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The Roles of MIST1 and RAB26 in Zymogenic (Chief) Cell Differentiation and Subcellular Organization

Ramon Jin
Washington University in St. Louis

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The Roles of MIST1 and RAB26 in Zymogenic (Chief) Cell Differentiation and Subcellular Organization

by

Ramon Jin

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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This thesis is the culmination of five years of life. To call it “work” would be unfair, as this single pursuit has permeated into every possible corner of my life. As scientific research, this dissertation must strive to remain emotionless, objective, uncontaminated. But how can one simply ignore the hours of soul crushing frustration, those pit-of-your stomach feelings of disappointment, the muffled when-you-are-alone-in-the-darkroom screams of anger, and most importantly, the fleeting elation that comes when you see those far too brief glimpses of Nature’s masterplan? I guess the real answer is with learned scientific maturity. It is the important lesson that has taken me five years to properly absorb. It means possessing the stoicism that comes with finally knowing what the goal of scientific research is. I realize that the aim of this thesis work is not to answer but to foster more questions; these results do not signify an ultimate truth or a final say, but beg for further study, counter examples, and alternative explanations. Science is selfless. This is what I have learned and it is with this mindset that I present this work.

All that I have done and the wonderful life in which I now live would not be possible without many instrumental people. First, I would like to thank Dr. Palmert and Dr. Drumm for opening my eyes to the world of science. The foundation from which I have built this work was laid by the mentorship of these two great men.

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To Tianyi, may you not be limited by the heavens.
ABSTRACT OF THE DISSERTATION

The Roles of MIST1 and RAB26 in Zymogenic (Chief) Cell Differentiation and Subcellular Organization

by

Ramon Jin

Doctor of Philosophy in Biology and Biomedical Sciences (Molecular Cell Biology)
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Professor Jason C. Mills, Chair
Professor Paul H. Taghert, Co-Chair

Little is known about how differentiating cells reorganize their cellular structure to perform specialized physiological functions. Specifically, exocrine cells like pancreatic acinar and zymogenic chief cells have a highly developed secretory architecture that is rapidly established upon differentiation. What is known in these cells is that the evolutionarily conserved transcription factor MIST1 is required for final maturation. We hypothesized that MIST1 directly regulates specific subcellular components that facilitate functional maturation of secretory cells.

We show that MIST1 binds to conserved CATATG E-boxes to activate transcription of 6 genes, including the small GTPases RAB26 and RAB3D. We next demonstrate that RAB26 and RAB3D expression is significantly downregulated in Mist1\(^{+/−}\) ZCs and upregulated in gastric cancer cell lines stably expressing MIST1 and induced to form secretory granules. Moreover, granule formation in these MIST1-expressing cells requires Rab activity because treatment with a Rab prenylation inhibitor or transfection of dominant negative RAB26 abrogates granule formation.
We next sought to elucidate how a scaling factor like MIST1 rearranges cellular architecture through transcriptional targets, specifically RAB26. We confirm that RAB26 expression was tissue specific and confined to acinar secretory cells. Functional studies in gastric cell lines showed RAB26 association with lysosomes but not with secretory granules. In addition, increasing RAB26 expression caused lysosomes to coalesce in a central, perinuclear region, causing redistribution of other organelles including mitochondria into distinct subcellular neighborhoods.

In mouse exocrine cells that are null for Mist1 and lack RAB26, we found lysosomes to be similarly abnormally distributed. Normally, lysosomes cluster centrally and basally away from secretory granules, whereas in Mist1−/− cells, they rapidly accumulate apically and degrade secretory vesicles. We confirmed active granule degradation when much of the vesicle phenotype in Mist1−/− mice was rescued upon crossing with mice deficient in the lysosome acid hydrolase processing enzyme, GlcNAc-1-phosphotransferase, alpha and beta subunits (Gntpab−/−).

Taken together, we propose that MIST1 promotes normal maturation of secretory granules through blocking their targeting by the cellular degradation/recycling machinery. These results illustrate how a transcription factor can regulate cell architecture and have implications for disease processes like acute pancreatitis where MIST1 is lost, and secretory vesicles are targeted to lysosomes.
CHAPTER 1: Preface
**Introduction**

As a whole, this work will examine the cell biology of two functionally interconnected molecules. In this Preface, I will give the context and background for the studies I will describe. Specifically, I will introduce the concepts of vesicular trafficking, Rab proteins, and transcriptional control of cellular architecture. Then, in Chapter 1, I will focus on applying this molecular knowledge towards an understudied organ, the stomach. I will strive to convince the reader of the continued need for translational cell biological discoveries with a clinical perspective. By exploring the congenital and acquired diseases of the stomach, I will show that our known understanding, be it molecular mechanistic or pathophysiologic, is still far exceeded by what is unknown. Bench to bedside translation has slowed, and far too often the treatment is found on the surgical table. Each major cell type of the gastric epithelium will be introduced and examined pathophysiologically with a focus on the specific importance of gastric zymogenic chief cells. Chapter 2 will describe the importance of the transcription factor MIST1, an exocrine secretory tissue specific functional maturation factor. Experiments will elucidate novel direct transcriptional targets and assay their function in secretory cell maturation. In Chapter 3, I will elucidate the novel lysosomal localization and function of RAB26, one of the novel direct MIST1 targets. From these experiments, I will return to an *in vivo* gastric system in Chapter 4 to apply these novel findings of lysosome vesicle organization to MIST1 function and zymogenic cell development. Finally, in Chapter 5, I will interpret these results and give my suggestions to future experimental considerations. This will be highlighted by novel findings that suggest MIST1, RAB26, and lysosomal dysfunction may play important complementary roles during the pathologic transition and dedifferentiation steps that occur during gastric carcinogenesis.
Secretory Vesicle and Lysosome Trafficking

The subject of vesicle trafficking involves answering the simple question of how cells develop compartmentalization as a response to ever increasing complex physiological functions. Specifically, I will focus on two common pathways that all cells share; which, at first glance, appear to be antitheses of each other. Lysosomes are responsible for the undertaking of cellular degradation, and secretory vesicles are charged with delivering and releasing the major synthetic products. But both vesicular compartments begin life at the endoplasmic reticulum (ER) (Cooper, 2000). From this organelle, the unsorted proteins that have been properly folded and glycosylated progress via COPII coated vesicles to the Golgi apparatus where they begin the process of vesicular differentiation (Benham, 2012).

Secretion can occur through a baseline constitutive pathway and an enhanced regulated version. In fact, giving a regulated secretory load to a cell with only constitutive pathway capabilities will induce neither accumulation of vesicles filled with that cargo nor secretion of those proteins; indicating that regulated secretory components are uniquely expressed and regulated (Molinete et al., 2000). In cells using both routes, the two pathways must be sorted and maintained separately because they simultaneously shuttle different secretory contents (Burgess and Kelly, 1987). It is thought that this division of secretory contents occurs at the trans-Golgi, where regulated secretory proteins are processed through protein aggregation, lipid raft formation, and organized budding (Tooze et al., 2001). From the Golgi, these immature secretory granules are processed by prohormone convertases as they mature (Taylor et al., 2003). In addition to sorting-for-entry into maturing secretory vesicles, a sorting-for-retention process (Arvan and Castle, 1998) also must occur for secretory granule maturation. Active events ensure proper vesicular identity by removal of constitutive secretory and lysosomal proteins and vesicle
membrane constituents through clathrin-coated vesicles (Brodsky et al., 2001). SNARE mediated vesicle fusion events form and enlarge the maturing secretory vesicles (Waters and Hughson, 2000). Once mature, secretory vesicles are maintained as reserve and ready-to-release pools that tether, dock, fuse, and release their contents upon secretagogue signaling in a complex process that is ATP-dependent and involves actin remodeling and calcium signaling (Burgoyne and Morgan, 2003).

The constitutive secretory pathway of trafficking from the trans-Golgi to the plasma membrane may actually involve a combination of several pathways (Ponnambalam and Baldwin, 2003) that are all distinct from one another and from the regulated secretory vesicle route (Tooze and Huttner, 1990). Thus, there may be direct sorting events at the Golgi or subsequent separation of constitutive secretory components from forming regulated secretory vesicles (Arvan and Castle, 1998). These continuously secreted vesicles may enter an endosomal intermediate compartment or directly progress to the cell surface (Arvan and Castle, 1998).

The itinerary for lysosomes also begins at the ER with subsequent Golgi processing and sorting. In formation, lysosomes require membrane proteins and acid hydrolases. The best understood pathway involves sorting of acid hydrolases by the Mannose-6-Phosphate Receptor (M6PR) (Kornfeld and Mellman, 1989). These hydrolases are bound by M6PR at the trans-Golgi and mediate adapter protein and subsequent clathrin coat protein binding and budding (Ghosh et al., 2003). As these mature and acidify, M6PR releases its cargo and is recycled back to the Golgi by interaction with the TIP47 protein (Diaz and Pfeffer, 1998). These late endosomes continue to mature and fuse with other similar endosomal vesicles to eventually become functional lysosomes. Lysosomal membrane protein trafficking is less well understood. They are surmised to reach mature lysosomes either by an indirect plasma membrane recycling
endocytic route or the more direct route taken by acid hydrolases through recognition of their sorting motifs (Saftig and Klumperman, 2009). Once formed, lysosomes are able to fuse with other trafficked membrane compartments including endosomes, vacuoles, and autophagosomes to regulate subcellular degradation (Luzio et al., 2000).

The diverse destination of lysosomes and the secretory pathway in light of the intimacy of their formation highlights the important task of ordered and organized vesicle trafficking. However, to confuse matters somewhat, a combined secretory lysosome organelle also exists in some cells. These hybrid vesicles share features of both lysosomes and regulated secretory vesicles, and have unique properties that enable cell specific physiologic function (Marks et al., 2013). They are expressed only in a particular subset of cells (Blott and Griffiths, 2002), highlighting the ability of cells to adapt their subcellular structure and components for specialized function. This concept of differential organization of subcellular compartments to suit differing cell physiological needs is a key theme of this dissertation and will be revisited many times.

**Rab Proteins as Membrane Organizers**

The Rab small protein GTPases are important intracellular regulators of vesicle positioning and trafficking. Of the more than 70 members identified (Schwartz et al., 2007), they all define and organize specific subcellular membrane domains. By guanine nucleotide cycling and binding membrane specific effector proteins, these small GTPases function as mechanical switches that mediate vesicle trafficking events (Grosshans et al., 2006). They become associated with cell membranes by enzymatic attachment of hydrophobic geranylgeranyl groups to carboxy-terminal cysteine residues (Seabra et al., 1992). The sequence of bound GTP
and GDP is facilitated and determined by membrane specific guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) (Stenmark, 2009). In its GTP bound form, Rab proteins are considered “active”; they can bind many unique effectors to coordinate membrane trafficking and transport (Zerial and McBride, 2001). Upon performing its cell biological task, Rabs hydrolyze to GTP to GDP and bind to GDP-dissociation inhibitor (GDI), which initiates retrieval back to the membrane of origin (Stenmark, 2009). Specifically, we will focus on the ones important in the secretory and lysosomal membrane trafficking pathways (Fig. 1).

Both routes begin in all cells with vesicle progression from the ER through the Golgi; many ubiquitously expressed Rab proteins mediate this traffic. Among the most extensively studied is RAB6, which has been found to regulate intra-Golgi and retrograde ER trafficking (Martinez et al., 1994; White et al., 1999). RAB33B complements the function of RAB6 in all cells by co-coordinating Golgi homeostasis and ER trafficking (Starr et al., 2010). Anterograde trafficking from ER to Golgi and through the Golgi cisternae is facilitated by RAB1 and RAB2 (Plutner et al., 1991; Tisdale and Balch, 1996). Additional ER and Golgi characterized Rabs include RAB43 and RAB18 (Liu and Storrie, 2012). The number of Rab proteins that all cells must dedicate to this initial pathway clearly indicates the complexity and importance of early protein sorting and processing.

The best characterized secretory Rabs are the RAB3 and RAB27 families, both playing important roles in regulated secretion (Fukuda, 2008). As discussed, this pathway is found only in certain cells; thus, RAB3 and RAB27 expression is limited. RAB27 functions in the transport and docking of mature vesicles (Fukuda, 2013) in highly secretory cells including cytotoxic T lymphocytes (Menasche et al., 2000), melanocytes (Hume et al., 2001), and exocrine secretory
cells (Gomi et al., 2007). RAB3 has four isoforms: RAB3A, B, C, and D; the first three isoforms are predominantly neuronal involved in neurotransmitter and neuroendocrine hormone release (Johannes et al., 1994; Schluter et al., 2004; Weber et al., 1994), while RAB3D is expressed in a variety of extraneuronal cells including exocrine secretory cells (Millar et al., 2002; Ohnishi et al., 1996). Functionally, RAB3 isoforms all share a similar role in mature secretory vesicle docking (Fukuda 2008). Constitutive secretion has been found to be coordinated by the widely expressed RAB8 (Chen et al., 1993). In particular, RAB8 facilitates basolateral transport from the trans-Golgi to the plasma membrane (Huber et al., 1993).

The endolysosomal trafficking pathway is found in all cells, and contains likewise more ubiquitously expressed Rab proteins (Gurkan et al., 2005). RAB4, 5, and 11 localize to the endocytic pathway where RAB5 controls early endosome fusion, and RAB4 and RAB11 mediate endosomal recycling events back to the cell surface (Mohrmann and van der Sluijs, 1999). Maturation of early to late endosome has been found to be mediated by a RAB5-RAB7 conversion (Rink et al., 2005). RAB7 further coordinates delivery to lysosomes, while RAB9 traffics M6PR in a retrograde fashion back to the Golgi (Stenmark, 2009).

Rab protein constitution reflects the diverse physiological functions of that cell (Zerial and McBride, 2001). Differential expression and control of Rab proteins may represent a yet unappreciated method for cells to adapt and change subcellular vesicle compartments to suite developing physiological needs.

**Developmental Regulation of Vesicle Compartments**

It has long been recognized that upon maturation, secretory cells change morphologically, characterized by increase stores of secretory vesicles (Boquist, 1970; Caro and
Palade, 1964). However, the direct relationship between developmental signals and cellular architecture has only recently been appreciated, and with the advent of modern molecular techniques we have begun to unmask the developmental regulators that govern these changes in subcellular organelles (Mills and Taghert, 2012). For example, as lymphocytic B cells differentiate into antibody secreting plasma cells, expression of XBP1, a b-ZIP transcription factor is increased and maintained at high levels. Induced XBP1 expression directly “scales-up” ER components to accommodate secretory capacity (Shaffer et al., 2004). In fact, this developmentally regulated transcription factor is a feature of many diverse mature secretory cells (Acosta-Alvear et al., 2007) including exocrine secretory cells (Huh et al., 2010; Lee et al., 2005). Remarkably, its function in these different tissues remains conserved in expanding ER to adjust to increasing secretory demands. XBP1 mediated ER expansion is accompanied by Golgi expansion during secretory cell differentiation (Clermont et al., 1993; Kirk et al., 2010). In fact, the same transcriptional signals may be involved in amplifying both compartments (Travers et al., 2000).

Concomitant with enlarging the ER and Golgi, XBP1 also functions to enhance secretory vesicles by direct induction of the transcription factor, MIST1 (Acosta-Alvear et al., 2007; Huh et al., 2010). This bHLH transcription factor is again highly expressed in mature secretory cells (Johnson et al., 2004; Lemercier et al., 1997; Pin et al., 2001), but it functions to control direct components of secretory vesicle maturation (Capoccia et al., 2013; Garside et al., 2010; Rukstalis et al., 2003; Tian et al., 2010). Analogously, in *Drosophila*, the expression of the bHLH transcription factor and MIST1 ortholog, DIMM are developmentally restricted to mature neurosecretory neurons and endocrine cells (Hewes et al., 2003; Park and Taghert, 2009). DIMM also controls a subset of direct effectors important for secretion (Park et al., 2011; Park et al.,
2008). Other secretory vesicle developmental transcription factor effectors include: REST control of neurosecretion (D'Alessandro et al., 2008), FOXQ1 mediated mucus granule formation in the stomach (Verzi et al., 2008), and FOXA2 regulation of β cell insulin secretion (Gao et al., 2007). In sum, these transcriptional control networks function in developing cells to enhance secretion logically and stepwise through the major organelles of the secretory pathway.

Lysosomes and degradative function have similarly been shown to be induced during development/differentiation. In epidermis development, keratinocytes terminally differentiate into keratinized corneocytes (Sotiropoulou and Blanpain, 2012); a process which involves a dramatic cell architectural change. Critical for this process is the upregulation of lysosomes, in particular the need for cathepsin D mediated terminal differentiation (Egberts et al., 2004). The autophagy pathway, a lysosome degradative and recycling process for organelles and intracellular proteins, is activated upon and required for differentiation in many cells including erythrocytes, adipocytes, and certain hematopoietic cell lineages to mediate subcellular reorganization (Mizushima and Levine, 2010). Maintained lysosome function is crucial for the sustained health of long lived terminally differentiated cells (Nixon, 2013).

Analogous to secretory vesicles, many transcriptional controllers of lysosomes have also been recently revealed. The best characterized of these is the bHLH transcription factor, TFEB (Sardiello et al., 2009). Through direct activation of a gene network, TFEB has also been found to be a master enhancer of lysosome function and autophagy (Settembre et al., 2011), and linked to important developmental growth signals (Settembre et al., 2012). Conversely, the zinc finger family transcription factor, ZKSCAN3, has been show to repress lysosomes and associated degradative and autophagocytic function (Chauhan et al., 2013). In addition, at least one factor critical for early tissue development/specification have also been found to have dual roles as
factors that scale lysosomal activity in terminal differentiation of cells. CDX2, a homeobox transcription factor required for intestinal specification, has been found to activate an endosome-lysosome trafficking gene network in enterocytes (Gao and Kaestner, 2010). Interestingly, the importance of developmental regulation of cell function is even seen in hybrid secretory lysosomes. Melanocyte differentiation is dependent on the bHLH transcription factor, MITF (Bertolotto et al., 1998), and upon expression in these cells, it activates secretory lysosome components and granule trafficking effectors (Chiaverini et al., 2008; Levy et al., 2006).

Clearly, the dramatic morphologic changes that occur rapidly and are maintained for the lifetime of differentiated cells depend on transcriptional regulation. In essence, these transcription factors and the network of genes they control, allow the cell to quickly and efficiently adjust physiological function. In disease states in which physiological function is decreased, manipulation of these factors may represent novel therapeutic targets (Decressac et al., 2013; Settembre et al., 2013).

**Summary**

The belief of all cells having the same unregulated baseline functions is changing into the realization that cells have precisely controlled, highly adaptable, and fully integrated networks that can be scaled up and down along different cell lineages and over time in the adult in response to changing environmental conditions. Elucidating the control points and the components of these networks will further advance our knowledge of how cells change upon physiological and pathological cues. In summary, this dissertation is founded on the following cell biological tenets (Fig. 2):
1. Vesicular compartments of cells are complex, dynamic, and reflect the specific function of the cell.

2. As function changes during cellular differentiation or changing environmental conditions, so must these vesicular compartments.

3. Developmental transcription factors have direct control over a network of subcellular components and are responsible, in part, for regulating vesicular compartments.
References


Figure Legends

Figure 1. Rab proteins coordinate trafficking of secretory vesicles and lysosomes. Important Rab proteins are highlighted with arrows indicating their canonical routes of effector mediated trafficking. Pertinent organelle and vesicular compartments are shown: the endoplasmic reticulum in blue, the Golgi in orange, secretory vesicles in red, and the endo-lysosomal pathway in green.

Figure 2. Transcriptionally controlled network of subcellular effectors. Schematic depicting the differentiation of a cell through augmentation of particular organellar and vesicular structures to meet new physiological functional needs. Known developmentally regulated transcription factors are shown with the particular subcellular compartment that they control. Black arrows signify gene network activation, and red arrows denote gene network repression.
Figure 1

Diagram showing the interaction of various cellular compartments such as secretory vesicles, early endosomes, late endosomes, Golgi apparatus, and lysosomes, with Rab GTPase proteins RAB1, RAB2, RAB3, RAB4, RAB5, RAB6, RAB7, RAB8, RAB9, RAB11, RAB18, RAB27, and RAB43.
CHAPTER 2: Congenital and Acquired Diseases of the Stomach

This chapter is adapted from the published work:

Congenital and Acquired Diseases of the Stomach

Ramon U. Jin and Jason C. Mills

Division of Gastroenterology, Departments of Medicine, Developmental Biology, Pathology & Immunology, Washington University School of Medicine, St. Louis, MO 63110

Address correspondence to:

Jason C. Mills
Department of Medicine
Washington University School of Medicine, Box 8124
660 So. Euclid Ave.
St. Louis, MO 63110
Phone: 314 362-4258
FAX: 314 362-7487
e-mail jmills@wustl.edu
Introduction

The basic function of the stomach is to facilitate initial digestion of food contents arriving from the esophagus and modulate their transport to the intestines. Interestingly, the basic tenets of stomach function have been known for more than 5,000 years, as traditional Chinese medicine has always taught that the qi (energy) of the stomach needs to be in balance for proper downward flow. In short, it has been believed that insufficient secretory function or “dryness” disturbs the function of the stomach, in effect causing upward movement (“reflux”) and dyspepsia. In this chapter, I will focus on the clinical disorders of the stomach, using lessons learned from modern science to help elucidate the mechanisms and gaps in traditional wisdom.

Conceptually, I will approach this task by examining first the congenital or developmental stomach disorders and then subsequently discuss the acquired or adult pathologies. Throughout, I will examine the mechanisms behind the pathology and discuss associated diagnostics and therapeutics. The focus of this chapter is to situate and justify our cell biological study of the stomach. Accordingly, I will adopt a human-centric perspective throughout.

I. Developmental/Congenital Clinical Disorders

A. Anatomic Development of the Stomach

The gross anatomic development of the stomach can be said to begin with the embryonic folding of the endodermal germ layer into the gut tube. Functional and developmental specification of this tube occurs next with formation of the foregut, midgut, and hindgut. During the fourth week of embryonic growth, endodermal foregut gives rise to a bulge that will form the primitive stomach. As the bulge grows, its dorsal region outpaces its ventral such that by the
sixth week of development, this asymmetric growth establishes the greater and lesser stomach curvatures and, subsequently, a complex, two-step reorientation. The stomach initially rotates 90 degrees clockwise about its longitudinal axis. Then it turns about its anterior-posterior axis such that the pylorus and cardiac regions tip upwards and downwards, respectively. These spatial movements end around the 8th week of development with the greater curvature of the stomach facing the left side of the body, and the lesser curvature facing right (Fig. 1) (Carlson, 1999; Sadler, 2012).

The histologic cell types that constitute the epithelium of the stomach arise around the time the stomach settles into its final position. Mucus secreting cells are the first to emerge, as the single cell epithelial lining invaginates from the surface of the gastric lumen towards the submucosal layers. These early invaginations are the gastric pits, lined by mucinous pit (aka foveolar cells). During the next couple of weeks, the other major cells of the gastric mucosa begin to develop with narrower gastric glands elongating downward from the pit region until birth (Angioi et al., 2001; Kataoka et al., 1984; Menard et al., 1993; Nomura et al., 1998). Once established, the glands and the differentiated cells that constitute them are continually renewed throughout adult life. Hence, I will discuss each specific lineage and associated diseases in the Adult/Acquired disorders section. As for the early developmental disorders, I will show that many of them are due to gut tube formation defects or problems occurring during stomach rotation. In addition, because of the shared foregut embryonic origins of the stomach with the esophagus and the duodenum, many of the disorders are shared with those organs. Congenital clinical disorders are summarized in Table 1.

B. Disorders
1. Motility

**Gastric ENS primer**

Motility of the stomach is dictated by the enteric nervous system. This autonomic gut “brain” is divided into the submucosal (Meissner’s) plexus and the myenteric (Auerbach’s) plexus. Developmentally, the ENS forms via migration of vagal, truncal and sacral enteric neural crest-derived cells (ENCCs) that colonize and innervate the developing gut (Heanue and Pachnis, 2007). This ENS development is complete by 13 weeks of embryonic development (Gariepy, 2004). The proximal stomach and esophagus is colonized by truncal ENCCs while the vagal ENCCs populate the rest of the stomach and distal GI tract (Heanue and Pachnis, 2007).

a. **Cardiac Achalasia**

Primary achalasia is characterized by diminished esophageal peristalsis with impaired LES relaxation and presents clinically as difficulty swallowing (Francis and Katzka, 2010). As mentioned above, the ENS that populates the esophagus also innervates the proximal or cardiac portion of the stomach. Accordingly, the cardiac region of the stomach has been shown to have similar impaired relaxation in patients with esophageal achalasia (Mearin et al., 1995). Thus, the disease causing mechanism will likely be shared between the esophagus and proximal stomach. In fact, loss of nitric oxide producing ganglion cells (De Giorgio et al., 1999) and diminished Interstitial Cells of Cajal (Vanderwinden and Rumessen, 1999) have both been found in the gastric cardia of patients with primary achalasia. Genetically, some insight has been gained from patients with mutations in the Triple A Syndrome (*AAAS*) gene. These patients present clinically
with a congenital autosomal recessive disorder characterized by the three A’s of achalasia along with ACTH resistance and alacrima (Handschuag et al., 2001; Tullio-Pelet et al., 2000).

b. Infantile Hypertrophic Pyloric Stenosis (IHPS)

IHPS is a common condition (2 per 1000 live births) that presents during the first weeks of life as gastric outlet obstruction that causes projectile vomiting (Gariepy, 2004; Peeters et al., 2012). Hypertrophy of the muscularis encircling the distal or pylorus region of the stomach prevents the stomach from emptying. The hypertrophy can be anatomical (i.e. actually increased muscle density) and/or functional (decreased ability of that muscle to relax normally). The underlying cause of this disease may be similar to that described for achalasia as ENS defects, nitric oxide deficiency, and loss of Interstitial Cells of Cajal have also been observed in IHPS patients (Guarino et al., 2000; Vanderwinden et al., 1992; Vanderwinden and Rumessen, 1999). In rodent models in which the enzyme responsible for nitric oxide generation, one of the nitric oxide synthases (NOS), is disturbed either genetically (Huang et al., 1993) or pharmacologically (Voelker et al., 1995) a similar anatomic defect is detected. Over-exuberant muscularis growth has also been suggested as a possible cause (Shima et al., 2000; Shima and Puri, 1999). Extensive genetic linkage, association, and candidate gene approach studies have elucidated a complex network of genes and loci with associated syndromes and environmental factors (Peeters et al., 2012). Briefly, the diagnosis of this condition is still made clinically through imaging modalities including barium studies and ultrasonography (Poon et al., 1996; Taylor et al., 2013) and the standard treatment for IHPS is still surgical pyloromyotomy (St Peter et al., 2006) while other interventions have been tried including endoscopic balloon dilatation (Ogawa et al., 1996).
2. Gastric Atresia

Gastric atresia is a rare condition seen only in 1 out of 100,000 live births. Clinically, the symptoms are similar to those seen in other gastric outlet obstructions comprising postprandial vomiting and abdominal distension presenting in early infancy. Anatomically, this obstruction occurs in the distal stomach and is likely due to aberrant outpouchings of the stomach wall termed membranes or fibrous cords. It has been theorized that gastric atresias arise from aberrant endodermal fusion or fetal ischemic events (Berrocal et al., 1999; Semrin, 2010). Understanding the molecular basis of this disease has benefited from the clinical association of pyloric atresias with epidermolysis bullosa (EB) (Hayashi et al., 1991; Pfendner et al., 2007). Several genes have been linked to this autosomal recessive disorder including α6 integrin (ITGA6) (Ruzzi et al., 1997), β6 integrin (ITGB6) (Niessen et al., 1996; Vidal et al., 1995), and plectin (PLEC1) (Nakamura et al., 2011; Pfendner and Uitto, 2005). These genes all encode components of the hemidesmosome, a cellular anchor that allows tight attachment of cells to the basement membrane. Generation of rodent models of this syndrome (Jiang and Uitto, 2005) should lead to continued understanding of the basic mechanisms of these adhesional complexes (Sterk et al., 2000). Diagnosis of gastric atresias is typically made using imaging modalities including contrast radiography and ultrasonography, and treatment is still largely by excision (Al-Salem, 2007; Ilce et al., 2003). Excitingly, chorionic villus biopsies or amniotic fluid samples are being used in novel genetic diagnostic techniques for the subset of gastric atresias that are associated with epidermolysis bullosa and genetically based on hemidesmosome defects (Pfendner et al., 2003). In addition, these same patients might benefit from new gene, protein,
and cell based therapies aimed at restoring the structural defects being developed for EB (Tamai et al., 2009).

3. Gastric Volvulus

**Gastric Mesentery Primer**

During embryonic development, the tubular foregut is attached to the front and back of the body wall by the ventral and dorsal mesogastria. As the stomach rotates and enlarges, the ventral mesogastrium moves to the right connecting the lesser curvature and the superior duodenum with the developing liver. The dorsal mesogastrium moves to the left, enlarges to become the greater omentum, and encompasses the developing pancreas and spleen (Carlson). These mesenteries mature to form the four major ligaments that anchor the stomach intraperitoneally. The gastrophrenic, gastroplenic, and gastrocolic form from the dorsal mesogastrium or greater omentum, while the gastrichepatic ligament develops from the ventral mesogastrium or lesser omentum (Gray, 2008).

Gastric volvulus is a rare medical emergency defined as an abnormal 180-degree twisting of the stomach that can ultimately result in vascular compromise and necrosis. When occurring in adults, this disorder is usually secondary to hernias of the stomach through the diaphragm (Wasselle and Norman, 1993). In children, twisting occurs primarily due to laxity of the gastric mesentery ligaments (Cribbs et al., 2008). Specifically, the stomach may twist about its longitudinal axis (organoaxial), its short axis (mesenteroaxial), or a combination of the two (Sevcik and Steiner, 1999). The clinical presentation of acute gastric volvulus in adults is often chest pain, unproductive vomiting, vomiting of blood, and inability to pass a nasogastric tube.
(Miller et al., 1991), while in children symptoms may include vomiting, distension, and pain (Cribbs et al., 2008). Diagnosis is made clinically based on symptoms and confirmed by barium studies and CT scans (McElreath et al., 2008). Treatments remains surgical with the strategy to decompress and reduce the volvulus, correct concomitant factors such as hernias and necrotic tissue, and anchor the stomach to the anterior abdominal wall or perform an anastomotic bypass of the twist (antral-fundal gastrogastrostomy) to prevent future movement (Rashid et al., 2010). Gastronomy tubes are usually placed in children with volvulus to prevent future movement and allow feeding (Cribbs et al., 2008).

4. Congenital Microgastria

Congenital microgastria is another extremely rare (only about 60 known cases) (Vasas et al., 2011) developmental gastric condition characterized by failure of foregut growth during the 5th week of embryonic development (Ramos et al., 1996). These patients present with obstruction-like symptoms including vomiting, reflux, and esophageal dilation (Kroes and Festen, 1998). Retarded stomach growth also manifests as iron and vitamin B12 deficiencies due to decreased acid and intrinsic factor production. This condition is usually associated with VACTERL (vertebral defects, anal atresia, cardiac defects, tracheo-esophageal abnormalities, renal anomalies, and limb malformations) and other developmental abnormalities including asplenia due to improper rotation and development of the dorsal mesogastrrium (Aintablian, 1987; Filippi et al., 2008; Hernaiz Driever et al., 1997). The role of Sonic hedgehog (Shh) and in particular the Gli transcription factors with VACTERL syndrome (Kim et al., 2001) may shed light on the underlying molecular mechanisms associated with congenital microgastria. Diagnosis is made using imaging modalities including contrast radiography and ultrasonography,
and treatment is individualized. Milder cases are treated with feeding strategies, while more severe cases are treated with a double lumen distal Roux-en-Y jejunal reservoir (Hunt-Lawrence pouch) operation allowing for increased stomach volume (Neifeld et al., 1980; Velasco et al., 1990).

5. **Gastric Diverticulum**

Gastric diverticulum disease consists of outpouchings of the stomach wall that typically present asymptptomatically in adults with an incidence of about 0.04% (Rashid et al., 2012). These diverticula are classified as either: a) true congenital, containing all gastric tissue layers and usually located on the posterior wall of the cardia; or b) partial acquired, lacking the muscularis and usually located in the antrum (Rodeberg et al., 2002). A congenital muscle weakness, lack of a peritoneal membrane, and invading arterioles all may be contributing factors in this condition (Mohan et al., 2010). Diagnosis is usually incidental upon radiological studies, and surgical excision is only needed if the diverticulum is at risk to bleed, perforate (Rodeberg et al., 2002), or become malignant (Fork et al., 1998).

6. **Gastric Duplication Cysts**

Duplication cysts are defined as heterotopic epithelium lined by muscle located in or near the wall of the GI tract. Less than 10% of all GI duplication cysts consists of aberrant gastric epithelium (Macpherson, 1993). When found in the stomach proper, they are located on the greater curvature (Macpherson, 1993). Symptoms vary depending on the type of heterotopic epithelium and location in the GI tract, with gastric cysts often presenting with symptoms of peptic ulcer disease. Because of their rarity (an incidence of 17 per 1 million births (Bonacci and
Schlatter, 2008)), the research into this disorder is limited, and currently a handful of hypotheses exist involving formation of invaginations during embryonic development that aberrantly persist into duplication cysts (O'Donnell et al., 2005). Imaging modalities including CT scan and transabdominal ultrasound are used for diagnosis, and surgical excision is usually the recommended treatment (Semrin, 2010).

7. Teratomas

Teratomas are germ cell tumors defined as having all three germ cell layers: endoderm, mesoderm, and ectoderm. These rare tumors are further classified into immature and mature depending on differentiation status with immature teratomas more likely to become malignant (Peterson et al., 2012). Gastric localization of teratomas is extremely rare with only about 100 documented cases, and when they do occur in the stomach, teratomas tend to be exophytic and on the posterior greater curvature side (Corapcioglu et al., 2004). The causative mechanism of teratomas remains unknown (Oosterhuis and Looijenga, 2005); however, interesting work implicates the origins of this germ cell tumor to primordial germ cells using genetic imprinting (Bussey et al., 2001) and animal model techniques (Kimura et al., 2003). Occurring mostly in males (Yoon et al., 2000), gastric teratomas may cause distension, vomiting, hematemesis, and perforation depending on the growth pattern (Sharif et al., 2010). CT scan is the gold standard for diagnosis (Singh M, 2012), and use of serum tumor markers (α-fetoprotein (AFP) and human beta-choriogonadotropin (HCG)) is important in the diagnosis and follow-up (Gobel et al., 2000). Treatment is usually surgical excision for the mature variety, while chemotherapy is used in conjunction with surgery for immature teratomas (Romagnani et al., 2006). Understanding of this rare tumor type will become increasingly important in the future as basic science findings
involving human embryonic stem cells and induced pluripotent stem cells are being translated into the clinical realm (Blum and Benvenisty, 2009).

II. Adult/Acquired Clinical Disorders

A. Histological Development of the Stomach

The acquired clinical disorders of the stomach occur in the “adult” stomach. Because the gastric epithelium is continuously renewed throughout life from multipotent stem cells, adults can acquire developmental disease based on abnormal differentiation of progeny cell lineages (Fenoglio-Preiser C.M., 1999). I have divided the adult clinical disorders discussion into lineage specific categories and, in doing so, I am tasked with explaining in detail the cells of each lineage. Karam, Lee, and Leblond did much of the seminal work describing the dynamics of stomach epithelium differentiation in the mouse using electron microscopy and 3H-thymidine pulse labeling. Despite small differences as will be described including lineage turnover times (about twice that seen in mice (Fenoglio-Preiser C.M., 1999; Karam and Leblond, 1995; Karam et al., 2003; Macdonald et al., 1964)), the mouse and human stomachs seem to share remarkable similarities in development, physiology and cell biology.

Grossly, the human stomach is divided into four anatomic regions: the cardia, the fundus, the body/corpus, and the antrum (Fig. 2A). The cardia is the short transition region of predominantly mucous glands on the border with the esophagus closest to the gastroesophageal junction, the fundus (the outpouching, hunchback portion of the stomach along the greater curvature) and body make up the major portion of the stomach, and the antrum (or pylorus) is the distal area leading to the pyloric sphincter. A key anatomic difference in mice is that they have
an enlarged proximal gastric region called the forestomach, which can be considered, in part, an extension of the esophagus and is, similarly, lined by mature stratified squamous epithelium.

Each subanatomic region of the human stomach contains unique gastric invaginations or glands comprised of distinct histological cell types. The cardiac glands comprise simple columnar, mucus-secreting epithelial cells. Architecture in the antrum is similar, though, in addition to mucus glands, there are abundant endocrine cells (specifically, gastrin secreting G cells and somatostatin-secreting D cells). In the mouse, careful nucleotide pulse-chase labeling studies have shown that antral glands are continuously renewed bi-directionally from the isthmal region with pit or surface mucus gland cells turning over every 3 days, and basal gland cells turning over with a more variable rate of 1-60 days (Fig. 2B) (Lee, 1985; Lee and Leblond, 1985a; Lee and Leblond, 1985b).

The glands of the fundus/body or oxyntic glands contain four distinct regions, from surface to base: the pit, isthmus, neck and base (Fig. 2C). Eleven separate cell types (Karam and Leblond, 1995) have been found in the oxyntic glands but for our purposes I will characterize and examine these cell types in terms of their developmental lineages. In the isthmus resides the self-renewing stem cell, which, as in the antrum, bidirectionally differentiates and migrates (Karam and Leblond, 1993a). Interestingly, in mice a clear “granule free” stem cell was identified by electron microscopy, but all isthmal cells in humans seem to have at least some granules (Karam et al., 2003). Those cells that migrate up from the stem cell towards the lumen of the stomach become the surface pit (foveolar) mucus secreting cells; turnover of this lineage is rapid occurring every 3 days in mice just as in the antrum (Karam and Leblond, 1993b). The acid secreting parietal cells terminally differentiate and move in both directions, luminally and basally; these cells survive for 54 days in mice (Karam, 1993). In humans, parietal cells seem
only to migrate towards the base, as they are not observed in the pit region (Karam et al., 2003). Cells destined to become pepsinogen- secreting zymogenic cells (chief cells), mature from isthmal stem cells through a mucus neck cell intermediate as they migrate towards the base. These neck cells take 1-2 weeks to migrate and terminally differentiate into chief cells (Ramsey et al., 2007); cells in this lineage turn over once every 190 days in mice (Karam and Leblond, 1993c). It is important to note that in mice, intrinsic factor is not typically produced in parietal cells as in humans but rather in chief cells (Shao et al., 2000). Finally, enteroendocrine cells (predominantly histamine-producing enterochromaffin like, ECL cells) differentiate and mostly migrate towards the base with a lineage turnover rate of 60 days (Karam and Leblond, 1993d; Thompson et al., 1990). It is in this complex setting of multiple simultaneous cell movement and maturation that acquired adult disorders occur. Adult/Acquired clinical disorders are summarized in Table 1

**B. Disorders**

1. **Surface Pit Cells**

   These mucus secreting cells populate the most apical region of the gastric gland and undergo rapid turnover and renewal (estimated at 4-6 day half-life in human (Macdonald et al., 1964)). Functionally, their mucus and bicarbonate secretory product protects the stomach epithelium from the damaging effects of luminal acid and digestive enzymes (Bhaskar et al., 1992).

   a. **Foveolar Hyperplasia**
Foveolar hyperplasia is defined histopathologically as an overgrowth of surface pit cells associated with underlying mucosal injury such as chronic gastritis. There are multiple disease states/underlying causes, so I will not discuss foveolar hyperplasia diagnostic and therapeutic interventions in isolation. However, newly gained mechanistic insights have shed light on how pit cell growth is hyperstimulated. Helicobacter colonization can induce IL-1β release from activated macrophages in areas of foveolar hyperplasia, which in turn stimulates neutrophils and mesenchymal cells to release the growth factor, HGF (Yasunaga et al., 1996). Mouse models in which HGF activator is genetically ablated do not display the reactive foveolar hyperplasia post gastric injury (Yamagata et al., 2012). TGFα has been implicated in foveolar hyperplasia in a mouse model (Goldenring et al., 1996) and TGFα/EGF signaling in humans with Ménétrier disease, which will be discussed later.

Foveolar hyperplasia occurs usually in the setting of atrophic gastritis (i.e., where there is chronic inflammation and atrophy of parietal cells, discussed in more detail below). Mouse models have shown that parietal cells express and need the morphogen Sonic hedgehog (Shh) for acid secretion (Stepan et al., 2005). Genetic ablation of Shh in mice caused decreased acid secretion, hypergastrinemia, foveolar hyperplasia, and a reduced ability for the stomach mucosa to heal (Xiao et al., 2013; Xiao et al., 2010). Loss of Shh has been associated with neoplastic progression in humans as well, thus making Shh signaling a valid clinical target post gastric injury (Shiotani et al., 2005). Importantly, gastrin secreted from antral G cells (to be discussed in detail in the enteroendocrine section) has been shown to have a similar causative role in foveolar hyperplasia (Nomura et al., 2005b; Wang et al., 1996)

b. Ménétrier Disease
Ménétrier disease is a chronic adult disease characterized by enlarged rugal folds due to surface mucus pit cell overgrowth. Symptoms include pain, vomiting, bloating, weight loss, and edema in large part due to hyper mucus secretion leading to hypoproteinemia (Fenoglio-Preiser C.M., 1999; Rich et al., 2010). The definitive diagnostic procedure is gastroscopy and confirmatory biopsy (Lambrecht, 2011). Unlike foveolar hyperplasia, in which the foveolar overgrowth is secondary to epithelial damage response and a potentially complex set of associated signaling pathways, the causative process in Ménétrier disease has long been thought to be specifically increased TGF-α signaling in foveolar pit cells (Dempsey et al., 1992; Goldenring et al., 1996). Activation of EGF receptors that bind EGF and TGFα as ligands in the setting of normal gastrin levels, redirects lineage specification towards the surface mucus cells at the expense of parietal and zymogenic cell lineages (Coffey and Tanksley, 2012; Nomura et al., 2005a). These observations have spurred exciting novel treatments of this disorder targeting this particular signaling cascade. The chemotherapeutic drug cetuximab (Erbitux), that blocks TGF-α activation of its receptor, is now being developed as a promising first line treatment, shown to be effective clinically to increase parietal cell numbers and stomach acidity (Burdick et al., 2000; Fiske et al., 2009; Settle et al., 2005).

2. Enteroendocrine Cells

Enteroendocrine cells are located at the base of the gastric gland. Their function is primarily to control parietal cell acid secretion through release of gastrin, histamine and somatostatin. In response to distention, digested proteins, and postprandial cholinergic innervation, antral G cells release the peptide hormone gastrin (Schubert and Makhlouf, 1992). Gastrin primarily mediates fundic ECL cell histamine release, although it may also directly
stimulate parietal cell acid release. Histamine from the ECL cells stimulates parietal cell \( H-2 \) receptors to activate cAMP signaling, the ultimate effect of which is to recruit \( H,K \) ATPase transporters to membranes that ultimately communicate with the glandular lumen, thereby initiating and augmenting \( H^+ \) release (Schubert, 2009). The principal inhibitory hormone is somatostatin from the D cells, which directly inhibits G cell gastrin, ECL histamine, and parietal cell acid secretion (Schubert, 2009).

a. **Hypergastrinemia**

Hypergastrinemia is a condition caused either directly by gastrin-secreting tumors (Zollinger-Ellison syndrome, discussed below) or, indirectly, by increased gastric pH secondary to atrophic gastritis (and other abnormal epithelial differentiation states like intestinal metaplasia and cancer), vagotomy, or pharmacological inhibition of gastric acid secretion. Hypergastrinemia is characterized by serum gastrin levels above the normal range of 150 pg/mL (Orlando et al., 2007). When hypergastrinemia is secondary to increased gastric pH, clinical symptoms will vary depending on the primary cause. Importantly, symptoms may be masked when acid production is inhibited pharmacologically (usually through proton pump inhibitor therapy). In these patients, discontinuation of therapy can cause rebound dyspepsia and sudden impairment of gastric emptying (McCarthy, 2010). Histologically, hypergastrinemic states present with increased epithelial hyperplasia of gastrin’s cellular targets: parietal cells and ECL cells. The underlying effects of increased gastrin levels have been extensively studied using genetic tools available in rodent models (Jain and Samuelson, 2006). In \( H,K \) ATPase deficient mice, increased cell proliferation caused by impaired parietal cell acid secretion was rescued upon genetic ablation of gastrin (Franic et al., 2001). In mouse models overexpressing gastrin,
increased levels of heparin-binding epidermal-like growth factor (HB-EGF) and TGF-α (Sinclair et al., 2004; Wang et al., 2000) have been found in parietal and surface mucus cells. It is still unclear how gastrin acts to initiate proliferation; whether it is through induction of growth factors like EGF/TGFα or via more direct signaling to stem/progenitor cells (Kazumori et al., 2001). Serum gastrin measurements, secretin test of gastrin release, and basal acid output are used diagnostically to differentiate gastrinomas (i.e. gastrin-producing tumors causing direct hypergastrinemia) from other, secondary hypergastrinemic states (Del Valle J, 2003a). Therapies should be aimed at treating the underlying cause, usually the agent causing the gastritis (e.g., Helicobacter) or causing the acid inhibition in non-neoplasia-related hypergastrinemia.

b. Zollinger-Ellison Syndrome

Zollinger-Ellison syndrome is a rare disorder characterized by excessive gastrin secretion from a neuroendocrine tumor resulting in aberrant mucosal growth and hypersecretion of acid (aka a gastrinoma). These neoplasias are usually located in a triangular area marked by the cystic and common bile ducts, the second and third parts of the duodenum, and the pancreas (Del Valle J, 2003a). Patients with gastrinomas usually present with peptic ulcer disease and diarrhea. The growth promoting effects of gastrin coupled with the difficulty in recognizing this disease (Corleto et al., 2001), make early diagnosis an important factor in preventing malignant progression and improving outcomes (Weber et al., 1995). Current clinical diagnostic logarithms involve measuring serum gastrin with concomitant pharmacologic control of peptic ulcer disease (e.g. via proton pump inhibitors). After endoscopic confirmation of peptic ulcer disease healing, PPI treatment is discontinued and gastric acid levels are measured. Once
hypergastrinemia is confirmed with gastric pH <2, other conditions can be excluded and curative surgical resection of the gastrinoma can be performed (Ito et al., 2012; Metz, 2012). The causative mechanism for the formation of these neoplasias is currently unknown, but they have been associated with several genetic defects (Yu et al., 2000) including mutations in the tumor suppressor gene \( p16 \) (Muscarella et al., 1998; Serrano et al., 2000) and the oncogene \( HER-2/neu \) (Evers et al., 1994). Interesting work has associated Zollinger-Ellison syndrome with the autosomal dominant disorder, multiple endocrine neoplasia type 1 (MEN1). Mutations in the MEN1 gene cause inactivation of the tumor suppressor Menin that has many roles in inhibiting cell proliferation (Wu and Hua, 2011). Classically, MEN patients present with hyperparathyroidism, pancreatic endocrine tumors, and pituitary and adrenal adenomas. Gastrinomas with Zollinger-Ellison syndrome are the most common endocrine tumor type seen in MEN1 patients occurring in 21-70% of those patients (Gibril et al., 2004). Genetic linkage testing can be used diagnostically in MEN1 patients (Larsson et al., 1992), and new gene therapies aimed at replacing MEN1 gene function are promising in reducing cancer cell proliferation rates (Walls et al., 2012).

c. Carcinoid Tumors

Carcinoid tumors are rare endocrine tumors that arise from fundic ECL cells and usually occur in the setting of hypergastrinemia associated with chronic gastritis and pernicious anemia (type 1), or MEN1 syndrome (type 2); an aggressive sporadic form (type 3) that is gastrin-independent also exists (Borch et al., 1985; Lehy et al., 1992; Rindi et al., 1993). Carcinoid tumors spawned by excessive gastrin secretion are usually asymptomatic aside from the underlying condition. Sporadic carcinoids are often also asymptomatic and are usually
discovered incidentally. They can, however, cause symptoms such as bleeding, diarrhea, weight loss, and atypical carcinoid syndrome (characterized by flushing, headaches, shortness of breath, and lacrimation) (Borch et al., 2005; Burkitt and Pritchard, 2006). Diagnosis is based on gastroscopy, biopsy and histological analysis for ECL cell markers (chromagranin A), and somatostatin receptor scintigraphy and ultrasound or abdominal CT to look for metastasis (Scherubl et al., 2010). Therapy for type 1 and type 2 gastric carcinoid tumors is surgical resection, which can be augmented by antrectomy (to decrease gastrin levels by removing G cells) and octreotide (a somatostatin analog and inhibitor of gastrin) (Lawrence et al., 2011). Type 3 carcinoids demand more aggressive treatment with gastrectomy and chemotherapy (Lawrence et al., 2011). The strong association with MEN1 syndrome independent of sporadic Zollinger Ellison Syndrome (Lehy et al., 1992) has led to the theory that loss of heterozygosity at the MEN1 gene loci plays a role in gastric carcinoid formation (Cadiot et al., 1993; Debelenko et al., 1997). Further insights into the pathogenesis of this disease have been made using unique rodent models including the cotton rat (*Sigmodon hispidus*), which spontaneously develop gastric carcinoids (Cui et al., 2000), and the Natal multimammate mouse (*Mastomys natalensis*) (Nilsson et al., 1993). Experiments using the *Mastomys* model have confirmed the ECL cell growth-promoting roles of gastrin (Kidd et al., 2000) and histamine (Modlin et al., 1996). Specifically, recent reports have shown that gastrin and histamine may mediate AP-1 transcriptional activation (Kidd et al., 2004), increase growth factor expression (TGF-α and EGFR (Tang et al., 1996), CCN2/CTGF (Kidd et al., 2007), and polyamines (Kidd et al., 1998)), and induce the expression of anti-apoptotic proteins (mcl-1 (Pritchard et al., 2008), clusterin (Fjeldbo et al., 2012)). Ultimately, these animal models have proven to be useful to test and validate potential new
clinical therapies including the novel gastrin (cholecystokinin-2) receptor antagonist Netazepide (YF476) (Kidd et al., 2010; Martinsen et al., 2003).

3. Parietal Cells

Parietal cells are the primary gastric acid and intrinsic factor secreting cells located in the body and fundic regions of the stomach. In the unstimulated state, the H,K ATPase proton pumps are stored in an elaborate tubulovesicle membrane system within the cell. Upon stimulation, these membranes are trafficked to fuse with the plasma membrane to form the intercanalicular system which increases surface area for increased acid release. Stimulation of acid secretion is mediated by histamine, gastrin, and acetylcholine. Briefly, as previously discussed, histamine released from fundic ECL cells and gastrin released from antral G cells stimulates parietal cell H2 and CCK2 receptors, respectively. Postganglionic vagal or ENS stimulation releases acetylcholine to stimulate M3 muscarinic receptors. Both gastrin and acetylcholine lead to phospholipase C mediated mobilization of intracellular calcium (Del Valle J, 2003b). Alternatively, histamine acts to increase intracellular cAMP levels through adenylate cyclase activation (Del Valle J, 2003b). Both pathways lead to well established cell biological events that result in proton pump mobilization and acid secretion (Yao and Forte, 2003). The principal inhibitor of acid secretion is somatostatin, produced by endocrine D cells, which, in addition to inhibiting gastrin release from G-cells and histamine release from ECL cells, directly inhibits parietal cell cAMP levels (Del Valle J, 2003b). Occasional studies have also found that intrinsic factor secretion is stimulated by the same factors that increase acid secretion (Del Valle J, 2003b)
a. Peptic Ulcer Disease

Peptic ulcer disease is characterized by mucosal erosions that are caused by imbalances between acid secretion and the mechanisms that protect against acid-induced damage to the mucosa. Acid hypersecretion, NSAID toxicity, and *H pylori* infection are the most common etiologies. In cases of pure acid hypersecretion, the cause is usually hypergastrinemia in the setting of Zollinger-Ellison syndrome, which has already been discussed. Here, I will focus on the latter two etiologies. Although I discuss it in the “parietal cell” section, peptic ulcer disease is a multifaceted mucosal disorder that affects multiple cell lineages. Peptic ulcer disease can be viewed simply as a breakdown in mucosal protection. The barrier in the most general sense comprises the interplay among stomach acid, surface mucus production, the ability of the epithelium to self-renewal, blood flow, inflammation, and molecular regulators, such as prostaglandins (PGE2 and PGI2) (Wallace, 2008). Clinically, peptic ulcer disease manifests as pain, bloating satiety, nausea and/or vomiting (Yuan et al., 2006). NSAIDs are thought to cause peptic ulcer disease via inhibition of the enzyme COX1 (PTGS1), which produces protective prostaglandins. Thus, selective inhibitors of the other prostaglandin-producing enzyme COX2 (PTGS2) have been adopted (Laine et al., 1999) to spare the gastric mucosa from barrier effects. However, recent evidence has revealed that COX2 inhibition might also have deleterious effects on mucosal health (Chan et al., 2004; Hippisley-Cox et al., 2005; Mizuno et al., 1997). This has led to the current hypothesis that both COX-1 and COX-2 have mutually protective effects in the stomach through synthesis of prostaglandins that reduce gastric hypermotility, vascular damage, and neutrophil activation (Laine et al., 2008; Taha et al., 1999; Wallace et al., 1990).

Colonization by the gram negative bacterium, *H pylori*, causes chronic gastritis. *H pylori* generally favors the antrum or transitional regions between the fundus/body and antrum, though
it also can chronically infect the fundus/body, too (see below). *H pylori* is estimated to infect half the world, and infected individuals are usually asymptomatic; however, when symptoms arise, patients with predominantly antral colonization tend to experience overproduction of acid in the fundus/body (Genta, 2003). Thus, *H pylori* is one etiology for hypersecretion of acid and the ultimate cause of the vast majority of duodenal peptic ulcers. On the other hand, fundus/body predominant colonization increases risk of gastric peptic ulcers and carcinogenesis (to be discussed in the neck cell/zymogenic cell lineage section) (Suerbaum and Michetti, 2002). *H pylori* causes pathology through interplay between toxins it produces and the inflammation induced in the host. The ability of *H pylori* to survive, navigate, and adhere in the acidic gastric lumen is facilitated by the bacterial enzyme urease, bacterial flagella, and the bacterial surface protein BabA binding to Lewis B blood group antigen on host cells (Suerbaum and Michetti, 2002). Once bound to the gastric epithelium, pathogenic strains of *H pylori* usually harbor the toxin CagA on a pathogenicity island that also encodes a type IV secretion system to inject CagA into the host cell. CagA in turn elicits a variety of responses including disruption of cell junctional complexes (Amieva et al., 2003), transduction of MAPK pathway growth signals (Mimuro et al., 2002), activation of SHP-2 phosphatase (Higashi et al., 2002), and initiation of host transcriptional activity (Hirata et al., 2002). In addition, *H pylori* strains can also secrete another toxin, VacA, a vacuolating toxin that inserts in the host cell membrane, forms anion-selective channels (Szabo et al., 1999), and induces a cytochrome c release mediated cell death response (Galmiche et al., 2000). The host inflammatory response to these microbial factors is mediated by cytokines (interleukin-1β, interleukin-2, interleukin-6, interleukin-8, and tumor necrosis factor alpha (TNF-α)) and involves recruitment of neutrophils, lymphocytes, plasma cells, and macrophages (Suerbaum and Michetti, 2002). Specifically, NF-κB mediated IL-8
production leads to the neutrophil recruitment classically noted by pathologists during \textit{H pylori} induced gastritis (Brandt et al., 2005; Sharma et al., 1998). This Th1 predominant inflammatory response (Mohammadi et al., 1996) allows persistent colonization of the microbe (Tummala et al., 2004). An antecedent Th17 response has been shown to be induced by \textit{H pylori} to trigger the Th1 and subsequent inflammatory response (Luzza et al., 2000; Shi et al., 2010); studies have also suggested that \textit{H pylori} may suppress this Th17 activation in order to promote immune tolerance and persistent colonization (Kao et al., 2010; Oertli et al., 2013). This ability of \textit{H pylori} to persist may also be due to its ability to survive intracellularly (Amieva et al., 2002; Oh et al., 2005) and to evade host autophagy defenses through VacA toxin action (Raju et al., 2012; Terebiznik et al., 2009). Importantly, due to their overlapping effects on the inflammatory response, \textit{H pylori} infections and NSAID use can synergistically increase the development and progression of peptic ulcer disease (Huang et al., 2002).

Endoscopy is currently the gold standard in diagnosing peptic ulcer disease with simultaneous testing for \textit{H pylori} (assayed by culture, histological examination, the rapid urease test (RUT), and polymerase chain reaction (PCR)). Treatment involves the first line, so-called, triple therapies (proton pump inhibitor, amoxicillin, and clarithromycin) and secondary quadruple (proton pump inhibitor, bismuth, tetracycline, and metronidazole) therapies to eradicate the bacterium (Luther et al., 2010; Malfertheiner et al., 2011; Malfertheiner et al., 2002) Additionally, administration of misoprostol (a PGE1 analog), use of H2-receptor antagonists, proton pump inhibitor therapy, and switching to a selective COX2 inhibitor (Laine et al., 2008; Yuan et al., 2006) can all aid treatment, depending on exact etiology (e.g., NSAID use with \textit{H pylori} infection). Several new classes of NSAIDs are in development that capitalize on insights gained from basic science. As previously discussed, it is neutrophil activation that is
thought to be the common mechanism for mucosal damage. Nitric oxide has been shown to regulate epithelial barrier permeability, increase gastric blood flow, inhibit neutrophil invasion, and facilitate gastric mucosal healing (Lanas et al., 2000; Wallace and Miller, 2000). To address both prostaglandin and nitric oxide mechanisms, several new cyclooxygenase-inhibiting nitric oxide donators are being developed (Fiorucci et al., 2003; Lohmander et al., 2005) including Naproxinod (Baerwald et al., 2010). In addition, inhibitors of 5-lipoxygenase, an enzyme that produces pro-inflammatory leukotrienes from arachidonic acid as a result of COX inhibition, are being packaged with COX inhibitors (Bias et al., 2004). Additional lines of therapy being develop involve using phosphatidylcholine to enhance mucosal barrier function (Lichtenberger et al., 2009) and hydrogen sulfide to reduce inflammation (Wallace, 2007). With regard to *H pylori*, new treatment algorithms are being tested (Malfertheiner et al., 2011) with alternative therapies including probiotics and phytomedicine (Vitor and Vale, 2011). The ultimate goal for much of the research into eradication has now shifted towards effective vaccine production, given the rising incidence of antibiotic resistant variants (Megraud, 2004). Several candidate vaccines are in development based on animal studies aimed at circumventing host immunoregulation, which seems to suppress the natural immune response that would be sufficient to eradicate *H Pylori* (Czinn and Blanchard, 2011).

b. Autoimmune Gastritis/Pernicious Anemia

Autoimmune gastritis is an autoimmune disease characterized by autoimmune destruction of parietal cells due to autoantibodies to the parietal cell H/K ATPase (Karlsson et al., 1988) and intrinsic factor (Bardhan et al., 1968). Clinically, these patients may present with achlorhydria or hypochlorhydria from parietal cell damage, decreased pepsinogen levels, hypergastrinemia with
ECL and G cell hyperplasia, iron deficiency and cobalamin (B12)-deficient (i.e. pernicious anemia) (Genta, 2003). Autoimmune gastritis patients often show a genetic HLA association (Oksanen et al., 2010) and possibly an inciting role of *H pylori* infection (Veijola et al., 2010). Immunologists have long studied this disease, because it is an autoimmune disease with well-established auto antigen target, the H,K ATPase proton pump (D'Elios et al., 2001). In a mouse model with a transgenic T cell receptor specific for H,K ATPase, scientists were able to induce autoimmune gastritis after transferring T cells to immunodeficient mice; Th1, Th2, and Th17 CD4 subtypes were all shown to play a role in development of gastritis (Stummvoll et al., 2008). Peripheral extra-thymic deletion and Treg suppression have been found to mechanistically prevent these H/K ATPase–specific T cells from becoming pathogenic (DiPaolo et al., 2007; DiPaolo et al., 2005; Read et al., 2007). Despite these insights, diagnosis of this condition is made usually after presentation and diagnosis of megaloblastic anemia due to vitamin B12 deficiency. Noninvasive serum screens for low pepsinogen, high gastrin, low serum ghrelin (Checchi et al., 2007), and auto antibodies are first line diagnostic tests followed by subsequent endoscopic confirmation of mucosal atrophy and surveillance for advanced stage disease complications like polyps, carcinoids, and cancer (Annibale et al., 2011; Toh et al., 2012). Conventional treatment involves vitamin B12 replacement (Annibale et al., 2011; Toh et al., 2012). Several potential areas of treatment are being pursued including extrathymically induced T regulatory cells (iTregs) that can be induced, expanded, and administered to attenuate stomach pathology in a mouse model of autoimmune gastritis (Nguyen et al., 2011). An alternatively novel approach being investigated in all autoimmune diseases is the use of gene therapy to introduce autoantigens into bone marrow stem cells to promote immune tolerance (Alderuccio et al., 2009).
b. Atrophic Gastritis

Chronic atrophic gastritis is a pathologic condition secondary to chronic infection with *H. pylori* characterized by loss of parietal cells and consequent changes in the chief cell lineage (to be discussed in detail in the chief cell lineage section), usually also associated with secondary foveolar hyperplasia and hypergastrinemia produced by endocrine cell response to loss of parietal cell acid production. As mentioned, *H pylori* infection can be asymptomatic, induce peptic ulcer disease, and also cause atrophic gastritis with increased risk for progression to gastric adenocarcinoma or lymphoma. In general terms, disease typically starts in the antrum with hypersecretion of acid often causing duodenal ulcers, as discussed. Several theories have served to explain the hyperchlorhydria associated with this initial phase. It is believed that *H pylori* infection causes alkalization of the antrum, loss of G cell inhibitory mechanisms, and ultimately hypergastrinemia-induced acid secretion (Gillen et al., 1998; Hamlet and Olbe, 1996; Moss et al., 1992; Olbe et al., 1996). If the disease progresses, it can spread to cause loss (atrophy) of parietal cells in the fundus/body, which allows the bacteria to colonize the fundic and corpus portion of the stomach, because pH increases to levels tolerated by the bugs (El-Zimaity, 2008). *H pylori* initiates death and dysfunction of parietal cells through many proposed mechanisms, including: direct cagE pathogenicity island mediated NF-κB activation of the inducible nitric oxide synthetase iNOS (Neu et al., 2002), upregulation of proapoptotic factors (Hagen et al., 2008; Houghton et al., 2000), and disruption of cell junctions and architecture (Murakami et al., 2013; Wang et al., 2008). Indirect effects of *H pylori* on parietal cells include T cell cytotoxicity where T cells are induced to react against parietal cell antigens as in autoimmune gastritis (Amedei et al., 2003; Claeys et al., 1998) and neutrophil activation as
previously discussed. In addition, *H pylori* has been found to directly (Saha et al., 2010) and indirectly (Beales and Calam, 1998; Wolfe and Nompleggi, 1992) decrease parietal cell acid secretion. In fact, even the hypergastrinemia caused by infection has been found to induce parietal cell death (Cui et al., 2006). Interestingly, recent work has shown that the *H pylori* induced cytokine IL-1β blocks Sonic hedgehog expression (Waghray et al., 2010; Xiao et al., 2010), thus providing a mechanistic link between parietal cell loss and subsequent foveolar hyperplasia and chief cell metaplasia. Diagnosis and treatment are the same as those discussed in peptic ulcer disease, mostly involving reducing acid secretion and eradication of *H pylori*.

4. **Zymogenic (Chief) Cells**

The zymogenic (aka Chief) cells are the major digestive enzyme secreting cells of the stomach. These cells terminally differentiate from TFF2 (Trefoil factor family 2 or spasmolytic polypeptide) and mucus-secreting cells in the neck of the gastric gland that migrate towards the bases of the glands in the fundus/body. Large secretory vesicles containing precursor forms of digestive enzymes like pepsinogen C are stored apically in zymogenic cells until regulated secretion is signaled mainly through vagal cholinergic stimulation and additional histaminergic, adrenergic, and peptide hormonal inputs (Gritti et al., 2000). These stimulatory signals are transduced through phospholipase C activation of PKC and mobilization of intracellular calcium stores, and cAMP activation of protein kinases. Once secreted, the inactive proenzyme form of pepsinogen is catalyzed by luminal acidity into the active proteolytic pepsin enzyme form. Physiologically, pepsin functions as an endopeptidase to initiate the first steps in luminal food digestion; peptides liberated by this digestion in turn facilitate subsequent digestive motility, secretions, and absorption (Del Valle J, 2003b).
a. Atrophic Gastritis/SPEM

In the setting of chronic gastritis and atrophy of parietal cells, mature chief cells become metaplastic, lose mature chief cell functions, and start re-expressing mucous neck cell markers, like TFF2. This phenomenon has been extensively studied using mouse models, with the initial observation made with a model where infection with the Helicobacter pylori relative Helicobacter felis recapitulates many aspects of human H pylori infection, including parietal cell atrophy, foveolar hyperplasia, and mucus metaplasia (Wang et al., 1998). TFF2 has proven to be a good marker for this chief cell mucous metaplasia in humans and rodent models (Schmidt et al., 1999; Yamaguchi et al., 2002). Hence, the sequence of parietal cell loss with chief cell metaplasia has been called Spasmolytic Polypeptide Expressing Metaplasia (SPEM), though it is also known as pseudopyloric metaplasia or antralization, because the resulting glandular morphology (with TFF2 expressing cells at the base and decreased or absent parietal cell) resembles that of the normal antrum/pylorus. Additional mouse models based on parietal cell ablation genetically (Ohnishi et al., 1996) or pharmacologically (Goldenring et al., 2000; Huh et al., 2012) have demonstrated the requirement of parietal cells for proper chief cell maturation and maintenance. Recent evidence has shown that upon oxyntic atrophy, not only do chief cells begin re-expressing immature markers, but they also become proliferative (Huh et al., 2012; Nam et al., 2010; Nozaki et al., 2008). These proliferative events are likely independent of the foveolar hyperplasia that also occurs during parietal cell loss (Nomura et al., 2005b). Mechanistically, mouse models have shown that it is loss of parietal cells and reduction in homeostatic differentiation signals: Sonic hedgehog (Xiao et al., 2010), TGF-β (Li et al., 2002; Yano et al., 2006), Bone Morphogenic Protein (BMP) (Maloum et al., 2011; Shinohara et al., 2010) that
initiate zymogenic cell dedifferentiation. Aberrant enteroendocrine signals may also play a role in chief cell metaplasia (Nozaki et al., 2009; Wang et al., 2000).

**MIST1 primer**

A developmentally regulated transcription factor, MIST1 was initially characterized in highly secretory tissues including the pancreas, salivary glands, digestive tract, and the testes (Lemercier et al., 1997; Pin et al., 2000). Subsequent studies have elucidated that MIST1 functions as a homodimer (Zhu et al., 2004) and binds to CATATG-type E-boxes to transcriptionally activate its targets (Tran et al., 2007). Genetically engineered mice deficient in MIST1 expression exhibited disruption of cellular polarity and secretory defects in specifically the exocrine tissues (Pin et al., 2001). Additional studies in the pancreas have further characterized these secretion defects and have revealed deficiencies in calcium mediated exocytosis (Luo et al., 2005) and decreases in granule size and organization (Johnson et al., 2004). Recent work has revealed that loss of MIST1, in these tissues, is a necessary and consequential event during injury response and subsequent tumorigenesis events (Alahari et al., 2011; Capoccia et al., 2013; Huh et al., 2012; Kowalik et al., 2007; Lennerz et al., 2010; Shi et al., 2009). While some purport that MIST1 may act through direct transcriptional targets in a traditional tumor suppressor role (Jia et al., 2008), there remain many unanswered questions about the importance of MIST1 in these secretory tissues (Goldenring et al., 2011; Mills and Taghert, 2012; Pin, 2012).

Many reports indicate the same molecular and cellular changes in zymogenic cell differentiation that occur in response to parietal cell atrophy in mouse models occur in humans
(Capoccia et al., 2013; Goldenring et al., 2010; Lee et al., 2010). If the insult to parietal cells is temporary (e.g., by a toxin), and parietal census is restored, than normal chief cell differentiation is also restored (Capoccia et al., 2013; Huh et al., 2012; Khurana et al., 2013); thus, it is generally thought that SPEM is a normal repair response to decrease elaboration of potentially harmful substances (acid, digestive enzymes) and increase protective agents (mucus, trefoil factor), while increasing proliferation to replace lost cells. Diagnosis of atrophy and metaplasia is made on biopsy histopathologically. Clinical diagnosis and treatments have been previously discussed. Specifically, \textit{H pylori} eradication has been found to reverse SPEM (Ley et al., 2004; Sung et al., 2000b; Zivny et al., 2003). Improved diagnosis of early stages of chief cell differentiation changes may result from histopathological biomarkers of chief cell damage, like loss of the transcription factor MIST1 (see primer) and its direct targets (Lennerz Am J Path 2010; Capoccia JCI 2013); diagnostic tests of serum pepsinogen levels (in particular the ratio between pepsinogen A, normally produced only in corpus zymogenic cells, and C, normally produced in antral glands, corpus zymogenic cells, and the duodenal bulb Brunner’s glands, can inform about loss of normal chief cell function) have been found to be predictive of atrophy, metaplasia, and cancer initiation (Miki, 2006).

b. Intestinal Metaplasia

The ectopic expression of intestinal type epithelium in the stomach defines this pathologic condition. A clear association between a sequence of predisposing events \textit{H pylori} infection, chronic gastritis, formation of SPEM, development of intestinal metaplasia, and ultimate carcinogenesis has been made (Correa, 1988; Uemura et al., 2001). In a Mongolian gerbil animal model, infection with \textit{H pylori} induced SPEM formation that later progressed into
intestinal metaplasia (IM) (Yoshizawa et al., 2007). Corroborating these findings, a mouse model deficient in amphiregulin also develops SPEM, and those same metaplastic glands further progress into intestinal metaplasia (Nam et al., 2009). The idea of IM arising from a SPEM precursor is supported by the finding that intestinal genes start to become expressed in subpopulations of SPEM cells (Weis et al., 2012) and that IM is formed by monoclonal conversion of a multipotent proliferative precursor (McDonald et al., 2008). Mechanistically, formation of intestinal metaplasia must arise from a re-expression of primitive intestinal factors. Among these factors are the master intestinal transcription factors, CDX-1 (Fujii et al., 2012) and CDX-2 (Barros et al., 2012) whose increased expression has been found in human IM (Almeida et al., 2003). Genetic mouse models have proven that ectopic stomach expression of both factors is sufficient to cause intestinal metaplasia (Mutoh et al., 2004; Silberg et al., 2002) with CDX-2 acting as the initiating factor (Mutoh et al., 2009). *H pylori* induced expression of BMP signaling has been shown to induce CDX-2 expression and represses the stomach specifying transcription factor SRY-related HMG-box 2 (SOX-2) (Asonuma et al., 2009; Barros et al., 2008; Camilo et al., 2012). *H pylori* CagA has also been shown to effect E-cadherin association and activate β-catenin mediated CDX1 expression (Franco et al., 2005; Murata-Kamiya et al., 2007). In addition, loss of parietal cells and hypochlorhydria has also been shown to induce inflammation and expression of intestinal genes (Zavros et al., 2005). Diagnosis of IM remains a key prognostic in the future development of stomach cancer; current surveillance options include endoscopic biopsy and use of serum biomarkers to monitor atrophy (Correa et al., 2010). Treatment remains focused on HP eradication. However, as opposed to atrophy and SPEM, whether elimination of *H pylori* reverses IM has been controversial (Zivny et al., 2003) with reductions in IM (Ley et al., 2004; Sung et al., 2000b; Zhou et al., 2003) and reductions only in
atrophic gastritis (Rokkas et al., 2007; Wang et al., 2011) seen upon treatment. In fact, CDX2 expression levels have been found to remain elevated despite eradication (Satoh et al., 2002). Other pharmacologic agents such as antioxidants and COX-2 inhibitors may play beneficial roles in preventing IM addition to *H pylori* eradication (Dinis-Ribeiro et al., 2012).

d. Gastric Cancer

The study of gastric cancer defines a vast and ever expanding field of knowledge, as is fitting for the world’s second most common cause of cancer death. For this review, I will specifically focus on adenocarcinomas, which represent the bulk of the cancers in the stomach. In particular, I choose to focus on the non-cardia type of gastric cancer, as adenocarcinomas of the cardia overlap in etiology, epidemiology, and prognosis with mechanisms of carcinogenesis in the distal esophagus and gastro-esophageal junction. Gastric adenocarcinomas of the body/antrum (so-called distal gastric adenocarcinomas) are generally classified roughly by their histological features: either intestinal (which resemble to varying degrees, colorectal tumors and are largely glandular) or diffuse (which are poorly differentiated, and defined by invasion of isolated, single cells) types (Leung, 2003). The former is the most common type, tends to occur in older individuals, displays geographic variations, and is generally decreasing in incidence throughout the world (Peleteiro et al., 2012; Wu et al., 2009b). Gastric cancer of any type tends to have terrible prognosis in the absence of active, endoscopic screening protocols of populations at risk, because it usually remains asymptomatic, and symptoms are non-specific until the tumor becomes large or metastatic (Leung, 2003). *H pylori* infection is thought to be at the root of the vast majority of intestinal-type adenocarcinomas, but only about 1% of patients with chronic infection will go on to develop cancer (Huang et al., 2003; Uemura et al., 2001). Thus, given the
regional variation in prevalence, additional environmental factors (Larsson et al., 2006; Torres et al., 2013; Tsugane and Sasazuki, 2007) and genetic susceptibilities (Gonzalez et al., 2002; Loh et al., 2009) have been proposed to play roles in carcinogenesis.

The majority of work exploring the mechanistic causes of gastric cancer has focused on the intestinal-type cancers, with study of the multiple underlying host and pathogen signaling pathways (Wu et al., 2010). In general, as opposed to pancreatic cancer (where mutant K-RAS plays a predominant role in carcinogenesis) or colorectal cancer (where APC mutations and aberrant Wnt signaling predominate), gastric cancer does not seem to have any predominant pattern of aberrant/mutant signaling at its root (Capoccia et al., 2009). I will briefly touch on some of the many aberrant signaling pathways that have been shown to be involved in at least some gastric cancers (Fig. 3). As previously discussed, *H pylori* mediates activation of the NF-κB transcriptional signaling pathway (Brandt et al., 2005; Sharma et al., 1998). In addition to its role in inducing inflammation, NF-κB has been shown to upregulate COX2 (Sung et al., 2000a) mediated prostaglandin E2 (Oshima et al., 2004) expression. Sustained COX2 mediated PGE2 activation leads to increased growth factor release and gastric carcinogenesis in a mouse model of cancer formation (Oshima et al., 2004). *H pylori* through its CagA-containing pathogenicity island can initiate activation of β-catenin (Franco et al., 2005; Murata-Kamiya et al., 2007). Wnt pathway signaling has been shown to be important in a population of antral gastric stem cells (Barker et al., 2010), and its activation has been correlated with gastric cancer (Kurayoshi et al., 2006; Oshima and Oshima, 2010). In fact, the effects of COX2 and Wnt pathway activation are synergistic and act to further promote carcinogenesis (Oshima et al., 2006). The role of TGF-β signaling has also been explored. Smad phosphorylation transduces TGF-beta signals to transcriptional activation of RunX3 induced apoptosis pathways (Yano et al., 2006). Mouse
models deficient for RunX3 (Li et al., 2002) or Smad4 (Xu et al., 2000) show cancer formation, as does increased STAT3 signaling induced by mutation of the common cytokine receptor GP130 (Jenkins et al., 2005; Judd et al., 2004; Judd et al., 2006). However, paradoxically, TGF-β is re-expressed in cancers with complex roles in repressing immune surveillance, initiating EMT, and inducing angiogenesis (Achyut and Yang, 2011). Similarly, BMP signaling also seems to have a bi-functional role in carcinogenesis and in cancer. In mouse models, disruption of BMP pathway function resulted in aberrant epithelium homeostasis with increased proliferation (Maloum et al., 2011; Shinohara et al., 2010). But BMP also can induce CDX-2 expression as previously described (Asonuma et al., 2009; Barros et al., 2008; Camilo et al., 2012) and promote carcinogenesis (Thawani et al., 2010). As discussed previously, the parietal cell morphogen Sonic hedgehog is often lost during atrophic gastritis (Shiotani et al., 2005) and may be downregulated directly by H pylori induced IL-1β (Waghray et al., 2010; Xiao et al., 2013). Hedgehog signaling also follows a similar trend of reemergence during cancer progression (El-Zaatari et al., 2007; Saze et al., 2012). Mechanistically, HP mediates NF-κB activation resulting in upregulation of the hedgehog targets Gli-1 and Bcl-2 to promote growth and inhibit apoptosis (El-Zaatari et al., 2013; Martin et al., 2010). The interdependence and crosstalk of these cancer promoting pathways is again evidenced by the finding that the hedgehog pathway may signal through the TGF-β pathway during cancer progression (Yoo et al., 2008). Notch pathway activation is also associated with gastric cancer (Sun et al., 2011) and has been shown to induce COX-2 expression (Yeh et al., 2009). In fact, a mouse model aberrantly overexpressing Notch in the stomach resulted in dedifferentiation, proliferation, and tumor formation (Kim JEM 2011). Upregulation of fibroblast growth factor signaling through receptor overexpression and activation has been found in gastric cancer (Shin et al., 2000; Ye et al., 2011)
along with direct activation of this pathway by *H pylori* CagA mediated SHP2 signal transduction (Katoh and Katoh, 2006). Clearly, reactivation of these important developmental pathways is important in carcinogenesis. The finding of developmental pathways reactivation in cancer has presented new therapeutic pathways to target with new TGF-β inhibitors (Kano et al., 2007) and FGF receptor inhibitors (Zhao et al., 2010) being developed.

Outside the realm of developmental growth factors, gastrin and chronic inflammation have been shown to be major components in gastric cancer formation. A mouse model overexpressing gastrin showed increased susceptibility to developing gastric cancer with a synergistic effect upon subsequent Helicobacter infection (Takaishi et al., 2009b; Wang et al., 2000). However, another model lacking the SHP2-binding site on the gp130 receptor family, as mentioned above, has shown that tumorigenesis occurs even in the absence of hypergastrinemia (Jenkins et al., 2005; Judd et al., 2004; Judd et al., 2006). These mice display a chronic inflammatory state due to hyperactivation of the STAT3 signaling pathway. Thus, the independent role of inflammation to mediate gastric cancer was established. The gastrin-deficient mouse model recapitulated many of the findings regarding the gastrin-independent transformative potential of chronic (Huang et al., 2012) inflammation through IFNγ activated STAT3 pathway activation (Zavros et al., 2005). Studies have even postulated a protective role of gastrin to cancer formation through its ability to upregulate the tumor suppressing and healing trefoil factors, TFF1 and TFF2 (Khan et al., 2003; Tu et al., 2007). Thus gastrin and STAT3 pathway activation both are being targeted for cancer therapy (Ajani et al., 2006; Giraud et al., 2012).

The molecular events that lead to diffuse-type gastric carcinomas, many of which are characterized by mucus-stuffed so-called signet ring cells, have been more clearly elucidated
under a common signaling theme. Seminal work established a hereditary form of diffuse-type gastric cancer that resulted from a germline mutation in the \textit{E-cadherin} gene (Guilford et al., 1998). Mouse models mimicking the human genetics by expressing only one copy of the \textit{E-cadherin} gene showed increased susceptibility to cancer initiation independent of \(\beta\)-catenin/Wnt pathway activation (Humar et al., 2009). In a murine system, it was shown that complete genetic ablation of E-cadherin in adult mice was sufficient to produce signet ring lesions (Mimata et al., 2011), and those progressed to cancer if p53 was also ablated (Shimada et al., 2012). In humans, E-cadherin promoter methylation leads to a second hit inactivation (Humar et al., 2009) in patients heterozygous for functional E-cadherin that results from \textit{H pylori} induced IL-1\(\beta\) stimulation of NF-\(\kappa\)B transcriptional activation of DNA methyl transferase activity through iNOS and NO production (Huang et al., 2012).

While discussing the cellular signals involved in tumorigenesis it is often easy to ignore which cells become responsive and receptive to those signals or, in other words, the cell of origin for cancer. I have included the cancer discussion in the chief cell section because there is evidence that chief cells dedifferentiate to become the proliferating cells in SPEM (Huh et al., 2012; Nam et al., 2010; Nozaki et al., 2008). Tracking of IM has shown that it develops from SPEM and clonally progresses to dysplasia (Gutierrez-Gonzalez et al., 2011; McDonald et al., 2008). Thus, it is possible that the dedifferentiation of mature chief cells gives rise to the cancer. However, other gastric cell lineages with the right growth impetus have been shown to have the ability to dedifferentiate and become multipotent in mouse models (Kim and Shivdasani, 2011; Quante et al., 2010). In fact, studies in mice have found that bone marrow-derived cells can be recruited to sites of stomach injury and give rise to adenocarcinoma (Houghton et al., 2004). This “dedifferentiation” of mature cells into cancer is at odds with alternative theories that
stomach cancer may progress from an undifferentiated stem cell precursor. Antral tumors in mice have been shown to develop clonally from multipotent cells marked by expression from the \textit{Lgr5} gene locus (Barker et al., 2010) marked by expression from a transgenic \textit{villin} promoter (Li et al., 2012). The difficulty in studying these cells is the lack of markers. Several markers have been proposed including CD44 (Khurana et al., 2013; Takaishi et al., 2009a), CD90 (Jiang et al., 2012), and CD133 (Fukamachi et al., 2011). Identification of these markers will lead to therapies that specifically target these cancer stem cells (Jiang et al., 2012; Khurana et al., 2013; Smith et al., 2008).

Early detection and \textit{H pylori} eradication therapy has been demonstrated to be effective at cancer prevention (Ito et al., 2009; Wu et al., 2009a), but as discussed previously, the benefits start to decrease with advancement of carcinogenesis towards SPEM and IM, indicating diffuse repatterning of the gastric epithelium that is often referred to as a “point of no return” (Peleteiro et al., 2012; Wong et al., 2004). Unfortunately, the early detection protocols involve endoscopic surveillance and biopsy, which are too expensive to initiate in regions where gastric cancer incidence is relatively much lower (e.g., the US, northern Europe) than in the far east where it is by far the most common cancer killer (e.g., Japan, South Korea) (Dan et al., 2006). New chemotherapy combination regimes are being implemented (Wagner et al., 2010) along with radiotherapy and biological agents targeting classic cancer pathway targets including HER2, EGFR, and VEGF (Price et al., 2012). Novel immunotherapies are also being developed utilizing dendritic cell-based vaccines, priming of natural killer cells, T cell based therapies to recognize and attack cancer cells (Amedei et al., 2011).

Conclusions
It is clear that our knowledge of the pathology and pathophysiology of the stomach has and continues to increase since ancient times. With the advent of modern genetics, cell biology, and biochemistry we are building new avenues exploring the molecular mechanisms behind congenital and acquired diseases. However there is still much we do not know. Much light has been shed on the developmental pathways that are important in formation and differentiation of the stomach, but an overarching interconnected model that might be clinically useful has yet to be proposed. And therein lies the complexity of the problem. Multiple signals to multiple receptors on multiple cell types all respond simultaneously during stomach development and disease. The beauty of modern science has been the ability to study complex systems in isolation and there again is its greatest weakness. For example, it is clear that during chronic inflammation every stomach lineage is affected and responds. How can we tease out which cell is the cell of origin that proceeds towards carcinogenesis? Efforts have started to concentrate on identifying gastric stem cell markers. But many new studies have demonstrated the plasticity of terminally differentiated cells in the stomach to transdifferentiate and possibly even dedifferentiate. In fact, much of our knowledge comes from work using genetic murine models. With fundamental differences between mice and men, do we have the best physiological model to study gastric diseases? Despite these unknowns, exciting new gene stem cell markers and gene expression studies are being performed currently that seek to unify and create a systems-based network for understanding normal development and disease.

Clinically, the challenges are now greater than ever. Our knowledge about the causes of rare developmental disorders has remained largely unchanged since their discoveries. Increased survival rates have largely benefited from advances in radiological and surgical interventions. Clearly, there is a need to further understand these congenital diseases. In fact, the same can be
said about the adult diseases. Stomach cancer remains a leading cause of mortality throughout the world, and despite near eradication of *H pylori* here in the United States, stomach cancer rates are actually increasing among certain demographics (Anderson et al., 2010). A growing demand exists for new noninvasive screening modalities including new biomarkers for diagnosis and therapeutic efficacy monitoring. Current therapy is bimodal; from mitigation of symptoms on one end of the spectrum to aggressive surgery and cancer pharmacotherapy on the other end. New safe treatment options that utilize our basic science insights must focus on the stomach as an interconnected network. We must continue to translate our findings from the bench back to the clinics. In this review, we have taken a developmental biology centric view, however future studies must apply our knowledge of stomach development and integrate those insights with new epidemiological evidence and modern genetic approaches. The traditional Chinese medicine holistic belief of interdependence and interconnectivity hold true and are concepts that we would be wise to continue to apply in our modern pursuits of stomach clinical cures.

**Acknowledgements**

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Figure Legends

Figure 1. Anatomic development of the human stomach. Embryonic gastric developmental milestones of enlargement, rotation, and asymmetric curvature establishment.

Figure 2. Histologic development of the human stomach. A. Gross anatomic regionization of the human stomach. B. Antrum and C. Corpus gastric unit architecture with regions, direction of migration and differentiation, major cell types, turnover times (* based on murine data), and main secretory products.

Figure 3. Re-emergent developmental pathways during gastric cancer progression. Well understood developmental pathways important in gastric carcinogenesis.

Table 1. Developmental/congenital clinical disorders with underlying disease mechanisms, diagnostic approach, and current therapies.

Table 2. Differences between human and murine stomach anatomy and histology.

Table 3. Adult/acquired clinical disorders with underlying disease mechanisms, diagnostic approach, and current therapies.
Gastric Cancer Progression
<table>
<thead>
<tr>
<th>Disorder</th>
<th>Etiologies</th>
<th>Diagnostic Approach</th>
<th>Therapies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric Atresia</td>
<td>Stomach Wall Outpouchings, association with epidermolysis bullosa (<em>ITGA6, ITGB6, PLEC1</em> gene mutations)</td>
<td>Contrast Radiography and Ultrasonography, novel genetic testing</td>
<td>Surgical excision and novel gene replacement therapy</td>
</tr>
<tr>
<td>Gastric Volvulus</td>
<td>Secondary to hernia, laxity of mesentary ligaments</td>
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</tr>
<tr>
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<td>Contrast Radiography and Ultrasonography</td>
<td>Surgical enlargement of stomach</td>
</tr>
<tr>
<td>Gastric Diverticulum</td>
<td>Congenital muscle weakness, lack of peritoneal membrane, invading arterioles</td>
<td>Radiological studies</td>
<td>Surgical excision if complications</td>
</tr>
<tr>
<td>Gastric Duplication Cysts</td>
<td>Persistant embyronic invaginations</td>
<td>CT scan and Ultrasonography</td>
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</tr>
<tr>
<td>Teratomas</td>
<td>Aberrant primordial germ cells</td>
<td>CT scan and monitoring of AFP and HCG serum tumor markers</td>
<td>Surgical excision and chemotherapy</td>
</tr>
<tr>
<td>Motility</td>
<td></td>
<td>See Chapter 3 and Chapter 6</td>
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<tr>
<td></td>
<td>Mouse</td>
<td>Human</td>
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<tr>
<td><strong>Anatomic Regions</strong></td>
<td>3 (Forestomach, Body/Corpus, and Antrum)</td>
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<tr>
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<td>See Figure 5.2</td>
<td>About twice that seen in Mice</td>
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<td><strong>Isthmal stem cell ultrastructure</strong></td>
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<td><strong>Parietal Cell Differentiation</strong></td>
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<tr>
<td>Disorder</td>
<td>Etiologies</td>
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<td>Surface Pit Cells</td>
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<td>Foveolar Hyperplasia</td>
<td>Injury induced growth factors, loss of Shh, hypergastrinemia</td>
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<td>Enteroendocrine Cells</td>
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<tr>
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<td>Serum gastrin measurements, secretin test of gastrin release, and basal acid output</td>
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<tr>
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<td>Carcinoid Tumors</td>
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<tr>
<td>Parietal Cells</td>
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<tr>
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</tr>
<tr>
<td>Atrophic Gastritis/SPEM</td>
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<td>HP eradication (triple and quadruple therapies), vaccine development</td>
</tr>
<tr>
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<tr>
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<td>SPEM progression, re-expression of CDXs and repression SOX2</td>
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<td>HP eradication</td>
</tr>
<tr>
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<td>HP infection, reemergence of developmental pathways, aberrant growth signals, and chronic inflammation</td>
<td>Early detection endoscopy and biopsy</td>
<td>Surgery, chemotherapy, radiotherapy, targeting specific developmental pathways, blocking growth signals, immunotherapies</td>
</tr>
</tbody>
</table>
CHAPTER 3: RAB26 and RAB3D are Direct Transcriptional Targets of MIST1 That Regulate Exocrine Granule Maturation

This chapter is adapted from the published work:


XT and RUJ contributed equally to this manuscript.
RAB26 and RAB3D are direct transcriptional targets of MIST1 that regulate exocrine granule maturation

Xiaolin Tian*,1,2,3, Ramon U. Jin*,1, Andrew J. Bredemeyer1,4, Edward J. Oates1, Katarzyna M. Błażewska5, Charles E. McKenna5, and Jason C. Mills1,2

*Contributed equally

Departments of 1Pathology and Immunology and 2Developmental Biology, Washington University School of Medicine, St. Louis, MO 63110; 3Current address: Neuroscience Center of Excellence, Louisiana State University Health Science Center, New Orleans, LA 70112; 4Current address: Partners HealthCare System, Boston, MA 02199; 5Department of Chemistry, University of Southern California, Los Angeles, California 90089-0744

Address correspondence to:

Jason C. Mills
Department of Pathology and Immunology
Washington University School of Medicine
Box 8118
660 So. Euclid Ave.
St. Louis, MO 63110
Phone 314 362-4258
FAX 314 362-7487
e-mail jmills@pathology.wustl.edu

Running title: MIST1, RABs and exocrine secretory granules
Key words: Bhlha15; phosphonocarboxylate; intronic enhancer; neck cell
Abstract

Little is known about how differentiating cells reorganize their cellular structure to perform specialized physiological functions. MIST1, an evolutionarily conserved transcription factor, is required for the formation of large, specialized secretory vesicles in gastric zymogenic (chief) cells (ZCs) as they differentiate from their mucous neck cell progenitors. Here, we show that MIST1 binds to highly conserved CATATGE-boxes to directly activate transcription of 6 genes, including the small GTPases RAB26 and RAB3D. We next show that RAB26 and RAB3D expression is significantly downregulated in Mist1−/− ZCs, suggesting that MIST1 establishes large secretory granules by inducing RAB transcription. To test this hypothesis, we transfected human gastric cancer cell lines stably expressing MIST1 with RFP-tagged pepsinogen C, a key secretory product of ZCs. Those cells upregulate expression of RAB26 and RAB3D to form large secretory granules, whereas control, non- MIST1-expressing cells do not. Moreover, granule formation in MIST1-expressing cells requires RAB activity because treatment with a RAB prenylation inhibitor and transfection of dominant negative RAB26 abrogate granule formation. Together, our data establish the molecular process by which a transcription factor can directly induce fundamental cellular architecture changes by increasing transcription of specific cellular effectors that act to organize a unique subcellular compartment.
Introduction

Developmentally regulated transcription factors (TFs) play central roles in cell fate specification and differentiation of all cell types. During the complex process of specified cellular differentiation, certain TFs are turned on only at the final steps of the transcription regulation hierarchy. Presumably, those TFs directly activate major cell effector genes and in turn govern the establishment of a differentiated cell’s designated morphology and function. There is emerging evidence that there might indeed be a limited number of TFs that are employed in diverse tissues to induce specific gene cassettes that regulate cell structure. For example, it was recently shown that differentiation in general, no matter the tissue, induced preferential expression of the same types of genes that govern secretion and communication with extracellular space (13). Further, it has been shown that X-box binding protein 1 (XBP1), a transcription factor that is also developmentally regulated and required for wholesale changes in cell structure in certain cell lineages, directly activates multiple secretory pathway genes that help establish the abundant rough endoplasmic reticulum and mitochondria needed by specialized secretory cells, such as plasma cells, pancreatic acinar cells, and intestinal Paneth cells (30, 33, 56, 59). Another example of a developmentally important TF that regulates cell structure is TFEB, a bHLH recently shown to upregulate a cohort of target genes that specifically regulate lysosome function and biogenesis(54).

The corpus of the adult murine stomach is a useful system for studying the role of developmentally regulated TFs in coordinating cell structural changes because the gastric epithelium comprises multiple secretory cell lineages that are constantly renewed in a spatiotemporally organized fashion (6, 28, 39). The zymogenic (aka chief) cell (ZC) lineage occupies the base of the gastric unit, migrating from the stem cell zone and passing first through
a distinct mucous neck cell progenitor phase, before rapid terminal differentiation that includes dramatic expansion of the rER and apical accumulation of large secretory vesicles filled with pepsinogen and other digestive enzymes (5, 29).

The bHLH transcription factor Mist1 is critical for ZC architectural maturation (50) (Fig. 1). Mist1 is a developmentally regulated and highly cell lineage specific TF, with onset of expression only during terminal differentiation of – along with ZCs – a handful of secretory cells in mammals (26, 48, 49). Mist1-expressing cells, ranging from immunoglobulin-secreting plasma cells (4) to alveolar breast lobular cells (69), are scattered in diverse tissues and have little in common in either their developmental origins or in the specific substances they elaborate. However, they do share a specialized physiological function: high-capacity secretion of proteins, indicating that Mist1 may be the transcriptional architect initiating the subset of structural changes required for such functionality. Indeed, loss of Mist1 leads to smaller cell cytoplasms, with smaller secretory granules, reduced secretory protein stores, and a decrease in secretory capacity (5, 34, 50). Modulating cell architecture is likely a critical feature of normal development because Mist1 is a highly conserved gene from Drosophila to Zebrafish to mammals, reflected not only by the high degree of homology among Mist1 orthologs but also the specific sequence of the genomic cis-element they bind, CATATG-type E-boxes (18-20, 45, 46).

To coordinate specific changes in cell structure, Mist1 must directly activate transcription of a group of cellular effector genes that coordinate structural changes. This cassette of structure-modifying genes would likely be conserved across tissues and species. However, no Mist1 targets that might coordinate such structural changes have yet been identified. Ras-like, small GTPases are important regulators of vesicular formation and transport in eukaryotic cells, making them potential structure-regulating genes (15, 17). It has been shown that Rab3d
expression is decreased in Mist1−/− pancreatic acinar cells, but Rab3d has not yet been shown to be a direct target of mist1, and Rab3d is expressed in non-Mist1-expressing cells as well. Furthermore, transgenic expression of Rab3d was insufficient to restore the defects caused by loss of Mist1 function in pancreatic acinar cells (26), indicating that other, as yet unidentified, Mist1 targets must also play a role. A paralogous RAB to Rab3d, Rab26, has not been extensively studied, but its expression is restricted to a limited number of highly secretory cells where it has been proposed to be involved in regulated vesicular secretion (40, 64, 68).

Here, we show that Mist1 is sufficient to upregulate 16 genes in 2 different gastric cell lines. We further show that it directly associates with CATATG E-boxes in 6 of those genes that have highly evolutionarily conserved CATATG E-boxes within the first intron. Two Mist1–induced genes, Rab3d and Rab26, stood out as representing a possible mechanism by which Mist1, acting at the level of transcription, could regulate formation of exocrine secretory granules in differentiating cells. We show that Rab3d and Rab26 are expressed in gastric ZCs in the corpus of mouse stomach in a Mist1-dependent manner. We next developed a system to test the functional importance of Mist1 induction of these RABS. We show that gastric epithelial cells stably expressing Mist1, but not control cell lines, produce large ZC-like secretory granules when they are transfected with RFP-tagged pepsinogen C (PGC). Expression levels of Rab26 and Rab3d were also specifically induced in those cell lines. Finally, we show that formation of large granules in these cells is dependent on RAB activity, because granule formation is abrogated by: 1) inhibition of Rab26 and Rab3d function by disrupting Rab prenylation and 2) transfection with a dominant negative Rab26 construct. We therefore propose that Mist1 regulates large secretory granule formation, a key aspect of differentiated secretory cell function, via activating transcription of Rab26 and Rab3d. Together, our data demonstrate how a developmentally
regulated TF can induce secretory cell architectural changes by transcriptionally regulating specific membrane trafficking genes.
Materials and Methods

Mice

All experiments involving animals were performed according to protocols approved by the Washington University School of Medicine Animal Studies Committee. Germline Mist1−/− mice were generated as described previously (49) and maintained in a specific pathogen free barrier facility. Control mice were the littermates of Mist1−/− mice resulting from heterozygote by heterozygote crosses.

Immunofluorescence

Stomachs were prepared and stained as described (50). Briefly, stomachs were inflated with freshly prepared methacarn fixative and suspended in fixative for 15-30 mins at RT, followed by multiple rinses in 70% ETOH, arrangement in 2% agar in a tissue cassette, and routine paraffin processing. Sections (5 μm) were deparaffinized and rehydrated, then antigen retrieval was performed by boiling in 50 mM Tris-HCl, pH 9.0. Slides were blocked in 1% BSA, 0.3% Triton-X100 in PBS, then incubated in primary followed by secondary antibodies (see below). Slides were incubated 5 mins in 1 μg/ml bisbenzimide (Invitrogen, Carlsbad, CA) prior to mounting in 1:1 glycerol:PBS.

For immunofluorescent analysis of cultured cells, cells were grown in Lab-Tek Chamber Slide 4 Well Permanox slides (Thermo Fisher Scientific, Rochester, NY) and fixed in 4% paraformaldehyde for 10 mins at RT. For antibody staining, slides were permeabilized by 0.1% Triton-X100 in PBS and blocked in 2% BSA, 0.05% Triton-X100 in PBS. Fluorescence microscopy and imaging were performed using a Zeiss Axiovert 200 microscope with Axiocam MRM camera at RT. Optical sectioning was performed using the Apotome adaptor following
calibration. In most cases, 1 μm sections were taken from cell-substrate interface to the full height of the cell. Contrast (maximal, minimal, and midtone) adjustment and fluorescent channel overlay, and pseudocoloring were performed in Adobe Photoshop (Adobe Systems, San Jose, CA). All adjustments were performed on the entire image equally. Cartoon cell traces and illustrations were performed using Adobe Illustrator (Adobe Systems, San Jose, CA).

The following primary antibodies were used for immunostaining: goat (1:2000) anti-human gastric intrinsic factor (GIF) (gift of Dr. David Alpers, Washington University); sheep anti-PGC (1:10,000, Abcam), rabbit anti-actin (1:200, gift of Dr. Thaddeus Stappenbeck, clone AC-40; Sigma, St Louis, MO). Secondary antibodies used were: Alexa Fluor 488, 594 or 647 conjugated donkey anti-goat, anti-rabbit, anti-sheep, or anti-mouse (1:500, Invitrogen).

**Immunofluorescent quantification**

Immunofluorescent quantification to determine cytoplasmic fluorescence intensity was performed in ImageJ software. For tissue sections, the cytoplasm was divided into regions fully apical to and fully basal to the nucleus. Mean fluorescent intensity was determined in each region after subtraction of background; background was determined by averaging PGC fluorescence in 4 parietal cells – which do not express PGC – in the same unit. Results were compiled from 51 cells from 7 gastric units in two separate knockout mice and 50 cells from 6 units in two control mice.

For cell line vesicle quantification, MIST1 stably expressing lines were transfected with PGC-RFP and appropriate treatment. Number of large vesicular cells (defined as cells containing 3 or more vesicles, each ≥ 1.5 μm), diffuse vesicular cells (cells with no visible vesicles), and total number of PGC-RFP transfected cells were scored.
fluorescence intensity quantification, MIST1 stable cell lines were co-transfected with PGC-RFP and eGFP-hRAB26T77N. 16 bit images captured in Zeiss Axiovision software were analyzed in ImageJ as follows: mean green and red cytoplasmic fluorescent intensities were determined for large vesicular cells (defined as above) and diffuse vesicular cells (defined as ≥135 intensity units above background, no vesicles ≥1 μm) in each region after subtraction from the median background (green and red fluorescence in an area with no cells) and normalization to average nuclear green fluorescence (nuclei were green due to expression of MIST1-eGFP) for ≥10 cell nuclei/field. 245 cells were scored across 3 transfections.

Electron microscopy

For transmission electron microscopic studies, stomachs were fixed, sectioned, stained, and imaged as described (50). For tEM of cell lines, cells were plated on Lab-Tek Chamber Slide 4 Well Permanox slides (Thermo Fisher Scientific). After rinsing in PBS, they were fixed in modified Karnovsky’s fixative (2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 mmol/l cacodylate buffer). tEM thin sections were cut directly from cell cultures embedded on the original Permanox substrate. To quantify vesicle size, fields of MIST1-stable and eGFP-control cells were randomly selected, digital images captured at 4000x magnification, and ImageJ was used to determine areas of all vesicle profiles in all cells in the field in each TIFF image. 20 cells were analyzed from each cell line (~500 total vesicles scored).

Cell lines and transient transfection

HGC-27 cells (HPACC, Porton Down, UK) were maintained at 37°C in 5% CO2 in DMEM supplemented with 10% fetal bovine serum, 1% non essential aminoacids, 1% glutamine and 100
ng/ml each of penicillin and streptomycin. 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. Cells were passaged every 3 days using Trypsin-EDTA. For transient transfection, 1 x 10^6 AGS or HGC-27 cells were transfected via electroporation using the Nucleofector I (Amaxa-Lonza, Basel, Switzerland), program B-023, and cell line transfection solution V. The following plasmids were used per electroporation: 3 μg PGCRFP (see below), 5 μg eGFP-hRAB26T77N (see below), or 3 μg pmaxGFP (Amaxa-Lonza). Cells were replated on Lab-Tek Chamber Slide 4 Well Permanox slides and routinely analyzed 24 hours post transfection.

**GeneChip analysis**

Untransfected HGC and AGS control plates were grown to 70-80% confluency on 10 cm dishes. For cells transfected with either MIST1-eGFP or Control eGFP vector, 1 x 10^6 AGS or HGC-27 cells were transfected with 1.5 μg pmaxGFP plus either 3 μg MIST1 cDNA or 3 μg empty vector using the transfection conditions described in the text. The GFP^+ population of each transfectant was isolated by flow cytometric cell sorting using a FACSvantage (Becton-Dickinson, San Jose, CA) at 24 hours post-transfection and then replated for an additional 24 hours before harvesting. Cells were harvested with trypsin-EDTA. RNA was extracted using a QIAGEN RNAeasy kit, and RNA quality was verified by visualization on an agarose gel. 5 ug total RNA was used to prepare labeled target using Affymetrix GeneChip One-Cycle Target Labeling reagents. 10 μg labeled target cRNA was hybridized to HGU133_Plus_2 GeneChips. For both AGS and HGC-27 cells, expression profiles of MIST1 + pmaxGFP-transfected cells were compared with profiles of empty vector + pmaxGFP-transfected cells. An initial, inclusive candidate MIST1 target list was
generated by determining by dChip analysis (70) the transcripts that had a 90% confidence lower bound fold-change increase of $\geq 1.2$ and an intensity greater than 100 in 4 independent comparisons, combined as follows: (AGS-MIST1 vs. vector transfected AND vs. untransfected) OR (HGC-27 vs. vector AND vs. untransfected). This list of 211 genes was then filtered to generate AGS- and HGC- MIST1-induced transcripts by filtering genes whose expression intensity was <100 in MIST1-transfected cells and whose mean fold-change was <1.5 in MIST1-transfected cells vs. either control population. 32 AGS and 59 HGC probe sets met those criteria, and 18 of those, representing 16 individual genes were shared (Fig. 2A). GeneChip datasets have been deposited in GEO (GSE16924).

**Bioinformatic analysis of MIST1 targets**

ECR Browser (44) was used to analyze the human genomic sequence for each gene 5’ to the transcription start. Sequence was analyzed up to 50 kb or until an exon from the neighboring gene was reached. The first intron up to 50 kb was also analyzed. When alternate splice forms exist, the farthest documented 5’ exon was used, and the intron following it was considered the first intron. CATATG sequences were identified within analyzed sequences using the dreg application in the EMBOSS suite. Conservation among species aligned in the ECR Browser was then determined at each of these CATATG sites. Note that only human, chimp, cow, dog, rat, mouse, opossum, and chicken were available in the ECR Browser at time of analysis, so conservation undoubtedly extends across numerous species not diagrammed in Fig. 3. All 16 putative MIST1 targets were analyzed in this way. 6 genes showed the conserved intronic pattern depicted in Fig. 3A,B. 7 genes showed scattered or no CATATG sequence with any conservation restricted to primates. Two genes (MAP2K5 and FIGN) were slight variants. MAP2K5 was highly
conserved throughout and showed sporadic CATATGs that were conserved with no single, predominant site and no particular pattern to the species conservation (not shown). FIGN has a short intron 1 and has two highly conserved CATATGs in the second intron (not shown). In addition to the 16 putative targets, we also analyzed the following RAB family members for conserved CATATGs: RAB3A,B,C; RAB27A,B; RAB37; RAB9A,B. Many of those had no CATATG sites; none had sites conserved outside primates.

Establishment of MIST1-eGFP and eGFP stable lines

1 x 10^6 HGC-27 cells were transfected with 3 μg MIST1-eGFP cDNA or 3 μg eGFP cDNA using the transfection conditions described above. After 24 hours, transfectants were replated at limiting dilution under selection with 500 ng/μl Geneticin (Gibco-Invitrogen). Wells that grew out over 14-21 days were analyzed by flow cytometry for level and homogeneity of GFP signal.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed after Im and coworkers (22). ~10^8 HGC cells that stably express MIST1GFP fusion proteins were harvested for one ChIP experiment. 15 μl of MIST1 antiserum (rabbit polyclonal anti-human MIST1) or serum from the rabbit prior to immunization (“pre-immune” control) together with protein A/G plus agarose (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the cell lysate for immunoprecipitation. Regular PCR and quantitative-real-time PCR were performed (these and all other primer sequences available upon request) to assess quantity of genomic sequences immunoprecipitated by either pre-immune or MIST1 antiserum, as well as a 1:10 dilution of the cell extract prior to immunoprecipitation.
**RAB inhibitor studies**

3-PEHPC, an analog of the bisphosphonate drug risedronate, was generated as previously described (10). 3-PEHPC was stored in a stock solution of 106 mM at 4°C in aqueous solution at pH 6.3. For cell culture experiments, 3-PEHPC was added directly to the appropriate dilution in tissue culture media. Multiple concentrations were tested up to 5mM, and effectiveness was determined by dispersion of transfected eGFP-RAB26 plasmid (Jin et al., unpublished observations). Based on these experiments, a final concentration of 5mM was used in all experiments described in this manuscript.

**In situ hybridization**

*Rab26* cDNA was obtained from OpenBiosystems (MMM1013-98478848). *Rab3d* cDNA was obtained via amplification from a mouse stomach cDNA library and cloned into T-easy vector (Promega, Madison, WI). DIG-labeled RNA probes were synthesized via *in vitro* transcription following plasmid linearization (Roche Applied Sciences, Indianapolis, IN; 11277073910). The anti-sense RNA probe was synthesized with T7 RNA polymerase, the sense using Sp6 RNA polymerase. Adult B6 strain mice were perfused with 0.9% NaCl and 4%PFA sequentially, the stomachs was removed and further fixed in 4% PFA at 4°C for 8 h or more. Tissue was blocked, frozen, and sectioned as previously described (50). Sections (20 μm) were dipped in water several times to remove OTC, fixed in 4% PFA, treated with Proteinase K, refixed in 4% PFA and then treated with 0.1 M triethanolamine before incubating in hybridization buffer (50% Formamide, 5x SSC, 1x Denhart’s, 250 μg/ml yeast tRNA, 500 μg/ml Salmon Sperm DNA, 100ug/ml Heparin, 5mM EDTA and 0.1% Tween-20) for 1-2h at 65°C. The tissue sections were
incubated with DIG-labeled RNA probes in hybridization buffer overnight at 65°C. After RNA hybridization, slides were washed in 2x SSC at 65°C for 15 min and then at room temperature for 5 min, followed by Rnase (1µg/ml in 2x SSC) incubation at 37°C for 30 min and washes with 2x SSC (RT) for 5 min twice and 0.2x SSC (60°C) for 30 min twice. DIG-RNA hybridized to tissue sections was visualized by incubating the slides first in blocking solution (1% BSA in PBS) for 1h and with anti-digoxigenin antibody (Roche; 1:2000) at 4°C overnight, followed by washing in PBT (PBS, 0.1% Tween-20), equilibrating in alkaline phosphatase (AP) staining buffer (100 mM Tris, pH 9.5; 50 mM MgCl₂; 100 mM NaCl and 0.1% tween-20) and color development with NBT/BCIP (Roche) until a strong signal was detected in the sections hybridized to anti-sense probes; specificity was confirmed by lack of signal in sense-hybridized sections developed in the same experiments for the same length of time. Reactions were stopped by PBT, and sections were mounted in 1:1 glycerol:PBS.

**Graphing and statistics**

All graphs and statistics were performed in GraphPad Prism, except the Venn diagram which was generated using BioVenn(21) and then traced using Adobe Illustrator. Statistical analysis was, in the case of simple control vs experimental comparison by student’s t test. Otherwise, significances determined by one-way ANOVA test with Dunnett’s Multiple Comparison correction.

**Laser-capture microdissection (LCM) and qRT-PCR**

Preparation of stomach frozen sections for LCM has been described previously (50). Briefly, stomachs were excised immediately following sacrifice, quickly flushed with RT PBS, inflated
by duodenal injection of O.C.T. (Sakura Finetek, Torrance, CA), frozen in Cytocool II (Richard-Allen Scientific, Kalamazoo, MI) and cut into serial 7 µm-thick cryosections which were mounted on Superfrost slides (Fisher Scientific), fixed in 70% ETOH, rehydrated in nuclease-free water (nuclease-free solutions from Ambion, Austin, TX) and then incubated in Alexa Fluor 488 conjugated Griffonia simplicifolia-II (GS-II) (diluted 1:500 in nuclease-free water) for 15 mins. Sections were washed in nuclease-free water and dehydrated in graded ethanol, followed by xylene. ZCs were identified as corpus cells that were basal to GS-II labeling and which did not show the dark silhouettes and characteristic shape of parietal cells following xylene dehydration. Four wildtype and 5 Mist1−/− of mice were used for dissection (PixCell II LCM apparatus, 7.5 µm spot diameter; CapSure HS LCM caps, Arcturus, Mountain View, CA) to generate two caps per mouse. RNA was purified by PicoPure kit (Arcturus), and RNA integrity was confirmed by Agilent 2100 Bioanalyzer (Palo Alto, CA). QRT-PCR was only performed on RNA that had sharp 18s and 28s bands. All RNA from each cap was treated with DNAse I (Invitrogen) and then reverse transcribed using the SuperScript III (Invitrogen) standard protocol (most cDNA syntheses started with 10 ng of total RNA). Measurements of cDNA levels were performed by qRT-PCR using a Stratagene (La Jolla, CA) MX3000P Detection System, and Absolute QPCR SYBR Green Mix (Thermo Scientific) fluorescence was used to quantify relative amplicon amount of mouse RAB3D, RAB26, CCPG1, and 18S. Data analysis to allow expression of qRT-PCR data in terms of cycles above background, normalized to 18s amplicon intensity, was performed as described in detail (5).

QRT-PCR from stable Mist1-expressing and eGFP-control HGC cells was performed after RNA isolation using TRIzol-solubilized tissue extract (Invitrogen) following the manufacturer's protocol, quantification using the Beckman Coulter DU 640 Spectrophotometer.
(Fullerton, CA), and cDNA synthesis as described (5) (1 µg of starting total RNA was used). Measurements of cDNA levels for Rab3D, Rab26 and 18S were performed and analyzed as described above.

Plasmid preparation

phMist1-EGFP-C1 expression plasmid was constructed with the coding region of human MIST1 cDNA (Open Biosystems, Huntsville, AL, IMAGE ID: 8322448) followed by a 30 aa peptide linker added in-frame to the amino terminus of EGFP in pEGFP-C1 (Clontech Laboratories, Inc., Mountain View, CA) by the restriction site-free PCR method of ribocloning ((1), http://barnes1.wustl.edu/~wayne/riboclon.htm) using Klentaq-LA enzyme (2), a kind gift of Wayne Barnes, Washington University, and accurate PCR conditions. The riboprimers used did not match the vector or target products so they were first elongated on longer bandaid primer templates. The vector region was amplified by PCR using primers DNA3as and pEGFP-DNA3sb with V5s and EGFP-V5asb on pEGFP-C1 DNA. The hMist1 target was PCR amplified using DNA3s and hMist-DNA3b with V5as after sequential elongations on bandais hMist-V5b3, hMist-V5b2, and hMist-V5b1. The construct coding region pMT6-5 (5416 bp) was verified correct by DNA sequencing.

The ‘pcDNA3.1 PGCRFP’ fusion construct was generated using a modified sequence overlap extension PCR strategy. The PGC fragment was amplified by PCR from a ‘pcDNA3.1PGC’ plasmid (a kind gift of Dr. Susan Guttentag, University of Pennsylvania; (16)) with T7 primer (forward) and a reverse primer that contained both the PGC 3’end and RFP 5’ end coding sequence separated by a small linker sequence. The RFP sequence was amplified by PCR from a ‘pCS2 Notch-RFP’ plasmid (kind gift of Dr. Rafi Kopan, Washington University)
and a forward primer (complementary to the reverse primer used to amplify the PGC fragment) and a reverse primer. The PGC and RFP fragments were then purified and mixed together with T7 and RFP reverse primer to amplify the PGC-RFP fusion fragment. This PCR fragment was then digested with EcoRI and XhoI and cloned into pcDNA3.1 vector that was linearized with the same pair of restriction enzymes.

pEGFP- hMist1-C1 (designed by a similar method to phMist1-EGFP-C1) was monomerized to pmEGFP-hMist1-C1 by site-directed mutagenesis using reverse and forward primers (58) to convert leucine 222 to lysine and introduce a new restriction site, AflIII. meGFP-hRab26 expression plasmid was constructed with the coding region of hRab26 cDNA (Open Biosystems, IMAGE ID: 5262795) added in-frame to the carboxyl terminus of EGFP replacing the hMist1 coding region in pEGFP-hMist1-C1 by the restriction site-free PCR method of ribocloning, (1), http://barnes1.wustl.edu/~wayne/riboclon.htm) using Klentaq-LA enzyme (2) and accurate PCR conditions as above. The vector region was PCRed using primers DNA3as and pcD3LKs on pmEGFP-hMist1-C1 DNA. The hRab26 target was PCR amplified using DNA3s and hRab26-DNA3b with pcD3LKas and hRab26-pcD3LKs. The construct coding region (5583 bp) was verified correct by DNA sequencing.

meGFP-hRab26 was mutagenized to meGFP-hRab26T77N by site-directed mutagenesis to convert threonine at amino acid position 77 to an asparagine and introducing a new BbsI restriction site. The construct coding region was verified correct by DNA sequencing.
Results

Identifying MIST1 direct targets that might regulate secretory cell granule formation and establishment of apical-basal polarity

During maturation, ZCs undergo three dramatic changes in cell biology; they: 1) greatly upregulate their rER network, 2) form large exocrine secretory granules; 3) expand their apical cytoplasm, with their nuclei moving basally. Mist1 expression begins as zymogenic cells (ZCs) start to differentiate from their mucous neck cell precursors (50); it is involved in both formation/maintenance of the secretory granules and in the apical-basal reorganization. In the absence of Mist1, ZCs exhibit markedly smaller zymogen-containing, secretory vesicles relative to wildtype (Fig. 1A,C,E). Vesicle size, as measured on tEM from multiple ZCs in multiple gastric units, decreases from a mean of 0.40±0.03 μm² (wildtype) to 0.14±0.02 μm² (Mist1−/− mice; p<0.001; Fig. 1B,C). The defects in apical-basal organization are illustrated in Fig. 1B,D. The ratio of supranuclear to infranuclear secretory vesicle fluorescence decreases from an apical predominance of 4.2±0.7:1 to essentially equal distribution of apical and basal granules (ratio of 1.49±0.06:1) in Mist1−/− ZCs (Fig. 1D). The changes reflect inappropriate basolateral trafficking of granules and/or overly apical positioning of the nucleus. Mist1 loss of function defects are summarized in the diagram in Fig. 1E. Loss of Mist1 does not result in decreased rER(50).

To determine the genes that might be activated by Mist1 to regulate cellular structural changes, we transiently transfected either Mist1 or empty vector control plasmid into two human gastric cell lines (AGS and HGC-27). eGFP plasmid was cotransfected under both conditions, and high GFP-expressing (i.e., highly transfected cells) were isolated by fluorescence activated cell sorting (FACS). Global gene expression changes specific to Mist1 transfection were
assayed by Affymetrix HGU133_Plus_2 GeneChips. A total of 16 genes exhibited increased expression (90% confidence interval ≥1.2-fold change, intensity difference ≥100) in both MIST1-transfected cell lines relative to respective parental and empty vector-transfected control populations (Fig. 2 and Table 1).

*MIST1 directly associates with conserved CATATG E-boxes in 6 MIST1–induced genes*

To determine which MIST1-induced genes were likely direct targets of MIST1, we first examined the entire genomic region upstream to the transcription start sites and the entire first intron of each gene for conserved E-box sequences of the CATATG type, shown previously to be the binding site for both mammalian MIST1 (62) and DIMMED, its fly ortholog (19, 45, 46). *ARRDC3, CCPG1, FNDC3A, and SERPINI1* all contain remarkably conserved CATATG sequences within the first intron (Fig. 3A). The *RAB3D* gene has the canonical MIST1 binding site in a region between 167 and 205 bp downstream of the end of the first exon in multiple species (Fig. 3B). *RAB26* has a similarly located CATATG that is conserved in multiple mammalian genomes; however, the only CATATG in the 5’ upstream and intronic genomic DNA we examined is in a region 11.3 kb upstream of the *RAB26* transcription start (Chr16: 2127505), a location also conserved in chimpanzee (Fig. 3C). The remainder of the 16 MIST1-activated genes showed no conserved CATATG sites in the first intron.

To confirm direct MIST1 binding to the conserved CATATG sites, we performed chromatin immunoprecipitation (ChIP) on HGC-27 gastric cells stably expressing a MIST1-eGFP plasmid (henceforth, HGC-MIST1). Primers closely flanking the conserved CATATG sites shown in Fig. 3A,B,C generated amplicons in the MIST1-immunoprecipitated genomic DNA fragments with greater efficiency than from preimmune-precipitated DNA in all 6 genes (Fig. 4). In
contrast, primers to control regions without CATATG sequences generated little to no amplicon in the MIST1-immunoprecipitated DNA. We also examined non-conserved CATATG sites in CCPG1, SERPINI1, and FNDC3A (Fig. 4). Only the site in CCPG1 showed possible occupation by MIST1, though this site was within 5 kb of the conserved CATATG, and genomic DNA was fragmented to an average size of 1 kb in these experiments. Hence, we cannot rule out that a small population of longer genomic DNA fragments derived from precipitation of MIST1 at the conserved site might account for amplification at this region. Overall, the results indicate that MIST1 occupies specific transcription regulatory elements in ARRD3, CCPG1, FNDC3A, SERPINI1, RAB26, and RAB3D genes.

Rab3d and Rab26 are ZC-specific and Mist1-dependent in vivo

Of the 6 MIST1-regulated genes, two, RAB3D and RAB26, have been previously proposed to play a role in regulated secretion (15). Thus, we reasoned that these two RABs might be the cellular effectors that MIST1 induces to govern maintenance and formation of large secretory granules in exocrine secretory cells. We examined other related RAB genes purported to be involved in regulated exocytosis (RAB3A,B,C; RAB27A,B; and RAB37) and did not find similarly conserved CATATG sequences (not shown), nor was expression of these other RAB family members affected by forced MIST1 expression (Fig. 2B). Thus, RAB3D and RAB26 appear to be specific and direct targets of MIST1 transcriptional regulation.

If RAB3D and RAB26 regulate secretory granule formation, they should be expressed in ZCs and be MIST1-dependent in vivo. In situ hybridization showed that RAB26 and RAB3D expression in mouse stomach was confined to MIST1–expressing ZCs in wildtype mice (Fig. 5A,B). To determine whether either Rab depended on MIST1 expression, we laser-capture
microdissected ZCs from the bases of Mist1−/− mice and their wildtype littermates. In Mist1−/− mice, RAB26 message was barely detectable above background signal (1.9 cycles above water; n=3 independent mice and dissections), whereas it was expressed at 11-fold higher levels in wildtype mice (p<0.01; Fig 5C). Mist1−/− mice also showed a significant decrease (8-fold, p<0.001) in RAB3D expression (Fig 5C). Hence Rab26 and Rab3d are transcriptionally regulated by MIST1 in vivo as well.

Expression of pepsinogen-RFP in mist1-expressing stable lines causes formation of large exocrine secretory granules

Our results so far showed that: 1) loss of MIST1 leads to small secretory granules and defects in the apical compartment where those granules mature; 2) expression of two putative regulators of exocrine granule homeostasis, RAB3D and RAB26, is directly regulated in vitro by MIST1; and 3) RAB3D and RAB26 expression is MIST1-regulated in vivo specifically in Mist1 expressing cells. Thus, the data indicate that MIST1 regulates vesicle maturation via direct transcriptional activation of RAB3D and RAB26. To functionally test that hypothesis, we needed an in vitro system where the role of RABs in large secretory vesicle dynamics could be assessed mechanistically. We designed a construct comprising one of the principal constituents of ZC secretory granules, pepsinogen C (PGC) fused at the carboxyl terminus to red fluorescent protein (RFP). Fig. 6 shows that stable HGC-MIST1 cells transiently transfected with this construct developed multiple large PGC-containing granules. The results of multiple (n=8) separate transfections showed, highly reproducibly, that 23.0±1.2% of the cells with RFP fluorescence detectable above background had ≥3 vesicles of ≥1 μm in size. In control HGC cultures stably expressing eGFP alone (without MIST1), cells with these large secretory granules were rarer.
(7.5±1.5%, \(p<0.001\) by one-tailed t-test between \text{MIST1} and control), whereas cells with bright diffuse PGC-RFP were far more common (4.3±0.2% \text{MIST1} vs. 11.2±0.6% control, \(p<0.001\); Fig. 6A,B).

When examined on their tissue culture substrate using transmission EM, both \text{MIST1}-expressing and control cells with PGC-RFP contained multiple secretory vesicles (Fig. 6C), whereas non-transfected cells did not (not shown). However \text{HGC-MIST1} cells formed vesicles that tended to cluster together and were each over twice as large as the vesicles found in control cells (0.70±0.12 \(\mu m^2\) vs. 0.34±0.11 \(\mu m^2\), \(p<1x10^6\); Fig. 6C-E). Fig. 6D demonstrates how vesicles could be seen undergoing exocytosis. We observed no consistent difference in cell proliferation, apoptosis, or cell morphology between \text{MIST1}-expressing cell lines and controls (not shown). Finally, we treated cultures of \text{MIST1}-expressing, PGC-RFP transfected cells with the secretagogue, carbachol (100 \(\mu m\)), which greatly increased formation and trafficking of large PGC-RFP granules (not shown), indicating that these cell lines can respond to a physiologic, muscarinic agonist that induces release of zymogen-containing granules in ZCs \textit{in vivo}.

\textit{Large exocrine granule formation requires RAB prenylation and RAB26 activity}

Using qRT-PCR, we confirmed that cells stably expressing \text{MIST1}, as expected, constitutively expressed higher levels of \text{RAB} transcripts. \text{RAB26} was constitutively expressed at 4.1±0.9 and \text{RAB3D} at 15.0±8.2- fold higher levels (\(n=6\) independent experiments; \(p<0.001\) and \(<0.005\)) in \text{HGC-MIST1} cells relative to those stably expressing a control eGFP vector alone (Fig. 7A).

We next sought to ascertain whether formation of large PGC-RFP granules required the \text{RAB} expression induced by \text{MIST1}. We transfected \text{HGC-MIST1} cells with PGC-RFP in the
presence of 5mM 3-PEHPC (3, 10), an inhibitor of RAB geranylgeranyl transferase (RGGT), the enzyme that catalyzes the addition of geranylgeranyl groups to the carboxy terminal di-cysteine (CC or CXC) motifs in \textit{RAB3D} and \textit{RAB26} (55). We used the minimal concentration of 3-PEHPC that completely dispersed the normal cellular localization of an eGFP-RAB26 construct. Once RABs lose the ability to localize to their defined cellular compartments, they no longer function. As hypothesized, inhibition of RAB function led to loss of the large PGC-RFP granules (Fig. 7B,C). Cells with large granules decreased ~4-fold in the presence of inhibitor (p<0.001), equivalent to levels in the non-MIST1-expressing control cells (Fig 7E).

The cellular localization and expression of RAB3D in exocrine secretion has been relatively well studied (38, 66). RAB3D is important for secretory-granule homeostasis in diverse secretory cells. Although there is some controversy about its primary function (51, 63), it is generally thought that RAB3D regulates release of mature secretory granules, likely by tethering them to elements of the cortical actomyosin mesh (7, 37). Given that RAB3D already had an established role in exocrine granule homeostasis, we decided to further examine the role of RAB26 in this process.

To determine if more targeted disruption of RAB26 function could inhibit large zymogenic vesicle formation, we designed a point mutant RAB26 (eGFP-RAB26T77N) based on close conservation of the RAB26 GTP binding region with the analogous RAB3D sequence (RAB3DT36N), which acts as a dominant negative when the equivalent threonine is mutated to asparagine in the G2 box region responsible for coordinating Mg\textsuperscript{2+} binding (7); for structure of RAB26, see: (65)). As expected, eGFP-RAB26T77N did not localize to a specific membrane compartment like wildtype RAB26, which is perinuclear (Jin et al., unpublished observations).
Rather eGFP-RAB26T77N distributed throughout the cytoplasm in a pattern similar to that of wildtype RAB26 in the presence of 3-PEHPC (not shown).

Transfection of the dominant negative construct inhibited large granule formation in HGC-MIST1 cells cotransfected with PGC-RFP (Fig. 7D). Overall, the percentage of cells expressing large granules (n=3 independent experiments) decreased 28±5% (p<0.01; Fig. 7E). When the effects of transfection of the dominant negative construct on the large, PGC-RFP granular phenotype were correlated on a cell-by-cell basis, almost none of the cells with large zymogenic granules were seen to express cytoplasmic eGFP-RAB26T77N (Fig. 7F, H). Conversely, among those cells transfected with eGFP-RAB26T77N above background, almost none showed the large vesicular phenotype (Fig. 7F), confirming that expression of eGFP-RAB26T77N prevented formation of the large PGC-RFP vesicles. In a control experiment, we transfected a plasmid with eGFP alone in lieu of eGFP-RAB26T77N (Fig. 7G, H). Expression of eGFP alone had no effect on formation of large PGC-RFP granules, indicating that the effects of eGFP-RAB26T77N were specific to its function as a dominant negative RAB. Together, the data demonstrate that RAB26 function is required for the formation of the large PGC granules.

A previous study showed that RAB3D was not sufficient to rescue granulogenesis in Mist1−/− cells(26). We tested whether RAB26 was sufficient to induce large granules in control HGC cells, stably transfected with eGFP and not MIST1. Transfection of eGFP-RAB26 in these cells in three independent experiments led to a higher fraction of cells with large PGC granules (from 7.5±2.3% to 10.9±0.4%; not shown) that approached a statistically significant increase (p<0.06 by one-tailed T-test) but did not approach the level seen in HGC-MIST1 cells. Thus, although required for maturation of granules, RAB26, like RAB3D, does not seem to be sufficient to rescue the loss of MIST1 function phenotype in the absence of other MIST1 targets.
Discussion

Here, we demonstrate the role of MIST1 in establishing the characteristic secretory phenotype of zymogen exocrine cells through direct transcriptional activation of RAB3D and RAB26. These two RABS were the only ones increased following MIST1 transfection and the only RABS of those thought to be associated with secretory granule homeostasis that had conserved MIST1-binding E-box elements. Furthermore, ChIP confirmed that MIST1 directly associated with RAB3D and RAB26 E-boxes. We next showed that RAB3D and RAB26 expression was specific to MIST1–expressing cells in vivo and was also Mist1-dependent. Finally, we established an in vitro system to test the functional importance of RAB3D and RAB26. Cells stably expressing MIST1, as expected, have constitutively increased levels of both RAB3D and RAB26. When transfected with an RFP-tagged zymogenic granule component PGC, these cells, but not controls, formed large zymogenic secretory granules. Large granule formation required increased RAB activity, because inhibition of RAB prenylation resulted in cells with smaller granules, indistinguishable from those of non MIST1-expressing control cells. Previous studies have shown that RAB3D participates in exocrine granule homeostasis but is not itself sufficient to induce large granule formation in MIST1-expressing cells (26, 38, 42, 51, 66). Here we show that forced expression of a RAB26 dominant negative construct completely abrogates large vesicle formation, yet transfection of RAB26 alone does not substantially rescue the mist1 loss of function granule phenotype. Thus, like RAB3D, RAB26 is required but not sufficient for exocrine granulogenesis.

MIST1 is expressed by a limited number of specialized secretory cells in diverse tissues. Loss of Mist1, in all reported cases, does not lead to a decrease in steady-state survival or formation of these cell lineages, nor does it lead to a change in their cell fate. The fundamental feature of loss of Mist1 function is that the cells that would express Mist1 are still generated,
occupy their same *in vivo* niches, and secrete the same substances (32, 50, 73). However, loss of *MIST1* has profound effects on cell shape/architecture and cell function. With loss of *MIST1*, zymogen-secreting cells in stomach, pancreas, and salivary gland don't achieve their full size, and their zymogenic granules become smaller as well. Pancreatic acinar cells grown *in vitro* show smaller zymogen stores, altered Ca\(^{2+}\) currents, and loss of normal apical, perinuclear Golgi localization (34).

Mist1 protein is highly conserved across species. The *Drosophila* ortholog of *Mist1*, *DIMMED*, shares 78% identity through the bHLH region and binds the same CATATG E-box sequence in its known target gene (46, 62). Conservation between the two genes in these distantly related organisms is such that expression of *MIST1* can induce a *DIMMED* target gene *in vitro* (45). The zebrafish *Mist1* orthologue shares 77% identity with mouse *MIST1* in the bHLH domain (18). Loss of zebrafish *Mist1* leads to morphologic abnormalities in pancreatic, enzyme-secreting cells (18). Gain of function experiments show that *DIMMED* is sufficient to orchestrate increased secretory vesicle size (19, 20, 45) analogous to *MIST1* gain of function in the current manuscript. Thus, *MIST1* is a developmentally regulated TF whose expression is induced during terminal differentiation of selected secretory cells in diverse tissues with highly evolutionarily conserved function.

Which genes might a single TF activate to effect complete remodeling of cellular architecture? Two previously reported targets of *MIST1* *in vitro* in pancreatic cells are the gap junction gene *Connexin32 (GJB1)* (52) and the cell cycle regulating *p21(CIP1/WAF1; Cdkn1a)(24)*. We do not detect *GJB1* expression in the gastric cell lines used in this manuscript by GeneChip or qRT-PCR (Tian et al., unpublished observations), so we do not think this is a *MIST1* target in our system. *CDKN1* is expressed in ZCs *in vivo* and in gastric cell lines, but
expression is not MIST1-dependent in cell lines. Furthermore, *in vivo*, expression of CDKN1 is lower in ZCs than in other MIST1-negative gastric epithelial cells (Tian et al., unpublished observations), so this also seems an unlikely target to mediate the morphological changes induced by MIST1.

It is possible that MIST1 activates both tissue-specific and universal sets of genes. In this work, we identified 16 genes whose expression is activated by MIST1 in two different gastric cell lines. The majority of these genes are thought to function in secretion or membrane trafficking, reinforcing our understanding of MIST1 as a TF that enables terminally differentiating cells to upregulate cellular effectors that establish a high capacity secretory architecture. In addition to RAB3D and RAB26, the subjects of the current manuscript, four other genes, including CCPG1 were also direct targets of MIST1 as indicated by our chromatin immunoprecipitation studies (Fig. 4). CCPG1 did not appear to be regulated by MIST1 in gastric ZCs (Figure 5C, and data not shown); however, CCPG1 is a large gene with multiple splice variants, and we have assayed only one variant so far. We are currently analyzing the 10 genes without conserved CATATGs to determine whether any of them bind MIST1 in a non-canonical fashion.

Are the increases in granule size and trafficking sufficient in themselves to effect the apical-basal cell shape reorganization also induced by MIST1, or are other MIST1 targets involved? We already know from previous studies that the cytoskeletal adaptor, CD2AP, is critical in the absence of MIST1 for maintaining cell shape, specifically cell height and ordered cell-cell adhesion (Fig. 8), though CD2AP does not appear to be a target of MIST1(5). The experiments do suggest, in any case, a role for other genes besides just Rab26 and Rab3d in the cell shape changes MIST1 induces.
RAB26 clusters by sequence homology with the RAB37, RAB27 and RAB3 subfamilies, which are critical for regulated secretion. However, whereas RAB27 and RAB3 family members (such as RAB3D) are relatively ubiquitously expressed in specialized secretory cells in numerous tissues, expression of RAB26 appears to be more limited, with highest previously reported expression in pancreatic and submandibular enzyme-secreting acinar cells, both of which express MIST1 (40, 64, 68). A functional genomic screen also identified RAB26 as a normal gastric gene whose expression is downregulated in human cancer (67). The only previous functional study of RAB26 showed that in salivary gland zymogen-secreting cells, RAB26 was associated with secretory granules (by cell fractionation and western blot), and injection of RAB26 antibodies inhibited exocytosis, consistent with our findings (40).

It is currently unclear what mechanisms RAB3D and RAB26 use to induce formation of larger secretory granules. Our current results and those of previous studies indicate that both RAB26 and RAB3D are required but not individually sufficient for the formation of large secretory granules. The simplest model would be that MIST1 mediated vesicle maturation and trafficking is dependent on a balance between RAB26 and RAB3D activity (Fig. 8), though we cannot currently rule out a role for other direct targets. In a series of ongoing experiments (Jin et al., unpublished observations), we have learned that RAB26 helps organize the apical cellular compartment where immature secretory granules form and mature. RAB3D has been implicated downstream in tethering vesicles to the apical cytoskeleton. Tethering vesicles would encourage vesicle enlargement, because it would delay vesicle secretion and facilitate fusion of smaller, immature vesicles. Thus, RAB3D and RAB26 might coordinate sequential membrane compartments, RAB26 encouraging homotypic fusion of immature granules and/or larger budding from the trans-Golgi and RAB3D tethering maturing granules to the cortical cytoskeleton to await a signal for granule
fusion with the plasma membrane. Together, these two RABs may represent the key cellular effectors that carry out the developmental signal encoded by MIST1.

Prospectus

The process of cellular differentiation is typically understood as a sequence of transcription factor inductions, each of which specifies the next step in the developmental program of a given cell lineage. Any given terminally differentiated cell is thus ultimately specified by its unique history of sequential transcription factor induction steps. That view explains well how each differentiated cell in an organism ultimately comes to produce the unique set of proteins that distinguish it from all other cells. But what induces the genes that high-capacity, secretory cells all share, no matter what they actually secrete—the genes that lay down the secretory architecture? Given the parsimony of evolution, it would make sense if these secretory cassettes were induced by a limited number of tissue-independent but still developmentally regulated transcription factors. Indeed, our recent survey of gene expression across multiple adult tissues indicates this is so (13). In the current paper, we show how MIST1, a transcription factor critical for enacting cellular architecture, activates RAB26 and RAB3D, which in turn organize vesicle trafficking to maximize apical production and maintenance of large secretory granules. The mechanism we describe here will undoubtedly be only part of a much larger story, as we continue to dissect the mechanisms of transcriptional regulation of cell architecture change.
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Abbreviations Used: TF – Transcription Factor; ZC – Zymogenic Cell; PGC – Pepsinogen C; EM – electron microscopy; eGFP – enhanced green fluorescent protein; RFP – red fluorescent protein, LCM – laser-capture microdissection; rER – rough endoplasmic reticulum; tEM – transmission electron microscopy; qRT-PCR – quantitative real time polymerase chain reaction; RGGT - Rab geranylgeranyl transferase
References


**Figure Legends**

**Figure 1.** Mist1 is required for development and maintenance of large zymogenic secretory granules and apical-basal orientation. *A*, tEMs of ZCs from wildtype and Mist1−/− mice. Note central position of nucleus along with reduced size of mature apical secretory vesicles in the Mist1−/− ZC. "SV", mature secretory vesicles “N”, nucleus. *B*, Epifluorescence of wildtype and Mist1−/− ZCs in the base of a gastric unit (oriented with stomach lumen to left, base to right). Cell and granule orientation observed by immunolabeling with anti-gastric intrinsic factor (GIF, red) labeling granules, anti-β-actin (green) labeling mesenchymal cells at base of cells and gastric unit lumen, anti-E-cadherin (purple) labeling basolateral cell-cell borders, and bisbenzimide (blue) labeling nuclei. Dashed white line: basement membrane. **Right-** cartoons of fluorescently labeled cells. Note that Mist1−/− ZCs have nuclei closer to the lumen with fewer, more basally localized secretory granules. *C*, Secretory vesicle areas from multiple ZCs were quantified from tEM photomicrographs ("**" – p<0.01; "***" – p <0.001, for all figures). *D*, Multiple ZCs from multiple mice were quantified for apical (luminal to the nucleus) and basal (basal to nucleus) secretory granule immunofluorescence in immunolabeling experiments like those in panel B. Each point is the ratio of apical to basal mean fluorescent intensity determined by anti-pepsinogen C (PGC, a principal component of zymogenic granules) in an individual ZC. Note that wildtype ZCs have predominately apical distribution of granules, whereas apical and basal granule distribution is nearly equal in Mist1−/− ZCs. *E*, Cartoon showing ZCs arising from mucous neck cell progenitors in the neck of the gastric unit. In the absence of Mist1, ZCs migrating into the base exhibit apical-basal organization and granule formation/maintenance deficits (panels C,E adapted from ref (50)).
Figure 2. Transient transfection with MIST1 in AGS and HGC-27 gastric cell lines increases expression of a common cohort of genes. A, Venn diagram showing the number of Affymetrix HGU133_Plus_2 GeneChips probe sets increased in two different gastric epithelial cell lines (HGC-27 and AGS) following transient transfection with MIST1. B, The expression intensities on the GeneChips for the 18 probe sets (16 genes) that were increased in both cell lines relative to vector-transfected and untransfected controls.

Figure 3. Several genes activated by MIST1 overexpression have evolutionarily conserved, first intronic canonical MIST1 E-box sequences. A, All MIST1-type E-boxes (CATATG) were identified in the first introns and 5’ to transcription start for the 16 human genes from Fig. 2 (see methods). Evolutionary conservation was then determined relative to the other vertebrate species listed in legend (species identified by 2 letter abbreviations of species names; eg, ‘Gg’ = Gallus gallus). Genes are schematized to scale with numbered exons in blue and region of gene analyzed in bold line. Note dramatic conservation of CATATGs located within 1 kb of end of the first exon in these 4 genes (red brackets). Primers to each of these conserved intronic sequences were analyzed for MIST1 binding in Fig. 4; control primers were generated to assess MIST1 binding to regions with no CATATG (amplicons indicated by arrows with boxed “C”s). In some cases, MIST1 binding to regions with non-conserved CATATGs (denoted by arrows with boxed “M”s) was also assessed. B,C Schematic as in panel A for the two RAB genes induced by MIST1 transfection but with detailed sequence alignment at each CATATG. “*”-depicted consensus sequence runs on antiparallel strand for the given species. Note that the conserved intronic CATATG in RAB26 has a single point mutation in humans and chimpanzees destroying the E-
box. The genomes of those species have another MIST1 binding site ~11.5 kb upstream of the transcription start; bovine genomes have both intronic and 5’ sites.

**Figure 4.** Conserved MIST1 E-box sequences are directly bound by MIST1. Gel electrophoresis images of PCR amplicons representative of ≥3 independent chromatin immunoprecipitation (ChIP) experiments using anti-MIST1 antibody followed by primers either to the conserved intronic CATATG (“Mist1 site conserved”) or to non-conserved CATATG and control regions denoted in Fig. 3. "Input 1:10" – genomic DNA prior to immunoprecipitation diluted 1:10; "Preimmune" – immunoprecipitation from non-specific rabbit antiserum. All images taken from PCR reactions run for 35 cycles, though each reaction was sampled at multiple cycles to ensure amplification had not plateaued. Note conserved CATATG sequences show much stronger amplification of MIST1-bound DNA than pre-immune-bound DNA, and no MIST1-bound DNA was detectable at control sites without CATATGs. Non-conserved CATATG sites showed little to no amplifiable MIST1-bound DNA.

**Figure 5.** Rab3d and Rab26 expression are zymogenic cell-specific and MIST1-dependent in vivo. A,B In situ hybridization of mouse fundic gastric gland sections showing antisense RAB3D and RAB26 signal confined to the ZCs at the base of the glands. Note the absence of signal in parietal cells (arrowheads in the higher magnification view below each main panel). C, Quantitative RT-PCR of LCM-dissected ZCs from Mist1−/− mice. RAB3D and RAB26 expression are significantly decreased in these mice. Note y-axis expressed as Log₂ relative to levels in wells containing no cDNA following 18s normalization; hence, 0=essentially no detectable levels of given amplicon; RAB26 expression in the Mist1−/− mice is nearly completely abrogated. Primers to another
potential MIST1 target, CCPG1, indicate no significant change in expression, indicating loss of MIST1 specifically affects levels of the two RABs.

Figure 6. HGC-MIST1 cells form large exocrine granules upon PGC-RFP transfection. A, Fluorescent microscopic image of HGC-27 cells stably expressing MIST1-eGFP (green nuclear staining) showing large PGC vesicles (arrowheads) that do not form in control cells (stable expressing eGFP alone) upon transfection with PGC-RFP. B, The fraction of cells with multiple (≥3), large (≥1 μm) vesicles and diffuse, bright vesicles is quantified across multiple experiments (note the remaining cells showed above-background RFP but were too dim to categorize). C, tEM of ZCs from HGC-MIST1 and control cells. Note the reduced size of secretory vesicles in the (control) HGC-27 cells. “SV” – secretory vesicle. D, tEM showing exocytosis of vesicle contents (arrowhead) in an HGC-MIST1 cell. E, Vesicle sizes were quantified from tEM of multiple HGC-MIST1 and control cells.

Figure 7. Large PGC-RFP vesicles in HGC-MIST1 cells are RAB function dependent. A, qRT-PCR from 6 separate cultures of stable, HGC-MIST1 cells showing constitutively increased expression of RAB26 and RAB3D in MIST1-expressing cells relative to cells stably expressing eGFP instead of MIST1-eGFP. B, Fluorescent microscopy showing PGC-RFP (red) vesicles after transient transfection in HGC-MIST1 cells. C, Dispersion of PGC-RFP vesicles in HGC-MIST1 cells treated with 5 mM of the RAB inhibitor 3-PEHPC. D, Dispersion of vesicles in HGC-MIST1 cells transfected with RAB26T77N, a dominant negative RAB26 construct. E, Effects of inhibition of RAB function on fraction of cells with large PGC vesicles is quantified. (p values by one-way ANOVA test with Dunnett’s Multiple Comparison correction). “eGFP Ctrl” denotes stable
eGFP-expressing control cells.  

**F,** The effects of dominant negative RAB26 transfection on PGC-RFP vesicle formation were assessed by quantifying green (RAB26T77N) fluorescence intensity in each cell, correlated with the distribution of red (PGC-RFP) fluorescence in the same cell. “Large vesicular” cells were those with multiple (≥3), large (≥1 μm) vesicles; small vesicular cells were those with substantial (see methods) red fluorescence that was not distributed in large vesicles. The large vesicular phenotype occurred almost exclusively in cells with limited to undetectable expression of dominant negative RAB26: only 5.16% of such cells had green mean fluorescence intensity (mfi) ≥300 in 16 bit images, following background subtraction (blue line). In contrast, 64.4% of cells effectively (≥300 mfi) co-transfected with RAB26T77N had the diffuse or “small vesicular” phenotype, indicating those cells did not form large secretory vesicles (red line).  

**G,** Transiently transfected eGFP has minimal effects on PGC-RFP vesicle formation, indicating inhibition of large PGC granules is specific to effects of RAB26, not cytoplasmic GFP.  

**H,** Graphical representation of mean green pixel intensity above background in HGC-MIST1 cells with either small (diffuse) or large PGC-RFP vesicular phenotype following transient transfection with meGFP-RAB26T77N or eGFP. Note that the large vesicular cells show little to no meGFP-RAB26T77N expression. There is no correlation between expression of eGFP alone and PGC vesicle phenotype.

**Figure 8.** Schematic illustrating how MIST1 expression is induced as mucous neck cells differentiate into zymogenic cells. MIST1 is modeled as acting primarily through RAB3D and RAB26 to regulate large zymogenic granules, though a role for other MIST1 targets in this process has not necessarily been ruled out. Other targets of MIST1 are probably involved in the apical-basal cell shape reorganization induced by MIST1. Based on an earlier study, the cytoskeletal
adaptor Cd2ap plays a role in this process but is not a target of \textit{MIST1} (5). Expansion of rER is not affected by loss of Mist1 expression.

\textbf{Table 1. Function of genes upregulated by MIST1 transfection}
Tian_Fig. 1

A

Wildtype

Mist1<sup>−/−</sup>

SV  Apical  Basal

N

2 microns

B

Wildtype

Mist1<sup>−/−</sup>

SV  Apical  Basal

N

5 μm

C

Vesicle size

Mean vesicle size/cell (μm<sup>2</sup>)

WT  Mist1<sup>−/−</sup>

D

Ratio of apical to basal granules

Normalized apical/basal PQC MFI

WT  Mist1<sup>−/−</sup>

E

Mucous Neck Cell

−MIST1

+MIST1

Small, zymogenic vesicles

Altered apical basal organization

Mature Zymogenic Cells
Tian_Fig. 4

**Mist1 site**
- (conserved)
- (non-conserved)

**CATATG site**
- Control site

- CCPG1
- SERPIN11
- RAB3D
- FNDC3A
- ARRDC3
- RAB26
Tian Fig. 6

A: Images showing MIST1 and Control conditions.
B: Bar graph showing percentage of cells with 1 μm vesicles and diffuse PGC.
C: Electron micrographs of MIST1 and Control conditions.
D: Additional electron micrograph showing MIST1 condition.
E: Bar graph showing mean vesicle area in MIST1 and Control conditions.
**Fig. 7**

**A** RAB3D & RAB25 mRNA expression in MIST1-stable lines

**B** MIST1-stable

**C** MIST1 & 3-PEHPC

**D** MIST1 & RAB26T77N

**E** Fraction of cells with small vesicular or large vesicular MIST1

**F** Number of cells with different RAB26T77N fluorescence intensities

**G** MIST1-eGFP, eGFP control, PGC-RFP

**H** Mean pixel intensity above background for vesicular categories

**Notes:**
- **Fig. 7** illustrates the expression and localization of RAB3D, RAB25, and MIST1 in different cell lines.
- **Fig. E** shows the fraction of cells with small or large vesicular MIST1.
- **Fig. F** presents the distribution of cells based on their RAB26T77N fluorescence intensity.
- **Fig. H** displays the mean pixel intensity for different vesicular categories.
Tian_Fig. 8

Mucous Neck Cell → RAB26 → Large protein-containing granules

Mucous Neck Cell → RAB3D → Cell shape changes

Mucous Neck Cell → CD2AP → Expansion of rER

Mucous Neck Cell → ?

Zymogenic Cell

140
Table 1. Function of genes upregulated by MIST1 transfection

<table>
<thead>
<tr>
<th>Gene</th>
<th>Potential Functions</th>
<th>Reference</th>
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<tr>
<td><strong>Genes that directly regulate vesicular formation and transport</strong></td>
<td></td>
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<tr>
<td>RAB3D</td>
<td>Regulates vesicular docking and fusion during regulated exocytosis</td>
<td>Millar et al. (38)</td>
</tr>
<tr>
<td>RAB26</td>
<td>Involved in regulated vesicular secretion</td>
<td>Yoshie et al., Wagner et al., Nashida et al. (40, 64, 68)</td>
</tr>
<tr>
<td><strong>Factors that are secreted or associate with specialized membrane compartments</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACP2 acid phosphatase 2, lysosomal</td>
<td>Regulates lysosomal functions. Its deficiency causes abnormal lysosomal storage and inclusions.</td>
<td>Saftig et al., Mannan et al. (53, 36)</td>
</tr>
<tr>
<td>SLC31A2 solute carrier family 31 (copper transporters), member 2</td>
<td>Cellular Copper uptake</td>
<td>Zhou and Gitschier (71)</td>
</tr>
<tr>
<td>SERPIN1 serpin peptidase inhibitor, clade I (neuroserpin), member 1</td>
<td>Secreted protein. Mutations associate with two families with FENIB, an autosomal dominant form of dementia.</td>
<td>Stoeckli et al., Davis et al. (11, 12)</td>
</tr>
<tr>
<td>DISP1 dispatched homolog 2</td>
<td>Multitransmembrane protein, hedgehog transporter</td>
<td>Ma et al. (35), Cohen et al. (8)</td>
</tr>
<tr>
<td><strong>Other genes that may potentially regulate vesicular transportation and cellular architecture</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCPG1 cell cycle progression gene 1</td>
<td>Novel scaffolding protein that regulates guanine nucleotide factor Dbs and promote CDC42 activation by Dbs</td>
<td>Kostenko et al. (31)</td>
</tr>
<tr>
<td>FNDC3A fibronectin type III domain containing 3A</td>
<td>Cell adhesion migration</td>
<td>Obholz et al. (41), Shan et al. (57)</td>
</tr>
<tr>
<td>AQP3 aquaporin 3</td>
<td>Water channel in many cell types</td>
<td>Inase et al. (23)</td>
</tr>
<tr>
<td>Gene/Description</td>
<td>Function</td>
<td>Reference(s)</td>
</tr>
<tr>
<td>---------------------------------------------------------------------------------</td>
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<tr>
<td><strong>ARRDC3</strong>&lt;br&gt;Arresting domain-containing 3</td>
<td>Associated with the plasma membrane, endosomes, and lysosomes during endocytosis; Plays a regulatory role in cell proliferation</td>
<td>Oka et al.(43)</td>
</tr>
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<td><strong>FIGN</strong>&lt;br&gt;Fidgetin</td>
<td>One of the “Meiotic” or subfamily-7 group of AAA proteins. AAA proteins are molecular chaperones involved in a variety of activities like proteolysis, peroxisome biogenesis, membrane fusion, endosome sorting, and meiotic spindle formation.</td>
<td>Patel and Latterich(47), Cox GA et al.(9)</td>
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<td><strong>Signaling molecules</strong></td>
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<tr>
<td><strong>TIFA</strong>&lt;br&gt;TRAF-interacting protein with forkhead-associated domain</td>
<td>Associates with TRAF and regulates tumor necrosis factor receptor signaling</td>
<td>Ea et al.(14), Kanamori et al.(27), Takatsuna et al.(61)</td>
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<td><strong>MAP2K5</strong>&lt;br&gt;Mitogen-activated protein kinase kinase 5</td>
<td>Interacts with MAPK7. MAP2K5/MAPK7 protein cascade is a novel signaling pathway</td>
<td>Zhou et al.(72)</td>
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<td><strong>Genes whose molecular function are unclear</strong></td>
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<tr>
<td><strong>BRP44L</strong>&lt;br&gt;Brain protein 44 like</td>
<td>Unknown</td>
<td>Jiang et al.(25)</td>
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<tr>
<td><strong>MGC13057</strong>&lt;br&gt;Chromosome 2 open reading frame 88</td>
<td>Unknown</td>
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<tr>
<td><strong>FAM63A</strong>&lt;br&gt;Family with sequence similarity 63, member A</td>
<td>Unknown</td>
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CHAPTER 4: RAB26 Coordinates Lysosome Traffic and Mitochondrial Localization

This chapter is adapted from the published work:

RAB26 Coordinates Lysosome Traffic and Mitochondrial Localization

Ramon U. Jin¹ and Jason C. Mills¹,²,³

Division of Gastroenterology, Departments of ¹Medicine, ²Developmental Biology, ³Pathology & Immunology, Washington University School of Medicine, St. Louis, MO 63110

Address correspondence to:

Jason C. Mills
Department of Medicine
Washington University School of Medicine, Box 8124
660 So. Euclid Ave.
St. Louis, MO 63110
Phone: 314 362-4258
FAX: 314 362-7487
e-mail jmills@wustl.edu

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Author Contributions

RUJ performed all experiments and drafted the manuscript. JCM edited the manuscript and provided funding. RUJ and JCM conceptualized, designed, and interpreted the experiments.
SUMMARY

As they mature, professional secretory cells like pancreatic acinar and gastric chief cells induce the transcription factor MIST1 (BHLHA15) to substantially scale up production of large secretory granules in a process that involves expansion of apical cytoplasm and redistribution of lysosomes and mitochondria. How a scaling factor like MIST1 rearranges cellular architecture simply by regulating expression levels of its transcriptional targets is unknown. RAB26 is a MIST1 target whose role in MIST1-mediated secretory cell maturation is also unknown. Here, we confirm that RAB26 expression, unlike most Rabs which are ubiquitously expressed, was tissue specific and largely confined to MIST1-expressing secretory tissues. Surprisingly, functional studies showed RAB26 predominantly associated with LAMP1/cathepsin D lysosomes and not directly with secretory granules. Moreover, increasing RAB26 expression – by inducing differentiation of zymogen-secreting cells or by direct transfection – caused lysosomes to coalesce in a central, perinuclear region. Lysosome clustering in turn caused redistribution of mitochondria into distinct subcellular neighborhoods. The data elucidate a novel function for RAB26 and suggest a mechanism for how cells could increase transcription of key effectors to reorganize subcellular compartments during differentiation.

INTRODUCTION

Rab proteins are the largest members of the Ras family of small protein GTPases with more than 70 members identified in humans (Schwartz et al., 2007). They are important regulators of intracellular membrane and vesicle positioning and trafficking. By cycling between GTP- and GDP-bound forms, they work like simple machines to define specific membrane-associated compartments within cells. Many Rabs - like RAB4, RAB5, RAB7, and RAB11 - are
ubiquitously expressed (Gurkan et al., 2005; Jin et al., 2012; Zhang et al., 2007) performing essentially the same function in diverse cells. RAB7, for example, aids the maturation of early endosomes into late endosomes and ultimately coordinates transport to lysosomes (Bucci et al., 2000; Feng et al., 1995).

In contrast, expression of some Rabs is largely confined to cells that perform specific physiological functions. Among the best studied are the RAB27 and RAB3 families which are expressed highly in specialized secretory cells. RAB27A and B function in the transport and docking of mature vesicles destined for secretion (Fukuda, 2013) in melanocytes (Hume et al., 2001) and T lymphocytes (Menasche et al., 2000). The RAB3 family includes RAB3A, B, C, and D; the first three isoforms are predominantly neuronal, involved in neurotransmitter and neuroendocrine hormone release (Geppert et al., 1994; Johannes et al., 1994; Schluter et al., 2004; Weber et al., 1994), while RAB3D is expressed in a variety of extraneuronal secretory cells, in particular exocrine serous cells in the pancreas, salivary glands, and stomach (Chen et al., 2002; Millar et al., 2002; Ohnishi et al., 1997). Functionally, RAB3 isoforms all share a similar role in mature secretory vesicle docking (Fukuda, 2008).

The primary function of serous exocrine cells is to synthesize, store, and secrete digestive enzyme precursors. MIST1 (BHLHA15) is a bHLH transcription factor that acts as a scaling factor in these cells (Mills and Taghert, 2012). MIST1 expression is a feature of all serous exocrine cells (Lemercier et al., 1997; Pin et al., 2000). Recent work has begun to identify the molecular targets that it scales up to establish a high-capacity, polarized secretory apparatus (Capoccia et al., 2013; Direnzo et al., 2012; Garside et al., 2010; Jia et al., 2008; Johnson et al., 2004; Pin et al., 2001; Rukstalis et al., 2003; Tian et al., 2010). For example, RAB3D was identified as a target in both pancreatic and gastric exocrine cells (Johnson et al., 2004; Tian et
We also identified RAB26, which is phylogenetically related to RAB3D (Fukuda, 2008; Pereira-Leal and Seabra, 2001) as a direct MIST1 target that is required for establishment of large secretory granules (Tian et al., 2010). However, the mechanism of RAB26 function in secretory cells has not been characterized (Azouz et al., 2012).

Here, we present work showing that RAB26 is not like other members of its phylogenetic family because it does not directly associate with secretory vesicles, but rather with lysosomes. Accordingly, scaling up expression of RAB26 causes increasing centripetal coalescence of lysosomes to the perinuclear region in a GTP cycling dependent fashion. Furthermore, using electron microscopy, immunofluorescence microscopy, and immunoblotting, we demonstrate that the lysosomal coalescence caused by increased RAB26 also results in redistribution of mitochondria into distinct cellular neighborhoods. Taken together, our data establish a role for RAB26 as a novel lysosome-associated Rab whose expression is transcriptionally induced specifically in secretory tissues to reorganize the cellular distribution of lysosomes and, indirectly, mitochondria.

RESULTS

RAB26 Expression is Induced in Acinar Secretory Cells by MIST1

Previous reports have shown RAB26 to be expressed in secretory tissues (Nashida et al., 2006; Tian et al., 2010; Wagner et al., 1995; Yoshie et al., 2000). To further elucidate the role of RAB26, we assayed the expression pattern of this gene in a human tissue panel (Ge et al., 2005) (http://sbmdb.genome.rcast.u-tokyo.ac.jp/refexa/) (Fig. 1A). In contrast to a more ubiquitously expressed Rab (e.g., RAB7), RAB26 showed variable expression with high expression levels in exocrine secretory tissues and in parts of the brain. The two RAB26 probesets showed strong
correlation among the tissues examined (Pearson’s correlation coefficient of 0.9145) and mirrored the known tissue distribution of the secretory cell transcription factor MIST1 (Lemercier et al., 1997; Mills and Taghert, 2012; Pin et al., 2000). Moreover, within specific organs, RAB26 expression was confined to MIST1 expressing cell lineages like the serous exocrine, digestive-enzyme-secreting zymogenic (chief) cells of the stomach, but not their precursors, the mucus-secreting “neck cells” ((Capoccia et al., 2013; Geahlen et al., 2013); Fig. 1B). RAB26 expression was not scaled up in the absence of the transcription factor MIST1 in either gastric units from Mist1−/− mice (Fig. 1B) or in another tissue populated by digestive-enzyme secreting cells, the pancreas (Fig. 1C). We next decided to investigate RAB26 scalability in a cell culture system that would facilitate analysis of RAB26 expression level relative to its subcellular distribution and function. First, we analyzed the well-established secretory pancreatic cell line, AR42J, which expresses MIST1 (Jia et al., 2008) and can be differentiated with dexamethasone treatment to upregulate MIST1 target gene expression (Limi et al., 2012; Qiu et al., 2001) and increase amylase containing secretory vesicles (Logsdon, 1986; Rinn et al., 2012) (Fig. 1D). In these cells, we found that upon differentiation, as in the stomach and pancreas in vivo, RAB26 expression was induced substantially (Fig. 1D). We confirmed this relationship between MIST1 and RAB26 in human gastric cell lines (Fig. 1E). Expression of RAB26 was endogenously low in both AGS and HGC-27 cells (absent in non-epithelial U937 monocyte cells), but increased in a scalable manner upon MIST1 (not GFP control) transfection (Fig. 1E; Pearson’s coefficient of 0.9660, indicating strong correlation). These data confirm that RAB26 expression is MIST1-dependent and, based on our earlier studies showing direct MIST1 binding to the RAB26 promoter (Tian et al., 2010), we conclude that RAB26 is a direct transcriptional target whose expression is scaled up by MIST1.
To study the functional role of RAB26, we performed experiments in HGC-27 cells because 1) they express low-level endogenous RAB26, even without MIST1 transfection (Fig. 1E); 2) we have previously shown that co-transfection of MIST1 and a cargo of digestive enzyme induces a network of large secretory granules that would allow us to study the interaction between RAB26 and those vesicles (Tian et al., 2010); and 3) they are more easily transfected and larger than AGS or AR42J cells, facilitating detailed microscopy. We engineered an N-terminus monomerized EGFP fused version of RAB26 (EGFP-RAB26) to aid in subsequent localization and trafficking studies.

We had previously shown that interfering with RAB26 function inhibited MIST1-mediated granulogenesis (Tian et al., 2010) and hypothesized, based on the initial descriptive publications (Nashida et al., 2006; Wagner et al., 1995; Yoshie et al., 2000), that RAB26 would function somehow to traffic nascent or maturing secretory granules. To study RAB26-secretory granule interactions, we induced a network of secretory granules by transfecting secretory cargo, RFP-tagged Pepsinogen C, in cells stably expressing MIST1, a system we have previously described (Tian et al., 2010). Using live cell, timelapse confocal microscopy, we observed, unexpectedly, that the smaller EGFP-RAB26 vesicles did not fuse, or move in concert with the larger PGC-RFP containing secretory granules (Movie 1). In addition, RAB26 vesicles showed no overlap with immature secretory vesicles labeled with antibody against the prohormone convertase, Furin (Fig. S1A). Finally, EGFP-RAB26 did not interact directly with amylase secretory granules in AR42J cells (not shown).

RAB26 associated vesicles similarly did not overlap substantially with markers of the
following other organelles: the ER (Calregulin), early endosomal (EEA1), Golgi (giantin), cis-Golgi (GM130), or trans-Golgi markers (TGN46) compartments (Fig. S1B-F). The lack of RAB26 association with the Golgi was of interest, because recent studies demonstrated that RAB26 may occupy a recycling endosomal (Chan et al., 2011) or giantin positive Golgi compartment (Li et al., 2012). Because of the proximity, if not substantial direct overlap, of RAB26 vesicles with our trans-Golgi marker, we next examined membrane compartments that interact with the trans-Golgi. RAB26 showed slight overlap but were often also proximal to vesicles of the post Golgi sorting compartment, as marked by antibodies against CI-M6PR, AP1, and GGA2 (Fig. S1G-J). The only other principal destination for microtubule associated vesicles emerging from the Golgi – besides secretory vesicles – is lysosomes, so, by process of elimination, we reasoned RAB26 was likely associated with lysosomes. Accordingly, antibodies against the late endosome/lysosome marker, LAMP1, substantially co-labeled RAB26-associated vesicles (Fig. 2A, B). Confocal microscopy in RAB26-transfected HGC-27 cells showed near complete colocalization of RAB26 with LAMP1 (Fig. 2B). Furthermore, confocal live imaging of EGFP-RAB26 with LysoTracker revealed nearly identical movement of the two fluors (Movie 2), indicating they both clearly defined the same, dynamic vesicle population. In addition, transfection of a plasmid that was independently designed to encode endogenous, full-length, non-fluorescent tagged RAB26 revealed colocalization with anti-RAB26 and anti-LAMP1 antibodies (Fig. 2C). Finally, using immunoelectron microscopy, we found RAB26-transfected cells showed significant labeling of large vesicular structures with both 12 nm anti-RAB26 and 18 nm anti-LAMP1 gold particles. 82.4% of total cellular RAB26 particles and 91.9% of LAMP1 associated with these structures, which sometimes contained identifiable cellular debris, consistent with lysosomes (Fig. 2D, E).
As a control for potential mislocalization caused by overexpression, we next surveyed endogenous RAB26 localization. Though expression of native RAB26 in HGC cells is relatively low (Fig. 1E, 3C), we found endogenous RAB26 associated with LAMP1 lysosomes in rare untransfected cells with higher levels (Fig. 3A, B). Immunoelectron microscopy showed that, although untransfected cells lacked the larger grouped LAMP1-associated vesicular complexes found in RAB26 overexpressing cells, the LAMP1-labeled vesicles that were present were also decorated with lower-level RAB26 labeling (Fig. 3D). Cell fractionation density gradients further confirmed that both EGFP-RAB26 (detectable by antibodies to both GFP and RAB26) and endogenous RAB26 protein were enriched in the purified lysosomal fraction as defined by positive LAMP1 immunoreactivity (Fig. 3E). Finally, as an additional control, we designed and transfected an N-terminal EGFP fused RAB3D plasmid, a close family member of RAB26 and also a MIST1 target (Johnson et al., 2004; Tian et al., 2010). RAB3D had a distinct cellular distribution apart from RAB26 with no lysosomal colocalization (Fig. S2A).

Our findings were applicable beyond HGC-27 cells as similar lysosome-RAB26 colocalization occurred in another gastric cell line (Fig. S2B), and epithelial cells from other tissues including 5637 bladder cells (Fig. S2C). Likewise, in pancreatic AR42J cells, we found that RAB26 vesicles formed a unique vesicle population separate from amylase containing secretory vesicles (not shown), and again overlapping nearly completely with LAMP1 (Fig. S2D, E).

There was also significant overlap of RAB26 and LAMP1 with the lysosomal acid hydrolase, cathepsin D (CTSD) (Fig. 4A-C). RAB26 and LAMP1 often defined the membrane portion of vesicles, whose interiors were filled with cathepsin D, as expected (Fig. 4A). Moreover, we designed an RFP-tagged cathepsin D protein (CTSD-RFP) that also showed...
significant colocalization with EGFP-RAB26 upon co-transfection (Fig. 4C), providing an independent non-antibody confirmation of the direct association of RAB26 with the lysosomal compartment. EGFP-RAB26 also overlapped with another known Rab protein, RAB7 (Fig. 4D), whose role in lysosomal trafficking has been well-established (Bucci et al., 2000). Combined, these experiments definitively show that RAB26 defines the lysosomal subcellular location in multiple cells.

To confirm that RAB26 associates with lysosomes actively, based on normal GTP/GDP cycling and trafficking, we next designed single point mutant EGFP-RAB26 variants: one with a threonine to an asparagine (EGFP-RAB26T77N) and another with glutamine to a leucine (EGFP-RAB26Q123L) (Fig. 5A). Based on published RAB26 structural models (Protein Data Bank accession code 2G6B) (Wang et al., 2006) and previous studies of Ras (Barbacid, 1987; Feig, 1999; Scheffzek et al., 1997) and orthologous Rabs (Bromdyk et al., 1993; Burstein et al., 1992; Chen et al., 2002), EGFP-RAB26T77N would abolish Mg\(^{2+}\) coordinated GTP binding in the G2 box region, and EGFP-RAB26Q123L would disrupt G3 box region mediated GTP hydrolysis (Fig. 5B). When transfected, EGFP-RAB26T77N showed a diffuse cytoplasmic distribution (Fig. 5C), and EGFP-RAB26Q123L showed a more dispersed vesicular distribution (Fig. 5D). Neither EGFP-RAB26T77N nor EGFP control localized with LAMP1, and EGFP-RAB26Q123L showed some correlation with lysosomes. Specifically, while most lysosomes still co-localized with EGFP-RAB26Q123L, some EGFP-RAB26Q123L overlapped with non-LAMP1 labeled structures (Fig. 5D, E; Pearson’s coefficient of 0.384 compared to wildtype EGFP-RAB26 with substantial co-labeling and Pearson’s coefficient of 0.702). We next analyzed RAB26-EGFP transfected cells in the presence of a prenylation inhibitor and found wildtype RAB26 no longer trafficked to lysosomes, and we blocked GTP hydrolysis with non-
hydrolyzable GTP analogs and found that RAB26-EGFP trafficked only partially to lysosomes, phenocopying the Q123L GTP hydrolysis deficient mutant (not shown). To further investigate extra-lysosomal EGFP-RAB26Q123L vesicle localization, we co-stained with EEA1, giantin, and the CI-M6PR (not shown) revealing no substantial co-localization with Golgi, but partial overlap with the endosomal compartments. Thus, we conclude RAB26 requires prenylation and GTP binding to target to lysosomes, and dynamic GTP cycling to maintain localization on lysosomes and avoid trafficking to other membranes as lysosomes recycle (Sridhar et al., 2013).

**Increased RAB26 is Sufficient to Traffic Lysosomes Centripetally**

To determine the consequences of RAB26 association with lysosomes, we analyzed the effects of scaling up RAB26 expression, which occurs in tissues as differentiating secretory cells increase RAB26 in response to the transcription factor MIST1. To analyze the effects specifically of scaling up RAB26 without the effects of other MIST1 targets, we took advantage of the varying levels of expression induced by transient transfection in HGC-27 cells, whose endogenous RAB26 levels are usually low. We noticed that HGC-27 cells with the highest levels of RAB26 by EGFP fluorescence intensity showed lysosomal coalescence into a central, perinuclear region, whereas cells with lower levels showed more diffuse, peripheral lysosomes (Fig. 6A, B). Moreover, when we quantified this on a cell-by-cell basis, we found cells with coalesced lysosomes almost always showed higher levels of RAB26 (MFI > 1000 on normalized, 16 bit scale) than cells retaining diffuse lysosomes (MFI < 1000; Fig. 6B). Transfection of the two RAB26 point mutants did not recapitulate this lysosome repositioning phenomenon (Fig. 6C, D), indicating that the ability of RAB26 to bind and cycle GTP is necessary for this process. In addition, cells transfected with EGFP or EGFP-RAB3D also failed
to show centripetal lysosomal movement (Fig. 6C, D), revealing that this process is independent of GFP overexpression and RAB26-specific. Quantification showed that, of cells with detectable EGFP-RAB26, 30% displayed this central perinuclear lysosomal phenotype (Fig. 6D), whereas only about 5-10% of EGFP+ cells transfected with various control constructs (EGFP alone, EGFP-RAB26T77N, EGFP-RAB26Q123L, EGFP-RAB3D) showed this pattern of lysosome distribution. These other transfected cells showed no change in lysosome distribution no matter the levels of expression.

In addition, we examined the AR42J pancreatic acinar cell system, in which we were able to differentiate, increase MIST1 and, scale-up RAB26 levels from moderate to high detectable levels. Increasing RAB26 (marked by anti-RAB26) correlated with striking centripetal coalescence of LAMP1 vesicles to the perinuclear region (Fig. 6E). In other words, endogenous RAB26 scaled up by cellular differentiation phenocopied transfection-mediated RAB26 scaling.

Localization of other vesicular structures including endosomes, the Golgi, and late endosomes (not shown and Fig. S3A, B) were unaffected by transfection of EGFP-RAB26 or any of its mutant forms. Transfection of RAB26 and RAB26 mutants had no significant effect on: 1) overall levels of LAMP1 protein in cell cultures, 2) levels of cathepsin D; 3) lysosome-mediated processing of cathepsin D to its smaller active form; 4) mTOR/S6K signaling, which depends in part on lysosomal function (Fig. S4A). In sum, RAB26 specifically localizes predominately with lysosomes in a GTP/GDP dependent manner, and, as RAB26 levels are scaled up, it reorients only lysosomes and not other vesicular compartments. Although global lysosomal function does not appear affected by RAB26, more experiments would be needed to analyze how RAB26 may mediate the many additional aspects of lysosome trafficking and function.
RAB26 Coordinates Reorganization of Mitochondria

To begin to address the potential cellular effects of scaling up RAB26, we returned to ultrastructural analyses of transfected cells. Supporting our immunofluorescence and immunoelectron microscopy data, we found large clusters of electron-lucent vesicles, morphologically consistent with lysosomes (Fig. 2D), only in the RAB26 transfected cells (Fig. 7A-E). In our control cells, or in cells transfected with EGFP-RAB26T77N, EGFP-RAB26Q123L, or EGFP, lysosomes were smaller and dispersed throughout the cytoplasm (Fig. 7C-E). We noticed that the lysosome clusters in the EGFP-RAB26 expressing cells largely excluded mitochondria (Fig. 7A, B). Thus, instead of the diffuse mitochondrial network seen in cells transfected with various control constructs (Fig. 7C-E), the mitochondria in RAB26-transfected were clustered into distinct cellular neighborhoods. In fact, upon returning to our initial immunoelectron micrographs, we found RAB26 occasionally labeling mitochondrial membranes (3.17% of total labeling) adjacent to LAMP1-labeled lysosomes. Thus, RAB26 might also directly or indirectly affect cellular mitochondrial organization.

Accordingly, in cells expressing abundant EGFP-RAB26, we found mitochondria, like lysosomes, were also clustered but into distinct subcellular regions that were specifically free of lysosomes (Fig. 8A, B). Control cells showed mitochondria organized in the expected diffuse, lattice-like network throughout the cell (Fig. 8A). Using live cell confocal microscopy, we were able to observe mitochondrial interaction on the borders of the EGFP-RAB26 and LAMP1 lysosomal clusters (Movie 2,) including temporary areas of overlap with apparent mitochondrial-lysosomal fusion or membrane exchange highlighted in frames from Movie 2 (Fig. 8C). When we quantified the cell area of mitochondria as a percentage of total cell area in EGFP-RAB26
transfected cells, we found it to be significantly reduced relative to controls (Fig. 8D). In fact, when whole cellular populations were analyzed for mitochondrial density by western blot for the mitochondrial outer membrane marker, TOM20, we found a significant reduction (n = 3 independent transfections per construct) only when functional RAB26 was transfected, indicating trimming of mitochondria across the whole population (Fig. 8E). However, when mitochondrial function was analyzed using dyes sensitive to mitochondrial membrane potential (CMXRos (Poot et al., 1996)) and superoxide damage production (MitoSOX Red (Mukhopadhyay et al., 2007)), we found that while mitochondria were reduced in cells overexpressing RAB26, those that remained were largely intact and functional, as was the case for all controls analyzed (Supp Fig. 4B, C). Taken together, our data show that RAB26 repositions lysosomes and reorganizes mitochondria.

**DISCUSSION**

Here, we find, in multiple cell types, that RAB26 associates with and traffics lysosomes, which is surprising both because lysosome-associated Rabs are rare and because RAB26 was initially characterized in the rat pancreas as being associated with secretory vesicles (Wagner et al., 1995). However, these earlier results were generated using antibody staining in fixed tissue sections and cell fractionation density gradient enrichment of granules (Nashida et al., 2006; Yoshie et al., 2000). In fact, secretory granules and lysosomes are not easily distinguishable by density gradient properties (Pasquali et al., 1999), and the mature secretory granule markers, syntaxin 6 and γ-adaptin, used as markers of secretory granules in previous analyses of RAB26, are also involved in Golgi sorting to lysosomes and late endosomes (Ghosh et al., 2003; Robinson, 1990). Those markers have been found to be specifically removed from mature
secretory granules (Klumperman et al., 1998). Moreover, in a large screen of mast cell Rabs that regulate secretion, transfected RAB26, which mast cells do not normally express, was also found to be perinuclear and associated with a vesicular compartment that was expected to be LAMP1 positive (Azouz et al., 2012).

Recent studies found RAB26 to be associated with RAB11 recycling endosomes (Chan et al., 2011) in Drosophila neurons, and a giantin-positive Golgi sorting compartment (Li et al., 2012) when transfected into HEK293 cells. These results would seem to contradict those of the current manuscript, though we have focused here exclusively on exocrine secretory cells, trying to mimic the effects of scaling RAB26 as occurs in differentiating cells in tissue. Thus, RAB26 may traffic differently depending on cell type; however, there are other possible explanations as well for our seemingly differing findings. There is overlap between Golgi, endosome, and lysosome markers as lysosomes mature through sorting and trafficking (Ghosh et al., 2003; Griffiths et al., 1990; Luzio et al., 2000; Moore et al., 2004; Peden et al., 2004; Saftig and Klumperman, 2009). RAB7, the most well studied lysosomal Rab protein (Bucci et al., 2000), has also been implicated in endosomal trafficking (Feng et al., 1995). In fact, its close relative, RAB7B, is found to co-localize with both Golgi and lysosomal markers (Progida et al., 2010). The transient nature of these dynamic and constantly communicating compartments (Saraste and Goud, 2007) complicate all subcellular localization studies. In the current manuscript, we have used multiple secretory cell types and multiple modalities to examine both endogenous and transfected RAB26, which we argue reinforces our conclusions that RAB26 is predominantly lysosome-associated in dedicated secretory cells.

That lysosomes cycle between peripheral and central regions has been well documented with various motor proteins (Hollenbeck and Swanson, 1990; Lin and Collins, 1992), and
cytoskeletal filaments (Cordonnier et al., 2001; Matteoni and Kreis, 1987) implicated in this traffic. Much work has also elucidated the role of the Rab family in lysosomal movement and function. Specifically, RAB4, 5, 9, and 11 have been shown to coordinate endosomal traffic into and out of maturing lysosomes (Saftig and Klumperman, 2009). However, only RAB7 has been well established as the key cellular regulator of late endosome to lysosome trafficking, and overexpression of RAB7 causes central lysosome movement, while T22N and N125I point mutants leave lysosomes dispersed (Bucci et al., 2000). There is some evidence that two other ubiquitously expressed Rabs, RAB34 (Wang and Hong, 2002) and RAB36 (Chen et al., 2010), also function to redistribute lysosomes centrally (Colucci et al., 2005). Interestingly, these Rabs share a common effector, RILP (RAB7-interating lysosomal protein), crucial for inducing dynein mediated movement (Cantalupo et al., 2001; Jordens et al., 2001; Wang and Hong, 2005). RAB26 may also mediate lysosomal rearrangement through RILP interactions.

Mitochondria are constantly pruned and recycled in post-mitotic cells. Under conditions of oxidative stress, the normal steady-state mitochondrial recycling (Lipsky and Pedersen, 1981; Terman et al., 2010) is scaled up to maintain physiological function and forestall apoptosis in a process that depends on lysosome-mediated pruning (Green and Kroemer, 2004). Mitochondria are delivered to lysosomes via many routes (Ashrafi and Schwarz, 2013). For example, damaged mitochondria, as tracked by their membrane marker TOM20, can be delivered directly to lysosomes (Soubannier et al., 2012).

Our findings indicate that scaling up RAB26 might enhance the ongoing baseline direct trafficking events between lysosomes and mitochondria. Little is known about lysosome-mitochondria interactions in tissues that are not under constant oxidative damage. In exocrine tissues, mitochondria play a crucial role in secretagogue mediated calcium signaling (Tinel et al.,
that depends on their subcellular positioning (Park et al., 2001). Increasing RAB26 may be an inherent mechanism for secretory cells to reorganize mitochondria and to focus them in areas where they can facilitate calcium delivery to secretory granules. We observed only partial reduction in mitochondrial numbers, no matter how high expression of RAB26 in a given cell; thus, RAB26 may prune or cluster, rather than destroy, mitochondria.

Our studies have confirmed that RAB26 is not ubiquitous but, rather, is specifically expressed in only certain cell lineages. Thus, we expect that it would be sufficient but not required for lysosome mediated mitochondrial reorganization because other more universal mechanisms must be present in all cells. In fact, our two point mutants do not act as dominant negatives in affecting lysosomal movement or preventing oxidative damage initiated mitochondrial degradation (not shown). In addition, siRNA mediated knockdown of endogenous RAB26 similarly had no effect on lysosomes or mitochondria (not shown). RAB3D, another direct MIST1 target and secretory cell specific Rab protein shows a similar ability to “scale-up” vesicle dynamics; when overexpressed in acinar cells, RAB3D enhances regulated secretion (Ohnishi et al., 1997). However, when Rab3D is deleted regulated secretion is unaffected (Riedel et al., 2002). Thus, these exocrine cell specific Rabs represent a subset of “scale-able” cellular effectors that enhance cellular dynamics.

In digestive-enzyme secreting tissues such as the pancreas and stomach, RAB26 expression is almost wholly dependent on the transcription factor, MIST1 (Tian et al., 2010). In the absence of MIST1 and, thus RAB26, exocrine secretory cells display aberrant secretory vesicle organization, maturation, and secretion (Direnzo et al., 2012; Johnson et al., 2004; Luo et al., 2005; Pin et al., 2001; Ramsey et al., 2007; Tian et al., 2010). Interestingly, Mist1−/− exocrine cells also exhibit aberrant lysosomal trafficking (Capoccia et al., 2013; Direnzo et al.,
Furthermore, in wildtype MIST1-expressing cells mitochondria are organized into distinct subcellular neighborhoods (similar to what we see with increased RAB26), while in Mist1−/− cells, mitochondria are dispersed throughout the cell, resulting in abnormal calcium flux (Luo et al., 2005). From our previous study, no other direct MIST1 targets seem to have clear roles in lysosome movement, mitochondria reorganization, or Rab protein function (e.g., neither guanine nucleotide exchange factors, GTPase-activating proteins, nor other identified Rab effectors are known MIST1 targets) (Tian et al., 2010). Thus, RAB26 function may be an important and crucial unrecognized aspect in MIST1 regulated cell secretory function.

Loss of MIST1 results in increased susceptibility to pancreatitis (Alahari et al., 2011; Kowalik et al., 2007; Zhu et al., 2004), and, in wildtype mice, pancreatitis leads to silencing of Mist1 (Shi et al., 2013; Shi et al., 2009). Acute pancreatitis is caused by inappropriate interaction between lysosomal enzymes and zymogen constituents of secretory granules (Gaiser et al., 2011; Halangk et al., 2000; Saluja et al., 1997), which leads to mitochondrial disorganization (Mareninova et al., 2006; Odinokova et al., 2009). Thus, one mechanism for how MIST1 might suppress acute pancreatitis is by trafficking of lysosomes and mitochondria through RAB26. Future experiments will have to address whether loss of RAB26 in MIST1-expressing cells causes disrupted lysosome/mitochondrial mediated damage, though deletion of tissue-specific Rabs in vivo has not been that informative (e.g., Rab3d null mice have only a mild phenotype (Riedel et al., 2002)) perhaps because they are sufficient for scaling up specific aspects of secretion but not absolutely required.

Our data clearly show that coalescence of lysosomes and mitochondria is caused by expression of higher levels of RAB26. The changes occur whether endogenous RAB26
expression is increased by MIST1 during differentiation or if it is directly transfected to higher levels. Thus, our studies exemplify how a transcription factor like MIST1 can scale up a specific target gene during maturation to redistribute organelles in a way that enhances the specific physiological function the cell must perform in the adult organism. In other words, we provide mechanistic evidence for how transcription factors like MIST1 can act as scaling factors to control cell architecture simply by increasing expression of specific genes that encode cellular effectors (Mills and Taghert, 2012). It will be interesting to determine how the scaling of RAB26 by MIST1 might work in more detail, whether RAB26 acts as a rheostat (i.e., the more RAB26 expression increases, the more centralized lysosomal trafficking there is) or more like a binary switch (i.e. low levels do not change lysosomal distribution, high levels cause lysosomes to coalesce centrally).

In summary, we propose a mechanism whereby a transcriptionally controlled novel Rab protein can regulate critical, physiologically relevant cell structural adaptations. RAB26 through its ability to reposition lysosomes can, in turn, effect mitochondrial reorganization. Mitochondria and lysosomes are critical for the formation, maintenance, and secretion of zymogen granules. Therefore, we posit that RAB26 is a novel, lysosome-associated, tissue and cell-type specific, small protein GTPase that can be “scaled-up” by the transcription factor MIST1 to enhance the secretory function of exocrine cells.

**MATERIALS AND METHODS**

*GeneChip analysis.*

Human tissue expression data was obtained from the LSBM RefExA database (http://sbmdb.genome.rcast.u-tokyo.ac.jp/refexa/) (Ge et al., 2005). Human cell line expression
data prepared as previously discussed (Tian et al., 2010). Mouse gene chip arrays were generated as previously discussed (Capoccia et al., 2013). Heat maps of RAB26 expression levels were generated by dChip analysis (Zhong et al., 2003). Correlation between RAB26 probesets was calculated using GraphPad Prism to generate Pearson’s correlation coefficients based on raw read values.

Cell Lines / Transfection

HGC-27 and AGS cells were maintained as previously described (Tian et al., 2010). 5637 cells (gift from Dr. Indira Mysorekar, Washington University) were maintained at 37°C in 5% CO2 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. AR42J cells (ATCC, Manassas, VA) were maintained in F12K media supplemented with 20% fetal bovine serum, 1.0% glutamine, and 100 ng/mL each of penicillin and streptomycin. Dexamethasone (100 nM, Sigma-Aldrich, St. Louis, MO) was added to cells for 48 hours to induce secretory differentiation. For transient transfection, AGS or HGC-27 cells were transfected via electroporation using Nucleofector II (Lonza, Basel, Switzerland), program B-023, and cell line transfection solution V. For each electroporation, 3 µg PGC-RFP (see below), 5 µg EGFP-RAB26 (see below), 5 µg EGFP-RAB26T77N (see below), 5 µg EGFP-RAB26Q123L (see below), 5 µg EGFP-RAB3D (see below), 3 µg CTSD-RFP (see below), or 3 µg pmaxGFP (Lonza) plasmid was used. In some experiments and other cell lines, we also used TransIT-2020 and TransIT-LT1 Transfection Reagent (Mirus, Madison, WI) according to manufacturer’s protocol. For visualization, cells were replated on Lab-Tek Chamber Slide 4-well Permanox slides (Thermo Fisher Scientific, Rochester, NY) and routinely analyzed 24-72h posttransfection.
MIST1-EGFP expressing stable cell lines were established as previously described (Tian et al., 2010).

**Immunofluorescence / Cell Imaging**

For immunofluorescence analysis, cultured cells were transfected and stained as described previously (Tian et al., 2010). Fluorescence microscopy and imaging were performed using a Zeiss Axiovert 200 microscope 20X (Plan-Apochromat, 0.8 NA), 40X (Plan-Neofluar 0.85 NA) and 63X objectives (Plan-Apochromat, 1.4 NA) with Axiocam MRM camera and AxioVision software. Additional, confocal microscopy and imaging were performed using a Zeiss LSM510 microscope with 40X (EC Plan-Neofluar, 0.75 NA) and 63X (Plan-Apochromat, 1.4 NA) objectives using LSM510 software. Live cell imaging was performed using an Olympus FluoView FV1000 microscope equipped with 60X objective (PLAPON 60XO, 1.42 NA) maintained at 37°C in 5% CO2 with images captured using the FV10-ASW software or using a Zeiss LSM510 confocal microscope with 63X objectives (Plan-Apochromat, 1.4 NA) maintained at 37°C in 5% CO2 with images captured using the Zeiss LSM510 software. Contrast (maximal, minimal, and midtone) adjustment and fluorescent channel overlay and pseudocoloring were performed with Photoshop (Adobe Systems, San Jose, CA). All adjustments were performed on the entire image equally.

The primary antibodies used for cell immunostaining: rabbit anti-amylase (1:100, Calbiochem, CA), rabbit anti-RAB26 (1:100, ProteinTech Group, Chicago, IL), rabbit anti-giantin (1:1000, Covance), rabbit anti-EEA1 (1:250, Abcam), rabbit anti-furin (1:500, Thermo Scientific), goat anti-calregulin (1:100, Santa Cruz), rabbit anti-GM130 (1:250, BD Biosciences, San Jose, CA), sheep anti-TGN46 (1:250, Serotec, Oxford, UK), rabbit anti-CI-M6PR (1:500);
mouse anti-AP1 (1:500); rabbit anti-CTSD (1:150); and mouse anti-GGA2 (1:500) (gifts from Dr. Stuart Kornfeld, Washington University), mouse anti-LAMP1 (1:40, Santa Cruz, Santa Cruz, CA), mouse anti-LAMP1 (1:50, clone H4A3, developed by J. Thomas August and James E. K. Hildreth and obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD maintained by The University of Iowa), rabbit anti-RAB7 (1:150, Cell Signaling Technology, Danvers, MA). Secondary antibodies used were Alexa Fluor 488-, 594-, and 647-conjugated donkey anti-goat, anti-rabbit, anti-sheep, and anti-mouse antibodies (1:500; Invitrogen, Carlsbad, CA). LysoTracker and MitoTracker (Invitrogen) were used at 75 nM and 250 nM concentrations, respectively, according to manufacturer’s instructions. MitoTracker CMXRos and MitoSOX Red (Invitrogen) were used at 100 nM and 2 µM concentrations, respectively, according to manufacturer’s instructions.

**Immunofluorescence Quantification**

For pixel-to-pixel overlap of EGFP-RAB26, EGFP-RAB26T77N, EGFP-RAB26Q123L, and GFP with LAMP1, cells were transfected and stained for LAMP1 as described and confocal images were taken from at least 10 cells per condition. Thresholded Pearson’s correlation coefficients were calculated using Volocity image analysis software (PerkinElmer, Waltham, MA).

For lysosome distribution quantification, cells were transfected as described with EGFP-RAB26, EGFP-RAB26T77N, EGFP-RAB26Q123L, EGFP-RAB3D, or GFP plasmids. Post transfection, lysosomes were stained with a LAMP1 antibody as described. Blinded to treatment conditions, sixteen-bit images captured in Zeiss Axiovision software were analyzed with ImageJ software as follows. Central lysosomes (bright concentrated staining around perinuclear region),
diffuse lysosomes (disperse cytoplasmic staining), transfected cells, and total cells determined by total nuclei counted using technique developed by (Selinummi et al., 2005) were scored. 65 low power fields comprising three independent experiments were examined totaling approximately 10,000 cells counted per condition.

For enhanced green fluorescent protein EGFP-RAB26 fluorescence intensity quantification, cells were transfected with EGFP-RAB26 and stained for LAMP1. Sixteen-bit images captured in Zeiss Axiovision software were analyzed with ImageJ software as follows. We determined mean green cytoplasmic fluorescence intensities in each region after subtraction from the median background (green in an area with no cells). These same cells were then scored individually for their lysosome distribution (as defined above). A total of 180 cells were scored across three transfection experiments.

For mitochondrial area quantification, cells were transfected and stained as previously described. Sixteen-bit images captured in Axiovision software were analyzed with ImageJ software as follows. Area selection tool was used to outline mitochondria, and area was measured using the Analyze area measurement tool. Multiple cells were scored across three transfection experiments.

*Plasmid Preparation*

PGC-RFP, EGFP-RAB26, and EGFP-RAB26T77N mammalian expression plasmids were constructed as previously detailed (Tian et al., 2010). To generate EGFP-RAB26Q123L, EGFP-RAB26 was mutagenized by site-directed mutagenesis to convert the glutamine at amino acid position 123 to a leucine, and a new XhoI restriction site was introduced. pcDNA3.1-RAB26 plasmid was constructed using the same approach as EGFP-RAB26 using backbone plasmid
pcDNA3.1/V5-His-TOPO (Invitrogen) with the first amino acid of the His-tag modified to a stop codon. The monomeric EGFP-RAB3D expression plasmid was constructed with the coding region of human RAB3D cDNA (IMAGE identification number 3861912; Open Biosystems) added in frame to the carboxyl terminus of EGFP, replacing the RAB26 coding region in EGFP-RAB26 by ribocloning (Barnes, 1994; Barnes, 2006). PCR was performed on the vector region by using primers DNA3as (GGCAATTCCACCACACTGGACTAGGu) and pcD3LKs (GGGCAATTCTGCAGATATCCAGCAc) on EGFP-RAB26 DNA. The RAB3D target was PCR amplified using DNA3s (ACTAGTCCAGTGTGGGTAATGGC) and hRAB3D-DNA3b (GGGTGTCTCCAGCTGATGCCATGGCGCAATTCACCACACTGGACTAGTG) with pcD3LKas (GTGCTGGATATCTGCAGAATTGCCc) and hRAB3D-pcD3LKs (GATGCTCCAGCCCCCAGGCCCTCCTCTTGTAGCTGCTAGGGCAATTCTGCAGATATCCAGCAC). The pcDNA3.1 CTSD-RFP fusion construct was generated with the human CTSD region of pcDNA3.1-hCTSD-myc (gift from Dr. Stuart Kornfeld) added in frame to the amino terminus of RFP, replacing the PGC coding region in PGC-RFP by ribocloning. PCR was performed on the vector region by using primers DNA3as and RFPs (GCCTCCTCCAGGACGTCAu). The hCTSD target was PCR amplified using DNA3s and hCTSD-DNA3b (GCTGGAGGGCTGATGCGCAATTCACCACACTGGACTAGT) with RFPas (ATGACGTCCCTCGAGGAGGe) and hCTSD-RFPs (CGAGGCTGCCCGCTCGCTCCTCCAGGAGGACGTCA). All construct coding regions were verified to be correct by DNA sequencing.

*Density Gradient Ultracentrifugation Lysosomal Enrichment*
Lysosomes were isolated with the Pierce Lysosomal Enrichment Kit of Tissue and Cultured Cells (Rockford, IL) according to manufacturer’s protocol. Briefly, cells were transfected as described, pelleted, and homogenized using a Dounce tissue grinder. Nuclei were removed and then lysate was ultracentrifuged on prepared Optiprep density gradients using Beckman Coulter Optima L-100 XP ultracentrifuge (Fullerton, CA). Fractions were removed and subjected to western blot analysis.

**Western Blot Analysis**

Mouse tissue was flash frozen in liquid nitrogen and homogenized in RIPA buffer using a PowerGen700 (Fischer Scientific, Pittsburg, PA). Cells were transfected as described and lysed in RIPA buffer. Proteins were quantified by DC protein assay (Bio-Rad) and then separated on NuPAGE Bis-Tris gels (Invitrogen), transferred to Immobilon polyvinylidene difluoride (Millipore, Bedford, MA) or Amersham Hybond ECL nitrocellulose (GE Healthcare, Buckinghamshire, United Kingdom) membranes, and detected by Immobilon chemiluminescence (Millipore). Primary antibodies used were mouse anti-LAMP1 (1:1000, Developmental Studies Hybridoma Bank), rabbit anti-GFP (1:1500, Santa Cruz), rabbit anti-RAB26 (1:800, ProteinTech Group Chicago, IL), rabbit anti-MIST1 (1:500, described previously in (Pin et al., 2000)), mouse anti-TOM20 (1:1000, BD Biosciences), rabbit anti-cathepsin D (1:2000, gift from Dr. Stuart Kornfeld, Washington University), rabbit anti-mTOR (1:1000, Cell Signaling Technology), rabbit anti-phospho-p70 S6 kinase (1:1000, Cell Signaling Technology), rabbit anti-p70 S6 Kinase (1:1000, Cell Signaling Technology), and rabbit anti-α/β tubulin (1:1500, Cell Signaling Technology). Secondary antibodies were horseradish peroxidase-conjugated donkey anti-rabbit, and anti-mouse (Santa Cruz Biotechnology, Santa Cruz, CA).
Quantifications of immunoblots were done by scanning sixteen-bit images into ImageJ. Band intensities for TOM20 and α/β tubulin were selected and calculated using Analyze mean gray value measurement tool. Standardized values were calculated by the ratio of TOM20 signal to α/β tubulin signal.

**Protein Structure Analysis**

RAB26 protein structure was obtained from Protein Data Bank, accession code 2G6B (Wang et al., 2006). Image was generated using Protein Workshop (Moreland et al., 2005) with surfaces (Xu and Zhang, 2009).

**Electron Microscopy**

For transmission electron microscopy studies, cells were transfected as previously described and plated on Lab-Tek Chamber Slide 4-well Permanox slides (Thermo Fisher Scientific). After being rinsed in PBS, they were fixed in modified Karnovsky’s fixative (2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 mmol/liter cacodylate buffer). TEM thin sections were cut directly from cell cultures embedded on the original Permanox substrate.

For immunoelectron microscopy studies, EGFP-RAB26 or EGFP controltransfected HGC-27 cells were trypsinized, pelleted, and fixed on ice for 1 hour in PBS, 4% paraformaldehyde, 0.05% glutaraldehyde. Subsequent sample processing was performed as previously described (Beatty, 2008). After incubation with primary antibodies against RAB26 (1:50, ProteinTech Group) and LAMP1 (1:25, Developmental Studies Hybridoma Bank) sections were then washed and probed with anti-rabbit conjugated to 12 nm and anti-mouse conjugated to 18 nm colloidal gold (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).
Quantification of labeling specificity of RAB26 and LAMP1 was performed by calculating cellular areas of lysosomes, mitochondria, nucleus, cytoplasm, and plasma membrane using ImageJ Area selection tool and Analyze area measurement tool. Areas were compared to counted 12 nm RAB26 and 18 nm LAMP1 labeling within 100 nm of the same cellular areas. Multiple cells from 5 fields were counted.

*Graphing and Statistics*

All graphs and statistics were determined with GraphPad Prism and then visualized using Adobe Illustrator. Statistical analysis was, in the case of simple control-versus-experimental condition comparison, by Student’s t-test. Otherwise, significances were determined by one-way analysis of variance (ANOVA) test with Dunnett’s multiple-comparison correction.

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REFERENCES


Figure Legends

Figure 1. Expression of RAB26 is cell and tissue-dependent, and inducible by the transcription factor MIST1. A. Expression of RAB7 and RAB26 in REFEXA database of human tissues ([http://sbmdb.genome.rcast.u-tokyo.ac.jp/refexa/](http://sbmdb.genome.rcast.u-tokyo.ac.jp/refexa/)). All tissues are labeled with highly RAB26 expressing secretory tissues highlighted below. Gene expression is shown with relative scale (0 to 200) with red=high, blue=low expression. B. Microarray analysis of RAB26 gene expression from isolated populations of gastric ZCs and their precursor neck cells from wild-type and Mist1−/− mice. Arrows indicate location of isolated cell populations in representative H&E gastric gland images. Gene expression for microarray analyses are shown with relative expression scale (-3.0 to 3.0) with red=high, blue=low expression. C. Western blot analysis of indicated proteins from two wildtype and two Mist1−/− mice. D. Immunofluorescence of AR42J acinar cell differentiation upon treatment with dexamethasone (Dex); amylase secretory vesicles are red; endogenous RAB26 are green. E. Gene expression analysis of RAB26 expression from AGS and HGC-27 gastric cell lines before and after transfection with either GFP or MIST1; a non-epithelial monocyte control cell line is also shown (U937). Scale bars = 20 µm.

Figure 2. RAB26 localizes to LAMP1 positive lysosomal vesicles. A. Immunofluorescence of HGC-27 cells transfected with EGFP-RAB26 (green) and co-immunostained with LAMP1 (red). B. Confocal image of EGFP-RAB26 (green) transfected HGC-27 cells stained for LAMP1 (red). Insets show unmerged RAB26 (green) and LAMP1 (red) positive membranes. (Cell border outlined, “N” – nucleus). C. RAB26 without GFP tag (pcDNA3.1-RAB26)
transfected HGC-27 cells co-stained with anti-LAMP1 (green) and anti-RAB26 (purple).  

D. Immunoelectron micrographs of HGC-27 cells transfected with EGFP-RAB26. Representative low magnification cell shown with insets and additional panel highlighting large labeled vesicles with anti-RAB26 (12 nm gold particles, yellow arrowheads) and anti-LAMP1 (18 nm gold particles, white arrowheads).  

E. Graph with left bar showing average percent total cellular area of lysosomes, mitochondria, nucleus, cytoplasm, and plasma membrane in multiple quantified cells. Right bars show fraction of total RAB26 or LAMP1 gold particles associated with each of those compartments. Scale bars = 20 µm unless indicated.

**Figure 3. Endogenous RAB26 is also lysosome-associated.**  

A. Immunofluorescence imaging of endogenous RAB26 staining (green) in a rare HGC-27 cell with moderate RAB26 levels co-stained for LAMP1 (red).  

B. Confocal immunomicroscopy of endogenous RAB26 (green) and LAMP1 (red) ("N" – nucleus). Arrowheads indicate colocalization of RAB26 and LAMP1 vesicles.  

C. Lower magnification epifluorescence of HGC-27 cells with more representative, low-level endogenous RAB26 (green) and LAMP1 (red).  

D. Immunoelectron micrographs of control GFP transfected HGC-27 cells. A representative cell is shown at low magnification with several panels showing electron lucent structures labeled with anti-LAMP1 (18 nm, white arrowheads) and sparsely labeled with anti-RAB26 (12 nm, yellow arrowheads).  

E. Density gradient cell fractionation experiment with numbered density fractions analyzed for lysosomes with anti-LAMP1 antibody; EGFP-tagged RAB26 by both anti-GFP and anti-RAB26 antibodies (middle and lower panel); and endogenous RAB26 by anti-RAB26 immunoblotting (lower panel). Scale bars = 20 µm unless indicated.
Figure 4. RAB26 localizes with other lysosomal markers. A. Confocal image of a HGC-27 cell transfected with EGFP-RAB26 (green) and immunostained for LAMP1 (red) and cathepsin D (blue) (Cell border outlined, “N” – nucleus). Inset highlights higher magnification of a lysosomal vesicle (arrowhead) that contains membranous RAB26 and LAMP1 with interior cathepsin D. B. Immunofluorescence staining of EGFP-RAB26 (green) transfected HGC-27 cells with cathepsin D (red). C. Epifluorescence microscopy of HGC-27 cells co-transfected with EGFP-RAB26 (green) and RFP-tagged cathepsin D (CTSD-RFP, red). E. Immunofluorescence staining of EGFP-RAB26 (green) transfected HGC-27 cells with RAB7 (red). Scale bars = 20 µm.

Figure 5. Design and characterization of RAB26 point mutants. A. Amino acid sequence of EGFP-RAB26 plasmid with EGFP sequence (green) and human RAB26 sequence (black) labeled. The EGFP-RAB26T77N and EGFP-RAB26Q123L were constructed by the indicated point mutations (shown in red) in the RAB26 sequence converting a threonine to an asparagine, and a glutamine to a leucine, respectively. B. Ribbon diagram of the crystal structure of RAB26 generated by FirstGlance in Jmol (ver. 1.03) from http://molvis.sdsc.edu/fgij/fg.htm?mol=2G6B (PDB ID: 2G6B; (Wang et al., 2006)). GNP bound RAB26 with the mutated threonine (T77) and glutamine (Q123) labeled and emphasized with their atomic structures. C. Confocal fluorescence microscopy of the distribution of EGFP-RAB26T77N (green) transfected HGC-27 cells immunostained with LAMP1 antibody (red) (Cell border outlined, “N” – nucleus). Right panels show epifluorescence microscopy of EGFP-RAB26T77N (green) co-stained for LAMP1 (red). D. Confocal fluorescence microscopy of the localization of EGFP-RAB26Q123L (green) transfected HGC-27 cells immunostained with LAMP1 antibody (red). Inset indicates areas of
overlap (solid white arrowheads) of EGFP-RAB26Q123L vesicles with lysosomes (transparent arrowhead) (Cell border outlined, “N” – nucleus). Right panels show epifluorescence microscopy of EGFP-RAB26Q123L (green) co-stained for LAMP1 (red). E. Pearson’s correlation coefficient quantification of pixel-to-pixel colocalization of multiple cells transfected with EGFP-RAB26, EGFP-RAB26T77N, EGFP-RAB26Q123L, and GFP with LAMP1 lysosomes. (“***” – p < 0.001, one-way ANOVA test with Dunnett’s correction). Scale bars = 20 µm.

Figure 6. Scaling of RAB26 expression leads to perinuclear localization of lysosomes. A. Fluorescence microscopy of HGC-27 cells transfected with EGFP-RAB26 (green) and stained for anti-LAMP1 (red). A long and short exposure is shown to highlight the differences in RAB26 levels with the cells expressing highest (“*”) and more moderate (solid white arrow) levels of RAB26 indicated. B. Histogram of EGFP-RAB26 transfected cells immunostained for LAMP1 with either diffuse (red line) or clustered (blue line) LAMP1 lysosomal distribution plotted vs. binned fluorescence intensity of EGFP-RAB26. C. Epifluorescence microscopy of HGC-27 cells transfected with EGFP-RAB26T77N, EGFP-RAB26Q123L, EGFP-RAB3D, and EGFP expression plasmids (green) co-stained with anti-LAMP1 (red). D. The percentage of EGFP-RAB26, EGFP-RAB26T77N, EGFP-RAB26Q123L, EGFP-RAB3D, and EGFP transfected HGC-27 cells from multiple cell fields with coalesced perinuclear LAMP1 structures quantified by cell count and plotted (“***” – p <0.001, one-way ANOVA test with Dunnett’s correction). E. Immunofluorescence microscopy of untreated (-Dex) or dexamethasone treated (+Dex) AR42J cells stained for endogenous RAB26 (green) and LAMP1 (red). Scale bars = 20 µm.
Figure 7. **RAB26 overexpressing cells show ultrastructural rearrangements.**  
A.  
F.  
Panels of immunoelectron microscopy of EGFP-RAB26 transfected HGC-27 cells labeled with LAMP1 (18 nm, white arrowheads) and RAB26 (12 nm, yellow arrowheads) with mitochondria (‘M’) and lysosomes (‘L’) highlighted (scale bars as shown).

Figure 8. **RAB26 induced lysosomal clustering leads to mitochondrial reorganization.**  
A.  
Fluorescence microscopy of EGFP-RAB26, EGFP-RAB26T77N, EGFP-RAB26Q123L, and EGFP transfected HGC-27 cells (green) co-stained for lysosomes (anti-LAMP1, red) and mitochondria (MitoTracker, purple). B.  
Confocal section of EGFP-RAB26 expressing cell (green) with stained for lysosomes labeled in red (anti-LAMP1) and mitochondria labeled in purple (MitoTracker). Perinuclear mitochondria free zones are indicated (‘*’) (Cell border outlined, “N” – nucleus). C.  
Live confocal timelapse microscopy of EGFP-RAB26 (green), LysoTracker labeled lysosome (red), and MitoTracker labeled mitochondria (purple) vesicle dynamics. Insets highlight RAB26 (green, filled white arrowhead) and mitochondria (purple, open white arrowhead) interactions (time stamps of images from Movie 2 shown, “N” – nucleus). D.  
Quantification of mitochondrial area/total cell area measured from multiple HGC-27 cells transfected with EGFP-RAB26, EGFP-RAB26T77N, EGFP-RAB26Q123L, and EGFP (‘*’- p <0.05, one-way ANOVA test with Dunnett’s correction). E.  
Densitometry quantification
of western blots (triplicate experiments) of mitochondrial membrane density using TOM20 and α/β tubulin markers for EGFP-RAB26, EGFP-RAB26T77N, EGFP-RAB26Q123L, and EGFP transfected HGC-27 cells ("*** – p < 0.001, one-way ANOVA test with Dunnett’s correction). Scale bars = 20 µm.
Supplemental Figure Legends

Supplemental Figure 1. RAB26 does not localize to the immature secretory vesicle, endosomal, Golgi, or post-Golgi sorting compartments. EGFP-RAB26 (green) transfected HGC-27 cells immunostained for: A. immature secretory vesicle marker, Furin (red); B. endoplasmic reticulum marker, Calregulin (red); C. early endosomal marker, EEA1 (red); D. Golgi marker, Giantin (red); E. cis-Golgi marker, GM130 (red); F. trans-Golgi marker, TGN46 (red); and G. post-Golgi/late endosome marker, CI-M6PR (red). Confocal microscopy of EGFP-RAB26 (green) transfected HGC-27 cells immunostained for post-Golgi/endosomal markers: B. CI-M6PR (red) C. AP1 (red), D. GGA2 (red). Insets indicate RAB26 vesicles (white filled arrowheads) overlap with stained markers (open arrowheads) (Cell border outlined, “N” – nucleus). Scale bars = 20 µm.

Supplemental Figure 2. RAB26 not RAB3D localizes to lysosomes. A. Immunofluorescence microscopy of HGC-27 cells transfected with EGFP-RAB3D and co-stained for LAMP1 (red) and cathepsin D (purple). Epifluorescence images of EGFP-RAB26 transfected: B. AGS, gastric cells; C. 5637, bladder cells; and D. AR42J, pancreatic cells co-labeled for LAMP1 (red). Panels indicate unmerged green and red channels. E. Confocal microscopy of an AR42J cell transfected with EGFP-RAB26 and immunostained for LAMP1 (red). Arrowheads indicate areas of overlap (Cell border outlined, “N” – nucleus). Scale bars = 20 µm unless indicated.

Supplemental Figure 3. RAB26 does not affect Golgi or late endosomal distribution. Fluorescence microscopy of EGFP-RAB26, EGFP-RAB26T77N, EGFP-RAB26Q123L, and
EGFP transfected HGC-27 cells (green) co-stained for: **A.** giantin (red) and **B.** CI-M6PR (red). Low magnification panels of EGFP-RAB26 transfected cells highlight giantin and CI-M6PR distribution in transfected (green) and untransfected cells. Scale bars = 20 µm.

**Supplemental Figure 4. RAB26 does not affect lysosomal or mitochondrial function.** **A.** Western blots (triplicate experiments) of EGFP-RAB26, EGFP-RAB26T77N, EGFP-RAB26Q123L, and EGFP transfected HGC-27 cells comparing levels of LAMP1, cathepsin D, mTOR, phosphorylated S6 kinase, total S6 kinase, and tubulin. **B.** Fluorescence microscopy of EGFP-RAB26, EGFP-RAB26T77N, EGFP-RAB26Q123L, and EGFP transfected HGC-27 cells (green) co-stained for: **B.** MitoTracker CMXRos (red) and **C.** MitoSOX Red (red) and MitoTracker Deep Red (purple). Scale bars = 20 µm.

**Movie 1. RAB26 does not co-localize with secretory vesicles.** Live confocal timelapse microscopy of HGC-27 cells transfected with EGFP-RAB26 (green) and PGC-RFP (red). Images were taken at 3 second intervals and movie is displayed at 5 frames/second. White arrows highlight RAB26 vesicle movement. Yellow arrows and circles show vesicle fusion or fission events. Time stamp is shown in upper left corner and scale bar = 20 µm.

**Movie 2. RAB26 localizes with lysosomes in mitochondrial free subcellular areas.** Live confocal timelapse microscopy of HGC-27 cells transfected with EGFP-RAB26 (green) stained for lysosomes (LysoTracker, red) and mitochondria (MitoTracker Deep Red, Blue). Images were taken at 40 second intervals and movie is displayed at 3 frames/second. White arrows
highlight RAB26/LysoTracker vesicle movement. Time stamp is shown in upper left corner and scale bar = 20 μm.
CHAPTER 5: MIST1 Facilitates Secretory Vesicle Maturation by Regulation of Lysosomal Trafficking in Exocrine Secretory Cells
MIST1 facilitates secretory vesicle maturation by regulation of lysosomal trafficking in exocrine secretory cells

Ramon U. Jin¹, Greg Sibbel¹,², Stephen F. Konieczny³, Stuart Kornfeld¹, and Jason C. Mills¹,²,⁴

Division of Gastroenterology, Departments of ¹Medicine, ²Developmental Biology, ⁴Pathology & Immunology, Washington University School of Medicine, St. Louis, MO 63110

³Department of Biological Sciences, Purdue University, West Lafayette, IN 47907

Address correspondence to:
Jason C. Mills
Department of Medicine
Washington University School of Medicine, Box 8124
660 So. Euclid Ave.
St. Louis, MO 63110
Phone: 314 362-4258
FAX: 314 362-7487
e-mail jmills@wustl.edu

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Abstract

Exocrine cells like pancreatic acinar and zymogenic chief cells have a highly developed secretory architecture that is rapidly established upon maturation. The evolutionarily conserved transcription factor MIST1 governs normal apical secretory vesicle organization in these cells. These effects have been attributed to MIST1 inducing transcriptional targets that directly interact with the regulated secretory pathway. How MIST1 may influence trafficking of non secretory vesicles has yet to be explored. In mouse exocrine cells that are null for Mist1, we found disorganized subcellular distribution of lysosomes. Normally, lysosomes cluster in a centralized basal compartment away from secretory granules, whereas in Mist1\(^{-/-}\) exocrine cells, they accumulate apically, associate with secretory vesicles, and ultrastructural examination shows partially degraded cargo in lysosomes. Secretory vesicles in Mist1\(^{-/-}\) exocrine cells are smaller, though vesicle size is partially rescued by disrupting normal lysosomal hydrolase trafficking. Thus, we propose MIST1 promotes normal accumulation of secretory granules in part by protecting them from the cellular degradation/recycling machinery. These results illustrate how a transcription factor can regulate cell architecture and have implications for disease processes like acute pancreatitis where MIST1 is lost, and secretory vesicles are targeted by lysosomal enzymes.
Introduction

Survival of the whole organism is crucially tied to the function of each cell type that constitutes its tissues and organs. These functions are inherently tied to the form or subcellular architecture of these cells. The dramatic importance of this concept is best illustrated in single celled prokaryotes, in which all aspects of shape are actively honed for the survival of the cell in response to its environment (Young, 2006). In eukaryotes, form and function are collectively referred to as “differentiation”. In the last decade, it has become clear that a cell can dedifferentiate, effectively stripping it of its identity and function (Takahashi and Yamanaka, 2006). In fact, this cellular identity crisis is a common link in many diseases including diabetes, heart disease, and cancer (Kubin et al., 2011; Schwitalla et al., 2013; Talchai et al., 2012).

Specifically, loss of form and function is apparent in gastric and pancreatic cancer tumorigenesis. In both cases dedifferentiation has been well studied and shown to progress stepwise (Correa, 1992; Hezel et al., 2006) as mature cellular structure and function is lost.

In the pancreas and the stomach, studies have shown that the bHLH transcription factor, MIST1, is one of these factors that maintains a secretory cell’s functionality (Pin et al., 2001; Ramsey et al., 2007). Its loss has been found to directly render the cell more susceptible to injury, metaplasia, and carcinogenesis ((Alahari et al., 2011; Kowalik et al., 2007; Lennerz et al., 2010; Nam et al., 2010; Nozaki et al., 2008; Shi et al., 2013; Shi et al., 2009). Studies from our lab using an inducible model of gastric atrophy and metaplasia show that MIST1 is one of the first factors that is lost as mature zymogenic chief cells respond to injury and dedifferentiate in a process known as pseudopyloric metaplasia (Huh et al., 2012). Additional studies in the pancreas using an inducible MIST1 knockout model, reveal that within hours of MIST1 loss,
mature pancreatic acinar cells began to remodel their granules, cytoskeleton, and cellular junctions (Direnzo et al., 2012).

Functionally, MIST1 does not act as a traditional differentiating factor in that in its absence, cells still acquire mature characteristics but have compromised functionality (Capoccia et al., 2013; Direnzo et al., 2012; Johnson et al., 2004; Pin et al., 2001; Ramsey et al., 2007). In other words, MIST1 does not impart lineage-specific properties to those maturing cells that express it. For example, in the stomach, in the absence of MIST1, mature gastric zymogenic cells still express their usual array of endogenous secretory products (Bredemeyer et al., 2009). However, these products are no longer packaged, maintained, or secreted normally (Capoccia et al., 2013; Ramsey et al., 2007; Tian et al., 2010). Analogously, the MIST1 ortholog in *Drosophila* (called DIMM) is sufficient to confer many aspects of the regulated secretory pathway when expressed ectopically but does not change the content of the vesicles (Hamanaka et al., 2010; Park et al., 2011). Thus, MIST1 functions more to enhance the baseline secretory machinery that all cells already possess (i.e., it is a Scaling Factor (Mills and Taghert, 2012)).

To understand its importance in physiology and pathophysiology, the critical question becomes how MIST1 can effect substantial changes in cellular architecture by regulating expression of specific genetic targets. Separate studies have identified various direct MIST1 targets (Garside et al., 2010; Jia et al., 2008; Rukstalis et al., 2003) but how the targets come together to regulate an interconnected pathway is still unclear. MIST1 does control two small GTPases, RAB3D and RAB26 (Direnzo et al., 2012; Johnson et al., 2004; Tian et al., 2010). RAB3D has been shown to function in secretory vesicle maturation, and preliminary work based only on subcellular localization indicated a similar role for RAB26 (Millar et al., 2002; Nashida et al., 2006; Yoshie et al., 2000). However, recent studies from our lab have implicated a novel
role of RAB26 in lysosomal movement and trafficking (Jin and Mills, manuscript in preparation). Thus, MIST1 might directly affect the degradative compartment in secretory exocrine cells.

Here we dissect how a transcription factor might regulate the exocrine secretory apparatus by suppressing the degradative lysosomal machinery to prevent premature destruction of developing secretory granules. We show that, in addition to the reduction in secretory vesicle size and general granule disorganization seen in exocrine tissues in Mist1−/− mice, there is a dramatic increase in functional lysosomes. Normally in gastric zymogenic cells, these degradative vesicles are small, limited in number, and reside basally to large apical secretory vesicles. But in the absence of MIST1, they expand apically out of the basal compartment to engulf and degrade many of the forming vesicles. The phenomenon occurs rapidly upon MIST1 loss and persists for the lifetime of mature zymogenic cells in the absence of MIST1. Lysosome up-scaling occurs in all another exocrine secretory tissue, the pancreas, once MIST1 is lost. Lysosomal enzyme trafficking defective Gnptab−/− mice crossed to Mist1−/− showed partial rescue of secretory vesicle size through prevention of the lysosomal mediated secretory vesicle destruction in that there was a dramatic increase in undegraded secretory content inside the accumulated lysosomes. We conclude that MIST1 is a transcription factor that governs trafficking in more than one vesicular compartment. In other words, MIST1 in part increases stores of large secretory granules by suppressing their targeting to cellular degradative pathways.

Results

The bHLH Transcription Factor, MIST1, is Required for Large Granule Homeostasis
Previous reports have shown that MIST1 (Bredemeyer et al., 2009; Capoccia et al., 2013; Ramsey et al., 2007; Tian et al., 2010) is required for the formation of large secretory vesicles in gastric zymogenic cells. To confirm these results, we examined the structure of gastric units from WT and Mist1<sup>−/−</sup> mice. Both by H&E and transmission electron microscopy we found aberrant secretory architecture in Mist1<sup>−/−</sup> ZCs (Fig. 1A). WT mice displayed the typical apical cytoplasm containing large secretory vesicles (Fig. 1B, C). Mist1<sup>−/−</sup> mice lack this prominent apex and, displaying smaller, apically localized secretory vesicles and more basally mislocalized granules (Fig. 1B, C). In fact, when whole stomach levels of secretory vesicle constituents are examined, Mist1<sup>−/−</sup> mice show an almost fifty percent reduction in global quantities of pepsinogen C and gastric intrinsic factor (Fig. 1B, C). When quantified on a vesicle by vesicle basis, WT mice have a greater proportion of large secretory vesicles and bigger mean vesicle size (Fig. 6C, D) compared to vesicles seen in Mist1<sup>−/−</sup> mice.

**Lysosomes Accumulate and Engulf Secretory Vesicles in the Absence of MIST1**

To address this possibility that the granular aberrations could be due to increased destruction, we assayed for lysosomal distribution using the well-established LAMP1 membrane marker. We found that in WT mice, lysosomes were sparse in mature zymogenic cells, and those present were mostly basal, localized away from the apically maturing secretory vesicles (Fig. 2A-D). In Mist1<sup>−/−</sup> ZCs, however, we saw a dramatic increase in LAMP1 lysosomes. Again, most of these lysosomes were seen basal to the nucleus. But the basal compartment was greatly expanded by accumulations of large lysosomes, labeled with LAMP1, LAMP2, or Cathepsin D (Fig. 2A-D), while the nucleus was displaced to nearly the apical plasma.
membrane. Thus, in addition to secretory vesicular aberrations, Mist1−/− ZCs also display an alteration in their lysosomes.

We next investigated whether accumulation of lysosomes was responsible for the smaller secretory vesicles. Using transmission electron microscopy we surveyed multiple Mist1−/− ZCs and consistently found engulfed secretory vesicles characterized by electron density in various stages of degradation inside lysosomal structures, whereas neither degrading secretory vesicles nor increased lysosomes were seen in WT ZCs (Fig. 3A, B). Based on these results, we used confocal immunofluorescence microscopy to look for enhanced secretory vesicle-lysosome overlap in Mist1−/− ZCs. Consistent with our tEM findings, Mist1−/− ZCs displayed many regions, especially basal to the nucleus, where PGC containing vesicles resided within LAMP1 marked lysosomes (Fig. 3C, D). When quantified, almost 30% of all secretory vesicles in Mist1−/− ZCs displayed LAMP1 overlap (Fig. 6E). WT mice showed small dispersed lysosomal staining with no colocalization with PGC secretory vesicles (Fig 3D, 6E).

**Lysosome Accumulation occurs immediately upon MIST1 loss**

To test if lysosome accumulation was directly concomitant with MIST1 loss, first we took advantage of Mist1−/− mice in which the Mist1 null allele has a LacZ cassette knock-in (Pin et al., 2001), thereby allowing us to track when and where MIST1 would normally be expressed by charting expression of the Mist1 promoter (Fig. 4A). Importantly, during normal differentiation, ZCs terminally differentiate towards the base of each gastric unit from a non-MIST1 expressing mucous neck cell precursor. In the Mist1−/− animals the basic elements of this terminal differentiation step occur despite absence of MIST1 expression (Bredemeyer et al., 2009; Ramsey et al., 2007).
Our data show that upon emergence of these MIST deficient ZCs from their mucous secreting precursors, they accumulated lysosomes, and they did so as early as the Mist1 promoter became active (Fig. 4A). Furthermore, aberrant lysosomes persisted for the lifetime of the cell, because ZCs from the earliest to most mature at the very base of the gastric unit showed the same lysosomal phenotype (Fig. 2A-D, 3C, 4A).

In parallel experiments, we assayed the effects of conditionally knocking out MIST1 on the two vesicle compartments using the Mist1<sup>BT/BT</sup> mouse model. Briefly, these mice have knocked in an N-terminal BT tagged, C-terminal 6XHis-Myc tagged Mist1 and two loxP sites introduced via homologous recombination into the endogenous Mist1 locus. Upon crossing to Mist1<sup>CreERT</sup> mice (Shi et al., 2009), Mist1<sup>BT/Cre</sup> mice were generated in which the remaining allele of Mist1 is susceptible to tamoxifen induced cre recombinase excision to generate induced functional nulls. The Mist1<sup>BT/BT</sup> mouse served as a control in which both transgenic alleles of myc-tagged MIST1 are expressed (Fig. S1A, 4B, C). One week after tamoxifen induced Mist1 deletion in the Mist1<sup>BT/Cre</sup> mice, we saw a mosaic pattern of secretory vesicle and lysosome changes in cells losing MIST1 (Fig. S1B, 4B, C). Units that retained MIST1 expression, as marked by myc staining, also preserved robust secretory vesicle apical distribution and scarce basal scattered lysosomal as per WT and Mist1<sup>BT/BT</sup> mice (Fig. S1B, 4B, C). However, at two weeks after Mist1 deletion, all gastric units exhibited MIST1 loss, scant apical secretory vesicles, and accumulation of lysosomes (Fig. S1C, 4B, C). When quantified, average secretory vesicle size decreased and overlap of those vesicles with lysosomes increased to over 90% (Fig. 4C). These data show that the process of stomach lysosomal accumulation in Mist1<sup>−/−</sup> exocrine cells is not a gradual, additive, or indirect effect that occurs as the cells age. But rather, it is a rapid defect that ensues directly concomitant to failed induction of MIST1 expression.
Other Acinar Tissues also exhibit Lysosome Accumulation upon MIST1 loss

Because MIST1 is expressed and functions analogously in all exocrine secretory tissues (Lemercier et al., 1997; Pin et al., 2000), we investigate if this aberrant lysosomal accumulation occurred in these other tissues. To this end, we surveyed LAMP1 staining in formalin fixed paraffin embedded pancreatic sections using immunofluorescence microscopy. Consistent with gastric zymogenic cells, pancreatic acinar cells also display an increase in lysosomes and associated secretory vesicle disorganization (Fig. 5A). Upon closer examination, we observed increased lysosomal structures in the Mist1−/− mice by high-magnification, single cell confocal microscopy (Fig. 5B). By western blot we confirmed that in pancreatic acinar cells, Mist1−/− mice showed dramatic increase in lysosomal markers, LAMP1 and CTSD (Fig. 5C). CTSD has a distinctive pattern of degradation on western blot, which was increased in the absence of MIST1 indicating increased global lysosomal activity. Finally, we found using the Mist1BT/Cre mice that conditional knockout of MIST1, as in the stomach, induced rapid lysosomal accumulation in the pancreas (Fig. 5D). Of note, the pattern of MIST1 loss (mosaic loss at one week and complete acinar cell loss at two weeks post tamoxifen induced cre recombinase excision) paralleled the results we found in the stomach. Thus, loss of MIST1 causes the same increase in lysosomes in multiple exocrine secretory tissues, suggesting a shared mechanism of MIST1 mediated lysosomal suppression.

Disruption of Lysosome Degradative Capacity Rescues Mist1−/− Secretory Vesicle Destruction

If MIST1 functions to suppress lysosomes in acinar secretory tissues to protect maturing secretory vesicles, then if we disrupted lysosomal function in these cells we reasoned we should
see rescue of secretory vesicles from degradation (but not initial lysosome targeting), that would manifest as 1) an increase in vesicle size and 2) an increase in undegraded secretory vesicles inside lysosomes. Importantly, the degradative effects exist in concert with the secretory vesicle formation defects previously reported in the absence of MIST1 (Capoccia et al., 2013; Direnzo et al., 2012; Johnson et al., 2004; Pin et al., 2001; Ramsey et al., 2007), so we did not anticipate complete restoration to WT levels of secretory vesicle appearance. We decided to investigate mice with reduced lysosomal function due to genetic ablation of the α/β catalytic subunits of GlcNAc-1-phosphotransferase (Gelfman et al., 2007). This enzyme is involved in the addition of mannose 6-phosphate moieties to lysosomal acid hydrolases, and in its absence, mice exhibit reduced lysosomal function (Boonen et al., 2011; Vogel et al., 2009). Intriguingly, these Gnptab⁻/⁻ mice exhibit accumulation of undegraded vesicles at baseline, most predominantly in the exocrine secretory cells (Boonen et al., 2011; Vogel et al., 2009).

Histologic examination showed that the basal zymogenic cell regions of Mist1⁻/⁻Gnptab⁻/⁻ gastric units exhibited an increase in eosin staining compared to WT or Mist1⁻/⁻ (Fig. 6A), suggesting increased intracellular vesicular membranes. Western blot examination of whole stomach secretory vesicle components and markers of lysosomal function revealed that Mist1⁻/⁻Gnptab⁻/⁻ mice partially restored WT levels of secretory components (Fig. 6B). Accumulation of LC3 (indicative of autolysosomes) and aberrant cathepsin D processing was observed consistent with reported findings of lysosome and autolysosome abnormalities in Gnptab⁻/⁻ mice. Partial restoration to WT levels of secretory proteins in Mist1⁻/⁻Gnptab⁻/⁻ mice would be expected as MIST1 also coordinates secretory vesicle formation and maturation (Capoccia et al., 2013; Direnzo et al., 2012; Johnson et al., 2004; Pin et al., 2001; Ramsey et al., 2007).
To tease out the effects of lysosomal inhibition on secretory vesicles, we used confocal microscopy to examine multiple mice of each genotype (WT, \(\text{Mist1}^{−/−}\), \(\text{Gnptab}^{−/−}\), and \(\text{Mist1}^{−/−}\text{Gnptab}^{−/−}\)) for PGC granules and LAMP1 lysosomes (Fig. 6C). Subsequently, we analyzed each genotype for vesicle number, secretory vesicle area size, and engulfment by LAMP1 marked membranes (Fig. 6C-E). For WT mice, as expected, we saw that the majority (>58%) of secretory vesicles were large (1 µm or greater) with mean vesicle size of 1.17 µm, and essentially no lysosomal interaction (< 1% of vesicles) (Fig. 6C-E). In \(\text{Mist1}^{−/−}\) mice, in line with our reported findings, we saw a shift of vesicle distribution to smaller sizes (>52% of vesicles were 0.1-0.3 µm) with mean vesicle size dropping to 0.34 µm and an increase in lysosome overlap (28% of vesicles) (Fig. 6C-E). Scaled up lysosomal attack on secretory vesicles was more apparent for the larger secretory vesicles such that only 20% of secretory vesicles smaller than 0.7 µm were engulfed by lysosomes, whereas 75% of secretory vesicles larger than 0.7 µm were targeted to lysosomes (Fig. 6C-E). Interestingly, \(\text{Gnptab}^{−/−}\) mice alone exhibited a subtle shift towards smaller secretory vesicles and reduction in mean vesicle size (0.87 µm). Interactions with lysosomes increased to 15% compared to WT hinting at a possible baseline interdependent relationship of secretory vesicles and lysosomes (Fig. 6C-E). The lysosomal interactions in \(\text{Gnptab}^{−/−}\) mice occurred with all sizes of secretory vesicles uniformly. Finally, \(\text{Mist1}^{−/−}\text{Gnptab}^{−/−}\) mice displayed an upwards shift in vesicle size distribution (all binned vesicle sizes above 0.3 µm showed proportional increase in number compared to \(\text{Mist1}^{−/−}\)) and increased mean vesicle size (0.44 µm) compared to \(\text{Mist1}^{−/−}\) mice (Fig. 6C, D). A dramatic increase in secretory vesicle-lysosome interaction also occurred in these mice with 59% of secretory vesicles positioned inside lysosomes (Fig. 6E). In addition, the preferential overlap of larger secretory vesicles with lysosomes seen in \(\text{Mist1}^{−/−}\) mice was abrogated in
Mist1−/−Gnptab−/− mice (55% of vesicles smaller than 0.7 µm and 76% of vesicles larger than 0.7 µm were lysosome associated). These data suggest that in Mist1−/− all vesicles are targeted to lysosomes and degraded, especially larger vesicles. But small vesicles are more rapidly and efficiently degraded because of their size. Upon inhibition of lysosomal function, this entire process is slowed, and emergence of these smaller group of vesicles is apparent as evidenced by increased lysosomal overlap and a size distribution shift for the small vesicles in the Mist1−/−Gnptab−/− mice. These results were corroborated by staining for other markers of secretory vesicles (gastric intrinsic factor) and lysosomes (cathepsin D) (Fig. S2A, S3A), and examination of another exocrine secretory tissue (Fig. S2B, S3B). Taken together, our data show that MIST1 functions to suppress lysosomal accumulation and resulting degradative function in maturing exocrine cells. Loss of MIST1 resulted in immediate and active degradation of secretory vesicles. These vesicles are restored upon inhibition of lysosome function in the Mist1−/−Gnptab−/− mice revealing the extent of Mist1−/− vesicle destruction.

Discussion

The idea that specific cellular organelles and/or subcellular pathways might be under direct transcriptional control is not a novel concept (Nunnari and Walter, 1996); however only recently have clear examples been elucidated (Mills and Taghert, 2012). In fact, new advances have revealed elegant examples of this concept as applied to lysosomes. The basic helix-loop-helix (bHLH)-Zip transcription factor, TFEB, was demonstrated to control the expression of a network of lysosomal genes that augments lysosomal number and degradative function (Sardiello et al., 2009). Elegantly, the same group showed that levels of TFEB, and thus degradative capacity, are directly regulated by extracellular nutrient signaling pathways
GATA1, an important hematopoietic transcription factor, has been shown to induce several lysosomal genes and the degradative function of maturing erythroblasts (Kang et al., 2012). In addition, the *caudal*-type homeo box transcription factor, CDX2, with known roles in gut maturation (Verzi et al., 2011), induces lysosomal genes and trafficking proteins to establish proper cell polarity (Gao and Kaestner, 2010). These examples highlight that all mature cells might need to strictly regulate autodegradative function. Regulation of intracellular degradative pathways may be especially crucial during times of development when cells engage in dramatic reorganizations of cellular architecture, and, conversely, it may be critical for mature, long-lived cells to have mechanisms to suppress autodegradation.

For example, as exocrine secretory cells assemble into high-output secretory machines, it would be expected that they would need to strictly regulate cellular degradative processes. The transcription factor, MIST1, is a critical mediator of multiple, mostly exocrine, long-lived secretory cell function (Capoccia et al., 2011; Lemercier et al., 1997; Pin et al., 2000). In all tissues, those cells retain the ability to terminally differentiate even when MIST1 is lost. However, MIST1-deficient cells exhibit a dramatic disorganization in secretory vesicle homeostasis, with markedly smaller secretory granules and decreased response to stimulated exocytosis. The secretory apparatus-regulating, scaling factor function of MIST1 is highly conserved evolutionarily. Loss of function experiments in Zebrafish (Guo et al., 2007) and gain of function studies with the *Drosophila* ortholog of MIST1, DIMMED (Hamanaka et al., 2010; Hewes et al., 2003; Park et al., 2011; Park et al., 2008), confirm MIST1/DIMM is sufficient and required to establish pro-secretory cell function across evolution. We and others have assumed that MIST1/DIMM would establish/maintain stores of secretory granules by regulating levels of targets directly involved in granule formation or trafficking (Capoccia et al., 2013; Johnson et al.,
2004; Rukstalis et al., 2003; Tian et al., 2010). Understandably, accumulation of degradative vesicles in the pancreas and seminal vesicles of MIST1-deficient mice was previously attributed as secondary to aberrant regulation of secretion (Pin et al., 2008; Pin et al., 2001).

Thus, our results here are unexpected and are the first to suggest that a key, if not the salient, way that MIST1 maintains secretory granules is by suppressing their destruction. That mechanism of MIST1 would explain at least two previous observations. Clearly, it suggests that the induction of degradation in Mist1−/− acinar cells (Pin et al., 2008; Pin et al., 2001) might be directly due to aberrant lysosomal trafficking, rather than a secondary phenomenon. Also, it would explain why transitional cells in Mist1−/− mice that are just beginning to emerge from neck cell progenitors with Mist1 promoter expression show abundant, if not increased, stores of normal-sized granules, in Mist1−/− mice relative to wildtype (Bredemeyer et al., 2009; Huh et al., 2010; Ramsey et al., 2007). It is possible that MIST1 is not required solely for granule formation, but rather functions in concert to also suppress the normal cellular autodegradative attack on long-lived vesicles needed for mature granule maintenance (Fig. 7).

Whatever the teleological reason, there is clearly aberrant interaction between the secretory and the degradative compartments in Mist1−/− mice. There are several possible explanations why this occurs, based on our in vitro finding that RAB26 repositions lysosomes centrally. Interestingly, centralization of the degradative machinery has been recently found to augment secretory function and facilitate efficiency of secretion (Narita et al., 2011). Lack of MIST1 and thus, RAB26, as a centralizing force, will by default move lysosomes further to the cell periphery, where their proximity with mature secretory vesicles would increase, resulting in direct degradation of the secretory granules by the process deemed crinophagy (Klionsky et al., 2007; Marsh et al., 2007) or possibly by a microautophagic like process (Mijaljica et al., 2011;
Similarly, MIST1, through RAB26, may inhibit direct autophagosome targeting of zymogenic granules for ultimate autolysosome degradation, recently termed zymophagy (Grasso et al., 2011) by separating the macroautophagy and lysosomal machinery. Alternatively, lysosomal machinery may normally associate with secretory vesicles, and RAB26 may be required for sorting lysosomal components from maturing secretory vesicles. Multiple reports have shown that mechanistic defects or alterations in the normal maturation process or trafficking of various vesicles or membrane-associated proteins will result in aberrant interaction of contents across vesicular compartments that are normally spatiotemporally separate (Arvan and Castle, 1998; Blott and Griffiths, 2002; Boonen et al., 2011; Hunziker and Geuze, 1996; Kogel and Gerdes, 2010; Meister et al., 2010; Morvan and Tooze, 2008; Saftig and Klumperman, 2009).

Furthermore, our work with RAB26 has also implicated its role in mitochondrial reorganization. These effects are also seen in vivo. In the absence of MIST1, both mitochondrial organization and mitochondria mediated calcium signaling were effected resulting in secretory defects (Luo et al., 2005). Because calcium signaling is intimately tied to the secretory pathway (Rizzuto et al., 2012), the effects of MIST1 on secretory vesicles may be even more complex then suppression of lysosome degradation. The ultimate function of MIST1 may be to enhance some compartments (secretory pathway), shrink and sequester other parts (degradative lysosomes), and re-organize ancillary components to facilitate and maintain physiological operation (mitochondria mediating calcium flux).

It is unlikely that RAB26 is the sole direct target to facilitate the MIST1 mediated lysosomal effects. Other direct MIST1 targets may play complementary roles in these same pathways. In fact, studies have found that the MIST1 target SPCA2 mediates calcium signaling
and work from our lab has shown the MIST1 target MIB1 coordinates digestive enzyme cargo accumulation in secretory vesicles – but not their size – via its effects on endolysosomal trafficking (Capoccia et al., 2013). Additional, yet uncharacterized direct MIST1 targets may also be important. From our previous studies, we identified targets with potential roles in vesicle maturation and trafficking, including ACP2 (a regulator of lysosome function), CCPG1 (promotes CDC42 activation by regulating DBS), ARRDC3 (membrane protein with known roles in cell proliferation), and FIGN (AAA protein involved in membrane fusion and endosome sorting) (Tian et al., 2010). Clearly, MIST1 induces expression of multiple factors to effect various vesicle, cytoskeletal, and organelle compartments through direct and indirect interactions, all in order to facilitate and maintain the dynamic and highly organized secretion apparatus.

In a differentiated exocrine cell, aside from protecting maturing secretory vesicles, what other purpose does MIST1 mediated suppression of lysosomal function and localization serve? Perhaps, MIST1 functions in mature cells to suppress the innate cellular defense mechanism that cells mount against intracellular pathogens that hijack vesicles for latent infection (Levine et al., 2011). In gastric epithelial cells, *Helicobacter pylori*, the pathogen linked to gastritis and stomach cancer development has been postulated to persist within autophagic vesicles via its VacA toxin (Raju et al., 2012; Terebiznik et al., 2009; Terebiznik et al., 2006); likely this occurs through subversion of lysosome fusion. In the exocrine pancreas, acute pancreatitis involves damaging auto activation of secretory enzymes (Gaiser et al., 2011). Lysosomal enzymes have been shown to activate zymogen constituents of secretory granules (Halangk et al., 2000; Saluja et al., 1997). Interestingly, loss of MIST1 and thus the lysosome suppression, results in increased susceptibility to pancreatitis (Alahari et al., 2011; Kowalik et al., 2007; Zhu et al.,
Those examples demonstrate that precise control of lysosomal function should be critical for all acinar cells. The mechanism described here will likely be a component of a larger network of MIST1 regulated structural and organizational genes that influence additional aspects of acinar cell secretory function.

Materials and Methods

Mice

All experiments involving animals were performed according to protocols approved by the Washington University School of Medicine Animal Studies Committee. Germ line Mist1−/− mice (Pin et al., 2001) were maintained in a specific-pathogen-free barrier facility. Control mice were the littermates of Mist1−/− mice resulting from heterozygote-by-heterozygote crosses. Mist1BT/BT mice (a kind gift from Dr. Stephen Konieczny) were generated via inserting an N-terminal BT tagged, C-terminal 6XHis-Myc tagged Mist1 and two loxP sites into the endogenous Mist1 locus. Mist1CreERT mice were generated as previously described (Shi et al., 2009). Mist1BT/Cre mice were generated by het crossing of Mist1BT/Cre mice generated from breeding Mist1BT/BT mice with Mist1CreERT mice; mice at 8 weeks of age were given 5 consecutive daily intraperitoneal injections of tamoxifen (1 mg/20 g BW; Sigma-Aldrich). Littermate Mist1BT/BT mice served as the controls in these experiments. Gnptab−/− mice (a kind gift from Dr. Stuart Komfeld) were generated as described (Gelfman et al., 2007). Mist1−/−Gnptab−/− were generated from crossing Mist1+/−Gnptab+/− mice. Other genotypes (Mist1+/+Gnptab+/+, Mist1−/−Gnptab+/+, and Mist1+/+Gnptab−/−) served as littermate controls. Mist1+/+Gnptab+/+ and Mist1−/−Gnptab+/+ were phenotypically the same as WT and Mist1−/− mice, respectively.
**Immunofluorescence / Imaging**

Stomachs were prepared and stained as described previously (Ramsey et al., 2007). Briefly, stomachs were inflated with freshly prepared methacarn fixative and suspended in fixative for 15 to 30 min at room temperature or 10% formalin fixative for 12 hrs at 4°C, followed by multiple rinses in 70% ethyl alcohol (EtOH), arrangement in 2% agar in a tissue cassette, and routine paraffin processing. Pancreases were dissecting and suspended in 10% formalin fixative for 12 hrs at 4°C, 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 antigen retrieval was performed by boiling in Trilogy (Cell Marque, Hot Springs, AR) or 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 primary antibodies followed by secondary antibodies (see below). Slides were incubated for 5 min in 1 µg/ml bisbenzimide (Invitrogen, Carlsbad, CA) prior to mounting in Cytoseal XYL (Rishard-Allan Scientific, Kalamazoo, MI).

Fluorescence microscopy and imaging were performed using a Zeiss Axiovert 200 microscope 20X (Plan-Apochromat, 0.8 NA), 40X (Plan-Neofluar 0.85 NA) and 63X objectives (Plan-Apochromat, 1.4 NA) with Axiocam MRM camera and AxioVision software. Additional, confocal microscopy and imaging were performed using a Zeiss LSM510 META microscope with 40X (EC Plan-Neofluar, 0.75 NA) and 63X (Plan-Apochromat, 1.4 NA) objectives using LSM510 software. Contrast (maximal, minimal, and midtone) adjustment and fluorescent channel overlay and pseudocoloring were performed with Photoshop (Adobe Systems, San Jose, CA). All adjustments were performed on the entire image equally. Cartoon cell traces and illustrations were produced using Illustrator (Adobe Systems, San Jose, CA).
The primary antibodies used for mouse tissue immunostaining: rat anti-LAMP1 (1:250, clone 1D4B, developed by J. Thomas August and obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD maintained by The University of Iowa), mouse anti-β-Gal (1:500, Promega, Madison, WI), sheep anti-PGC (1:1000, Abcam, Cambridge, MA), mouse anti-c-Myc (1:200, clone 9E 10, developed by J. Michael Bishop and obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD maintained by The University of Iowa), rabbit anti-amylase (1:100, Calbiochem, CA), rabbit (1:10,000) and goat (1:2000) anti-human gastric intrinsic factor (gifts of Dr. David Alpers, Washington University), rat anti-LAMP2 (1:40), rabbit anti-CTSD (1:200), and rabbit anti-CI-M6PR (1:500) (gifts of Dr. Stuart Kornfeld, Washington University). Secondary antibodies used were Alexa Fluor 488-, 594-, and 647-conjugated donkey anti-goat, anti-rabbit, anti-sheep, and anti-mouse antibodies (1:500; Invitrogen).

For immunohistochemical analyses, stomach sections were deparaffinized as stated above and subsequently immersed in 3% H$_2$O$_2$ to quench endogenous peroxidase activity. Antigen retrieval was performed as above. Avidin and biotin were applied consecutively to prevent endogenous biotin related background staining. Sections were incubated with 1:250 mouse anti-LAMP1 (clone 1D4B) overnight followed by incubation for one hour with biotinylated goat anti-mouse Abs (Santa Cruz). After rinsing (0.1% Tween-20), tissue sections were incubated with horseradish peroxidase-conjugated streptavidin (Dako) for 20 minutes at room temperature. Slides were washed (0.1% Tween-20), and the chromogen was developed for 5 minutes with liquid 3,30-diaminobenzidine (DiNonA, Seoul, Korea) before counterstaining with Meyer’s hematoxylin. After dehydration sections were mounted as described.
**Immunofluorescence Quantification**

Secretory vesicle quantification was performed on immunostained stomachs for PGC and LAMP1 as prepared above. Images were analyzed in ImageJ as follows. PGC secretory vesicles were traced using circle tool and tracked using ROI manager. After all vesicles were traced, individual areas were measured using the Analyze area measurement tool. Using ROI manager, traced secretory vesicles were overlayed onto LAMP1 stained images and vesicle/LAMP1 overlap was noted for each of the traced vesicles. Three WT, Mist1\(^{-/-}\), Gnptab\(^{-/-}\), and five Mist1\(^{-/-}\)Gnptab\(^{-/-}\) mice were analyzed.

**Western Blot Analysis**

Tissue was flash frozen in liquid nitrogen and homogenized in RIPA buffer using a PowerGen 700 (Fischer Scientific, Pittsburg, PA). Proteins were then separated on NuPAGE Bis-Tris gels (Invitrogen), transferred to Immobilon polyvinylidene difluoride (Millipore, Bedford, MA) or Amersham Hybond ECL nitrocellulose (GE Healthcare, Buckinghamshire, United Kingdom) membranes, and detected by Immobilon chemiluminescence (Millipore, Billerica, MA). Primary antibodies used were mouse anti-LAMP1 (1:1000, clone H4A3, developed by J. Thomas August and James E. K. Hildreth and obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD maintained by The University of Iowa), rabbit anti-CTSD (1:2000, gift of Dr. Stuart Kornfeld, Washington University), sheep anti-Pepsinogen C (1:3000, Abcam), mouse anti-LC3 (1:1000, Cosmo Bio, Carlsbad, CA), and rabbit anti-Tubulin (1:2000, Cell Signaling Technology, Beverly, MA). Secondary antibodies were horseradish peroxidase–conjugated donkey anti-rabbit, anti-rat and anti-mouse (Santa Cruz Biotechnology, Santa Cruz, CA).
PGC and GIF Western blot intensity were quantified using ImageJ and normalized to actin control band intensities.

Transmission Electron Microscopy

For transmission electron microscopy (TEM) studies, stomachs were fixed, sectioned, stained, and imaged as described previously (Ramsey et al., 2007).

Graphing and Statistics

All graphs and statistics were determined with GraphPad Prism and then visualized using Adobe Illustrator. Statistical analysis was, in the case of simple control-versus-experimental condition comparison, by Student’s t test. Otherwise, significances were determined by a one-way analysis of variance (ANOVA) test with Dunnett’s multiple-comparison correction.

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REFERENCES


Figure Legends

Figure 1. MIST1 is required for large secretory granule homeostasis. A. H&E micrographs of representative gastric units (outlined in black) from WT and Mist1−/− mice with transmission electron micrographs highlighting the basal zymogenic cell zones. Immunofluorescence staining of WT and Mist1−/− gastric units with zymogenic cell secretory markers: B. pepsinogen C (PGC, red) and C. gastric intrinsic factor (GIF, purple). Representative units are outlined in black and parietal cells which do not express ZC secretory markers are shown in yellow. Western blots for PGC and GIF levels between WT and Mist1−/− stomachs from triplicate experiments are shown below with quantification of signal normalized to actin. (“*” – p <0.05, “**” – p <0.01, two-tailed Student’s t-test). Scale bars = 20 µm unless indicated.

Figure 2. Gastric zymogenic cells show accumulation of lysosomes in the absence of MIST1. A. Fluorescence microscopy of an outlined WT gastric unit showing only light, perinuclear LAMP1-positivity (green) in ZCs (stained red for PGC and purple for GIF expression) compared to the bright diffuse LAMP1 staining ZCs from a Mist1−/− gland. Yellow tracing demarcates parietal cells which do not express MIST1 and do not change in LAMP1 staining between WT and Mist1−/− gastric glands, thus serving as internal controls for relative immunofluorescence intensity. B. Immunohistochemistry for LAMP1 in WT and Mist1−/− gastric glands. Two representative units highlighted in black and parietal cell staining control outlined in yellow. Epifluorescence staining of WT and Mist1−/− gastric glands for additional lysosomal markers: C. cathepsin D (CTSD, green) and D. LAMP2 (green) co-stained with GIF (red). Glands are demarcated in white with parietal cells shown in yellow. Scale bars = 20 µm.
Figure 3. *Mist1*−/− ZCs display degraded secretory vesicles inside accumulating lysosomes.  
A. Transmission electron micrograph of WT ZCs with large apical secretory vesicles and basally located nuclei.  
B. tEM of *Mist1*−/− ZCs with a reduction in large apical vesicles and an increase in degrading secretory granules. Panels represent higher magnification of boxed regions containing vesicles (arrows) in various states of degradation (later stages “*”)).  
C. Confocal sections of WT and *Mist1*−/− basal gastric units (outlined) stained for LAMP1 (green) and PGC (red).  
D. Higher magnified region of *Mist1*−/− ZCs through multiple serial optical sections. “*” indicates LAMP1 labeled lysosomal structures with various stages of degraded PGC entrapped. Scale bars = 20 µm unless indicated.

Figure 4. Lysosomes accumulate rapidly in the absence of MIST1.  
A. In *Mist1*−/− mice, which have a LacZ cassette inserted into the endogenous *Mist1* locus, the transitional cells where MIST1 should be expressed can be identified by nuclear β-galactosidase expression (red).  
Left inset – Higher magnification of the boxed region with nuclei in blue. Note that staining for LAMP1 (green) is absent in mucus precursor neck cells (NC, “*” marks their nuclei), becomes stronger in the earliest transitional cells that begin to express the *Mist1* promoter (arrows) and continues to be strong in all mature ZC cells trying to express *Mist1* (arrowheads).  
B. Confocal images of *Mist1*BT/BT and *Mist1*BT/Cre mice (see Materials and Methods) treated with tamoxifen after one or two weeks stained for LAMP1 (green) and PGC (red). Units are highlighted.  
C. Quantification of confocal immunofluorescent microscopy gastric units shown in B. PGC secretory vesicles were counted and areas measured, and percentage of vesicles with LAMP1 overlap was determined. Mean vesicle size is indicated in red. Scale bars = 20 µm.
Figure 5. **Mist1**\(^{-/-}\) pancreatic acinar cells have lysosomal accumulations.  

A. Fluorescence microscopy of WT and Mist1\(^{-/-}\) mice stained for amylase (red) and LAMP1 (green). Acini are outlined in white.  

B. Confocal image of a single Mist1\(^{-/-}\) pancreatic acinar cell showing the large vesicular nature of the lysosomes (“*” in lumen of individual vesicles, “N” – nucleus).  

C. Western blot analysis of two wildtype and two Mist1\(^{-/-}\) mice showing protein levels of LAMP1, cathepsin D, and tubulin.  

D. Immunofluorescence of Mist1\(^{BT/BT}\) and Mist1\(^{BT/Ct}\) mice treated with tamoxifen after one or two weeks stained for LAMP1 (green) and c-Myc (purple). Acini are outlined in white. Scale bars = 20 µm.

Figure 6. Inhibition of lysosome degradation partially rescues Mist1\(^{-/-}\) secretory vesicles.  

A. H&E histology panel of WT, Mist1\(^{-/-}\), Gnptab\(^{-/-}\), and Mist1\(^{-/-}\)Gnptab\(^{-/-}\) gastric units (labeled in black, parietal cells outlined in yellow).  

B. Western blots of multiple mice for each of the indicated genotypes (WT, Mist1\(^{-/-}\), Gnptab\(^{-/-}\), and Mist1\(^{-/-}\)Gnptab\(^{-/-}\)) probed for PGC, LC3, cathepsin D, and tubulin.  

C. Confocal immunofluorescence images of WT, Mist1\(^{-/-}\), Gnptab\(^{-/-}\), and Mist1\(^{-/-}\)Gnptab\(^{-/-}\) mouse stomachs stained for LAMP1 (green) and PGC (red). Gastric units highlighted in white. For each genotype, multiple mice were stained, PGC containing secretory vesicles were counted and sizes measured, and PGC LAMP1 vesicle overlap was quantified (see Materials and Methods). Binned percentage of total secretory vesicles (red bars) for each binned size (from 0.1 to 1.0 and greater µm) is graphed below. For each red bar, proportion of those secretory vesicles engulfed by LAMP1 lysosomes is shown to the right (green bars).  

D. Graphed PGC vesicle areas and numbers for multiple WT, Mist1\(^{-/-}\), Gnptab\(^{-/-}\), and Mist1\(^{-/-}\)Gnptab\(^{-/-}\) immunostained murine stomachs (Averaged vesicle size for
multiple mice for each genotype shown in red).  E. Percentage of PGC vesicles enclosed in LAMP1 lysosomes for multiple WT, Mist1^{−/−}, Gnptab^{−/−}, and Mist1^{−/−}Gnptab^{−/−} immunostained murine stomachs. Scale bars = 20 µm.

**Figure 7. MIST1 induces direct targets to reorganize subcellular architecture.** All WT ZCs (left) initiate and maintain expression of MIST1. Subsequent expression of Rab26 and Rab3D along with other yet unidentified MIST1 transcriptional targets (labeled “A” and “B”) is also induced. RAB26 functions to maintain lysosomes in a basal perinuclear distribution and RAB3D serves to mature secretory vesicles. This ensures proper segregation of the destructive basal lysosomes from the maturing apical secretory vesicles. In the absence of MIST1 (right), and thus no direct target induction, ZCs have lysosomes that are no longer maintained basally coupled with a secretory vesicle formation defect. Together, this manifests as more aberrant lysosomal-secretory vesicle interactions and overall smaller, more disorganized secretory vesicles in the Mist1^{−/−} ZCs.
Supplemental Figure Legends

Supplemental Figure 1. Conditional deletion of Mist1 results in lysosomal accumulation. Immunofluorescence of a conditional Mist1 knockout model (See Materials and Methods) stained for LAMP1 (green), PGC (red), and c-Myc (purple). A. Mist1\textsuperscript{BT/BT} represents the control condition, in two copies of an N-terminal BT tagged, C-terminal 6XHis-Myc tagged Mist1 and two loxP sites were introduced via homologous recombination into the endogenous Mist1 locus. Mist1\textsuperscript{BT/Cre} mice were generated by crossing Mist1\textsuperscript{BT/BT} with Mist1\textsuperscript{CreERT} mice, the Mist1 loci in excised by 5 consecutive intraperitoneal injections of tamoxifen (1 mg/20 g BW; Sigma-Aldrich), and phenotypes were examined at B. one and C. two weeks. Units expressing Mist1 detectable by c-Myc staining (purple) are outlined in white (present in all Mist1\textsuperscript{BT/BT} stomach; mosaic pattern of deletion in one week post tamoxifen injected Mist1\textsuperscript{BT/Cre} mice) and parietal cells are outlined in yellow. Scale bars = 20 µm.

Supplemental Figure 2. Inhibition of lysosome degradation partially rescues secretory vesicles in Mist1\textsuperscript{−/−} gastric ZCs and pancreatic acinar cells. A. Immunofluorescence images of WT, Mist1\textsuperscript{−/−}, Gnptab\textsuperscript{−/−}, and Mist1\textsuperscript{−/−}Gnptab\textsuperscript{−/−} mouse stomachs stained for LAMP1 (green) and PGC (red). Gastric units are outlined in white. B. Immunofluorescence images of WT, Mist1\textsuperscript{−/−}, Gnptab\textsuperscript{−/−}, and Mist1\textsuperscript{−/−}Gnptab\textsuperscript{−/−} murine pancreases stained for amylase (red) and LAMP1 (green). Acini are outlined in white. Scale bars = 20 µm.

Supplemental Figure 3. Other markers show partial rescue of secretory vesicles in Mist1\textsuperscript{−/−} gastric ZCs and pancreatic acinar cells. A. Immunofluorescence images of WT, Mist1\textsuperscript{−/−},
Gnptab<sup>−/−</sup>, and Mist1<sup>−/−</sup> Gnptab<sup>−/−</sup> mouse stomachs stained for cathepsin D (green), LAMP1 (red), and GIF (purple). Gastric units are outlined in white. **B.** Immunofluorescence images of WT, Mist1<sup>−/−</sup>, Gnptab<sup>−/−</sup>, and Mist1<sup>−/−</sup> Gnptab<sup>−/−</sup> murine pancreases stained for cathepsin D (green) and LAMP1 (red). Acini are outlined in white. Scale bars = 20 µm.
Supplemental Figure 1

A

Mist1^{BT/BT} LAMP1 PGC

B

Mist1^{BT/CreER} 1 week

C

Mist1^{BT/CreER} 2 week
CHAPTER 6: Conclusions and Future Directions
As a whole, this work has focused on the role of MIST1 and its direct transcriptional target RAB26 in zymogenic chief cells of the stomach. Chief cells are an ideal system to study transcriptional regulation of subcellular organization because 1) they are organized spatio-temporally within the stomach, 2) they have a well-structured secretory architecture, and 3) establishment of their secretory function occurs rapidly from a relatively long-lived developmental precursor. Using complementary in vivo mouse genetic approaches and in vitro cell culture techniques, I have uncovered a MIST1 regulatory network that is crucial for establishment and maintenance of secretory cell architecture in all exocrine tissues.

**MIST1 Induces a Gene Network for Integrated Control of Secretion**

In Chapter 2, I show that MIST1 directly induces the expression of 16 genes and that it directly binds highly-conserved first intronic CATATG E-boxes in 6 of those genes. Focusing on two targets, RAB3D and RAB26, I show that they are expressed in gastric ZCs in the corpus of mouse stomach in a MIST1-dependent manner. To address the function of these two small protein GTPases, I developed a MIST1 dependent secretory vesicle formation system, in which gastric epithelial cells stably expressing MIST1 produce large ZC-like secretory granules upon transfection with RFP-tagged pepsinogen C (PGC). In contrast, control cell lines expressing GFP lacked the ability to form large granules when given the same secretory load. Using this system, I confirm that expression levels of RAB26 and RAB3D were also specifically induced in the MIST1 overexpressing cell lines. Finally, I show that formation of the large granules in these cells is dependent on MIST1-induced Rab activity. Granule formation is abrogated by disrupting Rab prenylation and overexpression of dominant negative RAB26 mutant. These data show that MIST1 activates a network of genes including RAB26 and RAB3D, to enact mature secretory
cell architecture, organize vesicle trafficking, and maintain production of large secretory granules.

**Future Directions**

From the list of direct MIST1 targets, we only have yet explored the functions of two of those genes. I chose these two genes, RAB3D and RAB26, because they stood out as representing a possible mechanism by which MIST1 could regulate formation of exocrine secretory granules in differentiating cells. Because these targets were generated from two gastric cell lines, I wanted to confirm their *in vivo* relationship with MIST1 expression. To this end, I performed laser capture microdissection (LCM) to isolate zymogenic cells (ZCs) and precursor neck cells (NCs) from nine WT and nine *Mist1*−/− mice (Capoccia et al., 2013). A pooled sample of RNA from four mice for each group (WT NC, WT ZC, KO NC, and KO ZC) was sent for gene expression profiling with Affymetrix Mouse Gene 1.0ST arrays. Upon analyses, I found MIST1 dependent expression patterns, in which expression was preferentially restricted to WT ZCs, for 8 of the 16 genes (Fig. 1A).

Among these *in vivo* MIST1 targets were two genes, CCPG1 and SERPINI1, for which we had previously shown direct MIST1 binding by chromatin immunoprecipitation and that they were induced in cell culture by MIST1 overexpression (Tian et al., 2010). In collaboration with Dr. Xiaolin Tian, I confirmed these Genechip results using *in situ* hybridization to show specific expression of both CCPG1 and SERPINI in zymogenic cells from wildtype mice only (Fig. 1B). Further support for these two targets includes immunofluorescence microscopy indicating protein expression patterns that match our gene expression and *in situ* hybridization results (Fig.
These data indicate that CCPG1 and SERPINI1 are direct MIST1 targets that are expressed in mature zymogenic cells.

The published functions of both these genes warrant further study of their roles in zymogenic cell biology. In the only study of its function, CCPG1 was found to bind and regulate the activity of the RhoA and CDC42 guanine nucleotide exchange factor (Kostenko et al., 2006). Rho GTPases are key regulators of the actin cytoskeleton (Hall, 1998), and may reveal a yet unstudied function of MIST1. The morphology of Mist1−/− zymogenic cells includes a disorganized and contracted apical cytoplasmic region resulting in luminal expansion (Bredemeyer et al., 2009; Tian et al., 2010). CCPG1 may represent a mechanism by which MIST1 controls the cytoskeletal rearrangements necessary to expand the apical cytoplasm.

SERPINI1 or NEUROSERPIN functions as a secreted serine proteinase inhibitor and regulator of cell adhesion whose role is has been extensively studied in the nervous system (Kinghorn et al., 2006; Lee et al., 2008; Osterwalder et al., 1998). Mutations in SERPINI1 affecting conformational stability results in a familial form of dementia (Davis et al., 2002; Davis et al., 1999). These studies suggest the possibility of novel roles of MIST1 in the brain. Unpublished work from our group and other studies (Luo et al., 2005) have shown MIST1 expression in the brain where it retains its ability to regulate secretory function. In addition, our collaborator, Dr. Paul Taghert, has comprehensively studied the role of DIMM, the Drosophila ortholog of MIST1, in the fly brain (Hamanaka et al., 2010; Hewes et al., 2003; Park et al., 2011; Park et al., 2008a; Park and Taghert, 2009; Park et al., 2008b). On top of its well established neuronal function, SERPINI1 has recently been implicated to have tumor suppressive effects in gastric cancer (Yamanaka et al., 2012). Clearly, these data suggest that SERPINI1 may have an important physiological role in MIST1 expressing tissues including the stomach.
Additional work should examine CCPG1 and SERPINI1 using the tools that we have already developed. The roles of these two proteins should be examined in secretory vesicle formation using the MIST1-stably expressing PGC-RFP system. Overexpression and knockdown experiments should reveal novel secretion effects. In addition, other subcellular compartments should be examined including lysosomes and mitochondria. We can also apply our AR42J differentiation system to these two MIST1 targets by using these polarized cells to mimic \textit{in vivo} development. This system will further confirm and reveal structural effects (e.g., actin cytoskeletal and cell-cell junctional defects) that might be induced by overexpression or knockdown of these two targets. These functional experiments will augment our preliminary data (Fig. 1) to assign functional roles to these two proteins; adding two more genes to our network of MIST1 regulated genes. Ultimately, a murine model for CCPG1 deficiency should be engineered, and the gastric phenotype for the SERPINI1 knockout mice (Gelderblom et al., 2013) needs to examined to see if any component of the \textit{Mist1}^{−/−} phenotype is recapitulated.

An additional intriguing target that has been confirmed in our gastric cells and our \textit{in vivo} studies is Lysosomal Acid Phosphotase (ACP2). This enzyme is involved in lysosome homeostasis with a role in dephosphorylation of M6P containing acid hydrolyases (Makrypidi et al., 2012). Deficiency in mice leads to formation of lysosomal accumulations (characterized by enhanced LAMP1 and cathepsin D immunostaining) in many tissues (Mannan et al., 2004; Saftig et al., 1997).

Finally, the \textit{in vivo} Genechip data might prove to more informative than our \textit{in vitro} genechip results, specifically by providing us with a more physiologically relevant list of MIST1 targets (Capoccia et al., 2013). More work needs to be done with this data to analyze, refine, and add to our current list of MIST1 regulated genes.
RAB26 Functions to Reorganize Lysosomes and Prune Mitochondria

In Chapter 3, I present work showing that RAB26 expression in tissues and cells depends on MIST1 induction. In contrast to previous studies, I show direct association of RAB26 not with secretory vesicles but with lysosomes. By immunofluorescent microscopy, both endogenous and EGFP-tagged RAB26 localize to a LAMP1 and cathepsin D positive lysosome compartment. These data were confirmed by immunoelectron microscopy, gradient density cell fractionation, and live imaging of vesicle dynamics. Furthermore, forced expression of RAB26, but not the GTP cycling null point mutants (T77N, Q123L), specifically repositions lysosomes from the cell periphery to a perinuclear central region. As a consequence of this RAB26 mediated lysosomal reorganization, I demonstrate by electron microscopy, immunofluorescence microscopy, and immunoblotting that mitochondria are concomitantly pruned and reorganized into distinct cellular neighborhoods. Taken together, our data establish a role for RAB26 as a novel lysosome-associated Rab whose expression is transcriptionally induced by MIST1 specifically in a subset of highly secretory tissues to reorganize the cellular distribution of lysosomes and, indirectly, mitochondria.

Future Directions

Many unanswered and interesting questions remain from this work involving RAB26 and the mechanistic details required for it to effect lysosome positioning, degradative function, and mitochondrial organization. Further characterization of Rab mechanistic function has usually meant assaying for additional Rab effectors. Preliminarily, I have observed that EGFP-RAB26 in transfected HGC-27 cells is trafficked along microtubules in a nocodazole-dependent manner
(Fig. 2A). This is consistent with the known mechanism of Rab mediated lysosome movement. It has been shown that RAB7, RAB34, and RAB36 all function to reposition lysosomes upon overexpression (Bucci et al., 2000; Chen et al., 2010; Wang and Hong, 2002). Interestingly, all three of these Rab proteins interact with the same effector, RILP (RAB7 interacting lysosomal protein) (Cantalupo et al., 2001; Colucci et al., 2005; Jordens et al., 2001; Wang and Hong, 2005). In fact, RILP has been well established in its ability to recruit the microtubule motor protein, dynein, and induce trafficking of lysosomes towards the minus ends of microtubules adjacent to the microtubule organizing center near the center of the cell. (Cantalupo et al., 2001; Jordens et al., 2001). Thus, RILP is an intriguing Rab effector that might also regulate RAB26 mediated lysosome movement. In addition to this specific protein, other RAB26 effectors can be assayed in unbiased screens including well-established yeast two-hybrid (Fukuda et al., 2008) or mass spectroscopy (Seals et al., 2000) assays for protein-protein interactions. These screens will be augmented by using the point mutants (T77N and Q123L) or non-hydrolyzable GTP analogs (GTPγS or Gpp(NH)p) to elucidate nucleotide binding dependent effector interactions.

In addition to further RAB26 biochemical analyses, I propose to study the effects of “scaling up” RAB26 on the degradative process of autophagy. Briefly, this pathway involves the sequestration of proteins and organelles in a double membrane autophagosome for degradation upon fusion with lysosomes (Klionsky and Emr, 2000). RAB7, a protein with homologous function and localization as RAB26, has been found to be critical in the final fusion of autophagosomes with lysosomes (Jager et al., 2004). I posit that RAB26 mediated repositioning of lysosomes might affect the degradative capacity of the cell through a similar mechanism. In fact, I have found that overexpression of RAB26 in HGC-27 cells causes a similar aggregation of autophagosome markers (LC3 and p62) (Fig. 2B, C). These aggregations of LC3 marked
vesicles remained upon induction of the pathway by rapamycin treatment (Fig. 2B) or serum starvation. Immunofluorescence staining for p62 (a marker for autophagocytic flux) in these rapamycin treated cells revealed less p62 in the EGFP-RAB26 overexpressing cells, suggesting that autophagocytic degradation is induced in these cells (Fig. 2B). I confirmed this finding using a mCherry-EGFP-LC3 plasmid in which fluor intensity indicates progression along this degradative pathway. Specifically, both EGFP and mCherry mark the autophagosome, but upon lysosome fusion, EGFP is degraded, and more stable mCherry remains. Co-transfection of this plasmid with untagged-RAB26 (marked by increased RAB26 immunostaining) revealed that cells overexpressing RAB26 clustered autophagosomes (red and green) along with lysosomes (red) (Fig. 2C). These preliminary data suggest a role of RAB26 in regulating autophagy.

Additional studies would need to address if RAB26 overexpressing increases autophagosome formation, trafficking, or fusion with lysosomes. Specifically, we can use well established assays to measure levels of each of these steps (Klionsky et al., 2008a). It would also be interesting to address if RAB26 has the ability to function as a complement to RAB7 by knocking down RAB7 and assaying for rescue of degradative capacity upon restoration with RAB26. Based on our previous findings with lysosomal function (Chapter 3), it would be unlikely that reducing cellular levels of RAB26 would influence autophagy, but this would be another avenue needing further study. Involvement in any of the steps in autophagy would be an important finding, and would mean that autophagy as a process may be scaled up in secretory cells in response to MIST1 induction.

How RAB26 prunes and reorganizes mitochondria is also an area that merits further study. Two separate pathways have been found to mediate the actual delivery of mitochondria to lysosomes (Ashrafí and Schwarz, 2013). Mitophagy is a subset of autophagy that involves
formation of isolation membranes around damaged mitochondria before subsequent delivery to, fusion with, and degradation in lysosomes (Youle and Narendra, 2011). Cells may also use an autophagy independent route that involves direct vesicular transport of damaged mitochondria to lysosomes (Soubannier et al., 2012). Both pathways of lysosome mediated mitochondrial degradation can be induced pharmacologically (Jin and Youle, 2012; Soubannier et al., 2012). Mitophagy can be induced by addition of the mitochondrial membrane uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) in cells that express the ubiquitin ligase, Parkin, which functions to mark damaged mitochondria for degradation (Narendra et al., 2008). Using HGC-27 cells, which do not express much endogenous Parkin, I show that mitophagy is induced upon CCCP treatment and mCherry-Parkin transfection (Fig. 2D). This process is characterized by Parkin and LC3 (an autophagosome marker) recruitment to mitochondria and co-localization. Upon addition of RAB26, I find that these Parkin positive structures (damaged mitochondria) become marked with RAB26 and LAMP1 vesicles (Fig. 2E). Recruitment of lysosomes to these damaged Parkin bound mitochondria was not affected by transfection of RAB26 point mutants (Fig. 2E).

Conveniently, the autophagy-independent mitochondria degradation pathway is also inducible chemically (Soubannier et al., 2012). Glucose oxidase can stimulate direct vesicular interactions between lysosomes and the TOM20 mitochondrial membrane marker. Interestingly, this is the same mitochondrial marker that I used to monitor RAB26 induced mitochondrial reorganization (Chapter 3). In sum, I have provided preliminary data indicating that RAB26 is recruited to damaged mitochondrial through both autophagy (i.e., mitophagy) dependent and independent processes.
Using the tools that I have developed, specifically live cell imaging, it would be interesting to visualize the timing and capacity of RAB26 recruitment to mitochondria upon pharmacologic damage. At baseline, overexpressing RAB26 is already accelerating the pruning of mitochondria, it would also be important to quantify the effects of RAB26 overexpression in the setting of mitochondrial damage. Are damaged mitochondria more efficiently degraded upon RAB26 transfection? Using confocal live imaging, I can measure the decrease in mitochondrial area as a function of time after addition of drug. I can then compare the effects on timing and reduction of mitochondrial area upon overexpressing RAB26 or the point mutants.

In addition, the contribution by both pathways can be teased out pharmacologically using drugs which can block autophagosome-lysosome fusion (bafilomycin A1, (Klionsky et al., 2008b)) or inhibit lysosome proteolysis (leupeptin, (Haspel et al., 2011)). I would look for abrogation of RAB26 mediated baseline mitochondrial pruning (shown in Chapter 3) or prevention of any additional RAB26 mediated effects upon damaging mitochondria by the methods previously discussed. If treatment with bafilomycin does not inhibit baseline or pharmacologic damage induced RAB26 mediated mitochondrial degradation, then this process would be autophagy independent (i.e., RAB26 shuttles damaged mitochondria directly to lysosomes without an autophagosome intermediate). Treatment with leupeptin should delay mitochondrial degradation no matter the pathway they take to reach the lysosomes. In summary, all proposed experiments would further elucidate the sufficiency and necessity of RAB26 in lysosome mediated mitochondria degradation, and the exact vesicular trafficking pathways that are involved.

The relationship between RAB26 and secretory vesicles remains to be fully investigated. My initial characterization of RAB26 showed that the T77N point mutant inhibited formation of
large PGC-RFP secretory vesicles in MIST1 overexpressing cells (Tian et al., 2010). I assume based on subsequent studies that this effect is an indirect one. In these cells, as in chief cells in vivo, lysosomes must be moved and organized away from the secretory vesicles. MIST1-induced RAB26 mediated perinuclear lysosomal movement would shuttle lysosomes away from developing secretory vesicles. To prove this, I would use live imaging in this PGC-RFP expressing secretory vesicle system to monitor lysosome-secretory vesicle interactions upon transfection of RAB26 or any of its point mutants. Specifically, I would monitor for increased lysosome-secretory vesicle interaction upon T77N or even Q123L overexpression. In addition, the AR42J differentiation system would be an independent method to test the effects of RAB26 on secretion. These cells would be induced with dexamethasone treatment to induce expression of MIST1 and RAB26, increase amylase secretory vesicles, and cluster their lysosomes. Using siRNA knockdown or point mutant transfection, I would again assay by microscopy for increased lysosome-secretory vesicle interaction. This system would also be amenable to assays for secretion by measurement of amylase in the media upon RAB26 overexpression or knockdown either by siRNA or functionally through transfection with point mutants. The effects of RAB26 on secretory vesicle maturation may be indirectly related to its ability to position lysosomes.

Ultimately, a murine model of RAB26 deficiency and RAB26 overexpression should be engineered. The former should recapitulate the lysosomal phenotype of Mist1−/− mice, and the latter should rescue the lysosomal phenotype in the setting of MIST1 loss. Discouragingly, murine Rab knockout models have been difficult to interpret. For example, when RAB3D, another MIST1 target with well-established roles in secretory vesicle docking was overexpressed in Mist1−/− mice, secretory vesicle disorganization was not rescued (Johnson et al., 2004). When
exocrine tissues were examined in RAB3D deficient mice, no secretion defect was found (Riedel et al., 2002). As a result, results from in vivo genetic models of RAB26 deficiency or overexpression should be carefully interpreted, because redundancy among Rabs might mask loss of function effects.

**MIST1 Suppresses Lysosome Mediated Degradation in Mature Exocrine Cells**

In Chapter 4, I demonstrate that MIST1 regulates the exocrine secretory apparatus by suppression of the degradative lysosomal machinery. Specifically, I show that, in addition to the reduction in secretory vesicle size and general granule disorganization seen in Mist1−/− mice, there is a dramatic increase in functional lysosomes. Normally in these exocrine secretory tissues, lysosomes are small, diffuse, and organized away from larger maturing secretory vesicles. But upon MIST1 loss, this degradative compartment expands apically engulfing and degrading many of the forming vesicles. In collaboration with Greg Sibbel, I assayed the timing of this lysosome accumulation using an inducible mouse model of MIST1 loss. We found that lysosomes accumulate rapidly upon loss of MIST1 in all exocrine tissues. I confirm the functionality of these increasing lysosomes and the severity of secretory vesicle degradation by crossing Gnptab−/− mice with Mist1−/− mice. Because these Gnptab−/− mice exhibit impaired acid hydrolase trafficking and reduced lysosomal capacity, we were able to partially rescue the secretory destruction in Mist1−/− mice as measured by increased vesicle size and undegraded secretory vesicle content inside lysosomes. In other words, we were able to rescue subsequent destruction of the vesicles once inside lysosomes but did not effect on the aberrant targeting of vesicles to lysosomes. These data demonstrate the effects of MIST1 on other vesicular compartments and hint at the role of RAB26 in exocrine cells to effectively move and organize
lysosomes. In cells that lack MIST1, and have nominal levels of RAB26, lysosomes are ineffectively trafficked away from secretory vesicles causing promiscuous interactions between the two compartments.

**Future Directions**

These proposed experiments would shed light as to the exact pathway secretory vesicles take to reach lysosomes in the absence of MIST1 *in vivo*. Previous work from our lab has shown that in the absence of MIST1, as precursor mucous secreting neck cells differentiate into zymogenic chief cells, there is a transient increase of secretory vesicles (Ramsey et al., 2007). These “transition cells” express both precursor neck cell and mature zymogenic cell markers, and, in wildtype mice, would be increasing expression of MIST1 and its gene targets. Because MIST1 is not expressed, lysosomal degradation is not suppressed, and subsequent secretory vesicles show fewer secretory vesicles by immunofluorescence microscopy compared to these Mist1−/− “transition cells”. Interestingly, I have found that in WT mice, most mature secretory vesicles are marked with markers of autophagosomes (Fig. 3A). However, in contrast, in the absence of MIST1, only these “transition cells” with their transient increase in secretory vesicles exhibit the same staining for LC3 and p62 autophagosome markers (Fig. 3A, B). Once lysosomes are increased in Mist1−/− mice, these markers along with secretory vesicle proteins are all decreased as visualized by immunostaining (Fig. 3B). Therefore, this data suggest that at baseline mature secretory vesicles are tagged and poised to be degraded by lysosomes. It is only MIST1 suppression of the degradative compartment that prevents this process. So, when MIST1 is lost, these vesicles that are formed and tagged at the transition (arrowheads, Fig. 3B) become readily degraded as shown in Chapter 4.
Why would mature zymogenic cells maintain their secretory vesicles in a state of high-alert, ready to be immediately destroyed? I suggest that the answer lies in the evolutionary relationship between pathogen and host. *Helicobacter pylori* is the main constituent of the gastric microbiome (Fox and Sheh, 2013). Recent studies have revealed that people with defects in the degradative pathway increases susceptibility to *H. pylori* infection (Raju et al., 2012). In fact, intracellular survival has been found to be an important part of the pathogenesis of this bacterium (Deen et al., 2013; Terebiznik et al., 2009). *H. pylori* intracellular survival is dependent on entry into these autophagosomes and prevention of subsequent lysosome fusion by vacuolating toxin binding and sequestration of RAB7 (Terebiznik et al., 2006). As a whole, these studies indicate that the careful balance between formation of secretory vesicles and their degradation to prevent *H. pylori* infection is an important task for mature secretory cells in the stomach.

To test the involvement of autophagy in the degradation of secretory vesicles in Mist1\(^{-/-}\) mice, I propose to use a mouse model in which autophagy can be conditionally downregulated. Using the well characterized *Atg7\(^{flint}\)\(^{fl revealed in the previous paragraphs. Preliminary results show that *Atg7* is deleted by message and protein levels in the stomach by induction with *CAGGCreERT\(^{TM}\)* (Fig. 3C). Interestingly in these whole mouse inducible ATG7 knockouts, by immunofluorescence microscopy, we see aggregation of undegraded LC3 and p62 (consistent with previous reports (Riley et al., 2010)) preferentially in the exocrine secretory cells of the stomach (Fig. 3D, E) and the pancreas (Fig. 3F). Crossing these MIST1 expressing cell specific and whole mouse ATG7 knockouts to Mist1\(^{-/-}\) mice and then assaying for the...
effects on secretory vesicle degradation through established assays (Chapter 4) will reveal the effects and necessity of autophagy in lysosome mediated secretory vesicle destruction. If in the absence of MIST1 and the ATG7, secretory granules are rescued from degradation, specifically no longer targeted to lysosomes, this would prove that autophagy is indeed the intermediate pathway by which secretory vesicles are targeted to lysosomes. Further exploration of autophagy in MIST1 regulated secretory vesicle maintenance will have critical importance for our cell biological and microbiological understanding of zymogenic chief cells.

**Loss of MIST1 Induces Lysosome Mediated Downscaling During Gastric Metaplasia**

In Chapter 1, I highlighted the zymogenic cell lineage and its involvement in many of the critical diseases that affect the stomach. Specifically, these post mitotic mature secretory cells have been shown to possess the ability in response to tissue damage to dedifferentiate, become metaplastic (Goldenring et al., 2011; Nam et al., 2010), and regain stem cell-like properties (Stange et al., 2013). Thus, understanding the process by which these cells initiate this dedifferentiation program may be critical to furthering our knowledge of gastric carcinogenesis.

**Future Directions**

MIST1 and the maintenance of secretory architecture may be a key factor in preventing these cells from this metaplastic transformation. In fact, this may be a theme for all exocrine tissues including the pancreas. Specifically, loss of MIST1 has been found to directly render gastric chief and pancreatic acinar cells susceptible to injury, metaplasia, and carcinogenesis (Alahari et al., 2011; Kowalik et al., 2007; Lennerz et al., 2010; Nam et al., 2010; Nozaki et al., 2008; Shi et al., 2013; Shi et al., 2009). Studies using a pharmacologic (high dose tamoxifen)
inducible model of gastric atrophy and metaplasia show that MIST1 is one of the first factors that is lost as mature zymogenic cells respond to injury (Huh et al., 2012). Additional studies in the pancreas using an inducible MIST1 knockout model revealed that within hours of MIST1 loss, mature pancreatic acinar cells began to remodel their granules, cytoskeleton, and cellular junctions (Direnzo et al., 2012).

In the stomach, I have found that concomitant with early MIST1 loss upon high dose tamoxifen induced injury, LAMP1 stained lysosomes significantly increased. This increase in lysosomes is accompanied by loss of mature PGC secretory vesicles and re-expression of mucous neck cell markers (GSII) (Fig. 4A). By higher magnification confocal microscopy, I observed increasing lysosome and secretory vesicle interactions until 24 hours post tamoxifen injury (Fig. 4B). These degradation events were restricted to mature native PGC secretory vesicles and excluded newly formed metaplastic GSII-marked mucous vesicles. After 24 hours, the majority of zymogenic cells had turned over all their PGC vesicles, replaced with GSII vesicles. These data show that MIST1 loss and LAMP1 mediated lysosomal attack on secretory vesicles occur as early events during gastric tissue injury response, prior to and independent from synthesis of neck cell mucous granules.

These animal data are supported by observations made in human gastric biopsy samples containing regions of normal stomach adjacent to metaplastic areas. In the normal areas, large PGC secretory vesicles are preserved apically and lysosomes are small and diffusely distributed basally, consistent with our mouse model (Fig. 4D). However, in these areas of metaplasia characterized, as in our murine model, by metaplastic re-expression of precursor markers (GSII) and associated decrease in mature PGC vesicles, we find aberrant lysosome-secretory vesicle interactions (Fig. 4E). These lysosome mediated degradation events only occur for the PGC
vesicle population; the newly forming metaplastic mucous granules are excluded from this destruction.

Based on these findings, this process of lysosome mediated vesicle attack during injury response warrants further characterization. First, other injury models of murine gastric metaplasia (elicited by Helicobacter species (Nomura et al., 2004), other pharmacologic agents (Goldenring et al., 2000), or genetically (Li et al., 1996; Nomura et al., 2005)) should be examined to confirm this lysosome mediated attack on secretory vesicles is a universal feature of early gastric metaplasia. Next, as with the increase in lysosomes seen in our Mist1−/− mice, the role of autophagy should be investigated by looking for co-localization of autophagy markers with secretory vesicles in these early time points after injury induction.

All findings should be related to pancreatic metaplasia models (Habbe et al., 2008; Shi et al., 2013) to establish lysosome downscaling of mature secretory cell architecture as a common first step during injury response in MIST1 expressing tissues. Encouragingly, in the exocrine pancreas, acute pancreatitis (an injury state preceeding metaplasia and carcinogenesis (Hezel et al., 2006)) often involves damaging auto activation of secretory enzymes (Gaiser et al., 2011). Studies have shown that this process involves fusion of lysosomes and activation of secretory zymogens by lysosomal hydrolases (Halangk et al., 2000; Saluja et al., 1997).

After these experiments that characterize the process, I propose a series of functional experiments to address the requirement of MIST1 and upregulation of degradation in the metaplastic response of zymogenic chief cells. The former will be addressed by using an inducible model of MIST1 overexpression (Direnzo et al., 2012). Pharmacologic provocation of gastric metaplasia will be studied in the setting of continued MIST1 overexpression through Cre recombinase excision of a Lox-STOP-Lox cassette allowing constitutive CAG promoter driven
expression of MIST1. These experiments will assay the requirement of MIST1 loss during gastric metaplasia. In other words, if loss of MIST1 is needed for the metaplastic dedifferentiation of zymogenic cells, then these constitutive MIST1 expressing mice will retain morphologically normal chief cells upon gastric injury.

In addition, the mechanistic requirement for lysosomal degradation of secretory vesicles will also be addressed. Metaplasia will be induced in mice defective in lysosomal hydrolases, the *Gnptab<sup>-/-</sup>* mice, and ability of mature zymogenic cells to degrade mature PGC vesicles and form mucous precursor granules will be tracked. Complementary experiments exploring the role of autophagy in this process are also possible using *ATG7<sup>fl/fl</sup>* mice as described or additional well-established genetic models of autophagy inhibition (Hara et al., 2006; Ichimura and Komatsu, 2011).

Ultimately, the role RAB26 in this process should also be addressed. As previously mentioned, a murine model of RAB26 overexpression must first be constructed. With this tool in hand, metaplasia can be induced in gastric zymogenic cells overexpressing RAB26 and the lysosomal degradation of secretory vesicles can be examined. If RAB26 is the critical MIST1 target whose maintained expression is needed to suppress lysosome-secretory vesicle interactions, then these mice should exhibit increased PGC vesicles and resiliency against metaplasia. In summary, these proposed experiments will explore the physiologic role of MIST1, RAB26, and degradative cell architectural reorganization.

**Final Remarks**

The goal of this thesis was to determine the direct transcriptional targets of the exocrine cell specific transcription factor, MIST1. From this process, I found and focused on a specific
MIST1 target, RAB26, and elucidated its novel role in lysosome movement and mitochondrial reorganization. These findings led me to uncover the function of MIST1 in suppression of lysosome mediated degradation of mature secretory vesicles in exocrine cells. As a whole, this work demonstrates the importance of basic transcriptional factor controlled gene networks in the establishment of cellular form and function. I hope these findings will elucidate a physiologic correlate in and uncover a previously unrecognized feature of gastric pathologies.
References


Figure Legends

Figure 1. The expression of CCPG1 and SERPINI1 is MIST1 dependent. A. Microarray analysis from isolated populations of gastric ZCs and their precursor neck cells from wild-type and Mist1−/− mice. MIST1 expression is shown atop as a positive control validation of the microarray data. Expression of previously identified MIST1 targets from gastric cell lines are indicated and separated into groups exhibiting a MIST1 dependent pattern or a MIST1 independent pattern. Two of these MIST1 dependent targets, CCPG1 and SERPINI1 are highlighted in red. Gene expression for microarray analyses are shown with relative expression scale (-3.0 to 3.0) with red=high, blue=low expression. B. In situ hybridization of mouse fundic gastric gland sections showing antisense CCPG1 and SERPINI1 signal confined to the ZCs at the base of the glands in wild-type mice, and lack of staining in Mist1−/− mice. C. Immunofluorescence microscopy of wild-type and Mist1−/− mice stained for CCPG1 and SERPINI1 protein expression (green). Lysosomes (LAMP1, red) and secretory vesicles (GIF, purple) are also shown. Insets contain high magnification confocal images of CCPG1 and SERPINI1 vesicle staining in basal zymogenic cells. Gastric units are outlined in white and scale bars = 20 µm.

Figure 2. RAB26 traffics along microtubules and co-localizes with autophagy and mitophagy markers. A. Live confocal timelapse microscopy of EGFP-RAB26 vesicles after treatment with 10 µg/mL of nocodazole. Three panels indicate images taken at one minute intervals with arrowheads indicating the static nature of previously motile RAB26 vesicles post treatment. B. Epifluorescence of HGC-27 cells transfected with EGFP-RAB26 (green) and
stained for LC3 (red) and p62 (purple) after 6 hour treatment with 10 µm rapamycin to induce autophagy. C. Immunofluorescence staining of HGC-27 cells co-transfected with mCherry-EGFP-LC3B (red and green) and RAB26 without GFP tag (pcDNA3.1-RAB26) stained with anti-RAB26 (purple). D. mCherry-Parkin (red) transfected HGC-27 cells stained for LC3 (green) upon 1 hour treatment with carbonyl cyanide m-chlorophenylhydrazone (+CCCP). E. Fluorescence microscopy of CCCP treated EGFP-RAB26, EGFP-RAB26T77N, EGFP-RAB26Q123L, and EGFP HGC-27 cells (green) co-transfected with mCherry-Parkin (red) stained for LAMP1 (purple). Scale bars = 20 µm.

Figure 3. Autophagocytic vesicles mark secretory vesicles and accumulate upon deletion of Atg7. A. Fluorescence microscopy of outlined WT and Mist1−/− gastric units showing LC3 (green) and p62 (red) staining in ZCs (stained purple for PGC expression). Arrowheads indicated higher expression levels of LC3, p62, and PGC in the transitional cells of Mist1−/− gastric units. Parietal cells are shown in yellow. B. Immunofluorescence staining of a Mist1−/− gastric unit showing LAMP1 (green), LC3 (red), and GIF (purple) staining. Arrowheads indicated higher expression levels of LC3 and GIF in the Mist1−/− transitional cells before expansion of the LAMP1 lysosome compartment. C. Western blot and PCR analysis of the knockdown of ATG7 protein and mRNA for CAGGCreERTM+ATG7fl/fl (+) and CAGGCreERTM−ATGfl/fl (-) mice after tamoxifen induction. D. Immunofluorescence microscopy of gastric units from CAGGCreERTM+ATG7fl/fl (-) and CAGGCreERTM+ATG7fl/fl (+) mice 2 weeks after tamoxifen induction stained for LC3 (green), p62 (red), and GIF (purple). E. Inset shows confocal image of GIF (purple) stained basal zymogenic cells of CAGGCreERTM+ATG7fl/fl tamoxifen induced mice with arrows highlighting LC3 (green) and p62 (red) aggregations. F. Immunofluorescence
and confocal microscopy of pancreatic acini from \(CAGGC\text{Re}ER^{TM-}\text{ATG7}^{\text{fl/fl}}\) (-) and \(CAGGC\text{Re}ER^{TM+}\text{ATG7}^{\text{fl/fl}}\) (+) mice 2 weeks after tamoxifen induction stained for LC3 (green) and p62 (red). Arrows show LC3 (green) and p62 (red) aggregations. Gastric units and pancreatic acini are outlined in white and scale bars = 20 µm.

**Figure 4. Lysosome mediated secretory vesicle destruction is an early event during gastric metaplasia.** A. Gastric units from untreated (control) mice and mice 12 and 24 hours post tamoxifen induced metaplasia showing immunofluorescence staining of LAMP1 (green), GIF (red), and the mucous neck cell marker (GSII, purple). Gastric units are outlined in white. Higher magnification confocal microscopy of basal zymogenic cells B. 12 hours and C. 24 hours post tamoxifen treatment stained for LAMP1 (green), GIF (red), and GSII (purple). Arrows indicate GIF stained secretory vesicles inside LAMP1 labeled lysosomes. D. Immunofluorescence imaging of human gastric cancer biopsy samples immunostained for LAMP1 (green), PGC (red), and GSII (purple). Normal and adjacent metaplastic regions are outlined. E. Confocal sections (basal to apical) of a metaplastic zymogenic cell stained for LAMP (green), PGC (red), and GSII (purple). GSII vesicular regions devoid of LAMP1 overlap indicated (‘*’).
Ramon Jin, MSTP  
Washington University School of Medicine

15 N. Boyle Ave.        Email: jinr@wusm.wustl.edu  
St Louis, MO 63108        Phone: 216-544-2529

EDUCATION

Washington University School of Medicine        St. Louis, MO
M.D. / Ph.D. (expected graduation date: 2015)        08/06-present

Case Western Reserve University        Cleveland, OH
Summa cum laude, B.S.        08/02-05/06
Major: Biology

HONORS AND AWARDS

Travel Award        04/2012
ASCI/AAP/APSA Annual Meeting

Viktor Hamburger Award        06/2011
Department of Developmental Biology
Washington University School of Medicine

Travel Award        08/2009
FASEB Summer Research Conference: Gastrointestinal Tract
XII: The Molecular and Integrative Basis for GI Development,
Homeostasis and Disease

Trustees’ Scholarship Award        2002-2006
Case Western Reserve University

Case Alumni Association Junior/Senior Scholarship        2005-2006
Case Western Reserve University

PUBLICATIONS


Translational Gastroenterology, from Development to Disease, edited by Deborah L. Gumucio, Linda C.
Samuelson, Jason R. Spence, 57-72.


*co-first author


**POSTERS AND PRESENTATIONS**

"RAB26, a Direct Transcriptional Target of MIST1, is a Novel Regulator of Lysosome Positioning”
FASEB Summer Research Conference entitled: Gastrointestinal Tract XIV: Stem Cells, Adaptation, Inflammation and Cancer
Steamboat Springs, CO 08/2011

"RAB26 is required for formation of large exocrine secretory vesicles and is sufficient to reposition lysosomes”
Gordon Research Conferences entitled: Proprotein Processing, Trafficking & Secretion
New London, NH 07/2010

"RAB26, a direct transcriptional target of MIST1, is required for formation of large exocrine secretory vesicles”
FASEB Summer Research Conference entitled: Gastrointestinal Tract XII: The Molecular and Integrative Basis for GI Development, Homeostasis and Disease
Snowmass, CO 08/2009

"The cystic fibrosis transmembrane conductance regulator (Cftr) modulates the timing of puberty in mice”
The Endocrine Society’s 87th Annual International Meeting
San Diego, CA 06/2005

**LEADERSHIP/TEACHING/MENTORING EXPERIENCE**

Representative, MSTP Student Committee Washington University School of Medicine
St. Louis, MO 10/10-present

Research Mentor, Lydia Espinoza, Summer Research Student Opportunities in Genomics Research Undergraduate Scholars Program The Genome Institute, Washington University School of Medicine
St. Louis, MO 06/10-08/10

Research Mentor, Xavier Jirau, Summer Research Student Opportunities in Genomics Research Undergraduate Scholars Program The Genome Institute, Washington University School of Medicine
St. Louis, MO 06/09-08/09

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Teaching Assistant, Cell and Organ Systems Biology Histology  
Washington University School of Medicine  
St. Louis, MO  
08/08-04/09

**RESEARCH EXPERIENCE**

**Jason Mills Lab, Washington University School of Medicine**  
St. Louis, MO  
Graduate Student  
07/08 - Present  
The role of MIST1 in zymogenic (chief) cell secretory maturation

**Anthony Muslin Lab, Washington University School of Medicine**  
St. Louis, MO  
Rotational Student  
06/07-08/07  
Cellular localization of AKT1/2 upon Insulin and IGF stimulation

**Paul Hruz Lab, Washington University School of Medicine**  
St. Louis, MO  
Rotational Student  
06/06-08/06  
Metabolic disturbances associated with antiretroviral therapy

**Mark Palmert and Mitchell Drumm Labs, Case Western Reserve University**  
Cleveland, OH  
Undergraduate Research Student  
2002-2006  
The role of CFTR in modulating the timing of puberty