, 2013). The General RNA Pol II driven TFs (TFII(X) make up the basal transcription apparatus and there are seven core promoter element sequences found within 50 bps of the TSS, which allow them to bind at the promoter forming the transcription preinitiation complex (PIC).

Less than 1/3 of human genes contain an Adenine (A) and Thymine (T) rich sequence known as the TATA box [TATA(A/T)A(T/A)(A/G)] which is found 25 to 30 bps upstream of the TSS which allows binding of TATA-binding protein (TBP) and formation of the PIC at this region. In TATA-less promoters TBP is associated with other DNA binding proteins allowing it to be recruited to the promoter. The Initiator (Inr) element is found surrounding about 80% of TSS and is a pyrimidine rich sequence 

\[(C/G)(C/G)AAN(A/T)(C/G)(C/G)]\] that recruits TBP associated factors (TAF)s 1 and 2 to the PIC. The downstream promoter element (DPE) \[(A/G)G(A/T)CGTG\] is found +29 to +35 bps from the TSS in about 25% of promoters and recruits TAF6 and TAF9. Additionally the upstream transcription factor-RNA Pol II driven element B (BRE_U) \[(G/C)(G/C)(G/A)CGCC\] and the downstream transcription factor-RNA Pol II driven element B (BRE_d) \[(G/A)T(T/G/A)(T/G)(G/T)(T/G)(T/G)]\] can also be found in the
promoter of about 28% of genes to aid in transcription initiation by recruiting \( \text{TF}_{II} \text{B} \).

Additionally, the motif ten element (MTE) \([C(G/C)A(A/G)C(G/C)(G/C)AACG(G/C)]\) and the downstream core element (DCE) are also found downstream of the TSS. The DCE is composed of three subelements \( S_{I} \) (CTTC) found 6 to 11 bps downstream, \( S_{II} \) (CTGT) at 16 to 21 bps downstream, and \( S_{III} \) (AGC) found 30 to 34 bps downstream of the TSS and acts to recruit TAF1 (Thomas & Chiang, 2006).

Assembly of the PIC begins with binding of \( \text{TF}_{II} \text{D} \), which is made up of TBP and multiple tissue-specific TAFs. \( \text{TF}_{II} \text{A} \) and \( \text{TF}_{II} \text{B} \) are then recruited to stabilize \( \text{TF}_{II} \text{D} \) binding. RNA Pol II and \( \text{TF}_{II} \text{F} \) are then recruited forming a stable complex. \( \text{TF}_{II} \text{E} \) and \( \text{TF}_{II} \text{H} \) are then recruited and transcription can begin. Transcription of tissue-specific genes is controlled by upstream sequence elements that bind specific TFs allowing assembly of the PIC only in tissues expressing the necessary TFs (Lewin, 2008; Thomas and Chiang, 2006).

1.7 Epigenetic Transcriptional Regulation

Epigenetics is the study of heritable changes in gene regulation and expression without any changes to DNA sequence (Capell & Berger, 2013). Epigenetic changes are the basis for varied gene expression profiles allowing for diverse cell types and tissue types in complex organisms (Moore, Le, & Fan, 2013). The basis of epigenetic control results from the organization of DNA into chromatin. DNA is highly compacted into chromatin by first wrapping it around eight histone proteins (H) including two of each of the core histones H2A, H2B, H3, and H4. This histone-wrapped DNA is referred to as a nucleosome, which is further, compacted into chromatin by further coiling that requires
H1 (Lewin, 2008). Epigenetic modifications occur through direct modifications to the DNA such as methylation or through posttranslational modifications to histone proteins affecting the interaction between DNA and histones (Capell & Berger, 2013).

**DNA Methylation**

DNA can be epigenetically altered by methylation of cytosines, which often occurs at CpG sites. Four types of cysteine methylation exist: 5-methylcytosine, 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxylycytosine. DNA methylation is associated with several important physiological processes including genomic imprinting, X-chromosome inactivation, gene regulation, and chromatin stabilization. Gene methylation levels have been shown to change in response to environmental factors subsequently resulting in altered gene expression. Increased DNA methylation has been associated with gene silencing and recruitment of RNA Pol II is associated with demethylation (Jiang et al., 2013).

Three functional DNA methyltransferases (DNMTs) enzymes have been identified in eukaryotic cells: DNMT1 which maintains DNA methylation patterns during DNA replication, and DNMT3a and DNMT3b, which methylate DNA during development and form new methylation patterns on unmodified DNA (Moore et al., 2013). DNMT3a methylation also prevents the addition of gene silencing histone marks, suggesting it plays a role in maintaining transcriptional activation (Jiang et al., 2013).
Histone Modifications

Chromatin exists in active and inactive states. Activity can be conferred by post translation histone modifications, such as methylation, acetylation, ubiquitylation, glycosylation, and phosphorylation that regulate the accessibility of DNA (Capell & Berger, 2013; Lelli, Slattery, & Mann, 2012). Accessible DNA is more open to TF binding allowing increased transcriptional activity. Methylation of histones can be associated with both active and repressed genes depending on its location. Phosphorylation of histones weakens bonds the interaction between DNA and histones having roles in DNA repair, development and altering affinity of chromatin binding proteins. Glycosylation of histones by β-N-acetylglucosamine has been observed on histones H2A, H2B, and H4 but the effect of glycosylation on gene expression remains to be elucidated. Histone acetylation is a highly dynamic process that is carried out by histone acetylases and deactylases, both of which are linked to transcriptional activation. Acetylation of histones loosens their interaction with DNA making DNA more available for binding of polymerase and transcriptional machinery, thereby increasing the potential for transcription (Zentner & Henikoff, 2013).

Histone 3 is an important core histone involved in creating nucleosome structure and modifications of this histone mark active and silenced promoters. Active TSSs are marked by acetylation (H3Ac), methylation of Histone 3 Lysine (K) 4 (H3K4Me1, H3K4Me2, and H3K4Me3), and histone 3 lysine 36 trimethylation, (H3K36Me3) while silenced promoters are marked by trimethylation of lysine 27 (H3K27Me3) and Histone 3 lysine 9 trimethylation (H3K9Me3) (Rye, Sætrom, Håndstad, & Drabløs, 2011). These marks are found upstream of the transcriptional start site (TSS) (Heintzman et al., 2007).
Despite being associated with active genes loss of H3K4Me3 and H3K36Me3 does not appear to have a direct effect on transcriptional activity, suggesting that these modifications may not promote transcription but rather prevent the addition of silencing marks. H3K27Me3 and H3K9Me3 on the other hand promote formation of heterochromatin and stabilize nucleosome structure which inhibits binding of polymerase and other transcriptional machinery (Zentner & Henikoff, 2013).

### 1.8 Hypothesis and Objectives

The loss of MIST1 is associated with disruptions in Ca\(^{2+}\) movement and decreased acinar regulated exocytosis. Expression of the Ca\(^{2+}\) ATPase Atp2c2/SPCA2 is lost in the absence of MIST1 and SPCA2 expressed in the pancreas is much smaller than previously characterized suggesting that this represents a splice variant or novel isoform transcribed from an alternative transcriptional start site.

*I hypothesize that Atp2c2-c found in pancreatic acinar cells is transcribed from a novel TSS and that its expression is regulated by MIST1.*

The aims of my project are to define the transcriptional start site and promoter of the truncated Atp2c2 (Atp2c2-c) isoform and define factors involved in Atp2c2c gene regulation in pancreatic acinar cells.
METHODS
1.9 Mice

All of the mice used were of a C57Bl/6 background. The targeted deletion of the Mist1 gene has previously been described (Pin et al., 2001). For all studies, 2 to 4 month old male animals were used. The mice were handled according to the regulations set by the protocol for The University of Western Ontario by The Canadian Council of Animal Care (protocol #2008-116).

1.10 Chromatin Isolation, and ChIP

Acinar Cell Chromatin Isolation

Mice were sacrificed with 95% CO₂ with 5% O₂ and the pancreata were carefully dissected out with the removal of any residual fat or non-specific tissue and placed in 5 mL of oxygenated DMEM media containing 0.1 mg/ml collagenase (Sigma, St. Louis MO). Pancreata were injected 6 times with oxygenated DMEM using a syringe. The injected pancreata were then cut into small pieces, oxygenated for 1 minute, and incubated in collagenase/DMEM solution for 40 minutes in a 37°C water bath, shaking at 100 rpm. A plastic transfer pipette was used initially and then a 5 mL pipette to gently break apart the tissue. Tissue in collagenase/DMEM was gravitationally passed through a nylon filter (Millipore, Temecula CA) into a 50 mL conical tube (BD Falcon, Pittston PA). The nylon filter was washed with 40 mL DMEM containing 4% BSA. Tubes were centrifuged at 500 rpm for 4 minutes using the Eppendorf Centrifuge 5804 R. The supernatant was gently pipetted off.
To crosslink proteins to the DNA, 10 mL of DMEM with 1% formaldehyde (Ricca Chemical Company, Arlington TX) was added to the cells. This mixture was placed on the Thermolyne RotoMix Type 48200 shaker for 10 minutes. After 10 minutes 0.13 M glycine was added and tubes were placed on the shaker for 5 minutes. After 5 minutes, the cell pellet mixture was centrifuged for 3 minutes at 1500 rpm. The supernatant was removed and the cell pellet washed two times with 10 ml of phosphate buffered saline (PBS) and spun down for 3 minutes at 1500 rpm. PBS was removed and 500 mL of cytoplasmic extract (CE) buffer was added to cells to isolate the nuclei. CE buffer cell mixture was homogenized for 5 rounds with the tissue homogenizer and then incubated on ice for 15 minutes in a 1.5 ml Eppendorf tube. Tubes were centrifuged at 4°C at 5000 rpm for 10 minutes. The supernatant was removed and nuclei resuspended in 500 µl sodium dodecyl sulfate (SDS) lysis buffer with 2.5 µg/µl of each, aprotinin, and pepstatin A. Samples were incubated on ice for 15 to 20 minutes and then split into volumes of 200-300 µl per tube to be sonicated as stated below.

**Chromatin Isolation from Whole Pancreas**

Mice were sacrificed with 95% CO₂ with 5% O₂ and the pancreata were dissected out with the removal of any residual fat or non-specific tissue and placed in DMEM media warmed to 37°C. The injected pancreata were then cut into small pieces, added to 40 mL of media with 1% formaldehyde, and gently rocked at room temperature for 10 minutes. To stop the crosslinking process, 2 ml of 2.5M glycine was added and the sample gently rocked for 5 minutes at room temperature. Samples were centrifuged at
720 rpm for 2 minutes at 4°C. Supernatant was removed and the pellet was washed with 40 ml of ice cold PBS containing 5 µg/µl of each leupeptin, aprotinin, and pepstatin A. The samples were centrifuged at 720 rpm and the wash was repeated. Samples were then treated with 1 ml of CE buffer containing 5 µg/µl of each leupeptin, aprotinin, and pepstatin A, and incubated on ice for 5 minutes. Samples were transferred to a glass tissue homogenizer and homogenized for 5 rounds. The samples were centrifuged at 720 rpm for 2 minutes at 4°C. The supernatant was removed and the pellet was resuspended in 1 ml of CE buffer containing protease inhibitors and spun at 720 rpm for 2 minutes at 4°C. Supernatant was removed and the pellet was resuspended in 1 ml SDS lysis buffer with 5 µg/µl of each leupeptin, aprotinin, and pepstatin A and incubated on ice for 30 minutes. The sample was then treated to an additional 5 rounds with the tissue homogenizer. Samples were split into volumes of 200-300 µl per tube to be sonicated as stated below.

**Chromatin Sonication and Sonication Check**

Chromatin samples were sonicated 13 times on level 4 for 10 seconds using the Fisher Scientific Sonic Dismembrator Model 100. Tubes were centrifuged at 13000 rpm for 10 minutes at 4°C and combined into one new tube and 15-20 µL was aliquoted to check the sonication and the remainder was placed into the -80°C freezer. To check the sonication 80 µl of dH2O and 6 µl of 5 M NaCl were added to the chromatin sample for reverse crosslinking and incubated in a heating block at 65°C overnight. Samples were removed from the heating block and when they reached room temperature, 2 µL of 10 µg/µl proteinase K was added and samples were incubated for 1 to 2 hours at 42°C.
Phenol chloroform extraction was then performed and RNase was added to samples for 30 minutes at room temperature. Samples were then run on a 1% agarose (Fisher Scientific, Fair Lawn NJ) gel and visualized using the UVP BioDoc-IT™ System.

2.2.4 Chromatin Immunoprecipitation (ChIP)

Information for all buffers used in this protocol is found in Appendix 6.1.

Each ChIP reaction used 40 µg of chromatin, isolated as stated above. Samples were diluted to a concentration of 400 ng/µL at a volume of 100 µL for each antibody ChIP reaction, using lysis buffer containing protease inhibitors. Lysis buffer and chromatin solution was diluted with 300 µL of dilution buffer to give a final volume of 400 µL per antibody reaction. Protein A agarose (Millipore, Temecula CA) or magnetic beads were used. Agarose beads were washed twice using 500 µL of PBS and given a final wash with 300 µL of dilution buffer. When agarose beads were used samples were pre-cleared with 35 µL of beads for 1½ to 2 hours on a rotator at 4°C. The chromatin sample solution was centrifuged at 6000 rpm for 1 minute at 4°C and the supernatant collected. An aliquot 30 µL of chromatin sample solution was used for the input control. Antibody was added to each sample except input and samples placed on the rotator for 12-18 hours at 4°C. Antibodies used included anti-Histone 3 (H3) tri-methyl K36 antibody (H3K36Me3; Abcam, Cambridge MA), Anti-RNA polymerase II (Millipore, Temecula CA), Anti-H3K4Me3, and Anti-H3K27Me3. The antibody sample was mixed with 35 µL of prewashed agarose beads or 17 µL of magnetic beads and placed on the rotator for 1 hour at 4°C. After 1 hour, the bead solution was centrifuged down for 1 minute at 6000 rpm for 1 minute at 4°C and supernatant was removed using a magnet to retain magnetic
beads. Beads were successively washed with 1 mL of a low salt wash buffer, a high salt wash buffer, a Lithium Chloride buffer and twice with a Tris-EDTA buffer pH 8.1. For each wash beads were placed on the rotator for 10 minutes at 4°C followed by centrifugation at 6000 rpm for 1 minute at 4°C, and then supernatant was removed. Beads were placed on the rotator for 15 minutes at room temperature with 75 µL of elution buffer and then centrifuged at 6000 rpm for 1 minute. The supernatant was collected and the process repeated. Input samples had 150 µL of elution buffer added. Samples of chromatin and elution buffer has 6 µL of 5 M NaCl added, and placed at 65°C for 4 to 18 hours. Following incubation, 1 µL of 10 µg/µl RNAse A was added and tubes placed at 37°C for 30 minutes. 10 µL of EDTA-Tris HCl-Proteinase K solution was then added to each sample. Samples were placed at 45°C for 1 hour. Chromatin was isolated using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison WI).

1.11 Primer Design, PCR, and qPCR

Primer Design

All primers were designed using sequences from the University of California Santa Cruz (UCSC) Genome Browser and were generated by Sigma (Oakville, ON). Primer3 (Whitehead Institute and Howard Hughes Medical Institute) was used for choosing primer sequences from the UCSC Genome browser sequences. Primers for Atp2c2 mouse gene were designed based on the UCSC Genome browser identification Atp2c2 (uc009nqg.1). For all sequences see Appendix 6.2.
Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was carried out using a Bio Rad T100™ Thermal Cycler with 1 µl of cDNA or ChIP sample added to the master mix solution containing 10X Dream Taq Green Buffer (Promega, Madison, WI), 0.5 mM dNTP, 0.8 pM forward and reverse primers, diluted in dH₂O to a final volume of 23.85 µL per reaction. Samples were placed at 95°C and 0.15 µl of DreamTaq DNA Polymerase (Promega, Madison, WI) enzyme was added to each reaction. Products were amplified through 40 cycles, with each consisting of a melting step at 95°C for 30 seconds, annealing at 55-62°C for 30 seconds, and an elongation step at 72°C for 30 seconds. An additional elongation step of 72°C for 5 minutes was included in the final cycle. Temperature gradients were performed for each primer set to determine the optimal annealing temperature. All samples were resolved on a 1% agarose gel and visualized.

Quantitative ChIP Polymerase Chain Reaction

All ChIP qPCR experiments were performed following manufacturer’s directions using the ViiA™ 7 Real Time PCR System (Life Technologies) with the GoTaq® qPCR Master Mix (Promega, Madison WI). All reactions were performed in triplicate and contained 1 µl ChIP sample, 5 µl GoTaq® qPCR Master Mix containing Sybr green, 0.30 µM of forward and reverse primers, 2.5 mM dNTPs, and diluted to a final volume of 10 µL with nuclease-free water. Products were amplified through 40 cycles, with each consisting of a melting step at 95°C, annealing at 60°C, and an elongation step at 72°C, each for 30 seconds. Signal was recorded during the annealing step and a melting curve was also generated. Data was analyzed using the Viia7 Ruo software (Life Technologies).
Temperature gradients were performed for each primer set to determine optimal annealing temperature using PCR.

1.12 Bioinformatics

All Encyclopedia of DNA Elements (ENCODE) RNA-Sequencing and ChIP-Sequencing data was analyzed using the UCSC Genome browser (http://genome.ucsc.edu/encode/).

Protein sequences were predicted using the National Center for Biotechnology Information (NCBI) Open Reading Frame finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Protein structure was predicted using the European Molecular Biology Laboratory (EMBL)-European Bioinformatics Institute (EBI) InterProScan 4 program (http://www.ebi.ac.uk/Tools/pfa/iprscan/).

1.13 Cell Culture

Cell lines including the rat pancreatic acinar cell line AR42J (ATCC, Manassas VA), and Human embryonic kidney 293 adherent (HEK293A) cell line were maintained in an incubator at 37°C with 5% CO₂ and 95% O₂. AR42J cells were maintained in F12 Kaighn’s Modification (F12-K) media (Gibco, Grand Island NY) containing 1% penicillin (1000 units/mL) Streptomycin (1000 units/mL) (Penstrep) (HyClone, Logan UT) and 20% fetal bovine serum (FBS) (Fisher Scientific, Ottawa ON). HEK293A cells were maintained in 1X Dulbecco’s Modified Eagle Medium (DMEM) high glucose media (HyClone, Logan UT) containing 10% FBS and 1% Penstrep. Media was changed every two days and cells expanded using 0.25% w/v trypsin (HyClone, Logan UT) when
the cells reached 60-70% confluency in a T-75 flask (BD Falcon, Mississauga, ON). For immunofluorescence cells were grown on coverslips (Fisher Scientific, Ottawa ON) coated with 1mg/mL Poly-L-lysine (Sigma, St. Louis MO) and for RNA isolation cells were grown in 60 mm dishes (Corning, Big Flats NY).

1.14 Transfections and Construct Design

Construct Design

Constructs pMK−2286/+1544 ATG-FLAG-SPCA2-c, pGL3−1238 ATG 24-Promoter, pGL3−619 ATG 24-Promoter, pGL3−309 ATG 24-Promoter, and pGL3-Alternate −1238 ATG 23-Promoter, were designed based on the UCSC Genome browser identification Atp2c2 (uc009nqg.1) and were constructed by Invitrogen GeneArt® (Regensburg, Germany). For all sequences see Appendix 6.3-6.7.

FuGENE Transfections

Transfections were performed on HEK293A and AR42J cells at 70-80% confluency in 6-well plates for immunofluorescence (IF), 24-well plates for luciferase assays, and 60 mm plates for RNA isolation. Cells used for IF, RNA and protein isolation were transfected with pcDNA3.0-Green Fluorescent Protein (GFP) or pcDNA3.0-MIST1-VP16-HA or pcDNA3.0-FLAG-SPCA2916-1077 or pMK−2286/+1544 ATG-FLAG-SPCA2-c, or pMK−2286/+1544 ATG-FLAG-SPCA2-c and pcDNA3.0-MIST1-VP16-HA. Cells used for luciferase protein isolation were transfected with pRL-SV40 and one of pGL3−1238 ATG 24-Promoter, pGL3−619 ATG 24-Promoter,
pGL3–309 ATG 24-Promoter, or pGL3-Alternate −1238 ATG 23-Promoter, in addition to either pcDNA3.0-Green Fluorescent Protein (GFP) or pcDNA3.0-MIST1-VP16-HA. For 6-well plates, a total of 2 µg of DNA was added per well in a 100 µL solution of serum free media, with 7 µL of FuGENE transfection reagent (Promega, Madison WI). The media/FuGENE solution vortexed, and allowed to form a transfection complex for 15 minutes at room temperature while 2 mL of fresh media was added to each well. The transfection complex was gently agitated and added to each well in a drop wise manner. For 60 mm plates a total of 4 µg of DNA was added per plate in a 200 µL solution of serum free media with 14 µL of FuGENE transfection reagent. RNA was isolated, protein was isolator cells were fixed for immunofluorescence 48 hours after transfection.

1.15 Immunofluorescence

Cells were seeded on 6-well plates (BD Falcon, Mississauga ON) with cover slips at a density of 70% or higher and fixed two days later for IF. Cell lines were washed twice with 1x PBS (Hyclone, Logan UT) and fixed in the 6-well dish with cover slip with 4% formaldehyde for 20 minutes on ice. After fixation samples were washed 3x5 minutes in PBS. Samples were permeabilized with 0.1% Triton X-100 (BDH, Mississauga ON) in PBS for 10 minutes followed by a 1-2 hour incubation in 5% Bovine Serum Albumin (BSA) (EMD Sciences, Gainsburg, NJ) + 0.1% Triton X-100 in PBS (blocking solution). Samples were incubated with primary antibody diluted with blocking solution. The primary antibodies used included mouse anti-FLAG (1:500, Sigma), rat-anti-HA (1:500, Roche), anti-rabbit MIST1 (ProSci Inc.), and rabbit anti-SPCA2 (1:500, ProSci Inc.). Following a 1 hour incubation at room temperature, samples were washed 3x5 minutes
with PBS and then incubated for 1 hour at room temperature with secondary antibodies, including fluorescein isothiocyanate (FITC) goat anti-rat IgG (Jackson Immunoresearch Labs, West Grove, PA), FITC goat anti-rabbit IgG, tetramethylrhodamine isothiocyanate (TRITC) goat anti-rabbit IgG, and TRITC goat anti-mouse IgG (Sigma). Samples were washed with PBS 3x5 minutes and incubated with 4,6 diamidino-2-phenylindole dihydrochloride hydrate (DAPI) in PBS (1:1000, Sigma) for 10 minutes.

1.16 RNA Isolation

RNA was isolated from AR42J and HEK293A cell lines. Cells were washed twice with PBS. 1 mL ice cold Isol RNA Lysis Reagent (5prime, Gaithersburg MD) was added and cells were homogenized by pipetting up and down and using a cell scraper. Samples were left on ice for 5 minutes. 200 µL of chloroform was added, each sample was shaken vigorously for 15 seconds and left on ice for 3 minutes. After incubation, the samples were centrifuged at 5000 rpm for 15 minutes at 4°C. The aqueous phase (containing RNA) was transferred to a new tube and precipitated using 500 µL of isopropanol. Samples were placed at -20°C overnight and then centrifuged at 5000 rpm for 15 minutes at 4°C. The resulting pellet was washed with 1 mL of 75% ethanol. Once the pellet was dislodged, the samples were centrifuged at 5000 rpm for 10 minutes at 4°C. The supernatant was removed and the pellet air dried for approximately 10 minutes. RNA was resuspended in RNAse-free water and stored at -80°C.
# 1.17 Protein Isolation and Western Blot Analysis

Protein was isolated from AR42J and HEK293A cells. Cells were washed twice with cold PBS. Cells were scraped into an isolation buffer containing 50mM Tris (pH 7.2), 5 mM MgCl₂, 1mM CaCl₂, 10 mM dithiothreitol (DTT), 1% NP-40, 100 units/ml DNAse 1, 50 µg/ml RNAse A, 30mM sodium fluoride, 20mM sodium orthovanadate, 10 mM sodium molybdate, 75 µg/ml PMSF and 5µg/ml of each leupeptin, pepstatin, and chymostatin. Cells were collected using a cell scraper, sonicated for 15 seconds, and centrifuged at 6000 rpm for 5 minutes at 4°C. The supernatant was transferred to a new tube and the pellet discarded.

For Western blot analysis, 10-50 µg of protein was added to the sample buffer containing 1% β-mercaptoethanol and bromophenol blue and boiled at 95°C for 5 minutes. Samples were resolved at 100 volts on a 12% SDS polyacrylamide gel (PAGE) until the dye front ran off the bottom of the gel. The protein was transferred to a polyvinylidene difluoride (PVDF) membrane (BioRad, Hercules CA) at 200mA for 90 minutes. Following the transfer, the PVDF membranes were incubated in blocking solution consisting of 5% non-fat dried milk (NFDM) in Tris buffered saline (TBS) with 0.1% Tween-20 (TBS-T) for 1-2 hours at room temperature. Membranes were incubated with primary antibodies overnight at 4°C with mild rocking. Primary antibodies used included rabbit anti-SPCA2 (1:250) and rabbit anti-MIST1 (1:1000) diluted in blocking solution. Following incubation, PVDF membranes were washed 5x5 minutes with TBS-T and incubated in horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:10000, Jackson ImmunoResearch Labs, West Grove PA) diluted in blocking solution for 1 hour at room temperature. Membranes were then washed 6x5 minutes with TBS-T and
chemiluminescent substrate (PerkinElmer, Boston MA) was added to the blots according to the protocol provided by the manufacturer. Membranes were developed using the VersaDoc (BioRad, Hercules CA).

1.18 Luciferase Assay

Protein was isolated using the Dual-Luciferase® Reporter Assay Kit (Promega, Madison WI). Transfections were performed in triplicate and each 20 second triplicate read was performed in triplicate using the Lumat LB 9507 Luminometer (Berthold Technologies, Oakridge TN). Firefly luciferase reads were normalized using renilla luciferase reads, and this ratio was normalized to the ratio for the negative control pGL3-Basic. Data was analyzed using GraphPad Prism software version 6.0 by two way analysis of variance (ANOVA) with a Bonferroni post hoc test. All graphs show mean values + standard error of measure (SEM).
RESULTS
1.19 Identification of Atp2c2c Transcriptional Start Site

Bioinformatic analysis suggests Atp2c2c differs between human and mouse

Bioinformatic analyses of the ENCODE data was conducted to explore ATP2C2 RNA transcripts using the RNA- Sequencing (RNA-Seq) data. Bioinformatics analysis of the ENCODE RNA-Seq suggests that the ATP2C2 is transcribed from a unique TSS or alternative splicing. Through analysis of the ENCODE RNA-Seq data, it appears that in human there is an ATP2C2 isoform that begins in the intronic region between exon 24 and 25. The first exon of this human ATP2C2c isoform includes most of the 24\textsuperscript{th} intron and the 24\textsuperscript{th} exon of the longer ATP2C2 isoform, a similar transcript has not been identified in mice [Figure 3.1 A]. Alignment between the human SPCA2c predicted protein sequence with the protein sequences of SPCA2 from human, mouse, rat, and dog were compared. The predicted ATG initiating codon in the human transcript is in frame with the carboxy terminus of the full-length protein. In other words, if this is a novel TSS, it still encodes a truncated SPCA2, not a completely new protein [Figure 3.1 B].

Mouse bioinformatics data does not include a transcript that starts at the same place as the transcript identified in human. However if a TSS were in a similar area, the initiating ATG would be in frame with the full-length protein. Using EBI Open Reading Frame finder a transcript corresponding to the sequence originating from the ATG found in exon 24 was predicted in mouse. Protein structure was predicted for the mouse and human truncated isoforms using the European Bioinformatics Institute-Interpro Scan program and the human protein was predicted to be approximately 28 kDa while the
mouse protein was predicted to be approximately 15 kDa. The proteins also showed highly similar predicted structure with both proteins containing a $\text{Ca}^{2+}$ ATPase domain and a signal peptide, suggesting they may localize to a membrane. The human protein contains 4 predicted transmembrane domains while that of the mouse contains only 2 transmembrane domains, though the mouse signal peptide domain appears to line up with the transmembrane domain 2 in the human protein [Figure 3.1 C and D].
Figure S1. Human and Mouse SPCA2-c protein domains predicted using European Bioinformatics Institute (EBI).

Human: KELCVGLTGSFSFTVCGTDDGAGQLP
Human: KDPGSAGGSRCRGGSQVRCGLAFSCL
Human: ALDPPFWPGRSLHSCFCRLPLHSNF

Human: ISRCLSLVESLQVEFVEDAFLRQP
Human: ELLKLCEKFLCSSPKRVMHPEDV
Dog: YLFSLGGSRLQVEFVEDAFLRQP
Dog: YLFSLGGSRLCSSPKRVMHPEDV

Human: RSVGDTILRSLIKILMSAAYIGG
Human: RSVGDTILRSLIKILMSAAYIGG
Dog: RSVGDTILRSLIKILMSAAYIGG
Dog: RSVGDTILRSLIKILMSAAYIGG

Human: TLFIPWEKFMEDRRPFTMTTFTC
Human: TLFIPWEKFMEDRRPFTMTTFTC
Dog: TLFIPWEKEKAPGRPFTMTTFTC
Dog: TLFIPWEKEKAPGRPFTMTTFTC

Mouse: TLFIPWEKEKAPGRPFTMTTFTC
Mouse: TLFIPWEKEKAPGRPFTMTTFTC

Rat: TLFIPWEFEPHTSTFTTTMFTC
Rat: TLFIPWEFEPHTSTFTTTMFTC

Ca²⁺/ATPase Domain
Signal Peptide
Transmembrane Domain
Figure 3.1 Atp2c2 gene and Secretory Pathway Ca$^{2+}$ ATPase protein

(A) Atp2c2 gene schematic, exons in black and introns in grey. (B) Atp2c2 truncated carboxy gene (Atp2c2c) (C)SPCA2 protein structure in mouse. Cation binding site in green, cation ATPase domain in purple, hydrolase domain in blue, and transmembrane domains in black. (D) Truncated SPCA2 carboxy protein (SPCA2c).
Histone modifications of Atp2c2 indicate a Novel Transcriptional Start Site

The bioinformatics data suggest that a novel transcriptional start site exists within the 23rd intron of Atp2c2. To investigate this possibility in more detail, ChIP was performed on isolated murine acinar cells for H3K4Me3 (active TSS) and H3K27Me3 (repressive) using primers for specific to the full length Atp2c2 (L) and the 23rd intron (III) in wild type and Mist1\(^{-/-}\) mice. H3K4Me3 and H3K27Me3 enrichment was examined in Mist1\(^{-/-}\) tissue to look for modifications affecting gene expression because SPCA2 is not expressed. The region upstream of the full length Atp2c2 (L) gene shows limited expression in wild-type or Mist1\(^{-/-}\) animal pancreatic acinar cells, and no enrichment of either H3K4Me3 or H3K27Me3. Conversely, H3K4Me3 enrichment was observed only in the genomic region corresponding to the intron 23 in pancreatic acinar cells of wild type animals by ChIP-PCR [Figure 3.2]. Consistent with the loss of Atp2c2c expression in Mist1\(^{-/-}\) animals, no H3K4Me3 enrichment was observed in intron 23. Enrichment of H3K27Me3 was not observed around the Atp2c2 gene in wild type mice but was observed in Mist1\(^{-/-}\) chromatin in the region of intron 23, suggesting that gene silencing of Atp2c2 occurred in these mice. These observations were supported by ChIP analysis followed by Next Generation Sequencing, which revealed little enrichment of H3K4Me3 in the region of exon 1 in both Mist1\(^{-/-}\) and wild type mice, but a robust enrichment of H3K4Me3 in the area of exon 24 only in wild type tissue. Again, in Mist1\(^{-/-}\) mice the increase in enrichment for H3K27Me3 and decreased H3K4Me3 at this region suggests gene silencing. Combined these results suggests that there is a TSS just 5' of exon 24 in
the Atp2c2 gene in pancreatic acinar cells [Figure 3.2 A]. More in depth analysis of the ENCODE ChIP-Seq data indicated that the H3K4Me3 enrichment observed in pancreatic acinar cells of wild type mice appears to be restricted to a few cell types. H3K4Me3 and H3K36Me3 enrichment were not observed in human heart, liver or cerebellum [Figure 3.3 A]. Interestingly, enrichment of H3K4Me3 was observed in erythroblasts and megakaryocytes even though the peak was shifted slightly downstream to the region of intron 25. [Figure 3.3 B]. Combined these results suggests that there is a TSS just 5' of exon 24 in the Atp2c2 gene in pancreatic acinar cells. However, the presence of H3K4Me3 enrichment alone does not confirm a TSS.
H3K4

H3K27

Atp2c2
(Full-Length Isoform)

Atp2c2-c
(Truncated Isoform)
Figure 3.2. H3K4Me3 and H3K27Me3 enrichment on Atp2c2 in pancreatic acinar cells.

(A) Chromatin Immunoprecipitation for trimethylated histone 3 at lysine 4 (H3K4Me3) followed by Next Generation Sequencing (ChIP-seq) showing peak lining up to genomic region spanning exons 23 and 24. Genomic structure is shown in blue below the ChIP-seq results. (B) ChIP PCR for full length Atp2c2 (C) ChIP PCR for truncated Atp2c2.
H3K4Me3 WT C57Bl6 Pancreatic Acinar cells

H3K4Me3 Erythroblast

H3K36Me3 Erythroblast

H3K4Me3 Megakaryocyte

H3K4Me3 Liver

H3K36Me3 Liver

H3K4 Heart

H3K4Me3 Cerebellum

A

H3K4Me3 WT C57Bl6 Pancreatic Acinar cells

H3K4Me3 Erythroblast

H3K4Me3 Megakaryocyte

B
Figure 3.3. Truncated *Atp2c2c* appears to be pancreas specific.

(A) H3K4Me3 and H3K36Me3 enrichment from ChIP-Seq on pancreatic acinar cells, erythroblasts, megakaryocytes, liver, heart and cerebellum. (B) Zoom to show enrichment for H3K4Me3 only observed at the 24 exon and 24 intron in pancreas.
Histone modifications define the TSS of *Atp2c2c* downstream of exon 24

To further validate whether the genomic region around exon 24 of the *Atp2c2* gene represents a novel TSS, other epigenetic modifications specific to TSSs were examined. ChIP was performed on whole pancreas of 4 to 5 month old C57Bl6 male mice for histone modifications H3Ac, and H3K36Me3. Enrichment for modifications was observed in the upstream region of gene corresponding to a TSS located downstream of exon 24, this verified what was seen in the ChIP-Seq [Figure 3.4]. Enrichment of H3K4Me3 and H3Ac were observed most robustly in the area of intron 23 to exon 26 [Figure 3.4 D-E]. Enrichment of H3K36Me3 was observed beginning at intron 24 and continuing down the gene [Figure 3.4 C]. ChIP-PCR and qPCR for Mist1 and RNA Pol II was performed from whole pancreas as described above. Mist1 binding to the *Atp2c2c* gene was observed in the area of intron 24, suggesting that Mist1 may regulate *Atp2c2c* expression in the pancreas. Additionally RNA Pol II enrichment was observed in the area of intron 24 suggesting this is a TSS and that general transcriptional machinery is being recruited to this region of the gene [Figure 3.5]. E-box consensus sites (CAGCTG) where Mist1 would bind are found three times in intron 24, supporting the ChIP data showing Mist1 binding in this region [Figure 3.5 C]. The location of these epigenetic modifications, in combination with the ChIP-Seq data suggests there is a novel TSS for *Atp2c2c* in pancreas located in the region just downstream of exon 24.
A

B

Wild Type

<table>
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<th>Input</th>
<th>IgG</th>
<th>H3K4</th>
<th>H3K36</th>
<th>H3Ac</th>
<th>DNA ladder</th>
</tr>
</thead>
</table>

C

Atp2c2 H3Ac

D

Atp2c2 H3K4

E

Atp2c2 H3K36
Figure 3.4. Analyses of pancreatic Atp2c2 epigenetic modifications suggesting a novel TSS in region of Exon 24.

(A) Schematic of primer sets in Atp2c2 gene (B) PCR showing enrichment of H3K4, H3K36, and H3Ac at the III primer set. (C) ChIP qPCR for H3K4 showing peak enrichment at primer set III, suggesting a TSS (D) ChIP qPCR for H3Ac showing peak enrichment at primer set III, suggestive of a TSS (E) ChIP qPCR for H3K36 showing peak enrichment at primer set III, suggestive of a TSS in this area.
Figure 3.5. Enrichment of RNA Polymerase II and MIST1.

(A) ChIP PCR with primer set III shows enrichment for RNA Pol II and MIST1 (B) ChIP qPCR for RNA Pol II and MIST1 shows enrichment in the area 54kb downstream of the pre-established TSS suggesting a novel TSS (C) Schematic of the Atp2c2 gene in the region enriched for MIST1, with arrows indicating E-box consensus sites for MIST1 binding, red arrows indicate CAGCTG E-boxes which has been shown to be MIST1 preferred.
1.20 Expression of Spca2c

Expression of \textit{Atp2c2c} in vitro

To investigate whether the region around exon 24 represents a novel TSS I assessed the ability of the region to drive expression. I approached this in two ways. The first was to generate a construct containing a 2286 bp region upstream of the putative ATG initiation codon located in exon 24, which encompasses a potential promoter, and place this upstream of exons 24 to 27 with introns removed. The resulting construct \((pMK-2286/+1544\ ATG-FLAG-SPCA2c)\) also places a FLAG antigenic tag just before the stop codon \([\text{Figure 3.6 A}]\). \(pMK-2286/+1544\ ATG-FLAG-SPCA2c\) was transfected into AR42J (acinar-like) and HEK293A (non-acinar) cells alone or co-transfected with \(pcDNA3.0-MIST1-VP16-HA\), which contains the VP16 transcriptional activation domain allowing Mist1 to bind to and activate transcriptional complexes. Expression of SPCA2c was observed in AR42J cells when transfected alone or with enhanced MIST1 accumulation \([\text{Figure 3.6 B}]\). Unexpectedly, similar transfections in HEK293A resulted in SPCA2 accumulation, with expression of SPCA2 slightly increased in the presence of MIST1 \([\text{Figure 3.6 C}]\). Analyses of SPCA2 expression by Western blot in AR42J cells showed expression of Spca2 in the 17 to 20 kDa size expected. Expression of this protein appeared to increase slightly with \(pMK-2286/+1544\ ATG-FLAG-SPCA2c\) compared to untransfected and \(pcDNA3.0\)-GFP transfected cells. No shift was observed in the size of this protein suggesting that this construct was resulting in expression of a similar Spca2 to that expressed in the acinar cell endogenously. This protein was identified with and without Mist1 overexpression \([\text{Figure 3.6 D}]\). The IF and protein expression both suggest
that the region upstream of ATG contains a TSS and that this region has promoter activity allowing it to express a protein that is the correct size and recognized by a SPCA2 antibody.
B

<table>
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<tr>
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<td><img src="spacar2c_spacar2c.png" alt="Image" /></td>
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Figure 3.6. Expression of -2286/+1544 ATG FLAG SPCA2c.

(A) -2286/+1544 ATG SPCA2c FLAG construct schematic. IF of cells untransfected or transfected with: pMK -2286/+1544- AT- FLAG-SPCA2c (SPCA2c), pcDNA3.0-MIST1-VP16-HA (Mist1), or both pMK -2286/+1544- AT- FLAG-SPCA2c and pcDNA3.0-MIST1-VP16-HA for expression of SPCA2c and Mist1 (green) and merged with DAPI in (B) AR42J cells (scale bar = 25 μm) and (C) HEK293A cells (scale bar = 25 μm) (D) Western blot for SPCA2 and Mist1 and Actin (loading control) on AR42J cells transfected as above or with pcDNA3.0-Green Fluorescent Protein.
The *Atp2c2* promoter is located downstream of exon 24 ATG

The second approach to validating this novel *Atp2c2* TSS was to generate constructs in which the genomic regions upstream of the putative ATG initiation codon were placed in front of luciferase reporter genes. Constructs were designed to contain decreasing lengths of sequence upstream of the ATG in exon 24 to identify regions of the promoter that may contain important regulatory elements. Constructs were named according to the length of sequence they contained upstream of the ATG in exon 24 and as a control a similar construct containing sequence found upstream of an ATG located in exon 23 was also designed. The following constructs were created: *pGL3−1238 ATG 24-Promoter*, *pGL3−619 ATG 24-Promoter*, *pGL3−309 ATG 24-Promoter*, and *pGL3-Alternate −1238 ATG 23-Promoter* [Figure 3.8]. In non-acinar HEK293 cells, construct *pGL3−619 ATG 24-Promoter* shows the greatest luciferase activity in the presence of Mist1. This demonstrates that this sequence has the strongest promoter activity and that Mist1 plays a role in activating expression since it displays significantly greater expression than all of the other constructs and significantly greater expression in the presence of Mist1 compared to its absence [Figure 3.9 A].

All of the constructs containing sequence upstream of ATG in exon 24 showed luciferase activity. HEK293A cells show greater luciferase expression overall, likely due to their better transfection efficiency. In HEK293A cells *pGL3−619 ATG 24-Promoter* and *pGL3−309 ATG 24-Promoter* show the greatest luciferase expression compared to the other constructs. However these constructs do not differ significantly from one another suggesting they both have strong promoter ability. The addition of Mist1 to these
constructs expression also appears to have no significant effect on expression. Interestingly the pGL3-Alternate –1238 ATG 23-Promoter has significantly decreased promoter activity compared to both the -619 and -309 constructs, and even significantly lower expression than the negative control pGL3-Basic, suggesting that perhaps this region may contain a repressor domain [Figure 3.9 B]. In AR42J cells none of the constructs showed significant expression compared to the negative control. However in the presence of Mist1 construct pGL3–619 ATG 24-Promoter luciferase expression was significantly higher than control and all other constructs in both with and without Mist1 [Figure 3.9 C]. These results suggest that the region upstream (5′) of exon 24 shows promoter activity in AR42J and HEK293A cells, and in AR42J cells the addition of Mist1 may enhance activity.
A

XXIII → XXIV → XXV

-1238 ATG Promoter
-619 ATG Promoter
-309 ATG Promoter
-1238 Alt. ATG Promoter
B

AR42J

Relative Luciferase Expression

-1238 ATG  -619 ATG  -309 ATG  Alt-ATG 23  pGL3-Basic

-N=3

-M1

+M1
C

**Hek293A**

Relative Luciferase Expression

-1238 ATG
-619 ATG
-309 ATG
Alt-ATG 23
pGL3-Basic

N=3

- M1
+ M1
Figure 3.7. Relative Luciferase Expression of Atp2c2c promoter constructs.

(A) Gene Schematic showing regions contained in luciferase ATG promoter constructs. Construct numbering refers to base pair position relative to the ATG in exon 24. Alternate ATG promoter contains 1238 base pair region upstream of the ATG located in exon 23. Luciferase promoter activity of constructs in (B) AR42J cells and (C) HEK293A cells. Luciferase expression is a measure of firefly luciferase normalized to renilla luciferase expression, this ratio is then normalized to the negative control pGL3-Basic luciferase expression.

* P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001
DISCUSSION
1.21 Brief Summary of Conclusions

The experiments conducted in this thesis came to four specific conclusions:

1. Identification of a novel transcriptional start site for Atp2c2 in mice.
2. This transcriptional start site for Atp2c2c is limited to a subset of tissues.
3. Determined that 619 bp is required for at least some expression from this TSS.
4. MIST1 binds to the Atp2c2c gene near to this novel TSS in pancreatic tissue. In its absence, the Atp2c2 gene becomes enriched for silencing marks.

1.22 Summary

Pancreatic acinar cells must highly regulate Ca\textsuperscript{2+} levels to allow for proper secretion of digestive enzymes. They control Ca\textsuperscript{2+} levels through store operated Ca\textsuperscript{2+} and Ca\textsuperscript{2+} ATPases. This thesis describes the identification of a novel TSS for Atp2c2 and identification of MIST1 as a regulator of Atp2c2c expression in pancreatic acinar cells. Bioinformatics analyses identified a potential difference between human and mouse Atp2c2c and subsequently predicted SPCA2c. Bioinformatics analysis also suggests that expression of Atp2c2c is restricted to pancreas, with the possible exception of megakaryocytes and erythroblasts. The TSS of this isoform was also identified as being in the area of exon 24 by enrichment of histone marks indicating transcriptional activation. This region was further confirmed to have promoter activity through luciferase assays and MIST1 was shown to enhance promoter activation \textit{in vitro}. ChIP also confirmed that MIST1 was binding to the Atp2c2 gene in the region of the novel TSS, in addition to RNA Pol II binding. This strongly suggests there is transcriptional activity of this region. Therefore Atp2c2c is a novel isoform of Atp2c2, which, may be regulated by
MIST1. Understanding the transcriptional regulation of this gene is important for defining the full length of Spca2c and understanding how Spca2c expression is altered during pancreatitis. Additionally understanding how \textit{Atp2c2} is transcriptionally regulated in the pancreas allows it to be targeted for ablation. The ablation of \textit{Atp2c2c} will provide insight into whether aspects of the \textit{Mist1} \textsuperscript{−/−} phenotype result from the loss of \textit{Atp2c2c}. In particular the dysfunction in regulating cytoplasmic Ca\textsuperscript{2+} levels and disruptions in regulated exocytosis might be the result of lost \textit{Atp2c2c} expression, giving insight into its function and role in pancreatic pathology (Garside et al., 2010; Pin et al., 2001).

To date the \textit{Atp2c2} gene has not been targeted for ablation though it is being increasingly recognized as an important player in Ca\textsuperscript{2+} regulation of secretory cells, and as a potential contributor to pathological processes such as pancreatitis and tumorigenesis (Capoccia et al., 2011; Cross et al., 2013; M.-Y. Feng & Rao, 2013; M. Feng et al., 2011; Garside et al., 2010).

\textbf{1.23 Identification of a novel transcriptional start site for \textit{Atp2c2} in mice.}

Previous experiments from our lab identified a truncated SPCA2c protein, which could be the result of either alternative splicing or an alternative TSS. By identifying histone modification patterns and luciferase promoter analysis \textit{Atp2c2c} appears to be the result of a novel TSS. Enrichment of H3K4Me3, H3K36Me3 and H3Ac are all found in the area of the ATG located in exon 24 demonstrating that this region is likely a TSS, though transcriptional activity is not guaranteed by enrichment of these marks (Zentner & Henikoff, 2013). Mist1 and RNA Pol II are also shown to bind in this region suggesting transcriptional machinery is being recruited to this area [\textbf{Figure 4.1}]. Mist1 binding in
this area was also observed by ChIP-Seq in mouse pancreas, which identified Mist1 binding 54 kilo bases downstream of the full-length Atp2c2 TSS (Direnzo et al., 2012).

Additionally luciferase assays confirmed that the region upstream of the ATG in exon 24 demonstrates promoter activity in both AR42J and HEK293A cells. It is curious that this promoter was active in HEK293A cells, which would not be expected to express a pancreas specific gene. Though the HEK293A cells did not show increased activity in response to MIST1, suggesting the expression might be mediated by other factors in this cell line. The construct \( pMK−2286/+1544 \) containing a 2286 bp region upstream of the ATG in exon 24 followed by the sequence of exons 24-27 was able to produce a protein of equal size to the endogenous protein. Unfortunately this protein could not be detected using the FLAG tag because the FLAG tag in the construct was designed to express as DYKDDDK for recognition by a discontinued Millipore antibody (Cat. # MAB3118, Lot # JC1629909). Currently available FLAG antibodies recognize the DYKDDDDK sequence and will not recognize the above sequence. This protein was recognized by SPCA2 antibody suggesting it is the same protein but in future experiments it would be of interest to modify the FLAG sequence in this construct so expression can be definitively attributed to expression of the construct. This supports the existence of TSS and an active promoter in this area. Identification of the promoter of Atp2c2c is important to delineate SPCA2 protein structure in the pancreas and to allow Atp2c2c to be targeted for ablation, so we can begin to elucidate its function.

H3K27Me3 enrichment on Atp2c2 was observed in Mist1\(^{-/-}\) acinar cells, suggesting Atp2c2 is silenced in the absence of Mist1. Future experiments should include investigating whether other silencing marks such as H3K9Me3 are enriched in this area
as well (Rye et al., 2011). In addition to whether knock-down of MIST1 would cause
silencing of \textit{Atp2c2} similar to what is observed in Mist1\(^{-/-}\) animals (Garside et al., 2010).
Figure 4.1 Overview of Atp2c2-c Epigenetic Modifications, RNA Pol II, and MIST1 Enrichment.
1.24 *Atp2c2c* expression in human

The carboxy terminus of SPCA2 appears to be quite highly conserved between species. An isoform similar to mouse *Atp2c2c* appears to be present in human but this isoform begins in the intronic region between exon 24 and 25. It is unlikely that the mouse isoform includes this intronic sequence because in the mouse genome there is no ATG codon that would give a functional protein. The addition of this intronic region results in a predicted protein that contains an additional 80 amino acids at the amino terminus in the human, which are not found in the mouse. Despite this difference in sequence the structure and protein domains found in mouse and human, SPCA2c appear to be highly similar, suggesting that these isoforms may share a similar function. Future experiments should aim to identify the expression of this transcript in human secretory tissues as this transcript was identified as part of an effort to identify novel secreted proteins in the human genome using cDNA libraries and has not been confirmed to produce a protein or to exist *in vivo* (Clark et al., 2003). Preliminary efforts were made in our laboratory to identify this transcript in human pancreas and in human H1 embryonic stem cells, but the results were inconclusive. Given recent findings for the role of SPCA2 in lactating mammary gland I think investigating this tissue for *ATP2C2* expression can provide additional information on *ATP2C2* gene regulation (M.-Y. Feng & Rao, 2013; M. Feng et al., 2011). It is important to identify this *ATP2C2* human transcript and investigate whether it produces a protein product and if so to determine its size, tissue expression, and cellular localization in order to understand the role it may play in Ca^{2+} regulation and pancreatic pathology.

Interestingly when sequence consensus was investigated, the sequence that
appears to differ the most among the species is that of the canine. In dogs the methionine (ATG) that is found in rat, human, and mouse is not conserved. This is of interest because dogs are particularly sensitive to pancreatitis. This leads to the hypothesis that SPCA2c may have a role that is protective against injury in pancreatic acinar tissue. Preliminary results in our laboratory also suggest that Atp2c2 expression is decreased during injury, though this remains to be definitively confirmed. Prolonged SPCA2 activity, also results in constitutively high cytoplasmic Ca\(^{2+}\) levels in breast cancer cells, promoting tumorigenesis and proliferation (M. Feng et al., 2011). These findings suggest that excessive activity by Spca2 is damaging. Considering these findings on the effects of over activity in addition to preliminary observations of the effects of reduced Atp2c2 expression, it seems that Atp2c2 expression must be tightly regulated and maintained in the physiological range. Any deviation from this in either direction has pathological effects on the cells ability to maintain basal cytoplasmic Ca\(^{2+}\) levels leading to pathology such as tissue disorganization and increased proliferation (M.-Y. Feng & Rao, 2013; M. Feng et al., 2011; Garside et al., 2010). The requirement for strict regulation of Atp2c2 expression would not be surprising, as it is well known that the cell must strictly control Ca\(^{2+}\) levels (Clapham, 2007).

1.25 Atp2c2c expression is limited to a subset of tissues

Expression of Atp2c2 has been previously characterized as restricted to secretory tissues such as pancreas, lactating mammary gland, testes, and salivary glands (Garside et al., 2010). Though Atp2c2c expression has been observed only in pancreatic acinar cells thus far ENCODE ChIP-Seq data suggest that this isoform or a similar isoform may also
be expressed in human megakaryocytes and erythroblasts. While the ENCODE ChIP-Seq data available to date does not include data obtained from a cell type with high secretory function, it is interesting to note that megakaryocytes and erythroblasts have been shown to have some secretory function. They secrete cytokines, growth factors, and chemokines involved in regulating hematopoiesis (Majka, 2001). This suggests a role for Atp2c2c expression in megakaryocytes and erythroblasts because SPCA2c has been shown to play a role in regulating Ca\(^{2+}\) for secretion in the lactating mammary gland and likely in pancreatic acinar cells (Cross et al., 2013; Garside et al., 2010).

These findings suggest that SPCA2c expression may exist in other secretory cell types and enable cells with robust secretory function to regulate Ca\(^{2+}\) levels, though this requires further investigation. While the expression of SPCA2c has not been identified in cell types other than pancreatic acinar cells, this suggests SPCA2c expression should be explored in other cell types with secretory function.

It remains to be investigated whether SPCA2 functions similarly in different cell types. In the lactating mammary gland SPCA2 has been shown to interact the store operated Ca\(^{2+}\) channel Orai1 at the basolateral membrane, to mediate Ca\(^{2+}\) influx that is independent of Golgi and ER stores. In these mammary cells, SPCA2 is localized to the perinuclear region, similar to the localization of SPCA2 in pancreatic acinar cells. In the mammary gland however SPCA2 also shows some punctate localization to the plasma membrane. At the plasma membrane SPCA2 colocalizes with Orai1 to mediate Ca\(^{2+}\) influx but it appears that the full length SPCA2 is more effective in interacting with Orai1 than is the SPCA2 carboxy terminus alone (Cross et al., 2013; M. Feng et al., 2011). Unpublished results from Feng and Rao however suggest that the carboxy terminus of
SPCA2 alone is able to interact effectively with Orai1 (M.-Y. Feng & Rao, 2013). Orai1 has been shown to colocalize with STIM1 at the basal membrane in pancreatic acinar cells. However the majority of Orai1 found in acinar cells is located at the apical membrane and the role of Orai1 here is controversial. Hong et al. found Orai1 and STIM1 localizing at the apical membrane while Dingsdale et al. did not observe this interaction with STIM1 (Dingsdale et al., 2012; Hong et al., 2011). These results suggest that SPCA2c could possibly interact with Orai1 if it is able to localize to the plasma membrane. Interestingly it has been shown that movement to the plasma membrane of both Orai1 and SPCA2, from the perinuclear or ER region, requires Orai1 and SPCA2 interaction (Cross et al., 2013). It however remains to be elucidated whether Orai1 and SPCA2 interact in pancreatic acinar cells. Orai1 localization in pancreatic acinar cells suggests that if Orai1 and SPCA2 did interact it would likely be at the apical membrane where the bulk of Orai1 is localized, though this seems unlikely because SPCA2 is localized to the basolateral perinuclear region in acinar cells (Garside et al., 2010). Additionally, it has been suggested that SOCE and SICE may coexist, so it is possible that at the basolateral membrane some Orai1 could be interacting with SPCA2 and some with STIM1, allowing it to function in both a store operated dependent and independent manner to regulate Ca^{2+} levels (M.-Y. Feng & Rao, 2013). In conclusion whether SPCA2 colocalizes with Orai1 in the pancreas should be investigated, though this seems unlikely since SPCA2 is basolaterally located while Orai1 is largely apically located. It is possible that pancreatic SPCA2 could also interact with other channels to mediate Ca^{2+} levels and that its functions differs between pancreas and mammary gland. Future studies should aim to provide insight into SPCA2’s function in the pancreas.
1.26 Mist1 regulates Atp2c2c expression

MIST1 is a bHLH TF expressed in secretory tissues. MIST1 is necessary for development of secretory cells, as it regulates expression of a set of genes necessary for secretory function (Capoccia et al., 2011; Pin et al., 2001). Mice carrying a targeted ablation of the Mist1 gene (Mist1\(^{-/-}\)) show disorganization of pancreatic acinar tissue, disruptions in Ca\(^{2+}\) handling, and defects in regulated exocytosis. Previous work in our lab suggests that the disruptions in Ca\(^{2+}\) handling may be attributed to loss of SPCA2, as it is the only Ca\(^{2+}\) ATPase, which loses expression in the absence of Mist1. Interestingly the primary SPCA expressed in pancreas appears to be a novel isoform of the Atp2c2 gene consisting of the 3’ end of the gene, entitled Atp2c2c (Garside et al., 2010). In this thesis, through ChIP-qPCR and luciferase assays it has been shown that MIST1 may regulate Atp2c2c expression. Binding of MIST1 to Atp2c2 was observed in the region of exon 24, in the same area that binding of RNA Pol II was identified, suggesting that MIST1 is playing a role in activating gene transcription of Atp2c2c. A robust enrichment of the activation mark H3K4Me3 was also observed in this region of wild type mice, and this enrichment was lost and replaced with enrichment of the repressive mark H3K27Me3 in Mist1\(^{-/-}\) mice. This further supports the idea that MIST1 is a regulator of Atp2c2c expression.

Interestingly expression of MIST1 has been shown to play a role in differentiation of plasma cells in mouse and human downstream of XBP1. XBP1 also activates MIST1 expression in pancreatic acinar cells and other secretory cells. Plasma cells also have a secretory function and MIST1 plays a role in activating expression in plasma cells of the
same secretory associated genes, as MIST1 activates in the secretory Chief Cells of the stomach (Capoccia et al., 2011). Rab3d and Rab26 have also been observed to be downstream transcriptional targets of MIST1, which are involved in vesicular secretion in regulated exocytosis by Chief cells and show similar changes in expression when MIST1 is lost as those observed in *Atp2c2* (Garside et al., 2010; Tian et al., 2010). Therefore *Atp2c2c* appears to be regulated by MIST1 though this is likely not the only regulator. Future experiments should aim to identify other potential regulators of *Atp2c2* and explore where this gene is regulated differently in different tissues.

In conclusion, I have identified the TSS of *Atp2c2c* in pancreatic acinar cells and confirmed that Mist1 may be a regulator of *Atp2c2c* expression. It is important to understand how Ca$^{2+}$ is regulated in pancreatic acinar cells because aberrant Ca$^{2+}$ regulation has been associated with increased susceptibility and severity of pancreatic diseases (Voronina & Tepikin, 2012). Pancreatitis and pancreatic ductal adenocarcinoma severely reduce quality of life and life expectancy and insight into their potential mechanisms is crucial for improving outcomes (Anderson, Cotterchio, & Gallinger, 2009).
REFERENCES


Appendix
1.27 Chromatin Immunoprecipitation Wash Buffers

SDS Lysis Buffer
1% SDS
10mM EDTA
50mM Tris pH 8.1

Dilution Buffer
0.5% Triton X-100
2mM EDTA
20mM Tris pH 8.1
500mM NaCl

Low Salt Wash Buffer
0.1% SDS
1% Triton X-100
2mM EDTA
20mM Tris pH 8.1
150mM NaCl

High Salt Wash Buffer
0.1% SDS
1% Triton X-100
2mM EDTA
20mM Tris HCl pH 8.1
500mM NaCl

LiCl Wash Buffer
0.25M LiCl
1% IGEPAL (octylphenoxypolyethoxyethanol)
1% deoxycholate Na
1mM EDTA
10mM Tris pH 8.1

TE Wash Buffer
10mM Tris pH 8.1
1mM EDTA
1.28 Primer Sequences for ChIP-PCR and ChIP-qPCR

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Education

Masters of Science January 2012-January 2014
Pharmacology & Toxicology
Developmental Biology Collaborative Program
The Atp2c2 Gene as Transcribed from a Novel Transcriptional Start Site in Pancreatic Acinar Cells
Supervisor: Dr. Christopher Pin
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B. HSc Bachelor of Health Science Honors (4 year) April 2011
University of Western Ontario, London, ON
Major in Health Sciences
Major in Pharmacology

Certificate in Ethics April 2011
University of Western Ontario, London, ON
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Awards

Graduate Student Award January 2012-January 2013
Department of Paediatrics Graduate Studentship Award
University of Western Ontario

Poster Presentations

- Sullivan, Caitlin M., Mehmood, Rashid, & Pin, Christopher L., (October 2013)
The Atp2c2 gene as transcribed from a novel transcriptional start site in pancreatic acini. Poster presented at the American Pancreatic Association Annual Meeting, Miami, FL, USA.

- Sullivan, Caitlin M., Mehmood, Rashid, & Pin, Christopher L., (May 2013)
Characterization of the Atp2c2 Gene in Pancreatic Acinar Cells II. Poster
presented at the University of Western Ontario Developmental Biology Research Day, London, ON, CA.


**Teaching Experience**

- **Teaching Assistant**  
  2130 Human Physiology  
  Professor: Dr. John Ryan Gillespie  
  May 2013-Aug 2013

- **Teaching Assistant**  
  2130 Human Physiology  
  Professor: Dr. Sarah McLean  
  May 2012- Aug 2012