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WASHINGTON UNIVERSITY IN ST. LOUIS
Division of Biology and Biomedical Sciences
Molecular Cell Biology

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Transcriptional Regulation of the Endoplasmic Reticulum in Dedicated Secretory Cells

by

Benjamin D. Moore

A dissertation presented to the
Graduate School of Arts & Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

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Benjamin D. Moore

Dedicated to Dr. Greg Sibbel

ABSTRACT OF THE DISSERTATION

Transcriptional Regulation of the Endoplasmic Reticulum in

Dedicated Secretory Cells

by

Benjamin D. Moore

Doctor of Philosophy in Biology and Biomedical Sciences

Molecular Cell Biology

Washington University in St. Louis, 2015

Professor Jason Mills, Chair

Differentiating cells express subsets of genes to build the cellular machinery necessary to perform their specific function as they acquire their mature fate. These subsets of genes are regulated by networks of transcription factors as cells progress through their developmental program. In secretory tissue, highly-specialized cells establish an extensive secretory apparatus and scale up their cellular architecture to facilitate the production and secretion of large amounts of protein. Here, we identify a network of transcription factors required for the development of this cellular machinery in these cells, and develop new tools to elucidate the molecular networks that control the differentiation of secretory cell lineages.

We show that the expression of *Xbp1*, a transcription factor responsible for establishing and maintaining the ER in gastric zymogenic (chief) cells (ZCs), is enhanced by HNF4α. HNF4α directly binds the *Xbp1* promoter and is sufficient to enhance its expression in gastric epithelial cells. We observe that loss of HNF4α disrupts normal differentiation in the gastric epithelium,

and causes morphological changes similar to the loss of XBP1, suggesting it acts through *Xbp1* to maintain homeostasis in the mouse stomach.

To facilitate the study of the molecular underpinnings of ZC differentiation, we sought to develop a technique to isolate pure ZC populations from the gastric mucosa. Using a microarray-based screen, we identified ANPEP as a surface marker of mature ZCs that enabled us to sort homogenous ZC populations using flow cytometry. We found that ANPEP is rapidly lost as ZCs dedifferentiate in response to damage or disease in both mouse and human models, and our improved method allowed us to use flow cytometry to quantify these molecular changes to the ZC surface.

We found that HNF4α is required for maintenance of the gastric epithelium, but it has also been well-established that HNF4α plays a pivotal role in pancreatic beta-cell function. Mutations in HNF4α cause a prevalent subset of inheritable diabetes, MODYI. However, the mechanism through which mutated HNF4α causes beta-cell dysfunction has not yet been established. Our data shows that HNF4α is required for expression of both *Xbp1*, and downstream targets of XBP1, and that loss of HNF4α leads to diminished ER morphology in mouse beta-cells. We show data suggesting that the loss of glucose-stimulated insulin secretion in MODYI may be due to impaired calcium signaling from the ER, and that restoring *Xbp1* expression is sufficient to rescue insulin secretion in HNF4α-null beta-cells.

Taken together, the data presented below characterizes an important new transcriptional relationship in both the stomach and endocrine pancreas, and establishes new tools for the study of secretory cells. These results illustrate how the study of a basic transcriptional relationship can have broad implications in the development and treatment of prevalent human diseases.

CHAPTER ONE

Preface

Introduction

Mature secretory cells require extensive cellular machinery to synthesize, process, and secrete large amounts of proteins. In contrast, the progenitor cells that give rise to professional secretory cells are relatively smaller and less complex. The establishment and maintenance of this robust secretory apparatus is coordinated at a molecular level by signaling networks that activate or repress transcription factors (TFs); which in turn sculpt the differentiation landscape of each cell by tightly controlling gene expression. The following will define the unique role of a novel transcriptional cascade required for the development of mature secretory cells, describe an exciting new tool to study these cells, and finally show the consequences of disrupting this regulatory pathway in human disease (Fig 1).

The Secretory Pathway

Professional secretory cells are highly-specialized to secrete incredible amounts of protein. The pancreatic beta cell, for instance, secretes over one million molecules of insulin per minute when stimulated by glucose.(Henquin and Meissner, 1984) Expression of genes encoding proteins that function in the secretory pathway are tightly regulated during specific stages of cell differentiation.(Coutinho et al., 2004; Dunne et al., 2002; Schotman et al., 2009) This leads to a substantial increase in subcellular resources i.e. organelles. For instance the Golgi apparatus increases in volume during secretory cell maturation, enabling it to process, sort, and package polypeptides more efficiently. (Guo and Linstedt, 2006; Jackson, 2009) Intuitively, intracellular stores of secretory vesicles are also significantly increased in terminally differentiated secretory cells.(Boquist, 1970; Caro and Palade, 1964) Arguably the most important feature of secretory cells is a vast, highly-developed endoplasmic reticulum (ER). Secretory and transmembrane

proteins are synthesized and undergo post-translational modifications in the ER.(Palade, 1956) To accommodate the increase in cargo load during differentiation, a transcriptional cascade propagates both an increase in size and a change in composition of the ER.(Farhan et al., 2008; Forster et al., 2006; Iwakoshi et al., 2003) The well-regulated development and maintenance of the ER in the mature secretory cell is paramount to both form and function.

Maintenance of ER Homeostasis

Formation of the vast ER network required by secretory cells is regulated both developmentally and acutely, in response to ER stress. Synthesis, processing, and transport from the ER to the Golgi apparatus is the rate limiting step in secretion for many proteins. When the flux of nascent polypetides into the ER exceeds the capacity of the ER to fold, process, and transport protein to the Golgi, the acute- unfolded protein response (UPR) pathway is activated. The UPR increases the biosynthetic pathway and decreases the burden on the ER, maintaining homeostasis.(Selye, 1985) To increase the folding capacity of the ER, the UPR spurs an increase in the synthesis of molecular chaperones and foldases in the ER lumen,(Kozutsumi et al., 1988) and induces ER biogenesis, increasing the volume of the ER to dilute the increased unfolded cargo.(Cox et al., 1997; Dorner et al., 1989) The UPR also alleviates ER stress by decreasing the biosynthetic load, downregulating the transcription and translation of genes that encode secretory proteins (Harding et al., 1999) and removing misfolded proteins through ER-associated degradation (ERAD).(Travers et al., 2000) While this acute response to an increased cargo load is very well studied, the developmental regulation of the ER remains poorly understood.

XBP1 and the ER

A principal component of the UPR pathway is the bZIP TF, X-box binding protein 1 (XBP1, first identified in yeast as HAC1p). (Cox and Walter, 1996) Canonically, XBP1 activity is activated via a post-transcriptional splice event. Accumulation of unfolded/misfolded proteins activates the ER transmembrane endonuclease inositol requiring kinase 1 (IRE1).(Liu et al., 2002) Activated IRE1 splices a small intron from *Xbp1* mRNA, allowing translation of functional XBP1 protein (XBP1s) and subsequent translocation to the nucleus where it activates a large network of genes that encode proteins vital for ER biogenesis, protein folding, and formation of the cell's secretory apparatus(Fig. 2). (Acosta-Alvear et al., 2007) While the regulation of XBP1 in acute ER stress conditions has been extensively studied, regulation and function of XBP1 in the development and maintenance of the ER is not well defined. The transcriptional regulation of *Xbp1* may be as important to its role in secretory cell development as its activation by IRE1. *Xbp1* expression is significantly elevated in secretory cells, and large pools of unspliced *Xbp1* mRNA are required to restore homeostasis in chronic ER stress conditions, e.g. the large biosynthetic load of professional secretory proteins.(Ogawa and Mori, 2004) Additionally, IRE1 is basally activated in dedicated secretory cells at levels comparable to acute ER stress in non-secretory cells (>40%).(Yang et al., 2010) When the presumably UPR-activating biosynthetic load-stimulus is removed in B-cells differentiating to plasma cells by disrupting expression of IgM, XBP1 is still activated, indicating that XBP1 activation is differentiation-dependent rather than UPR-dependent in secretory cells.(Bonnefous et al., 2009) Together, these data suggest that transcription of *Xbp1* is the rate-limiting step in its activation during the differentiation of secretory cells.

Regulation of Secretory Cell Differentiation by XBP1

XBP1 has been characterized as an important transcriptional regulator of the differentiation of dedicated secretory cells in the pancreas, stomach, and intestine.(Huh et al., 2010; Kaser et al., 2008; Lee et al., 2005; Lee et al., 2011) As expected, because of its developmental regulation and its role in cell-differentiation, XBP1 has been shown to act in a developmental transcriptional cascade in secretory cells unrelated to ER function. XBP1 directly enhances the transcription of the TF *Mist1* in secretory tissue.(Huh et al., 2010) MIST1 is a TF that regulates a subset of genes encoding proteins that scale up cellular machinery and architecture dedicated secretory cells.(Pin et al., 2001) Loss of *Mist1* in gastric or pancreatic acinar cells leads to reduced cell and secretory granule size, altered subcellular organization, and disruption of the downstream secretory apparatus,(Jin and Mills, 2014; Ramsey et al., 2007; Zhu et al., 2004) however, MIST1 has no known role in ER function. The regulation of *Mist1* by XBP1 shows that XBP1 is not only a main component of the UPR pathway, but also an important factor in the differentiation of professional secretory cells.

Despite the significance of the transcriptional regulation of *Xbp1* in the secretory cell, and the key role XBP1 plays in development, little is known about what regulates *Xbp1* expression. In ER stress conditions, the TF ATF6 is activated and able to enhance *Xbp1* expression, (Lee et al., 2002) and XBP1 can synergistically stimulate its own expression, (Ogawa and Mori, 2004) though this regulatory pathway is likely more important to the UPR, and not to developmental patterning. In plasma cells, the TF *Blimp1* is required for XBP1 expression, and activation of this transcriptional cascade enlarges the ER and secretory apparatus.(Shaffer et al., 2004) Recently, the TF *Hnf1a* has been shown to enhance *Xbp1* expression in pancreatic beta-cells.(Kirkpatrick

et al., 2011) Delineating the mechanism of *Xbp1* regulation in secretory cells will lead to a better understanding of cell differentiation and homeostasis in both normal and pathological states.

Secretory Cell Differentiation in the Gastric Epithelium

Disruption of secretory cell development and homeostasis underlies many prevalent diseases and malignancies. Understanding the molecular pathways and signaling networks that guide normal secretory cell differentiation and how these pathways are altered in disease states will lead open new avenues to the treatment and prevention of myriad pathologies. In the following chapters, I characterize the developmental role of a novel transcriptional cascade, focusing on dedicated secretory cells in two distinct tissue; first the stomach, and finally the pancreas. The professional secretory cell in the gastric epithelium is the zymogenic (chief) cell (ZC).(Karam and Leblond, 1993) Damage or disease to the stomach leads to a rapid dedifferentiation event in ZCs, where these formally terminally differentiated secretory cells begin to re-express progenitor markers, disassemble their secretory machinery through autophagy, and re-enter the proliferative cycle.(Aikou et al., 2009; Nam et al., 2010; Nozaki et al., 2008) In environments of chronic stress in the gastric epithelium, this aberrant dedifferentiation leads to precancerous metaplasia.(Goldenring et al., 2010) Defining the molecular pathways that orchestrate the differentiation and maintenance of normal ZCs is key to demarcating the factors that drive the formation of this metaplasia, and may uncover the key to slowing or reversing the progression to gastric cancer.

Xbp1 is one of the few known transcriptional regulators of ZC development. Loss of *Xbp1* in mouse adult gastric corpus epithelium leads to a collapse of the ZC architecture; the ER degrades from a tightly-packed, well organized network to a sparse, free structure, *Mist1*

(another TF important to ZC differentiation)(Ramsey et al., 2007) expression is no longer induced, and new ZCs fail to mature from precursor cells.(Huh et al., 2010) Because of the indispensable role of *Xbp1* in ZC development, and the evidence discussed above demonstrating the importance of the transcriptional regulation of *Xbp1*, I sought to identify a TF that directly regulates *Xbp1* expression. In "Chapter 1", I provide evidence that hepatic nuclear factor 4-alpha (HNF4α) is a direct regulator of *Xbp1*, and characterize its role in the gastric epithelium. The novel transcriptional regulation of *Xbp1* by HNF4α is the cornerstone of this thesis, and has many important implications in the field of secretory cell development.

The Master Developmental Regulator, HNF4α

The nuclear hormone receptor HNF4α is a master developmental regulator of several secretory tissues. Loss of *Hnf4α* results in embryonic lethality in mouse models, as it is required for proper gastrulation.(Chen et al., 1994) Tissue-specific knockouts of the *Hnf4α* gene have established its importance in the development and maintenance of many secretory tissues. HNF4α is required for both the development of the intestine,(Babeu et al., 2009; Garrison et al., 2006) and maintenance of its secretory cell-homeostasis, architecture, and function.(Cattin et al., 2009) Interestingly, human patients with inflammatory bowel (Crohn's) disease have low levels of HNF4α expression, and loss of HNF4α has been used as a model of colitis in mice.(Ahn et al., 2008; Darsigny et al., 2009) Similarly, tissue-specific knockouts have shown that HNF4α is a crucial component in the initial embryonic development and maintenance of the adult liver.(Battle et al., 2006; Hayhurst et al., 2001; Parviz et al., 2003) In the adult pancreatic beta-cells, HNF4α is required for proper secretion of insulin in response to glucose.(Gupta et al., 2005; Miura et al., 2006) Correspondingly, mutations in HNF4α in human patients have been shown to cause a subset of diabetes called Mature-Onset Diabetes of the Young Type-1 (an

aspect of HNF4α biology that will be described at length below).(Yamagata et al., 1996) The fundamental role of HNF4α in these tissues and human disease has led to an incredible wealth of studies defining its structure,(Chandra et al., 2013) preferred binding sequences and partners,(Fang et al., 2012; Misawa et al., 2003) expression variants,(Drewes et al., 1996) and its regulatory interplay with other related transcription factors.(Odom et al., 2004) Despite these efforts, the molecular mechanisms through which HNF4α maintains secretory cell identity and function are not well understood. Additionally, despite the high level of *Hnf4α* expression in the gastric epithelium and its pivotal role in the maintenance of closely related secretory tissue e.g. the intestine and pancreas,(Dean et al., 2010) the function of HNF4α has never been explored in the stomach. In chapter 1, I present data suggesting that HNF4α acts through enhancing *Xbp1* expression to maintain secretory cell identity, and that HNF4α is required for the maintenance of the gastric epithelium.

Gastric cancer is the third-leading cause of cancer-related mortalities worldwide.(Ferlay et al., 2015) However, the events that drive the progression from a healthy gastric epithelium, to precancerous metaplasia, and finally to the development of adenocarcinoma are not well understood. A major obstacle to elucidating the molecular underpinnings of secretory cell development in the normal and metaplastic stomach is the difficulty of isolating individual cell populations from the epithelium. Like all secretory tissues, the stomach comprises a number of distinct cell lineages. Unlike other secretory tissues, however, the tools to isolate these lineages are cumbersome and underdeveloped. These difficulties are compounded by a lack of molecular markers with which to identify each cell type. In chapter 2, I describe a novel surface marker of fully differentiated ZCs in mouse and human stomach, and describe an improved method to isolate pure populations of ZCs from the gastric epithelium using this marker. This approach

allows not only efficient isolation of gastric ZCs, but also enables the characterization of molecular changes in response to damage or disease.

Mature-Onset Diabetes of the Young Type-1 and the ER

The transcriptional regulation of *Xbp1* expression by HNF4α is likely important to the development of secretory cells in several tissues, perhaps most importantly in the endocrine pancreas. Polymorphisms in *Hnf4α* are the cause of MODYI, a subset of diabetes characterized by diminished glucose-stimulated insulin secretion (GSIS) in pancreatic beta-cells. MODY accounts for 2-5% of total diabetes cases, though often it remains undiagnosed.(Porter et al., 2006; Shields et al., 2010) The mechanism by which HNF4α dysfunction causes MODYI pathology is unclear. The data presented in chapter four suggests that the transcriptional activation of *Xbp1* expression by HNF4α is necessary for beta-cell function.

The pancreatic beta-cell is a finely-tuned secretory machine. Once stimulated with glucose, the beta-cell increases insulin synthesis 10-fold, accounting for 50% of the cell's total protein synthesis.(Schuit et al., 1988) This biosynthetic load creates an incredible burden on the ER, and subsequently small aberrations in beta-cell ER function lead to dysfunction and apoptosis.(Eizirik et al., 2008; Gurzov et al., 2009) A rare genetic form of type I diabetes, Wolcott–Rallison syndrome, is caused by a loss of function mutation in a key protein (PERK) in the UPR signaling cascade. (Rubio-Cabezas et al., 2009) This disruption of PERK function causes a diminished UPR, and subsequently β-cell apoptosis. Wolfram syndrome, another rare genetic form of diabetes, is usually caused by mutations in the WFS1 gene. (Fonseca et al., 2010) The protein encoded by this gene is important for proper ER stress response, and the pathogenesis of Wolfram syndrome is most likely due to chronic, unmitigated ER stress that

leads to β-cell death. The pathology of type II diabetes is driven by peripheral insulin resistance, which provokes a sustained, increased synthesis and secretion of insulin in β-cells. This increased biosynthetic load in the ER of β-cells instigates prolonged ER stress, and likely leads to decreased β-cell function in patients genetically susceptible to ER stress. Interestingly, recent genomic studies have shown patients with point mutations in ER stress factors have an increased susceptibility to type-II diabetes.(Sandhu et al., 2007) Pathological ER stress induced by long-term exposure to high glucose levels alters ER-calcium homeostasis, disrupting intracellular signaling and causing cell death.(Hara et al., 2014) Similiarly, genome-wide association studies have also linked polymorphisms in *Hnf4α* to increased susceptibility to type-II diabetes. Similar to MODYI mouse-models, beta-cell-specific knockouts of *Xbp1* in adult mice are unable to secrete insulin in response to a glucose stimulus.(Lee et al., 2011) Chapter four outlines the importance of the relationship between *Xbp1* and HNF4α to beta-cell function, and outlines the first-clear molecular mechanism of MODYI pathology.

Summary

The transformation of a cell from a small multipotent progenitor to a large and complex professional secretory cell is meticulously orchestrated by a network of developmental regulators. Defining the signaling networks and factors that guide this differentiation is pivotal to understanding how these pathways are disrupted in disease, and to providing new therapeutic avenues for the many maladies associated with secretory cell dysfunction, from cancer to diabetes. Here, we identify one such signaling cascade, HNF4α →*Xbp1*, responsible for the development and homeostasis of secretory cells in multiple tissues. In this thesis, we:

1. Define HNF4α as a direct transcriptional regulator of *Xbp1*, required for secretory cell homeostasis in the gastric epithelium.
2. Identify a novel surface marker of ZCs in the gastric epithelium, allowing cell-lineage specific isolation and characterization in normal and diseased stomachs.
3. Establish that HNF4α directly enhances *Xbp1* expression in beta-cells, and that this relationship is required for ER homeostasis, glucose-stimulated insulin secretion, and intracellular signaling, defining the first clear molecular mechanism for MODYI pathology.

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Figure Legends

Figure 1. Overview. The differentiation from a relatively simple progenitor cell to a large, complex professional secretory cell is orchestrated by transcriptional network that coordinate the establishment of secretory machinery, and remodel cellular architecture to facilitate production and secretion of large amounts of protein. We have A) identified one of these networks, HNF4α→*Xbp1*, and found it to be indispensable to normal differentiation in the gastric epithelium B) developed a new set of tools to isolate and better characterize secretory cells in the stomach, and C) identified the role of HNF4α→*Xbp1* in beta-cell dysfunction during human disease.

Figure 2. Regulation of *Xbp1* in response to ER stress. In response to an increased secretory load, misfolded proteins accumulate in the ER lumen. This activates the UPR by causing phosphorylation and dimerization of IRE1, which in turn splices and activates XBP1. Once activated XBP1enters the nucleus and enhances expression of a genes that increase the secretory capacity of the ER and alleviate ER stress. Adapted from (Pandol et al., 2011)

Figure 1

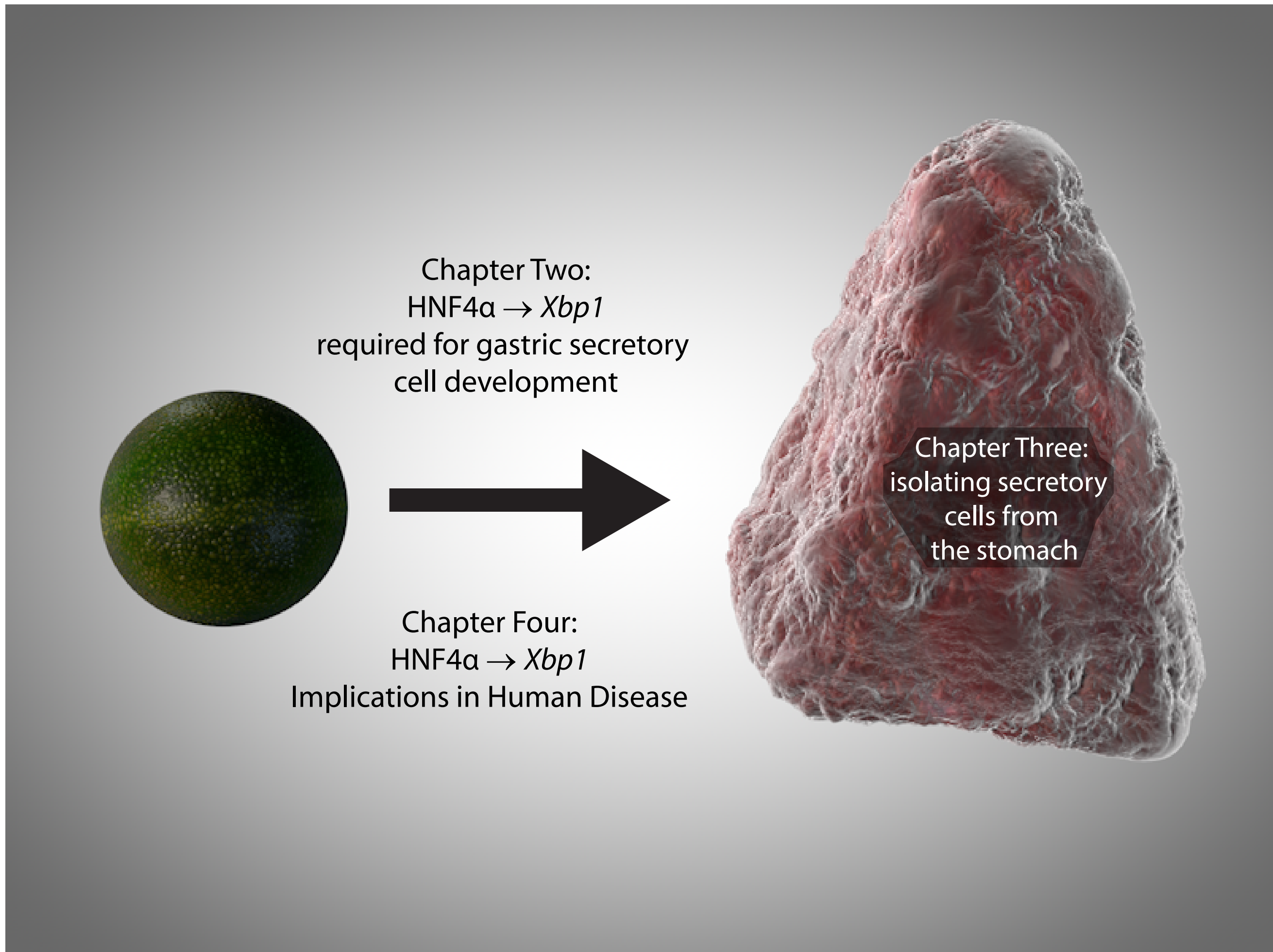
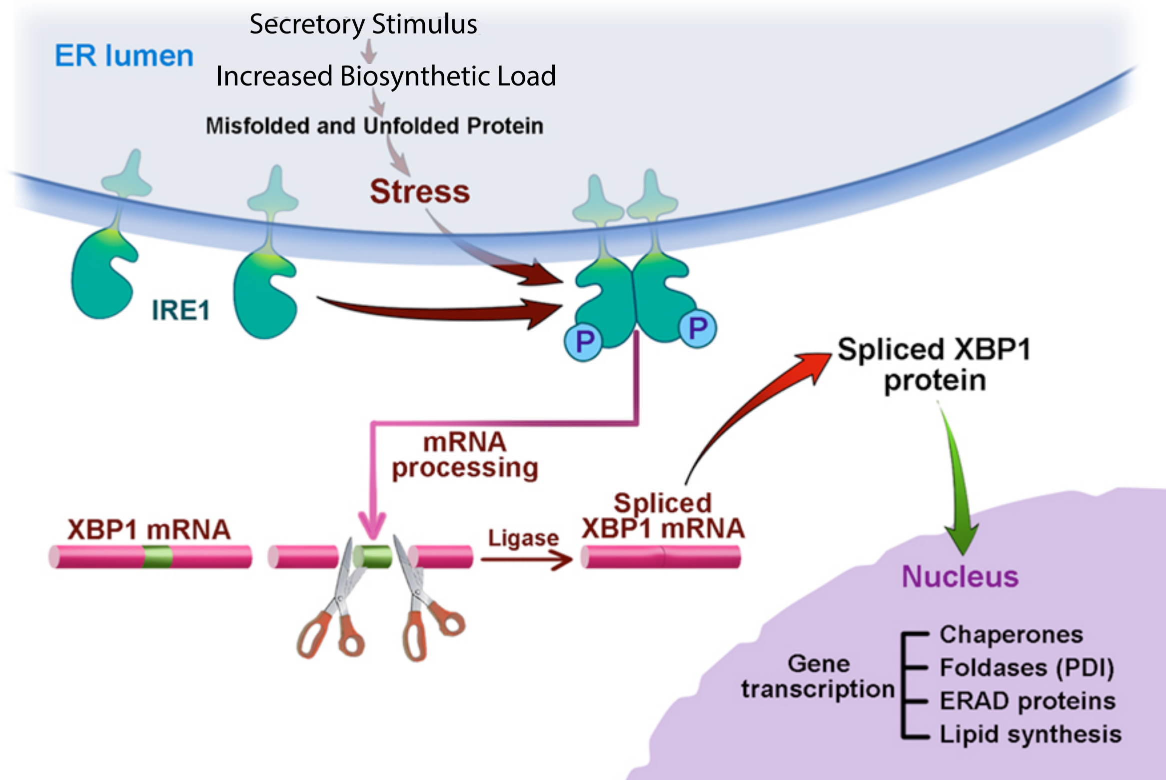


Figure 2



CHAPTER TWO

HNF4α is required for Normal Cell-Differentiation

in the Adult Mouse Gastric Epithelium

HNF4α IS REQUIRED FOR NORMAL CELL DIFFERENTIATION IN THE ADULT MOUSE GASTRIC EPITHELIUM

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Abstract

Little is known about the molecular pathways that orchestrate the differentiation of the gastric epithelium. Our lab has identified a transcriptional cascade, XBP1→*Mist1*, which regulates the development of gastric-enzyme secreting ZCs in the gastric unit. Here, we show that HNF4α acts upstream of *Xbp1* in ZC development. Using an in silico screen of evolutionarily conserved regions of the *Xbp1* promoter, we identified two potential HNF4α binding sites. Using chromatin immunoprecipitation, we show that HNF4α directly occupies the *Xbp1* locus. We found that by overexpressing HNF4α in a gastric cancer cell line, *Xbp1* expression increased, suggesting that HNF4α is sufficient for *Xbp1* expression. Immunofluorescent staining of HNF4α showed that it is expressed in gastric pit, isthmal progenitor, neck, and zymogenic cells, but not in parietal cells. Using a tamoxifen-inducible cre system, we show that loss of HNF4α in the adult stomach led to increased proliferation, reduced ZC size and ER formation, and aberrant differentiation. These data show that HNF4α is an important regulator of differentiation and a transcriptional enhancer of *Xbp1* expression in the stomach.

Introduction

The mammalian gastric epithelium is an organized network of cells which function to secrete mucus, acid, and digestive enzymes into the gastric lumen. These cells are located in repeating glandular invaginations called gastric units. Based on anatomy and cell function each unit can be divided into four distinct sections: the pit zone opens into the gastric lumen and contains mucus-secreting pit cells; the isthmus region houses stem cells and early progenitors; the neck zone contains both acid-secreting parietal cells (PC) and mucus-secreting neck cells; and the base zone contains digestive-enzyme secreting zymogenic cells (ZCs). (Fig. 1A) The differentiation of each lineage has been characterized by detailed morphological studies and analysis of gene expression patterns, which are conserved in each gastric unit (Karam,1993; Karam and Leblond, 1993a, b, c, d). The gastric unit is a uniquely well
suited system to study transcriptional regulators of cell differentiation and morphology, because development of each cell type is tightly controlled along a well-defined spatiotemporal gradient. The molecular mechanisms that control these changes in expression and morphology are largely uncharacterized in the stomach. The best example of this process is the formation of ZCs, which arise from mucus neck cells. As ZCs differentiate from their progenitor neck cells, a cascade of transcription factors is activated that include XBP1 and MIST1.(Huh et al., 2010; Ramsey et al., 2007) Elucidation of the factors that orchestrate epithelial differentiation will shed light on fundamental cellular processes, and give insight on how pathological conditions like metaplasia and gastric atrophy develop. Understanding these transcriptional regulators is essential to developing new ways to treat and prevent gastric cancer.

Our lab has previously identified the transcription XBP1 as a regulator of the maturation of ZCs that occupy the base of the gastric unit.(Huh et al., 2010) XBP1 controls the

differentiation of ZCs by induction of the TF MIST1 and the expansion of the rough endoplasmic reticulum. When Xbp1 is conditionally deleted in mice using a tamoxifen/Cre-loxP system, Xbp1-null ZCs decrease in size, and MIST1 expression is lost; the gastric units resemble those of mice with pseudopyloric or spasmolytic polypeptide expressing metaplasia (SPEM), a well-established precursor to stomach cancer. To further explore the molecular networks that drive ZC differentiation, we sought to elucidate TFs that act upstream of XBP1 in the ZC signaling cascade. We found that *Xbp1* expression is enhanced by the TF HNF4α, a master regulator of secretory tissue development.

HNF4α is required for proper secretory cell differentiation and maintenance in many tissues with similarities to the stomach. In the adult intestinal epithelium, loss of HNF4α results in altered secretory cell architecture, aberrant cell differentiation, proliferation, and impaired barrier function.(Cattin et al., 2009) HNF4α is also required to maintain cell differentiation in the colon, and disruption of its expression leads to inflammation, proliferation, and cell dedifferentiation.(Darsigny et al., 2009) Similarly, loss of HNF4α in the adult mouse liver leads to altered cell differentiation, causing hepatocytes to dedifferentiate into progenitor-like cells, and disrupting lipid synthesis.(Hayhurst et al., 2001) Though HNF4α is known to be expressed at high levels in the stomach, and is alternatively spliced in early gastric carcinomas,(Dean et al., 2010; Takano et al., 2009) nothing is known about its function or importance to gastric epithelial development and maintenance. Here, we show that HNF4α is required for the maintenance of the ZC lineage and normal differentiation in the gastric unit.

Results

HNF4α directly enhances *Xbp1* expression. To identify potential regulators of *Xbp1*expression, we performed an *in silico* screen, scanning evolutionarily conserved regions of the *Xbp1* promoter locus for known TF binding sites.(Ovcharenko et al., 2005) We identified two putative HNF4α binding sites upstream of the *Xbp1* transcription start site. To determine whether HNF4α directly bound these sites, we performed chromatin immunoprecipitation. Cells from a gastric cancer cell line (AGS) were transiently transfected with an expression vector containing *HNF4α*. These cells were then crosslinked and immunoprecipitated with an HNF4α antibody or serum control. HNF4α occupied both predicted sites in the *XBP1* promoter (Fig. 1A). The site located -1.6 kb from the transcription start site showed strong occupation of HNF4α as assayed with two different antibodies. The site -2.4 kb upstream showed weaker occupation, and the intronic control site showed no HNF4α occupation. To determine the function of HNF4α in *XBP1* regulation, we used isolated protein from AGS cells transiently transfected with two common splice variants of HNF4α and measured the expression of XBP1 by immunoblot. XBP1 expression was dramatically increased in cells transfected with HNF4α compared to a GFP control plasmid (Fig. 1B,C), indicating that HNF4α is a direct transcriptional enhancer of *XBP1*.

HNF4α is expressed in the isthmus, pit, neck, and zymogenic cells in the gastric corpus. Similar to the situation in other endoderm-derived secretory tissues, HNF4α has been shown to be highly expressed in the gastric epithelium.(Dean et al., 2010) However, the specific cell lineages that express HNF4α have not been determined. Understanding which cell types express HNF4α will give us a better understanding of the molecular mechanisms of HNF4α in the maintenance and development of the stomach. We used immunofluorescent microscopy on mouse stomach tissue to determine the pattern of HNF4α expression, and found that HNF4α was expressed highly in progenitor cells in

the isthmus and pit cells, and at lower levels in cells expressing the neck cell marker and ZC-marking GIF (Fig. 2A,B). HNF4α was not, however, expressed in parietal cells.

HNF4α is required for maintenance of the gastric epithelium. Because HNF4α was expressed highly in the stomach, we sought to determine its function by knocking out HNF4α expression in the adult mouse gastric epithelium. We generated a mouse model of acute loss of HNF4α by crossing the tamoxifen inducible $CAGCre^{ERT}$ mice, previously shown by our group to be an efficient driver of cre recombination in the gastric epithelium,(Huh et al., 2010) to mice containing a floxed *Hnf4α* allele.(Hayhurst et al., 2001) This model allowed us to induce loss of HNF4α via injection of low-dose(1mg/20g mouse) tamoxifen. Loss of HNF4α led to a ~4 fold increase in proliferation, measured by ki67 expression and Brdu staining (Fig. 3A,B). This increased proliferation did not, however, lead to an increase in unit length (data not shown) or overall area (Fig. 4A), as we had expected. The most striking phenotype was a dramatic change in ZC morphology. The base/ZC region was significantly diminished (Fig. 4B), and the ZCs in ΔHNF4α mouse stomachs were half the size of WT ZCs (Fig. 4C). Additionally the overall census of ZCs was ~25% lower in the HNF4α KO (Fig. 4D). These changes in unit morphology are evident when comparing WT and ΔHNF4α hematoxylin and eosin stained tissue from the gastric corpus (Fig. 5A). The dark-staining ZC region is noticeably collapsed (highlighted by white arrowheads, Fig. 5A) when HNF4α is lost. Interestingly, this collapse mimics loss of XBP1 observed in previous work (Fig. 5B, adapted from (Huh et al., 2010)). Because of the transcriptional relationship between *Xbp1* and HNF4α, and the role of XBP1 in maintaining the ER, we expected loss of HNF4α to result in diminished ER in the gastric epithelium. Accordingly, the ER (as measured by expression of the ER-localized protein, Calregulin) was significantly reduced in size upon loss of HNF4α (Fig. 5C).

The ZC transitions from a proliferative progenitor cell, to a neck cell, and finally to its definitive form as the stomach's professional secretory cell in the normal gastric epithelium. Because of its role in other tissues as a master regulator of secretory cell differentiation, and its regulation of *Xbp1*, an important player in ZC differentiation, we hypothesized that disrupting HNF4α in the stomach would result in aberrant differentiation in the gastric unit. As expected, we observed precursor neck cells in the base of the gastric unit in ΔHNF4α mice (white arrowhead, Fig. 6A), a phenomenon that is rarely observed in WT mice. Both the average distance of neck cells from the basal membrane (Fig. 6B) and the percentage of ZC-regions (which generally contain no neck-cells in WT mice, Fig. 6C) were significantly different upon loss of HNF4α, with almost a quarter of ΔHNF4α ZC regions exhibiting neck cell intrusion. These data indicate that HNF4α is required for the normal differentiation of the gastric epithelium.

Discussion

We show here that HNF4α is expressed in pit, isthmal, neck, and zymogenic cells in the gastric corpus, and that it is required for normal differentiation. Additionally, HNF4α is a direct transcriptional regulator of *Xbp1* expression, suggesting that upregulation of *Xbp1* is one of the mechanisms through which HNF4α regulates ZC development. To our knowledge, this is the first time the role of HNF4α in the stomach has been described.

Relatively few transcriptional regulators responsible for gastric epithelial development have been previously identified. Characterizing such factors and the signaling networks which they govern is key to understanding how to target maladies resulting from their dysregulation, e.g. cancer. The transcriptional enhancement of *Xbp1* by HNF4α in the stomach is a potentially important finding for 3 reasons: 1) it adds an upstream component to the only well-defined signaling cascade that orchestrates ZC differentiation HNF4α→XBP1→MIST1, aberrations in which lead to metaplastic-like gastric mucosa, 2) it builds further upon and the developing concept that tight regulation of *Xbp1* expression is not just important for the unfolded protein response but is also pivotal to its role as a developmental coordinator of secretory cell differentiation, and 3) many of the effects of loss of HNF4α in other secretory tissues (i.e. liver-dyslipidemia, intestine-secretory cell architecture, β-cell-secretory dysfunction) may be caused in part by this novel relationship. Future experiments further characterizing this signaling cascade, and the molecular mechanisms by which HNF4α orchestrates other aspects of the development and maintenance of the gastric epithelium have potential to uncover fundamental mechanisms of disease development in the stomach and other secretory tissues.

Methods

Cell lines and Transient Transfection

AGS cells (from ATCC, Manassas, VA) were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 0.9% glutamine, 0.4% HEPES, 1% Na pyruvate, 2.5% glucose, and 100 ng/ml each of penicillin and streptomycin. For overexpression of myc-tagged HNF4α2 or HNF4α8, coding regions (obtained from addgene) were subcloned into a pcDNA3.1expression vector, and 5μg of each plasmid or the pmaxGFP(lonza) control plasmid were transiently transfected using TransIT-2020 (Mirus, Madison, WI). For siRNA we transfected MIN6 and INS-1 cells with 10nM HNF4α siRNA (silencer select Invitrogen) using Lipofectamine 2000 according to the manufacturer's protocol.

Western Blot

Cells for western blot analysis were lysed in RIPA buffer. Proteins were quantified by DC protein assay (Bio-Rad) and then separated on NuPAGE Bis-Tris gels (Invitrogen), transferred onto Amersham Hybond ECL nitrocellulose (GE Healthcare, Buckinghamshire, UK) membranes, and detected by Immobilon chemiluminescence (Millipore). Primary antibodies used were rabbit anti-XBP1(Santa Cruz), mouse anti-c-myc (dshb), and rabbit anti-α- and β-tubulin (Cell Signaling). Secondary antibodies were horseradish-peroxidase-conjugated donkey anti-rabbit and anti-mouse Ig (Santa Cruz Biotechnology, Santa Cruz, CA). Quantifications of immunoblots were performed by scanning 16-bit images into ImageJ. Band intensities for XBP1 and α/β tubulin were selected and calculated by using the 'Analyze mean gray value' measurement tool. Standardized values were calculated determining the ratio of XBP1 signal to α/β tubulin signal.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described previously.(Im et al., 2004) Approximately 10^6 AGS cells transiently transfected with HNF4α expression vector as described above were used for this ChIP experiment. Ten microliters of anti-HNF4α (rabbit anti-human MIST1) or whole rabbit serum (preimmune control) together with protein A/G plus agarose (Santa Cruz Biotechnology) was added to the homogenized tissue for immunoprecipitation. Quantitative real-time PCR (qRT-PCR) was performed to assess the quantity of genomic sequences immunoprecipitated by either preimmune control or HNF4α antiserum, as well as a 1:10 dilution of the cell extract prior to immunoprecipitation (input). Two predicted HNF4α binding sites[8] were probed in addition to an intronic control region with no predicted HNF4α binding sites nearby.

Mouse Studies

All experiments involving animals were performed according to protocols approved by the Washington University School of Medicine Animal Studies Committee. Floxed *Hnf4α*, *CAGGCreER*^{TM} transgenic mice were generated by crossing *Hnf4α*$^{floxed/floxed}$ mice (a gift from Frank Gonzalez, NIH)(Hayhurst et al., 2001) with *CAGGCreER*^{TM}*;Hnf4α*$^{floxed/+}$(Hayashi and McMahon, 2002) mice to allow systemic, tamoxifen-inducible knock out of HNF4α. 6-8 week old *CAGGCreER*^{TM};*Hnf4α*$^{floxed/floxed}$ mice and *CAGGCreER*^{TM};*Hnf4α*$^{floxed/+}$ littermate controls were injected intraperitoneally with tamoxifen (1mg/20g body weight, 5 consecutive days) to induce cre-mediated *Hnf4α* deletion. Mice were sacrificed 4 weeks after first tamoxifen injection.

Immunofluorescence and Quantification

Stomachs were prepared and stained as described previously.(Ramsey et al., 2007) Stomachs were inflated with 10% formalin fixative and suspended in fixative for 4 hours at room temp. Tissue was rinsed with 70% EtOH multiple times, arranged in 2% agar in tissue cassettes, and paraffin processed. Sections (5µm) were deparaffinized and rehydrated, and antigen retrieval was performed by boiling in Trilogy Buffer (Cell Marquee Corporation). Slides were blocked in 1% BSA, 0.3% Triton X-100 in PBS, then incubated in primary followed by secondary antibodies and with fluorescently labeled lectin Griffonia simplicifolia-II (neck cell-specific GS-II; 1:1,000; Invitrogen). Finally, slides were incubated for 5 min in 1µg/mL bisbenzimide (Invitrogen) prior to mounting in 1:1 PBS-glycerol. Primary antibodies used for immunostaining were: goat anti-Calregulin (1:200, Sigma), goat anti-human gastric intrinsic factor (GIF) (1:2,000; gift of Dr. David Alpers, Washington University), rabbit anti-HNF4α (1:100 Cell Signaling), goat anti-BrdU (1:2,000; gift of Dr. Jeff Gordon, Washington University), rabbit anti-Ki67 (AbCam) and sheep anti-PGC (1:10,000; Abcam).

For morphological analysis, sections were stained with hematoxylin and. Whole slides were scanned with Nanozoom microscope and every 5th unit was measured across each slide using Nanozoom Digital Pathology software (Hamamatsu). Samples were randomized, and the scorer was blinded to ensure unbiased quantification.

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Figure Legends

Figure 1. HNF4α directly enhances *Xbp1* expression. A) Chromatin Immunoprecipitation from AGS cells transiently transfected with an HNF4α expression plasmid. Two different antibodies (α-HNF4α-1, α-HNF4α-1) both showed significant binding of HNF4α to two predicted sites in the *Xbp1* promoter when compared to serum control. B,C) Western blot and band intensity quantification of protein isolated from AGS cells transiently transfected with one of two HNF4α expression plasmids or a GFP control plasmid shows XBP1 expression is enhanced by HNF4α.

Figure 2. Expression of HNF4α in the gastric epithelium. A) HNF4α (green) is expressed in isthmal progenitor, pit, neck(purple), and zymogenic (red) cells of the mouse gastric corpus. B) Pit/isthmal region shows intense staining of nuclear HNF4α

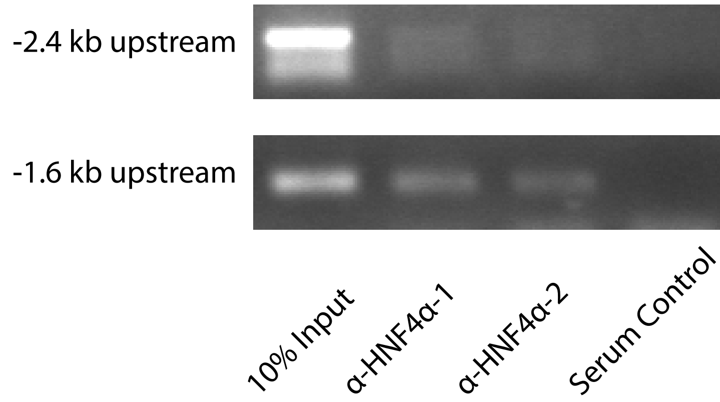
Figure 3. Loss of HNF4α leads to increased proliferation in the gastric epithelium. A) Proliferation marker Ki67 (green) in WT and ΔHNF4α mouse gastric corpus. B) Quantification of BrdU incorporation in WT and ΔHNF4α mouse stomachs

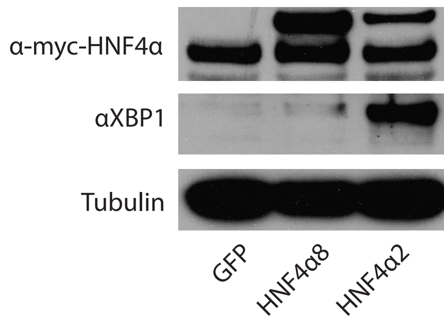
Figure 4. Loss of HNF4α causes altered ZC morphology. A) Quantification of total area of the gastric unit. B) Quantification of the ZC-containing region at the base of the gastric unit, normalized to total unit area. C) Quantification of the total area/ZC obtained by dividing the area of the ZC region by the number of ZCs. D)Total number of ZCs/unit. All experiments represent Mean±SEM of three biological replicates.

Figure 5. Loss of HNF4α leads to diminished ER, resembling loss of XBP1. A)H&E staining of mouse gastric epithelium in WT and ΔHNF4α mice. White arrowheads highlight the ZC-containing base region, which is noticeably collapsed in ΔHNF4α mice. B) E staining of mouse gastric epithelium in WT and ΔXBP1 mice. Like HNF4α, the base region is noticeably collapsed upon loss of XBP1. C) Immunofluorescent stain of the ER marker, Calregulin (red), in ZCs of WT and ΔHNF4α mice. ΔHNF4α mice have significantly reduced ER.

Figure 6. Neck cell intrusion in ΔHNF4α mice. A) Neck cells (green) in ΔHNF4α mice are inappropriately present in the ZC region (red) of the gastric unit. B) Quantification of the average distance from the base of the unit to the unit's first neck cell. C) Quantification of the percentage of ZC regions containing neck cells.

Figure 1





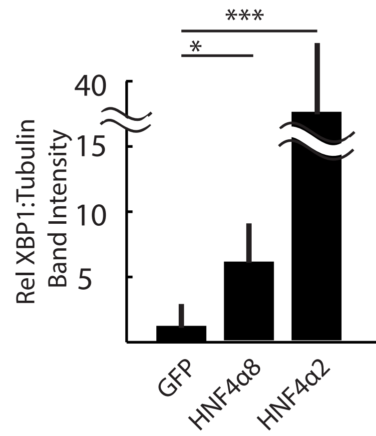
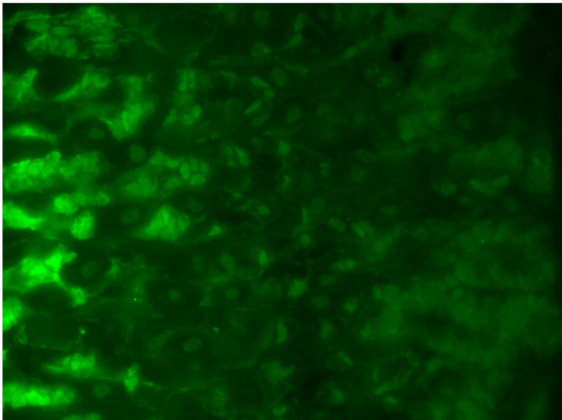
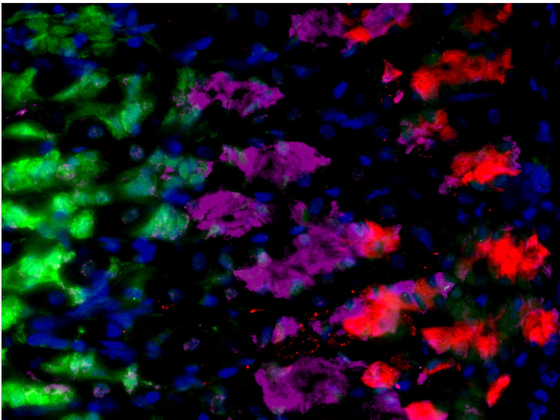


Figure 2

A





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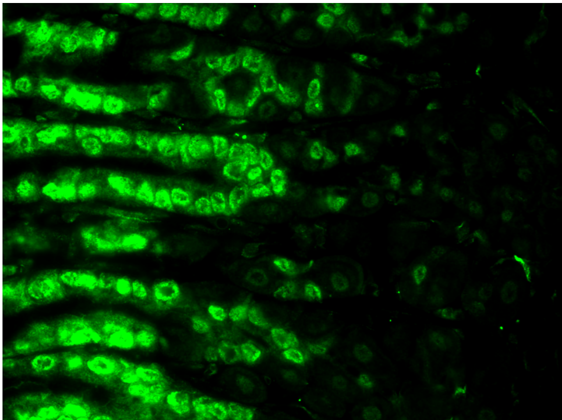
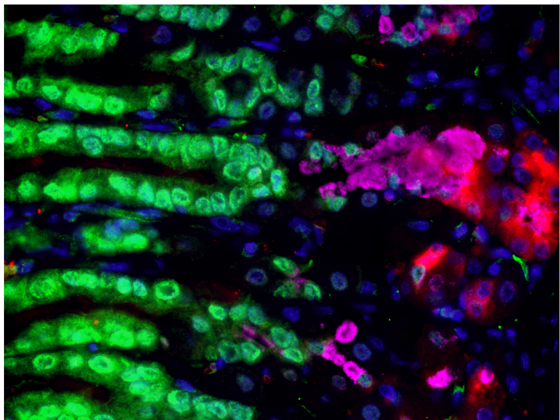
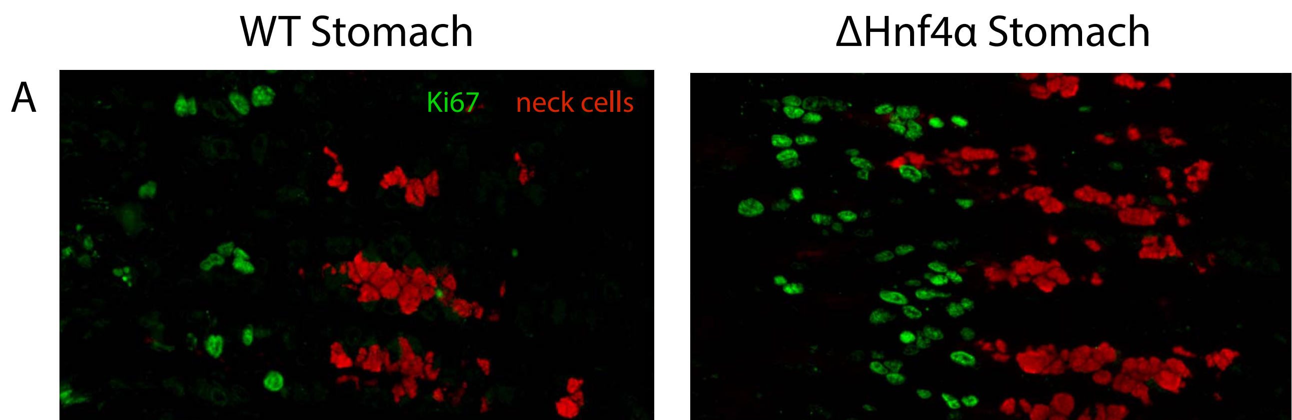




HNF4α neck cells ZCs nuclei

Figure 3



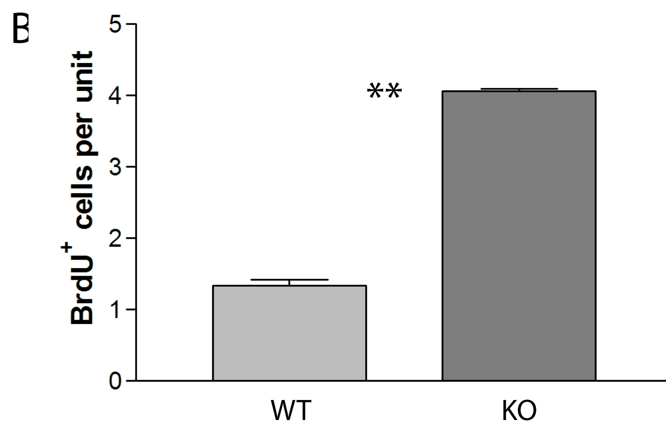


Figure 4

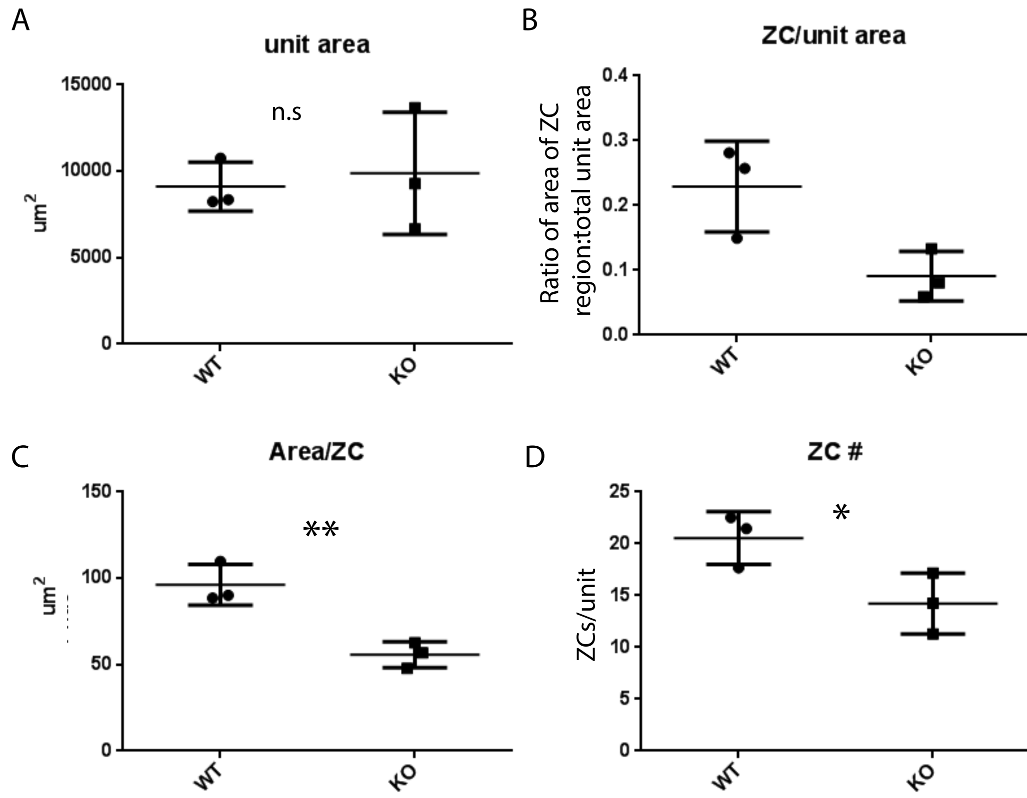


Figure 5

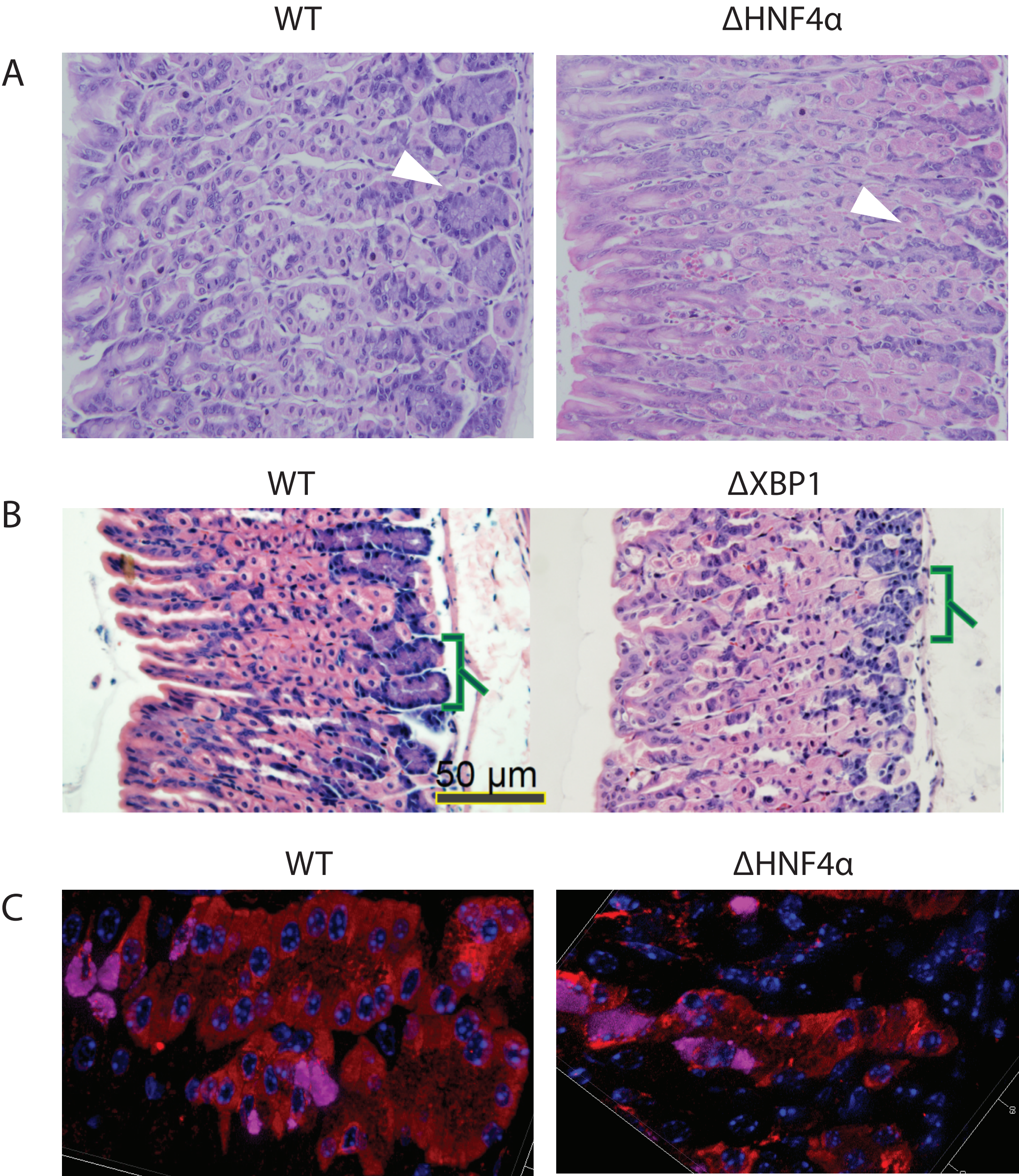
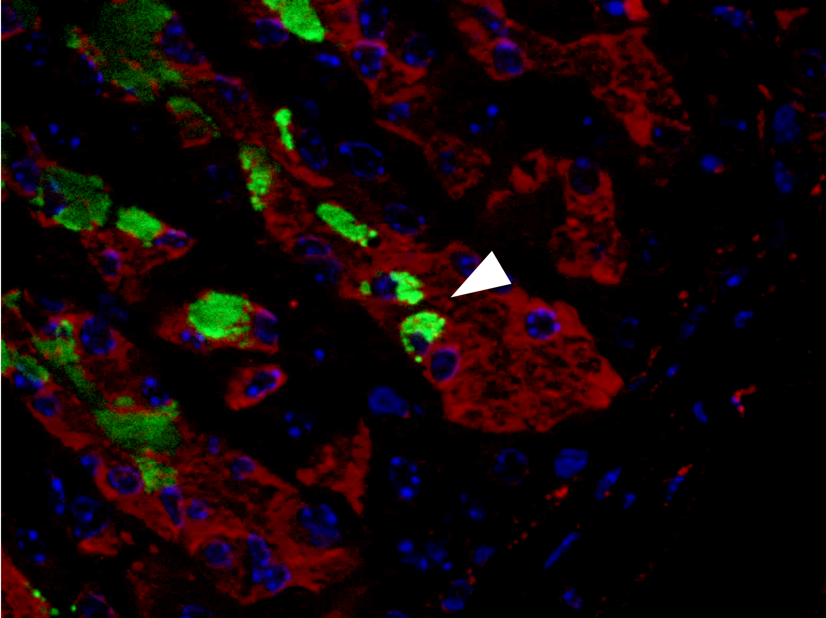


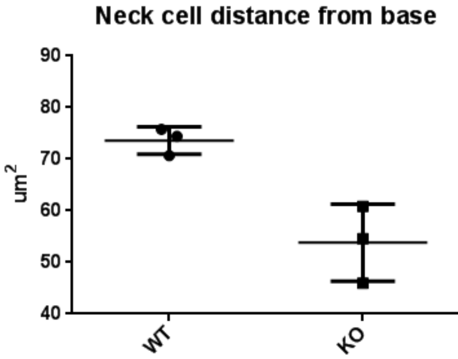
Figure 6

A



neck cells ZCs nuclei

B



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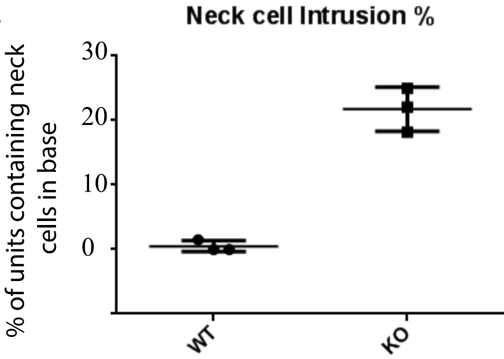

CHAPTER THREE

Identification of ANPEP as a Surface Marker

for Isolation of Mature Gastric ZCs

Identification of Alanyl Aminopeptidase (CD13) as a Surface Marker for Isolation of Mature Gastric Zymogenic Chief Cells

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Abstract

Injury and inflammation in the gastric epithelium can cause disruption of the pathways that guide the differentiation of cell lineages, which in turn can cause persistent alterations in differentiation patterns, known as metaplasia. Metaplasias that occur in the stomach are associated with increased risk for cancer. Methods for isolating distinct gastric epithelial cell populations would facilitate dissection of the molecular and cellular pathways that guide normal and metaplastic differentiation. Here, we identify Alanyl Aminopeptidase (ANPEP; aka CD13) as a specific, surface marker of zymogenic chief cells (ZCs) in the gastric epithelium. We show that: 1) among gastric epithelial cells, ANPEP expression is confined to mature ZCs, and 2) its expression is lost en route to metaplasia in both mouse and human stomachs. With this new marker coupled with new techniques we introduce for dissociating gastric epithelial cells and overcoming their constitutive autofluorescence, we are able to reliably isolate pure populations of ZCs, and observe changes in ZC differentiation ex vivo in response to epithelial damage.

Keywords: Aminopeptidase N/CD13, Zymogenic Chief Cell, Gastric Epithelium, Splasmolytic Polypeptide Expressing Metaplasia

Introduction

The differentiation and maintenance of each lineage in the epithelium of the body of the mammalian stomach is orchestrated by signaling networks and molecular pathways. (Aikou et al., 2009; Bredemeyer et al., 2009; Leys et al., 2006)The 3 most abundant differentiated cell lineages are: the mucus-secreting pit cells near the luminal surface, the acid-secreting parietal cells located mostly in the middle (neck) portion of each gastric unit, and the digestive enzyme-secreting zymogenic chief cells (ZCs) in the base of the unit. Each cell in the gastric epithelium is thought to derive from the same, undifferentiated population of stem cells and is replenished throughout adulthood.(Karam and Leblond, 1993b; Werbowetski-Ogilvie et al., 2011) The ZC has an unusual differentiation pattern, deriving from the stem cell by an intermediary form, the mucous neck cell, which migrates amongst parietal cells for ~ 2 weeks (in mice) towards the base before terminal differentiation into ZCs.(Bredemeyer et al., 2009; Karam and Leblond, 1993a; Ramsey et al., 2007)

Disruption of the molecular pathways regulating the differentiation of those cell lineages through damage or disease can cause a chronic, aberrant differentiation state known as metaplasia. In mice and humans, one of the most common such aberrations is associated with increased risk for progression to gastric cancer and is termed, splasmolytic peptide-expressing metaplasia (SPEM; named because the progenitor marker Spasmolytic Peptide, aka TFF2, becomes re-expressed in ZCs, as they reprogram into a metaplastic lineage).(Nozaki et al., 2008) SPEM in humans is associated with further potential aberrations in epithelial differentiation patterns, like intestinal metaplasia, as well as progression to gastric cancer. (Nam et al., 2010)

Metaplasia in the stomach, especially SPEM, is hypothesized to derive largely via the cellular reprogramming of ZCs from a post-mitotic, terminally differentiated cell back into a proliferative, regenerative state.(Goldenring et al., 2011; Mills and Sansom, 2015; Nozaki et al., 2008) In certain injury/inflammatory states (in particular, in response to infection with the bacterium *Helicobacter pylori*), ZCs can reprogram, meaning, they reexpress markers of their precursor neck-cell phase and also re-enter the cell cycle to become proliferative.(Aikou et al., 2009; Nam et al., 2010) The molecular pathways underlying ZC reprogramming may be similar to those that govern reprogramming of other cells in other tissues during injury/repair (e.g. pancreatic acinar cells in acinar-to-ductal metaplasia) and is currently an area of intensive research.(Mills and Sansom, 2015) Isolating pure populations of ZCs to analyze the pathways that lead to reprogramming would help us understand the molecular underpinnings of this newly recognized, fundamental cellular process. Here, we identify a surface marker of ZCs that allows the isolation of a pure population from the normal gastric epithelium, Alanyl Aminopeptidase (ANPEP).

ANPEP is a membrane-associated protein involved in the metabolism of peptides by diverse cell types. ANPEP null mice have significantly impaired angiogenesis in pathological conditions,(Rangel et al., 2007) and ANPEP is required for endothelial cell adhesion/invasion.(Mina-Osorio et al., 2008; Petrovic et al., 2007) ANPEP is thought to function in the final digestion of peptides in the digestive tract, though its specific function in the gastric epithelium is unknown.(Kruse et al., 1988) Intestinal absorption of cholesterol is impaired when ANPEP is pharmacologically inhibited.(Kramer et al., 2005) In disease states, ANPEP expression in colonic tumors is associated with a poor prognosis for node-positive patients with colon cancer, and aberrant expression of ANPEP is a marker for multiple

leukemias/lymphomas.(Dalal et al., 2014; Saxena et al., 2010) We show that ANPEP is expressed exclusively in mature ZCs in the gastric epithelium, exploit this to both isolate pure populations of ZCs, and document changes in the gastric epithelium in normal and metaplastic states.

Materials and Methods

Bioinformatic Analysis

Affymetrix Mouse Gene 1.0ST microarrays were used to analyze gene expression in each cell lineage from RNA captured with LCM in previously performed experiments.(Capoccia et al., 2013; Huh et al., 2012; Ramsey et al., 2007) Affymetrix Mouse Genome 430 2.0 microarrays were used to analyze gene expression in stomachs from treated with tamoxifen or vehicle from 3 pooled mice as described below. Chip quality control and gene specific ANOVA analysis were performed using Partek® Flow® software, version 3.0 Copyright ©; 2014 (Partek Inc., St. Louis, MO, USA). Zymogenic cell lineage-specific expression was determined by identifying genes that were significantly increased in ZCs (fold change >1.5) compared to other gastric epithelial cell lineages. To enrich for potential surface markers, ZC specific genes were filtered with Gene Ontology (GO) terms “membrane”, “integral component of membrane”, and “external side of plasma membrane”.

Animals

Experiments involving animals were conducted according to protocols approved by the Washington University School of Medicine Animal Studies Committee. Mice were maintained in a specific-pathogen-free barrier facility. Stomachs from Germline *Mist*$^{-/-}$ mice and wild-type C57BL/6 mice of both sexes (Jackson Laboratory), were used at 6 weeks age. Metaplasia was induced with daily intraperitoneal injection of Tamoxifen (5mg/20g body weight) dissolved in a vehicle of 10% ethanol and 90% sunflower seed oil (Sigma) as described previously. (Huh et al., 2012)

H. Pylori growth and murine infection

H. pylori growth conditions and murine infection. The WT cag+ H. pylori strain, PMSS1, was cultured on trypticase soy agar with 5% sheep blood agar plates (BD Biosciences). It was then cultured in Brucella broth (BB, BD Biosciences) supplemented with 10% FBS (Atlanta Biologicals) for 16 to 18 hours at 37°C with 5%CO2. Male C57BL/6 mice were purchased from Jackson Laboratories and housed in the Vanderbilt University Animal Care Facilities in a room with a 12-hour light-dark cycle at 21°C to 22°C. Mice were orogastrically challenged with either Brucella broth (BB), as an uninfected (UI) control (referred to here as "Mock infected"),or with the mouse-adapted wild-type cag + H. pylori strain PMSS1. Mice were euthanized at 4 and 8 weeks post challenge and gastric tissue was harvested for immunohistochemistry as described below.

Patient samples

Examination of human gastric pathological tissue specimens was approved by the Institutional Review Board of Washington University School of Medicine, the Comité de Bioetica of Nicaragua for Universidad Nacional Autonoma De Nicaragua-Facultad De Ceincias Medicas Managua, and the Research Ethics Board Manager for Health Sciences at the University of Toronto. Serial sections (4–6 μm thick) obtained from paraffin-embedded tissue samples (H&E and alcian blue–periodic acid–Schiff stains) were reviewed by two pathologists with expertise in gastrointestinal diseases. Diagnoses and selection of specific regions of transition among normal stomach and SPEM stomach were performed by a third pathologist.

Immunofluorescence

Stomachs were prepared and stained as described previously.(Ramsey et al., 2007) Stomachs were inflated with 10% formalin fixative and suspended in fixative for 4 hours at room temp. Tissue was rinsed with 70% EtOH multiple times, arranged in 2% agar in tissue cassettes, and paraffin processed. Sections (5µm) were deparaffinized and rehydrated, and antigen retrieval was performed by boiling in Trilogy Buffer (Cell Marquee Corporation). Slides were blocked in 1% BSA, 0.3% Triton X-100 in PBS, then incubated in primary followed by secondary antibodies and with fluorescently labeled lectin Griffonia simplicifolia-II (neck cell-specific GS-II; 1:1,000; Invitrogen). Finally, slides were incubated for 5 min in 1µg/mL bisbenzimide (Invitrogen) prior to mounting in 1:1 PBS-glycerol. Primary antibodies used for immunostaining were: mouse anti-CD13 (1:200, Sigma), goat anti-human gastric intrinsic factor (GIF) (1:2,000; gift of Dr. David Alpers, Washington University), and sheep anti-PGC (1:10,000; Abcam). Secondary antibodies were AlexaFluor (488, 594)-conjugated anti-mouse or anti-goat (1:500, Invitrogen)

Single Cell Isolation

Epithelial cells were isolated by methods modified from previously described work.(Zavros et al., 2000) Stomachs are removed and washed multiple times in PBS. Forestomach and antrum were excised, and corpus was sliced into ~1mm^2 fragments. In some experiments, tissue was then placed in 50 µm Medicon (Beckman, a chamber designed for efficient cutting of tissue), and mechanically dissociated with 2, 30 second pulses in a Medimachine (Beckman). Tissue was removed from the Medicon and incubated in 10mL HBSS with 5mM EDTA and 1mM DTT for one hour at 37° with vigorous shaking. Tissue was

subsequently passed through a 50 μm mesh filter (Partec). Sections of tissue too large to pass through the filter were placed in 10 mL RPMI 1640 with 5% BSA (Sigma) and 1.5 mg/mL Dispase II (Stem Cell Technologies) for 1.5 hours at 37° with vigorous shaking and then passed through 50 μm mesh again. The dissociated cells that passed through the 50 μm mesh at either stage were then pooled, washed twice with cold PBS, filtered through a 50 μm mesh filter once more, and stained for Flow cytometry analysis/sorting.

Flow Cytometry Cell Sorting and Analysis

Single cells from the epithelial isolation were counted and suspended in PBS with 1% BSA and 5mM EDTA at 1 $x10^6$ cells/mL. Cells were stained with epithelial cell adhesion molecule (EpCAM)-Alexa 647 (1:100, Cell signaling), and CD13-FITC (1:200, BD Pharmingen) (20 minutes, 4°C). $EpCAM^+$ single cells from the $ANPEP^+$ and $ANPEP^-$ fractions were sorted using a MoFlo FACS machine (Dako/Cytomation) or analyzed by FACScan (Becton, Dickinson and Company). Flow cytometry data were analyzed with FlowJo 7.6 software.

Immunofluorescent characterization of Sorted Cells

$EpCAM^+$ single cells from the $ANPEP^+$ and $ANPEP^-$ fractions were plated on slides for immunofluorescent staining as follows: 1 $x10^5$ cells were pipetted into a plastic chamber, and centrifuged onto a slide using a Cystospin Slide Centrifuge (Cytospin) for 3 minutes at 800 RPM as previously described.(Pollock et al., 2013) Cells deposited on the slide were fixed with methanol (-20°C, 10 minutes), rinsed in PBS, and stained as described above. The number of GIF-positive cells was then quantified by an observer blinded to experimental condition in the $CD13^+$ and $CD13^-$ fractions of cells in 3 separate experiments.

qRT-PCR and Western Blot

RNA was isolated using RNeasy (Qiagen) per the manufacturer's protocol. RNA was treated with DNase I (Invitrogen) and then reverse transcribed using the SuperScript III (Invitrogen) standard protocol (most cDNA syntheses started with 1 μg of total RNA). Measurements of cDNA levels were performed by qRT-PCR using a Stratagene (La Jolla, CA) MX3000P detection system. Absolute QPCR SYBR green mix (Thermo Scientific) fluorescence was used to quantify relative amplicon amounts of *Gif*, *Atp4a*, and *18s*.

Cells for western blot analysis were lysed in RIPA buffer. Proteins were quantified by DC protein assay (Bio-Rad) and then separated on NuPAGE Bis-Tris gels (Invitrogen), transferred onto Amersham Hybond ECL nitrocellulose (GE Healthcare, Buckinghamshire, UK) membranes, and detected by Immobilon chemiluminescence (Millipore). Primary antibodies used were rabbit anti-ANPEP (Sigma) and rabbit anti-α- and β-tubulin (Cell Signaling). Secondary antibodies were horseradish-peroxidase-conjugated donkey anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA).

Graphing and Statistics

Experiments were performed at least 3 times independently. Values represent mean ± standard deviation or standard error of mean as indicated. All statistics and graphs were determined using GraphPad Prism and visualized with Adobe Illustrator. Statistical analysis was by one- or two-tailed Student's t test, depending on the hypothesis prior to commencing the experiment.

RESULTS

ANPEP is a membrane-associated protein expressed in fully differentiated ZCs. Given that ZCs have previously been purified by differential centrifugation and not by specific markers in published reports, and given our aim to develop flow cytometric techniques to isolate them, we sought to identify a surface marker exclusively expressed in ZCs. We identified potential ZC-specific markers with a screen using previously obtained microarray data coupled with gene ontology analysis.(Capoccia et al., 2013) We compared RNA isolated from pit, parietal, neck, and zymogenic cells previously obtained using Laser-capture microdissection and measured gene expression changes among these cell populations (Fig. 1A). We first looked for targets enriched in zymogenic cells vs. other cell types. Then, we sorted for targets associated with the plasma membrane, determined by gene ontology terms (Fig. 1B). Using this method, we found a short list of targets potentially expressed on the surface of the ZC that would allow isolation via flow cytometry.

The ZC in a normal stomach is a terminally differentiated, professional secretory cell residing in the base of the gastric unit. The ZC may act as a bellwether of damage or disease with the capacity to undergo SPEM (i.e., to reprogram to re-express progenitor markers and proliferate to repair damage to the gastric unit). To identify potential surface markers to enable the isolation of mature, fully-differentiated ZCs, we looked for those surface markers that were lost during SPEM induced by gastrotoxic doses of intraperitoneal tamoxifen. High dose tamoxifen causes rapid parietal cell death spurring ZC reprogramming to SPEM.(Huh et al., 2012) We performed Affymetrix GeneChip microarrays of the body of the stomach 12h after injection of tamoxifen (5 mg/20 g mouse weight) or vehicle control. As expected, markers of terminally differentiated parietal cells were rapidly lost upon treatment with tamoxifen,

consistent with parietal cells beginning to die: e.g. ATPase, H^+/K^+ exchanging, alpha polypeptide (*Atp4a*). A feature of ZCs undergoing SPEM is that, even though they begin to re-express progenitor markers, they maintain expression of some ZC genes, while turning off expression of others. One gene whose expression is known to be rapidly extinguished is basic helix-loop-helix family member a15 (*Mist1*).(Lennerz et al., 2010) We used this GeneChip screen to determine which of the potential ZC surface markers followed the *Mist1* pattern, indicating loss of expression in concert with ZC reprogramming. The only ZC specific surface marker (predicted from the screen in Fig. 1B) that followed that pattern was *Anpep* (aka CD13, Fig 1C), whereas the other markers were not changed significantly or actually increased during SPEM. Western blot of the mouse corpus gastric epithelium showed that ANPEP protein lingered at the 12h time point after tamoxifen, but the loss of *Anpep* mRNA did eventually correlate with decreased protein also by 3 days of daily injections (Fig. 1D). Thus, ANPEP is a surface marker, specific to ZCs in the normal stomach and lost upon ZC reprogramming in metaplasia.

ANPEP is expressed exclusively in mature zymogenic cells. To confirm that ANPEP expression is specific to ZCs, we used immunofluorescent staining in mouse and human stomachs. With confocal microscopy, we observe that ANPEP was expressed in ZCs but not in ZC-precursor cells (Fig. 2A-C). As neck cells move towards the base of the gastric epithelium, they transition into zymogenic cells, rapidly losing expression of neck cell markers like trefoil factor 2 (TFF2) and Griffonia simplicifolia II (GSII), and begin to express ZC markers eg. Pepsinogen (PGC) and Gastric intrinsic factor (GIF).(Karam and Leblond, 1993a; Ramsey et al., 2007) ANPEP expression distinctly marks cells expressing mature ZC markers anddoes not overlap with cells expressing ZC-precursor neck cell markers (Fig. 2C). Thus, ANPEP specifically identifies mature ZCs in a pattern that indicates expression on both intracellular and

plasma membranes supporting the possibility that it might be used as a surface marker to isolate pure ZCs from the gastric epithelium.

MIST1 is not required for ANPEP expression. MIST1 is a transcription factor responsible for scaling up the ZC secretory apparatus during terminal differentiation of these cells.(Mills and Taghert, 2012) Like ANPEP, in the stomach MIST1 is expressed exclusively in mature ZCs, and its expression is rapidly lost as ZCs scale down their secretory apparatus en route to SPEM.(Lennerz et al., 2010; Nam et al., 2010) Several of MIST1's transcriptional targets that help establish and maintain the cellular machinery that governs ZC secretion of digestive enzymes have been identified.(Capoccia et al., 2013; Tian et al., 2010) To determine if *Anpep* expression might be dependent on MIST1, we analyzed *Anpep* expression in RNA isolated from ZCs, and their mucous neck cell precursors, laser-capture microdissected from wild-type (WT) and *Mist1*$^{-/-}$ stomach bodies.(Capoccia et al., 2013) As expected, *Anpep* expression increased ~10-fold in mature ZCs compared to mucous neck cell precursors; however, this increase occurred in both WT and *Mist1*$^{-/-}$ mice. Thus, *Anpep* expression in ZCs was clearly not dependent on MIST1 (Fig. 3A). Additionally, ANPEP was clearly expressed at substantial levels in *Mist1*$^{-/-}$ ZCs and not mucous neck cells, just as occurs in WT (Fig. 3B). Note the ZC (and therefore the ANPEP-expressing) regions are significantly smaller, as previously characterized in mice lacking *Mist1*.(Ramsey et al., 2007)

ANPEP expression is lost in gastric disease. Recent studies have shown expression of ANPEP is altered during tumorigenesis.(Razvi et al., 2007; Wulfanger et al., 2012) For example, loss of ANPEP is an adverse prognostic factor in prostate cancer.(Sorensen et al., 2013) Because

of our data showing loss of ANPEP in ZCs upon induction of gastric metaplasia, we sought to determine if ANPEP expression was altered in other models of gastric disease. Infection by the bacterium, *Helicobacter pylori* (HP), can cause metaplasia in many people and thereby greatly increase risk for progression to gastric cancer.(Correa and Houghton, 2007) Indeed, the high-dose tamoxifen protocol discussed earlier is a rapid, reversible model for the effects of HP infection.(Huh et al., 2012) In mice, HP causes, over the course of 2-3 months parietal cell atrophy, and ZC reprogramming into SPEM.(Yoshizawa et al., 2007) In other words, in mice colonized by HP, ZCs re-express precursor neck cell markers and become proliferative. Eventually, HP infection causes hyperplasias and dysplasias.(Herrera and Parsonnet, 2009) We analyzed the base of gastric corpus units in 3 mice infected with a CagA+ strain of Helicobacter PMSS1 8 weeks after infection. As expected, infection caused parietal cell atrophy and SPEM in multiple regions throughout most of the corpus, by this timepoint (data not shown, but see (Capoccia et al., 2013; Khurana et al., 2013) for previous characterization of infections with this HP strain in these mice). Using immunofluorescent microscopy, we observed that mice colonized by HP labeled with both the neck cell marker GSII, and the ZC marker GIF in gastric ZCs that had reprogrammed into a SPEM differentiation state (Fig. 4A). Only cells that had not yet reprogrammed at this timepoint (i.e., did not label with GSII) retained their ANPEP expression.

To determine whether ANPEP expression was also lost in human gastric metaplasia, we looked at ANPEP expression in tissue sections from a database of tissues infected with HP that show regions of transition between normal and SPEM-type metaplasia that we have previously described (Capoccia et al., 2013; Khurana et al., 2013; Lennerz et al., 2010). (Fig. 4B) shows a representative region of transition to SPEM. As in completely normal human stomach, ZCs

labeled with antibodies against the ZC digestive enzyme pepsinogen (PGC) also labeled with antibodies against ANPEP (Fig. 4B). However, ZCs transitioning to SPEM, identified by labeling with both PGC and the mucous neck/precursor marker GSII, lose ANPEP expression (Fig. 4B). In glands showing complete SPEM, nearly every cell expresses both neck and zymogenic markers and has undetectable ANPEP. Even in units nearly completely transformed to SPEM, a rare PGC+/GSII- ZC (e.g, cell marked by arrow, Fig. 4B) will still be identifiable by its maintained expression of ANPEP.

Disassociation of Gastric Epithelial Cells and Analysis by Flow Cytometry. Because of strong cell-cell junctions and the high mucus environment of the stomach, gastric epithelial cells are challenging to dissociate into single-cell populations for isolation by differential centrifugation or flow/magnet-based sorting. In the past, we have used laser-capture microdissection to isolate cells from tissue, which has been useful for isolating smaller numbers of cells to generate RNA that can be amplified for qRT-PCR or microarray analysis (e.g., Fig. 1A,B). Laser-capture purification of cells is restricted to relatively small numbers of cells and is not the ideal technique for isolating pure populations of cell populations that are intermingled in tissues with complex organization like the gastric epithelium.(Bredemeyer et al., 2009) However, to isolate larger numbers of cells for biochemistry or for culture ex vivo in organoids, we experimented with multiple published protocols and with previous methods for dissociation used in our lab.(Mills et al., 2003; Mills et al., 2001; Zavros et al., 2000) We found that by mechanically disaggregating epithelial tissue before enzymatic digestion using a Medimachine,(Ottesen et al., 1996) we were able to reduce the fraction of cell aggregates and doublets ~5-fold when compared to tissue cut into small pieces with a razor blade before subsequent enzymatic digestion (Fig. 5A). Mechanical disaggreagation did not affect cell

viability as measured by Propidium Iodide incorporation (Fig 5B). We have also found that in addition to difficulty in achieving single cell dissociation, analysis of gastric epithelial cells with flow cytometry is complicated by high levels of autofluorescence of gastric epithelial cells. Dissociated single cells from normal mouse stomach fluoresce across the detectable spectrum in response to excitation at multiple wavelengths in the absence of any exogenous label. Indeed when light emitted from an unstained stomach is compared to that of an unstained spleen in (Fig 5C), it is evident that any positive signal from staining with antibody-conjugated fluorophores could be potentially obscured by non-specific signal from unstained cells.

When we sorted gastric epithelial cells based on their intrinsic autofluorescence and characterized them thereafter by lineage marker expression, we observed no consistent pattern in autofluorescence intensity relative to cell lineage or viability (not shown). We established a method that reliably detects true positive cells with specific staining by first analyzing emission of a gastric epithelial cell population in two close wavelengths on the same plot as shown (Fig 5D). The autofluorescence in both channels is similar, causing a linear plot of ~x=y light emission in each channel. When these cells are stained with an antibody conjugated with a fluor that emits at one of the wavelengths, the additional signal is detectable, so positive cells can be identified as a population distinct from the autofluorescent cells on the diagonal of the plot. By looking simultaneously at the detectors for APC (positive channel, stained with APC-conjugated EpCAM antibody) and APC-Cy7 (negative control channel, used to adjust for autofluroescence) in (Fig. 5D), we can see that a population of Epithelial Cell Adhesion Molecule (EPCAM) positive cells is distinguishable. This distinct population would not be readily observable by analyzing APC signal alone. Mechanical disaggregation and FACS analysis, carefully controlled

for autofluorescence, allowed for accurate and repeatable analysis of individual populations of gastric epithelial cells.

ANPEP can be used to isolate mature ZCs from the gastric epithelium. We used the above method with ANPEP as a surface marker to isolate a purified population of zymogenic cells (Fig 6A). We dissociated, then stained gastric epithelial cells with an APC-Cy7 conjugated anti-ANPEP antibody. We found ~14% of EPCAM$^+$ epithelial stomach cells were also ANPEP$^+$, consistent with the fraction of gastric epithelial cells that are differentiated ZCs.(Mills et al., 2003) Additionally, flow-sorted ANPEP positive cells expressed the ZC-specific marker GIF (Fig. 6B,C) but not the parietal cell-specific marker *Atp4a*, while the ANPEP negative fraction expressed *Atp4a* but not GIF (Fig. 6C). We next induced SPEM using tamoxifen, which led, as expected, to a decreased fraction of ANPEP-positive epithelial cells, consistent with earlier findings in tissue (Fig. 6D).

Discussion

Elucidation of the molecular mechanisms regulating normal homeostasis in the stomach as well as aberrant differentiation patterns like SPEM has been hindered by a limited toolkit for isolating and studying individual cell populations. There are few markers of mature gastric ZCs, and to our knowledge no ZC-specific surface marker has ever been characterized. Our data show that ANPEP is expressed exclusively in mature ZCs and that its expression is lost when they reprogram to SPEM-type metaplastic cells in both mice and humans. Here, we have also presented what has proven to be a useful for protocol for cell dissociation and flow cytometric sorting using ANPEP as a marker to purify ZCs. ANPEP-mediated isolation of ZCs can be used in “OMICS” studies in the future to determine, for example, ZC-specific gene expression, chromatin modifications, transcription factor binding sites under different conditions. Alternatively, we have shown that ZCs expressing specific promoters can serve as stem/progenitor cells ex vivo in organoid systems.(Stange et al., 2013) A method for isolating all ZCs that is not genetically based (i.e., not based on transgenic or knockin expression of inducible Cre recombinases) would be useful in determining the progenitor properties of ZCs in a parallel, complimentary manner.

ANPEP is an integral membrane protein that hydrolyzes peptides in multiple tissues and has varying functions that depend on the cells and tissues where it is expressed. In the small intestine, ANPEP hydrolyzes peptides from proteins partially digested by gastric and pancreatic proteases;(Kruse et al., 1988) it is also required for normal cholesterol absorption. In endothelial cells, ANPEP is required for cell motility and adhesion, and disruption prevents endothelial cell invasion in matrigel assays.(Ghosh et al., 2014; Mina-Osorio et al., 2008; Petrovic et al., 2007) ANPEP null mice don’t manifest any observable phenotypes in normal conditions, but have

exhibit impaired angiogenesis in pathological settings.(Rangel et al., 2007) We used several small molecule inhibitors of CD13 function (Ezetimibide, Bestatin, Tosedostat, Circumin),(Kramer et al., 2005; Rich et al., 1984; Shim et al., 2003; Wickstrom et al., 2011) but found no observable phenotype in the normal gastric epithelium (data not shown). ANPEP expression correlates negatively with progression to prostate cancer and with aggressiveness of disease.(Sorensen et al., 2013) Expression of ANPEP is also known as myeloid antigen, because it is expressed in myeloid lineages of the bone marrow, and has been proposed to correlate with worse prognosis in lymphoblastic leukemias.(Craddock et al., 2013; Dalal et al., 2014; Shim et al., 2014) Despite a large, and somewhat controversial literature on the topic, it is not clear there is functional significance for ANPEP expression in lymphoid leukemias; it may simply be a reflection of abnormal gene expression as a whole where genes normally restricted to the myeloid lineage are mis-expressed in lymphoid neoplasms.(Alfalah et al., 2006; Pasqualini et al., 2000; Saxena et al., 2010) ANPEP expression has been shown to be increased in gastric cancers with poor prognosis, though we are unaware of previous reports on its expression in normal fundic-type mucosal cells like ZCs.(Carl-McGrath et al., 2004) Given that ANPEP is a known intestinal protein, and gastric cancers frequently have intestinal differentiation,(El-Zimaity et al., 2002; Hattori, 1986) it is possible that ANPEP expression is correlated with progression from SPEM (thought to be the earliest lesion in gastric cancer tumorigenesis as it correlates with loss of parietal cells in the stage known as Atrophic Gastritis),(Nozaki et al., 2008) in which ANPEP is not expressed as we show in the current study, to later stage disease like intestinal metaplasia, dysplasia, and cancer where it returns.

The role of ANPEP is in ZCs is not clear. Its rapid loss during SPEM in multiple mouse models and human tissue indicates that it may play a role in their secretory function, as one of

the first events in ZC reprogramming during injury is scaling down MIST1 and the secretory apparatus.(Lennerz et al., 2010) ANPEP expression is on the plasma membrane and also seems to be distributed around secretory granules in ZCs. It has been shown to help regulate phagocytosis,(Villasenor-Cardoso et al., 2013) be required for development of secretory tissue like mammary glands(Kolb et al., 2013) and for secretion of cytokines.(Kuhlmann et al., 2009) An interesting function of ANPEP seems to be to specifically degrade the cytokine like IL-8. (Kanayama et al., 1995; Mishima et al., 2002) Perhaps ZC ANPEP helps reduce IL-8 abundance under homeostatic conditions. More severe damage that can lead to reprogramming of ZCs and loss of ANPEP would allow increased abundance of this cytokine to the gastric epithelium.

In summary, we have refined protocols for the isolation, and analysis of gastric epithelial cells have identified the first surface marker of mature ZCs useful for flow cytometry, and have described the pattern of this protein ANPEP in normal and metaplastic gastric epithelium. The tools should allow for additional characterization of other isolated gastric epithelial cells with other markers and help us better understand the biology of the elaborate but highly plastic digestive-enzyme secreting cells of the stomach.

Figure Legends:

Figure 1. **ANPEP is expressed in mature gastric zymogenic cells**. A) H&E stained gastric unit. Each circle represents a distinct cell lineage isolated for Microarray analysis. B) Heat map representing enrichment of surface markers of ZCs. Genes enriched in ZCs when compared to other cell populations were sorted by gene ontology terms for plasma membrane markers. 5 potential candidates were identified. C) Change in candidate gene expression in response to tamoxifen-induced metaplasia, measured by microarray of RNA isolated from corpus portion of mouse stomach. MIST1 is known to be lost in metaplasia. ANPEP was the only candidate from panel B with decreased expression. D) Western blot of mouse stomach. ANPEP expression is lost when metaplasia is induced with tamoxifen, though loss of protein trails loss of mRNA expression in panel C

Figure 2. **ANPEP is expressed exclusively in zymogenic cells**. A,B) Human and mouse gastric epithelium respectively stained with ANPEP (green), neck cell specific GSII (purple), and zymogenic cell specific PGC (red). In every mature zymogenic cell, PGC and ANPEP are expressed, but not GSII. C) Confocal image of ZC region of mouse gastric epithelium stained as described above. ANPEP expression is absent in cells that express GSII. Arrowhead marks a cell in transition between neck and ZC zones. ANPEP is not expressed where GSII epitope is maintained and marks only cells that express the ZC marker GIF (Gastric Intrinsic Factor, red). All scale bars represent 20μm

Figure 3. **MIST1 is not required for *Anpep* expression**. A) Affymetrix GeneChips generated from lasercapture microdissected ZCs and their precursor mucous neck cells from wild-type and

Mist1 null mice. *Mist1* and *Anpep* gene expression are enriched in the ZCs relative to neck cells. *Anpep* expression is not decreased when *Mist1* is deleted. B) Immunofluorescent staining of wild type and MIST1 KO mouse stomach showing no loss of ANPEP protein in ZCs despite the absence of MIST1. Note loss of MIST1 does make ZCs smaller.

Figure 4. **ANPEP is lost during ZC metaplasia**. A) ZCs in mouse stomachs infected with *H. pylori* undergo SPEM, evidenced by overlapping PGC and GSII expression in the base of units. SPEM cells that express both GSII and PGC do not express ANPEP (yellow arrowhead) B) Tissue from a patient infected with H. pylori in a region of transition between normal basal gastric gland architecture and SPEM-type metaplastic differentiation pattern, ANPEP is expressed only in mature ZCs; i.e., only in cells expressing the digestive enzyme PGC and not the SPEM/mucous neck cell marker GSII. Normal tissue, tissue transitioning to SPEM, and SPEM tissue is highlighted from left to right, respectively. Asterisk indicates a cell that expresses both neck and ZC markers (i.e. a SPEM cell), but not ANPEP, adjacent to a cell that expresses only ZC markers and has not lost ANPEP expression (i.e. a normal ZC). White arrowhead denotes the only cell in the SPEM unit that expresses only ZC markers and retains ANPEP expression.

Figure 5. **Optimization of FACS to sort and analyze gastric epithelium** A) Doublet discrimination analysis of mouse gastric epithelium shows mechanical disaggreagation is necessary to achieve single cell isolation (Mean±SEM, n=3 biological replicates). B) Additional mechanical disaggreagation does not increase cell death in mouse gastric epithelial cells. (Mean±SEM, n=3 biological replicates). C.) Autofluorescent histograms of unstained spleen

(grey) and stomach (red) throughout detectable fluorescent spectrum. High levels of autofluorescence are present throughout the spectrum in gastric epithelial cells. D) EpCAM staining of single gastric cells.

Figure 6. **ANPEP can be used to isolate ZCs** A) FACS plot of ANPEP stained gastric epithelium. EpCAM-positive cells were stained with an APC-Cy7 conjugated ANPEP antibody (x-axis) and plotted against autofluorescence in an unstained channel (APC) B) quantification of ZC marker positive cells in sorted fractions. ANPEP-positive mouse gastric epithelial cells were isolated using flow cytometry, and attached to a slide using cytospin. Cells were then stained for the ZC marker GIF, and quantified. (Mean±SEM, n=3 biological replicates) C) Semi-qPCR of ANPEP-positive and negative mouse gastric epithelial cell fractions for ZC (*Gif*) and parietal cell (*Atp4a*) specific genes. D) FACS quantification of ANPEP expression in EpCAM-positive gastric epithelial cells treated with vehicle or high-dose tamoxifen for 12 hours or 3 days to induce SPEM. (Mean±SEM, n=3 biological replicates)

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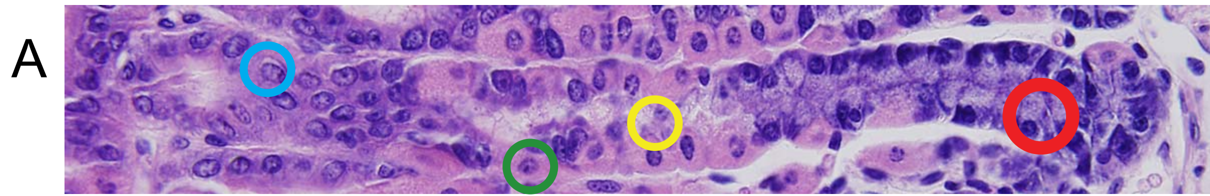
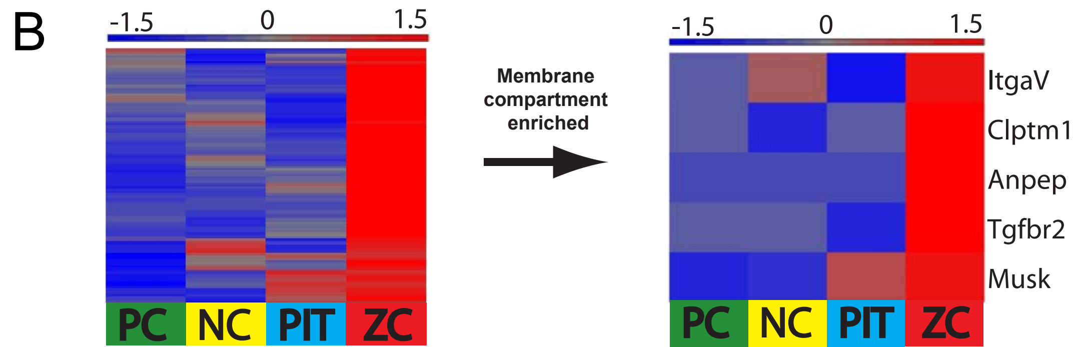
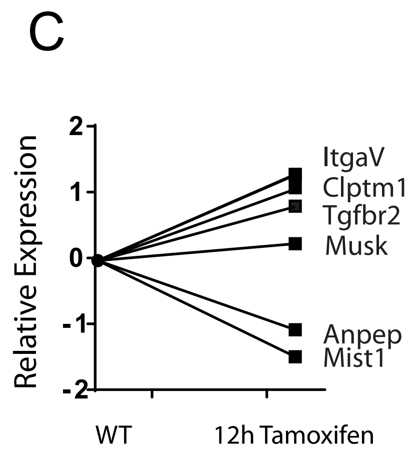
Figure 1







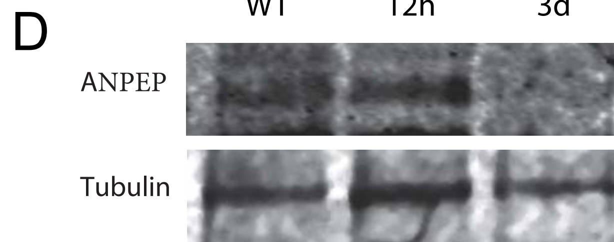


Figure 2

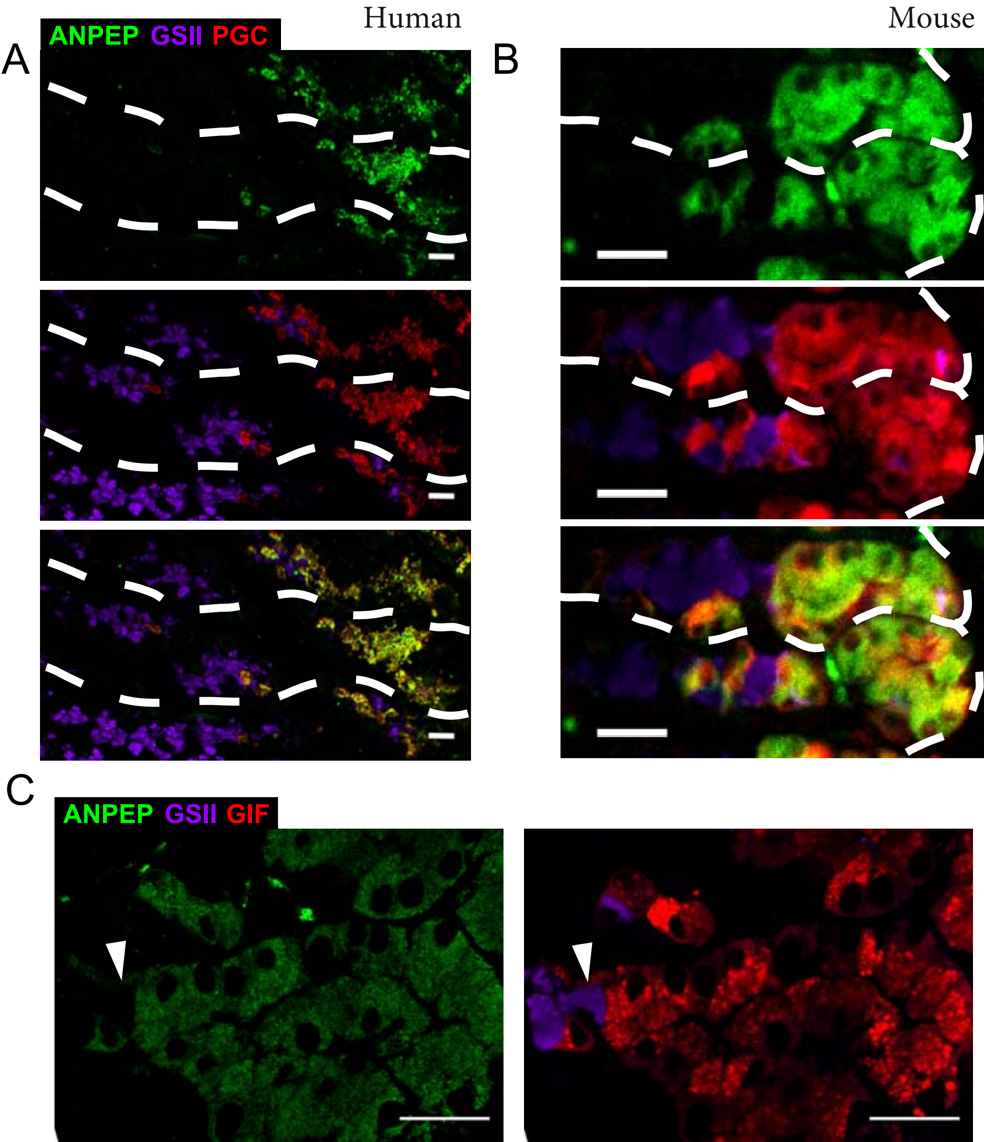
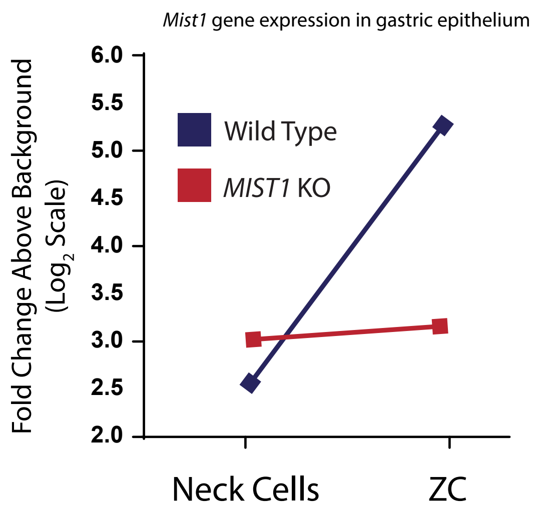
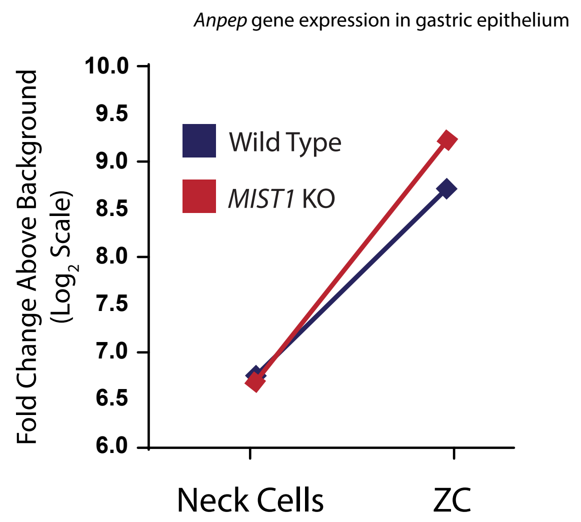


Figure 3

A





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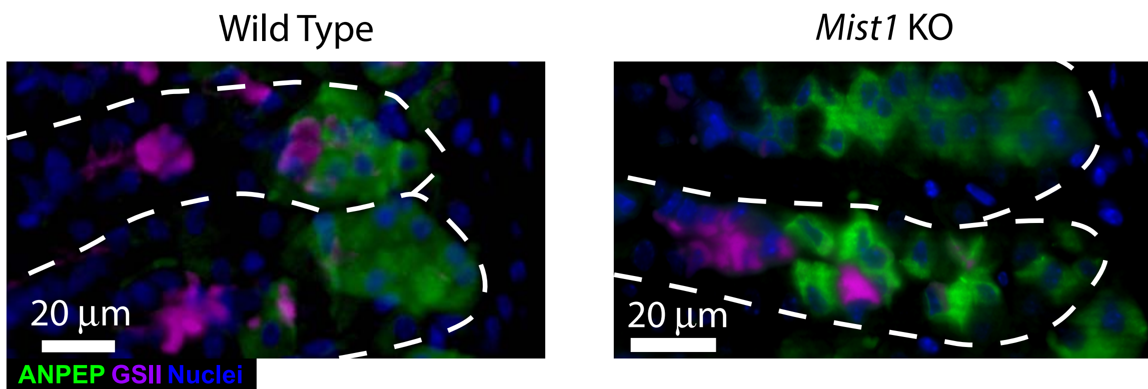


Figure 4

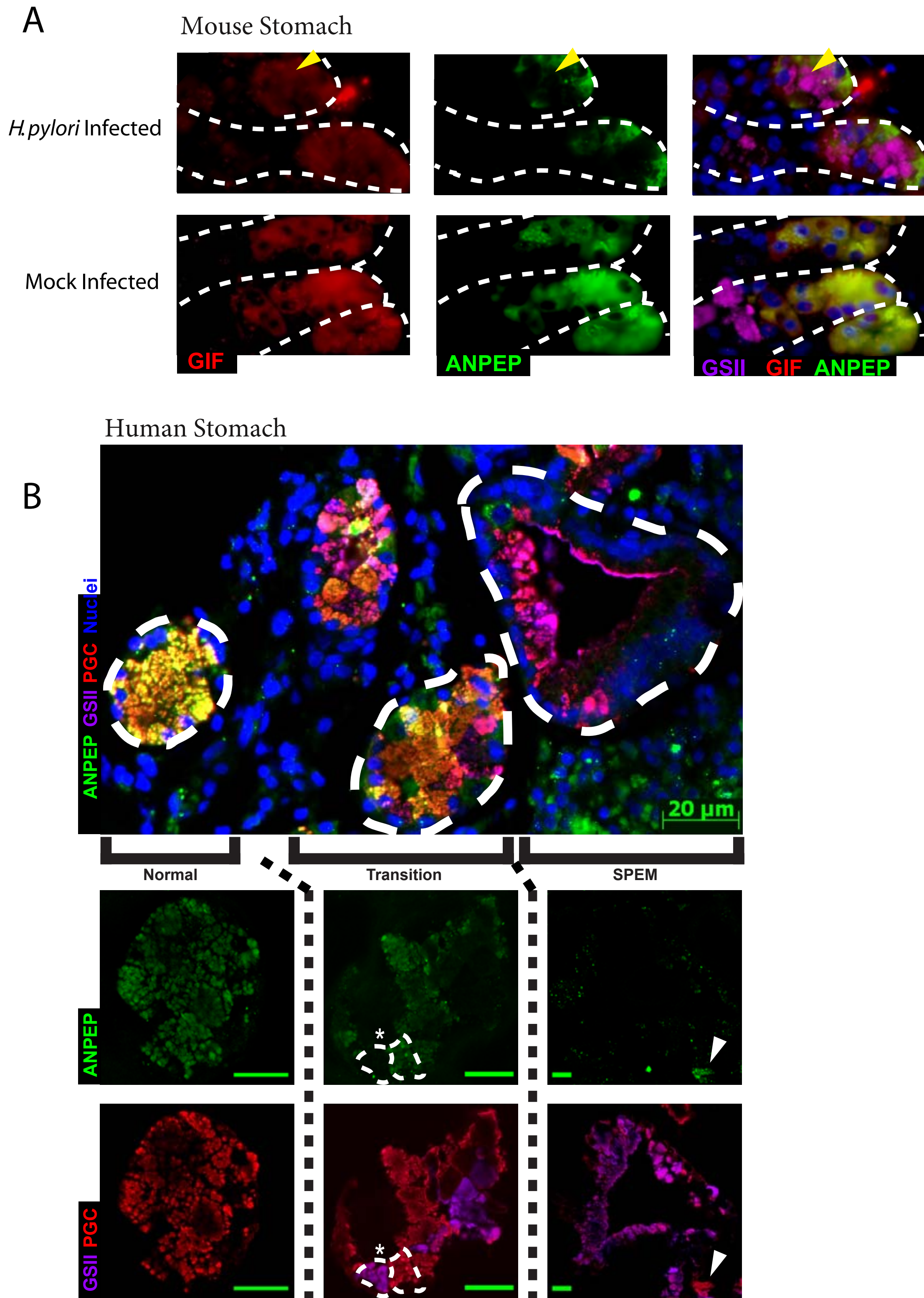


Figure 5

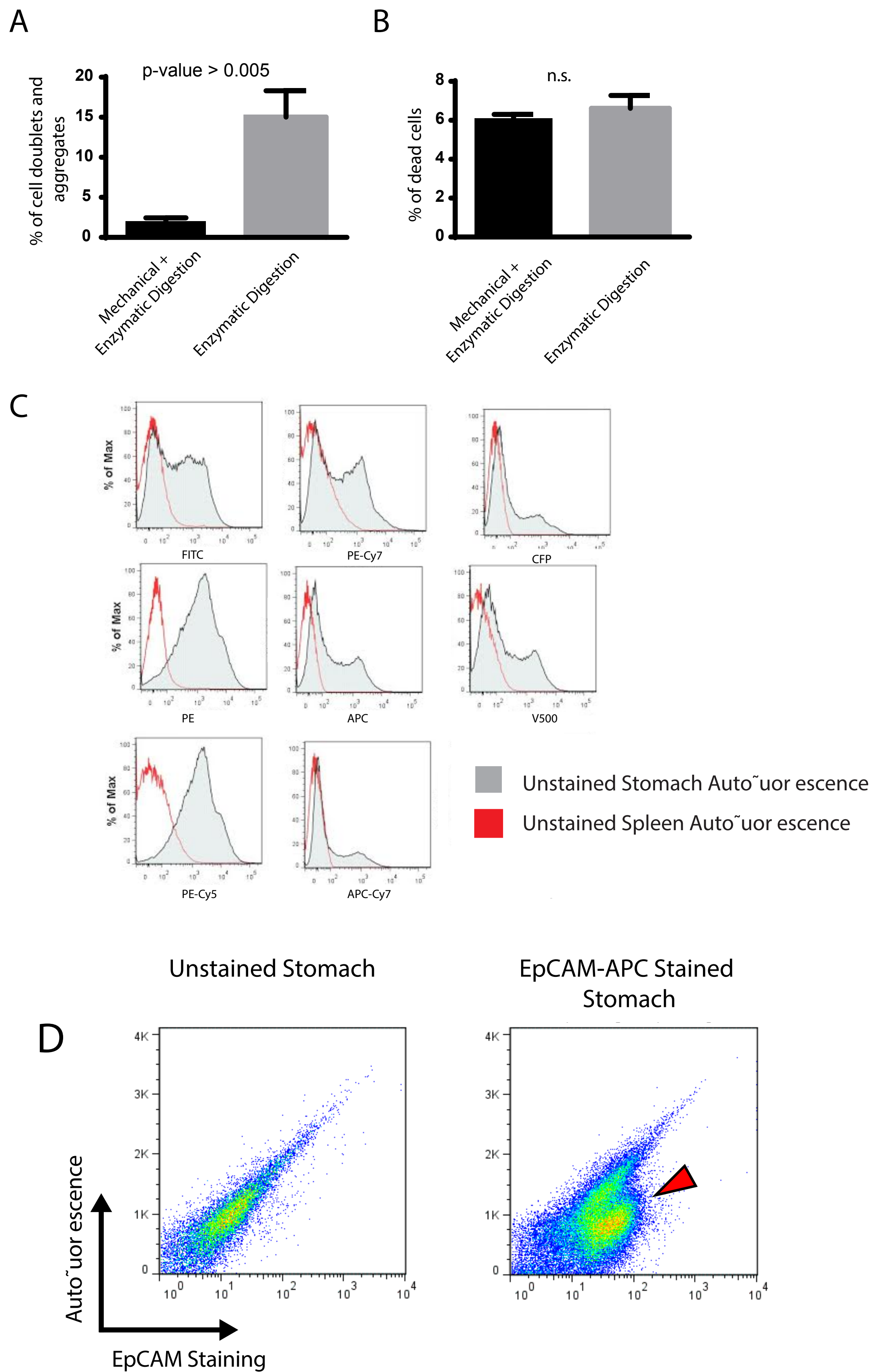
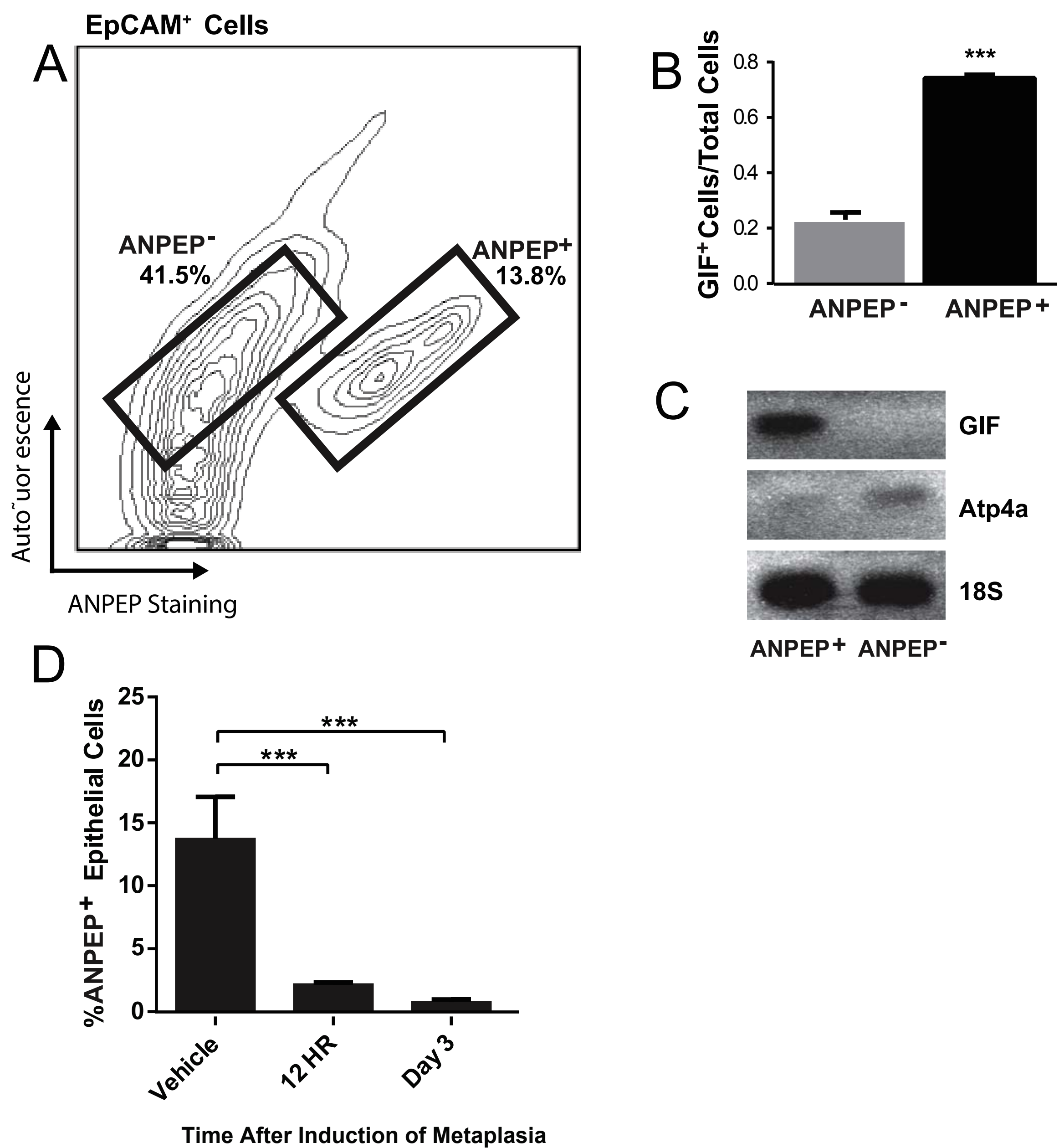


Figure 6



CHAPTER FOUR

Transcriptional Regulation of *Xbp1* by HNF4α is Vital to Beta-Cell Function

TRANSCRIPTIONAL REGULATION OF X-BOX-BINDING PROTEIN ONE (XBP1) BY HEPATOCYTE NUCLEAR FACTOR 4α (HNF4A) IS VITAL TO BETA-CELL FUNCTION.

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Abstract

The transcription factor, X-box Binding Protein-One (XBP1), controls the development and maintenance of the endoplasmic reticulum (ER) in multiple secretory cell lineages. We show here that Hepatocyte Nuclear Factor 4-alpha (HNF4α) directly induces *XBP1* expression. Mutations in *HNF4α* cause Mature-Onset Diabetes of the Young I (MODYI), a subset of diabetes characterized by diminished glucose-stimulated insulin secretion (GSIS). In mouse models, cell lines, and ex vivo islets, using dominant negative and human-disease-allele point mutants or knockout and knockdown models, we show that disruption of HNF4α caused decreased expression of XBP1 and reduced cellular ER networks. GSIS depends on ER Ca^{2+} signaling; we show that diminished XBP1 and/or HNF4α in β-cells led to impaired ER Ca^{2+} homeostasis. Restoring XBP1 expression in the absence of HNF4α was sufficient to completely rescue GSIS in β-cells. Our findings uncover a transcriptional relationship between HNF4α and *Xbp1* with potentially broader implications about MODYI and the importance of transcription factor signaling in the regulation of secretion.

Summary

HNF4α transcriptionally regulates XBP1, and loss of *HNF4α* causes diminished pancreatic β cell insulin secretion that is rescued by XBP1.

Introduction

Cells use transcription factors to regulate expression of gene cohorts that coordinate response to stress, determine specific developmental fates, and scale intracellular architecture during physiology and disease.(Huh et al., 2010; Mills and Taghert, 2012). It is well established that ER stress causes increased activity of the transcription factor x-box binding protein 1 (XBP1) via IRE1 splicing of the *XBP1* transcript.(Shen et al., 2001; Yoshida et al., 2001). However, XBP1 also establishes the subcellular machinery for synthesizing large quantities of protein during the normal development of professional secretory cells.(Huh et al., 2010; Lee et al., 2005). How XBP1 is induced during differentiation of secretory cells even in the absence of substantial ER stress is unclear, but an obvious mechanism is that XBP1 may also be transcriptionally regulated. Hepatocyte Nuclear Factor 4-alpha (HNF4α) is a highly-conserved transcription factor responsible for orchestrating the early development and maintenance of multiple adult organs. As a master developmental regulator, HNF4α likely acts upstream of the factors that establish the extensive cellular machinery required in professional secretory cell lineages within those organs. Despite overlapping expression and function, no direct relationship between HNF4α and XBP1 has yet been described.

HNF4α is vital for β-cell function, and indeed, human mutations in HNF4α cause Mature-Onset Diabetes of the Young 1 (MODYI), a subset of diabetes characterized by diminished glucose-stimulated insulin secretion (GSIS) in pancreatic β-cells.(Yamagata et al., 1996) While we know that β-cells require HNF4α to function, we understand little about the mechanistic/physiological role of HNF4α in these cells. Previous work showed that disrupting HNF4α expression in vivo in mouse islets resulted in diminished GSIS similar to that observed in MODY patients with HNF4α mutations. Loss of HNF4α also was observed to disrupt Ca2+

signaling, though the mechanisms underlying those defects remain unclear. Decreased ER function is a plausible mechanism for the loss of function in MODYI β-cells, because: insulin secretion in β-cells is diminished if ER homeostasis is disturbed, (Cardozo et al., 2005; Hara et al., 2014) defects in ER-related proteins contribute to multiple diabetic phenotypes in humans,(Inoue et al., 1998) and HNF4α has been shown to be important for maintaining ER stress response.(Luebke-Wheeler et al., 2008) In addition, knocking down XBP1 specifically in β-cells also leads to significantly reduced GSIS. Finally, disruption of calcium homeostasis in the ER leads to impaired GSIS, similar to the pathology observed in MODY1 islets.(Jacobo et al., 2009)

Here we have identified how XBP1 expression is governed at the transcriptional level and establish HNF4α as a direct transcriptional regulator of its expression. This implicates HNF4α in the maintenance and establishment of secretory-cell ER networks. Accordingly, we report for the first time that both HNF4α and XBP1 are required to maintain ER calcium homeostasis and GSIS in β-cells. In addition, we show that restoration of *XBP1* expression alone in islets lacking HNF4α is sufficient to rescue impaired GSIS. Thus, the results may provide new insight towards discerning why dysfunction in HNF4α causes the pathophysiological findings in MODYI patients.

Results

Regulation of *Xbp1* by HNF4α. To elucidate the potential transcriptional regulation of XBP1, we identified evolutionarily conserved binding sites in the human *XBP1* promoter by aligning regions of synteny, then screening them with the Transfac transcription factor binding site database.(Ovcharenko et al., 2005) Two regions with high conservation containing putative HNF4α binding sites, 1.4 and 2.6 kilobases upstream of the *Xbp1* transcription start site, were identified using first the Transfac transcription factor binding library and then affirmed using a previously published algorithm developed to search for sites of high HNF4α binding affinity.(Bolotin et al., 2010) These putative binding sites were constitutively occupied by HNF4α in mouse pancreas, measured via chromatin immunoprecipitation (Fig. 1A). Overexpression and knockdown experiments *in vitro* showed HNF4α was both sufficient and necessary for normal *Xbp1* expression in pancreatic β-cell derived-cell lines. Disrupting HNF4α either by siRNA knockdown (Fig. 1B,C) or by overexpressing a dominant-negative version of HNF4α (Fig. 1D,E) resulted in a 65-75% decrease in *Xbp1* expression in INS-1 and MIN-6 cells. Conversely, overexpression of HNF4α via transient transfection caused a five-fold increase in *Xbp1* expression (Fig. 1F). To further substantiate this transcriptional relationship, we analyzed the effects of *Hnf4α* deletion on *Xbp1* expression in other tissues by mining published microarray studies and by direct qRT-PCR analysis of adult and embryonic liver(Battle et al., 2006) as well as adult small intestine (Supplemental Fig. 1)(Bonzo et al., 2012; Cattin et al., 2009; Hayhurst et al., 2001). Again, the results showed a consistent trend toward correlation of *Xbp1* expression decrease with loss of *Hnf4α* in multiple secretory tissues.

Various single point mutations in the HNF4α locus have been identified in patients afflicted with MODYI. To better understand the impact of these mutations, we designed two

HNF4α expression vectors, each containing one of the most prevalent MODYI mutations.(Furuta et al., 1997; Lindner et al., 1997) Each of these mutations expressed both a fully functional homo-dimerization domain and DNA-binding domain but an altered or truncated ligand-binding domain (Supplemental Fig. 2A,B). Overexpression of each of these individual MODYI mutants in INS-1 cells resulted in a ~4-fold decrease in the expression of *XBP1* (Supplemental Fig. 2C), suggesting that prevalent MODYI mutations decrease XBP1 expression *in vitro*.

HNF4α is required for ER maintenance *in vivo*. The mechanisms whereby disruptions of HNF4α in MODYI cause β-cell dysfunction remain an area of open debate. As XBP1 is critical in scaling up and maintaining the ER of professional secretory cells, we hypothesized mutations in *HNF4α* may drive MODYI pathology via dysregulation of XBP1 and consequent ER dysfunction in the insulin-secreting β-cells. We also sought to study the relationship between HNF4α and XBP1 in vivo in mice with loss of *Hnf4α* induced in adulthood, mirroring the onset of MODYI. After global deletion of *Hnf4α* in adult mice, we observed significant loss (~60%) of *Xbp1* expression in islets and of XBP1 transcriptional targets like *Edem1* (Fig. 2A) when compared to littermate *Hnf4α*$^{floxed/+}$controls (referred to hereafter as "WT") (Lee et al., 2003). Supporting previous findings, Insulin and *Hnf1α* mRNA levels were unaffected by loss of *Hnf4α*.(Gupta et al., 2005; Miura et al., 2006) In accordance with XBP1's role in professional secretory cells of maintaining cell architecture and not as a predominantly ER-stress-response gene (Huh et al., 2010; Todd et al., 2009), decreased XBP1 in *Hnf4α*$^{Δ/Δ}$ mice did not cause increase in the unfolded-protein response genes *Chop*, *Bip*, or *Atf4,* nor a decrease in mRNA levels of the ER marking *Calregulin (CRP55)* (Fig. 2B). However, loss of XBP1 following deletion of *Hnf4α* did correlate with a nearly 7-fold reduction in ER network in β islet cells (Fig.

2C,D, Supplemental Fig. 3A-D). Despite decreased ER in each cell, islet area was not changed in *Hnf4α*$^{Δ/Δ}$ pancreata (Fig. 2E), indicating that *Hnf4α* is not required to maintain islet number or size. Thus, loss of *Hnf4α* caused diminished ER network, a phenotype similar to that caused by deleting *Xbp1* from existing adult secretory cells.(Huh et al., 2010; Lee et al., 2005)

HNF4α and XBP1 are necessary to maintain ER calcium homeostasis. In previous reports, constitutive deletion of *Hnf4α* from islets early in development, as opposed to in the adult, caused impaired GSIS.(Gupta et al., 2005; Miura et al., 2006). The mechanism of decreased GSIS was hypothesized to result from dysregulated cytoplasmic Ca^{2+} signaling in response to glucose, but the molecular mechanism driving this impairment has remained unclear. Ca^{2+} signaling depends on the ATP-dependent closure of KATP channels, triggering membrane depolarization and opening voltage-gated Ca^{2+} channels(Ashcroft et al., 1984). Thus, one mechanism that could mediate how loss of *Hnf4α* could cause GSIS could be via disruption of those channels. However, qPCR analysis showed levels of the KATP channel subunits *Sur1* (*Abbc8*) and *Kir6.2* (*Kcnj11*) in *Hnf4α*$^{Δ/Δ}$ islets were unchanged relative to littermate controls (Fig. 3A). The sulfonylurea Tolbutamide, which closes KATP channels leading to membrane depolarization, stimulated less insulin secretion in INS-1 cells expressing dnHNF4α than in normal INS-1 cells (Supplemental Fig. 4). The inhibition of Tolbutamide-stimulated insulin secretion caused by disrupted HNF4α was rescued by transduction of *Xbp1*, though transducing *Xbp1* alone did not increase tolbutamide-stimulated insulin secretion (Supplemental Fig. 4). These data, along with previous work showing impaired insulin secretion even upon depolarization with KCl,(Gupta et al., 2005) suggest the defect in the glucose response pathway

due to disrupted HNF4α is distal to KATP channel dependent-membrane depolarization and depends on loss of XBP1.

Though KATP channel expression was unchanged in *Hnf4α*$^{Δ/Δ}$ islets, expression of the putative XBP1 transcriptional target, *Serca2b(Atp2a2)*, was significantly reduced. (Park et al., 2010) SERCA2b is an ER Ca^{2+} pump, responsible for establishing and maintaining the large calcium gradient between the ER and cytoplasm.(Vangheluwe et al., 2005). , Intracellular stores of Ca^{2+} are critical for GSIS, because Ca^{2+} release from these stores triggers secretion of insulin granules. Accordingly, decreasing ER Ca^{2+} stores and/or flux has been shown to disrupt GSIS (Jacobo et al., 2009). Thus, in *Hnf4α*$^{Δ/Δ}$ mice, the decreased expression of a key molecular driver of the ER Ca^{2+} gradient suggested that disruption of ER Ca^{2+} stores may play a critical role in the GSIS abnormalities seen in the absence of normal HNF4α. We used an ER-specific FRET sensor to measure ER [Ca^{2+}] in *ΔHnf4α* β-cells (Hara et al., 2014; Palmer et al., 2004). The D1ER cameleon construct encodes two fluorophores conjugated to a calmodulin molecule that is targeted specifically to the ER lumen with a KDEL sequence. When it binds Ca^{2+}, the cameleon undergoes a conformational change that approximates the fluors to produce FRET activity quantitatively proportional to ER [Ca^{2+}] (Fig. 3B). In accordance with the mechanism of lost GSIS in MODYI being disruption of XBP1-mediated ER Ca^{2+} stores, both knockdown of HNF4α and pharmacological inhibition of XBP1 activation in INS-1 cells resulted in decreased ER [Ca^{2+}] (Fig. 3C). Simultaneously knocking down HNF4α and pharmacologically inhibiting XBP1 did not augment the decreased ER [Ca^{2+}]. Thus, it is likely that HNF4α and XBP1 work via the same pathway to maintain high ER [Ca^{2+}] in adult β-cells. To further explore the requirement of HNF4α for proper Ca^{2+} signaling in β-cells, we observed changes in cytoplasmic Ca^{2+} levels in response to various stimuli in *ΔHnf4α* and normal INS-1 cells using Fura 2AM-

based Ca^{2+} imaging. Expectedly, *ΔHnf4α* β-cells exhibited a diminished response to stimulation with 16.7mM glucose, as observed in other MODYI β-cell models (Fig. 3D, Supplemental Fig. 5A). To identify the cause of this deficit in Ca^{2+} signaling, and further explore our previous results indicating HNF4α is required for ER Ca^{2+} homeostasis, we exposed these cells to 20mM Caffeine. Caffeine is an agonist of the ryanodine receptor that stimulates release of Ca^{2+} from stores in the ER and thus an increase in cytoplasmic [Ca^{2+}].(Verkhratsky and Shmigol, 1996) Caffeine induced diminished cytoplasmic [Ca^{2+}] increase in *ΔHnf4α* β-cells (Fig. 3E, Supplemental Fig. 5B), indicating that ER Ca^{2+} homeostasis is disrupted. In short, loss of ER Ca^{2+} in *ΔHnf4α* β-cells may underlie their impaired GSIS.

XBP1 is sufficient to rescue insulin secretion in *ΔHnf4α* β-cells. We next sought to confirm the physiological relevance of the HNF4α→*XBP1* relationship by determining if we could rescue aberrant GSIS in the absence of HNF4α simply by restoring *XBP1*. If *XBP1* were decreased in the absence of HNF4α due to direct loss of transcriptional upregulation by HNF4α, as we hypothesized, then restoration specifically of unspliced *XBP1* (*XBP1u*) should rescue GSIS, because *XBP1u* is the unmodified mRNA directly generated from transcription of the *XBP1* gene. We used an *in vitro* model employing transgenic INS-1 cells containing a doxycycline inducible dominant negative-HNF4α (dnHNF4α) (Wang et al., 2000). As with other methods we used to examine how loss of HNF4α activity affected insulin-secreting cells (e.g., Fig. 1D), doxycycline treatment to induce dnHNF4α caused both decreased XBP1 and loss of GSIS (Fig. 4A). This decreased GSIS was completely rescued by adenoviral transduction of *Xbp1u*. As discussed above, forced expression of *Xbp1u* was also sufficient to rescue impaired insulin secretion in response to sulfonylurea treatment in these dnHNF4α β-cells (Supplemental

Fig. 4). We repeated this study *ex vivo*, using *Hnf4α*$^{Δ/Δ}$ islets isolated 3 weeks following tamoxifen-induced *Hnf4α*$^{Δ/Δ}$ deletion. As expected, cultured islets had impaired GSIS due to HNF4α deficiency. *Xbp1u* restoration increased the direct XBP1 transcriptional targets *Edem1* and *Serca2b* (Fig. 4B) confirming that transduction of *Xbp1u* restored functional XBP1-mediated transcriptional activity to scale up expression of its normal transcriptional targets. Remarkably, restoring unspliced *Xbp1* expression in these *ex vivo* cultured, HNF4α-deficient β-cells was also sufficient to completely rescue their GSIS, indicating that the impaired GSIS in the absence of HNF4α depends on transcriptional maintenance of *XBP1* expression by HNF4α (Fig. 4C). Because of the direct transcriptional regulation of *XBP1* by HNF4α and the lack of GSIS enhancement in WT β-cells transduced with *Xbp1u*, the ability of *Xbp1u* to rescue the phenotype caused by loss of HNF4α is likely because it corrects the diminished basal *Xbp1* expression in ΔHNF4α β-cells.

We also transduced spliced XBP1 (*Xbp1s*) in isolated ΔHNF4α mouse islets, bypassing the normal regulation of transcriptionally regulated *Xbp1u* by IRE1α splicing. Transduction of *Xbp1s* rescued the XBP1 targets, *Edem1* and *Serca2b* (Supplemental Fig 6A) but resulted in GSIS roughly 50% lower than that in control WT islets (Supplemental Fig 6B). That result is consistent with previous reports that β-cell homeostasis is compromised by forced expression of spliced *Xbp*1 because cells must be able to dynamically regulate XBP1 levels via the endogenous IRE1 splicing mechanism (Allagnat et al., 2010). Accordingly, *Hnf4α* $^{Δ/Δ}$ islets infected with *Xbp1s* exhibited GSIS rescue to the levels observed in WT islets infected with *Xbp1s*, suggesting that, while forced expression of XBP1s is detrimental to β-cell health, it is still able to compensate for GSIS defects in β-cellscaused by the absence of HNF4α.

Discussion

We report that *Xbp1* is a direct transcriptional target of HNF4α in multiple secretory tissues. Given the importance of *HNF4α* mutations in diabetes, we have focused on the relationship between HNF4α and XBP1 specifically in insulin-secreting β-cells. Deletion of HNF4α in β-cells causes them to lose XBP1, which in turn causes dismantling of ER. *HNF4α* point mutants designed to match mutations that cause human MODYI also resulted in loss of XBP1 in vitro. Loss of either HNF4α or XBP1 leads to disrupted ER Ca^{2+}, which in turn diminishes GSIS, a pathology that can be completely rescued by reestablishing normal XBP1 levels (Fig 4D, Supplemental Fig. 4,6). Together, our results identify a new transcriptional relationship between evolutionarily-conserved genes, *Xbp1* and $Hnf4\alpha^{\Delta/\Delta}$, involved in fundamental development and disease in multiple tissues.

We also demonstrate specific cellular contexts during which *Xbp1* expression is functionally regulated at the transcriptional level. XBP1 is induced in response to unfolded protein accumulation in the ER by splicing of its message via the endonuclease IRE1α, and the canonical view of how XBP1 abundance is modulated concern that mechanism. On the other hand, in multiple, long-lived professional secretory cells like antibody-secreting plasma cells and zymogenic chief cells, expression of the unspliced *Xbp1* transcript also increases many fold.(Huh et al., 2010; Reimold et al., 2001) Thus, there are likely transcriptional mechanisms that govern expression of *Xbp1* as well, though these have largely not been elucidated. It is somewhat surprising that HNF4α, which is largely studied in developmental contexts as a master regulator of differentiation in endodermal organs, is required for continued maintenance of XBP1 in differentiated, adult cells. However, unbiased, comprehensive screens for genes whose expression depends on HNF4α have previously identified XBP1 as a potential target*(14-17)*, and

chromatin immunoprecipitation followed by sequencing (ChIP-Seq) has also shown peaks indicating potential binding of HNF4α to the putative *XBP1* promoter.(Boyd et al., 2009) Thus, though the results of the previous screens have not been validated and the direct relationship between HNF4α and XBP1 has apparently never been specifically studied, our results are not entirely unprecedented. Indeed, HNF4α has also been shown to regulate expression of ankyrin repeat and sterile α motif domain containing 4b (*Anks4b*), a protein that binds ER chaperones and augments the ER stress response, supporting our hypothesis that HNF4α is required for the establishment and maintenance of the ER.(Sato et al., 2012)

ER Ca^{2+} homeostasis is important for myriad cellular processes and plays a pivotal role in intracellular Ca^{2+} signaling. Our data indicate that XBP1 and HNF4α are required for maintaining this homeostasis. The targets whose expression is dictated by XBP1/HNF4α and help maintain ER homeostasis are not clear, but one such candidate target may be SERCA2b. Transduction of XBP1u or XBP1s in β-cells increased expression of SERCA2b, the ER Ca^{2+} transporter, in isolated islets (Fig. 4B, Supplemental Fig. 6A), confirming previous studies performed *in vivo* in the liver.(Park et al., 2010) Thus, our results indicate that knocking down HNF4α causes disruption of the expression of the Ca^{2+} pump responsible for establishing the high [Ca^{2+}] in the ER, a functional decrease in ER [Ca^{2+}], and finally, a diminished release of ER Ca^{2+} when cells are stimulated with caffeine (Fig. 3A,C,E). These data outline a potential mechanism wherein altered ER Ca^{2+} homeostasis from loss of HNF4α function disrupts Ca^{2+} signaling and insulin release in β-cells of patients with MODYI (model in Supplemental Fig. 7).

There are no faithful animal models of MODYI. Some mutations in *HNF4α* in humans result in alleles though to cause diabetes via a dominant negative mechanism,(Furuta et al., 1997; Lindner et al., 1997) whereas others would be expected to act via haploinsufficiency.(Alam et

al., 1995; Thomas et al., 2001) Thus, it may be premature to reach any firm conclusions about MODYI mechanisms based on our results using known MODYI-inducing human polymorphisms and adult-onset knockout of *Hnf4α* in mice. However, if we are to speculate on the implications of our findings, we might suggest that they indicate consideration of a new angle on MODYI therapy. Currently, MODYI is responsive to treatment with sulfonylureas, though treatment often eventually involves insulin therapy to manage hyperglycemia, presumably because β-cells eventually become dysfunctional or die (Pearson et al., 2005). Our results suggest that various therapies targeting ER homeostasis, a rapidly developing therapeutic avenue with several drugs at various stages of development, may augment or improve existing approaches to managing MODYI.

Materials & Methods

Cell lines and Transient Transfection

Min6 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 25 mM Hepes, and 285 μM 2-mercaptoethanol, and penicillin and streptomycin. INS-1 832/13 cells were cultured in the RPMI 1640 containing 10% fetal bovine serum (FBS), penicillin and streptomycin, sodium pyruvate and β-mercaptoethanol. Human embryonic kidney (HEK)-293 cells (ATCC) were cultured in DMEM containing 10% FBS and penicillin and streptomycin. INS-1 cells containing doxycycline inducible dnHNF4α were treated with 500ng/mL doxycycline to induce expression as previously described.(Wang et al., 2000) All cells were passaged at 90% confluency using trypsin-EDTA. For overexpression of myc-tagged HNF4α2 coding regions (obtained from addgene) were subcloned into a pcDNA3.1expression vector, and 5μg of each plasmid or the pmaxGFP(lonza) control plasmid were transiently transfected using TransIT-2020 (Mirus, Madison, WI). For mutation analysis, site-directed mutagenesis was performed using the HNF4α overexpression vector described above as an initial template. Mutations were introduced for each mutant using primers listed in (Supplemental Fig. 9). Constructs were verified to be correct by DNA sequencing. For siRNA we transfected MIN6 and INS-1 cells with 10nM HNF4α siRNA (silencer select Invitrogen) using Lipofectamine 2000 according to the manufacturer's protocol.

in silico Identification of HNF4α binding sites in XBP1 promoter

Areas of high conservation among multiple mammalian species (human, rhesus, mouse, rat, dog) 10kb upstream and downstream of the XBP1 transcription start site were identified using ECR browser (http://ecrbrowser.dcode.org/). These areas were then scanned with the

Transfac transcription factor binding database for known transcription factor binding site sequences. Two *Hnf4α* sequences were identified; one 1.2 kb upstream (hg19 chr22:29,198,941) of the *Xbp1* transcription start site and one 2.4 kb upstream (hg19 chr22:29,197,731).

<u>qRT-PCR and Western Blot</u>

RNA was isolated using RNeasy (Qiagen) per the manufacturer's protocol. RNA was treated with DNase I (Invitrogen) and then reverse transcribed using the SuperScript III (Invitrogen) standard protocol (most cDNA syntheses started with 1 μg of total RNA). Measurements of cDNA levels were performed by qRT-PCR using a Stratagene (La Jolla, CA) MX3000P detection system. Absolute QPCR SYBR green mix (Thermo Scientific) fluorescence was used to quantify relative amplicon amounts of Primers listed in Supplemental Fig. 9.

Cells for western blot analysis were lysed in RIPA buffer. Proteins were quantified by DC protein assay (Bio-Rad) and then separated on NuPAGE Bis-Tris gels (Invitrogen), transferred onto Amersham Hybond ECL nitrocellulose (GE Healthcare, Buckinghamshire, UK) membranes, and detected by Immobilon chemiluminescence (Millipore). Primary antibodies used were rabbit anti-XBP1(Santa Cruz), mouse anti-c-myc (dshb), and rabbit anti-α- and β-tubulin (Cell Signaling). Secondary antibodies were horseradish-peroxidase-conjugated donkey anti-rabbit and anti-mouse Ig (Santa Cruz Biotechnology, Santa Cruz, CA). Quantifications of immunoblots were performed by scanning 16-bit images into ImageJ. Band intensities for XBP1 and α/β tubulin were selected and calculated by using the 'Analyze mean gray value' measurement tool. Standardized values were calculated determining the ratio of XBP1 signal to α/β tubulin signal.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described previously.(Im et al., 2004) Approximately 100 mg of tissue from the pancreata of 5, 6-8 week old WT mice were homogenized and used for this ChIP experiment. Ten microliters of anti-HNF4α (rabbit anti-human MIST1) or whole rabbit serum (preimmune control) together with protein A/G plus agarose (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the homogenized tissue for immunoprecipitation. Quantitative real-time PCR (qRT-PCR) was performed (the sequences used and all other primer sequences are available in Supplemental Fig. 9) to assess the quantity of genomic sequences immunoprecipitated by either preimmune control or HNF4α antiserum, as well as a 1:10 dilution of the cell extract prior to immunoprecipitation (input). Two predicted HNF4α binding sites were probed in addition to an intronic control region with no predicted HNF4α binding sites nearby. Data are graphed as a percentage of precipitated DNA:total input(genomic DNA).

Mouse Studies

All experiments involving animals were performed according to protocols approved by the Washington University School of Medicine Animal Studies Committee. Floxed *HNF4α*, $CAGGCreER^{TM}$ transgenic mice were generated by crossing $Hnf4\alpha^{floxed/floxed}$ mice (a gift from Frank Gonzalez, NIH)(Hayhurst et al., 2001) with $CAGGCreER^{TM};Hnf4\alpha^{floxed/+}$(Hayashi and McMahon, 2002) mice to allow systemic, tamoxifen-inducible knock out of HNF4α. 6-8 week old $CAGGCreER^{TM}$;$Hnf4\alpha^{floxed/floxed}$ mice and $CAGGCreER^{TM}$;$Hnf4\alpha^{floxed/+}$ littermate controls were injected intraperitoneally with tamoxifen (5mg/20g body weight, 5 consecutive days) to

induce cre-mediated *Hnf4α* deletion. Mice were sacrificed 4 weeks after first tamoxifen injection. No mouse samples were excluded from analysis in this study.

Beta-cell morphological characterization using Immunofluorescence

Pancreata were prepared and stained as described previously.(Tian et al., 2010) Briefly, they were fixed with freshly prepared formalin and suspended in fixative for 24 hours at room temperature, followed by multiple rinses in 70% ethyl alcohol (EtOH), arrangement in 2% agar in a tissue cassette, and routine paraffin processing. Sections (5 μm) were deparaffinized and rehydrated, and then antigen retrieval was performed by boiling in 50 mM Tris-HCl, pH 9.0. Slides were blocked in 1% bovine serum albumin (BSA) and 0.3% Triton X-100 in phosphate-buffered saline (PBS) and then incubated in goat anti-Calregulin (SantaCruz) followed by AlexaFluor594 antigoat. Fluorescence microscopy and imaging were performed using a Zeiss Axiovert 200 microscope with Axiocam MRM camera with Apotome.

For morphological analysis, 3, 5 μm sections taken 100 μm apart were stained with hematoxylin and eosin to allow identification of islets. Whole slides were scanned with Nanozoom microscope and the cross-sectional area of islet/total pancreas tissue was measured across each slide using Nanozoom Digital Pathology software (Hamamatsu). Samples were randomized, and the scorer was blinded to ensure unbiased quantification. Values are expressed as %β-cell area.

Immunofluorescent Quantification

For quantification of ER in islets, the pancreata of HNF4α KO and littermate control heterozygous mice were fixed, mounted and stained as described above. 16-bit images captured in Zeiss Axiovision software were analyzed with ImageJ software as follows; Insulin positive

regions (Santa Cruz rabbit anti-insulin) were measured as regions of interest, and mean fluorescence intensity of the global ER marker Calregulin in each region was determined and then subtracted from the median fluorescent intensity of acinar cell regions in the same image to normalize fluorescence intensity on each slide. The mean fluorescent intensity was measured in every islet in a 5μm section (three mice/condition). Analysis of Calregulin fluorescence was restricted to the β-cell cytoplasm by excluding Hoechst-positive (nuclear) regions. After capture, each image was assigned a random number so that subsequent fluorescent quantification was blind relative to condition. Nuclear areas were identified by Hoescht staining, and pixels with an intensity of >30 gray value as determined by the "plot profile" tool in ImageJ were excluded from measurement. Hoescht-negative, cytoplasmic pixels were measured and normalized by subtracting the mean fluorescence of the surrounding acinar tissue.

Endoplasmic Reticulum Calcium Fret measurement

INS-1 832/13 cells stably expressing the D1ER calcium sensor(Hara et al., 2014; Palmer et al., 2004) were cultured as described above. Cells were transiently transfected with 10nM HNF4α siRNA or scrambled control siRNA to knockdown HNF4α expression. Cells were also treated with vehicle or 16μM 4-methyl umbelliferone 8-carbaldehyde (4μ8C) to inhibit XBP1 splicing as previously described.(Cross et al., 2012) Five days post transfection/treatment, 100k cells were seeded in transparent bottom 96 well plates to achieve 70% confluency 6 hours pre-measurement. Cells were washed twice in PBS and incubated in Krebs-Ringer buffer supplemented with 3mM glucose immediately before measurement. To establish maximum and minimum ER Ca^{2+} levels, at this time, cells were respectively treated with 10μM membrane-

permeabalizing ionomycin and subsequently 10mM $CaCl_2$ or 5mM EGTA. As an additional control for low ER Ca^{2+} Cells were treated with 1µM Thapsigargin to specifically diminish ER Ca^{2+}. FRET ratio of the D1ER cameleon was measured using the Tecan Infinite M1000Pro microplate reader. Fluorophores were excited at 434 nm, and emission was quantified at 530nm (YFP) and 477nm (CFP). The ratios were measured across 4 fields/well, and the values were averaged from 4 wells per experimental condition. After FRET microplate measurement, RNA was isolated as described above and quantified by qPCR to measure *Hnf4α* and *Xbp1* knockdown efficiency (Supplemental Fig. 8).

Ratiometric calcium imaging and data analysis

Studies were performed 24 hours after plating INS-1 823/13 cells at 50% confluency and carried out at 37°C with 5% CO_2 in a perifusion chamber with a flowrate of 2mL/min. Cells were loaded with Fura-2AM by incubation at 37°C in Krebs-Ringer buffer supplemented with 3mM glucose, 1µM Fura-2AM, and 0.1% Pluronic F-127 for 30 min, washed in HBSS, and incubated for another 30 min to allow for ester hydrolysis. After loading, cells were imaged on an inverted microscope (Till Photonics; Munich, Germany) equipped with a cooled CCD camera (Cooke, Auburn Hill, MI) using a ×20/0.45 Plan Fluor objective (Nikon). The fluorescence excitation (340 and 380 nm) was provided by a Polychrome V Monochromator (Till Photonics). After the matching background was subtracted, the image intensities from each pair of images, measured at 520nm, were divided by one another to yield ratio values for individual cells. $[Ca^{2+}]_i$ in individual cells was estimated based on the formula: $[Ca^{2+}]_i = KD \times B \times (R - Rmin)/(Rmax - R)$, where KD is the indicator's dissociation constant for Ca^{2+} (0.22 µM); R is ratio of fluorescence intensity at two different wavelengths (340/380 nm); Rmax and Rmin are the ratios

of Ca^{2+}-free and Ca^{2+}-bound Fura-2, respectively; and B is the ratio of the fluorescence intensity of the second excitation wavelength at zero and saturating Ca^{2+} concentrations. The calibration constants were determined as previously described,(Grynkiewicz et al., 1985) and the ratio values were plotted against time.

Islet Isolation and Culture

Pancreatic islets from CAGGCreERTM;*Hnf4α*$^{floxed/floxed}$ mice and CAGGCreERTM;*Hnf4α*$^{floxed/+}$ littermate controls were isolated as previously described,(Li et al., 2009) by pancreatic duct injection of 1000 U/mL of collagenase solution (sigma) followed by digestion at 37°C for 15 minutes with mild shaking. Islets were washed several times with Hanks' balanced salt solution, separated from acinar cells by straining through a 100 µm filter, viewed under a dissecting microscope, and handpicked for culture (yield = 200-300 islets/mouse). Isolated Islets were maintained in RPMI1640 supplemented with 10% FBS and penicillin and streptomycin at 37° with 5% CO_2. All islets were allowed to recover from isolation for 24h before analysis. Islets were isolated from mice in random order relative to condition.

Adenoviral transduction

Unspliced XBP1 adenovirus (Applied Biological Materials), LacZ Adenovirus (Applied Biological Materials), and spliced XBP1 (a gift from Laurie Glimcher),(Lee et al., 2008) were amplified in HEK293 cells, cultured as described above. Infected cells were lysed by three

cycles of freezing and thawing and then centrifuged. Viral titer was determined by infecting HEK-293 cells with serially diluted viral stock and overlaying with agar and subsequently counting the resulting plaques. INS-1 cells containing dnHNF4α were treated with doxycycline or vehicle to induce expression of dnHNF4α as described above. Five days post-treatment, cells were infected with either XBP1u or LacZ adenovirus at a multiplicity of infection (MOI) of 100. Viral stock was replaced with complete medium after 2 hours of infection. Isolated murine islets were infected as described previously.(Muniappan and Ozcan, 2009) Briefly, 70 islets/condition were washed in cold PBS, pretreated with HBSS containing 2 mM EGTA at 37° with 5% CO_2 for 15 min, then infected with adenovirus in serum-free RPMI 1640. Following a15 min incubation, complete medium was added to islet culture. Islets were infected for 24 hr before GSIS assay and harvesting RNA. Adenovirus was used in isolated islets at the following MOIs (Supplemental Fig. 8): LacZ MOI=50, XBP1u MOI=50, XBP1s MOI=10.

Glucose-Stimulated Insulin Secretion Measurement

For INS-1 GSIS assay, 2 days post-adenoviral infection cells were washed with PBS, then incubated for one hour in Krebs-Ringer Buffer containing 3mM glucose. After one hour, cells were washed with PBS, and basal insulin secretion was measured by incubating cells for one hour in Krebs-Ringer Buffer containing 3mM glucose. Media were sampled, then replaced with media containing 16.7 mM glucose, or 200μM Tolbutamide for one hour. Media were collected and analyzed for insulin content by ELISA using the Singulex Erenna platform by the Washington University Diabetes Research Center Immunoassay Core. Static GSIS was similarly measured in isolated islets as previously described.(Nolan and O'Dowd, 2009) 24h post

infection, fifty islets were placed in Krebs-Ringer Buffer containing 3mM glucose to measure basal insulin secretion, then stimulated with 16mM glucose. Insulin secretion was measured in each condition as described above.

Graphing and statistic

All graph values represent the mean of the sample, and error bars represent SEM where indicated. Significance was determined using student's T-test or ANOVA with Dunnet's comparison as indicated. Wherever possible, samples were randomized and measurements were blinded to prevent the introduction of experimental bias. Sample sizes were determined based on statistical significance and practicality.

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Figure Legends

Figure 1. HNF4α is a direct transcriptional regulator of *XBP1* A) Immunoprecipitation of chromatin from 5 C57/B6 mouse pancreata with anti-HNF4α followed by qPCR (ChIP assay) showed significant occupancy at 2 predicted binding sites in the *Xbp1* promoter (but not at a downstream intronic control site lacking a predicted HNF4α-binding motif) when compared to normal preimmune serum controls. B) MIN6 and C.) INS-1 cells were transfected with siRNA targeting *Hnf4α* or scrambled control siRNA and *Hnf4α* and *Xbp1* mRNA was quantified by RT-qPCR at 48 hours post transfection and normalized to 18S. (means±SEM of n=6 experiments depicted, statistical significance by one-tailed Student's t test) D) INS-1 cells stably expressing a doxycycline-inducible *Hnf4α* construct that acts as a dominant negative were incubated in the presence or absence of doxycycline for 7 days (means±SEM of n=12 experiments depicted, statistical significance by one-tailed Student's t test). E) Representative western blot following activation of doxycycline inducible myc-tagged DN-*HNF4α* in INS-1 cells (label at right is at the level of the specific XBP1s protein). F) Transient transfection of INS-1 cells with an HNF4α expression vector or a GFP control plasmid and quantification of *XBP1* mRNA 48 hours post transfection. (means±SEM of n=3 experiments depicted, statistical significance by one-tailed Student's t test). For all figures the following symbols mean: "***" - p<0.001; "**" - p<0.01; "*" – p <0.05.

Figure 2. HNF4α is required for XBP1 expression *in vivo*. *Hnf4α*$^{floxed/floxed}$ mice under the control of a ubiquitously-expressed CAGGCre^{ERT} promoter were treated with tamoxifen to induce *Hnf4α* deletion. A) Pancreatic islets were isolated 28 days after beginning tamoxifen

treatment (see Methods) and their RNA harvested. Expression of *Xbp1*, *Hnf4α* and the downstream XBP1 target, *Edem1*, was assayed by qRT-PCR. B) Expression of other unfolded protein response pathway transcripts, *Chop*, *Bip*, and *Atf4* were assayed as for (A). C) Immunofluorescent images of the ER marker Calregulin reveal altered ER structure in ΔHnf4α mouse islets. Scale bars=20μm. D) Mean fluorescent intensity (arbitrary units from 16-bit images) of non-nuclear, β-cell-specific Calregulin staining in mouse islets was determined after normalizing each tissue section to neighboring acinar Calregulin mean fluorescence (see Supplemental Fig. 3 and Methods for more details) Data represent means±SEM from 3 mice/condition, significance determined by one-tailed Student's t-test. E) Total β-cell area in the pancreas was quantified by anti-insulin immunofluorescence from sections by completely sectioning through whole tissue blocks of the entire embedded pancreata from *ΔHnf4α* and controls (means±SEM from 6 mice/condition, significance determined by one-tailed Student's t-test).

Figure 3. HNF4α and XBP1 are required for ER Ca^{2+} Homeostasis. A) qRT-PCR performed on RNA harvested from ΔHnf4α islets as for Figure 2 for transcripts for the KATP channel genes KIR6.2 (*Kcnj11*)and SUR1 (*Abcc8*) and the ER Ca^{2+} pump SERCA2b (*Atp2a2*) (means±SEM from 6 mice/condition, significance determined by one-tailed Student's t-test). B) Cartoon of the ER Ca^{2+} FRET Sensor D1ER. In low Ca^{2+} conditions, calmodulin is conformed in a manner that does not allow photon transfer between the 405nm excitable CFP and the unexcitable YFP fluorophore. In high Ca^{2+} conditions, the CFP domain emits at a frequency to excite YFP. Measuring the YFP(FRET):CFP ratio determines relative Ca^{2+} levels in the ER as opposed to other cellular compartments. C) FRET:CFP ratio in INS-1 D1ER cells was determine to quantify

relative ER Ca^{2+} levels under the following conditions: five days after transfection of *Hnf4α* or scrambled siRNA; 5 days after incubation with vehicle or 4μ8c, an inhibitor of XBP1 activation; or combinations thereof. To determine maximal detectable Ca^{2+} levels, FRET:CFP was determined 1 hour following treatment with the Ca^{2+} ionophore Ionomycin + $CaCl_2$. The FRET/CFP of that condition was set to 1.0, and all other conditions were normalized to it. To determine the minimal detectable ER Ca^{2+} levels using this assay, cells were treated with 10μM Ionomycin + EGTA for 1 hours or 1 μM Thapsigargin. (means±SEM of n=6 experiments depicted, statistical significance by one-tailed Student's t test). D) Cytoplasmic [Ca^{2+}] of individual INS-1 cells 5 days after transfection of *Hnf4α* or scrambled siRNA determined by Fura-2AM emission levels in response to 16.7mM glucose. Plots are representative of (n>50 cells;4 biological replicates; see other representative plots in Supplemental Fig. 5). E) Cytoplasmic [Ca^{2+}] of individual INS-1 cells 5 days after transfection of *Hnf4α* or scrambled siRNA determined by Fura-2AM emission levels in response to 20mM caffeine, an agonist of the ryanodine receptor, added to induce release of ER Ca^{2+} stores. Plots are representative of (n>50 cells;4 biological replicates).

Figure 4. XBP1 is Sufficient for GSIS in ΔHnf4α β-cells. A) Glucose-stimulated insulin secretion (GSIS) was determined by harvesting supernatant from wildtype INS-1 and doxycycline-inducible DN-HNF4α INS-1 cells following incubation for one hour under high (16mM) glucose conditions. Induction of dominant negative HNF4α abrogates GSIS (insulin secretion of ~1 means no induction relative to baseline insulin secretion with 3mM glucose). All cells were transduced by adenovirus carrying either unspliced *Xbp1* or *LacZ* control vectors. Note that *Xbp1* transduction rescues GSIS in DN-HNF4α cells (means±SEM of n=6 experiments

depicted, statistical significance by one-tailed Student's t test), B) ΔHNF4α or heterozygote control islets were cultured for 24 hours after isolation and then transduced with either *LacZ* or *Xbp1* vector-containing adenovirus. 24 hours later, RNA was harvested and qRT-PCR performed for transcripts from *Hnf4α*, *Xbp1* and two downstream transcriptional targets of XBP1, *Serca2b* and *Edem11* (data represent means±SEM from 3 individual islet isolations and transduction experiments). C) Normalized GSIS was determined as for panel (A) with transduction of either *LacZ* or *Xbp1* vector-containing adenovirus into isolated islets. Note isolated islets from $Hnf4\alpha^{\Delta/\Delta}$ mice exhibit a complete lack of GSIS. (data represent means±SEM from 3 individual islet isolations and transduction experiments) D) Model of MODYI pathology. XBP1 regulates transcription of multiple genes like *SERCA2B* that induce and maintain normal ER and ER Ca^{2+} function. Loss of HNF4α in patients with MODYI causes reduced XBP1 expression.

Supplemental Fig. 1. **HNF4α is necessary for XBP1 in multiple tissues**. A) Quantification of mRNA in ΔHNF4α KO embryonic (E18.5) liver tissue relative to wildtype littermates by qRT-PCR.(Battle et al., 2006) (Significance was determined using student's t-test; error bars represent SEM of 3 mice/condition). B-D) Mouse microarray data archived in the Gene Expression Omnibus (GEO)(Cattin et al., 2009; Hayhurst et al., 2001; Lee et al., 2003) show reduced XBP1 expression upon loss of HNF4α in multiple tissues. Expression intensity values from redundant probes for *Xbp1* in each normal and ΔHNF4α tissue were averaged together. Error bars represent standard deviation of each biological replicate.

Supplemental Fig. 2 Transfection of H*nf4α* containing mutations corresponding to those in characterized human MODYI patients decrease *XBP1* expression *in vitro* A) Conserved domain analysis of HNF4α2 protein and location of mutations with respect to domains B) Structure of the HNF4α homodimer bound to DNA as characterized previously.(Chandra et al., 2013) Note the location of MODYI mutations, after the homo-dimer/DNA binding domain and before the ligand binding domain, presumably allowing mutated HNF4α to function as a dominant-negative. C) *Xbp1* mRNA expression levels in INS-1 cells transiently transfected with MODYI mutants and GFP cDNA as a control. Error bars represent standard deviation of 3 biological replicates. Significance determined by ANOVA with Dunnet's comparison.

Supplemental Fig. 3 Diminished ER in ΔHNF4α mouse islets. A) Epifluorescent images of the pan-ER marker calregulin in mouse islets. ΔHNF4α islets exhibit significantly altered ER. B) Calregulin (red) and Hoescht (blue) stain of mouse islet. C) Pixel intensity along a line bisecting

each islet shows dramatically reduced calregulin signal in the non-nuclear areas of the islet. Integration of the Hoescht negative areas was used to determine the cytoplasmic intensity of calregulin staining in (Fig. 2D)

Supplemental Fig. 4. Tolbutamide induced insulin secretion. Expression of a dominant negative form of HNF4α in INS-1 cells completely abrogates increased insulin secretion in response to tolbutamide exposure, suggesting the MODY1 secretion defect is distal to β-cell membrane depolarization in the GSIS signaling cascade. Infection with an adenovirus carrying *Xbp1u* expression vector is sufficient to rescue this GSIS defect. Dn-HNF4α INS-1 cells were treated with doxycycline as shown in (Fig. 4A) (means±SEM of n=3 biological replicates depicted, statistical significance by one-tailed Student's t test)

Supplemental Fig. 5. Cytoplasmic Ca^{2+} signaling in ΔHNF4α β-cells. A,B) Cytoplasmic [Ca^{2+}] of INS-1 cells upon addition of 16.7mM Glucose or 20mM caffeine, respectively, determined by Fura-2 excitation ratios. Plots represent cytoplasmic [Ca^{2+}] vs time of individual INS-1 cells, and are representative of (n>50) of each condition (4 biological replicates). Bars at the top of each plot indicate duration of treatment.

Supplemental Fig. 6. Rescue of GSIS in ΔHNF4α islets with spliced *Xbp1*. A) Disruption of *Hnf4α* expression reduces *Xbp1* expression and the expression of downstream XBP1 targets in *ΔHnf4α* mouse islets compared to heterozygous control islets. B) *ΔHnf4α* and heterozygote

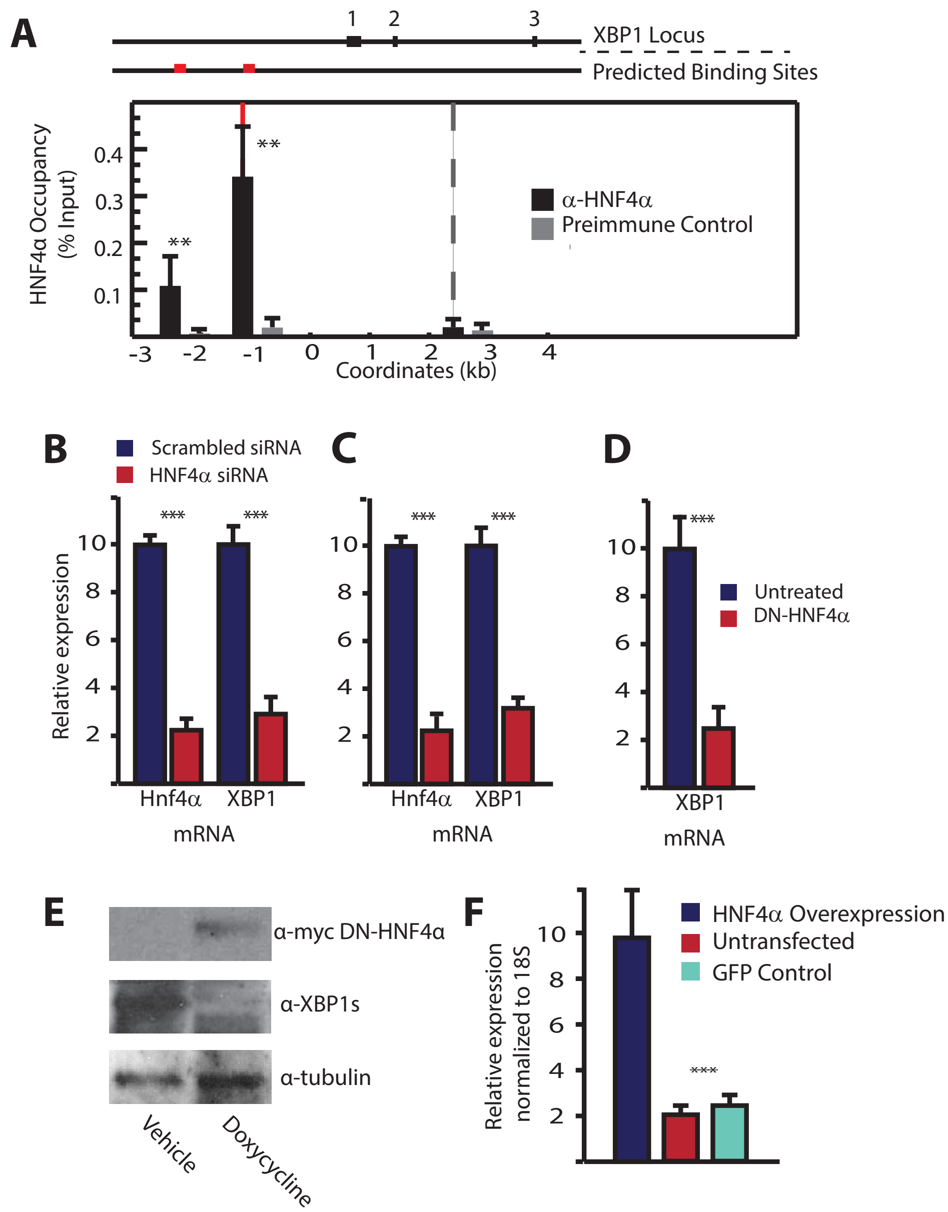
control islets were isolated and treated as in (Fig. 4B) but infected with adenovirus harboring plasmid encoding a transcript for spliced XBP1 (XBP1s). XBP1s expression is sufficient to partially rescue GSIS in isolated ΔHNF4α islets, but GSIS (i.e. insulin release following switching of islets to 16 mM glucose) is diminished in both WT and *ΔHnf4α* islets compared to WT GSIS. *LacZ* vector-containing adenovirus was used as a control for transduction (means±SEM of n=3 experiments depicted, statistical significance by one-tailed Student's t test)

Supplemental Fig. 7. Model: HNF4α enhances *XBP1* expression leading to robust ER and proper insulin secretion in normal conditions. Loss of HNF4α leads to reduced *XBP1* expression, diminished ER calcium levels, and impaired insulin secretion in response to glucose.

Supplemental Fig. 8. A) Titration of optimal MOI of control, unspliced, or spliced XBP1 adenovirus in isolated WT mouse islets shows efficient transfer of XBP1. Islets were isolated and cultured as described in methods, then treated with indicated levels of adenovirus before quantifying *Xbp1* expression by qPCR B) qPCR analysis of D1ER INS-1 cells used in (Fig. 3C). Transient transfection of HNF4α siRNA efficiently knocked down expression of both *Hnf4α* and *Xbp1*. Pharmacological inhibition of post-transcriptional *Xbp1* activation with the IRE1α inhibitor, 4µ8C had no effect on overall *Xbp1* levels.

Supplemental Fig. 9 Oligonucleotides used

Moore Figure 1



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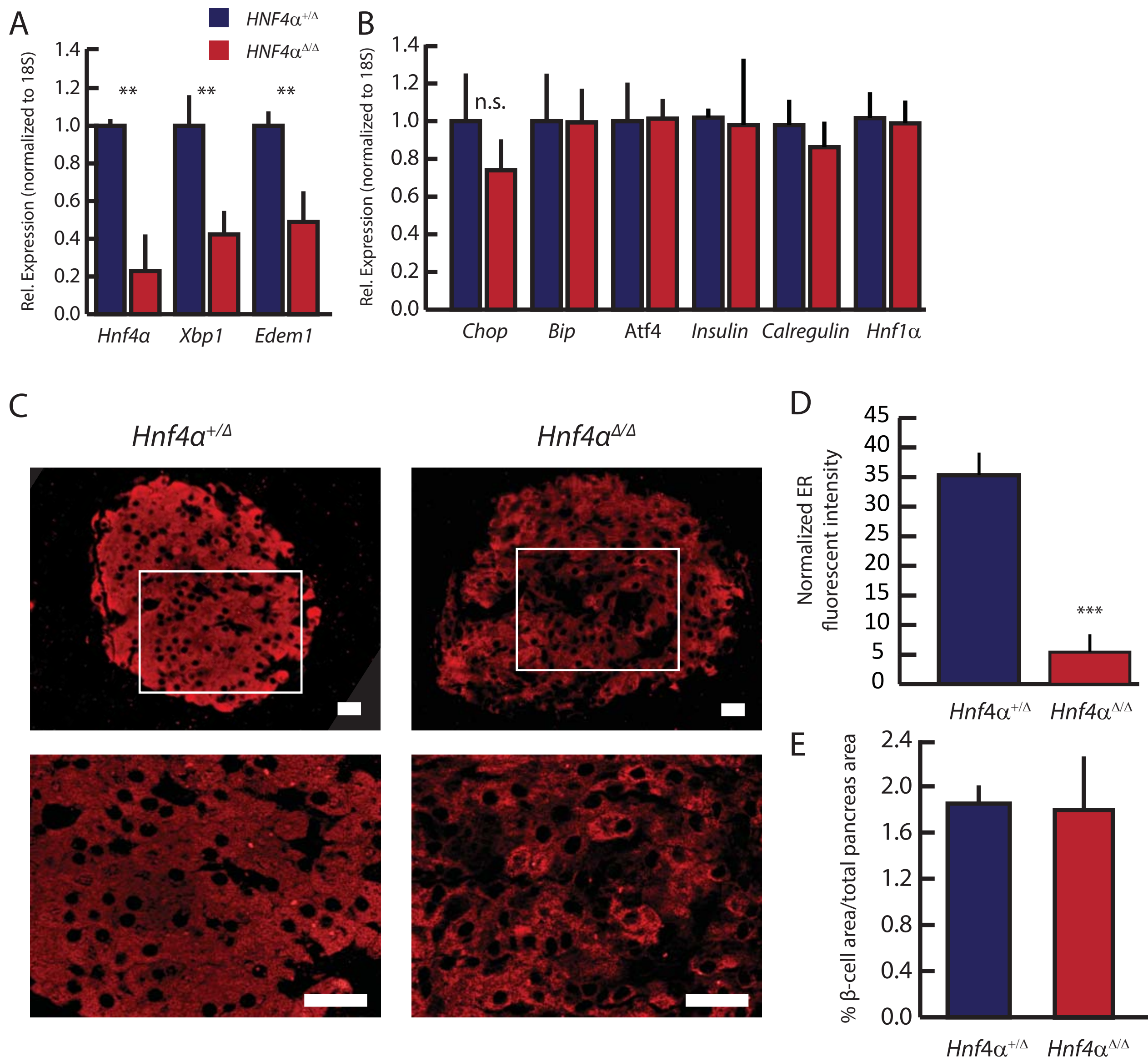

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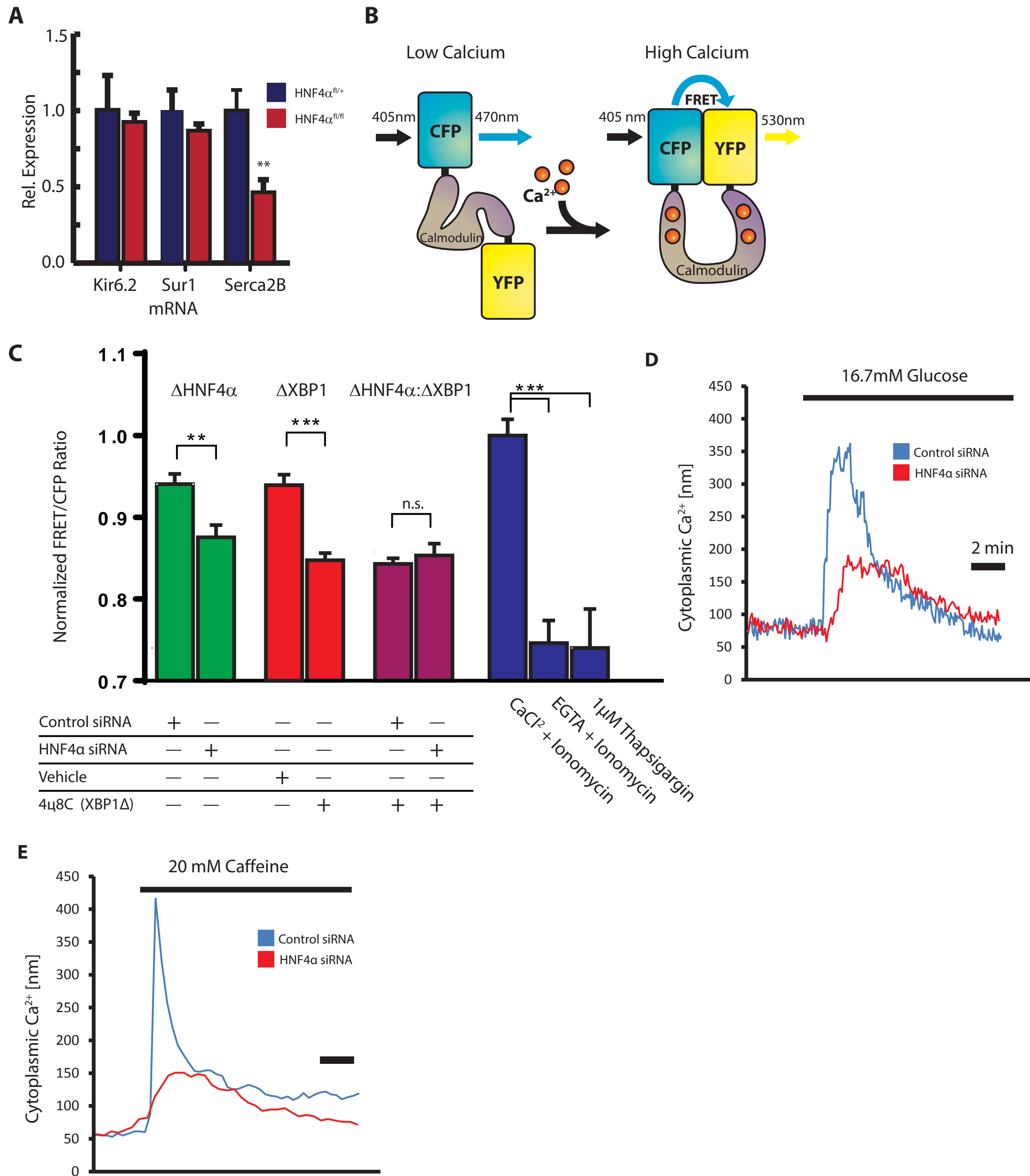

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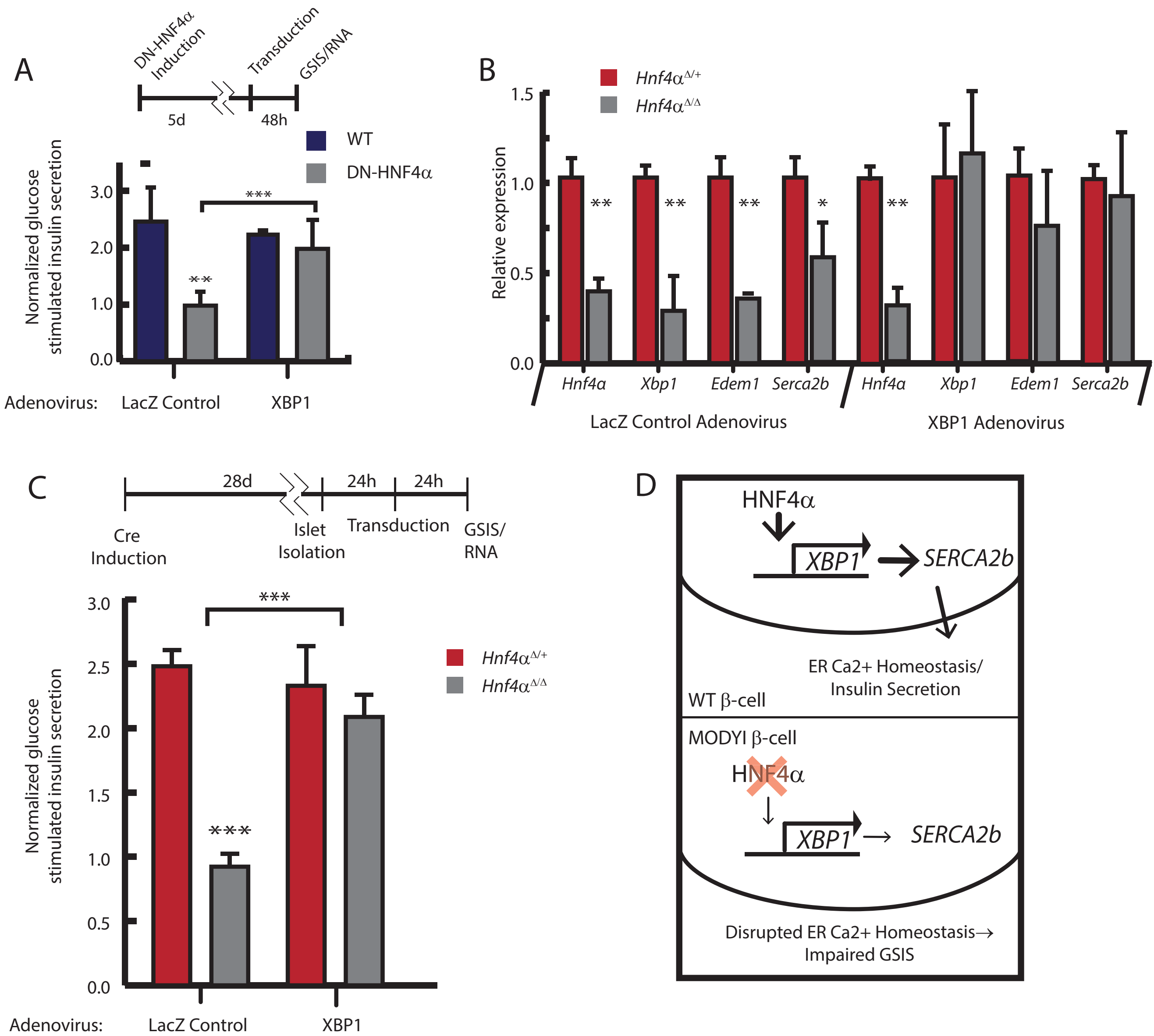

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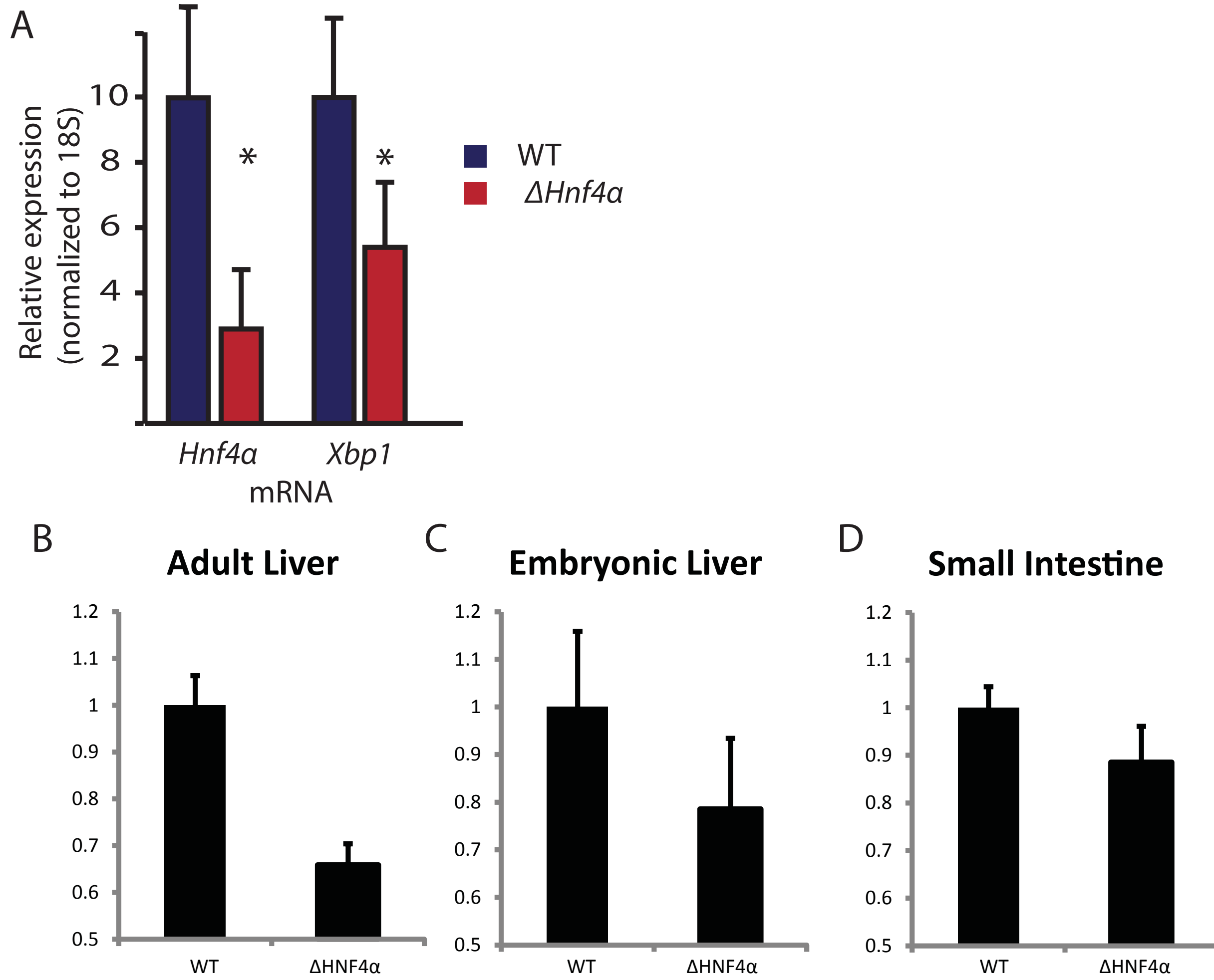

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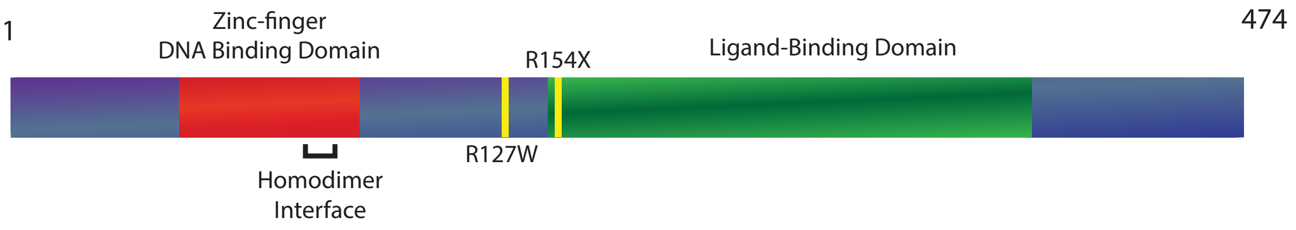

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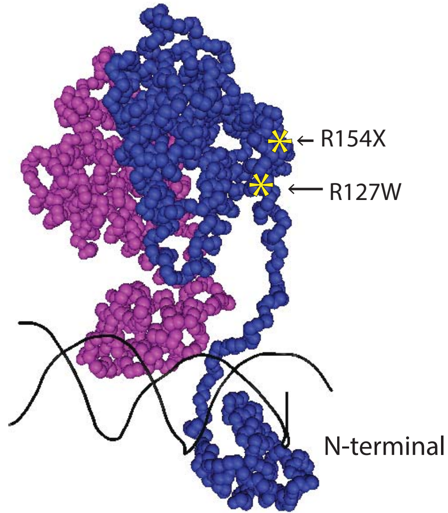

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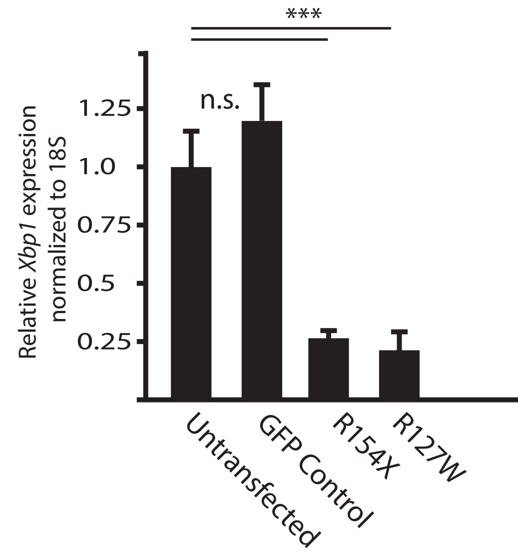

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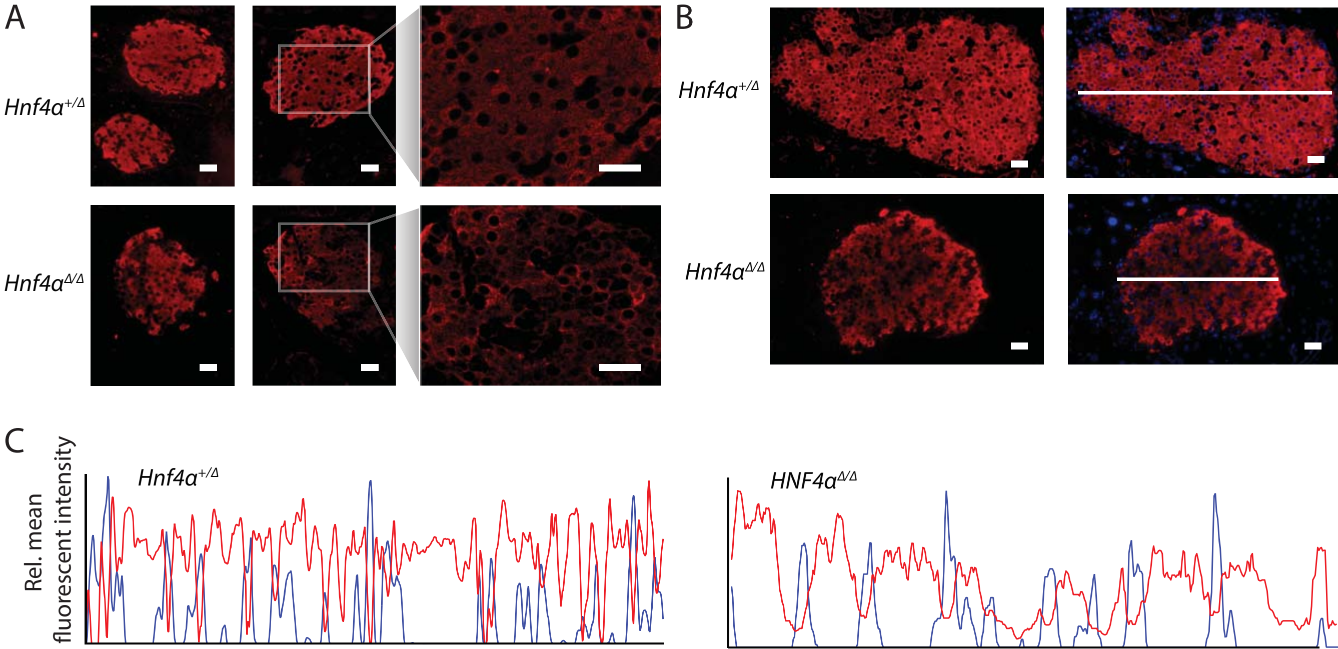

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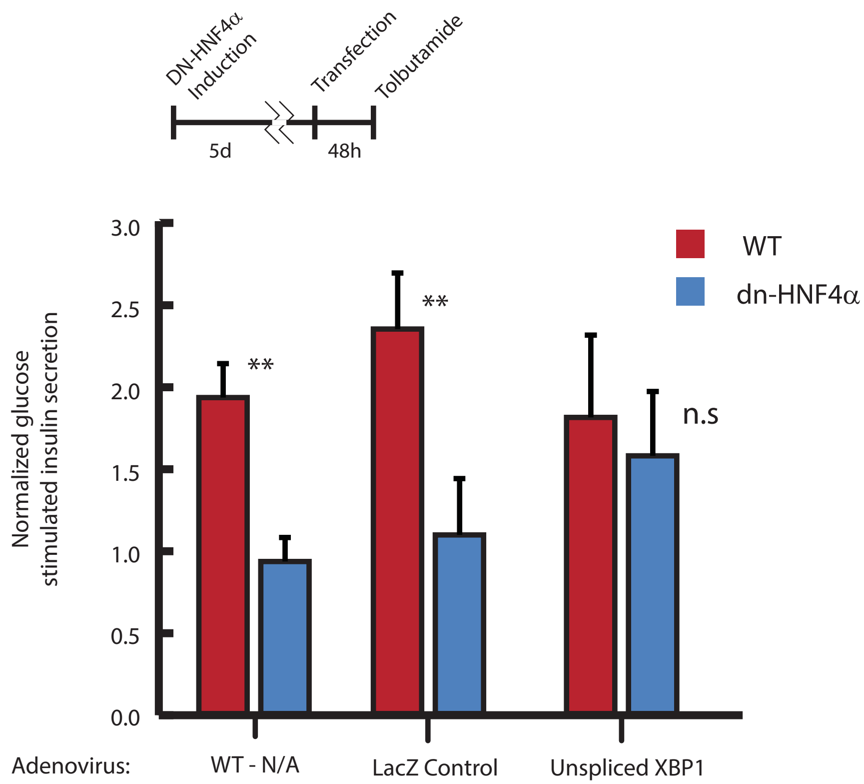

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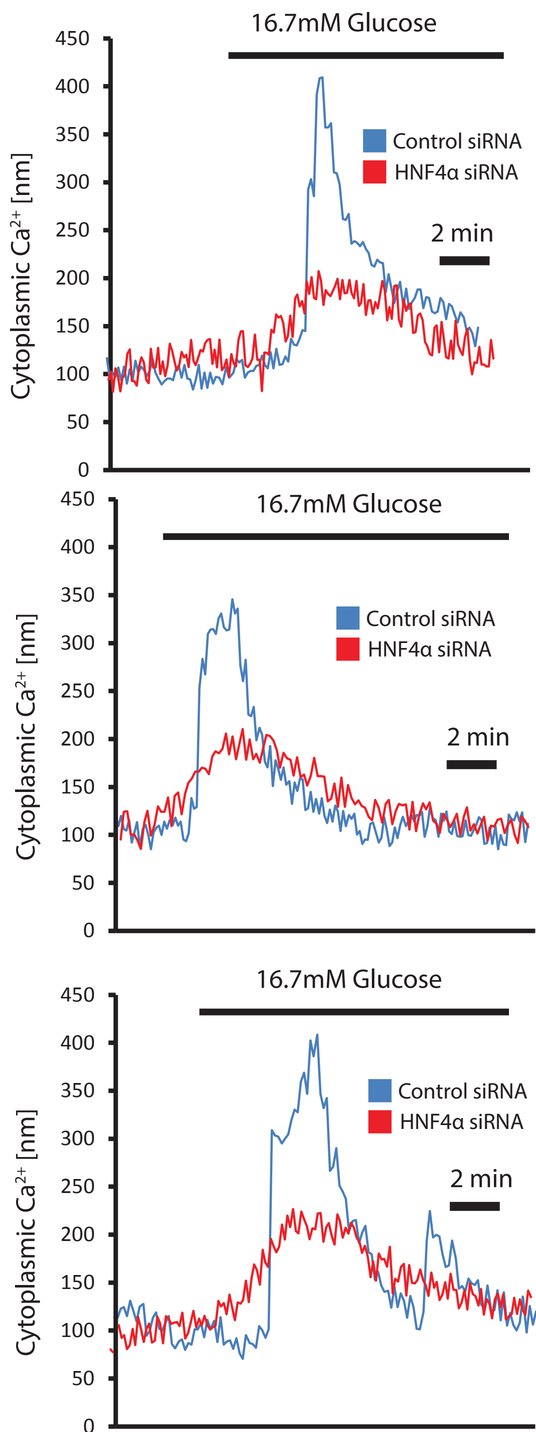

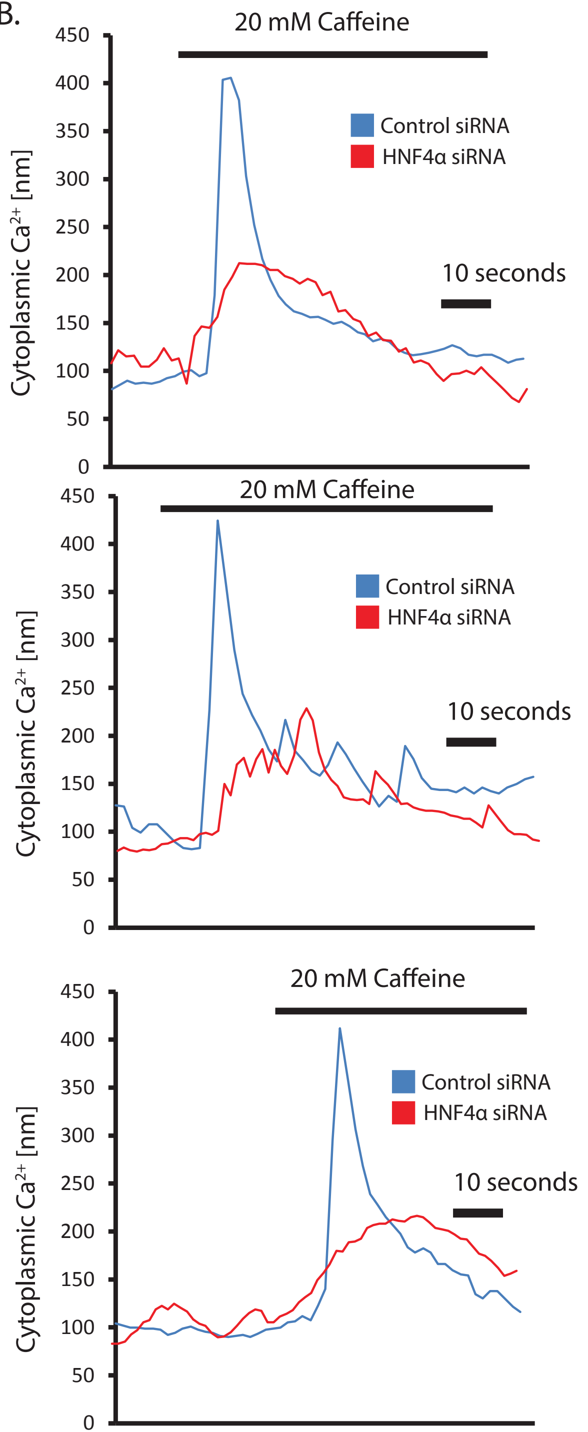

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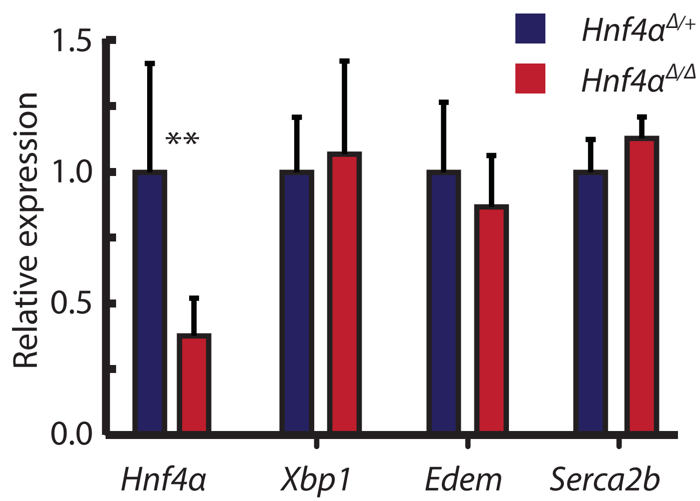

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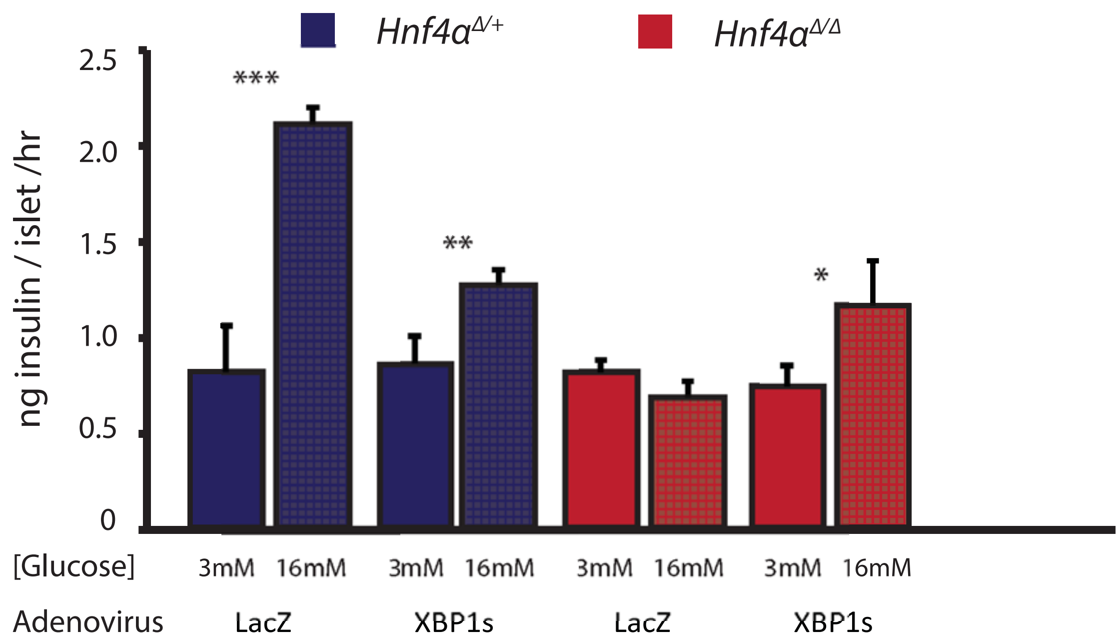

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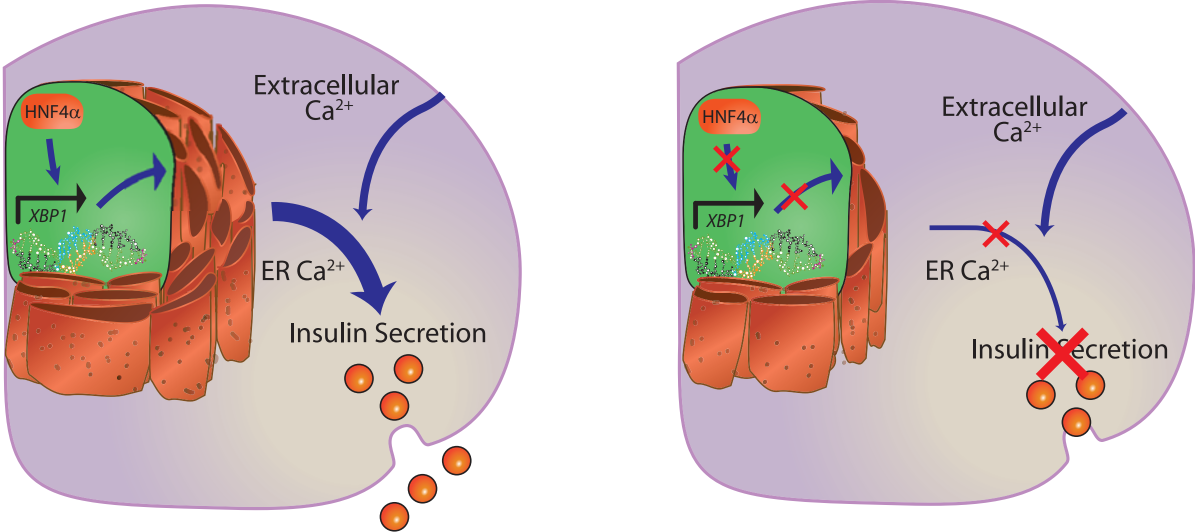

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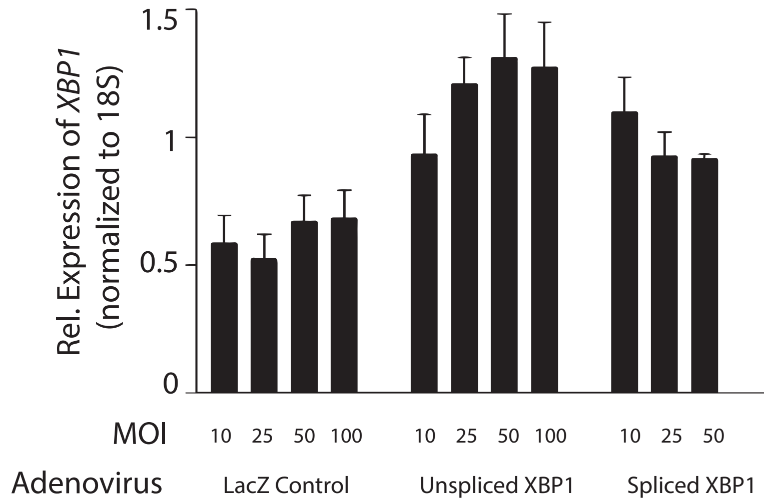

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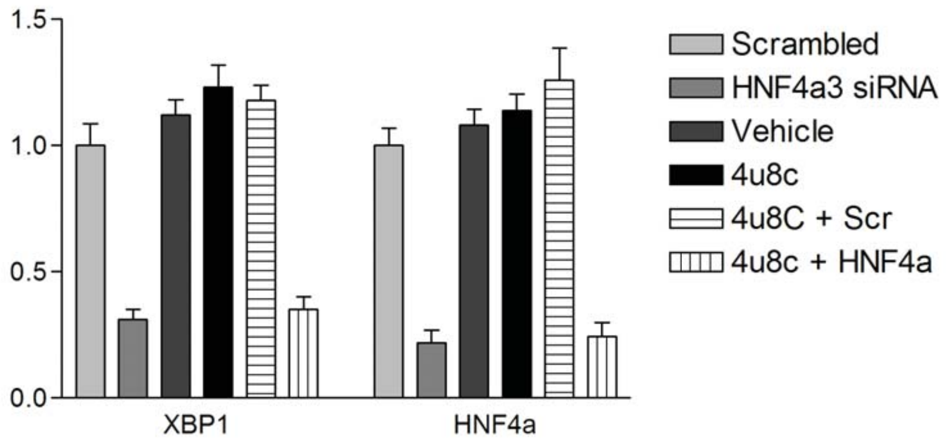

Moore Supplemental Figure 9

Primer	Forward Sequence	Reverse Sequence
ChIP -1.2 kb Hnf4-alpha site	GCCCCAAGGAGACATACAGA	GGGGGATAAGTTCAGCTCCT
ChIP -2.4 kb Hnf4-alpha site	AAGGGCGGATGAAAAGAGTT	TTGGCAATTGGAAATTATGC
ChIP intronic control site	GGCGAGTGGTACCTCACTGT	AAATGGTGGCAGCCTAGATG
Mouse *Hnf4α*	CTGAAGGTGCCAACCTCAAT	CCACACATTGTCGGCTAAAC
Mouse *Xbp1*	GAACCAGGAGTTAAGAACACG	AGGCAACAGTGTCAGAGTCC
Rat *Hnf4α*	CAAGAGGATTGCCAACATCA	GAGCAGCACATCCTTGAACA
Rat *Xbp1*	CACAGACTGCGCGAGATAGA	CCAAGCGTGTCCTTAACTCC
Mouse *Edem1*	CTACCTGCGAAGAGGCCG	GTTCATGAGCTGCCCACTGA
Mouse *Atf4*	GGGTTCTGTCTTCCACTCCA	AAGCAGCAGAGTCAGGCTTTC
Mouse *Chop*	CCACCACACCTGAAAGCAGAA	AGGTGAAAGGCAGGGACTCA
Mouse *Insulin*	TGGCTTCTTCTACACACCCAT	CTCCAGTGCCAAGGTCTGAA
Mouse *Calregulin*	AAGTTCTACGGTGACGAGGAG	GTCGATGTTCTGCTCATGTTTC
Mouse *Hnf1α*	ACCCATGGCGCGTGGCAAAG	CACCTGTGGGCTCTTCAATC
Mouse *Kir6.2*	CTGGCCATCCTCATTCTCAT	TTGGAGTCGATGACGTGGTA
Mouse *Sur1*	TCAGCAGCACATTCCGTATC	GGGCCAGGAACAGAAGTACA
R154X *HNF4α*	GAGGTCCTGTCCTGACAGATCACCTC	GAGGTGATCTGTCAGGACAGGACCTC
R127W *HNF4α*	GAATGAGCGGGACTGGATCAGCACTC	GAGTGCTGATCCAGTCCCGCTCATTC

CHAPTER FIVE

Conclusions and Future Directions

HNF4α is required for Normal Cell-Differentiation in the Adult Mouse Gastric Epithelium

In Chapter Two, we show that HNF4α is required for the development and maintenance of the gastric epithelium. We screened for enhancers of Xbp1 expression and found that HNF4α binds the *Xbp1* locus and enhances its expression. We show that HNF4α is expressed in the stem cell and progenitor zone, pit cells, neck cells, but not in parietal cells in the gastric unit. Loss of HNF4α results in increased proliferation and abnormal ZC differentiation in the gastric corpus. Despite this increased proliferation in ΔHNF4α stomachs, total gastric unit size was unchanged. We provide evidence that this loss of ZC architecture is likely due to lack of *Xbp1* expression. This is the first characterization of HNF4α in the normal stomach, and adds to our understanding of the transcriptional network that orchestrates secretory cell development in the gastric epithelium.(Nam et al., 2010)

The gastric unit differentiates in a highly-conserved, spatiotemporal manner.(Karam et al., 1997) Other dedicated secretory organs don't turn over constitutively and are arranged in more complex, three-dimensional structures, complicating analysis of changing differentiation patterns. In contrast, slight aberrations in gastric unit can be quantified using simple histological approaches. This makes it the ideal system to study the molecular mechanics of secretory cell development. In chapter two we illustrate this powerful approach by describing new functions of HNF4α in differentiating and maintaining secretory cell lineages.

Future directions:

We show that HNF4α is a transcriptional enhancer of *Xbp1* in the stomach, and that knockout models of both TFs have similar morphological defects. Future studies should establish whether this transcriptional relationship is directly required for ZC differentiation. First,

I propose measuring levels of XBP1 and downstream TF MIST1 in the gastric corpus using western blot. This will establish whether HNF4α is required for *Xbp1* expression, or whether loss of HNF4α is causing ZC defects through a separate XBP1-independent mechanism. To further test this, I would propose rescuing XBP1 in ZCs via ectopic expression, as previously described in the liver.(Lee et al., 2005)If the defects in ZC differentiation are rescued, it will be clear that HNF4α acts through enhancing *Xbp1* expression in ZCs.

Chapter two shows that HNF4α is necessary for normal gastric epithelial differentiation. While our data suggests it acts through *Xbp1* to regulate ZC differentiation, the cause of the increased proliferation in ΔHNF4α mice is unclear. To elucidate potential drivers of this proliferation, I propose gene expression analysis using RNA-seq to identify differences between stomachs from WT and ΔHNF4α mice. This would allow analysis of signaling pathways that are altered upon loss of HNF4α, and determination of the role of HNF4α in isthmal progenitor cells, where its expression is strong. Additionally, to determine if HNF4α coordinates the differentiation of ZCs by upstream patterning in progenitor cells, or directly, in the ZC, I propose crossing MIST1^{creERT} mice,(Shi et al., 2009) with HNF4α$^{floxed/floxed}$ mice.(Hayhurst et al., 2001) If ZC differentiation is disrupted, it is likely due to a cell-autonomous loss of HNF4α, rather than an upstream event.

Finally, an important next step is to understand the role of HNF4α in the damaged/diseased gastric epithelium. I propose to measure changes in the expression of HNF4α in WT mice in response to damage i.e. high-dose tamoxifen, *Helicobacter pylori* infection. Because loss of HNF4α stimulates proliferation, I expect that HNF4α expression would be decreased in response to damage, to allow enhanced proliferation and recovery. I further propose histological analysis of WT and HNF4α-null mouse stomachs upon damage, and their

subsequent recovery. If ΔHNF4α mice are more prone to severe metaplasia, it would mean that HNF4α is required to protect the epithelium. Observing the recovery from damage will also increase our understanding in the role of HNF4α in patterning the differentiation of specific cell lineages in the gastric unit. These proposed experiments will explore the molecular mechanisms of HNF4α in the regulation of gastric secretory cell development.

Identification of ANPEP as a Surface Marker for Isolation of Mature Gastric ZCs

In Chapter Three, we identify the first surface marker of mature ZCs in the gastric epithelium, ANPEP. Our data shows that neither neck cells neither transitioning to ZCs, nor dedifferentiated ZCs express ANPEP in human and mouse models. Importantly, by exploiting these properties of ANPEP expression and improving on single cell isolation/FACS sorting techniques, we are able to isolate pure populations of mature ZCs from normal mouse stomachs. This is a significant improvement upon the current more expensive and time consuming genetic manipulation and lineage tracing methods. Using flow cytometry to directly quantify molecular changes in the gastric epithelium using cell-surface markers is a powerful tool to characterize cellular changes in response to damage or disease. We observed a loss of $ANPEP^{+}$ epithelial cells in response to induction of SPEM, mirroring that observed in RNA expression, protein expression, and human immunofluorescence. Using this method, we were able to quantify changes in a population of progenitor cells known to proliferate in response to injury.(Khurana et al., 2013) CD44 expression in the gastric epithelium is significantly increased upon induction of SPEM. This observation is quantifiable by FACS, and, allows for isolation of this cell population for further analysis. Accordingly, we were able to isolate this population and analyze its gene expression profile using microarray analysis (Fig. 1A) We found that the isolated cells were highly enriched in CD44 mRNA, but contained significantly less markers of mature cell lineages

(*Muc5AC*-Pit, *Atp4a*-Parietal, *Pgc/Gif*-ZC, *Tff2*-Neck – Fig. 1B). This illustrates how powerful and versatile our improved method is in the analysis of molecular changes in the stomach, and enables future studies to characterize unique molecular pathways activated in specific cell lineages, and changes in these pathways in damage/disease.

Future directions:

The identification of ANPEP as a marker of mature ZCs that's lost in response to damage or disease invites further exploration as to its function in ZC biology. In other tissues, ANPEP acts as a mediator of cell-extrinsic signaling and cell migration.(Ghosh et al., 2014; Nam et al., 2010; Villasenor-Cardoso et al., 2013) In mouse knockout models, ANPEP is required for angiogenesis in hypoxic conditions, but no observable phenotype was found elsewhere.(Nam et al., 2010; Rangel et al., 2007) However, the stomach was not analyzed. Observing any disruptions in the spatiotemporally conserved differentiation of the gastric unit would be instrumental in characterizing the role of ANPEP in ZCs. In preliminary experiments, none of the four known inhibitors of ANPEP (Ezetimibide, Bestatin, Tosedostat, Circumin) had any morphological effect on the gastric epithelium (data not shown).

The sorting of ZCs using ANPEP is a proof of principle for how FACS can be applied to the characterization of the gastric epithelium. Other gastric epithelial cell lineages can be easily isolated and characterized using our improved method. Acid secreting parietal cells, for instance, may be sorted using the known surface marker encoded by the *Atp4a* gene. This technology complements recent advances in gastric organoid culture, which may allow the culture and characterization of organoids derived not from an entire unit, but rather from a single cell population.(Stange et al., 2013) I propose to study the effects of known pharmacological

methods of inducing metaplasia, e.g. Tamoxifen, DMP-777, on cultured distinct cell lineages to better understand the fundamental drivers of the formation of SPEM.

The gastric unit is thought to be replenished from a single, multipotent progenitor stem cell due to radiolabeling and genetic lineage tracing studies. (Bjerknes and Cheng, 2002; Thompson et al., 1990) This cell was first identified in 1966 as a small, undifferentiated cell with open chromatin and without granules in the isthmus of the gastric unit. (Corpron, 1966) No specific markers have been identified for this cell, preventing the study of the signaling pathways that govern gastric stem cell homeostasis in maintenance of the normal epithelium or that drive injury response pathways in damaged tissue. I propose exploiting the inherent qualities of stem cells in conjunction with our improved cell isolation technique to isolate, and characterize gastric stem cells. Fluorescent in situ hybridization,(Hultdin et al., 1998) detection of fluorescent hTERT (Ali et al., 2000), or identification by side population(Goodell et al., 1996) may mark the progenitor cell population, and allow isolation for RNA analysis using RNA-seq or gene expression microarrays. Alternatively, using flow cytometry to sort out and characterize individual cells that proliferate in response to damage, like $CD44^+$ cells (Fig 1A), will allow us to better understand the gastric epithelium's damage response pathways, and may uncover potential markers of stem, or at least early progenitor, cells in the normal epithelium. These proposed experiments will leverage this powerful new approach to better defining the molecular underpinnings of the gastric secretory cell.

Transcriptional Regulation of *Xbp1* by HNF4α is Vital to Beta-Cell Function

In Chapter four, we established that HNF4α directly binds the *Xbp1* promoter locus, and is both necessary and sufficient for *Xbp1* expression in pancreatic beta-cells. We recreated three of the most common HNF4α mutations responsible for MODYI in human patients, and upon overexpression found ~4 fold reduction in *Xbp1* expression *in vitro*. *in vivo*, loss of *Hnf4α* leads to loss of *Xbp1*, loss of downstream targets of *Xbp1*, and failure to maintain the extensive ER network required for insulin secretion. Interestingly, we found that loss of *Hnf4α* led to a reduction of *Serca2b* expression. We report that both *Hnf4α* and XBP1 are required to maintain calcium homeostasis in the ER. To the best of our knowledge no one has ever linked XBP1 activity to ER calcium, despite its important role in ER maintenance. Disruption of *Hnf4α* caused impaired cytoplasmic calcium signaling in response to both glucose, and caffeine, which specifically targets the ryanodine receptor to flood the cytoplasm with calcium stored in the ER. We hypothesize that loss of HNF4α in beta-cells leads to impaired ER calcium homeostasis, disrupting intracellular calcium signaling in response to glucose. As previously reported, loss of HNF4α completely ablated GSIS in isolated mouse islets. Rescuing *Xbp1* expression was able to completely restore GSIS in these islets, indicating that loss of *Xbp1* expression due to HNF4α dysfunction may be a driving factor in MODYI pathology.

It's important to note that, congruent with our hypothesis in Chapter One, restoring unspliced *Xbp1* expression was sufficient to rescue both downstream transcriptional targets of *Xbp1* (*Edem*, *Serca2b*), and the dysfunctional GSIS in MODYI islets. This suggests that in beta-cells, the transcriptional regulation of *Xbp1* expression is likely the major factor in the regulation of its function, and activation of XBP1 by IRE1 is likely not the rate-limiting step in XBP1 activity. Expressing constitutively activated XBP1 in islets, effectively bypassing IRE1, resulted

in reduced GSIS as previously reported.(Allagnat et al., 2010) These data indicate that in our *XBP1u* rescue experiment, overexpression of *Xbp1* is likely rescuing the deficient *Xbp1* levels caused by HNF4α disruption, and not promoting off-target, compensatory effects.

MODYI is currently treated with sulfonylureas, selective inhibitors of the KATP channel in beta-cells.(Thanabalasingham and Owen, 2011) Gupta et. al. found that in beta-cell specific HNF4α knockout mice, expression of the components of this KATP channel were reduced, and provided evidence that this was because they are direct transcriptional targets of HNF4α.(Gupta et al., 2005) Shortly thereafter, however, Miura et. al. showed that levels of both subunits remained unchanged upon loss of HNF4α in a nearly identical system.(Miura et al., 2006) Our data confirms these latter results. It would be paradoxical for a reduction in KATP channels to cause impaired GSIS, as fewer KATP channels would result in a more easily depolarized membrane and subsequent calcium signaling to release insulin.(Cartier et al., 2001) In fact, increased KATP channel activity is a well-studied mouse-model of diabetes. (Koster et al., 2000) We hypothesize that the mechanism of GSIS impairment in MODYI is the disruption of ER calcium homeostasis leading to impaired intracellular calcium signaling in response to an increase in glucose concentration.

Many type 2 diabetes patients, initially responsive to treatment with sulfonylureas, require insulin replacement therapy as their disease progresses.(Swinnen et al., 2009) Excessive and prolonged stimulation of insulin biosynthesis leads to beta-cell failure and death due to chronic ER stress.(Cnop et al., 2005; Prentki and Nolan, 2006) Similarly, while MODYI patients are responsive to sulfonylureas, many eventually require insulin therapy.(Pearson et al., 2005) Therapeutic agents that directly target the beta-cell ER are an area of intense research, and may improve existing approaches to managing MODYI.(Vetere et al., 2014)

Future Directions:

In Chapter four, we show that HNF4α is required to maintain Xbp1 expression in islets, and that this leads to a disruption of ER morphology and intracellular signaling. The next important step, is to characterize the effect of Xbp1 loss on other members of the UPR network, and the function of the ER. The UPR is an intricately regulated signaling network that regulates the size and efficiency of the ER in response to increased misfolded protein accumulation and various other cell-stress stimuli.(Schroder and Kaufman, 2005) Inhibiting different proteins in this signaling network can result in deleterious or enhanced beta-cell function. (Wang and Kaufman, 2012) For instance, deleting XBP1 ablates GSIS and impairs insulin processing,(Lee et al., 2011) while deleting another key TF in the UPR, CHOP, inhibits beta-cell apoptosis and enhances function in mouse models of diabetes.(Song et al., 2008) Because the IRE1-XBP1 arm of the UPR is canonically thought to be responsible for response to long-term or chronic ER stress, it is important to characterize the effect of its loss on the more acute arms of the UPR. Preliminarily, we found that levels of *Chop*, *Atf4*, and *Bip* were unaffected by deletion of *Hnf4α*. However, future directions should further characterize the state of activation of each arm of the UPR. Measuring IRE1α and PERK phosphorylation, and ATF6 cleavage in ΔHNF4α islets would be an ideal way to elucidate whether beta-cells activate the UPR to compensate for loss of *Xbp1*. Because disrupted ER calcium homeostasis often leads to an accumulation of misfolded proteins,(Fu et al., 2011) it is likely that ΔHNF4α beta-cells do have an active UPR, although our preliminary studies don't show any increase in apoptosis in these islets (data not shown).

While other MODYI mouse models disrupt *Hnf4α* expression from the early pancreatic development stage, our model disrupts *Hnf4α* acutely in adult mice. This more accurately reflects the juvenile onset of MODYI. Our model also uses a ubiquitous CAGCre^{ert} driver to knock out

HNF4α, mirroring the systemic mutation of HNF4α in MODYI patients. We assayed GSIS in this model *ex vivo* in isolated islets to allow adenoviral transduction of gene expression, and to control for non-beta-cell-specific effects of loss of HNF4α. However, mutations in HNF4α are also associated with dyslipidemia, renal defects, and other maladies.(Hamilton et al., 2014; Weissglas-Volkov et al., 2006) To determine whether the GSIS defect in MODYI is islet specific, I propose to induce islet death in WT mice with Streptozotocin, then transplant islets from our MODYI mouse to the kidney capsule of the WT mouse. GSIS could then be measured and compared between transplanted WT and MODYI islets. Additionally, WT islets could be transplanted into the kidney capsule of ΔHNF4α mice, to see if systemic disruption of *Hnf4α* has any non-islet-specific effect on glucose-tolerance or insulin secretion.

Calcium homeostasis in the ER is known to be important multiple human diseases e.g. diabetes, neurodegenerative disorders, cancer.(Sammels et al., 2010) Inflammation models have shown that activation of XBP1 leads to expansion of ER calcium stores in bronchial secretory cells.(Martino et al., 2009) Our data indicates that XBP1 activity is required for ER calcium homeostasis, a predictable, but a novel clue into how XBP1 functions the development and maintenance of the ER. Further studies should endeavor to define the molecular mechanism of this regulation. Previous work has shown that overexpression of *Xbp1* in the liver leads to an increase in expression of the main ER calcium pump, *Serca2b*.(Park et al., 2010) Our data shows that loss of *Xbp1* due to loss of HNF4α reduces *Serca2b* expression 2-fold, and that restoring *Xbp1* is sufficient to rescue this expression. Future experiments should characterize whether XBP1 is a direct regulator of Serca2b, or whether this observation is a non-specific effect of XBP1 activation. Modulation of ER calcium is a particularly attractive therapeutic target in many human pathologies. To define the role of ER calcium in our MODYI model,

ΔHNF4α islets should be treated with pharmacological agents to modulate ER calcium levels. Depleting ER calcium stores by inhibiting SERCA2b with thapsigargin reduces GSIS, similar to our MODYI model.(Vangheluwe et al., 2005) ER calcium release is mainly mediated by the ryanodine receptors.(Berridge et al., 2003) Modulators of these receptors such as small molecules derived from 1,4-benzothiazepines restore intracellular calcium stores and have been used therapeutically in mouse models to treat cardiac arrhythmias attributed to depleted ER calcium.(Lehnart and Marks, 2007; Lehnart et al., 2006) I propose treatment of our MODYI islets with these modulators of the ryanodine receptor to bolster ER calcium stores and observing their effect on GSIS and cytoplasmic calcium signaling. If these small molecules are able to rescue the beta-cell defects caused by loss of HNF4α, they may represent a new avenue of treatment for MODYI patients.

In addition to modulating ER calcium as a strategy to alleviate MODYI beta-cell dysfunction, I propose further study of the ability of these cells to handle ER stress, and the functionality of the ER itself. Preliminary data shows that insulin mRNA levels were unchanged in ΔHNF4α islets, but further work is needed to characterize the folding and processing of insulin. In MODYI patients, basal insulin secretion is unaffected, and in mouse models of MODYI islet insulin content is unchanged.(Gupta et al., 2005; Miura et al., 2006) Measuring the levels of insulin/proinsulin in WT and ΔHNF4α islets in our system would determine whether the major beta-cell secretory protein is being properly synthesized/processed, or whether abnormal insulin production contributes to the impaired glucose response. Small molecule chaperones, such as 4-PBA and TUDCA, have been used to alleviate the negative effects of ER stress in mouse models of diabetes,(Ozcan et al., 2006) obesity,(Basseri et al., 2009) and Alzheimer's disease.(Ricobaraza et al., 2009) They function by assisting proper protein folding

and processing, reversing the adverse effects of ER stress caused by accumulated misfolded proteins. Increasing ER capacity pharmacologically could partially rescue the diminished ER caused by lack of *Xbp1,* and may ameliorate impaired MODYI GSIS.

The work in Chapter Four exploits genetic tools to characterize the role of HNF4α in *Xbp1* regulation. The next step forward is to translate these findings into human MODYI models. The functional targets of HNF4α are thought to be highly-conserved between mouse and human,(Boj et al., 2009) and disrupting HNF4α in mouse islets mirrors the pathology of human MODYI patients, therefore we predict that our mechanistic findings will apply to human MODYI. The best system to test human MODYI would be in isolated islets from MODYI patients. However, the low diagnoses rates of MODYI make this approach unfeasible. I propose generating MODYI beta-cells from induced pluripotent stem cells. Well established methods (Hua et al., 2013; Stepniewski et al., 2015) could be combined with the recent advances in genome editing using the CRISPR-Cas system,(Musunuru, 2013) could be used to create beta-cells expressing HNF4α containing the most prevalent MODYI mutations, as we did in vitro in beta-cell cancer cell lines. ER development, Xbp1 levels, GSIS, and calcium signaling should all be characterized to determine whether our model mirrors human MODYI. This system could also be used to screen for pharmaceutical compounds with potential to restore GSIS in humans. In sum, these experiments will define the role of HNF4α in beta-cells, both in humans and mouse models, and may identify new strategies to treat beta-cell dysfunction in MODYI patients.

Final Remarks

The goal of this dissertation was to understand the molecular processes that guide the differentiation of a cell from a simple, proliferative-progenitor cell, to a highly-complex, dedicated secretory cell. This led me to uncover a novel transcriptional relationship with an important role in the gastric epithelium, and likely many other secretory tissues. This effort to better understand these professional secretory cells led to the development of new tools to isolate them from heterogeneous populations, which will open the doors to future work that will characterize their molecular underpinnings. Finally, uncovering this signaling cascade in the stomach, led to the answer of the decades-old question of what causes beta-cell dysfunction in MODYI, a finding which will hopefully open new avenues to the treatment and prevention of this prevalent human disease.

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Figure 1. Isolation of CD44-positive cells from the gastric epithelium. A) Comparison of unstained and stained, EpCAM-positive CD44 cells, plotted vs. autofluorescence. B) Gene expression enrichment analysis of markers of mature gastric epithelial cell lineages from $CD44^{+}$, $EpCAM^{+}$ cells isolated from (A) compared to $CD44^{-}$, $EpCAM^{+}$ cells.

Figure 1