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Cystine/Glutamate Antiporter (xCT) Is Required for Chief Cell Plasticity After Gastric Injury

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SUMMARY

We have determined that the cystine/glutamate antiporter, solute carrier family 7 member 11 (SLC7A11; also known as xCT), is required for the reprogramming of chief cells into mucus-secreting metaplasia after parietal cell loss in the stomach. This study indicates that xCT is crucial for cystine uptake, reactive oxygen species (ROS) detoxification, and survival of chief cells after gastric damage.

BACKGROUND & AIMS: Many differentiated epithelial cell types are able to reprogram in response to tissue damage. Although reprogramming represents an important physiological response to injury, the regulation of cellular plasticity is not well understood. Damage to the gastric epithelium initiates reprogramming of zymogenic chief cells into metaplastic cell lineage known as spasmolytic polypeptide-expressing metaplasia (SPEM). The present study seeks to identify the role of xCT, a cystine/glutamate antiporter, in chief cell reprogramming after gastric injury. We hypothesize that xCT-dependent reactive oxygen species (ROS) detoxification is required for the reprogramming of chief cells into SPEM.

METHODS: Sulfasalazine (an xCT inhibitor) and small interfering RNA knockdown were used to target xCT on metaplastic cells in vitro. Sulfasalazine-treated wild-type mice and xCT knockout mice were analyzed. L635 or DMP-777 treatment was used to chemically induce acute gastric damage. The anti-inflammatory metabolites of sulfasalazine (sulfapyridine and mesalazine) were used as controls. Normal gastric lineages, metaplastic markers, autophagy, proliferation, xCT activity, ROS, and apoptosis were assessed.

RESULTS: xCT was up-regulated early as chief cells transitioned into SPEM. Inhibition of xCT or small interfering RNA knockdown blocked cystine uptake and decreased glutathione production by metaplastic cells and prevented ROS detoxification and proliferation. Moreover, xCT activity was required for chief cell reprogramming into SPEM after gastric injury in vivo. Chief cells from xCT-deficient mice showed decreased autophagy, mucus granule formation and proliferation, as well as increased levels of ROS and apoptosis compared with wild-type mice. On the other hand, the anti-inflammatory metabolites of sulfasalazine did not affect SPEM development.
CONCLUSIONS: The results presented here suggest that maintaining redox balance is crucial for progression through the reprogramming process and that xCT-mediated cystine uptake is required for chief cell plasticity and ROS detoxification. (Cell Mol Gastroenterol Hepatol 2019;8:379–405; https://doi.org/10.1016/j.jcmgh.2019.04.015)

Keywords: Cellular Plasticity; xCT; CD44; Reactive Oxygen Species; Sulfasalazine; Metaplasia; Chief Cell; Reprogramming; Oxyntic Atrophy; Autophagy; SPEM.

Severe injury to the lining of the gastrointestinal tract or other epithelial-lined organs leads to reprogramming of differentiated cells and the emergence of metaplastic cell lineages. Metaplastic lineages often are characterized by the expression of mucins, adding a protective barrier to the mucosa. In fact, metaplastic lineages often acquire the characteristics of mucus-secreting cells found in the distal stomach: antral/pyloric glands or Brunner’s glands. Similar reparative lineages are observed in the esophagus, stomach, small bowel, colon, and pancreas. In this study, we focus on the development of metaplastic lesions in the oxyntic region (body) of the stomach.

Zymogenic chief cells in the oxyntic region of the stomach produce enzymes required for digestion, such as pepsinogen and lipases. Chief cells also show high cellular plasticity after injury to the gastric epithelium. Damage to the epithelial cell barrier, including loss of acid-secreting parietal cells or ulceration, promotes the reprogramming of chief cells into a metaplastic cell lineage known as spasmolytic polypeptide-expressing metaplasia (SPEM). SPEM expresses mucin 6 (Muc6) and trefoil factor 2 (TFF2, also known as the spasmolytic polypeptide) with a morphology similar to mucus-producing cells found deep within antral glands. Although chief cell plasticity is a key mechanism to promote repair and tissue regeneration in the stomach, the maintenance of metaplasia in the presence of chronic injury and inflammation can predispose an individual to develop dysplasia or cancer in the gastric mucosa.

The epithelium that lines the stomach faces harsh conditions, such as ingested food and the bacteria resident in our gut, making the stomach more susceptible to damage. Chronic infection with the bacterium Helicobacter pylori leads to the loss of acid-secreting parietal cells in the stomach. Gastric pathology can take months to develop in Helicobacter-infected mice, and years in human beings. To facilitate an accelerated model for induction of oxyntic atrophy, our laboratory has developed 2 acute models of parietal cell loss through the administration of the parietal cell toxic drugs L635 and DMP-777. Chief cell reprogramming is visualized within hours of the administration of a parietal cell toxic drug and includes the up-regulation of proteins required for the metaplastic transition.

Cluster-of-differentiation (CD)44 is widely known as the receptor for hyaluronic acid that influences cell motility, survival, and proliferation. CD44 regulates proliferation of normal progenitors and metaplastic cells in the stomach.

Alternative splicing can produce variant isoforms of CD44 with unique functions. CD44 variant isoform 9 (CD44v9) is a cell surface glycoprotein not normally expressed in the stomach. However, it recently was discovered that CD44v9 is up-regulated early in the transition to SPEM and CD44v9 now is considered a SPEM marker. One of the functions of CD44v9 is to interact with and stabilize xCT (SLC7A11), a subunit of the heterodimeric cystine-glutamate antiporter. CD44v9 stabilization of xCT on the cell membrane increases cystine uptake into the cell. Increased intracellular cystine promotes glutathione synthesis, an important molecule for the defense against reactive oxygen species (ROS). The oxidative stress response, including up-regulation of nutrient transporters, plays an important role in many biological processes and the pathogenesis of a variety of diseases. Perturbations to the CD44v9-xCT system often result in redox imbalance.

Sulfasalazine is a drug that was first synthesized in the 1940s from a combination of sulfapyridine (an antibiotic) and mesalazine (an anti-inflammatory agent) linked by an azo bridge. Recently, it was discovered that the parent compound sulfasalazine is a specific and potent inhibitor of xCT-mediated cysteine transport. Several studies have used sulfasalazine treatment to target xCT activity on cancer stem cells. Here, we used sulfasalazine as a tool to inhibit xCT-mediated cysteine transport, an event that is required for chief cell reprogramming after gastric injury. We determined that xCT activity is required for cysteine uptake and ROS detoxification by SPEM cells and metaplasia proliferation. Inhibition of xCT prevented zymogenic chief cell reprogramming in vivo. xCT-deficient mice showed increased levels of ROS and apoptosis and did not develop SPEM after acute parietal cell loss. Our results suggest that adaptation to oxidative stress and up-regulation of xCT activity is crucial for reprogramming of chief cells after gastric injury.

Results
Parietal Cell Loss Promotes SPEM and Up-Regulation of CD44v9-xCT

L635 is a parietal cell toxic drug that causes acute parietal cell necrosis. We used L635 treatment to...
elucidate the role of xCT in epithelial repair and chief cell reprogramming. Chief cell reprogramming can be visualized within hours of administering L635, and proliferative metaplasia subsequently develops within 3 days. Immunohistochemical analysis of tissue sections from wild-type C57Bl/6J mice treated with 3 days of L635 showed basolateral membrane expression of the metaplastic marker CD44v9 on chief cells at the base of glands. No CD44v9 staining was visualized in the stomach of untreated mice. Immunostaining showed increased nuclear expression of ESRP1 in gastric intrinsic factor (GIF)-positive chief cells from L635-treated mice (Figure 1C and D). In addition, we observed increased messenger RNA (mRNA) expression of Cd44v9, xCT, and Esrp1 after 3 days of L635 treatment (Figure 1B). These results suggest that alternative splicing of CD44 is up-regulated by ESRP1 after L635-induced injury to the stomach. Furthermore, increased CD44v9 stabilizes xCT on the plasma membrane.

**Figure 1. Increased expression of CD44v9, xCT, and ESRP1 in L635-treated mice.** (A) Immunohistochemical staining of serial sections from the body of the stomach for CD44v9 (top) and xCT (bottom) in untreated and L635-treated (3 days) C57Bl/6J mice. Scale bar: 100 µm. Magnified inset from corresponding chief cell regions in top right corner. (B) Relative mRNA expression of Cd44v9, xCT, and Esrp1 in untreated and L635-treated (3 days) C57Bl/6J mice determined by reverse-transcription quantitative PCR (P = .006**, .03*, and .001**, respectively). Statistical significance was determined by unpaired Student t test (n = 4 per group). (C) Immunofluorescent staining for the zymogenic granule marker GIF (red) and ESRP1 (green) in untreated and L635-treated (3 days) C57Bl/6J mice. Scale bar: 100 µm. Magnified inset of chief cell region with arrows indicating ESRP1 and GIF dual-positive cells (right). (D) Relative fluorescence intensity of nuclear ESRP1 in GIF-positive chief cells (P = .001**). Statistical significance was determined by unpaired Student t test (n = 4 per group).

Metaplastic Cells Are Dependent on xCT for Cystine Uptake, ROS Detoxification, Proliferation, and Survival In Vitro

To target xCT activity on the plasma membranes of metaplastic (SPEM) cells, we used sulfasalazine, an inhibitor
of xCT-mediated cystine transport, to treat previously characterized cell lines for chief cells (ImChief) and SPEM cells (ImSPEM) isolated from Immortomice. The relative expression of $Cd44v9$ and xCT were measured in ImChief and ImSPEM cells. ImSPEM cells showed increased expression of $CD44v9$ and xCT compared with ImChief cells (Figure 2A). ImChief and ImSPEM cells were immunostained for $CD44v9$ and xCT. No $CD44v9$ or xCT staining was visualized in ImChief cells, but $CD44v9$ and xCT were observed on the plasma membranes of ImSPEM cells, recapitulating what is observed in chief and SPEM cells in the stomach (Figure 2B–D).

To monitor xCT activity and cystine uptake into ImSPEM cells, we added fluorescently labeled cystine (cystine–fluorescein isothiocyanate [FITC]) to cultures. Abundant intracellular fluorescent signal was observed in ImSPEM cells 2 hours after the addition of cystine–FITC to culture. xCT blockade with sulfasalazine treatment significantly reduced the uptake of cystine–FITC by ImSPEM cells (Figure 2E and F). Through a multistep process, intracellular cystine can be converted to glutathione, a critical antioxidant for protection against ROS. We used an antibody that recognizes glutathione and ROS indicators (dihydroethidium [DHE] and CellROX) to measure glutathione.
production and ROS levels. Inhibition of xCT with sulfasalazine decreased glutathione in ImSPEM cells (Figure 2E and F). Similarly, sulfasalazine increased ROS in ImSPEM cells as shown by increased accumulation of oxidized DHE and CellROX (Figure 2E and F).

In addition, we observed that sulfasalazine treatment decreased the proliferation of ImSPEM cells by staining for the proliferation marker Ki67, a protein present in all active phases of the cell cycle, and monitoring cell number (Figure 3A–C). Sulfasalazine treatment also increased
apoptosis of ImSPEM cells, as assessed by staining for the apoptosis marker, cleaved caspase-3 (Figure 3E and F). Caspase-3 is activated when it is cleaved by an initiator caspase and mediates cell death by apoptosis. These results indicate our in vitro culture system mimics the expression pattern observed in the stomach and that sulfasalazine inhibits cystine uptake, ROS detoxification, and proliferation of metaplastic cells in culture. Likewise, increased ROS levels led to SPEM cell death.

Sulfasalazine is broken down to sulfapyridine and mesalazine through azo cleavage (Figure 4A). Therefore, we sought to confirm that the outcomes observed were owing to the specific inhibition of xCT by sulfasalazine, and not the anti-inflammatory properties of sulfasalazine metabolites. In contrast with sulfasalazine treatment, sulfapyridine or mesalazine treatment of ImSPEM cells in culture did not inhibit cystine uptake or proliferation (Figure 4B–E).

To further examine if the effects of sulfasalazine are mediated by inhibition of xCT, we transfected ImSPEM cells with control or xCT small interfering RNAs (siRNAs). Similar to sulfasalazine treatment, ImSPEM cells transfected with xCT siRNA showed decreased cystine uptake and glutathione production, increased ROS, less proliferation, and more apoptotic cell death compared with ImSPEM cells transfected with control siRNA (Figure 5). Taken together, these results suggest that sulfasalazine effectively blocks xCT activity on SPEM cells and SPEM cells are dependent on xCT for control of ROS.

**Figure 4.** Metabolites of sulfasalazine do not prevent cystine uptake, ROS detoxification, or proliferation of metaplastic cells.

(A) Diagram of sulfasalazine metabolism. Sulfasalazine is broken down to sulfapyridine (an antibiotic) and mesalazine (an anti-inflammatory agent) through azo cleavage. (B) ImSPEM cells were seeded at the same density plus sulfapyridine or mesalazine for 3 days. ImSPEM cell number with sulfapyridine or mesalazine at 24, 48, and 72 hours. (C) Representative transmitted light images monitoring cell density over 3 days. Scale bar: 100 μm. (D) ImSPEM cells were treated with sulfapyridine or mesalazine and then treated with cystine-FITC. Live cell imaging of intracellular cystine-FITC (green) in ImSPEM cells treated with sulfapyridine or mesalazine with nuclear counterstain Hoechst (blue). Scale bar: 100 μm. (E) Relative fluorescence intensity of intracellular cystine-FITC in ImSPEM cells treated with sulfapyridine or mesalazine (n = 3 per condition).

**Inhibition of xCT Blocks Chief Cell Reprogramming Into SPEM**

To elucidate the role of xCT in chief cell reprogramming in vivo, we combined sulfasalazine treatment with the paretial cell toxic drug, L635. Chief cell reprogramming into SPEM occurs through a coordinated process that involves disassembly of the chief cell protein secretory apparatus and transcriptional changes including the up-regulation of...
mucus granule proteins Muc6 and TFF2.5.9 We treated wild-type C57Bl/6J mice with sulfasalazine 2 days before and throughout 3 days of L635 treatment (Figure 6A). Stomachs were harvested from 4 experimental groups: (1) untreated, (2) sulfasalazine-treated, (3) L635-treated, and (4) L635 þ sulfasalazine–treated mice for histologic analysis. Treatment with sulfasalazine by itself did not alter the gastric mucosa (Figure 7). To visualize L635-induced parietal cell loss, we performed immunostaining for the proton pump, Hþ/Kþ–adenosine triphosphatase (ATPase), an integral membrane protein responsible for gastric acid secretion by parietal cells (Figure 6B). In untreated mice, a large number of Hþ/Kþ-ATPase–positive parietal cells were detected throughout the corpus glands. Treatment with L635 reduced the number of parietal cells by almost 80%. Sulfasalazine treatment did not affect L635-induced parietal cell loss (Figure 6C). To detect chief cell reprogramming, we immunostained for the zymogenic granule marker GIF and the mucus granule marker Griffonia simplicifolia (GSII)–lectin, which binds to a sugar modification on Muc6 (Figure 6B). Chief cells reprogram to mucus-secreting metaplastic cells to protect and fuel repair of the stomach when there is injury or cell loss in the gastric mucosa. During this process, SPEM cells contain both zymogenic (GIF-positive) granules and mucus (GSII-positive) granules. Sulfasalazine treatment decreased the number of GIF and GSII dual-positive SPEM cells by greater than 80% after L635-induced parietal cell loss (Figure 6D). Similarly, periodic acid–Schiff (PAS) staining, a stain that detects mucins, showed decreased mucus at the base of glands in L635 þ sulfasalazine–treated mice (Figure 7B). Collectively, these results suggest that xCT blockade prevents chief cell reprogramming after acute parietal cell loss.

**xCT Activity Is Required for Autophagy in Reprogramming Chief Cells**

The process of reprogramming requires chief cells to downscale their mature characteristics. The basic helix-loop-helix transcription factor Mist1 (Blhia15) governs the secretory architecture of chief cells including production of large protein-containing zymogen granules and cellular organization.3 Loss of Mist1 is a distinct feature of chief cell reprogramming into SPEM.34 Dual immunofluorescence staining for GIF and Mist1 allows for monitoring of Mist1 loss in zymogenic granule-containing chief cells (Figure 8A). In untreated mice, GIF-positive chief cells were positive for the transcription factor Mist1. L635 treatment resulted in almost complete loss of Mist1 expression in GIF-positive cells.
Interestingly, treatment with L635 + sulfasalazine did not rescue Mist1 loss by chief cells (Figure 8B). This suggests that xCT inhibition with sulfasalazine does not influence the initiating step of Mist1 loss in reprogramming chief cells.

In addition to loss of Mist1, autophagic and lysosomal pathways in chief cells are up-regulated acutely after injury to the stomach. In particular, rough endoplasmic reticulum, mitochondria, and secretory granules are targeted for degradation during early stages of SPEM development.
Furthermore, mice with defects in autodegradative function (Gnptab−/− mice) are unable to develop SPEM after gastric injury. To investigate autophagic and lysosomal pathways, L635-treated mice were killed 12 or 24 hours after L635 treatment. We performed immunostaining for the autophagosome marker microtubule-associated proteins 1A/1B light chain 3B (MAP1LC3B or LC3B) and the lysosome marker lysosomal associated membrane protein-2 (LAMP2) (Figure 8C and E). Very few LC3B or LAMP2-positive puncta were observed in GIF-positive chief cells from untreated mice. As expected, however, gastric injury induced by L635 treatment resulted in increased LC3B and LAMP2-positive puncta in close proximity to GIF-positive zymogenic granules. By contrast, L635 + sulfasalazine-treated mice showed fewer LC3B and LAMP2-positive puncta in GIF-positive cells compared with L635 treatment only (Figure 8D and F). Notably, large/dense zymogen granules were maintained in L635 + sulfasalazine-treated mice compared with the L635-treated mouse. Similarly, transmission electron microscopy (TEM) of tissue from L635-treated mice showed abundant double-membrane-bound (autophagic) structures engulfing granules and other cytosolic components in chief cells. Although similar double-membrane–bound structures were observed in L635 + sulfasalazine–treated mice, they were significantly less frequent (Figure 8G). Real-time quantitative polymerase chain reaction (PCR) analysis for several autophagy-related genes showed a significant decrease in the relative expression of Atg4, Atg7, Atg16L1, Lamp1, and Lc3 in sulfasalazine-treated mice (Figure 8H). Although there was Mist1 loss in L635 + sulfasalazine–treated mice, these findings suggest that xCT blockade decreases downscaling by autophagic and lysosomal pathways in reprogramming chief cells.

**Figure 7.** Histologic analysis of sulfasalazine–treated mice. (A) H&E– and (B) PAS-stained sections from untreated (n = 4), sulfasalazine–treated (n = 4), L635–treated (n = 4), and L635 + sulfasalazine–treated (n = 3) mice. Scale bars: 100 μm. Dark purple–magenta color represents PAS-positive, mucus-producing cells. Glands containing PAS-positive cells at the base are indicated with arrows.

**xCT Blockade Suppresses Proliferation of Reprogramming Chief Cells**

Sulfasalazine treatment inhibited proliferation of ImSPEM cells in culture, so we sought to determine the effect of sulfasalazine treatment on proliferation after gastric injury in vivo. To do this, we immunostained for the proliferation marker Ki67. In the normal oxyntic mucosa, Ki67 labeled stem/progenitor cells approximately a third of the way down the gland in the gland isthmus. Upon gastric injury, chief cells reprogram and are capable of re-entering...
Inhibition of xCT suppresses SPEM proliferation. (A) Immunostained sections from untreated (n = 4), L635-treated (n = 4), and L635 + sulfasalazine–treated (n = 3) C57Bl/6J mice killed after 3 days of L635 treatment for proliferation marker Ki67 (red), mucus-producing foveolar cell marker UEA1-lectin (green), and zymogen granule marker GIF (blue). Scale bars: 100 μm. Magnified inset of chief cell region with arrows indicating proliferative metaplasia (right). (B) Average thickness (μm) of UEA1-positive foveolar region. (C) Quantification of Ki67-positive (red) proliferating cells per 20× field. (D) Percentage of Ki67-only–positive cells or Ki67-positive cells dual-positive for UEA1 or GIF to determine distribution of proliferative cells (P = .002**). Statistical significance was determined by 1-way analysis of variance with the Bonferroni post hoc multiple comparisons test.

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xCT blockade inhibits autophagy of zymogenic granules in downscaling chief cells. (A) Immunostained sections from untreated (n = 4), L635-treated (n = 4), and L635 + sulfasalazine–treated (n = 3) C57Bl/6J mice killed after 3 days of L635 treatment for zymogenic granule marker GIF (red) and chief cell transcription factor Mist1 (green). Scale bars: 50 μm. (B) Quantification of Mist1-positive cells per 20× field. (C) Immunostained sections from untreated (n = 5), L635-treated (n = 5), and L635 + sulfasalazine–treated (n = 4) C57Bl/6J mice killed after 24 hours of L635 treatment for GIF (red) and autophagosome marker LC3B (green). Scale bars: 50 μm. Magnified inset of GIF-positive cell with arrows indicating puncta (right). (D) Average number of LC3B puncta per GIF-positive cell (P = .003**). (E) Immunostained sections from untreated (n = 5), L635-treated (n = 5), and L635 + sulfasalazine–treated (n = 4) C57Bl/6J mice killed after 24 hours of L635 treatment for GIF (red) and lysosome marker LAMP2 (green). Scale bars: 50 μm. Magnified inset of GIF-positive cell with arrows indicating puncta (right). (F) Average number of LAMP2 puncta per GIF-positive cell (P = .0003***). Statistical significance determined by 1-way analysis of variance with the Bonferroni post hoc multiple comparisons test. (G) Transmission electron micrographs of zymogenic chief cells 12 hours after L635 treatment ± sulfasalazine. Scale bars: 2 μm. Magnified inset of double-membrane autophagic structures (right). (H) Relative mRNA expression of autophagy-related proteins in L635-treated and L635 + sulfasalazine–treated mice (Atg4, Atg5, Atg7, Atg12, Atg16L1, Beclin1, Lamp1, Lamp2, and Lc3) (P = .02*, .005*, .03*, .01*, and .02*, respectively). Statistical significance was determined by an unpaired Student t test.
into the cell cycle and proliferating. In addition, surface mucus-producing (foveolar) cells located near the lumen also expand in response to injury and increases in gastrin. This gastric lesion is referred to as *foveolar hyperplasia*. Foveolar cells produce Muc5ac, a mucin recognized by *Ulex Europaeus Agglutinin I* (UEA1) lectin. Unlike reprogramming chief cells, foveolar cells do not express xCT. To classify the identity of the proliferating cells in each of our experimental groups we immunostained for Ki67, UEA1 lectin, and GIF (Figure 9A). To evaluate foveolar hyperplasia after L635 treatment, we measured the average thickness of the UEA1-positive foveolar region. L635-treated mice and L635 + sulfasalazine–treated mice showed similar levels of foveolar hyperplasia (Figure 9B). We also quantified the total number of Ki67-positive cells in the oxyntic mucosa of each experimental group. Although the total number of Ki67-positive proliferating cells was not significantly different between L635-treated mice and L635 + sulfasalazine–treated mice, the percentage of Ki67-positive cells that were dual-positive for GIF was decreased significantly in the sulfasalazine-treated group (Figure 9C and D). Together, these results show that proliferation of xCT-negative foveolar cells is not affected by xCT blockade. However, chief cells that express high levels of xCT as they reprogram do not proliferate after xCT blockade with sulfasalazine.

**Inhibition of xCT Leads to Oxidative Stress and Chief Cell Death After Gastric Injury**

Next, we examined evidence of cell stress or cell death. Clusterin (Clu) is a heterodimeric protein up-regulated in many diseases related to oxidative stress and is associated with clearance of cellular debris and apoptosis. Previous studies have shown clusterin expression in SPEM lineages through gene microarray analysis. Clu protein expression in SPEM was validated in DMP-777, L635, and *Helicobacter* infection mouse models. In the normal oxyntic mucosa, clusterin is expressed in some mucous neck cells along with the spasmolytic polypeptide TFF2. In L635-treated mice, clusterin and TFF2 are up-regulated in chief cells at the base of oxyntic glands as they reprogram into SPEM. Interestingly, although sulfasalazine inhibited TFF2 up-regulation, chief cells at the gland base still showed clusterin expression after L635 treatment (Figure 10). Thus, it is likely that Clu is not up-regulated as part of the metaplastic process, but rather as a result of increased oxidative stress. Similarly, staining with ROS indicators (DHE and CellROX) showed increased levels of ROS in L635 + sulfasalazine–treated mice compared with L635-treated mice, especially in GIF-positive chief cells (Figure 11). Furthermore, L635 + sulfasalazine–treated mice showed a few GIF-positive chief cells that were dual-positive for the apoptosis marker cleaved caspase-3 (Figure 12A). Taken together, these
Figure 11. xCT blockade leads to increased ROS after acute gastric damage. Sections from untreated (n = 3), L635-treated (n = 3), and L635 + sulfasalazine-treated (n = 3) C57Bl/6J mice killed after 3 days of L635 treatment were treated with ROS indicators DHE and CellROX. (A) Representative images of DHE staining with oxidized DHE (DHE-ox) (red), f-actin marker phalloidin (green), and unoxidized DHE (blue). Scale bars: 100 μm. Magnified inset of chief cell region (right). (B) Relative fluorescence intensity of nuclear DHE-ox (P = .02*). (C) Representative images of CellROX green staining with zymogenic granule marker GIF (red), CellROX Green Reagent (green), with nuclear counterstain Hoechst (blue). Scale bars: 100 μm. Magnified inset of chief cell region (right). (D) Relative fluorescence intensity of CellROX in GIF-positive cells (P = .01*). Statistical significance was determined by 1-way analysis of variance with the Bonferroni post hoc multiple comparisons test.
findings indicate that inhibition of xCT prevents chief cell reprogramming, but chief cells still initiate a stress response owing to increased ROS and, in some cases, undergo apoptosis.

Next, we examined in each of our experimental groups the expression of CD44v9, a metaplastic cell marker protein that interacts and stabilizes xCT (Figure 13A). Immunostaining for CD44v9 confirmed no expression in untreated mice. Similarly, no CD44v9 staining was observed in the sulfasalazine-only treatment group. In contrast, mice treated with L635 showed high expression of CD44v9 on the basolateral membranes of cells at the base of oxyntic glands. The majority of these cells were dual-positive for the mucus granule marker GSII lectin. Interestingly, reduced expression of CD44v9 was observed in L635 + sulfasalazine–treated mice (Figure 13B). The remaining CD44v9 staining in mice treated with L635 and sulfasalazine appeared to be intracellular and not on the basolateral membrane. We used real-time PCR to measure the relative expression of Cd44v9 mRNA transcript in each of the experimental groups (Figure 13C). No significant difference in Cd44v9 mRNA was observed between L635-treated mice ± sulfasalazine treatment. Therefore, these results indicate that sulfasalazine may disrupt the interaction of CD44v9 and xCT on the membrane and may result in mis-localization and possible degradation of CD44v9 protein. On the other hand, it also is possible that maintenance of redox balance is required for up-regulation of CD44v9 protein.
Metabolites of Sulfasalazine Do Not Alter Metaplasia Development

To validate further that our observations were owing to specific xCT blockade by sulfasalazine and not the anti-inflammatory properties of sulfasalazine metabolites, we treated mice with sulfapyridine or mesalazine 2 days before and throughout 3 days of L635 treatment (Figure 14A).36 Immunostaining for CD44v9, mucus granule marker GSII lectin (green), with nuclear counterstain 4',6-diamidino-2-phenylindole (DAPI) (blue). Scale bars: 100 μm. Magnified inset of chief cell region (right). (B) Number of cells per 20× field with basolateral membrane expression of CD44v9 (P < .0001***). (C) Relative mRNA expression of Cd44v9. Statistical significance was determined by 1-way analysis of variance with the Bonferroni post hoc multiple comparisons test.

xCT Knockout Mice Recapitulate the Phenotype of Sulfasalazine-Treated Mice

To examine further if the effects of sulfasalazine are mediated by inhibition of xCT we treated xCT knockout (xCTKO) mice with the parietal cell toxic drug L635. Stomachs were harvested from 2 experimental groups for histologic analysis: untreated xCTKO mice and L635-treated xCTKO mice (Figure 15A). H&E or PAS staining showed normal gastric histology in xCTKO mice (Figure 15B). To visualize L635-induced parietal cell loss, we performed immunostaining for the proton pump H⁺/K⁺-ATPase (Figure 15C). In untreated xCTKO mice, a large number of H⁺/K⁺-ATPase-positive parietal cells were detected throughout the corpus glands. Treatment with L635 in xCTKO mice reduced the number of parietal cells by more than 70% (Figure 15D). To detect chief cell reprogramming, we immunostained for the zymogenic granule marker GIF and the mucus granule marker GSII lectin (Figure 15C). L635-treated xCTKO mice did not show reprogramming chief cells, dual-positive for GIF and GSII (Figure 15E). Similarly, PAS staining showed no mucus staining at the bases of glands in L635-treated xCTKO mice (Figure 15B). Collectively, these results suggest that chief cells from xCT-deficient mice are unable to reprogram after acute parietal cell loss.

In addition, we examined xCTKO mice to determine ROS levels using the ROS indicators DHE and CellROX. Although untreated xCTKO mice had low levels of ROS, after L635
Inhibition of xCT Prevents DMP-777–Induced SPEM

To test the efficacy of xCT blockade on chief cell reprogramming in a different mouse model of acute parietal loss, we used another parietal toxic drug, DMP-777. Similar to L635, DMP-777 induces parietal cell necrosis. However, DMP-777 also functions as an elastase inhibitor and prevents inflammatory infiltration.16 With DMP-777 treatment, chief cell reprogramming and metaplasia development occur in the absence of inflammation. We treated mice with...
sulfasalazine 2 days before and throughout 10 days of DMP-777 treatment. Stomachs were harvested from 2 experimental groups for histologic analysis: DMP-777–treated and DMP-777 + sulfasalazine–treated mice (Figure 18A). H⁺/K⁺-ATPase staining showed similar levels of parietal cell loss in both groups (Figure 18B and C). We also performed immunostaining for the zymogenic granule marker GIF and mucus granule marker GSII lectin to detect chief cell reprogramming. Sulfasalazine treatment decreased the number of GIF and GSII dual-positive SPEM cells by greater than 75% after DMP-777–induced parietal cell loss (Figure 18B and D). Therefore, xCT inhibition with sulfasalazine prevents metaplasia development even in the absence of inflammation.

We further examined DMP-777–treated mice with ROS indicators (DHE and CellROX) and apoptosis markers. DMP-777 + sulfasalazine–treated mice showed slightly increased levels of ROS compared with DMP-777–treated mice (Figure 19). Furthermore, very few GIF-positive chief cells were dual-positive for the apoptosis marker cleaved caspase-3 (Figure 20A). It is likely that the generation of ROS intrinsic to the reprogramming process accounts for the increased ROS observed in DMP-777 + sulfasalazine–treated mice. However, in the absence of

Figure 15. Loss of xCT prevents the development of metaplasia after acute gastric damage. (A) Diagram of drug treatments. L635 was administered to xCTKO C57BL/6J mice for 3 days to induce acute gastric damage. Mice were killed 2 hours after the final dose of L635, and stomach tissue from untreated xCTKO mice (n = 4) and L635–treated xCTKO mice (n = 4) were harvested for histologic analysis. (B) H&E– and PAS-stained sections. (C) Immunostained sections for parietal cell marker H⁺/K⁺-ATPase (red), mucus granule marker GSII lectin (green), and zymogenic granule marker GIF (blue). Scale bars: 100 μm. Magnified inset of chief cell region (right). (D) Quantification of parietal cells as determined by the number of H⁺/K⁺-ATPase–positive (red) cells per 20× objective field (P = .0002***). (E) Quantification of GSII (green) and GIF (blue) dual-positive (SPEM) cells per 20× objective field. Statistical significance was determined by the unpaired Student t test.
inflammatory ROS, chief cells are able to endure the oxidative stress, at least over the 10-day time course.

**CD44v9 and xCT Are Up-Regulated in SPEM in Helicobacter-Infected Mice and Human Beings**

Given the prominent changes observed in the acute models of parietal cell loss, we sought to examine whether CD44v9 and xCT also were up-regulated after chronic SPEM induction in *Helicobacter felis*-infected mice or in clinical specimens. Figure 21A shows that staining for both CD44v9 and xCT was observed in SPEM from the stomach of mice infected with *H felis* for 12 months. Similarly, Figure 21B shows that both CD44v9 and xCT staining was observed in SPEM from human patients. These studies confirm the up-regulation of ROS detoxification mechanisms in both chronic induction of SPEM and SPEM associated with acute gastric damage.

**Discussion**

After significant gastric injury, chief cells reprogram to mucous cell metaplasia or SPEM. The present study identifies that xCT is required for the reprogramming of digestive enzyme–secreting chief cells after gastric injury. Increased membrane expression of xCT is observed in reprogramming chief cells expressing CD44v9 after gastric damage with parietal cell toxic drugs. Inhibition of xCT, through sulfasalazine treatment, or xCT siRNA knockdown blocks cystine uptake, ROS detoxification, and the proliferation and survival of metaplastic cells in culture. In addition, chief cells from sulfasalazine-treated mice or xCT
knockout mice were unable to reprogram after acute gastric injury. Importantly, we confirm that our findings were owing to specific inhibition of xCT by sulfasalazine, and not the anti-inflammatory characteristics of sulfasalazine metabolites. Although loss of xCT activity inhibited chief cell reprogramming, chief cells endured oxidative stress and in some cases apoptosis. Interestingly, the transcription factor Mist1 was down-regulated in chief cells after acute parietal cell loss, even with xCT blockade. In contrast, inhibition of xCT restricted the up-regulation of autosomal and lysosomal degradation machinery, a distinct step in the reprogramming process. Thus, although xCT inhibition does not alter initiation of reprogramming, it arrests the completion of the process. These results show that the process of reprogramming involves multiple discrete steps leading to metaplasia.

Recent studies have sought to understand the mechanisms used by mature postmitotic cells to change their differentiation state. Reprogramming promotes the regenerative capacity of differentiated cells. This is a critical process used to repair damage in tissues that lack an active adult stem cell compartment such as the pancreas. Still, in tissues with active adult stem cells such as the stomach and intestine, reprogramming differentiated cells can enable repair after tissue damage. It is evident that reprogramming of differentiated cells occurs through a stepwise process. First, differentiated cells must undergo autodegradation to downscale mature characteristics. Next, metaplastic genes are up-regulated, and mucus granules are formed. Finally, metaplastic cells have the capability of re-entering into the cell cycle and proliferating. Progression through this stepwise process can be blocked at intervening check points. The results presented here suggest that maintaining redox balance is crucial for progression through the reprogramming process in gastric chief cells and may represent a checkpoint for this process. It is likely that the downscaling of zymogen granules and up-regulation of mucus granule formation would engender the production of ROS. Furthermore, acute and chronic inflammation can add to increased ROS levels. Indeed, it is likely that decreased ROS levels are important for the undifferentiated status or stemness of reprogramming chief cells.

Oxidative stress refers to high levels of intracellular ROS that can cause damage to lipids, proteins, and DNA. Increased ROS act as signaling molecules that contribute to both physiological and pathologic conditions. Cells use several mechanisms to counteract oxidative stress by producing antioxidants such as glutathione. Increased expression of xCT is part of the oxidative stress response. In addition, molecules

Figure 17. Chief cells from xCT knockout mice apoptose after acute gastric damage. (A) Immunostained sections from untreated xCTKO (n = 4) and L635-treated xCTKO (n = 4) C57Bl/6J mice killed after 3 days of L635 treatment for apoptosis marker CC3 (red), zymogenic granule marker GIF (green), with nuclear counterstain 4',6-diamidino-2-phenylindole (DAPI) (blue). Scale bars: 100 μm. Magnified inset of chief cell region with arrows indicating CC3 and GIF dual-positive apoptotic chief cells.
that work to stabilize and increase the activity of xCT, such as CD44v9, are up-regulated as a consequence of oxidative stress. Increased xCT activity results in a build-up of intracellular cystine, which through a multistep process can be converted to glutathione. Disruption of xCT activity in cases of tissue injury leads to increased levels of ROS and oxidative damage. Excessive oxidative damage may cause cell death.
results here suggest that generation of ROS is inherent to the process of injury and reprogramming. Therefore, activation of antioxidant responses is required for the successful reprogramming of differentiated cells. Although the cell of origin for cancer in the stomach is still under debate, cellular reprogramming to metaplasia in the setting of chronic injury and inflammation may expose these cells to prolonged oxidative stress, which could promote neoplastic transformation. 46

In summary, our investigations have shown that a response to ROS and up-regulation of xCT activity is crucial for reprogramming of zymogenic chief cells into SPEM. Targeting xCT may prove an effective tool for arresting metaplasia development in the stomach as well as mucous metaplasia in other epithelial tissues for the analysis of cellular plasticity and oxidative stress response.

**Methods**

**Mouse Models**

C57BL/6J mice approximately 8 weeks old were purchased from Jackson Labs (Bar Harbor, ME). xCTKO mice were generously donated by Dr David E. Featherstone, MS, PhD (University of Illinois, Chicago, IL).24,47,48 Genotype was confirmed for the xCTKO mice using PCR as previously described.10 Each experimental group consisted of at least 3–5 mice. L635 (synthesized by the Chemical Synthesis Core of the Vanderbilt Institute of Chemical Biology, Nashville, TN), dissolved in dH2O was administered by oral gavage (350 mg/kg) once a day for 3 consecutive days. Three time points were used to analyze chief cell reprogramming: 12, 24, and 72 hours after L635 treatment ± sulfasalazine. DMP-777 (a gift from DuPont-Merck Co. Wilmington, DE) dissolved in 1% methylcellulose was
administered by oral gavage (350 mg/kg) once a day for 10 consecutive days. Intraperitoneal injection of sulfasalazine (10 mg/day) was administered 2 days before and throughout L635 and DMP-777 treatment. Sulfasalazine was freshly prepared each day in 0.1 mol/L NaOH adjusted with 1 mol/L HCl to pH 8. Intraperitoneal injection of the metabolites of sulfasalazine, sulfapyridine (3 mg/day), and mesalazine (5 mg/day) were administered 2 days before and throughout L635 treatment in the same manner as sulfasalazine.36 Archival sections of stomach from 12-month *H. felis*–infected mice and human clinical specimens were obtained from previous investigations.8,49 The care, maintenance, and treatment of animals in these studies adhere to the protocols approved by the Institutional Animal Care and Use Committee of Vanderbilt University.

**Cell Culture**

Cell lines were isolated from Immortomice as previously described.32 Immortomice are transgenic mice with interferon-γ (IFN-γ) inducible expression of temperature-sensitive T antigen. In cells that are isolated from Immortomice, addition of IFN-γ promotes the expression of immortalizing T antigen. At the permissive temperature (33°C), T antigen protein folds properly and immortalizes the cells. At the nonpermissive temperature (39°C) in the absence of IFN-γ, remaining T antigen protein misfolds and returns the cells to a differentiated state. Chief and SPEM cell lines were maintained at the permissive temperature in the presence of IFN-γ and were differentiated at 39°C for 48 hours with no IFN-γ before experiments were performed. Cells were cultured in ThermoFisher (Waltham, MA) Dulbecco’s modified Eagle medium/F12 (50:50) media supplemented with 10% Omega Scientific (Tarzana, CA) fetal bovine serum, 100 U/mL penicillin, and streptomycin (30-002-Cl; Corning, Corning, NY), 100 μg/mL MycoZAP Plus-PR (VZA-2021; Lonza, Basel, Switzerland), 1 ng/mL epidermal growth factor (AF-100-15; Peprotech, Rocky Hill, NJ), 1 ng/mL basic fibroblast growth factor (100-18B; Peprotech), 1 μg/mL hydrocortisone (H0888; Sigma, St. Louis, MO), 8 μg/mL insulin/transferring/selenium solution (41400045; ThermoFisher), and 5 U/mL IFN-γ (315-05; Peprotech). Cells were plated on collagen (PureCol Type 1 Advanced BioMatrix 5005, Carlsbad, CA)-coated plates (working solution, 0.03 mg/mL in phosphate-buffered saline [PBS]). For staining, cells were fixed in 4% paraformaldehyde (PFA) for 20 minutes. After fixation, cells were blocked and permeabilized in 1× PBS with 10% donkey serum and 0.3% Triton X-100 (Sigma) for 30 minutes. Cells were incubated in primary antibodies and diluted in 1× PBS with 1% donkey serum and 0.05% Tween-20 for 1 hour at room temperature. Cells then were incubated in fluorescent secondary antibodies, diluted in 1× PBS, for 1 hour at room
temperature, before 4',6-diamidino-2-phenylindole nuclear counterstain was added. A Zeiss (Oberkochen, Germany) Axio Imager M2 microscope with Axiovision digital imaging system was used to image fixed cells. For xCT blockade, cells were treated with 0.1 mmol/L sulfasalazine every day after 48 hours of differentiation. The pH level of media was monitored daily. To monitor cystine uptake, cells were treated with cystine-FITC (a gift from Dr Jeffrey Rathmell) for 2 hours. After 2 hours, Hoechst nuclear counterstain was added, and cells were washed 3 times with 1× PBS. The EVOS FL Cell Imaging System (ThermoFisher) was used to visualize intracellular cystine-FITC. To monitor proliferation, cell numbers were measured by a Bio-Rad (Hercules, CA) TC10 Automated Cell Counter. Three replicates were performed for each experiment.

**siRNA Transfection**

ImSPEM cells were plated on collagen-coated, 6-well plates the day before transfection to provide confluency of 60%–70% in 24 hours. ImSPEM cells were transfected with Slc7a11 Mouse siRNA Oligo Duplexes or Trilencer-27 Universal Scrambled Negative Control siRNA Duplex (SR416143; Origene, Rockville, MD) according to the manufacturer's recommendations using siTran 1.0 siRNA transfection reagent (TT300001; Origene).

**Quantitative Real-Time PCR Analysis**

Total RNA from the stomach (oxyntic region) was extracted from paraformaldehyde-fixed, paraffin-embedded tissue of 3–5 mice per experimental group to examine the expression of mRNA transcripts (Cd44v9, xCT, Esrp1, Atg4, Atg5, Atg7, Atg12, Atg16L1, Beclin1, Lamp1, Lamp2, and Lc3). Five-micrometer sections were taken for H&E stain to identify the oxyntic region in tissue block. A 2-mm biopsy punch then was used to extract tissue. The standard Qiagen (Hilden, Germany) RNasy FFPE Kit (73504) protocol was used for purification of total RNA. The ThermoFisher High-Capacity cDNA Reverse Transcription Kit (4368814) was used for complementary DNA synthesis. Quantitative real-time polymerase chain reaction was performed with the Bio-Rad SsoAdvanced Universal SYBR Green Super Mix (172-5270) and specific primers (Table 1) on the Bio-Rad CFX96 Touch Real-Time PCR Detection System. The expression of mRNA transcripts was normalized to TBP expression and shown as relative expression levels (2^D_Cq). All graphs and statistics were completed in GraphPad Prism (San Diego, CA) using an unpaired Student t test to determine significance.

**Immunohistochemical Staining**

Mouse stomachs were fixed in 4% PFA overnight at 4°C and then were transferred into 70% ethanol for subsequent paraffin embedding. Five-micrometer sections were used for all immunohistochemistry studies. Deparaffinization, rehydration, and antigen retrieval were performed as previously described. Tissue sections were blocked in Dako (Santa Clara, CA) Peroxidase Blocking Solution at room temperature for 20 minutes followed by Dako Protein Block Serum-Free at room temperature for 1.5 hours. The primary antibodies were added overnight at 4°C in Dako Antibody Diluent with Background Reducing Components. Horse-radish-peroxidase–conjugated secondary antibodies were added for 15 minutes at room temperature. 3,3'-Diaminobenzidine tetra hydrochloride chromogen was added for detection. A Leica (Wetzlar, Germany) SCN400 Slide Scanner in the Vanderbilt Digital Histology Shared Resource was used to image sections.
### Table 1. Catalog of Quantitative qPCR Primer Sequences

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<th>Oligonucleotides</th>
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<td><strong>mCd44v (Cd44v9) primers</strong></td>
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<tr>
<td>F: 5' GGCTTAATAGGCATGCACACACTTCTCT 3'</td>
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<td><strong>mScl7a11 (xCT) primers</strong></td>
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<tr>
<td>R: 5' TCACGATAGCAGCGGTCTCACA 3'</td>
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<tr>
<td><strong>mEsp1 (Esp1) primers</strong></td>
</tr>
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<td><strong>mAtg4 (Atg4) primers</strong></td>
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<td>R: 5' TCCACCTGCAAGTCTGAGCTTA 3'</td>
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<tr>
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<td><strong>mAtg16 (Atg16) primers</strong></td>
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<td><strong>mBeclin1 (Beclin1) primers</strong></td>
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<td>R: 5' TCAGTTGTTGGCTCTTATTAGTGCACT 3'</td>
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F, forward; R, reverse.

### Table 2. Catalog of Primary Antibodies

<table>
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<th>Antibodies</th>
<th>Supplier</th>
<th>Catalog No.</th>
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<tbody>
<tr>
<td>xCT (1:500)</td>
<td>Abcam</td>
<td>ab37185</td>
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<td>CD44v (1:25,000)</td>
<td>Cosmo Bio</td>
<td>CAC-LKG-M002</td>
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<tr>
<td>ESRP1 (1:200)</td>
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<td>NBP1-82201</td>
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<td>Glutathione</td>
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<tr>
<td>Ki67 (1:1000)</td>
<td>Cell Signaling Technology</td>
<td>9129</td>
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<tr>
<td>H “K” ATPase (1:10,000)</td>
<td>A gift from Dr Adam Smolka</td>
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<tr>
<td>GSII lectin (1:2000)</td>
<td>Invitrogen</td>
<td>L32451</td>
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<tr>
<td>GIF (1:2000)</td>
<td>A gift from Dr David Alpers</td>
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<tr>
<td>Mist1 (1:1000)</td>
<td>A gift from Dr Jason Mills</td>
<td>N/A</td>
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<tr>
<td>L3C/MAP1LC38 (1:500)</td>
<td>Novus Biologicals</td>
<td>NB100-2220</td>
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<tr>
<td>LMAP2 (GL27) (1:500)</td>
<td>Abcam</td>
<td>ab13524</td>
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<tr>
<td>UEA1-lectin (1:2000)</td>
<td>Sigma</td>
<td>L9006</td>
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<tr>
<td>Cleaved caspase-3 (Asp175) (1:200)</td>
<td>Cell Signaling Technology</td>
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<td>Clusterin-α antibody (M-18) (1:2000)</td>
<td>Santa Cruz Biotechnology</td>
<td>sc-6420</td>
</tr>
<tr>
<td>TFF2 (1:500)</td>
<td>A gift from Dr Nicholas Wright</td>
<td>N/A</td>
</tr>
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</table>

**Immunofluorescence Staining**

Five-micrometer tissue sections were blocked in Dako Protein Block Serum-Free at room temperature for 1.5 hours. For mouse primary antibodies, Mouse on Mouse blocking reagent (Vector Laboratories, Burlingame, CA) was added to slides for 20 minutes at room temperature. The primary antibodies (Table 2) were added overnight at 4°C in Dako Antibody Diluent with Background Reducing Components. Fluorescent donkey secondary antibodies were added in Dako Antibody Diluent at room temperature for 1 hour. A Zeiss Axio Imager M2 microscope with Axiosvision digital imaging system, Zeiss LSM 710, or the Leica Aperio Versa 200 Fluorescent Slide Scanner in the Vanderbilt Digital Histology Shared Resource was used to image sections. Immunostaining for LC3 was performed at Washington University (St. Louis, MO) as previously described.

**Immunofluorescence Quantitation**

Experimental groups contained 3–5 mice. Images were analyzed using CellProfiler (Cambridge, MA) to quantify objects and verified manually (nuclei, cells). At least 5 representative images (>150 glands) of proximal stomach corpus were taken from each mouse at 20× objective for quantification. Relative fluorescence intensity was determined using ImageJ (National Institutes of Health, Bethesda, MD) and was normalized to cell number. Relative fluorescence intensity for more than 250 cells per replicate was measured. To quantify autophagy of zymogenic granules, GIF-positive cells were selected. Images were analyzed using CellProfiler to quantitate objects (puncta). More than 250 cells per mouse were measured. All graphs and statistics were completed in GraphPad Prism using an unpaired Student t test or 1-way analysis of variance with the Bonferroni post hoc multiple comparisons test to determine significance. n.s. *P > .05, **P ≤ .01, ***P ≤ .001, ****P ≤ .0001.
Electron Microscopy

For TEM, freshly excised stomach (oxyntic region) tissue was washed briefly in 0.1 mol/L cacodylate buffer. Samples then were fixed in 2.5% glutaraldehyde (in 0.1 mol/L sodium phosphate buffer, pH 7.4, 0.1 mol/L cacodylate buffer) for 1 hour at room temperature, followed by overnight fixation at 4°C. Subsequent tissue preparation and imaging for TEM was performed as previously described.53

ROS Detection

Fresh stomach tissue was trimmed, embedded in Tissue-Tek optimal cutting temperature compound, and snap frozen in an acetone bath on dry ice. Five-micrometer cryosections were obtained on glass slides. DHE (D1168; Invitrogen Carlsbad, CA) was diluted to a final concentration of 10 μmol/L in 1× PBS with Phalloidin-iFluor 488 (Abcam Cambridge, United Kingdom). DHE/phalloidin solution was applied to slides in a light-protected humidified chamber at 37°C for 30 minutes. Slides were washed with 1× PBS and imaged immediately using a Zeiss Axio Imager M2 microscope with an Axiovision digital imaging system. Relative fluorescence intensity (red) of oxidized DHE was determined using ImageJ and was normalized to area. CellROX Green Reagent (C10444; Invitrogen Carlsbad, CA) was diluted to a final concentration of 5 μmol/L in 1× PBS. CellROX solution was applied to slides in a light-protected humidified chamber at 37°C for 30 minutes. Slides were washed with 1× PBS and fixed in 4% PFA for 15 minutes. After fixation, to block/extract the tissue it was incubated in 1× PBS with 10% donkey serum and 0.3% Triton X-100 for 30 minutes. GIF primary antibody was diluted in 1× PBS with 1% donkey serum and 0.05% Tween-20 and incubated for 1 hour at room temperature. Fluorescent donkey anti-goat secondary antibody was diluted in 1× PBS and incubated for 1 hour at room temperature and Hoechst nuclear counterstain was added. A Zeiss Axio Imager M2 microscope with an Axiovision digital imaging system was used to image fixed tissue within 24 hours. DHE (10 μmol/L) or CellROX Green Reagent (5 μmol/L) was added to ImSPEM cells plated on glass coverslips and incubated at 39°C for 30 minutes. A Zeiss Axio Imager M2 microscope with an Axiovision digital imaging system was used to image cells immediately. The relative fluorescence intensity (green) of CellROX in GIF-positive cells was determined using ImageJ and was normalized to the cell number.

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Author contributions
Anne R. Meyer designed and performed experiments, analyzed data, and drafted the manuscript; Amy C. Engevik, Spencer G. Willet, and Janice A. Williams performed experiments, analyzed data, and revised the manuscript; Yong Zou and Pierre P. Massion designed experiments and revised the manuscript; and Jason C. Mills, Eunyoung Choi, and James R. Goldenring designed experiments, analyzed data, and revised the manuscript.

Conflicts of interest
The authors disclose no conflicts.

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