Neurogenin 3 is essential for the proper specification of gastric enteroendocrine cells and the maintenance of gastric epithelial cell identity

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The notch signaling pathway is essential for the endocrine cell fate in various tissues including the enteroendocrine system of the gastrointestinal tract. Enteroendocrine cells are one of the four major cell types found in the gastric epithelium of the glandular stomach. To understand the molecular basis of enteroendocrine cell development, we have used gene targeting in mouse embryonic stem cells to derive an EGFP-marked null allele of the bHLH transcription factor, neurogenin 3 (ngn3). In ngn3−/− mice, glucagon secreting A-cells, somatostatin secreting D-cells, and gastrin secreting G-cells are absent from the epithelium of the glandular stomach, whereas the number of serotonin-expressing enterochromaffin (EC) cells is decreased dramatically. In addition, ngn3−/− mice display intestinal metaplasia of the gastric epithelium. Thus, ngn3 is required for the differentiation of enteroendocrine cells in the stomach and the maintenance of gastric epithelial cell identity.

[Key Words: Basic-helix-loop-helix [bHLH] protein; neurogenin 3 (ngn3); notch signaling; metaplasia; enteroendocrine cells; iFABP; Muc 2]

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The mouse stomach is divided into two domains, the proximal third, which is known as the forestomach and is lined with a keratinized squamous epithelium, and the distal two-thirds that make up the stomach proper, which is lined with a glandular epithelium containing four major cell types, that is, pit, parietal, zymogenic, and enteroendocrine cells (Lorenz and Gordon 1993; Gordon and Hermiston 1994). Endocrine cells produce peptide hormones that regulate various physiological functions [Skipper and Lewis 2000]. The specification of endocrine cells from a population of precursor cells is thought to be in part mediated by the Notch-signaling pathway [Artavanis-Tsakonas et al. 1999]. Upon activation by ligands such as Delta or Jagged on adjacent cells, the intracellular domain of the Notch receptor is cleaved and translocated to the nucleus with subsequent up-regulation of downstream targets including the Hairy/Enhancer of Split (HES) genes. The HES proteins then inhibit the expression of several bHLH transcription factors including the neurogenin genes [Sommer et al. 1996; Artavanis-Tsakonas et al. 1999].

The bHLH transcription factors are instrumental in the determination of various cell fates including that of many endodermal endocrine cells. For example, gene targeting experiments in mice have shown that Mash1 is required for endocrine cell differentiation in the lung [Borges et al. 1997], whereas loss of Math1 leads to depletion of the secretory cell lineage in the intestine [Yang et al. 2001]. Loss of BETA2/NeuroD results in diminished numbers of pancreatic α- and β-cells, as well as secretin- and cholecystokinin (CCK)-expressing cells of the gut [Naya et al. 1997], whereas deletion of neurogenin 3 results in the absence of all four pancreatic endocrine cell types [Gradwohl et al. 2000]. In addition, ablation of the repressor of bHLH gene expression, Hes1, leads to precocious and excessive differentiation of enteroendocrine cells in the gastrointestinal tract, underscoring the importance of the Notch-signaling pathway in the specification of enteroendocrine cells [Jensen et al. 2000]. In this study, we have generated mice lacking ngn3 and have analyzed the role of ngn3 during gastric epithelial development. Using this model, we have uncovered an important role for ngn3 in enteroendocrine cell differentiation and the maintenance of gastric epithelial cell identity.
Results

Derivation of ngn3–EGFP mice

To investigate the potential role of the bHLH transcription factor ngn3 during stomach development, we have derived mice homozygous for a null mutation of this gene by homologous recombination in mouse embryonic stem (ES) cells. We constructed a targeting vector that replaces the entire coding region of ngn3 with the enhanced green fluorescent protein (EGFP) gene [Fig. 1A]. Of 200 stably transfected ES cell clones obtained after G418 selection, two were homologous recombinants as identified by PCR screening [Fig. 1B; data not shown]. Germ-line chimeras and mice heterozygous for the ngn3 mutation were obtained. Heterozygous animals of the original mixed background (129Sv × C57BL6) were crossed, and the offspring were genotyped by PCR [Fig. 1C]. No gross differences were observed between heterozygous and wild-type animals in overall development, growth characteristics, and histology; therefore, mice of both genotypes were used as controls throughout this study.

Ngn3–EGFP marks the enteroendocrine cell lineage of the glandular stomach

During endocrine lineage development in the pancreas, ngn3 is expressed transiently in endocrine precursor cells, but extinguished in fully differentiated cells [Gradwohl et al. 2000]. To investigate the expression domain of ngn3 in the stomach, we made use of our ngn3–EGFP allele to follow the expression of ngn3 by green fluorescence. Ngn3–EGFP was located specifically in the glandular stomach, whereas no expression was detected in the squamous epithelium of the forestomach [Fig. 1F]. The image of the ngn3 heterozygous stomach shown in Figure 1F was obtained by imaging the entire organ on a confocal fluorescence microscope. Due to the low intensity of the ngn3–EGFP signal, the image was captured from multiple focal planes, thus giving the impression of a high density of enteroendocrine cells in the glandular stomach. To delineate the identity of these EGFP-positive cells, we examined the coexpression of the pan-enteroendocrine marker, Chromogranin A, with EGFP in the stomach of heterozygous mice. In addition to multiple single-label EGFP-positive cells that presumably mark enteroendocrine precursor cells, we also observed numerous EGFP/Chromogranin A double-positive cells. The long half-life of EGFP thus allowed us to trace the fate of the ngn3–EGFP-positive endocrine precursors to their mature descendants. These data suggest that ngn3–EGFP marks the enteroendocrine lineage in the stomach [Fig. 1G].

Ngn3−/− mice display a disorganized gastric mucosa

Ngn3−/− mice derived previously are born alive with reduced size and experience 100% mortality by postnatal day 3 due to severe diabetes [Gradwohl et al. 2000]. We observed similar abnormalities in our ngn3−/− mice [Fig. 1D], including the lack of the four enteroendocrine cell types of the pancreas. However, in addition to this pancreatic phenotype, ngn3−/− mice also have previously undescribed abnormalities in other endodermally derived organs, including smaller stomachs [Fig. 1E]. To further analyze the gastric phenotype of ngn3−/− mice, we examined gastric histology in 3-day-old control and ngn3−/− mice [Fig. 2A–H]. We utilized Alcian blue staining to detect potential transformation of the gastric epithelium to intestinal cell types. Alcian blue stains acidic mucins that are normally found exclusively in the goblet cells of the small intestine and colon. Whereas the gastric mucosa of control mice contained no Alcian blue staining cells [Fig. 2C,E], a significant number of stained cells were found in the ngn3−/− gastric mucosa [Fig. 2D,F], suggesting regional intestinal metaplasia of the stomach. At higher magnification, we noticed that the Alcian blue positive cells in the ngn3−/− gastric epithelium are elongated, resembling the shape of a goblet cell [Fig. 2F]. To assess whether these cells are goblet cells, we examined the ultrastructure of control and ngn3−/− stomachs by electron microscopy. As expected, no goblet cells were present in control stomachs [Fig. 2G], whereas goblet-like cells occurred frequently in the ngn3−/− stomach [Fig. 2H]. These goblet cells appear immature, as they exhibit relatively few mucin-containing vesicles.

To support these findings on the molecular level, two intestine-specific markers, Muc2 and iFABP, were analyzed for expression by RT–PCR, RNase protection assay, and immunohistochemistry. Muc2 is normally expressed in goblet cells in the intestine [van Klinken et al. 1999; Longman et al. 2000], and iFABP is restricted to both absorptive enterocytes and goblet cells of the intestine [Sweetser et al. 1988]. Muc2 expression was detected in ngn3−/−, but not in control stomachs as shown by RT–PCR [Fig. 3A] and RPA [Fig. 3B] analysis. As expected, no iFABP-positive cells were found in the gastric epithelium of controls [Fig. 3C], whereas iFABP expression was induced in the gastric mucosa of ngn3−/− mice [Fig. 3D]. Quantification of iFABP-positive cells showed a 13-fold increase in the number of iFABP-expressing cells in the gastric epithelium of ngn3−/− mice compared with littermate controls [Fig. 3E]. Taken together, data from histological and molecular analyses implicate ngn3 in the maintenance of the gastric mucosal identity in the developing stomach, as its absence leads to regional intestinal metaplasia of the gastric epithelium.

Proliferation and apoptosis are normal in the gastric epithelium of Ngn3−/− mice

The reduced size and expanded mucosal thickness of ngn3−/− stomachs prompted us to measure rates of proliferation and apoptosis in these mice using Ki67 and Caspase 3, respectively, as markers [Schluter et al. 1993; Yuan et al. 1993; Stenniche and Salvesen 1997]. The pattern and numbers of proliferating cells stained by Ki67 were similar in the gastric mucosa of control and ngn3−/− mice [Fig. 4A,B]. This suggests that the expanded gastric
epithelium observed in ngn3−/− mice is not caused by increased proliferation. Next, we examined the possibility of altered programmed cell death in the ngn3−/− mucosa by caspase 3 staining. As shown in Figure 4, C and D, we did not observe a significant difference in the number of apoptotic cells between control and ngn3−/− mice. Thus, it appears likely that the disorganization of the ngn3−/− mucosa causes the gastric epithelium to ap-
pear thinner than that of the control stomach. Finally, it is possible that the smaller size of the stomach in the 3-day-old ngn3−/− mice is an indirect result of the abnormal metabolic status of these animals, as there was no difference in the fetal development of the organ when stomachs of control and ngn3−/− animals were compared at embryonic day 17.5 (data not shown).

Gastric enteroendocrine differentiation is impaired in ngn3−/− mice

To determine whether enteroendocrine cells are affected in the ngn3−/− stomach, expression of a general enteroendocrine cell marker, Chromogranin A, was examined by immunofluorescence. All major endocrine cell types express Chromogranin A in the stomach (Norlen et al. 2001). As shown in Figure 5, B and C, there were fewer Chromogranin A-positive cells in the ngn3−/− stomach than in the control. To distinguish the subtypes of enteroendocrine cells affected by the lack of ngn3, we utilized markers specific for A-, D-, G-, and EC-cells (Fig. 5A). Whereas cells expressing glucagon, somatostatin, and gastrin were found in the control stomach, none of these peptide hormones was detected in the gastric epithelium of ngn3−/− mice [Fig. 5F–K]. In contrast, serotonin-positive cells were still present in the ngn3−/− stomach, but at reduced frequency (Fig. 5D–E; Fig. 6C). The expression of BETB2/NeuroD, a downstream target of ngn3 (Huang et al. 2000), which plays an important role during endodermal endocrine cell differentiation, was absent in the ngn3−/− stomach as assessed by quantitative RT–PCR analysis (data not shown). This suggests that ngn3 regulates the enteroendocrine lineage in the stomach through BETB2/NeuroD, similar to the transcription factor hierarchy that specifies pancreatic endocrine cells (Gradwohl et al. 2000).

Glucagon, somatostatin, and gastrin mRNAs are absent in the stomach of ngn3−/− mice

Although immunofluorescence is useful to investigate enteroendocrine cell differentiation, it is also crucial to determine the expression level of the marker genes quantitatively. Thus, we investigated glucagon, somatostatin, and gastrin mRNA levels in the control and ngn3−/− stomach by RT–PCR analysis. Consistent with what we have observed in our immunofluorescence studies, glucagon, somatostatin, and gastrin mRNA were completely absent in the ngn3−/− stomach, whereas these transcripts were detected in the control stomach [Fig. 6A]. Interestingly, we have found a broad range of gastrin mRNA levels in the control stomachs, which might reflect small differences in the age or nutritional status of these animals.

In addition, we performed microarray expression profiling using various platforms with total RNA extracted from both control and ngn3−/− stomach. As shown in Figure 6B, the mRNA levels for proglucagon, somatostatin, and gastrin were decreased by 22-, 7-, and 14-fold,
respectively, in ngn3−/− stomachs, consistent with our RT–PCR results. In contrast, the intestinal markers, Trefoil factor 3 (TFF3; Mashimo et al. 1995), and iFABP were found to be increased by 18- and 3-fold, respectively in the ngn3−/− stomachs, confirming the presence of regional intestinal metaplasia of the stomach in ngn3−/− mice described above. The expression of the H+/K+-ATPase, a marker for parietal cells, showed no difference between the control and ngn3−/− stomachs, again confirming our histological findings [see below].

As multiple attempts at establishing an RT–PCR protocol for serotonin were unsuccessful, and as serotonin is...
not included on the microarrays used for our study, we quantified the level of serotonin expression by counting cells that are immunostained with serotonin antibody from both control and ngn3−/− stomachs. We observed an overall sixfold decrease in the numbers of serotonin-positive cells in the ngn3−/− stomachs when compared with controls [Fig. 6C].

Parietal and mucous cells are not affected in the ngn3−/− stomach

In addition to enteroendocrine cells, other cell types such as zymogenic cells, mucin-producing pit cells, and parietal cells are also present in the gastric mucosa (Gordon and Hermiston 1994). As it has been shown that gastrin deficiency leads to a decrease in the number of parietal cells and an increase in mucous neck cell number in gastrin-deficient mice (Koh et al. 1997), we sought to determine whether these lineages were also affected by the loss of gastrin expression observed in the stomach of ngn3-deficient mice [Fig. 5K]. For this purpose, we utilized lectin staining, which is a sensitive tool for defining the differentiation program of gut epithelial cell lineages. Immunohistochemistry with DBA and AAA, which label parietal and mucous cells, respectively [Falk et al. 1994], revealed no differences between the control and ngn3−/− stomachs [Fig. 2I–J; data not shown]. These data indicate that the gastric parietal and mucous cell

Figure 5. Ngn3 is required for the differentiation of enteroendocrine cells in the gastric epithelium. (A) Classification of enteroendocrine cells by their main secretory products. Immunofluorescence was performed on paraffin sections from 2–3-day-old control [B,D,F,H,J] and ngn3−/− [C,E,G,I,K] glandular stomach. Immunostained cells are labeled in green for the enteroendocrine cell-specific antigen indicated at left (labeled by white arrows), and images were captured by confocal microscopy. Evan’s blue was used as counterstain for the tissues and is visualized in red in all sections [Beaulieu 1997]. (B) Chromogranin A, a general endocrine cell maker, labels all subtypes of enteroendocrine cells in the gastric epithelium of control mice. (C) The number of Chromogranin A-expressing cells is reduced in the ngn3−/− gastric epithelium. (D) An enterochromaffin (EC) cell-specific marker, serotonin, is normally expressed by the EC-cells in the control stomach. (E) The number of serotonin-positive cells is reduced in the ngn3−/− stomach. (F) An A-cell-specific enteroendocrine cell marker, glucagon, is normally present in the gastric epithelium of control stomach. (G) No glucagon-positive cells are found in the ngn3−/− gastric epithelium. (H) Somatostatin, a D-cell-specific enteroendocrine cell marker, is found in the control gastric epithelium. (I) Somatostatin expression is not present in the ngn3−/− stomach. (J) Gastrin expression is found in the G-cells of control gastric epithelium. (K) No gastrin-positive cells are found in the ngn3−/− gastric epithelium. Magnification, 400×.
lineages are not regulated by ngn3 and are not affected by the lack of gastrin in the stomach. The apparent discrepancy in the observations in gastrin-deficient mice cited above might be explained by the difference in the age of the animals investigated. Due to the early lethality of ngn3−/− mice, we had to limit our analysis to postnatal day 3, whereas the phenotype of gastrin null mice is only apparent in adult mice (7–8 wk old) [Koh et al. 1997].

Discussion

Models for the role of ngn3 during gastric enteroendocrine cell differentiation

Our analysis in ngn3−/− mice suggests the existence of both ngn3-dependent and independent enteroendocrine cell lineages. We propose two models to illustrate how ngn3 might specify gastric enteroendocrine development [Fig. 7]. The first model suggests that ngn3 is needed initially for the proliferation of all endocrine cells, and subsequently required for the terminal differentiation of A-, D-, and G-cells, but not EC-cells [Fig. 7A]. Thus, ngn3 deficiency leads to a smaller pool of enteroendocrine precursors from which EC-cells can differentiate, resulting in the observed reduction in the frequency of this cell type. The second model suggests that ngn3 is absolutely required for the specification of A-, D-, and G-cells, but not EC-cells, because the specification of EC-cells can also be orchestrated by factor X [Fig. 7B]. Factor X could be regulated by effectors in the Notch-signaling pathway or by factors in other signaling pathways. Other neurogenin family members such as ngn1 and ngn2 are potential candidates for factor X that might also be involved in governing enteroendocrine cell specification. In conclusion, we have shown that ngn3 is essential for the specification of enteroendocrine cells in the stomach and for the maintenance of gastric epithelial cell identity.

Materials and methods

Derivation of the ngn3–EGFP allele

Primers specific to the mouse ngn3 gene were used to screen a 129 SvEv mouse BAC [bacterial artificial chromosome] library.
(Research Genetics). Three BACs containing the ngn3 gene were identified and an 8-kb XbaI fragment of the final ngn3 BAC was subcloned into pBluescript [Stratagene; ngn3x3x8.0] and used for the construction of the targeting vector. Sequencing confirmed that the entire 642-bp ngn3-coding region was contained in ngn3x3x8.0. Furthermore, ngn3x3x8.0 also contains a 6.4-kb 5’ flanking sequence and 948 bp of 3’UTR. The ngn3-coding region was replaced by the EGFP cDNA and a loxp-pgk-neomycin-cin-loxP resistance cassette. The negative selectable marker Diphtheria Toxin A was cloned outside of the 3’ homology of ngn3 to enhance the targeting frequency in ES cells. The targeting scheme is detailed in Figure 1A. The targeting vector was linearized with Xhol and 20 µg of DNA was electroporated into 107 TL1 embryonic stem cells (Labosky et al. 1997). Stably transfected cells were isolated after selection in 350 µg/ml G418 (GIBCO), and 200 clones were screened for homologous recombinants by PCR using the following primers: ES cell screening primer 1, 5’-ATAGCGTTGGCCGTTTACGT-3’; and 5’-ATACTCTGGTCCCCC-3’. PCR reactions were carried out [95°C for 5 min, 42 cycles of 95°C for 45 sec, 60°C for 45 sec, 72°C for 2.5 min, and 72°C for 5 min] in a buffer containing 1.5 mM MgCl2. The homologously recombinant clones produced a band of 1.5 kb. ES cells from the correctly targeted clones were injected into blastocysts derived from C57BL/6 mice. Blastocysts were transferred to pseudopregnant females and chimeric offspring were identified by the presence of agouti hair. Chimeric males were mated to C57BL/6 females to obtain ES-derived offspring that were analyzed by PCR of tail DNA to identify the heterozygous (ngn3+/−) mice. Embryos and mice were also genotyped by PCR using genotyping primers as follows: ngn3-1, 5’-ATACCTCTGTGCCCTCCC GTG-3’; ngn3-2, 5’-TGTTTGCTGAGTGCCAACTC-3’; and ngn3-3, 5’-AGTCTCCCCTTGCTCCTCT CC-3’.

Histology

Tissues were fixed in 4% paraformaldehyde overnight at 4°C, embedded in paraffin, cut using a Leica Ultracut microtome and mounted on 200 mesh thin bar copper grids, stained in 7% aqueous uranyl acetate, and counterstained in bismuth subnitrate. Digital images were collected on a JEOL JEM 1010 equipped with a Hamamatsu CCD camera and AMT 12-HR imaging software.

RNA analysis

Total RNA from postnatal stomach was isolated after homogenization and processed using the Totally RNA extraction kit [Ambion]. RNase protection analysis was carried out using 10 µg of total RNA and the RPA II kit [Ambion] following the manufacturer’s protocol. The probes used were Muc2 [Silberg et al. 2001] and GAPDH [Ambion]. RT–PCR analysis was performed as described previously [Wilson and Melton 1994; Duncan et al. 1997]. To determine conditions for quantitative analysis, cDNA samples were diluted serially, and each primer pair was tested as above. Primers were designed as follows:

| Probe | Sense | Antisense |
|-------|-------|-----------|
| G418  | 5’-GAGCATC-3’ | 5’-TGCAGGAGGCTGCTGACC-3’ |
| Hprt  | 5’-GCTGGCCTATAGGCTCATAG-3’ | 5’-GGTGCCTGATACTAAGC-3’ |
| Muc2  | 5’-GCTGAGGAGGCTGCTGACC-3’ | 5’-GAACCTTGAAAGCT-3’ |
| SOMATOSTATIN | 5’-CTCGTGGAGAGCATGGTACCACCA-3’ | 5’-CTCGTGGAGAGCATGGTACCACCA-3’ |
| Alcian blue | 5’-GGTGAAGAGAAGCTACAGTTT GAAGCATC-3’ | 5’-GAACATGAGAAGCTACAGTTT GAAGCATC-3’ |
| Gastrin | 5’-GACAAATGAGAAGCTACAGTTT GAAGCATC-3’ | 5’-GAACATGAGAAGCTACAGTTT GAAGCATC-3’ |

For immunofluorescence, tissues were fixed, sectioned, and processed as described above. Slides used for Chromogranin A were subjected to microwave antigen retrieval by boiling for 6 min in a 10-mM citric acid buffer [pH 6.0] and allowed to cool for 10 min at room temperature. Tissues were fixed in 4% paraformaldehyde overnight at 4°C, embedded in paraffin, cut to 6-µm sections, and applied to rabbit (1:200; Vector Laboratories) for 30 min at 37°C. Slides were washed in PBS, then incubated with goat anti-rabbit IgG (1:50; Jackson). For immunohistochemistry, tissues were fixed and processed as described above, and quenched in 2.25% hydrogen peroxide at room temperature for 20 min. Slides were blocked with protein-blocking reagent (ImmunoTech) for 20 min at room temperature. The primary antibodies were diluted in PBS containing 0.1% BSA and 0.2% Triton X-100 [PBT] unless noted otherwise, and incubated overnight at 4°C. Slides were washed in PBT, then incubated with the appropriate secondary antibodies diluted in PBT for 2 h at room temperature. Slides were washed in PBS. To counterstain the tissues, slides were dipped in 0.01% Evan’s Blue solution for 20 sec, rinsed in PBS, mounted, and examined using confocal microscopy (Leica). For immunofluorescence, tissues were fixed, sectioned, and processed as described above. Slides were blocked with protein-blocking reagent (ImmunoTech) for 20 min at room temperature. The primary antibodies were diluted in PBT and incubated overnight at 4°C. Slides were washed in PBS and incubated with goat anti-rabbit IgG (1:200; Vector Laboratories) for 30 min at 37°C. Slides were blocked with protein-blocking reagent (ImmunoTech) for 20 min at room temperature.
were rinsed with PBS and incubated with HRP-conjugated ABC reagent (Vector Elite kit) for 30 min at 37°C. Slides were washed and colors were developed using a DAB substrate kit (Vector Laboratories). Slides for Ki67 immunohistochemistry were microwaved for antigen retrieval (see above). The following antibodies were used at the indicated dilutions for immunohistochemistry: rabbit anti-iFABP (1:2500; gift from J. Gordon, Washington University, St. Louis, MO), rabbit anti-human/mouse caspase 3 (activated [1:750; R&D Systems], rabbit anti-Ki67 [1:5000; Novoceastra], biotinylated-anti-rabbit IgG [1:200; Vector], and biotinylated-DBA lectin [1:20; EY Laboratories]. Ki67 and Caspase 3-labeled nuclei were counted manually in a blinded fashion and care was taken only to evaluate gastric mucosa.

Whole-mount immunostaining

Milk was removed from the stomach and the tissue fixed in 4% PFA at room temperature for 30 min. Tissues were washed in PBS/0.1% Triton X [PT] for 30 min at room temperature, then blocked in PT/5%BSA at 4°C overnight. Rabbit anti-Chromogranin A [Diasonin] was added at 1:500 and incubated overnight at 4°C. Tissues were washed in PT/1%BSA for 1.5 h and Goat anti-Rabbit-Cy3 was added at 1:200 in PT/5%BSA for 1.5 h. Tissues were washed in PBS and mounted in PBS/50% glycerol for confocal microscopy (Pepling and Spradling 1998).

Microarray analysis

RNA samples were extracted as described above. cDNA was reverse transcribed and fluorescently labeled from 2 µg of total RNA using Cy3 dendrimers (Genisphere) from control (n = 2) and mutant (n = 3) stomachs. Common controls were pooled from the controls and mutants and labeled with Cy5 dendrimers. These probes were hybridized to the PancChip2 (Scerace et al. 2002) plus 6912 mouse 70-mer oligonucleotides (Operon) at 45°C according to the Genisphere protocol. A Genepix Scanner was used to scan the slides and the signals were quantified using the Genepix software [Axon]. Concurrently, 10 µg of total RNA from control (n = 2) and mutant (n = 3) stomach were used to screen the Affymetrix MG_U74A version 1 chip. Data were acquired and analyzed according the Affymetrix protocol. Global normalization was used to obtain the normalized intensity levels.

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