Epithelial Sel1L is required for the maintenance of intestinal homeostasis

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ABSTRACT Inflammatory bowel disease (IBD) is an incurable chronic idiopathic disease that drastically decreases quality of life. Endoplasmic reticulum (ER)–associated degradation (ERAD) is responsible for the clearance of misfolded proteins; however, its role in disease pathogenesis remains largely unexplored. Here we show that the expression of SEL1L and HRD1, the most conserved branch of mammalian ERAD, is significantly reduced in ileal Crohn’s disease (CD). Consistent with this observation, laboratory mice with enterocyte-specific Sel1L deficiency (Sel1LΔIEC) develop spontaneous enteritis and have increased susceptibility to Toxoplasma gondii–induced ileitis. This is associated with profound defects in Paneth cells and a disproportionate increase of Ruminococcus gnavus, a mucolytic bacterium with known association with CD. Surprisingly, whereas both ER stress sensor IRE1α and effector CHOP are activated in the small intestine of Sel1LΔIEC mice, they are not solely responsible for ERAD deficiency–associated lesions seen in the small intestine. Thus our study points to a constitutive role of Sel1L-Hrd1 ERAD in epithelial cell biology and the pathogenesis of intestinal inflammation in CD.

INTRODUCTION

Inflammatory bowel disease (IBD) is a devastating disease that affects millions of people worldwide. Phenotypically, IBD is categorized as Crohn’s disease (CD), which is defined by transmural inflammation of the ileum and or colon, or ulcerative colitis (UC), which is a mucosal inflammation and ulceration restricted to the colon. The etiology of IBD is unresolved, but it is generally considered as a disturbed host–microbial symbiosis in a genetically susceptible individual, leading to aberrant proinflammatory immune responses (Wlodarska et al., 2015). The intestinal epithelium is the physical interface between the host and gut microbiota. Absorptive enterocytes and specialized cells such as Paneth and goblet cells, which actively secrete antimicrobial peptides and mucin glycoproteins, form an antimicrobial barrier that is critical for the maintenance of intestinal homeostasis (Bevins and Salzman, 2011).

Disturbance of endoplasmic reticulum (ER) homeostasis has been implicated in the pathogenesis of IBD (Heazlewood et al., 2008; Kaser et al., 2008, 2013; Adolph et al., 2013; Das et al., 2013). ER-associated degradation (ERAD) is a principal quality-control mechanism in the cell, targeting misfolded proteins for cytosolic degradation (Olzmann et al., 2013). Whereas the biochemical

Abbreviations used: CD, Crohn’s disease; DSS, dextran sodium sulfate; ER, endoplasmic reticulum; ERAD, ER-associated degradation; FISH, fluorescence in situ hybridization; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; IgG, immunoglobulin G; PBS, phosphate-buffered saline; RT-qPCR, reverse-transcription quantitative PCR; TEM, transmission electron microscopy; UC, ulcerative colitis; UPR, unfolded protein response.

Monitoring Editor
Reid Gilmore
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Received: Oct 20, 2015
Revised: Nov 23, 2015
Accepted: Nov 23, 2015
processes of ERAD have been well characterized, the physiological significance of ERAD and its role in the pathogenesis of IBD are underdefined. The E3 ligase Hrd1 and its cofactor Sel1L (Hrd3p in yeast) represent the most highly conserved branch of the mammalian ERAD (Travers et al., 2000; Yoshida et al., 2003; Kaneko et al., 2007; Christianson et al., 2012). The Sel1L-Hrd1 complex is responsible for the recognition and retrotranslocation of a subset of misfolded proteins in the ER directed for cytosolic proteasomal degradation (Ozmann et al., 2013; Christianson and Ye, 2014). The specific function of Sel1L-Hrd1 ERAD has been difficult to assess due to the embryonic lethality of Sel1L- or Hrd1-deficient mice (Yagishita et al., 2005; Francisco et al., 2010). Defining the role of the ERAD machinery in specific cell types could lead to the identification of molecular pathways underpinning ERAD-associated physiology and disease.

Conditional knockout mouse and cell models deficient in Sel1L provide in vivo evidence that Sel1L is an indispensable component of mammalian E3 ligase Hrd1 ERAD complex (Sun et al., 2014). In adipocytes, Sel1L is required for diet-induced obesity and the development of postprandial hypertriglyceridemia by regulating the ER exit of lipoprotein lipase (Sha et al., 2014). In a recent study, we identified IRE1α, the sensor of unfolded protein response (UPR), as an endogenous Sel1L-Hrd1 ERAD substrate (Sun et al., 2015). Sel1L-Hrd1 ERAD degrades IRE1α under basal conditions in a Bip-dependent manner. ER stress triggers the dissociation of IRE1α from the ERAD complex, leading to IRE1α accumulation and activation.

To dissect the physiological significance of Sel1L and Sel1L-Hrd1 ERAD-mediated IRE1α degradation, we generated intestinal epithelial cell-specific Sel1L-deficient (Sel1LΔIEC) mice (Sun et al., 2015). Surprisingly, epithelial Sel1L is dispensable for the overall morphology of the colon under basal conditions; however, Sel1L deficiency increases sensitivity to DSS-induced experimental colitis. This is in part mediated through stabilization and accumulation of IRE1α protein in colonic epithelium (Sun et al., 2015). How Sel1L and Sel1L-Hrd1 ERAD affect the function of the small intestine remains to be demonstrated. Here we show that expression of epithelial Sel1L and Hrd1 is inversely correlated with the severity of ileal inflammation in individuals with CD. Further mechanistic studies in mice provide definitive evidence for a critical role of epithelial Sel1L-Hrd1 ERAD in spontaneous enteritis and hypersensitivity to pathogens.

RESULTS

Expression of SEL1L and HRD1 in CD

To explore a possible role of SEL1L-HRD1 ERAD in CD, we determined the gene expression pattern of SEL1L and HRD1 in ileal tissues from CD patients. Intriguingly, expression of SEL1L was tightly correlated with that of HRD1 (Figure 1A) and was reduced by nearly 10-fold with inflammation (Figure 1B, Supplemental Figure S1A, and Supplemental Table S1). Similar observations were made for HRD1, albeit to a lesser extent (Figure 1B and Supplemental Figure S1A). Expression of SEL1L and HRD1 was also positively correlated with the expression of UPR effectors and ER chaperones, such as XBP1s, HSP5A, and CALRETICULIN (Supplemental Figure S1, B and C). These results demonstrate down-regulation of SEL1L-HRD1 in the inflamed small intestine of human patients with IBD, pointing to a possible role of ERAD in disease pathogenesis.

An epithelia-specific mouse model defective in Sel1L-Hrd1 ERAD

To test directly the pathophysiological role of Sel1L-Hrd1 ERAD in intestinal inflammation, we generated intestinal epithelial cell (IEC)-specific, Sel1L-deficient mice (Sel1LΔIEC) by crossing Sel1Lflox/fox mice (Sun et al., 2014) with Villin-Cre transgenic mice. Reduction of Sel1L and Hrd1 protein was observed in the small intestine of Sel1LΔIEC mice (Figure 2, A–C). Indeed, these mice were defective in ERAD function, as demonstrated by the accumulation of the known substrate OS9 in IEC (Sha et al., 2014; Sun et al., 2014; Figure 2, A and B). Thus Sel1LΔIEC mice are defective in Sel1L-Hrd1 ERAD function in the small intestine.

Epithelial Sel1L deficiency leads to spontaneous enteritis

Of interest, the small intestine villi of Sel1LΔIEC mice were blunted, and the lamina propria at the tip of the villi contained large clusters of plasma cells and many eosinophils (Figure 3, A and B), similar to those seen in lymphoplasmacytic (Ochoa et al., 1984) and eosinophilic enteritis (Baig et al., 2006) in humans (Odze, 2003). Immunofluorescence staining identified immunoglobulin G (IgG)-positive B-cells at the tip of intestinal villi of Sel1LΔIEC mice, whereas in wild-type (WT) mice, these cells were present at the base of the villi (Figure 3C). In line with elevated inflammation, the intestinal crypts in the duodenum of Sel1LΔIEC mice were longer and contained an expanded population of epithelial cells expressing Ki67 and...
the base of crypts (Figure 4, A and B). Paneth cells comprise a specialized cell population in the small intestine that secretes antimicrobial peptides from secretory granules. Sel1L deficiency dramatically reduced eosinophilic secretory granules in Paneth cells (Figure 5A), resembling variable Paneth cell abnormalities in human CD patients involving the terminal ileum (Liu et al., 2014; VanDussen et al., 2014). Protein levels of the antimicrobial peptides lysozyme C and RegIIIγ were greatly reduced (Figure 5, B–D). Indeed, expression of a panel of antibacterial peptides, including lysozyme and RegIIIγ, was reduced in the small intestine of Sel1LΔIEC mice (Figure 5E).

Transmission electron microscopy (TEM) of the ileum revealed dilated and fragmented ER cisternae, as well as “fried egg”–shaped secretory vesicles with centrally located granules in Paneth cells of Sel1LΔIEC mice (Figure 5F). Thus Sel1L-Hrd1 ERAD is required for the secretory function of Paneth cells.

Epithelial Sel1L deficiency alters gut microbiota composition

Studies have shown that the secretory function of Paneth cells influences intestinal bacterial overgrowth and gut microbiota composition (Ayabe et al., 2000; Vaishnava et al., 2008). To delineate how epithelial Sel1L deficiency affects gut microbiota composition, we performed microbiota sequencing of fecal samples from Sel1LΔIEC and cohoused WT littermates using culture-independent PCR amplification of variable region 4 (V4) of bacterial 16S rRNA genes (Ji et al., 2014). The multiplexed amplicons were subjected to Illumina

Epithelial Sel1L is required for resistance to pathogen infection

To determine whether Sel1L-Hrd1 ERAD deficiency influences intestinal response to pathogen infection, we infected mice with Toxoplasma gondii, which causes a granulomatous Th1-dominated ileitis and proliferation of Escherichia coli that recapitulates many of the features of CD ileitis (Egan et al., 2012). Both male and female Sel1LΔIEC mice exhibited increased disease susceptibility and lethality to T. gondii infection (Figure 3F). Histological analyses revealed more severe ileitis in Sel1LΔIEC mice, with generalized villous blunting, Paneth cell loss, and crypt hyperplasia associated with massive inflammatory cell infiltration (Figure 3G). Fluorescence in situ hybridization (FISH) analysis revealed increased adherence and invasion of eubacteria and E. coli in the ileal mucosa of Sel1LΔIEC mice compared with WT littermates (Supplemental Figure S2, A and B). Thus epithelial Sel1L-Hrd1 ERAD is required for protection against pathogen infection in the small intestine.

Epithelial Sel1L is required for Paneth cell function

In the small intestine, Sel1L was ubiquitously expressed in the epithelium and highly enriched in lysozyme C–positive Paneth cells at the base of crypts (Figure 4, A and B). Paneth cells comprise a specialized cell population in the small intestine that secretes antimicrobial peptides from secretory granules. Sel1L deficiency dramatically reduced eosinophilic secretory granules in Paneth cells in CD patients involving the terminal ileum (Liu et al., 2014; VanDussen et al., 2014). Protein levels of the antimicrobial peptides lysozyme C and RegIIIγ were greatly reduced (Figure 5, B–D). Indeed, expression of a panel of antibacterial peptides, including lysozyme and RegIIIγ, was reduced in the small intestine of Sel1LΔIEC mice (Figure 5E). Thus Sel1L-Hrd1 ERAD is required for protection against pathogen infection in the small intestine.

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in-depth analysis revealed a 10-fold increase of *Ruminococcus gnavus*, a Gram-positive anaerobic bacterium (Dethlefsen et al., 2006), in Sel1LΔIEC mice compared with cohoused WT littermates (Figure 5, G and H). Because *R. gnavus* is not overrepresented in other mouse strains bred in our colony (Ji et al., 2014), this expansion appears to be directly linked to the absence of epithelial Sel1L. Thus epithelial Sel1L deficiency influences microbiota composition.

**Epithelial Sel1L deficiency induces ER stress and cell death in small intestine**

In keeping with our previous report that IRE1α is an ERAD substrate (Sun et al., 2015), IRE1α protein level elevated by >20-fold in the small intestine of Sel1LΔIEC mice, whereas its mRNA level was not affected (Figure 6, A, B, and D). IRE1α was moderately activated, as measured by mobility shift of IRE1α protein in a Phos-tag gel (Sha et al., 2009; Yang et al., 2010; Qi et al., 2011) and its substrate Xbp1 mRNA splicing (Figure 6, A and E). IRE1α activation was in line with the activation of other UPR markers, including PERK and eIF2α phosphorylation, as well as elevated expression of various downstream effectors and ER chaperones (Figure 6, A–D). Furthermore, epithelial cell death in the small intestine was increased in Sel1LΔIEC mice under...
basal conditions, as demonstrated by caspase 3 cleavage (Figure 6F). Histological analyses revealed clustered cell death in both villi and the crypts of small intestine of Sel1LΔIEC mice but only at the tips of the villi in WT mice (Figure 6G). Thus epithelial Sel1L regulates ER homeostasis and cell survival in the small intestine.

**Lack of epithelial Sel1L haploinsufficiency**

To address whether Sel1L heterozygosity affects intestinal homeostasis, we generated and characterized Sel1LΔIEC/ΔIEC mice (HET) mice. Unlike Sel1LΔIEC littermates, Sel1LΔIEC/ΔIEC mice did not develop enteritis (Supplemental Figure S3A) or Paneth cell atrophy in the small intestine (Supplemental Figure S3, B–E). ER homeostasis in the epithelium of small intestines was not affected by Sel1L heterozygosity (Supplemental Figure S3B). This result suggests that one copy of Sel1L is sufficient to maintain Paneth cell function and homeostasis in the small intestine.

**IRE1α is dispensable for intestinal pathologies of Sel1LΔIEC mice**

UPR sensor IRE1α may link ER stress to inflammation and cell death (Hetz, 2012). IRE1α accumulated and was wildly activated in the small intestine of Sel1LΔIEC mice (Figure 6). To determine whether IRE1α activation is responsible, at least in part, for the intestinal abnormalities in Sel1LΔIEC mice, we generated and characterized epithelium-specific Sel1LΔIEC;Ire1aΔIEC mice (Figure 7, A and B). Both IRE1α protein and Xbp1s mRNA were abolished in the double-knockout mice (Figure 7, A and B). Surprisingly, loss of IRE1α failed to alter ERAD deficiency-induced enteritis (Figure 7C), Paneth cell atrophy (Figure 7, D–F), or cell death (Figure 7, G and H) in the small intestines of Sel1LΔIEC mice. Thus Sel1L deficiency-associated abnormalities of the small intestines are not mediated by IRE1α.

**C/EBP homologous protein is dispensable for intestinal pathologies of Sel1LΔIEC mice**

C/EBP homologous protein (CHOP), a downstream effector of the PERK pathway of the UPR, may link ER stress to cell death (Hetz, 2012). Chop mRNA was upregulated by fourfold in the small intestine of Sel1LΔIEC mice (Figure 6D). To determine whether Chop is responsible, at least in part, for the intestinal pathologies of Sel1LΔIEC mice, we generated and characterized Sel1LΔIEC mice on the Chop−/− background. Of interest, CHOP was not responsible for ERAD deficiency-induced enteritis (Supplemental Figure S4A), Paneth cell atrophy (Supplemental Figure S4, B and C), or cell death (Supplemental Figure S4, D and E). In aggregate, we conclude that Sel1L deficiency–associated abnormalities of the small intestine are not mediated by IRE1α and CHOP.

**DISCUSSION**

The physiological importance of ERAD complexes in vivo is largely unknown. We generated the first enterocyte-specific, Sel1L-Hrd1 ERAD–deficient mouse model, allowing the investigation of a new pathway in the pathogenesis of IBD. Of interest, Sel1LΔIEC mice develop spontaneous inflammation in the small intestine (as discussed here) but have few abnormalities in the colon (Sun et al., 2015). Differences in the regional distribution of inflammation between colon and ileum are frequently observed in human IBD, with CD frequently restricted to the small intestine and UC restricted to the colon. The decreased SEL1L and HRD1 expression in the ileal mucosa of patients with CD suggests a potentially causal effect of SEL1L-HRD1 deficiency–associated abnormalities. Whether R. gnavus is mucolytic (i.e., degrades mucin; Hoskins and Boulding, 1981; Hoskins et al., 1985) and is particularly overrepresented in human patients (Prindiville et al., 2004; Png et al., 2010; Willing et al., 2010; Joossens et al., 2011) is unclear. Whether R. gnavus is responsible for the pathology of Sel1LΔIEC remains to be established using monoassociation studies.

Different cell types have distinct requirements for protein folding and disposal and hence for Sel1L-Hrd1 ERAD. Comparative analysis of the cellular response to ERAD deficiency in different cell types may provide important insights into tissue- or organ-specific functions of ERAD. Paneth and pancreatic acinar cells have a strict requirement of Sel1L-Hrd1 ERAD (Sun et al., 2014; present results), whereas Sel1L-Hrd1 ERAD seems dispensable for the normal physiology of adipocytes and intestinal goblet cells (Sha et al., 2014; Sun et al., 2014, 2015). The differential requirement of ERAD is likely due to the diversity and amount of the proteins synthesized in specific...
cells. Cell type–specific ERAD function and endogenous ERAD substrates are open areas for further investigation.

Disturbance of ER homeostasis has been implicated in the pathogenesis of human CD and UC (Heazlewood et al., 2008; Kaser et al., 2008, 2013; Adolph et al., 2013; Das et al., 2013). In addition to ERAD, ER homeostasis is monitored by another highly conserved protein quality-control system known as UPR. In the last several years, the role of UPR in gut epithelium in the context of IBD has been increasingly appreciated, with the characterization of enterocyte-specific UPR-deficient mice such as Xbp1ΔEC, Ire1αΔEC, and CHOP−/− mice (Bertolotti et al., 2001; Kaser et al., 2008, 2013; Namba et al., 2009; Park et al., 2009; Zhao et al., 2010; Adolph et al., 2013; Niederreiter et al., 2013; Tsuru et al., 2013). Xbp1 mRNA is spliced by Ire1α in response to ER stress, leading to the generation of a stabilized transcription factor XBP1s to regulate ER homeostasis in the ER (Cadwell et al., 2010; Adolph et al., 2008; Kaser et al., 2009; Park et al., 2009; Zhao et al., 2010; Adolph et al., 2013; Niederreiter et al., 2013; Tsuru et al., 2013). Thus the physiological effect of Sel1L-Hrd1 ERAD in the small intestine is likely to be mediated by other UPR branches or UPR-independent mechanisms. Future investigations are warranted to identify other endogenous ERAD substrates or associated signaling pathways to elucidate their importance in the pathogenesis of IBD.

In addition to ERAD and UPR, autophagy has been implicated as an important regulator of Paneth cell biology and protein homeostasis in the ER (Cadwell et al., 2008; Adolph et al., 2013). The interplay among ERAD, UPR, and autophagy, however, has not been tested in vivo. The generation and characterization of compound mouse models will be critical to delineate how they function cooperatively in a cell type–specific manner in vivo.

MATERIALS AND METHODS

Mice

Cell type–specific Sel1LΔEC, Sel1LΔECΔΔIEC, Sel1LΔIEC, Chop−/−, and Sel1LΔIEC/ΔIEC mice were used in this study. Sel1Lflox/flox mice on the C57BL/6J background have been described (Sun et al., 2014, this study) and large intestines (Sun et al., 2014, 2015). In the colon, Ire1α heterozygosity partially rescues the DSS sensitivity of the Sel1LΔIEC mice, whereas CHOP was dispensable (Sun et al., 2015). By contrast, in the present study, neither Ire1α nor CHOP was involved in the pathogenesis of the small intestinal abnormalities observed in Sel1LΔIEC mice. Moreover, Sel1LΔIEC mice exhibit unique phenotypes, including spontaneous plasmacytic and eosinophilic enteritis and a disproportionate increase of mucolytic and potentially pathogenic R. gnavus compared with previous UPR models (Bertolotti et al., 2001; Kaser et al., 2008; Park et al., 2009; Zhao et al., 2010; Adolph et al., 2013; Niederreiter et al., 2013; Tsuru et al., 2013). In our studies, Sel1L-Hrd1 ERAD deficiency causes dramatic accumulation and activation of both Ire1α and CHOP in the epithelia of both small (this study) and large intestines (Sun et al., 2014). In the colon, Ire1α
and were crossed with villin 1 promoter–driven Cre mice (B6. SJL-Tg(Vil-Cre)997Gum/J, JAX 004586, Bar Harbor, ME), which have been backcrossed to the C57BL/6J background for more than five generations. Mice were housed under specific pathogen-free conditions and fed on a low-fat diet consisting of 13% fat, 67% carbohydrate, and 20% protein (Harlan Teklad 2914, Madison, WI). Cohoused age-matched adult littermates were used at the age of 8–12 wk in all in vivo experiments. Killing of animals was performed by cervical dislocation. Intestinal tissues were immediately harvested and either fixed in 10% neutralized Formalin for histology or flushed with phosphate-buffered saline (PBS) and snap-frozen in liquid nitrogen for protein and RNA analyses. Frozen tissues were stored at −80°C. All animal procedures were approved by the Institutional Animal Care and Use Committee at Cornell University.

Histological analysis

Tissues were fixed in 10% neutralized Formalin and processed by the Cornell Histology Core Facility as described (Sun et al., 2015). Sections of proximal small intestine were scored for the presence and distribution of lymphocytes, plasma cells, and eosinophils within the lamina propria of intestinal villi and crypts in thirty 400× fields (0, none; 1, rare; 2, few scattered; 3, many groups; 4, large numbers] blinded to treatment group (Garner et al., 2009). Crypt and villus length in the proximal small intestine was measured for 30 villous:crypt (V:C) units.

T. gondii infection

Pathogen infection was performed as previously described (Cohen and Denkers, 2015). Briefly, T. gondii cysts of the type II ME49 strain were passaged in vivo through sublethal infection of female Swiss Webster mice (6–8 wk of age, JAX). Cysts were harvested from chronically infected Swiss Webster mice by whole-brain homogenization in sterile PBS. For infection of SeI/1L^noc mice, 30 cysts of T. gondii were administered by oral gavage in 200 μl of PBS and monitored daily. Mice were killed on day 7, and intestines were fixed in Formalin for histology. For survival curve, mice were followed until the time indicated in the graph and killed.

FISH

Formalin-fixed, paraaffin-embedded intestine sections were mounted on Probe-On Plus slides (Fisher, Pittsburgh, PA) and evaluated by FISH with probes to all bacteria (EUB338-5′Cy3) or E. coli/ Shigella (E. coli-5′Cy3, 16S rRNA), in combination with a nonspecific binding control probe (non-EUB338-5′FAM; IDT, Coralville, IA), as previously described (Simpson et al., 2006; Baumgart et al., 2007). Sections were examined with an Olympus BX51 epifluorescence microscope, and images were captured with an Olympus DP-7 camera.

Microbiota sequencing and analysis

Fecal samples were collected from 8-wk-old mice and analyzed as previously described (Ji et al., 2014). To identify bacteria that were significantly altered, p values were calculated by unpaired two-tailed Student’s t-test and corrected by the Benjamini–Hochberg procedure. p < 0.05 was considered statistically significant.

Human IBD samples, RNA extraction, and quantitative PCR analysis

Terminal ileum tissue biopsies (n = 57) were obtained at endoscopy from 11 UC patients (7 noninflamed and 4 inflamed mucosa), 20 CD patients (11 noninflamed and 9 inflamed mucosa), and 7 miscellaneous and 19 healthy individuals (control group) as previously described (Sheng et al., 2011). Inflammation was scored by an in-house scheme as shown in Supplemental Table S1. Biopsies were considered “inflamed” with inflammation scores ≥3. All patients gave informed consent for the procedure. The study was approved by the Mater Health Services Health and Research Ethics Committee. Total RNA was extracted by RNasey Plus Mini Kit (Qiagen, Valencia, CA), and all RNA samples had RNA integrity number or RNA quality indicator value of >3. Reverse-transcription (RT) quantitative PCR (qPCR) analysis was performed with Bio-Rad Viia 7 (Life Technologies, Carlsbad, CA) using a SensiFASTTM SYBR Lo-Rox kit (Bioline, Meridian Life). Primer sequences are listed in Supplemental Table S2. The qPCR data for human tissues were normalized to β-actin (ACTB) in the corresponding sample. Fold changes were calculated by the ΔΔ method and normalized to the control group.

Statistical analysis

Results are expressed as mean ± SEM. Comparisons between groups were made by unpaired two-tailed Student’s t-test unless otherwise indicated. Survival curves were compared by the log-rank (Mantel–Cox) test. p < 0.05 was considered statistically significant. All experiments were repeated at least two to three times, and representative data are shown. For human studies, the Mann–Whitney test was used for comparisons to generate p values, and Spearman’s r values were calculated for correlations.

ACKNOWLEDGMENTS

We thank Lora Hooper (UT Southwestern, Dallas, TX) for generous gifts of antibodies and other members of the Qi lab for comments, suggestions, and technical assistance. This work was supported by National Institutes of Health Grants R21AI085332 (G.D.) and R21AI09061 (E.Y.K.), Chinese National Natural Science Foundation Grant 31371391 (to Q.L.), National Institutes of Health Grants 1R01GM113188 and 1R01DK105393, Juvenile Diabetes Research Foundation Grant 47-2012-767, and American Diabetes Association Grant 1-12-CD-04 (L.Q.). S.S. is an International Student Research Fellow of the Howard Hughes Medical Institute (59107338). R.L. is the recipient of a Betty McGrath Mater Practitioner Research Fellowship. L.Q. is the recipient of Junior Faculty and Career Development Awards from the American Diabetes Association.

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