XBP1 Controls Maturation of Gastric Zymogenic Cells by Induction of MIST1 and Expansion of the Rough Endoplasmic Reticulum

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BACKGROUND & AIMS: The transition of gastric epithelial mucous neck cells (NCs) to digestive enzyme–secreting zymogenic cells (ZCs) involves an increase in rough endoplasmic reticulum (ER) and formation of many large secretory vesicles. The transcription factor MIST1 is required for granulogenesis of ZCs. The transcription factor XBP1 binds the *Mist1* promoter and induces its expression in vitro and expands the ER in other cell types. We investigated whether XBP1 activates *Mist1* to regulate ZC differentiation. **METHODS:** *Xbp1* was inducibly deleted in mice using a tamoxifen/Cre-loxP system; effects on ZC size and structure (ER and granule formation) and gastric differentiation were studied and quantified for up to 13 months after deletion using morphologic, immunofluorescence, quantitative reverse-transcriptase polymerase chain reaction, and immunoblot analyses. Interactions between XBP1 and the *Mist1* promoter were studied by chromatin immunoprecipitation from mouse stomach and in XBP1-transfected gastric cell lines. **RESULTS:** Tamoxifen-induced deletion of *Xbp1* (*Xbp1*Δ) did not affect survival of ZCs but prevented formation of their structure. *Xbp1*Δ ZCs shrank 4-fold, compared with those of wild-type mice, with granulogenesis and cell shape abnormalities and disrupted rough ER. XBP1 was required and sufficient for transcriptional activation of *MIST1*. ZCs that developed in the absence of XBP1 induced ZC markers (intrinsic factor, pepsinogen C) but showed abnormal retention of progenitor NC markers. **CONCLUSIONS: XBP1 controls the transcriptional regulation of ZC structural development; it expands the lamellar rough ER and induces *MIST1* expression to regulate formation of large granules. XBP1 is also required for loss of mucous NC markers as ZCs form.**

Keywords: Maturation; Stem Cell; Cell Structures; Gastric Acid Secretion.

BASIC–ALIMENTARY TRACT

The corpus of the mouse stomach is an excellent tissue for studying developmentally regulated transcription factors (TFs) in generation of secretory cell architecture, because the epithelium turns over continuously throughout adult life. In addition, the gastric epithelial stem cell gives rise to several diverse secretory lineages. Zymogenic cells (ZCs), for example, reside at the base of gastric epithelial glands and develop after a prolonged (~2 weeks) phase as progenitor cells, known as mucous neck cells (NCs), which in turn differentiate from the gastric epithelial stem cell.[1,2] Thus, for the ZC lineage, distance from the progenitor zone corresponds to the differentiation stage.

The TF MIST1 is involved in ZC differentiation. In *Mist1*$^{-/-}$ mice, ZCs delay turning off progenitor markers as they arise from NCs, although fully differentiated ZCs eventually form in normal numbers. However, all *Mist1*$^{-/-}$ ZCs are structurally defective with deficient apical cytoplasms and small secretory vesicles, although they show normal deposition of elaborate, lamellar rough endoplasmic reticulum (rER).[3,4] The function of MIST1 as a secretory cell-specific structure-inducing TF is highly conserved: even in flies, the MIST1 orthologue DIMM mediates granule structure of peptide-secreting cells without affecting survival.[5,6]

Despite the complex and interesting developmental patterning in the gastric epithelium, little is known about the underlying transcriptional and molecular mechanisms. Some progress has been made in understanding morphogens in gastric patterning. For example, epidermal growth factor receptor ligands transforming growth factor α/epidermal growth factor/amphiregulin drive increased surface cell growth[7,8]; the Hedgehog pathway seems to be required for inhibiting surface cell growth and promoting NC transition into ZCs[9,10]; and various cytokines, such as interleukin-1β and interleukin-11, and in general the nuclear factor κB signaling pathway, seem to be key in regulating growth and multiple differentiation pathways.[9,11]

Other than MIST1, only a handful of other TFs play a known role in differentiation of adult corpus epithelial

Abbreviations used in this paper: ER, endoplasmic reticulum; NC, mucous neck cell; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; rER, rough endoplasmic reticulum; SPEM, spasmolytic polypeptide expressing metaplasia; TF, transcription factor; TM, tamoxifen; ZC, zymogenic cell.

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lineages: FOXQ1, which regulates granule maturation in mucus-secreting surface (aka pit/foveolar) cells[12]; NGN3 and MASH1,[13,14] which regulate development of hormone-secreting endocrine cells; and KLF4, which apparently regulates differentiation of multiple secretory lineages.[15]

XBP1 is a TF traditionally viewed as a key regulator of the unfolded protein response during endoplasmic reticulum (ER) stress. XBP1 messenger RNA is spliced and thereby activated by IRE1, which governs part of the unfolded protein response.[16] XBP1 has also been described as a developmentally regulated TF that induces ER expansion and may be required for differentiation of dedicated secretory cells, such as antibody-secreting plasma cells and intestinal Paneth cells.[17–19] It is unclear, however, whether XBP1 is required for cell survival and fate determination like other developmentally regulated TFs or whether it plays a special role in establishing differentiated cell function rather than cell identity.[20,21] Interestingly, an in vitro screen recently identified MIST1 as a transcriptional target of XBP1 in myoblasts, plasma cells, and pancreatic β cells.[22] It is not clear whether XBP1 is required for induction of MIST1 or whether XBP1 targets MIST1 in vivo.

Here, we examine the role of XBP1 in gastric epithelial differentiation. Using inducible deletion with tamoxifen-Cre-loxP, we show that XBP1 is required for nearly the entire structural development of ZCs, including elaboration of rER and formation of large secretory granules. XBP1 induces MIST1 in gastric epithelial cell lines and, in mice, is required for induction of *Mist1* expression in ZCs. Interestingly, ZCs arising in the absence of XBP1 still induce normal ZC differentiation markers such as gastric intrinsic factor, but they cannot extinguish expression of progenitor NC markers; in other words, they never terminally differentiate. Thus, XBP1 is absolutely required for structural differentiation and maturation of ZCs but is dispensable for survival and initial induction of the ZC fate. The results show for the first time that XBP1 is the principal governor of ZC structural maturation, plays a role in shutting off progenitor features, and is required for induction of *Mist1* in vivo.

Materials and Methods

Mice

All experiments involving animals were performed according to protocols approved by the Washington University School of Medicine Animal Studies Committee. Floxed *Xbp1,* CAGGCreER™ transgenic mice, and germline $Xbp1^{-/-}$ mice with liver *Xbp1* transgene ($Xbp1^{-/-}$; Liv^{XBP1}) were generated as described previously.[17,23–26] $Xbp1^{flox/flox}$ mice were crossed with CAGGCreER™ transgenic mice, and then CAGGCreER™ tg; $Xbp1^{flox/+}$ mice were crossed with $Xbp1^{flox/+}$ to generate CAGGCreER™ tg; $Xbp1^{flox/flox}$ as well as a variety of control mice. Tamoxifen (0.75 mg/20 g body wt; Sigma, St Louis, MO) was injected intraperitoneally for 7 consecutive days to induce gene deletion. Mice were killed 7 days, 14 days, 28 days, 5 months, 7 months, and 13 months after the first injection of tamoxifen.

Cell Imaging

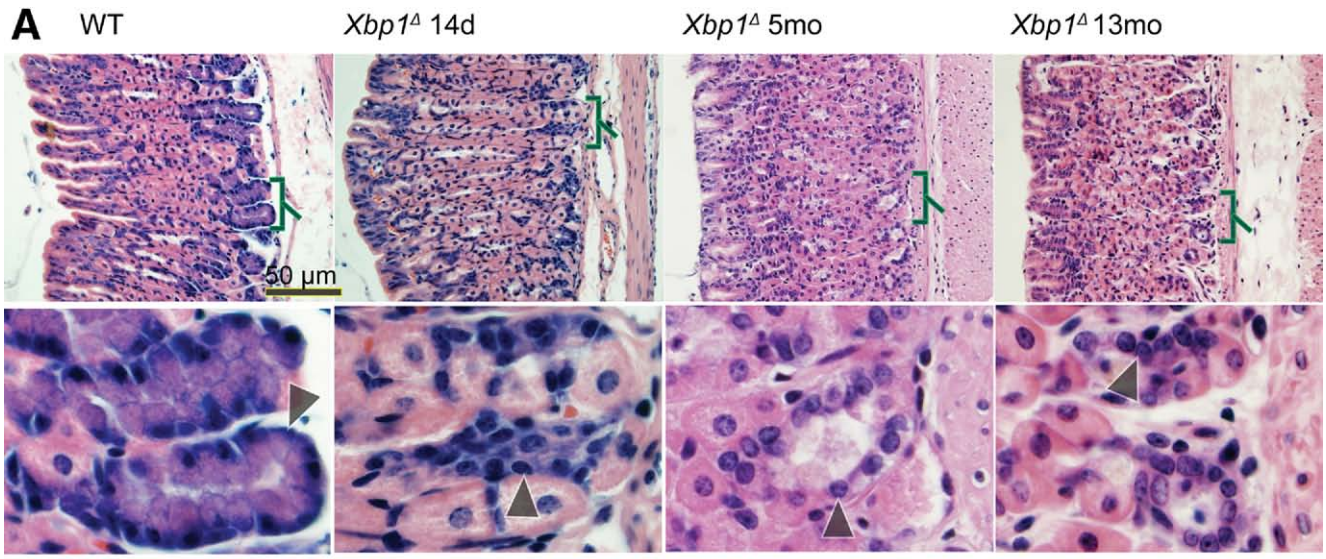
Transmission electron microscopy,[4] terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling, bromodeoxyuridine,[27] and other immunofluorescence studies were as described[3,4,28]; goat anti-calregulin (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 1:200. Immunofluorescent quantification to determine cytoplasmic fluorescence intensity was performed using ImageJ software with methods described previously[3]; however, for the current study, the mean fluorescent intensity for each cell was normalized to the maximum mean fluorescent intensity for that channel in each unit to generate the percent maximal mean fluorescent intensity. In $Xbp1^{\Delta}$ mice, 351 cells in 14 units from 2 mice were quantified; in controls, 225 cells in 9 units from 5 mice were quantified.

Stomach Chromatin Immunoprecipitation

Mouse stomach chromatin immunoprecipitation was performed following Wells and Farnham.[29] One stomach was dissected for one experiment, and the experiment was repeated with another mouse showing similar results. A total of 10 μL of rabbit anti-XBP1 antibody (Santa Cruz Biotechnology) with protein A/G plus agarose (Santa Cruz Biotechnology) were added to the tissue lysate for immunoprecipitation. A consensus XBP1 binding motif in the *Mist1* promoter was described previously,[22] and using ECR browser (http://ecrbrowser.dcode.org), we noted this site was conserved from human to mouse to opossum (not shown). Primers spanning the putative XBP1 binding site were designed with Primer3 (http://frodo.wi.mit.edu/primer3/) (Supplementary Table 1). Real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) with these primers was performed to assess the quantity of genomic sequences immunoprecipitated by anti-XBP1 antibody, as well as a 1:10 dilution of the cell extract before immunoprecipitation, and a control genomic region lacking consensus XBP1 binding sites.

Cell Line and Transient Transfection

AGS cells (a human gastric carcinoma cell line; American Type Culture Collection, Manassas, VA) were grown and transfected by Nucleofection as described[3] with 3 μg hXbp1(s) and 2 μg pmaxGFP (Lonza, Walkersville, MD). qRT-PCR analysis was as described.[30]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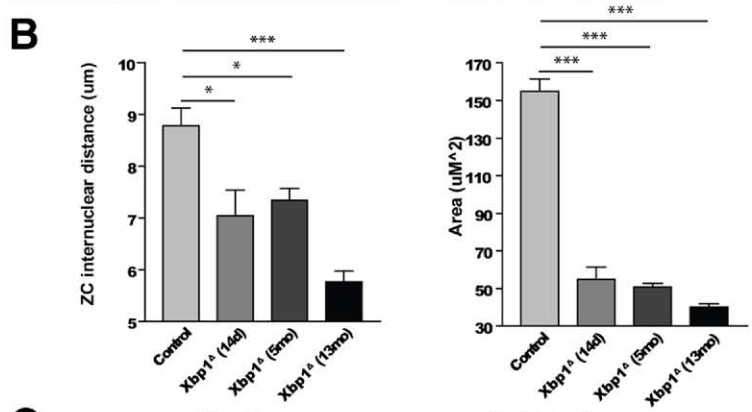
50 μm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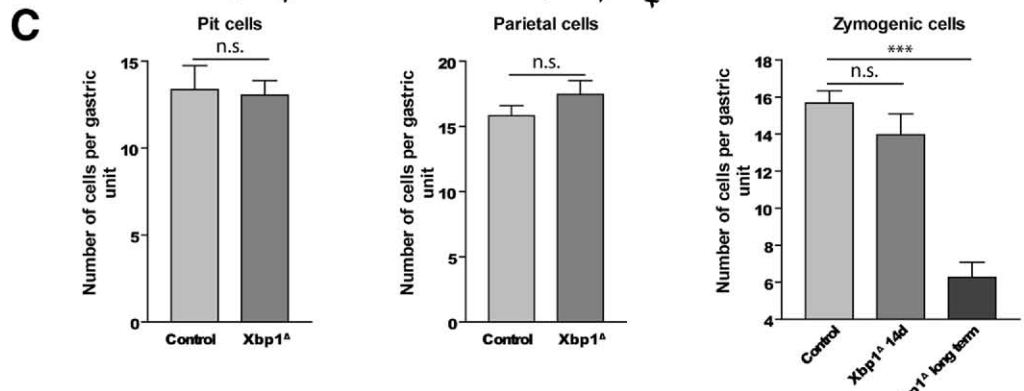
ZC internuclear distance (um)
Area (uM^2)
Control
Xbp1Δ (14d)
Xbp1Δ (5mo)
Xbp1Δ (13mo)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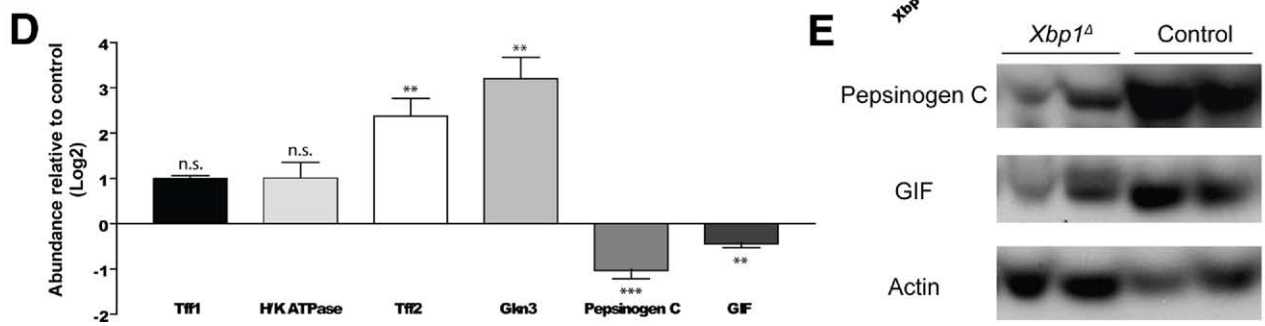
Parietal cells
Zymogenic cells
Number of cells per gastric unit
n.s.
Control
Xbp1Δ
Xbp1Δ 14d
Xbp1Δ long term


D
Abundance relative to control (Log2)
Tff1
H'K ATPase
Tff2
Gkn3
Pepsinogen C
GIF
E
Xbp1Δ
Control
Pepsinogen C
GIF
Actin

Western Blot and qRT-PCR for Stomach Tissue

For blots, corpus tissue was frozen in liquid nitrogen and ground with mortar and pestle with proteins separated on NuPAGE 4%–12% (Invitrogen, Carlsbad, CA), transferred to polyvinylidene difluoride, and detected by Immobilon chemiluminescence (Millipore, Billerica, MA). Primary antibodies were rabbit anti-MIST1 (1:200), sheep anti-PGC (1:1000), rabbit anti-GIF (1:20,000), and goat anti-actin (1:1000; Santa Cruz Biotechnology). Secondary antibodies were horseradish peroxidase–conjugated donkey anti-rabbit (1:2000; Jackson ImmunoResearch, West Grove, PA) and donkey anti-sheep and anti-goat (both 1:2000; Santa Cruz Biotechnology). For qRT-PCR, total RNAs from corpus were extracted and assayed as described.[27]

Results

Our earlier work showed that ZCs express the highest levels of *Xbp1* in the gastric epithelium.[4] To determine its role in ZC development, we deleted *Xbp1* in adult stomachs by tamoxifen injections in $Xbp1^{flox/flox}$ mice[23] expressing CAGGCreER™ (chicken β-actin promoter).[24] Based on our long-term experience with expression patterns of this promoter in stomach and other tissues,[27] we titrated tamoxifen concentration and frequency to the minimum needed to induce lacZ expression in all ZCs and their NC progenitors in CAGGCreER™/R26R mice (Supplementary Figure 1). The protocol resulted in loss of 79.1% ± 5.7% of *Xbp1* expression across the whole stomach by qRT-PCR (n = 7 mice, 4 experiments; Supplementary Figure 1).

Effects of Loss of Xbp1 *Are Specific to the Zymogenic Lineage*

Induced *Xbp1* deletion (hereafter designated $Xbp1^{\Delta}$) caused dramatic reorganization of the basal, ZC-containing zone of gastric units (Figure 1*A*). We examined every possible control: with or without *Cre* allele, with or without floxed *Xbp1* alleles, and with or without tamoxifen. The ZC phenotype was observed only in tamoxifen-treated CAGGCreER™; $Xbp^{flox/flox}$ stomachs. Deletion of *Xbp1* caused a substantial reduction in size and number of histologically identifiable ZCs. By 14 days after deletion, the average cross-sectional area of $Xbp1^{\Delta}$ ZCs was decreased 2.8-fold and 3.9-fold by 13 months (the longest time point studied). The census of NCs, the progenitors of ZCs, showed considerable variability across the gastric region and from mouse to mouse even in wild-type mice, precluding statistical analysis. However, there was a statistically significant increase of the NC markers TFF2 and GKN3[31] by qRT-PCR in the gastric corpus (Figure 1*D*). Other gastric lineages (eg, parietal, pit, endocrine cells) were not affected by *Xbp1* deletion histologically (Figure 1*A and C* and Supplementary Figure 2) or by qRT-PCR for specific markers (Figure 1*D*). At no time were inflammatory cells observed. Consistent with the reduction in ZC size, there was a modest but statistically significant decrease in 2 ZC markers, GIF and PGC, in the corpus by qRT-PCR and by Western blot (Figure 1*D* and *E*).

XBP1 Is Necessary for rER and Secretory Vesicles in Zymogenic Cells

XBP1 regulates rER organization and deposition; as wild-type ZCs arise, they show a dramatic increase in rER relative to their NC progenitors (Figure 2*A*, *upper panel*). In contrast, $Xbp1^{\Delta}$ ZCs showed nearly no increase in rER compared with progenitors (Figure 2*A*). Transmission electron microscopy confirmed sparse, disorganized rER in $Xbp1^{\Delta}$ and abundant, lamellar rER in wild-type ZCs (Figure 2*B* and *C*). Interestingly, we noted frequent free ribosomes, organized in a whorled pattern but no longer associated with ER, in $Xbp1^{\Delta}$ ZCs (Figure 2*C*). ER stress markers were either slightly (EDEM1 and DNAJB9; $P < .05$) or markedly (CHOP and HSPA5; $P < .001$) increased in corpora of $Xbp1^{\Delta}$ mice (Figure 2*D*).

XBP1 Directly Binds the MIST1 Promoter and Is Sufficient to Induce MIST1

The deficiency in rER in ZCs lacking *Xbp1* is consistent with the known role of XBP1 in direct transcriptional regulation of cellular effectors that establish ER.[17] However, we also noted defects in granulogenesis, which typifies loss of function of the only other TF known to regulate ZC development, MIST1.[3,4] *Mist1* was recently identified as a potential direct transcriptional target of XBP1 in vitro.[22] Hence, we reasoned that XBP1 might act through parallel pathways: one wherein it directly induces cellular effectors of rER biogenesis and one wherein it regulates vesicular structure indirectly by inducing *Mist1*.

To test that hypothesis, we first asked whether XBP1 binds the *Mist1* promoter in the stomach in vivo. Chro-

Figure 1. *Xbp1* is required for ZC lineage cellular structure and function. (*A*) H&E staining of adult gastric units (oriented with gastric lumen to *left*), with control, CAGGCreER™; $Xbp^{flox/flox}$ stomachs 14 days, 5 months, and 13 months after tamoxifen injection. *Lower panels* show magnification of bracketed regions. *Arrowheads* indicate individual ZCs. (*B*) Internuclear distance (μm) and cross-sectional area (μm^2) measured from H&E sections (n = 3 independent experiments; means ± SD; control denotes data from all mice in the given experiment that were not homozygous for floxed *Xbp1* or did not have a Cre allele or were treated with vehicle rather than tamoxifen). (*C*) Cell census scored from H&E (n = 3 independent experiments). (*D*) Transcripts in whole stomach corpora by qRT-PCR (n = 7 mice; means ± SD). (*E*) Western blot of ZC markers from 2 $Xbp1^{\Delta}$ and 2 control mice. n.s., not significant. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$.

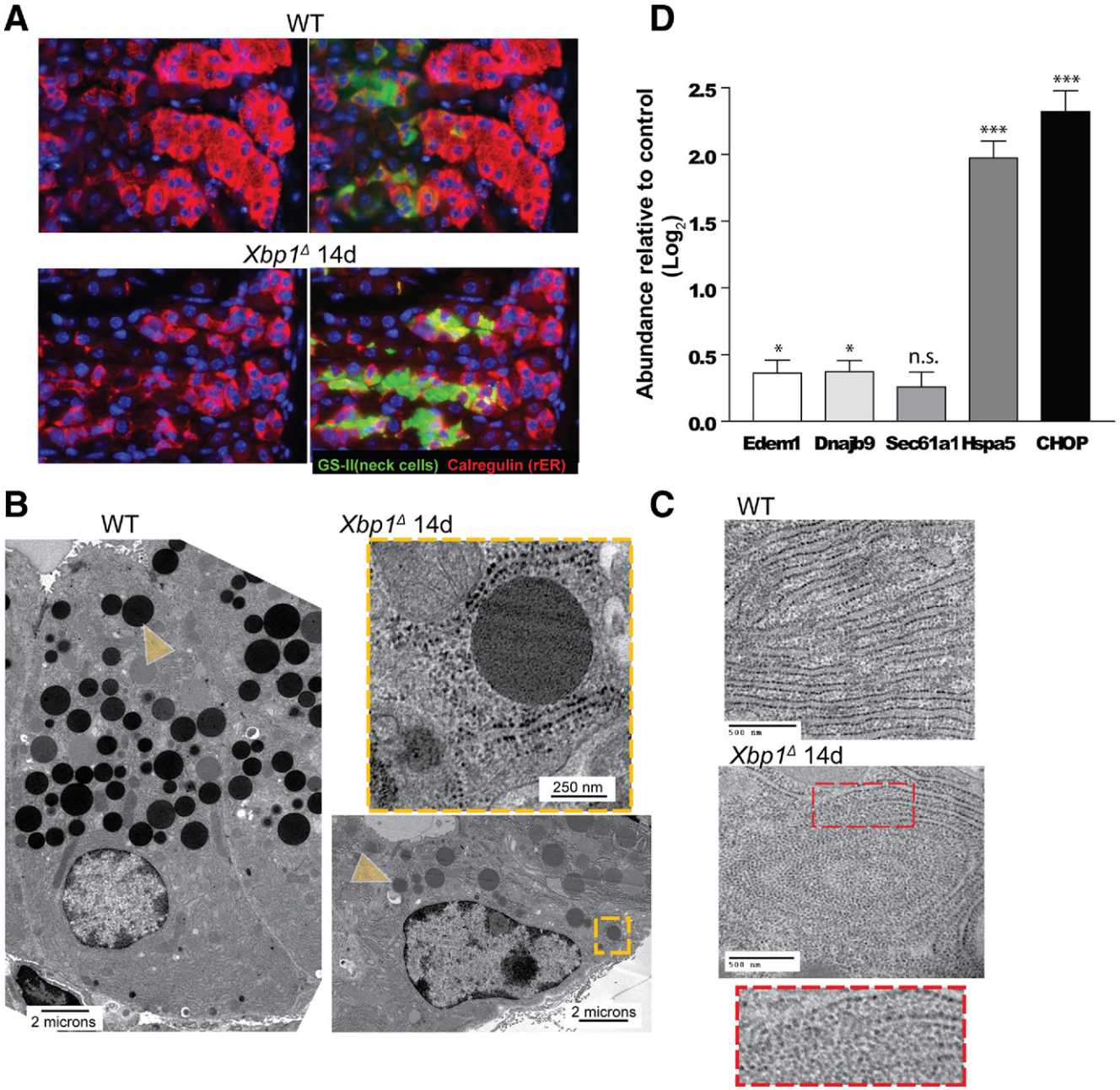


Figure 2. *Xbp1* is required for rER expansion and secretory vesicle formation. (*A*) Immunofluorescent staining for GSII (labels NCs, ZC progenitors, *green*) and calregulin (ER marker, *red*; nuclei, *blue* with bisbenzimide). Control (wild-type) ZCs induce abundant ER after emerging from neck progenitor cells. *Xbp1*$^{\Delta}$ ZCs lack rER expansion. (*B*) *Xbp1*$^{\Delta}$ ZCs have decreased secretory vesicles (eg, *yellow arrowhead*) by transmission electron microscopy. *Orange box* magnified in *inset*. (*C*) High-magnification view of rER in control vs *Xbp1*$^{\Delta}$ ZCs. *Inset* shows a magnified view of the box in the *middle panel*. Note loose whorls of free ribosomes with loss of associated ER in *Xbp1*$^{\Delta}$ ZC vs lamellar rER in control. (*D*) Expression levels of ER stress markers (n = 7 mice; means ± SD). n.s., not significant. $^{*}P < .05$; $^{***}P < .001$.

matin immunoprecipitation on adult mouse stomach indicated that XBP1 bound a consensus *cis*-regulatory sequence in the *Mist1* promoter but not a reference control site (Figure 3*A*). Next, we transfected a complementary DNA plasmid encoding the active, spliced form of *XBP1* into AGS cells. *MIST1* expression was significantly increased by *XBP1* transfection (Figure 3*B*, *left panel*). *XBP1* is expressed at high levels in cultured cells; hence, to circumvent confounding by endogenous *XBP1*, we repeated the transfections to varying final *XBP1* levels to confirm that *MIST1* levels always correlated positively with *XBP1* levels, independent of transfection efficiency (note the linear relationship [$r^2 = 0.81$; $P < .001$] between levels of *XBP1* and *MIST1* [Figure 3*B*, *middle panel*]). XBP1 is also induced by ER stress. Accordingly, untransfected cells treated with tunicamycin for 12 hours in-

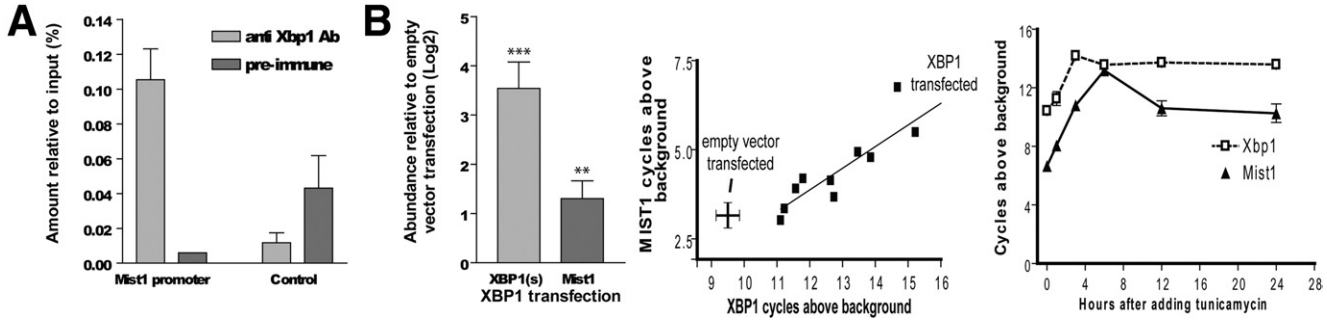


Figure 3. XBP1 directly binds *Mist1* promoter in the mouse stomach, and XBP1 is sufficient to induce MIST1 expression in gastric cells. (*A*) Chromatin immunoprecipitation from mouse stomach. Nuclear proteins from mouse stomach were immunoprecipitated with either anti-XBP1 or control preimmune antibody and fragmented genomic DNA amplified in qRT-PCR reactions (means ± SD). Note signal in the sample immunoprecipitated with anti-XBP1 antibody and amplified by primers flanking the XBP1 binding site in the *Mist1* promoter is enriched relative to control antibody immunoprecipitate; there is no such enriched amplification using primers flanking the control genomic site that lacks consensus XBP1 binding sites. (*B*) (*Left panel*) qRT-PCR for XBP1(s) (active, spliced form) and *MIST1* transcripts in *XBP1* transfected AGS normalized to empty vector transfected AGS cells (n = 11 independent experiments; means ± SD). (*Middle panel*) qRT-PCR for overall levels of *MIST1* and *XBP1* transcripts in the multiple independent *XBP1* transfection experiments summarized in the *left panel*. Note that despite high levels of endogenous *XBP1* (9.6 PCR cycles above background in vector transfected controls), increasing *XBP1* levels by transfection results in higher *MIST1* expression across a wide range of *XBP1* levels ($r^2 = 0.81$, $P < .0002$; crosshairs = SEM). Data represented as PCR cycles above control (water) wells after normalization to 18s ribosomal RNA (Bredemeyer et al[3]); hence, each axis represents $\log_2$ relative message abundance. (*Right panel*) *XBP1* and *MIST1* by qRT-PCR (means ± SD) following treatment with 1 μg/mL tunicamycin treatment (n = 3 independent experiments). $^{**}P < .01$; $^{***}P < .001$.

creased *XBP1* and *MIST1* expression (Figure 3*B*, *right panel*).

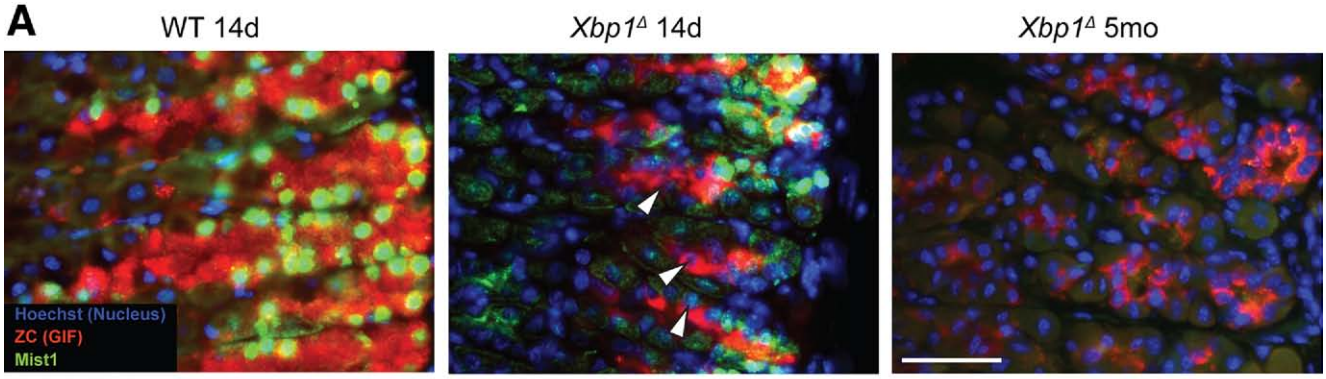
XBP1 Is Required for Induction of but Not Maintenance of MIST1

To determine whether XBP1 is necessary in vivo for induction of MIST1, we followed MIST1 expression by immunofluorescence in *Xbp1*$^{\Delta}$ ZCs. As expected, MIST1 levels were dramatically reduced by Western blot and immunofluorescence in *Xbp1*$^{\Delta}$ ZCs (Figure 4*A* and *B*). With qRT-PCR, *Mist1* expression was 25.2% of control levels by 14 days and absent by 5 months (not shown). However, we noted that some ZCs at the base at 14 days still expressed detectable MIST1 (Figure 4*A*). By transmission electron microscopy, those older, basally located ZCs were small and dramatically rER deficient but did not have as dramatic vesicular defects as the newly emerged ZCs closer to the progenitor zone. Vesicles in the first 5 ZCs closest to the progenitor zone were 60% smaller than wild-type, whereas basal ZC vesicles were essentially unchanged. In addition, although basal ZCs had 44% fewer vesicles per cell relative to wild-type (32.6 ± 9.0 vs 18.4 ± 4.2), emerging ZCs had even fewer vesicles (10.4 ± 0.8/cell, a 68% decrease) (Figure 4*C*).

One explanation for the more pronounced vesicular phenotype in newly emerged ZCs is that XBP1 is required for the induction of *Mist1* transcription but not for its maintenance. Normally, the transition from NCs to ZCs is characterized by induction of abundant MIST1, but ZCs forming from NCs in *Xbp1*$^{\Delta}$ mice would be *Xbp1* null, and, if XBP1 were absolutely required for direct activation of *Mist1*, the newly formed ZCs in the transition zone would always lack MIST1. On the other hand, ZC life span is several months,[1] so cells nearer the base would already be ZCs and already have MIST1 expression at the time of *Xbp1* deletion. If XBP1 was not required for maintenance of MIST1, one would expect that it would take several months for complete MIST1 loss in basal ZCs. Supporting that interpretation, *Xbp1*$^{\Delta}$ ZCs at 5 and 13 months showed complete loss of MIST1, even at the base (Figure 3*A*).

XBP1 Is Partially Required for ZC Differentiation

By histologic analysis, qRT-PCR, and Western blot, we observed a modest decrease in ZC markers in *Xbp1*$^{\Delta}$ mice, whereas markers for their progenitor NCs were increased. This phenotype could have resulted either from the inhibition of differentiation from NCs to ZCs or from increased proliferation of NCs with increased apoptosis of ZCs. Thus, we immunostained and quantified markers of proliferation, cell death, and NC/ZC differentiation. In control gastric units, differentiation from NCs to ZCs is abrupt. Thus, a vast majority of cells are either NCs or ZCs with only 13.3% of total cells in the lineage unit coexpressing progenitor and differentiated markers (Figure 5*A*–*C*; Bredemeyer et al[3] and Ramsey et al[4]). *Xbp1*$^{\Delta}$ units at 14 days, on the other hand, showed a large increase in such transitional cells coexpressing NC and ZC markers (26.4%), although there were still discrete NC and mature ZC populations that expressed predominately one marker and not the other (Figure 5*A*–*C*). At later time points, when all ZCs would have arisen in the absence of XBP1, there were essentially no definitive ZCs formed. There were large NC and transitional populations (42.7%) but only rare mature ZCs expressing the ZC marker and not the progenitor marker (Figure 5*A*–*C*). Thus, XBP1 is required for extinguishing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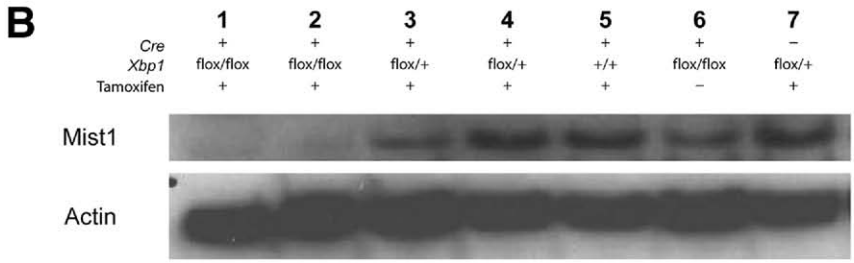
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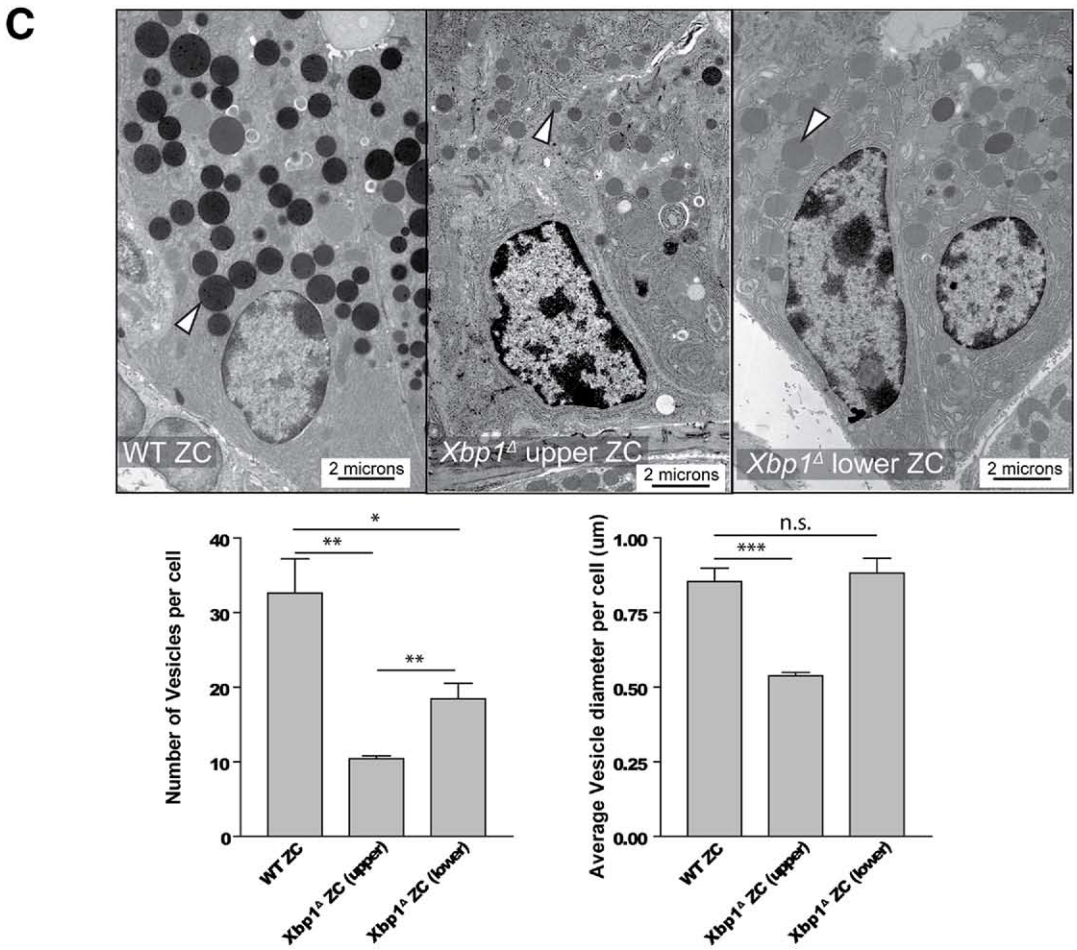
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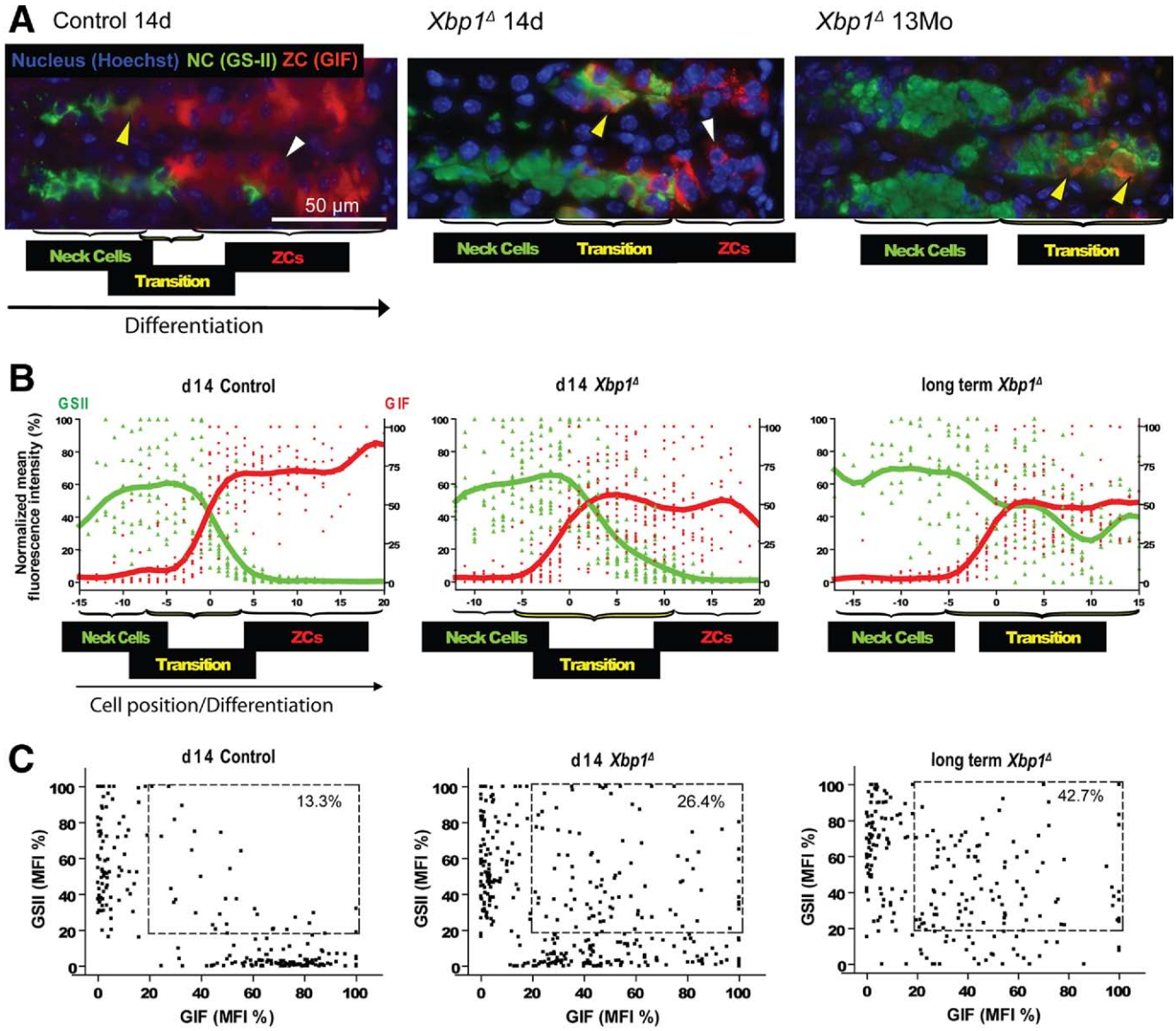
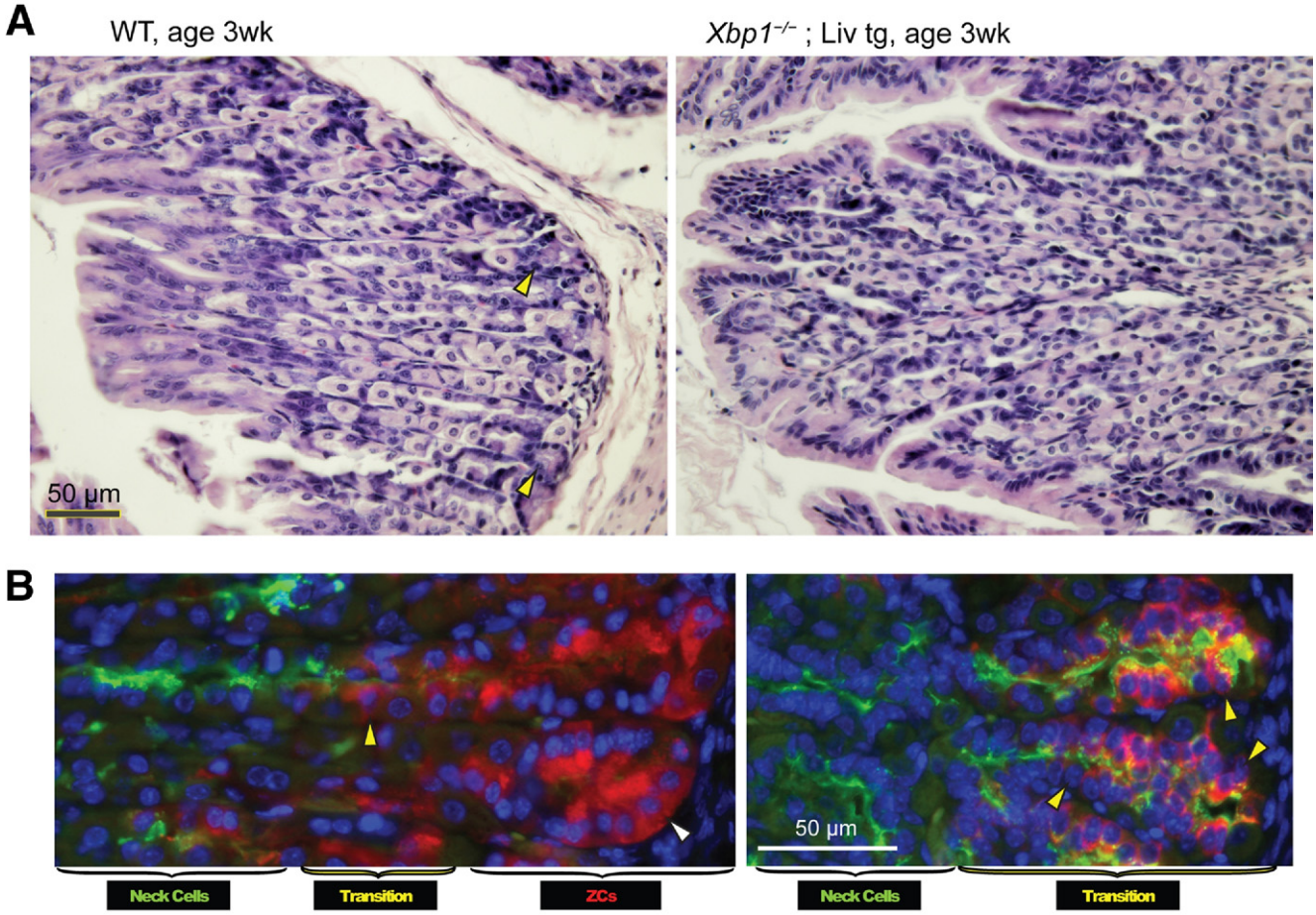


Figure 5. *Xbp1* is required for complete ZC maturation, specifically to turn off NC genes. (*A*) Immunofluorescent staining for GSII (progenitor cell marker, *green*), GIF (ZC marker, *red*), and Hoechst 33258 (nucleus, *blue*). Transitional cells (eg, *yellow arrowheads*) coexpressing progenitor and differentiated markers are increased in *Xbp1*$^{\Delta}$ stomachs by 14 days (*middle panel*) and completely fill the basal zone by 13 months (*right panel*) after tamoxifen injection. ZCs only expressing GIF (*white arrowheads*) are decreased by 14 days (*middle panel*) and rare by 13 months (*right panel*). (*B*) Compiled results from multiple gastric units as in *A*. Each point represents the normalized green or red channel mean fluorescent intensity for a given cell in a unit, expressed as a function of the maximal mean fluorescent intensity in that unit. Units are aligned by setting the first cell with background subtracted GIF mean fluorescent intensity ≥25 as cell #0. Cells to the *right* (ie, more differentiated) are given positive cell positions, with the highest number being the cell farthest toward the base of the unit (ie, the oldest). (*C*) The same data are expressed on a *scatter plot*, ignoring cell position. Cells with both progenitor and differentiated cell marker expression (ie, transitional cell phenotype; *boxed*) are increased in *Xbp1*$^{\Delta}$ units; note also that long-term *Xbp1*$^{\Delta}$ deletion leads to failure in formation of a cell population expressing exclusively ZC markers without NC markers.

Figure 4. *Xbp1* is necessary for activation of *Mist1* in ZCs, and *Xbp1* regulates number and size of secretory vesicles partially through *Mist1*. (*A*) All ZCs (*red*, GIF) express MIST1 (*green*) in control gastric units (nuclei, *blue*). In *Xbp1*$^{\Delta}$ units, early, immature ZCs (ie, those closest to the progenitor zone to the *left*) do not express MIST1 (*arrowheads*, *middle panel*), whereas mature ZCs (to the *right*) that were MIST1 positive at the time of tamoxifen treatment remain MIST1 positive. By 5 months, no ZCs expressed MIST1 (*right panel*). Green channel exposure was equivalent in all 3 *panels*. (*B*) Western blot using anti-MIST1 antibody with 2 individual 14-day *Xbp1*$^{\Delta}$ mice with various controls (actin as loading control). (*C*) Transmission electron microscopy of ZCs illustrating secretory vesicle (*white arrowheads*) phenotypes. Note that relative to wild-type ZCs, the upper (ie, newly formed) ZCs from an *Xbp1*$^{\Delta}$ stomach have scant, small vesicles; the lower (older) ZC formed before *Xbp1* deletion has fewer but similarly sized vesicles. (*Upper right panel*) Lower ZC from *Xbp1*$^{\Delta}$ stomach. The *graphs* quantify mean vesicle number (±SD) and diameter per cell (±SEM), scored from transmission electron microscopy micrographs. n.s., not significant. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$.


A
WT, age 3wk
Xbp1−/− ; Liv tg, age 3wk
50 μm
B
50 μm
Neck Cells
Transition
ZCs
Neck Cells
Transition
C
GSII
GIF
WT, age 3wk
Normalized mean fluorescence intensity (%)
Neck Cells
Transition
ZCs
Cell position/Differentiation
13%
GSII (MFI %)
GIF (MFI %)
Xbp1−/−; Liv tg, age 3wk
Neck Cells
Transition
Cell position/Differentiation
40%
GSII (MFI %)
GIF (MFI %)

progenitor cell features but not induction of ZC markers; without XBP1, NCs form only transitional cells. At no time point do *Xbp1*$^{\Delta}$ ZCs show significantly increased cell death either by morphology or terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling stain, and loss of XBP1 does not affect proliferation in the gastric unit, as assessed by bromodeoxyuridine immunolabeling (Supplementary Figure 3).

To further examine XBP1 in determining ZC cell identity, we examined ZC development in germline *Xbp1* null mice. *Xbp1*$^{-/-}$ mice die in utero; however, *Xbp1*$^{-/-}$ mice expressing liver-specific transgenic *Xbp1* (*Xbp1*$^{-/-}$;Liv^{XBP1}) survive to birth, although most die within a few days (Supplementary Figure 4).[17] We examined a rare 3-week-old *Xbp1*$^{-/-}$;Liv^{XBP1}. By 3 weeks, wild-type gastric units already showed substantial separation into progenitor, transition, and mature ZC zones (Figure 6*A* and *B*, *left panels*, quantified in Figure 6*C*). On the other hand, *Xbp1*$^{-/-}$;Liv^{XBP1} units lacked a distinct ZC zone and had abundant transitional cells (Figure 6*A* and *B*, *right panels*, quantified in Figure 6*C*), again consistent with a role of XBP1 in regulating terminal differentiation of ZCs.

Discussion

Here we show that XBP1 governs nearly the entire morphogenetic program in gastric ZCs (Figure 7). It likely acts through direct cellular effectors that regulate rER formation and via MIST1, which in turn regulates secretory vesicle maturation via RAB26/3D[30] (Figure 7).

That *Xbp1* is upstream of *Mist1* in vivo and is required for *Mist1* activation has not previously been shown. However, XBP1 is dispensable for maintenance of MIST1, because loss of XBP1 in mature ZCs did not uniformly lead to loss of MIST1 until months after tamoxifen-induced deletion. At such late time points, most, if not all, ZCs would have been generated in the absence of XBP1. Interestingly, despite large deficits in rER resulting from loss of XBP1 and despite the reduction in number of secretory vesicles, mature MIST1^{+}/XBP1^{-} cells at 14 days after administration of tamoxifen still had vesicles similar in size to wild-type. That suggests MIST1 regulates vesicle size independent of the secreted proteins available for packaging into those vesicles.

Although the terminal ZC fate is apparently initially specified in the absence of XBP1 (ie, ZC specific markers are still up-regulated), newly forming *Xbp1*$^{\Delta}$ ZCs cannot extinguish expression of progenitor cell proteins; thus, they remain stuck in transition. XBP1 is believed to be a transcriptional activator, so there might be downstream targets of XBP1 that themselves turn off progenitor cell gene expression. It is also possible that loss of XBP1 leads to defects in cellular structures, like the ubiquitin-proteasomal or autophagic machinery, that are needed to degrade NC proteins. However, ZCs live for months, so it is difficult to imagine that continued expression of NC proteins would not also involve continued transcription of the genes encoding those proteins.

The increase in cells expressing NC markers at the base is reminiscent of pseudopyloric or spasmolytic polypeptide expressing metaplasia (SPEM).[33,34] In SPEM, loss of parietal cells correlates with changes in zymogenic lineage differentiation; however, SPEM further correlates with increased foveolar cells and expansion of proliferating cells toward the base of the unit.[33,34] In SPEM, the entire zymogenic lineage (both progenitor NC and ma-

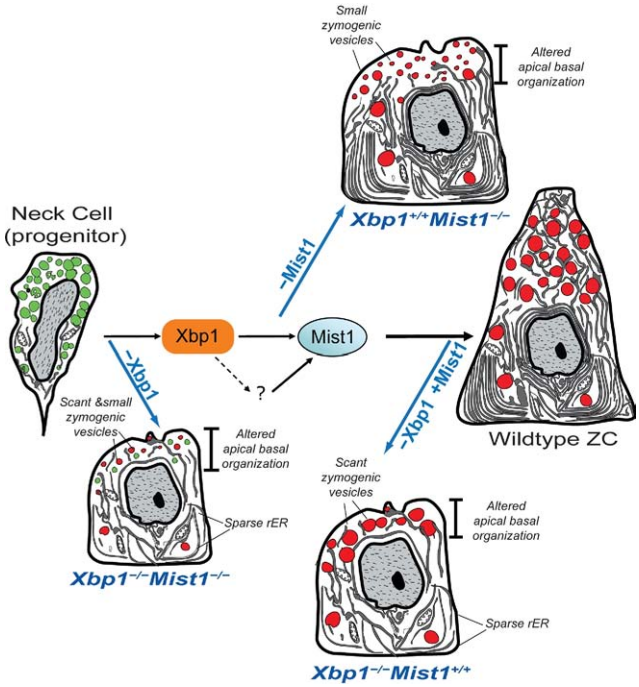


Figure 7. *Xbp1* regulates the principal aspects of ZC structural development: secretory vesicle size/number and rER expansion. Secretory vesicle size is regulated through *Mist1*, which is a direct transcriptional target of *Xbp1*. There is likely another factor(s) that maintains *Mist1* expression once induced ("?" in the diagram), because loss of *Xbp1* does not abrogate *Mist1* once *Mist1* is already expressed. If *Xbp1* is deleted before *Mist1* is expressed, ZCs have scant, small vesicles and sparse ER; if after, ZCs have scant, normal-sized vesicles and sparse ER. If *Mist1* alone is deleted, cells have normal rER but small vesicles.

BASIC–ALIMENTARY TRACT

Figure 6. *Xbp1* germline knockout mice confirm *Xbp1* is required for ZC maturation. (*A*) H&E staining showing wild-type stomach has abundant parietal cells and cytoplasm-rich ZCs in the base (*yellow arrowhead*, *left panel*) 3 weeks postnatally, whereas *Xbp1*$^{-/-}$;Liv^{XBP1} units show abundant parietal cells without obvious ZCs. (*B*) Immunofluorescent staining shows that, whereas definitive ZCs are already developed in control stomach (*white arrowheads*) in 3-week-old mice, *Xbp1*$^{-/-}$;Liv^{XBP1} mice show only cells coexpressing GIF/GSII (*yellow arrowheads*) at the base, similar to *Xbp1*$^{\Delta}$ stomach 13 months following tamoxifen injection (see Figure 5*A*). (*C*) Differentiation patterns across multiple units are quantified as for Figure 5*B* and *C* (*upper panels*, means ± SD).

ture ZC) assumes a transitional morphology; there are neither NCs expressing only NC markers nor mature ZCs expressing only ZC markers.[3] Thus, compared with previous studies, the current results are unusual in that only ZC terminal maturation is profoundly affected without dramatic effects on any other cell lineage in the gastric units. In some ways, the *Xbp1*$^{\Delta}$ phenotype is a more dramatic example of the *Mist1*$^{-/-}$ phenotype, because those mice show increased transitional cells, although all ZCs eventually turn off NC gene expression.[4]

The molecular underpinnings of SPEM are almost wholly unknown. Our recent analysis of hundreds of human gastric samples exemplifying the progression of changes from chronic gastritis to carcinoma showed that loss of MIST1 expression is one of the first molecular markers of altered NC/ZC differentiation in SPEM and that MIST1 expression is lost in more than 99% of gastric cancers.[35] Thus, it will be interesting to determine whether loss of *XBP1* expression is a key early event in SPEM, which might explain both decreased MIST1 levels and the coexpression of NC and ZC markers that characterize this precancerous lesion.

The effects of deletion of Sonic Hedgehog (Shh) in parietal cells in the stomach[10] are in some aspects similar to the *Xbp1*$^{\Delta}$ phenotype. For example, transitional ZCs accumulate in the base in both cases, with preservation of normal NCs. Aspects of the *Shh*$^{\Delta}$ mice not seen in *Xbp1*$^{\Delta}$ stomachs are expansion of foveolar cells and increased proliferation. Nonetheless, it is possible that Hh signaling is aberrant in *Xbp1*$^{\Delta}$ stomachs; indeed, we found increased levels of transcripts for the Hh signaling ligands and targets *Shh* (2.1-fold, $P < .001$), *Ihh* (1.7-fold, $P < .05$), *Ptc1* (2.9-fold, $P < .01$), and *Gli1* (2.7-fold, $P < .01$) in *Xbp1*$^{\Delta}$ mice. Hh signaling is complex in the gastric unit with multiple sources, age-dependent changes, and unclear cellular and molecular targets[9,36]; however, an easy interpretation of the results is that the ZC structural and differentiation defects lead to a compensatory increase in Hh signaling from other epithelial cells and thereby increase Hh target expression in the mesenchyme.[10] In other experiments, we have inducibly deleted the Hh ligand mediators *Ptc1* and *Smo,* but we have not seen effects on ZC differentiation (unpublished results; April 2009).

One caveat of our study is that the genetic tools are not currently available to target inducible *Xbp1* deletion specifically to NCs; thus, even though the phenotype we see is entirely restricted to ZCs, it is possible that some component is due to deletion of *Xbp1* in cells other than NCs and ZCs. We believe such non–cell-autonomous contributions to the phenotype are minimal for several reasons. First, *Xbp1* expression is highest in general in large secretory cells, and our previous studies showed that its gastric expression is highest in the zymogenic lineage.[4] Second, we have extensive experience with the tamoxifen levels necessary to induce deletion in this mouse pedigree, and the levels we use target the NC/ZC lineage[27] (Supplementary Figure 1). Third, at no time after administration of tamoxifen did we observe a change in mesenchymal cells, an influx of inflammatory cells, or a change in census or marker expression of other epithelial lineages in the stomach (Figure 1*C* and *D*). Fourth, an entirely different *Xbp1* deletion strategy, germline (as opposed to inducible) loss of *Xbp1*, also caused ZC-specific effects.

The next step in dissecting zymogenic differentiation and understanding gastric metaplasia will be to determine what is upstream of XBP1. What up-regulates *Xbp1* expression and what leads to the sudden increase in expression of zymogenic lineage markers that occurs as cells migrate out of the neck cell zone? There are few clues from other tissues about upstream regulation of *Xbp1*. In plasma cells, it is known that the transcription factor *Blimp1* is upstream and required for *Xbp1* expression,[32] but *Xbp1* is not a direct target. Clearly, there is a great deal more to learn about ZC differentiation, although with the *Xbp1* → *Mist1* sequence, we are beginning to parse that circuitry.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2010.08.050.

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Conflicts of interest

The authors disclose no conflicts.

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