

The bHLH transcription factor *Mist1* is required to maintain exocrine pancreas cell organization and acinar cell identity

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The pancreas is a complex organ that consists of separate endocrine and exocrine cell compartments. Although great strides have been made in identifying regulatory factors responsible for endocrine pancreas formation, the molecular regulatory circuits that control exocrine pancreas properties are just beginning to be elucidated. In an effort to identify genes involved in exocrine pancreas function, we have examined *Mist1*, a basic helix-loop-helix transcription factor expressed in pancreatic acinar cells. *Mist1*-null (*Mist1*^{KO}) mice exhibit extensive disorganization of exocrine tissue and intracellular enzyme activation. The exocrine disorganization is accompanied by increases in *p8*, *RegI/PSP*, and *PAP1/RegIII* gene expression, mimicking

the molecular changes observed in pancreatic injury. By 12 m, *Mist1*^{KO} mice develop lesions that contain cells coexpressing acinar and duct cell markers. Analysis of the factors involved in cholecystokinin (CCK) signaling reveal inappropriate levels of the CCK receptor A and the inositol-1,4,5-trisphosphate receptor 3, suggesting that a functional defect exists in the regulated exocytosis pathway of *Mist1*^{KO} mice. Based on these observations, we propose that *Mist1*^{KO} mice represent a new genetic model for chronic pancreas injury and that the *Mist1* protein serves as a key regulator of acinar cell function, stability, and identity.

Introduction

The development of the mammalian pancreas represents an attractive model system to study the molecular signals that direct the commitment and differentiation of endodermal epithelial cells along different endocrine and exocrine cell lineages (Slack, 1995; Debas, 1997; for review see Habener and Stoffers, 1998). The pancreas consists of two separate compartments of cells that develop from common precursors derived from an evagination of the primitive foregut (Kim and Hebrok, 2001). The endocrine compartment, which controls blood sugar levels, has been well characterized, and several key transcription factors have been identified that regulate the development and function of this tissue (Jonsson et al., 1994; Offield et al., 1996; Gradwohl et al., 2000; Schwitzgebel et al., 2000). The development of the exocrine compartment, which produces and secretes the majority of enzymes necessary for digestion, is not nearly as well defined.

Initial specification of the exocrine pancreas begins at embryonic day (E)*10 and follows endocrine determination (Slack, 1995). Beginning at E12.5, cells from the pancreatic buds migrate from the ducts and begin to express exocrine-specific markers such as amylase and carboxypeptidase. By E16, circular arrangements of pancreatic acini are first observed. After the establishment of cellular polarity, acinar cells exhibit a fully mature appearance shortly after birth. To date, only a few transcription factors have been identified with acinar-specific expression including pancreatic transcription factor 1, 48 kD subunit (PTF1-p48; Krapp et al., 1996), and *Mist1* (Lemerrier et al., 1997). Both PTF1-p48 and *Mist1* are members of the basic helix-loop-helix (bHLH) family of proteins. These proteins form active dimers that function as either activators or repressors of tran-

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*Abbreviations used in this paper: β -gal, β -galactosidase; bHLH, basic helix-loop-helix; CCK, cholecystokinin; CK, cytokeratin; CPA, carboxypeptidase A; E, embryonic day; ER, endoplasmic reticulum; ES, embryonic stem; IP₃R, inositol 1,4,5-trisphosphate receptor; SMA, smooth muscle actin; PTF-p48, pancreatic transcription factor, 48 kD subunit; ZG, zymogen granule.

scription by binding to E-box DNA elements (CANNTG) within gene regulatory regions (Molkentin and Olson, 1996). Dimer formation occurs typically between a ubiquitously expressed family member (for example, E12) and a tissue-specific family member (for example, MyoD in skeletal muscle). The bHLH family of transcription factors has been linked to the development of many different cell types including skeletal muscle (Rudnicki et al., 1993), neuronal systems (Schwab et al., 2000), and lymphocytes (Zhuang et al., 1994).

PTF1-p48 is the earliest exocrine-specific marker observed, detected initially at E9.5 in pancreatic primordial tissue (Krapp et al., 1998). The PTF1-p48 transcription factor is thought to promote digestive enzyme gene expression through the formation of a heterotrimeric complex (PTF1) with two other bHLH transcription factors, E12, and a truncated form of HEB (Roux et al., 1989). Subsequent binding of the PTF1 complex to acinar cell DNA target sequences leads to gene activation (Cockell et al., 1995). Targeted disruption of the *PTF1-p48* locus confirms its importance in pancreatic development, since *PTF1-p48*-null mice have no exocrine pancreas (Krapp et al., 1998). Whether the PTF1-p48 factor alone is sufficient for exocrine pancreas development and function remains to be determined.

Mist1 is the second known bHLH transcription factor exhibiting acinar cell-specific expression. Mist1 has been shown to negatively regulate bHLH-mediated transcription through an NH₂ terminus repressor domain (Lemerrier et al., 1998). Although PTF1-p48 is limited to the exocrine pancreas (Krapp et al., 1996), *Mist1* gene expression is observed in a wider array of tissues including the acinar cells of salivary glands and the serous secreting cells found in the stomach, prostate, and seminal vesicles (Pin et al., 2000). This restricted cellular specificity suggests that Mist1 may be involved in a more general regulatory pathway that is common to each of these cell types. An essential function that is shared by all Mist1-positive cells is regulated exocytosis, which involves the temporary storage of zymogen granules (ZGs) at the cell's apical surface and the establishment of specific signaling pathways through which external cues induce regulated secretion.

The exocytosis process is initiated through the recognition of secretagogues, such as cholecystokinin (CCK) (Williams et al., 1997; Burghardt et al., 1998), by G protein-coupled receptors located on the basal aspect of acinar cells (Wank, 1995; Williams, 2001). Binding of the CCK receptor high affinity sites leads to the release of Ca²⁺ from the endoplasmic reticulum (ER). The intracellular release of Ca²⁺ is mediated, in part, by the inositol 1,4,5-triphosphate receptor 3 (IP₃R3), which is situated at apical ER regions (Joseph, 1996). Increased intracellular Ca²⁺ levels trigger the movement of ZGs toward and through an actin terminal web, resulting in exocytosis (McNiven and Marlowe, 1999). Improper signaling through the CCK receptor low affinity site leads to impaired exocytosis and premature enzyme activation (Saluja et al., 1989). These changes in cell function are accompanied by changes in the expression patterns of specific secreted lectins (PAP/RegIII, RegI/PSP), transcription factors (p8), and cytokines (IL1) (Iovanna et al., 1991; Fink and Norman, 1997; Mallo et al., 1997). Similar alterations in gene expression, exocytosis, and enzyme activation are ob-

served in pancreatic injury and diseases such as pancreatitis (Iovanna et al., 1991; Steer, 1997).

In an effort to determine the role of Mist1 in the development and maintenance of pancreatic exocrine tissue, a mouse model was created in which the *Mist1* locus was replaced by the bacterial *LacZ* gene (*Mist1^{LacZ}/Mist1^{LacZ}*, denoted as *Mist1^{KO}*). Although *Mist1^{KO}* mice are viable and outwardly indistinguishable from their littermates, histological analysis reveals extensive disorganization of the exocrine pancreas. *Mist1^{KO}* mice show progressive deterioration of acinar tissue, wide spread dysplasia, and alterations in the regulated exocytosis pathway. In addition, expression of the *CCKAR*, *p8*, *RegI/PSP*, and *PAP1/RegIII* genes is increased greatly in *Mist1*-null mice. Eventually, *Mist1^{KO}* mice develop pancreatic damage in the form of focal lesions, which contain cells that coexpress acinar and duct cell-specific markers. These results suggest that Mist1 is a key regulator of acinar cell function, stability, and identity. In the absence of Mist1, overt pancreatic damage develops that mimics conditions of pancreatic injury.

Results

Embryonic expression of Mist1

In adult tissue the *Mist1* gene is transcribed exclusively in serous exocrine cells with high levels of expression, specifically in the acinar cells of the pancreas (Pin et al., 2000). Immunohistochemistry on staged embryos reveals that Mist1 protein is first detected in the nuclei of cells adjacent to pancreatic ducts as early as E13.0 (unpublished data). By E14.5, pancreatic ducts are surrounded by Mist1-positive cells that predominate eventually in the developing pancreatic tissue (Fig. 1 A). At birth (PN1) acini are clearly visible and all acinar cells are Mist1 positive (Fig. 1 B). The acinar-specific expression of Mist1 is maintained in adult pancreatic tissue as well. Dual fluorescence for cytokeratin (CK)-20 or CK-7 and Mist1 (Fig. 1, C–H) confirms that nuclear Mist1 is expressed only in acinar cells. There is a complete absence of Mist1 expression in intra- or interlobular pancreatic ducts and in all islet cells (see Fig. 3 A) in adult tissue.

In an effort to increase the sensitivity for detecting *Mist1* gene expression, mice were generated in which the coding region of the endogenous *Mist1* allele was replaced with the bacterial *LacZ* gene (Fig. 2, A and B). Embryonic stem (ES) cell clones identified as containing a correctly targeted allele were used for the generation of chimeric mice that were then mated to produce *Mist1⁺/Mist1^{LacZ}* (denoted *Mist1^{LacZ}*) heterozygous progeny. As shown in Fig. 2 C, β -galactosidase (β -gal) activity is not observed at E9.0, which is the embryonic stage when only endocrine pancreatic markers are detected (Gittes and Rutter, 1992). Beginning at E10.5, β -gal is found in the primitive foregut, caudal, and medial to the forelimb bud (Fig. 2, D and E). Examination of sections from E10.5 embryos reveals the pancreatic bud containing β -gal-positive cells. These cells originate in the wall of the foregut, suggesting that an exocrine-specific cell population exists before cell migration into the pancreatic bud (Fig. 2, F and G). By E12.5, β -gal staining is more prominent (Fig. 2 H), appearing as two separate buds (dorsal and ventral lobes of the pancreas) in the lower part of the foregut. This early *LacZ* expression suggests that the *Mist1* locus delineates aci-

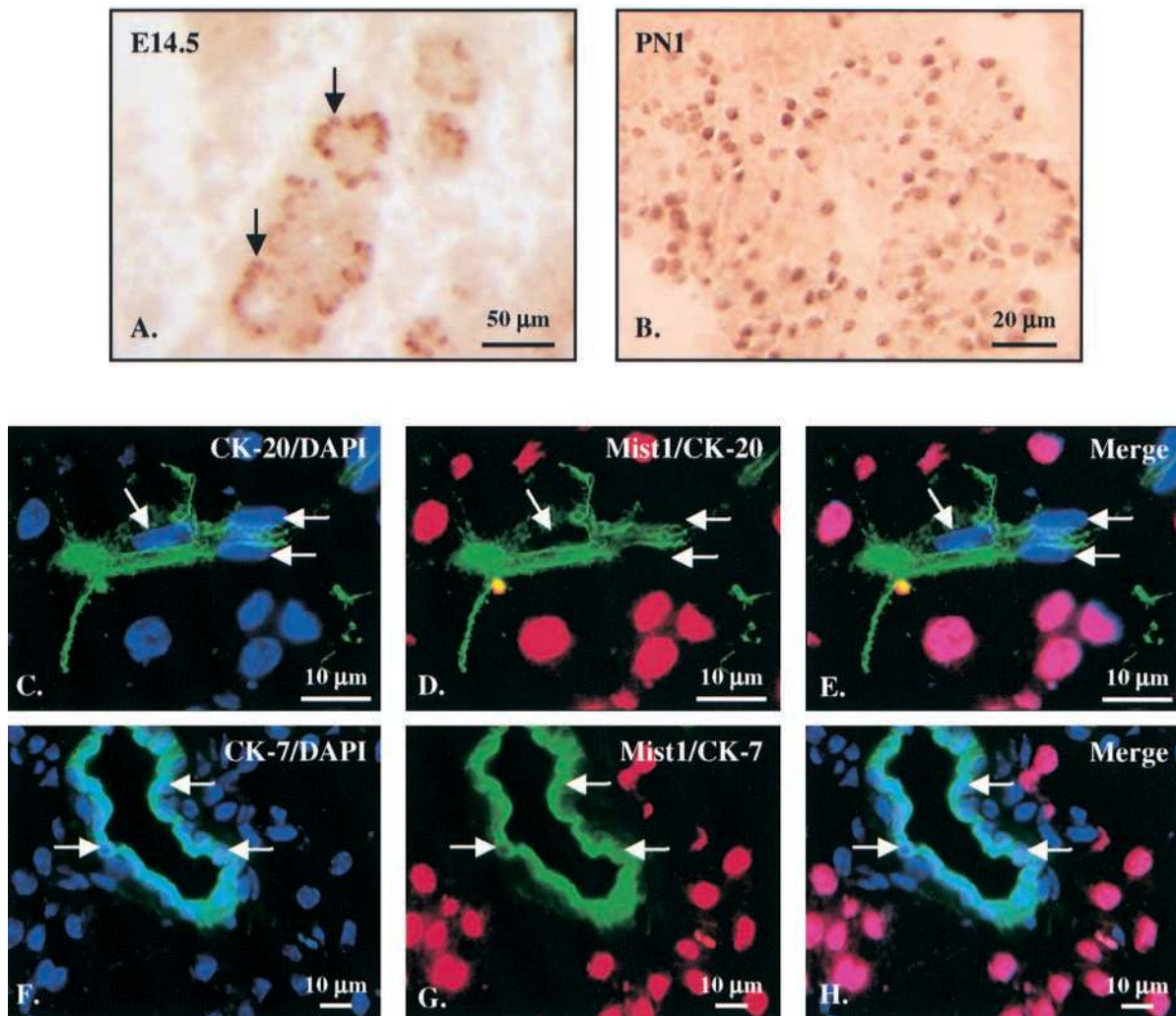


Figure 1. **Mist1 is expressed specifically in acinar cells during pancreatic development.** (A) Immunohistochemistry using a Mist1-specific antibody reveals nuclear expression in putative acinar cells surrounding duct tissue (arrows) at E14.5. (B) By postnatal day 1 (PN1), Mist1 expression is wide spread in the pancreas, remaining exclusively in acinar cells. (C–H) Colocalization of CK-20 (C) or CK-7 (F) with Mist1 (D and G, red) and DAPI (blue) in adult wild-type mice indicates that Mist1 is not expressed in intra- or interlobular ducts, respectively. Combining the staining patterns (E and H) reveals an exclusion of Mist1 from duct cell nuclei (arrows).

nar cell lineages and distinguishes Mist1 as one of the earliest exocrine-specific markers to be expressed in the developing pancreas. Analyses of older embryos (E14.5 and E15.5) confirm the pancreatic acinar cell-specific expression of the *Mist1* locus (Fig. 2, I–K). Similarly, detailed analysis of β -gal accumulation in *Mist1^{LacZ}* adult mice also confirms that the *LacZ* gene faithfully mimics endogenous *Mist1* gene expression, being expressed only in acinar cells and not in duct or endocrine cell types (unpublished data).

***Mist1^{KO}* pancreatic tissue exhibits disrupted acinar cell organization**

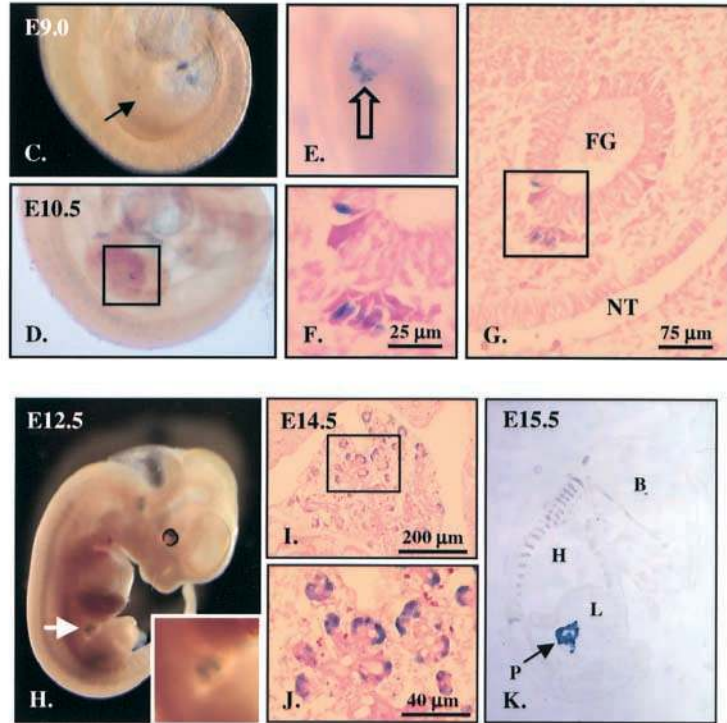
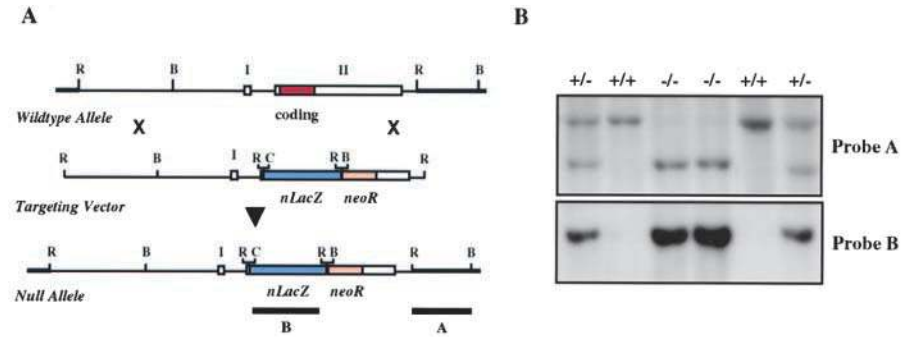
To determine a presumptive role for the Mist1 protein, acinar cell morphology was examined in mice in which both *Mist1* alleles were replaced by *LacZ* (*Mist1^{KO}*). Genotype analysis of the offspring from *Mist1^{LacZ}* matings revealed a gene distribution typical for Mendelian inheritance of single genetic loci (wild-type, 26.6%; *Mist1^{LacZ}*, 48.6%; *Mist1^{KO}*, 24.8%). The absence of Mist1 protein and gene expression

in *Mist1*-null mice was confirmed by immunohistochemical (Fig. 3, A and B), Western blot, and reverse transcriptase PCR analysis (unpublished data). *Mist1^{KO}* mice exhibit normal birth size, body weight, and feeding habits compared with wild-type and heterozygous littermates (unpublished data). These mice also develop pancreatic tissue expressing levels of digestive enzymes similar to wild-type mice, suggesting that Mist1 is not essential for the initial formation of the exocrine pancreas or for digestive enzyme expression. However, a closer examination of pancreatic tissue from 3-m-old *Mist1*-null animals shows that significant structural defects exist that are specific to acinar cells.

Hematoxylin and eosin staining of exocrine pancreas sections from wild-type mice reveals well defined inter- and intracellular levels of organization (Fig. 3 C). This is in contrast to the exocrine pancreas of *Mist1^{KO}* animals in which few presumptive acini are observed and acinar cells are highly disorganized. Nuclei are not basally located, and the distinct border between the ER and ZGs that exists in wild-

Figure 2. Insertion of *LacZ* into the *Mist1* locus reveals an expression pattern linked to exocrine pancreatic development.

(A) Recombination of the *Mist1* gene using a targeting vector in which the *LacZ* gene replaces the coding region of the *Mist1* gene (*Mist1^{LacZ}*). The wild-type allele of *Mist1* contains two exons (I and II; white boxes) with the entire coding region (red box) contained within the second exon. Probe fragments (A and B) used for Southern hybridization are indicated. B, BamHI; R, EcoRI; C, ClaI. (B) Southern blot analysis confirms the genotype of offspring from *Mist1^{LacZ}* mice. Digestion of tail DNA with BamHI produces a differential hybridization pattern with probe A. Using this external probe, the wild-type allele (10.5 kb) is distinguished from the null allele (6.7 kb). Similar blots were performed using the *LacZ* gene as a probe (Probe B). (C–G) β -Gal in *Mist1^{LacZ}* mice first appears at E10.5. Whole-mount staining reveals an absence of β -gal at E9.0 in the distal region of the foregut (C). At E10.5 (D), a circular rim of β -gal expression just caudal and medial to the forelimb bud appears. (E) Higher magnification suggests that this staining is specific to the distal region of the foregut. (F and G) Sections from whole-mount β -gal staining at E10.5 counterstained with eosin. β -Gal-positive cells are observed in the wall of the foregut and in a small bud of cells extending from the foregut to the neural tube (NT). (F) Higher magnification of a region from (G) shows that both the pancreatic bud and adjacent foregut contain β -gal-expressing cells, indicating *Mist1* expression at the beginning of exocrine pancreas morphogenesis. At E12.5 (H), β -gal staining highlights the two pancreatic buds (inset). Staining at E14.5 indicates that *LacZ* expression is limited to the budding acinar cells, whereas duct cells are negative (I and J). At E15.5 (K), sagittal sections show that β -gal is limited to only the pancreas (arrow). B, brain; H, heart; L, liver; P, pancreas.



type animals is not observed (Fig. 3 D). The absence of polarity in *Mist1^{KO}* acinar cells is accompanied by vacuolation with cellular and nuclear dysplasia. Toluidine blue staining of epoxy sections (Fig. 3, E and F) and immunohistochemistry for carboxypeptidase A (CPA) (Fig. 3, G and H) confirm the loss of cell polarity in the *Mist1^{KO}* mice. In normal acinar cells, ZGs accumulate towards the center of the acinus, whereas apical localization is lost completely in the *Mist1^{KO}* mice (Fig. 3, E compared with F). This change in organization results in nuclei surrounded completely by ZGs (Fig. 3 H). Pancreatic tissue from *Mist1^{KO}* mice at time points earlier than 3 m also exhibits a consistent absence of ZG accumulation and cellular polarity (unpublished data).

***Mist1^{KO}* mice develop pancreatic lesions characteristic of severe pancreatic injury**

Analysis of *Mist1^{KO}* acinar tissue in older animals indicates that the absence of *Mist1* leads to a progressively more severe tissue architecture over time. Up to 9–10 m of age, this phenotype is manifested as a gradual deterioration of the aci-

nar cells. At 12 m, focal lesions, specific to the exocrine tissue, are observed readily (Fig. 4 B). These lesions appear initially as acinar cells that have significantly lower levels of enzymes and surround slightly distended lumens. More progressive lesions exhibit circular structures that appear as distended acini or ducts (Fig. 4 C). In mice that exhibit extensive damage, there is a significant decrease in the amount of acinar tissue along with large accumulations of other cell types (Fig. 4 D) including duct cells, stellate cells (see below), and leukocytes (unpublished data). There also is a dramatic loss of enzyme expression limited to small regions within the pancreas (Fig. 4 J). Importantly, the endocrine component of the pancreas appears unaffected in *Mist1^{KO}* mice, indicating that the observed tissue damage is specific to the exocrine pancreas (Fig. 4 K). Null mice exhibit relatively normal levels of insulin and glucagon, and blood serum glucose levels are unaffected (unpublished data). These observations suggest that pancreatic damage in the *Mist1^{KO}* mice is limited to the cells that normally express *Mist1* (for example acinar cells). Interestingly, the relative wet weight of

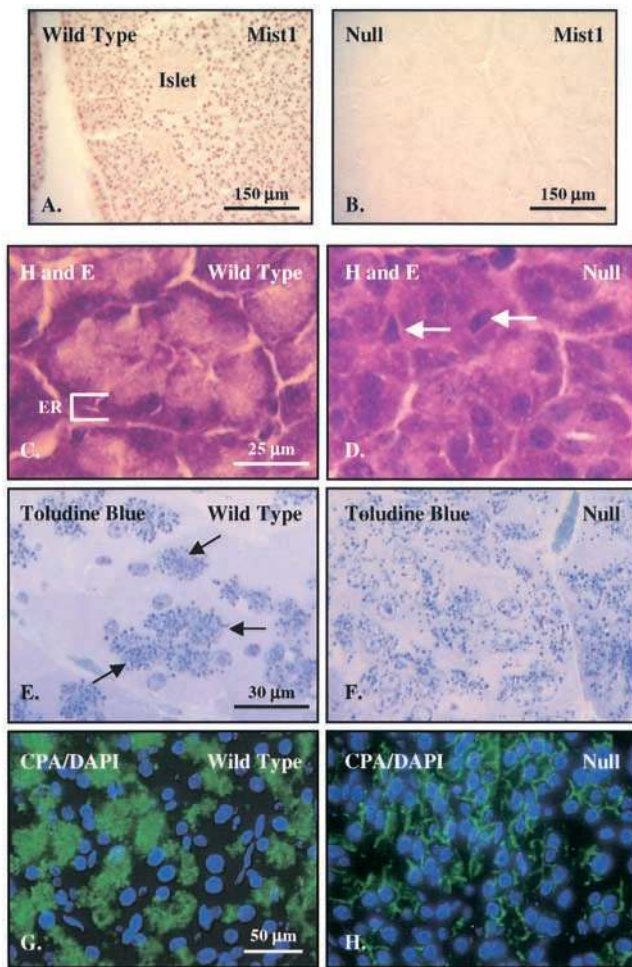


Figure 3. *Mist1*^{KO} mice have defective acinar cell organization and polarity. (A and B) The absence of *Mist1* expression is confirmed in *Mist1*^{KO} mice (B) using immunohistochemistry. Wild-type mice (A) show specific expression of *Mist1* in the nuclei of acinar cells. Islet and duct cells remain *Mist1* negative. (C–F) Histochemical analysis reveals acinar cell disorganization in 3-m-old *Mist1*^{KO} mice. (C) Stained cryostat sections from wild-type animals show exocrine acinus formation with well-defined cellular polarity and clearly demarcated ER borders (delineated by bracket). This organization is not detected in sections from *Mist1*^{KO} mice (D). At this magnification, nuclear dysplasia (arrows) is observed also. Epoxy sections stained with toluidine blue confirm the disorganization of the ZGs. In wild-type mice (E), ZGs accumulate in clusters (arrows) at the apical border of the acinar cells, whereas nuclei remain at the basal border of the cell. In *Mist1*^{KO} mice (F), ZGs extend throughout the cell with no specific localization observed. Immunolocalization of CPA (costained with DAPI) confirms the loss of ZG organization in *Mist1*^{KO} mice. The wild-type pancreas exhibits a well-defined cellular organization (G), whereas in null pancreatic tissue CPA localization completely surrounds individual nuclei (H).

pancreatic tissue from *Mist1*^{KO} mice does not differ significantly from wild-type mice.

In an effort to determine the fate of acinar cells within pancreatic lesions, individual pancreas sections were examined for *LacZ* expression. As shown in Fig. 4, E–H, cells within these lesions appear to represent acinar cells that exhibit a duct cell phenotype. Initially, β -gal expression is limited to acinar cells (Fig. 4 F). However, as lesion formation becomes more significant *LacZ* expression is observed in

cells lining putative duct structures (Fig. 4 G). Eventually, the lesions become extensive, and only a minority of the cells continue to express β -gal (Fig. 4 H).

Analysis of *Mist1*^{KO} pancreatic tissue with antibodies specific to CK-20 (Fig. 5, A and B) and CK-7 (unpublished data) confirms that these mice develop extensive duct cell accumulations throughout the tissue. CK staining is accompanied by a decrease in CPA (unpublished data) and amylase expression (Fig. 6). Many cells coexpress β -gal and CK-20 (Fig. 5, C–E), suggesting the possibility that acinar cells may be reverting to a duct cell phenotype. *Mist1*^{KO} pancreatic tissue also exhibits a significant increase in the number of desmin- and vimentin-positive cells (unpublished data) and the appearance of cells expressing smooth muscle actin (SMA) (Fig. 5 G). Many of the SMA-positive cells exist within the walls of small blood vessels and reveal an increased vascularity of the *Mist1*^{KO} pancreatic tissue. However, there also are many single cells that are not part of the vascularity. The expression of SMA in these cells suggests that they are activated stellate cells, which promote increased collagen deposition and are characteristic of several pancreatic diseases (Haber et al., 1999). Gomori's trichrome staining confirms an increase in connective tissue (Fig. 5 H), indicating that fibrosis is also occurring in the exocrine pancreas of these animals. The presence of activated stellate cells with increased ductal organization suggests that *Mist1*^{KO} acinar tissue exhibits significant pancreatic injury.

To confirm the coexpression of duct and acinar cell-specific markers within individual cells of the exocrine pancreas of *Mist1*^{KO} mice, antibodies specific to CK-20 and amylase were colocalized on cryostat sections. Regular fluorescence microscopy reveals the expression of amylase surrounding the lesion with many cells within the lesion also expressing amylase (Fig. 6 A). CK-20 expression is increased throughout the lesions (Fig. 6 B), and several cells appear to coexpress both markers (Fig. 6 C). To confirm this coexpression pattern, confocal microscopy was performed on 0.7- μ m thick optical sections. As shown in Fig. 6, D–I, coexpression of CK-20 and amylase is observed readily in many cells within the lesions, whereas cells from wild-type animals never coexpress these two markers (Fig. 1, C–H). The identification of cells coexpressing CK-20 and amylase suggests that these cells may represent a transition state between an acinar and duct cell phenotype.

Loss of proteins involved in cellular organization

To examine the underlying cause of acinar cell disorganization in *Mist1*^{KO} mice, proteins involved in adherens junction formation were analyzed. The adherens junction complex has been implicated as an important mediator of cellular polarity through maintaining cell–cell interactions and stabilizing the cytoskeleton (Petzelbauer et al., 2000). At 1 m of age, *Mist1*^{KO} animals contain normal levels of β -catenin expression (Fig. 7 A). However, 5-m-old *Mist1*^{KO} mice show a significant decrease in expression, and by 12 m of age β -catenin is diminished greatly. This gradual loss of expression is also observed for γ - and α -catenin (unpublished data) but not for E-cadherin (Fig. 7 B), suggesting that the catenin protein family is influenced specifically by the absence of *Mist1*. These results were further confirmed by immunohis-

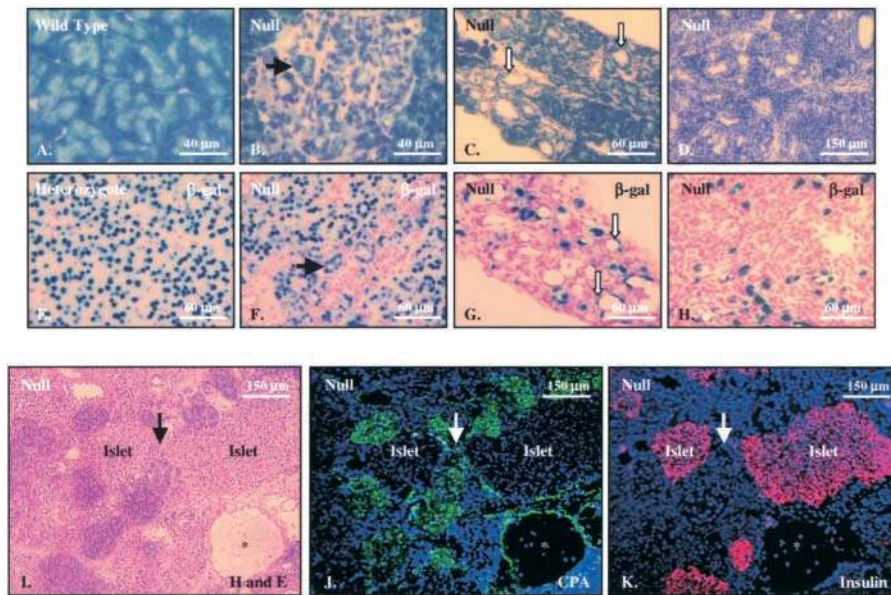
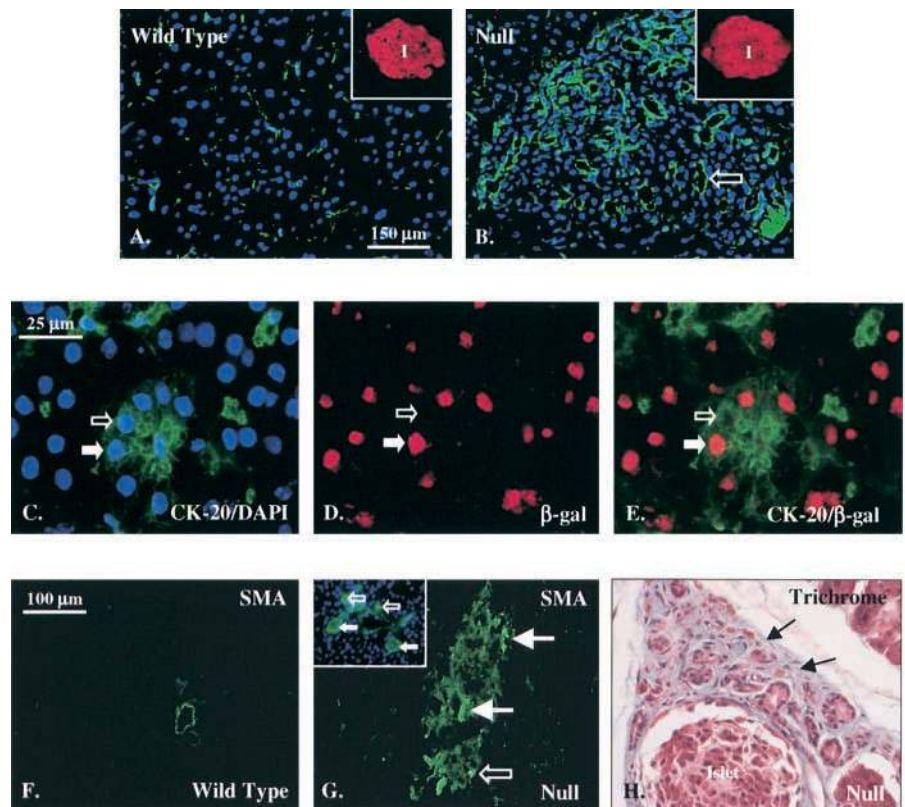


Figure 4. ***Mist1*-null mice develop exocrine pancreatic lesions by 12 m of age.** (A–H) To reveal the overall morphology of the tissue, cryostat sections were stained with methylene blue (A–D) or β -gal histochemistry and counterstained with nuclear fast red (E–H). Wild-type littermates exhibit typical acinar cell organization (A). In contrast, pancreatic tissue of *Mist1*^{KO} mice shows progressive deterioration, resulting in lesions throughout the exocrine tissue (B–D). Initially, lesions contain presumptive acinar tissue (arrow) that maintains expression of the *Mist1* gene locus (β -gal positive) (F). Eventually, circular duct-like structures are observed within the lesions (C). The appearance of presumptive ducts containing β -gal-positive cells (G, open arrows) suggests that acinar cells may have developed a duct cell phenotype. In $\sim 10\%$ of the *Mist1*^{KO} mice, the majority of pancreatic tissue is lost (D) with few *Mist1-LacZ*-expressing cells remaining (H). (I–K) Exocrine tissue in *Mist1*-null animals is selectively targeted for trauma. Pancreatic tissue from a severely affected animal (I) reveals extreme disruption of the exocrine tissue (J) while retaining islets and insulin expression (K). Arrows mark identical positions on the individual serial sections. The asterisks indicate a large area that is CPA and insulin negative.

Figure 5. **Lesions within *Mist1*^{KO} pancreatic tissue contain cells that coexpress the *Mist1* locus and duct cell markers.** (A and B) Immunohistochemistry with CK-20-specific antibodies reveals the normal accumulation of duct tissue within wild-type animals (A). In contrast, the *Mist1*^{KO} pancreas contains large accumulations of duct cells throughout the exocrine tissue (B, open arrow). Identical staining patterns are obtained with antibodies against CK-7 (unpublished data). Insets reveal normal insulin-positive islets in these animals. (C–E) Staining for CK-20 and β -gal reveals apparent coexpression of acinar- and duct-specific markers in the same cells. The filled and open arrows point to β -gal-positive and -negative nuclei, respectively, within CK-20-positive cells. (F–H) Immunohistochemistry for SMA on wild-type (F) and null (G) tissue sections. SMA expression is limited to blood vessels in the wild-type mouse. However, several SMA-positive cells are observed throughout the null tissue with one particular lesion shown (H). Higher magnification (inset, costained with DAPI) indicates that these cells likely represent small blood vessels (filled arrow) and stellate cells (open arrow). Staining of null sections with Gomori's trichrome (H) also reveals the vast accumulating connective tissue (green, arrows) found in many fibrotic lesions.



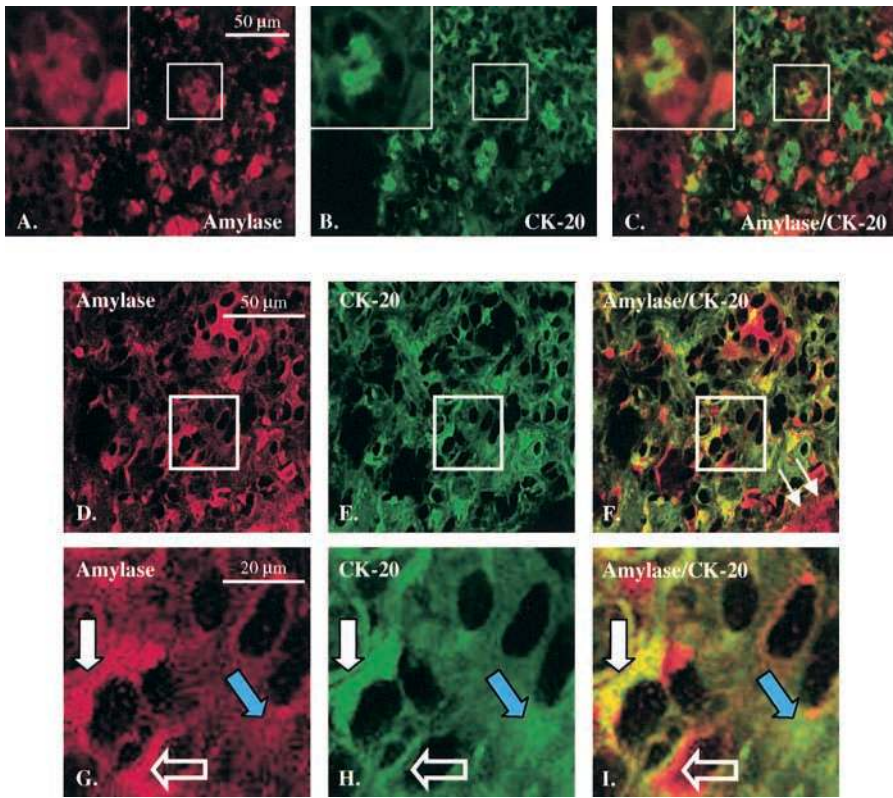


Figure 6. Lesions in *Mist1*^{KO} mice contain cells expressing both duct and acinar cell markers. (A–C) Staining for amylase (A) and CK-20 (B) reveals coexpression of acinar- and duct-specific markers in the same cells (combined in C). The insets show a higher magnification of one particular acinus that coexpresses CK-20 and amylase. (D–I) Confocal microscopy on a similar lesion at 0.7- μ m optical section resolution confirms the coexpression of amylase (D and G) and CK-20 (E and H). Cells can be observed that express either amylase (open arrow), CK-20 (blue arrow), or both cell markers (white arrow) (F and I). G–I show a higher magnification of the areas highlighted in D–F. The arrows in F demarcate the edge of the particular lesion. The cells outside the lesion are primarily amylase-positive acinar cells.

tochemical analysis on 12-m-old tissue in which β -catenin is not observed in acinar cells, although islet and ductal cells maintain appropriate levels and localization of the protein (Fig. 7, C–D). Analysis of the tight junction protein ZO-1 shows that it is maintained at appropriate levels with the protein localizing to presumptive apical borders of acinar cells (Fig. 7, E and F). However, the ZO-1 staining also reveals the expanded ducts that are characteristic of *Mist1*^{KO} pancreatic tissue, delineating the border of distended lumens in the center of acinar cell clusters. Although tight junctions continue to form, the loss of catenin expression may account for the increasing severity of the *Mist1*^{KO} phenotype and the eventual loss of the acinar cell phenotype.

Mist1^{KO} mice model chronic pancreas injury

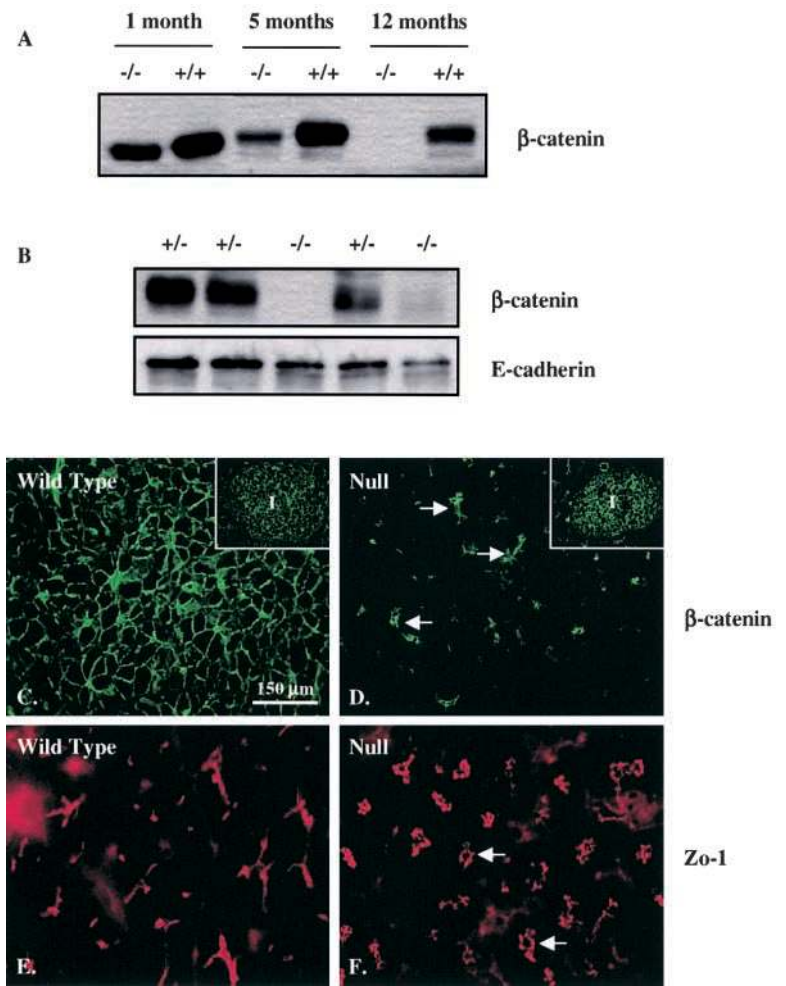
To assess the potential of *Mist1*^{KO} mice as a model for pancreatic injury, protein extracts from adult pancreatic tissues were analyzed for the expression of several digestive enzymes (Fig. 8 A). Western blot analysis reveals equivalent levels of amylase expression in 1-m-old wild-type and *Mist1*^{KO} mice. However, at later time points (4 and 12 m), there is a slight but consistent decrease in the expression of amylase. Similar analysis of CPA reveals relatively equal levels of expression throughout the first 12 m in wild-type and *Mist1*^{KO} animals. However, *Mist1*^{KO} mice also contain an active intracellular form of the enzyme. The inactive precursor to CPA (procarboxypeptidase) migrates at 47 kD and is the exclusive form found in wild-type mice. Active CPA, which should not be present within acinar cells, migrates at 33 kD and is found at all time points examined in *Mist1*^{KO} animals. Electron micrographs similarly reveal ultrastructural defects in cellular organization and confirm the intracellular digestion of indi-

vidual organelles, supporting the potential outcome of premature enzyme activation in *Mist1*^{KO} acinar cells (Fig. 8, B and C). Organelles are observed fusing readily to one another, mitochondria often are degraded (Fig. 8 B), and acinar cells contain large autophagocytic vesicles (Fig. 8 C), all of which are hallmarks of cells undergoing extensive intracellular degradation. Finally, to extend the comparison of the *Mist1*^{KO} mouse to other animal models we also examined the expression patterns of several genes known to be upregulated after the induction of pancreatic injury (Iovanna et al., 1991; Dusetti et al., 1996; Mallo et al., 1997). As expected, expression of the *RegI/PSP*, *PAP1/RegIII*, and *p8* genes are increased dramatically in *Mist1*^{KO} pancreatic tissue (Fig. 8 D).

Defects in the CCK signaling pathway are associated with the *Mist1*^{KO} phenotype

Several animal models of pancreatic injury suggest that defects in regulated exocytosis can lead to cellular disorganization and degradation (Leach et al., 1991). The exocytosis pathway of acinar cells is dependent on (a) the correct localization of ZGs to the apical border and (b) the existence of specific signaling pathways that identify and act upon external cues. Acinar cells of *Mist1*^{KO} mice exhibit no apical organization. Therefore, we examined the expression profiles of specific proteins involved in ZG localization (VAMP2) (Gaisano et al., 1996) and CCK signaling (CCK A receptor [CCKAR] and IP₃R 1–3) (Williams, 2001) to determine if these pathways are altered in *Mist1*-null mice. As shown in Fig. 9 A, *Mist1*^{KO} mice exhibit a complete loss of IP₃R3 expression, which is accompanied by slightly increased levels of VAMP2. Interestingly, IP₃R1 and IP₃R2 levels are unaffected in *Mist1*-null mice. Further analysis reveals that the

Figure 7. Progressive loss of β -catenin is associated with increased acinar cell disorganization in $Mist1^{KO}$ mice. (A) Western blot analysis of protein samples from wild-type (+/+) and $Mist1^{KO}$ (-/-) mice reveals a gradual loss of β -catenin expression in $Mist1^{KO}$ acinar tissue. (B) The loss of β -catenin expression is observed in all $Mist1^{KO}$ mice at 12 m of age, whereas $Mist1^{LacZ}$ mice (+/-) exhibit normal levels of β -catenin. E-cadherin expression remains relatively normal in $Mist1^{KO}$ mice. (C–F) Loss of β -catenin is specific to the acinar tissue in $Mist1^{KO}$ mice. The exocrine tissue of $Mist1$ -null mice exhibits a complete absence of β -catenin protein (compare C with D), whereas the islets (I, insets) and duct cells (arrows) continue to express normal β -catenin levels. Similar analysis with a ZO-1-specific antibody (E and F) reveals that tight junctions are maintained in $Mist1^{KO}$ mice, albeit surrounding severely distended lumens (arrows).



loss of IP₃R3 expression is accompanied by an increase in the level of the CCKAR at all ages examined (Fig. 9 B). These changes in the CCK signaling pathway coupled with premature activation of digestive enzymes, cellular disorganization, and conversion of acinar cells to duct cells supports the hypothesis that $Mist1^{KO}$ mice represent a genetic model for pancreatic injury, resembling early and advanced symptoms of pancreatitis.

Discussion

Cellular development involves key transition stages of establishing a specific lineage (determination), expression of cell-restricted gene products (differentiation), and the modification of a final adult phenotype (maturation). For the exocrine pancreas, complete development includes the expression of digestive enzymes, the appearance of cellular polarity, and the establishment of regulated exocytosis. To date, only one transcription factor has been identified as playing a primary role in any of these processes. PTF1-p48 is critical for acinar cell determination (Krapp et al., 1998) and regulates transcription of many digestive enzymes through a consensus PTF1 binding site (Cockell et al., 1989). However, the complete absence of pancreatic tissue in *PTF1-p48*-null mice prevents analysis of its role in acinar cell organization and regulated exocytosis. In this study, we provide evidence that

Mist1, a bHLH transcription factor expressed specifically in the exocrine pancreas, is necessary for the complete maturation and the maintenance and proper function of pancreatic acinar cells. In the absence of *Mist1*, extensive disorganization occurs, leading to pancreatic injury and loss of the acinar cell phenotype. We propose that $Mist1^{KO}$ mice represent a novel mouse model for chronic pancreatic disease.

Experimental animal models of pancreatic injury (pancreatitis) have involved increased exposure to secretagogues, dietary manipulation, and transgene overexpression (Saluja et al., 1989; Sanvito et al., 1995). The $Mist1^{KO}$ phenotype parallels these models in three important ways. First, these mice exhibit dramatic transcriptional increases in several genes known to be associated with pancreas injury and are linked to the development of pancreatitis, including *RegI/PSP*, *PAP1/RegIII*, and *p8* (Iovanna et al., 1991; Duseti et al., 1996; Mallo et al., 1997). Second, $Mist1^{KO}$ mice show classic histological signs of chronic pancreatitis, including increases in active digestive enzymes, that lead to the presence of autophagocytic vesicles (Leach et al., 1991), the activation of stellate cells (Haber et al., 1999), and the appearance of duct cell accumulations (Wildi et al., 1999). Finally, $Mist1^{KO}$ mice exhibit defects in the regulated exocytosis signaling pathway including loss of cellular organization and misexpression of IP₃R3 and CCKAR. IP₃R3 is a key regulator for intracellular calcium release (Joseph, 1996), which is

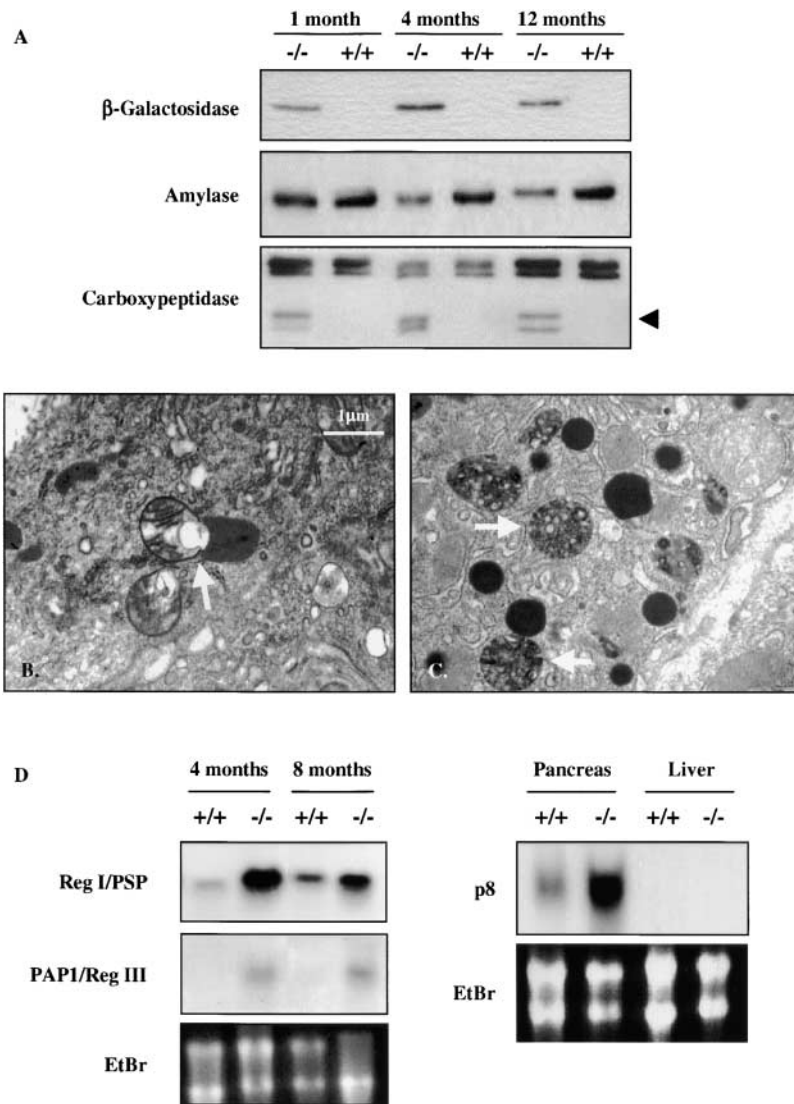


Figure 8. *Mist1*^{KO} mice present biochemical and molecular markers of pancreatic injury. (A) Western blot analysis for pro-CPA (47 kD) reveals the accumulation of the activated CPA enzyme (35 kD), specifically in *Mist1*^{KO} animals (arrowhead). (B and C) Intracellular digestion is observed in *Mist1*^{KO} mice. In B, a presumptive lysosome is observed fusing with a mitochondrion (arrow). Many digested mitochondria can be seen within each acinar cell, and autophagocytic bodies are observed commonly in the *Mist1*^{KO} samples (C, arrow). (D) Northern hybridizations were used to examine *RegI/PSP* and *PAPI/RegIII* gene expression at 4 and 8 m of age. In both cases, a significant increase in expression is observed in *Mist1*^{KO} mice. A similar increase is detected in *p8* transcript levels in *Mist1*^{KO} mice compared with wild-type litter mates. Liver RNA samples are included as negative controls.

essential for movement and correct targeting of ZGs to the acinar cell apical border (Ito et al., 1997), whereas the CCK pathway is critical to monitoring the degree of pancreatic enzyme release (Williams, 2001). Previous studies indicate that mistargeting of ZGs produces premature activation of enzymes within acinar tissue (Grady et al., 1998) with the active enzymes further disrupting the architecture of the acinar cells. The presence of activated enzymes may contribute to the eventual loss of catenin expression that, in turn, leads to a loss of adherens junctions and decreased coupling of individual acinar cells. The combined effects of enzyme activation and a disruption in cellular architecture may contribute to the loss of the acinar cell phenotype. This is the first example of a single gene deletion being linked to progressive pancreatic injury without external manipulation. Therefore, the availability of the *Mist1*^{KO} mouse model should be invaluable in determining the factors that initiate and promote pancreatic injury disease.

Whether the loss of organization in *Mist1*^{KO} acinar cells predates enzyme activation is still under investigation. Preliminary histological analysis of other cell types that express *Mist1* (for example, acinar cells in the salivary glands and

cells lining the seminal vesicles) reveals a similar disruption in cell organization (unpublished data). Although it is possible that enzymes within these other cell types are prematurely activated, the similar cellular phenotype suggests that the primary defect in *Mist1*^{KO} mice is a loss of correct cellular organization, potentially due to defects in the regulated exocytosis pathway.

It is somewhat surprising that *Mist1*^{KO} mice show no overt phenotypic abnormalities given the significant disruption of cellular organization. One would predict that this disruption would lead to a decrease in exocytosis and digestion with an eventual affect on the relative weight of *Mist1*^{KO} mice. However, the loss of pancreatic enzyme production must be extreme to produce noticeable phenotypic abnormalities (DiMagno et al., 1973; Gaskin et al., 1984). Significant trauma to the pancreas occurs in both pancreatic cancer and pancreatitis, and these diseases are detected at rather late stages when almost complete wasting of the pancreas has occurred. In addition, mice that overexpress TGF- β 1 exhibit a near complete loss of exocrine tissue with no overt abnormalities (Lee et al., 1995). Although there is a general reduction in ZGs in *Mist1*^{KO} mice, the relative amounts of enzyme are

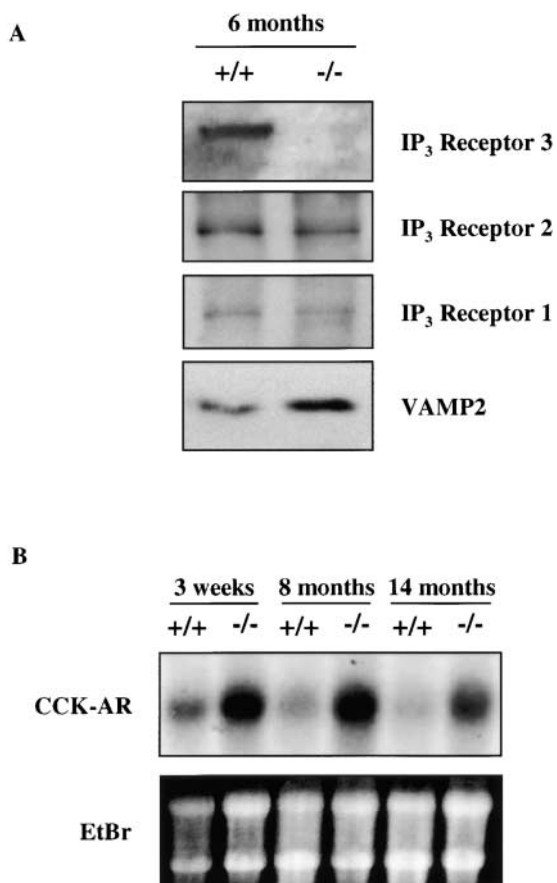


Figure 9. *Mist1*^{KO} mice exhibit alterations in the CCK signaling pathway. (A) Western blot analysis for IP₃R1, -2, and -3, and VAMP2 expression. A specific loss of IP₃R3 expression is observed in 6-m-old *Mist1*^{KO} mice, whereas VAMP2 protein levels are elevated slightly in null animals. (B) Northern hybridization of pancreatic RNA at 3 wk and 8 and 14 m of age reveals increased expression of CCK AR in *Mist1*^{KO} mice (-/-) compared with wild-type (+/+) animals.

consistent with wild-type levels. This is probably due to accumulations of activated enzymes in both intra- and extracellular locations after ZG disruption. Therefore, it is likely that sufficient amounts of enzymes still reach the intestine for digestion. On the other hand, premature enzyme activation and disrupted cellular organization likely predispose *Mist1*^{KO} mice to significant pancreatic disease under adverse conditions. The mice in this study were kept under strict environmental conditions, and current experiments are aimed at challenging these mice through a variety of dietary and experimental manipulations.

Although we have been able to document the phenotypic defects associated with adult *Mist1*^{KO} mice, it remains unclear if *Mist1* has a specific function during the initial stages of embryonic pancreas formation. The early appearance of *Mist1* in the foregut (E10.5) identifies *Mist1* as one of the first exocrine-specific markers to be expressed in the developing pancreas, and yet *Mist1* is clearly not required for the initial specification of the exocrine pancreatic lineage. The only other transcription factor that exhibits a similar exocrine pancreas specificity is PTF1-p48 (Krapp et al., 1996). Although *Mist1* has the ability to form heterodimers with

the bHLH factors associated with the PTF1 complex (unpublished data) and *Mist1* and PTF1-p48 are found within identical pancreatic cells, gene-targeting experiments show clearly that *Mist1* and PTF1-p48 remain functionally distinct. Although it is possible that *Mist1* could act as a repressor to modulate the effects of the PTF1 complex, this seems unlikely, since substantial increases in digestive enzyme expression are not observed in *Mist1*^{KO} mice. Thus, *Mist1* is likely positioned downstream of the regulatory pathways controlled by PTF1-p48.

In contrast, it seems likely that *Mist1* is necessary for full maturation of the acinar cell phenotype. In the absence of *Mist1*, cell polarity is not achieved, and this lack of organization likely contributes to the eventual loss of the acinar cell phenotype. The loss of the acinar cell phenotype is indicative of acinar cell dedifferentiation and occurs in many different pancreatic diseases (Steer, 1997). Dedifferentiation also is observed in mice expressing dominant negative forms of the TGF- β (Bottinger et al., 1997) or activin II (Shiozaki et al., 1999) receptors and is believed to delineate acinar cell hyperplasia. The maintained expression of *p8* (Vasseur et al., 1999) and *RegII/PSP* (Levine et al., 2000) in the *Mist1*^{KO} pancreas supports the lack of complete maturation of acinar cells, since these genes are normally expressed during embryonic pancreatic development and exhibit very low levels of expression in adult tissue. Currently, studies are underway to determine the precise molecular targets of *Mist1* and how alterations in the expression of these target genes lead to acinar cell dedifferentiation and the initiation of pancreatic injury.

Materials and methods

Creation of *Mist1*^{LacZ} mice

A 12-kb region of the mouse *Mist1* gene (including the entire coding region) (Pin et al., 1999) was used to generate a targeting plasmid containing the neomycin resistance gene cassette pGT29 (NEB) and pSKTnslacZ, which encodes a nuclear localized β -gal gene product (Fire et al., 1990) (details available upon request). The final targeting vector was analyzed carefully by restriction mapping and DNA sequencing to confirm that the entire 5' flanking region of *Mist1* was intact up to position +782, at which point the *LacZ* gene was inserted. The targeting construct was linearized by NotI digestion and electroporated into TL1 ES cells as described by Van Kaer et al. (1992). Individual targeted ES cell clones were injected into C57 B6 blastocysts to generate chimeric mice per standard procedures (Hogan et al., 1994).

RNA isolation and Northern hybridization

Pancreatic RNA was isolated according to Han et al. (1987) with modifications similar to Chirgwin et al. (1979). RNA from the liver was isolated using Trizol (GIBCO BRL) following the manufacturer's instructions. For Northern blot analysis, 30 μ g of total RNA was electrophoresed on a 1.0% agarose/formaldehyde gel, blotted onto Hybond membranes (Amersham Pharmacia Biotech) using 10 \times SSC and hybridized in 50% formamide, 5 \times SSPE, 2 \times Denhardt's solution, and 0.1% SDS for 18 h at 42°C. After hybridization, blots were washed at 65°C in solution I (2 \times SSC, 0.1% SDS) two times for 5 min, in solution II (1 \times SSC, 0.1% SDS) two times for 10 min, and in solution III (0.1 \times SSC, 0.1% SDS) two times for 5 min. Probes for Northern blot hybridizations included the full-length *p8* cDNA, a 400-bp fragment of the *CCK AR* cDNA, a 450-bp fragment of the *PAP1/RegIII* coding region, and a 400-bp fragment of the *RegII/PSP* coding region.

Antibodies

Antibodies were obtained from both commercial and individual suppliers. Primary antibodies included polyclonals specific for glucagon (1:1,000; Dako), insulin (1:1,000; Incstar), amylase (1:1,000; Calbiochem), CPA (1:1000; Biogenesis), β -catenin (1:2,000; Sigma-Aldrich), PTF1-p48 (1:250; a gift from R. MacDonald, University of Texas Southwestern Medical Cen-

ter, Dallas, TX), PDX1 (1:400; a gift from H. Edlund, University of Umeå, Umeå, Spain; and 1:5,000, a gift from C. Wright, Vanderbilt University Medical Center, Nashville, TN), VAMP2 (1:500; a gift from W. Trimble, Hospital for Sick Children, Toronto, Canada), IP₃R1 and IP₃R2 (1:20; a gift from R. Wojcikiewicz, State University of New York Upstate Medical Center, Syracuse, NY), and Mist1 (1:250; Pin et al., 2000). Monoclonal antibodies used were specific for SMA (1:100; Sigma-Aldrich), ZO-1 (1:100; Chemicon), vimentin (1:20; Sigma-Aldrich), desmin, CK-20 (Dako), CK-7, E-cadherin (1:1,000; BD Transduction Laboratories), α -catenin (1:1,000; BD Transduction Laboratories), β -gal (1:1,000; Sigma-Aldrich), and IP₃R3 (1:1,000; BD Transduction Laboratories). All secondary antibodies were obtained from Jackson ImmunoResearch Laboratories with the exception of the biotinylated anti-rabbit antibody, which was purchased from Vector Laboratories.

Immunohistochemistry, β -gal histochemistry, and Western analysis

Embryos were obtained from timed pregnant B6 mice, and observation of a vaginal plug was considered E0.5. Whole embryos obtained from E9 to E16 were embedded while the abdominal area in neonatal animals was dissected away from the rest of the animal before embedding. Embryos (E13 and older) and adult tissue were fresh frozen in OCT as described in Pin and Merrifield (1997). Embryos age E12.5 and younger were fixed in 2% formaldehyde in PBS and then assayed for LacZ expression following the protocol described in Pin et al. (1997). After β -gal histochemistry, embryos were incubated in 20% sucrose for 18 h and then embedded and sectioned. All tissues were sectioned at 5–10 μ m on a ZEISS cryostat at -20°C . Immunohistochemistry was carried out as described in Pin et al. (2000) using Texas red- or FITC-conjugated secondary antibodies (diluted 1:250). For confocal analysis, fluorescence localization was analyzed and images obtained using a ZEISS LSM 410 confocal microscope. 10–15 μ m sections were analyzed optically through a series of 0.7- μ m Z-sections. Sections also were stained with hematoxylin and eosin, Gomori's trichrome, toluidine blue, methylene blue, or processed for β -gal histochemistry as described in Pin et al. (1997). β -Gal-stained sections were counterstained with eosin (embryonic sections) or nuclear fast red (adult sections). Images were captured using a Sony video camera and the imaging program Northern Eclipse (Empix, Inc.).

Tissue protein extraction, electrophoresis, and Western blotting were performed as described in Pin et al. (2000). For Western blot analysis, 5 μ g (digestive enzymes) or 40–75 μ g (IP₃R1, IP₃R2, IP₃R3, VAMP2, E-cadherin, and β -catenin) of whole cell protein extracts were electrophoresed on acrylamide gels. Western blots were analyzed using an ECL kit (Pierce Chemical Co.) as per manufacturer's instructions.

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