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ER stress transcription factor Xbp1 suppresses intestinal tumorigenesis and directs intestinal stem cells

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Unresolved endoplasmic reticulum (ER) stress in the epithelium can provoke intestinal inflammation. Hypomorphic variants of ER stress response mediators, such as X-box-binding protein 1 (XBP1), confer genetic risk for inflammatory bowel disease. We report here that hypomorphic Xbp1 function instructs a multilayered regenerative response in the intestinal epithelium. This is characterized by intestinal stem cell (ISC) expansion as shown by an inositol-requiring enzyme 1α (Ire1α)–mediated increase in Lgr5+ and Olfm4+ ISCs and a Stat3-dependent increase in the proliferative output of transit–amplifying cells. These consequences of hypomorphic Xbp1 function are associated with an increased propensity to develop colitis–associated and spontaneous adenomatous polyposis coli (APC)–related tumors of the intestinal epithelium, which in the latter case is shown to be dependent on Ire1α. This study reveals an unexpected role for Xbp1 in suppressing tumor formation through restraint of a pathway that involves an Ire1α– and Stat3–mediated regenerative response of the epithelium as a consequence of ER stress. As such, Xbp1 in the intestinal epithelium not only regulates local inflammation but at the same time also determines the propensity of the epithelium to develop tumors.

Colorectal cancer (CRC) is the second most prevalent cause of death from cancer in the Western world (Lieberman, 2009). One third of the 147,000 patients diagnosed with CRC every year in the United States will succumb to the disease (Lieberman, 2009). Significant progress has been made in revealing somatic genetic alterations in CRC, ranging from the now classical adenoma–carcinoma sequence to insights into genomic instability and patterns of accumulation of somatic mutations at distinct genes and loci (Kinzler and Vogelstein, 1996; Cancer
CRC arises from the intestinal epithelium, a highly proliferative tissue which renews itself every several days under steady-state conditions. Upon intestinal epithelial injury and cell loss, such homeostatic renewal is supplanted by a regenerative response, which also represents an important defense strategy of the host (Amcheslavsky et al., 2009; Cronin et al., 2009; Jiang et al., 2009). However, an unabated regenerative, wound healing-like response to tissue injury may lay the ground for intestinal tumors (Kuraishy et al., 2011). This is clinically evident by the common occurrence of tumors at sites of chronic injury (e.g., colitis-associated cancer [CAC] in inflammatory bowel disease [IBD] or gastric cancer upon Helicobacter pylori infection). Hence, the remarkable paradox emerges that cell death caused by tissue injury augments the tumorigenic potential of adjoining cells (Kuraishy et al., 2011). Inflammatory signals from the chronically inflamed intestinal microenvironment, such as IL-6 and IL-11 among others, have been identified to contribute to such tumor-promoting activities in the injured intestine (Becker et al., 2004; Bollath et al., 2009; Grivennikov et al., 2009).

In mammals, the intestinal epithelial compartment consists of several subtypes of differentiated cells (absorptive, Paneth, goblet, and endoendocrine cells) that arise from rapidly cycling Lgr5+Olfm4+ intestinal stem cells (ISCs) at the crypt bottom (Barker et al., 2007). This Lgr5+ stem cell population also gives continuous rise to a quiescent label-retaining population, located at the +4 position and expressing Lgr5, that is committed to mature into Paneth and endoendocrine cells, but which can alternatively be recalled to the stem cell state within the crypt in instances of injury to the crypt (Sangiorgi and Capecchi, 2008; Montgomery et al., 2011; Takeda et al., 2011; Tian et al., 2011; Buczacki et al., 2013; Clevers, 2013). ISCs feed into transit-amplifying cells, which serve as the forerunners of the differentiated intestinal epithelial cell (IEC) types (Barker et al., 2007). Through a model of human sporadic and familial CRC, ISCs have been revealed as the cells of origin of intestinal cancer (Barker et al., 2009; Zhu et al., 2009; Schepers et al., 2012).

The unfolded protein response (UPR) is a cytoprotective response to ER stress that arises when misfolded proteins accumulate in this organelle (Schröder and Kaufman, 2005; Todd et al., 2008; Walter and Ron, 2011). In metazoans, three UPR-associated pathways coordinate an adaptive response to ER stress that results in expansion of the ER, promotion of ER–associated degradation and chaperone functions and, when unabated, cellular death by apoptosis. The evolutionarily most conserved UPR branch consists of inositol-requiring enzyme 1α (Ire1α; encoded by Emn1), an ER stress sensor, and the transcription factor X-box–binding protein 1 (Xbp1) as its effector (Schröder and Kaufman, 2005; Ron and Walter, 2007; Todd et al., 2008; Kohno, 2010; Walter and Ron, 2011). Ire1α activates Xbp1 by conversion of unspliced Xbp1 (Xbp1u) mRNA to spliced Xbp1 (Xbp1s) via its atypical endoribonuclease function, which removes 26nt within Xbp1u to generate an alternate reading frame (Xbp1s; Hetz et al., 2011; Walter and Ron, 2011). Unresolved ER stress in IECs has emerged as an important mechanism that initiates inflammation in the intestine (Kaser et al., 2008). Specifically, partial or complete Xbp1 deletion in mouse IECs leads to unresolved ER stress and consequently hypersensitivity of IECs to inflammatory and microbial signals, Paneth cell dysfunction with loss of their characteristic granules, increased epithelial apoptosis, spontaneous small intestinal enteritis, and increased susceptibility to colitis-inducing agents (Kaser et al., 2008). Fittingly, hypomorphic XBP1 variants confer genetic risk for both forms of IBD, Crohn’s disease and ulcerative colitis (Kaser et al., 2008). Additional genetic risk factors that impact the UPR have been discovered in IBD (e.g., ORMDL3 [McGovern et al., 2010] and AGR2 [Zheng et al., 2006]), and in some cases their genetic deletion in mice can lead to spontaneous IBD-like disease as well (Zhao et al., 2010). Notably, it appears that IECs in IBD generally experience unresolved ER stress, even in the absence of overt tissue-destructive inflammation (Heazlewood et al., 2008; Kaser et al., 2008; Tréton et al., 2011), with the effectiveness of the UPR being under the influence of primary (genetic) and secondary (environmental) factors (Kaser and Blumberg, 2011). Prompted by the increased turnover of IECs in mice that lack Xbp1 (Kaser et al., 2008), here we investigated the UPR’s role in epithelial regeneration and its implications for intestinal tumorigenesis.

## RESULTS

### Xbp1 deletion increases ISC numbers

The Xbp1−/− deficient small intestinal epithelium exhibits increased turnover (Kaser et al., 2008), which is similarly present in the colon (Fig. 1, A and B). A 2-h pulse of BrdU revealed expansion of the transit-amplifying zone in the ileum and colon, whereas a 24-h pulse demonstrated accelerated migration of IECs along the crypt–villus axis in IEC-conditional knockout Xbp1−/− mice compared with Xbp1−/+ littermates (Fig. 1, A–C). This corresponded with increased numbers of proliferating cell nuclear antigen (PCNA)+ cells along the crypt–villus axis in Xbp1−/− (IEC) mice compared with Xbp1−/+ (IEC) mice (Fig. 1, D and E). Moreover, deletion of Xbp1 resulted in a 57 ± 3% increase in Olfm4+ ISCs (Fig. 1, F and G). This correlated with an increased number of BrdU+ cells at the crypt base consistent with proliferating ISCs (Fig. 1 H). In situ hybridization (ISH) for Lgr5 indicated increased expression in Xbp1−/− (IEC) compared with Xbp1−/+ (IEC) both in the small intestine and colon (Fig. 1 I). This was also reflected by a trend toward increased Lgr5 mRNA expression in isolated crypts upon quantification by RT-PCR. (Fig. 1 J) and significantly increased expression of characteristic mRNAs that define the ISC signature (Fig. 1 J; Sato et al., 2011; Muñoz et al., 2012). Altogether, these data indicate an expansion of ISC numbers in Xbp1−/− (IEC) compared with Xbp1−/+ (IEC) mice. This increase in ISCs is interesting because Paneth cells, which contribute to the ISCs’ niche to a variable extent depending on the model system studied.
Figure 1. Xbp1 deletion increases ISC numbers. (A) Animals were injected with BrdU and sacrificed 24 h later. BrdU+ cells per total cells along the crypt–villus axis were counted (n = 3/4; two-tailed Student’s t test). (B) Anti-BrdU IHC of the ileum and colon 24 h after i.p. injection with BrdU (n = 3/4). (C) Similar experiment as A with a 2-h BrdU pulse to assess transit-amplifying cells (n = 4/4; two-tailed Student’s t test). (D) Anti-PCNA IHC of the small intestine (n = 5/5). (E) PCNA+ cells per total cells along the crypt–villus axis were counted (n = 5/5; two-tailed Student’s t test). (F) Olfm4+ ISCs (ISH; red arrowheads) in the small intestine of Xbp1+/+(IEC) and Xbp1−/−(IEC) mice. Lysozyme staining identified fully differentiated Paneth cells (black arrowheads) intermingled with ISCs (n = 3/4). (G) Quantification of Olfm4+ ISCs per 100 crypts (n = 3/4; two-tailed Student’s t test). (H) Sections of Xbp1+/+(IEC) and Xbp1−/−(IEC) mice from C were analyzed for BrdU+ cells at the crypt bottom up to position +4 (n = 4/4). (I) ISC identification by Lgr5 ISH in ileum and colon (Lgr5+ crypts marked by red arrowheads; n = 4/4). Bars: (B, D, and I) 20 μm; (F) 5 μm. (J and K) Analysis of isolated crypt mRNA of Xbp1+/+(IEC) and Xbp1−/−(IEC) mice for transcripts representative of the ISC signature (J; Muñoz et al., 2012) or Paneth cell signature (K; Sato et al., 2011) by RT-pPCR (n = 12/11; Student’s t test). Graphs show mean ± SEM. §, P = 0.0548; *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Hypomorphic Xbp1 leads to Jak1/Stat3 activation in IECs

In Drosophila melanogaster, injured enterocytes induce cytokines (Upd, Upd2, and Upd3) that activate Jak/Stat (dorso, hop, and STAT92E) signaling in intestinal epithelial stem cells, which promotes their division and initiates progenitor cell differentiation (Amcheslavsky et al., 2009; Jiang et al., 2009). In the mammalian system, this feedback mechanism that links enterocyte loss to stem cell output is less well understood. The mammalian orthologues of the Drosophila pathway (IL-6, IL-11, and Stat3) play an important role in IEC regeneration (Becker et al., 2004; Bollrath et al., 2009; Grivennikov et al., 2009; Pickert et al., 2009). Fig. 3 A shows substantial levels of Stat3 Tyr-705 phosphorylation (p) in the Xbp1+/− (IEC) epithelium, whereas only minimal p-Stat3 was observed in Xbp1+/+ (IEC) epithelium. Immunohistochemistry (IHC) localized p-Stat3 to the transit-amplifying zone with further extension into the villus epithelium, with barely any immunoreactivity demonstrable within the crypts (Fig. 3 B). Jak1 but not Jak2 phosphorylation was also induced in Xbp1+/− (IEC) compared with Xbp1+/+ (IEC) epithelium (Fig. 3 A). Total Stat3 as well as Jak1 and Jak2 was also increased (Fig. 3 A), which might reflect the known capacity of Stat3 to bind its own promoter and to transactivate its transcription (Narimatsu et al., 2001; Lam et al., 2008; Snyder et al., 2008). Increased p-Stat3 was independent of Ire1α as Xbp1+/− (IEC);Ern1+/− (IEC) and Xbp1+/− (IEC);Ern1+/− (IEC) epithelia exhibited similar levels (Fig. 3 C). These experiments provide evidence for Ire1α-independent Stat3 activation in Xbp1-deficient epithelia that localizes to the transit-amplifying zone and its downstream progeny.

Stat3 blockade abrogates epithelial hyperproliferation in Xbp1-deficient epithelium

S3I-201 selectively inhibits Stat3 transcriptional activities by blocking dimer formation and DNA binding ( Siddiquie et al., 2007). To test whether Stat3 is responsible for epithelial hyperproliferation in Xbp1-deficient IECs, we administered S3I-201 i.p. every other day for 14 d to Xbp1+/− (IEC) mice. Fig. 3 D demonstrates that Stat3 inhibition abrogated the increased turnover of the intestinal epithelium in Xbp1+/− (IEC) mice. Notably, S3I-201 did not affect ISC numbers in Xbp1+/− (IEC) mice (Fig. 3 E), which is consistent with an absence of p-Stat3 in crypts (Fig. 3 B). Of note, S3I-201 did not affect epithelial turnover in Xbp1-sufficient mice (not depicted), indicating that this Stat3-dependent mechanism is specifically engaged under conditions of ER stress. Altogether, these data establish that Xbp1 deficiency results in Stat3 activation in the transit-amplifying zone, which mediates the hyperproliferation of ER-stressed IECs.

Hypomorphic Xbp1 induces an autocrine activation loop in IECs via NF-κB, IL-6/IL-11, and Stat3

We chose the small IEC line MODE-K as a model system for studying the mechanisms underlying Stat3 activation and silenced Xbp1 expression via a lentivirus expressing a specific shRNA (Kaser et al., 2008). Stable Xbp1 silencing resulted in profound Stat3 Tyr-705 phosphorylation compared with...
Figure 2. ISC expansion is dependent on overactivation of Ire1α. (A) Immunoblot of total and phosphorylated Ire1α of indicated genotypes in total epithelial scrapings or after immunoprecipitation (IP) as indicated. Samples were immunoprecipitated with anti-Ire1α pAb H-190 (immunogen: amino acids 371–560) and immunoblotted with anti-Ire1α mAb 14C10 (immunogen: C-terminal fragment) or pAb H-190. Data are representative of four independent experiments. (B) Sections of the indicated genotypes were in situ hybridized for Olfm4 (red arrowheads), stained for lysozyme (black arrowheads), or both (n = 5 per group). Bars, 5 µm. (C) Quantification of Olfm4+ ISCs per 100 crypts (n = 5 per group with combined analysis of duodenum, jejunum, and ileum; one-way ANOVA with Bonferroni post-hoc test). (D) Quantification of Olfm4+ ISCs in Em1+/+(IEC), Em1−/−(IEC), and Em1−−(IEC) mice (n = 5 per group). (E) Western blot of Em1+/+(IEC) and Em1−−(IEC) (exons 20–21 floxed) mice immunoblotted with mAb 14C10 and pAb H-190 detects a truncated Ire1α protein in Em1−−(IEC) epithelial scrapings. (F) Xbp1 mRNA splicing in epithelial scrapings of the indicated genotypes. 171 bp, Xbp1u; 145 bp, Xbp1s. (E and F) Data are representative of two independent experiments. (G) Animals were injected with BrdU and harvested 24 h later to assess epithelial turnover. BrdU+ cells per total cells along the crypt–villus axis were counted (n = 4 per group with combined analysis of duodenum, jejunum, and ileum; one-way ANOVA with Bonferroni post-hoc test). Graphs show mean ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure 3. Hypomorphic Xbp1 leads to Jak1/Stat3 activation in the intestinal epithelium. (A) Immunoblot for p-Stat3, Stat3, p-Jak1, Jak1, p-Jak2, and Jak2 on epithelial colonic scrapings. Data are representative of more than four independent experiments. (B) IHC localizes p-Stat3 immunoreactivity to IECs in the transit-amplifying zone and villus, but largely spares the crypt bottom (n = 3/3). Boxed areas are shown at higher magnification on the right. Bars, 20 µm. (C) Small intestinal epithelial scrapings from the indicated genotypes were analyzed for p-Stat3, Stat3, p-Jnk, and Jnk. The experiment was performed with four mice per group. (D) Mice were treated with the Stat3 inhibitor S3I-201 or vehicle for 14 d, and BrdU was administered 24 h before harvest. The ratio of BrdU+ cells per total IECs along the crypt–villus axis in the small intestine is presented (n = 4/5/7; one-way ANOVA with Bonferroni post-hoc test). (E) Olfm4+ ISC counted in the same experiment as in D (n = 2/3/3; one-way ANOVA with Bonferroni post-hoc test). (F) MODE-K.iCtrl and MODE-K.iXbp1 cells were stimulated with the ER stress inducer tunicamycin and analyzed for Stat3 Tyr-705 phosphorylation. Data are representative of two independent experiments. (G) Il6 and Il11 mRNA expression in MODE-K.iXbp1 and MODE-K.iCtrl cells (n = 3; two-sided Student’s t test). Data are representative of two independent experiments. (H) Supernatants from MODE-K.iXbp1 and MODE-K.iCtrl cells were analyzed for IL-6 and IL-11 secretion by ELISA (n = 6; two-sided Student’s t test). (I) MODE-K.iXbp1 and MODE-K.iCtrl cells were incubated with anti–IL-11 and anti–IL-16 mAbs or respective
control-silenced cells (Fig. 3 F). Stat3 Tyr-705 phosphorylation was also induced in Xbp1-sufficient MODE-K cells after ER stress induction via tunicamycin (Fig. 3 F). The extent of Stat3 phosphorylation in Xbp1-silenced MODE-K cells was not further augmented by incubation with tunicamycin (Fig. 3 F) and, in fact, decreased at the latest time point studied, likely reflecting the exhaustion of the compensatory UPR (Fig. 3 F). These experiments establish that Stat3 activation in IECs is likely to be an IEC-intrinsic consequence of unresolved ER stress.

IL-6 and IL-11 are prototypical Stat3-activating cytokines (Yu et al., 2009). Xbp1 silencing induced mRNA expression and protein secretion of IL-6 and IL-11 in MODE-K cells (Fig. 3, G and H). Neutralization experiments with anti-cytokine antibodies revealed that combined administration of anti-IL-6 and anti-IL-11 mAbs abrogated increased Stat3 phosphorylation in MODE-K.iXbp1 cells (Fig. 3 I). Co-silencing of individual cytokine receptor–associated Janus tyrosine kinases (Jak1-3 and Tyk2; Yu et al., 2009) in MODE-K.iXbp1 cells revealed Jak1 as the critical kinase for Stat3 activation under ER stress conditions (Fig. 3 J). These data indicate that hypomorphic Xbp1 leads to induction of IL-6 and IL-11 secretion in IECs, which in turn activates Stat3 phosphorylation via a Jak1-dependent mechanism.

Il6 is typically transactivated by NF-κB (and, e.g., also by Stat3; Vallabhapurapu and Karin, 2009; Yu et al., 2009). ER stress can lead to NF-κB activation via several mechanisms (Vallabhapurapu and Karin, 2009). Xbp1 T−/− (IEC) IECs exhibited increased total NF-κB p65 and p-p65 compared with Xbp1 T+/+(IEC) mice (Fig. 3 K). Blocking NF-κB activation in Xbp1 T−/− (IEC) mice via the specific irreversible inhibitor of IkBα phosphorylation, BAY 11-7082 (Pierce et al., 1997), indeed abrogated Stat3 activation to baseline levels observed in Xbp1 T+/+(IEC) littermates (Fig. 3 L). Hence, hypomorphic Xbp1 function is associated with increased NF-κB–dependent activation of Stat3 in IECs.

Jnk inhibition in Xbp1 T−/− (IEC) mice does not affect ISC expansion or hyperproliferation

Hypomorphic Xbp1 function had previously been reported to result in activation of Jnk, and pharmacological blockade of Jnk phosphorylation with the specific inhibitor SP600125 resulted in abrogation of elevated CXCL1 secretion in Xbp1 T−/− IECs (Kaser et al., 2008). Biteau et al. (2008) reported that Jnk regulates proliferation and the regenerative capacity of somatic stem cells in the Drosophila gut. Moreover, Jnk can be recruited to IRE1 via the adapter molecule tumor necrosis factor receptor–associated factor 2 (Traf2) under certain conditions of ER stress (Urrano et al., 2000). To test the hypothesis that Jnk activation may contribute to the IRE1α-dependent increase in ISC numbers or has a role in determining the increased proliferative output of the transit-amplifying zone, we administered SP600125 i.p. every other day for 14 d to Xbp1 T−/− (IEC) mice. As depicted in Fig. 3 (M and N), SP600125 affected neither Olfm4+ ISC numbers nor IEC turnover along the crypt–villus axis. Furthermore, increased Jnk phosphorylation in IECs was indistinguishable between Xbp1 T−/− (IEC);Irm1 T−/− (IEC) and Xbp1 T−/− (IEC) mice and hence not under the control of IRE1α (Fig. 3 C). These observations let us conclude that Jnk does not have a critical role in the hyperregenerative phenotype observed in the ER-stressed, Xbp1 T−/−-deficient intestine.

Xbp1 protects from CAC

Long-standing IBD may lead to CAC, which has served as a paradigm for the relationship between inflammation and cancer (Grivennikov et al., 2010; Mantovani, 2010; Danese and Fiocchi, 2011). The mediators involved in regeneration of the ER-stressed, Xbp1 T−/−-deficient intestinal epithelium, such as Stat3, IL-6/IL-11, and NF-κB, also cooperate to drive CAC (Becker et al., 2004; Greten et al., 2004; Bollrath et al., 2009; Grivennikov et al., 2009; Grivennikov and Karin, 2010; Kuraishi et al., 2011). We therefore induced CAC by azoxymethane (AOM), followed by three cycles of dextran sodium sulfate (DSS; Fig. 4 A; Greten et al., 2004). Xbp1 T−/− (IEC) mice developed 10-fold more and larger colonic tumors compared with Xbp1 T+/+(IEC) littermate controls (Fig. 4, B–D). When analyzed at an early time point (day 15 after initiation), atypical regenerative lesions were only detected in Xbp1 T−/− (IEC), but not in Xbp1 T+/+(IEC) mice (Fig. 4 E). Similarly, reactive oxygen species (ROS) levels were increased fivefold in Xbp1 T−/− (IEC) relative to Xbp1 T+/+(IEC) epithelia at day 15 (Fig. 4 F), consistent with ROS generation in ER-stressed, Xbp1 T−/−-deficient cells (Liu et al., 2009) and increased severity of DSS colitis in Xbp1 T−/− (IEC) mice (Kaser et al., 2008). ROS can lead to DNA damage and thereby contribute to cancer initiation and progression (Sedelnikova et al., 2010). Indeed, at day 15, Xbp1 T−/− (IEC) epithelia exhibited nuclear staining for p53 (Fig. 4 E), evidence for a DNA damage response (Brady et al., 2011), together with a trend toward an increase in aneuploid cells compared...
with Xbp1+/+(IEC) IECs (Fig. 4 G). Moreover, Stat3 phosphorylation was increased in Xbp1+/−(IEC) compared with Xbp1+/+(IEC) mice (Fig. 4, H and I). These observations imply that epithelial Xbp1 deficiency is associated with a profound increase in tumorigenesis in CAC. Because IEC-specific Xbp1 deficiency is associated with increased severity of DSS-induced colitis (Kaser et al., 2008), increased tumorigenesis in Xbp1−/−(IEC) compared with Xbp1+/+(IEC) mice in the AOM/DSS model as observed here may result from a combination of inflammatory, tumor-promoting signals emanating from the more intense myeloid infiltrate (Greten et al., 2004; Bollrath et al., 2009; Grivennikov et al., 2009), as well as from IEC-intrinsic mechanisms.
**Figure 5. Epithelial Xbp1 suppresses tumor burden in Apc<sup>min</sup> mice.** (A) Representative macroscopic pictures of the colon with rectal tumors in the indicated genotypes analyzed at age 15 wk (n = 13/10). Boxed areas are shown at higher magnification on the right. (B) Representative H&E-stained sections, with tumors highlighted by dotted lines (n = 13/10). (C) Quantification of tumor numbers per mouse along the intestinal tract (n = 13/10; two-sided Student’s t test). (D) Peripheral blood count of the indicated genotypes at age 15 wk (n = 13/10; two-sided Student’s t test). (E) Ileal and colonic tumor counts in the indicated genotypes stratified by size of tumors (n = 13/10; two-sided Student’s t test). (F) Tumors from colon of Xbp1<sup>+/IEC</sup>;Apc<sup>min</sup> and Xbp1<sup>−/−IEC</sup>;Apc<sup>min</sup> mice were microdissected, and mRNA expression of the indicated targets was analyzed by qPCR (n = 5/5; two-tailed Student’s t test). (G) Olfm4+ ISCs (red arrowheads; IHC) and lysozyme<sup>+</sup> Paneth cells (black arrowheads; IHC) in the indicated genotypes (n = 4/4). Bars: (A) 5 mm; (B) 100 µm; (G) 5 µm. (H) Number of Olfm4<sup>+</sup> ISCs per 100 crypts in Xbp1<sup>−/−IEC</sup>;Apc<sup>min</sup> and Xbp1<sup>−/−IEC</sup>;Apc<sup>min</sup> small intestines. The occasional presence of crypts with lysozyme<sup>+</sup> mature Paneth cells among the vast majority of crypts with lysozyme<sup>−</sup> Paneth cell remnants in Xbp1<sup>−/−IEC</sup>;Apc<sup>min</sup> mice allowed crypt-specific stratification of Olfm4<sup>+</sup> cell enumeration in lysozyme<sup>+</sup> and lysozyme<sup>−</sup> crypts (n = 4/4; two-sided Student’s t test). Graphs show mean ± SEM. §, P = 0.0519; *, P < 0.05; **, P < 0.01; ***, P < 0.001.

**Epithelial Xbp1 suppresses tumor formation in Apc<sup>min</sup>s mice**

Inflammatory signaling is an important contributor to CAC but also to sporadic and familial CRC (Rakoff-Nahoum and Medzhitov, 2007; Lee et al., 2010), with overlapping and distinct inflammatory pathways being involved (Salcedo et al., 2010). ER stress in various organs, including the liver, skeletal muscle, adipose tissue (Ozcan et al., 2004; Gregor et al., 2009; Kars et al., 2010), and intestinal crypts (Hodin et al., 2011), is associated with obesity, which is epidemiologically closely associated with increased cancer incidence, most notably CRC (Calle et al., 2003). CRC and CAC exhibit distinguishing features with regard to their molecular genetic underpinning; e.g., inactivating somatic mutations in adenomatous polyposis coli (APC) are very common and appear early as a rate-limiting step in CRC pathogenesis (Kinzler and Vogelstein, 1996; Fearon, 2011; Cancer Genome Atlas Network, 2012) but late in CAC (Leedham et al., 2009). Germline mutations in APC also cause familial adenomatous polyposis. APC-associated CRC can be modeled in Apc mutant mice (Moser et al., 1990). Such a model has enabled the determination that ISCs are the cells of origin in CRC (Barker et al., 2009; Zhu et al., 2009; Schepers et al., 2012). The expansion of ISCs in Xbp1<sup>−/−IEC</sup> mice, and the desire to delineate the specific tumor-promoting role of Xbp1 deficiency specifically in IECs, prompted us to...
investigate whether unresolved ER stress augments tumorigenesis in this CRC model.

Xhp1−/−(IEC) mice crossed onto an Apeomin background (Xhp1−/−(IEC);Apeom) developed twofold more tumors than their Xhp1+/−(IEC);Apeom littermates (Fig. 5, A–C), along with lower blood hemoglobin levels reflecting intestinal blood loss (Fig. 5 D). Increased tumor numbers in Xhp1−/−(IEC);Apeom mice were detected in the small intestine, but also the colon, where only few tumors typically develop in the Apeomin model (Fig. 5, C and E). Colonic tumors are notable because Xhp1 deletion does not induce colonic, but only small intestinal inflammation (Kaser et al., 2008), suggesting that ER stress–induced tumor promotion is independent of overt inflammation in the colon. Nonetheless, colonic tumors from Xhp1−/−(IEC);Apeom mice exhibited increased Il6 and Il11 mRNA expression compared with those from Xhp1+/−(IEC);Apeom littermate controls (Fig. 5 F). Tumors in Xhp1-deficient mice exhibited Xhp1 deletion, excluding outgrowth from Xhp1-sufficient IECs (not depicted). Together, these experiments demonstrate that Xhp1 suppresses the growth of spontaneously arising intestinal tumors.

**ISC expansion is individually determined in each Xhp1-deficient crypt**

The Xhp1−/−(IEC);Apeom model serendipitously also afforded us the opportunity to study whether regulation of ISC numbers by Xhp1 was autonomously regulated within individual crypts or, alternatively, was a consequence of the inflammatory milieu of the inflamed small intestinal mucosa. Although lysozyme staining was absent in the vast majority of Paneth cells (i.e., Paneth cell remnants) in Xhp1−/−(IEC) epithelia (Fig. 1 F; Kaser et al., 2008), the occasional (∼5%) occurrence of crypts with intact lysozyme+ Paneth cells in Xhp1−/−(IEC);Apeom epithelia (which is presumably caused by inefficient Cre-mediated Xhp1 deletion and hence mosaicism or, alternatively, caused by effective adaptation to ER stress despite Xhp1 deletion) allowed us to quantify Ollm4+ ISC populations separately in crypts without (i.e., Paneth cell remnants) and with (i.e., normal, mature Paneth cells) lysozyme expression. Within the Xhp1−/−(IEC);Apeom genotype, ISC expansion was solely noted in crypts with Paneth cell remnants, whereas crypts with intact lysozyme+ Paneth cells (in immediate vicinity to those without) exhibited ISC numbers indistinguishable from those found in the Xhp1+/−(IEC);Apeom genotype (Fig. 5, G and H). These data suggest that expansion of ISCs is not merely a result of the surrounding proinflammatory milieu, which would similarly affect the occasional crypts with intact lysozyme+ Paneth cells, but an IEC-inherent, Xhp1- and ER stress–dependent, crypt-autonomous process.

**Tumor formation in Xhp1−/−(IEC);Apeom mice is dependent on Ire1α**

Analogous to its role in ISC expansion under ER stress, we hypothesized that tumor formation in Xhp1−/−(IEC);Apeom mice would be dependent on Ire1α. Hence, Ern1−/−(IEC);Xhp1−/−(IEC);Apeom mice would be predicted to be indistinguishable in tumor formation from Apeom mice. We generated mice that harbored floxed alleles for both Xhp1 and Ern1 that were also hemizygous for Apeom and compared littermates that expressed one allele of the Villin-Cre transgene with those that did not. We observed that deletion of Ern1 in the intestinal epithelium eliminated the increased tumorigenesis caused by Xhp1 deficiency in Apeom mice. Specifically, as depicted in Fig. 6 (A–D), tumor numbers in Ern1−/−(IEC);Xhp1−/−(IEC);Apeom mice were indeed no longer significantly different from Ern1+/−(IEC);Xhp1−/−(IEC);Apeom mice, and even tended to be lower. These data indicate that increased tumor formation in Apeom mice consequent to Xhp1 deletion is dependent on ER stress sensed by Ire1α and may, by inference, be related to the Ire1α-dependent activation of ISCs.

**DISCUSSION**

We report here that epithelial injury resulting from ER stress is a potent activator of IEC regeneration and promotes intestinal tumorigenesis. Hypomorphic Xhp1 causes ER stress that is sensed by Ire1α and increases ISC numbers, whereas Stat3 signaling, independent of Ire1α, increases the output of differentiated IECs. This organ-protective function, when
sustained, augments tumor formation in both an inflammatory (i.e., CAC after DSS/AOM) and noninflammatory (i.e., CRC in Ape<sup>min</sup> mice) context. Cytokines and other factors originating from the more prominent inflammatory infiltrate in Xbp<sup>1<sup>-/-</sup>(IEC)</sup> mice exposed to DSS (Kaser et al., 2008) will likely contribute to increased tumorigenesis in the AOM/DSS CAC model (Kuraishi et al., 2011) through effects on the Ire1α-dependent and -independent pathways described here. However, it is clear that the hyperregenerative response observed in the setting of hypomorphic epithelial Xbp1 function is not only a consequence of increased inflammation as increases in colonic tumor formation were observed in Ape<sup>min</sup>;Xbp1<sup>-/-</sup>(IEC) mice, which is not associated with appreciable inflammation in this locale when Xbp1 is deficient (Kaser et al., 2008). Moreover, increased tumor formation in Xbp1-deficient Ape<sup>min</sup> mice is dependent on Ire1α, linking ER stress sensing to increased intestinal tumorigenesis. This relationship has important ramifications as the UPR integrates microbial, environmental, and inflammatory signals at the mucosal surface and highlights an unexpected tumor-suppressive role of Xbp1 that is associated with the intestinal epithelium.

The UPR transcription factor Xbp1 is critical for IEC homeostasis in that its reduced function causes pathological ER stress and spontaneous enteritis in the small intestine (Kaser et al., 2008). As shown here, sensing of unresolved ER stress by Ire1α and its consequent overactivation is centrally related to increased ISC numbers observed in the Xbp1-deficient epithelium. Moreover, Ire1α does not notably affect homeostatic ISC function, but is only engaged as part of the regenerative response consequent to pathological ER stress. It might be speculated that this could involve regulated Ire1-dependent decay (RIDD) of mRNAs of as yet poorly defined proteins involved in restraining the ISC niche (e.g., Wnt or growth factor receptor antagonists or regulatory microRNAs [Upton et al., 2012]). RIDD is engaged upon pathological ER stress and results in endonucleolytic degradation of ER-localized mRNAs (Han et al., 2009; Hollien et al., 2009).

Paneth cells secrete factors that support the proliferation of intermingled ISCs (Sato et al., 2011). Paneth cells are particularly susceptible to impairment in the UPR as Xbp1 deletion in IECs results in loss of their characteristic secretory granules (resulting in Paneth cell remnants); however, Paneth cell remnants remain juxtaposed to ISCs (Kaser et al., 2008). The direct relationship between ISC expansion and the presence of Paneth cell remnants within discrete crypts observed in the Xbp1<sup>-/-</sup>(IEC);Ape<sup>min</sup> genotype (Fig. 5 H) also implies that ISC expansion may be autonomously regulated within individual crypts. Xbp1 splicing (i.e., active Xbp1s) localizes to Paneth cells, transit-amplifying cells, and cells further up the crypt–villus axis, whereas ISCs do not express appreciable levels of Xbp1s (Heijmans et al., 2013; Schwitalla et al., 2013), raising the possibility that ER stress in Paneth cell remnants may directly promote ISC expansion (Sato et al., 2011). Increased expression of the Paneth cell–specific (Sato et al., 2011) transcript Wnt11 in Xbp1<sup>-/-</sup>(IEC) compared with Xbp1<sup>+/+</sup>(IEC) crypts may have a role as a trans-acting factor and deserves further exploration in vivo. Wnt11 has been shown to be expressed at elevated levels in patients with ulcerative colitis (You et al., 2008), gastric carcinoma cell lines, and primary CRC cells (Uysal-Onganer and Kypta, 2012), and it can induce the proliferation and transformation of the IEC6 line in vitro (Ouko et al., 2004). Analogous to the model proposed in this paper, Paneth cells have recently been reported to sense calorie restriction (via mTORC1) and orchestrate ISC expansion in trans (Yilmaz et al., 2012). However, because Vil-Cre recombines in all IEC types (including ISCs), another possibility is that the effects of Xbp1 deletion could also have yet-to-be-defined direct effects on ISCs themselves. However, ISCs express only minute levels of Xbp1 and do not exhibit evidence of Xbp1 splicing (Heijmans et al., 2013; Schwitalla et al., 2013) under basal conditions. Neither of these hypotheses are mutually exclusive.

Although expansion of ISCs in the setting of ER stress was mediated by Ire1α, the increased turnover of Xbp1-deficient IECs was independent of Ire1α, suggesting different underlying mechanisms for both aspects of this regenerative response. An analogous discordant regulation of ISC expansion and proliferative output from transit-amplifying cells, and hence the engagement of separate mechanisms, has also been observed in the aforementioned study by Yilmaz et al. (2012), in which sensing of calorie restriction via mTORC1 in Paneth cells led to increased ISC numbers but decreased proliferative output of differentiated epithelial cells. IEC-specific Stat3 is essential for epithelial restitution and wound healing upon injury, where it localizes with increased epithelial proliferation (Pickert et al., 2009). Indeed, Xbp1 deletion, independently of Ire1α, leads to activation of Stat3 in the transit-amplifying zone. Consistent with this, pharmacologic blockade of Stat3 signaling abrogates heightened turnover of IECs in Xbp1<sup>-/-</sup>(IEC) mice. The secretion of the Stat3-activating cytokines IL-6 and IL-11 by Xbp1-silenced MODE-K cells suggests a feed forward loop instigated by ER-stressed IECs, which is relayed via Jak1. Il6 is a prototypical NF-κB target gene (Vallabhapurapu and Karin, 2009; Yu et al., 2009), and Il6 and Il11 can be transactivated by Stat3 (Yu et al., 2009). Given our observations here, it might be speculated that increased NF-κB activation as a consequence of pathological ER stress (Wu et al., 2004; Schröder and Kaufman, 2005) in the Xbp1-deficient epithelium might be an inciting event that leads to excessive production of IL-6, which activates Stat3, which may further transactivate Stat3, Il6, and Il11. Indeed, pharmacologic blockade of NF-κB signaling abrogated Stat3<sup>Tyr-705</sup> phosphorylation in Xbp1<sup>-/-</sup>(IEC) epithelium.

Malignant cells are commonly exposed to hypoxia, nutrient deprivation, and pH changes that impact protein folding (Ma and Hendershot, 2004; Hetz et al., 2011; Luo and Lee, 2013). Human tumor cells indeed exhibit evidence of ER stress, and reduced capacity to elicit an UPR within malignant cells results in decreased tumorigenesis in model systems (Ma and Hendershot, 2004; Bi et al., 2005; Fu et al., 2008; Luo and Lee, 2013). Xbp1 has been considered protumorigenic
(Luo and Lee, 2013); Xbp1-deficient tumor cells exhibit impaired growth when xenografted into Scid mice (Romero-Ramirez et al., 2004, 2009), and disruption of Ire1/Xbp1 signaling may be exploited pharmacologically for the treatment of multiple myeloma (Lee et al., 2003; Minnura et al., 2012). Our data imply an unexpected tumor-preventive role of Xbp1 in the intestinal epithelium. In this rapidly renewing tissue that is constantly exposed to noxious stimuli that may impact protein folding, Xbp1 may not only assist in resolving ER stress but may thereby keep the regenerative response, including stem cell activation, in check. However, overwhelming the remedial abilities of Xbp1 (e.g., through genetic hypofunction of Xbp1 or persistence of microbial or environmental ER stressors including those that inhibit Xbp1 function; Tashiro et al., 2007) may initiate a compensatory regenerative response, which is facilitative of neoplasias if persistent. In line with this, the architecture of ISCs in close proximity to Paneth cells is preserved in tumors (Scheppers et al., 2012), which we speculate might result in a situation in which the increase in ISCs and proliferative output in Xbp1−/−(IEC) tumors may outcompete any potential survival disadvantage of differentiated epithelial cells caused by Xbp1 deficiency.

Finally, the very mechanisms that appear critical for tumor initiation and promotion during CAC (NF-κB, IL-6, and Stat3; Becker et al., 2004; Greten et al., 2004; Bollrath et al., 2004) are engaged when Xbp1 function is relatively insufficient. In parallel, the Ire1α-dependent expansion of the ISC compartment increases the stochastic chance for genotoxic damage in these cells of origin of intestinal cancer (Barker et al., 2009; Zhu et al., 2009; Medema and Vermeulen, 2011; Scheppers et al., 2012). Indeed, the Ire1α dependence of increased tumorigenesis in Xbp1−/−(IEC);Apcmin mice critically implies a fundamental role of ER stress sensing as a facilitator of CRC. Apart from integrating environmental, microbial, genetic, and inflammatory inputs that affect ER stress levels at the hostile intestinal surface, Ire1α itself might also be affected by somatic mutations in tumors. Indeed, it has ranked prominently as carrying driver mutations in a large-scale survey of somatic mutations in tumors. Indeed, it has ranked prominently as carrying driver mutations in tumors. Indeed, it has ranked prominently as carrying driver mutations in tumors (Schepers et al., 2012), which we speculate might result in a situation in which the increase in ISCs and proliferative output in Xbp1−/−(IEC) tumors may outcompete any potential survival disadvantage of differentiated epithelial cells caused by Xbp1 deficiency.

In summary, this study demonstrates an unexpected role of Xbp1 in restraining the development of CAC and non-flammation–associated CRC. These Xbp1-dependent effects mechanistically derive from a role for Xbp1 in regulating the activity of Ire1α, which in turn determines the size of the ISC pool and its propensity to develop intestinal neoplasias when perturbed, and in restraining an IEC-intrinsic inflammatory response that is mediated by NF-κB, IL-6, IL-11, and Stat3, which are directly involved in promoting IEC proliferation and expansion of the transit-amplifying compartment. Together, the unrestrained activity of the Ire1α-regulated ISC compartment and Stat3-mediated hyperproliferation are central to the development of colorectal neoplasia in the presence of hypomorphic Xbp1 function.

**MATERIALS AND METHODS**

**Mice.** Xbp1fl/fl;VillinCre (Xbp1−/−(IEC)) mice have been described previously (Kaser et al., 2008). EnfVillinCre, Xbp1fl/fl;VillinCre;Apcmin;Ern1mice were generated by intercrossing Xbp1−/−(IEC);Apcmin mice with Apeo−/−(The Jackson Laboratory; Moser et al., 1990) and EnfVillinCre mice (Iwasaki et al., 2009). All mice were housed under specific pathogen-free conditions at Innsbruck Medical University, the University of Cambridge, and Harvard Medical School. The mating strategy involved keeping the Cre and Apeo−/−alleles hemizygous so that nondeleted or non-Apeo−/−offspring was generated at 50% and hence could be used as littermate controls. Mice were born at a Mendelian ratio, and mice on Apeo−/−background were regularly surveyed for rectal bleeding and general health status. Mouse protocols were approved by the relevant authorities, and all procedures were performed in accordance with institutional guidelines, using gender- and age-matched littermate controls whenever possible. S3I-201 (EMD Millipore), SP600125 (Sigma-Aldrich), and BAY 11-7082 (EMD Millipore) were injected i.p. at 10 mg/kg, 30 mg/kg, and 5 mg/kg, respectively, in 6–8-wk-old mice every other day for 14 d.

**Antibodies.** Antibodies directed to p-Stat3 (Tyr705; D3A7), Stat3 (7D9D), p-Jak1 (Tyr1022/1023), Jak3 (664), p-Jak2 (Tyr1007/1088; C803C), Jak2 (D2E12), p–NF-κB p65 (Ser536; 93H1), NF-κB p65 (D14E12), Gapdh (14C10), IRF1 (41C10; all Cell Signaling Technology), IRF1α (H-190, Santa Cruz Biotechnology, Inc.), p–IRF1α (Ser724; Abcam), anti-lysozyme (Dako), p53 (CM5; Vector Laboratories), DIG (Roche), PCNA (PC10; Thermofisher Scientific), IL-6 (MPS-2F3), IL-11 (188520, IgG1 isotype control (43414), and IgG2A isotype control (54447; all R&D Systems) were used.

**AOM/DSS model.** 6–8-wk-old Xbp1−/−(IEC) and Xbp1fl/fl;VillinCre mice were injected i.p. with 12.5 mg/kg AOM (Sigma-Aldrich). Colitis was induced by two cycles of 2.5% DSS (MP Biomedicals) in drinking water for 5 d, followed by a 16-d tap water period (Fig. 4 A; Greten et al., 2004). The final DSS cycle (2%) was administered for 4 d, followed by a 10-d tap water period. Tumor number and area were determined at day 61 in paraffin-embedded hematoyxin and eosin (H&E)–stained “Swiss rolls.” Serial sections every 200 µm were analyzed for tumor number and area using the section with the largest diameter for each individual tumor by Scion Image software (Scion Corporation).

**Apeo−/− model.** Longitudinally cut formalin-fixed intestines of 15-wk-old Xbp1fl/fl;VillinCre;Apeo−/− or EnfVillinCre;Apeo−/− mice were analyzed under a stereomicroscope (SZH-ILLD; Olympus) and categorized according to their size (Lee et al., 2010), complemented by H&E sections of the largest diameter for each individual tumor by Scion Image software (Scion Corporation).

**MODE-K cell cultures.** MODE-K IECs cultured in DMEM-10 were transduced with Xbp1-specific or control shRNA lentiviral vectors as described previously (Kaser et al., 2008), and stable clones were established. 105 cells were seeded in 6-well plates overnight and Jak1-3, Tyk2, or control shRNA (Life Technologies) transfected, and experiments were harvested 68–72 h after transfection. Tunicamycin (Sigma-Aldrich) was added at 2 µg/ml and anti–IL-6/–IL-11 antibodies or isotype control antibodies at optimal concentrations of 2 µg/ml and 20 µg/ml, respectively, and IL-6 and IL-11 in supernatants were measured by ELISA (BD and R&D Systems, respectively).

**Cryp isolation.** Crypt isolation was performed as previously reported (Munoz et al., 2012). In brief, the small intestine was flushed with cold PBS and cut longitudinally. Subsequent to snapping on ice, which removed most vili, the intestine was cut into 5-mm pieces and incubated for 5 min in 5 mM EDTA/PBS. Pieces were placed in 30 mM EDTA/PBS for 30 min, passed through a 100-µm cell strainer, and centrifuged, RNA was isolated from crypts, and RT-qPCR was performed as described below.
RNA extraction, RT-qPCR, and splicing assay. RNA was isolated by the RNeasy Mini kit (QiAGEN). Total RNA was reverse transcribed with M-MLV RT (Invitrogen), and SYBR–Green (Eurogentec) qPCR was performed using MX-3000 (Agilent Technologies). Target gene expression is expressed as ratio to housekeeping gene expression. Splicing assay was performed as described previously (Kaser et al., 2008). See Table S1 for primer sequences.

Western blotting. Total protein lysates from MODE-K, IEC scrapings, or tissue were prepared in RIPPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS). Equal amounts of lysates containing Lennuhi buffer were boiled at 95°C and resolved on 8–12.5% SDS-PAGE, and transferred, and Hybrid-B-Polyvinylidine fluoride membranes (GE Healthcare) were blocked with 5% milk in TBS-T, overnight incubated with primary antibody, detected with HRP-conjugated secondary antibody, and visualized with LumiGLO (Cell Signaling Technology).

Immunoprecipitations. 300 µg of protein lysate was incubated for 1 h with 1 µg anti-IRE1α pAb H-190 and pulled down with Sepharose-A. Pull-downs were washed three times with RIPPA buffer and eluted with 2x SDS loading buffer, followed by Western blot analysis.

IHC. Sections were deparaffinized in xylol and dehydrated in ethanol. Antigen retrieval was performed by citrate or EDTA buffer for 15 min at sub-boiling temperature in a microwave, followed by blocking of endogenous peroxidases activity. Primary antibody was incubated overnight at 4°C, and secondary biotinylated antibody was detected with streptavidin-HRP (Vector Laboratories). Sections were developed using a DAB Peroxidase Substrate kit (Vector Laboratories). Images were acquired using an Axio Observer Z.1 and AxioCam MRc5 and analyzed with AxioVision software (release 4.8; all Carl Zeiss).

ISH. DIG-labeled sense and antisense cRNA probes (Gregoire and Clevers, 2010) from full-length cDNA clones (I.M.A.G.E. Consortium) were hybridized for 48 h at 50°C and visualized using anti-DIG antibody (Roche).

Aneuploid cell fraction. Extroverted intestines were incubated in so- wood treatment in a microwave, followed by blocking of endogenous peroxidases activity. Primary antibody was incubated overnight at 4°C, and secondary biotinylated antibody was detected with streptavidin-HRP (Vector Laboratories). Sections were developed using a DAB Peroxidase Substrate kit (Vector Laboratories). Images were acquired using an Axio Observer Z.1 and AxioCam MRc5 and analyzed with AxioVision software (release 4.8; all Carl Zeiss).

ROS. Colon pieces (proximal, mid, and distal) were incubated in Luminol Assay buffer (10 mM luminol in 1 mM CaCl2, 5 mM glucose in PBS) for 3 min and luminescence was measured (Garrett et al., 2009). Mean RLU/mean wet tissue weight was calculated.

BrdU incorporation and quantification. 2.5 mg BrdU was injected 2 or 24 h before sacrificing mice (Kaser et al., 2008). BrdU- cells were revealed by a BrdU staining kit (BD). BrdU- cells per total cells along the crypt–villus axis were counted in five randomly selected crypt–villus axes per single sample.

Statistical analysis. Mean ± SEM is reported. Statistical significance was calculated using a two-tailed Student’s t-test, and significance was assumed for p-values <0.05. Where more than two groups were compared, one-way ANOVA with Bonferroni’s post-hoc testing was performed. Grubbs test was used to identify outliers. Data were analyzed using Excel (Microsoft) and Prism (GraphPad Software) software.

Online supplemental material. Table S1 shows primer sequences used in this study. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20122341/DC1.

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