NLRX1 Acts as an Epithelial-Intrinsic Tumor Suppressor through the Modulation of TNF-Mediated Proliferation

Highlights
- Epithelial NLRX1 protects against colorectal cancer
- NLRX1 dampens expression of intestinal wound healing factors
- NLRX1 inhibits proliferation during epithelial regeneration post-colitis
- In organoids, NLRX1 inhibits TNF-induced proliferation and signaling

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In Brief
NLRX1 protects against colorectal tumorigenesis. Tattoli et al. now examine the mechanism underlying this effect and find that epithelial NLRX1 protects against colitis-associated colorectal cancer. In the epithelium, NLRX1 controls the expression of intestinal wound healing factors following injury and inhibits the proliferative action of TNF.
NLRX1 Acts as an Epithelial-Intrinsic Tumor Suppressor through the Modulation of TNF-Mediated Proliferation

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SUMMARY

The mitochondrial Nod-like receptor protein NLRX1 protects against colorectal tumorigenesis through mechanisms that remain unclear. Using mice with an intestinal epithelial cells (IEC)-specific deletion of Nlrx1, we find that NLRX1 provides an IEC-intrinsic protection against colitis-associated carcinogenesis in the colon. These Nlrx1 mutant mice have increased expression of Tnf, Egf, and Tgfb1, three factors essential for wound healing, as well as increased epithelial proliferation during the epithelial regeneration phase following injury triggered by dextran sodium sulfate. In primary intestinal organoids lacking Nlrx1, stimulation with TNF resulted in exacerbated proliferation and expression of the intestinal stem cell markers Olfm4 and Myb. This hyper-proliferation response was associated with increased activation of Akt and NF-kB pathways in response to TNF stimulation. Together, these results identify NLRX1 as a suppressor of colonic tumorigenesis that acts by controlling epithelial proliferation in the intestine during the regeneration phase following mucosal injury.

INTRODUCTION

NLRX1 is a member of the Nod-like receptor (NLR) family of intracellular sensors of danger-associated molecular patterns (DAMPs) and microbial-associated molecular patterns (MAMPs) that function in innate immunity (Philpott et al., 2014). In contrast to other members of the NLR family, NLRX1 possesses an N-terminal addressing sequence that targets the protein to the mitochondrial matrix (Arnoult et al., 2009). The exact function of NLRX1 has remained poorly understood. It was proposed to act as a negative regulator of antiviral immunity through interaction with the protein MAVS (Moore et al., 2008), but these findings were not reproduced by other groups (Jaworska et al., 2014; Rebsamen et al., 2011; Soares et al., 2013). Overexpression of NLRX1 was also shown to regulate the formation of reactive oxygen species (ROS) by mitochondria (Tattoli et al., 2008). Further support for this observation was obtained in mouse embryonic fibroblasts (MEFs) from Nlrx1−/− mice, which displayed blunted tonic levels of ROS in resting condition as compared to wild-type (WT) MEFs (Soares et al., 2014). NLRX1 was also shown to inhibit inflammatory pathways triggered by lipopolysaccharide (Allen et al., 2011; Xia et al., 2011). Moreover, Nlrx1−/− mice display increased cerebral inflammation in a model of experimental autoimmune encephalopathy (Etas et al., 2014), higher lung inflammation in a model of cigarette smoke-induced chronic obstructive pulmonary disease (Kang et al., 2015), and enhanced intestinal inflammation in a colitis model induced by dextran sodium sulfate (DSS) (Soares et al., 2014). However, the underlying mechanism through which NLRX1 controls inflammation in these various models remains unclear.

Recently, findings from several groups have identified a role for NLRX1 in the control of cell death in various cellular systems (Imbeault et al., 2014; Jaworska et al., 2014; Singh et al., 2015; Soares et al., 2014). However, it remains unclear if the effect of NLRX1 on ROS or cell death signaling is direct or whether it is a consequence of an overarching function of the protein in the mitochondria that remains to be identified. In support of the latter hypothesis, it must be noted that NLRX1 expression is significantly repressed by glucose starvation (Soares et al., 2014); in addition, NLRX1 silencing is sufficient to upregulate the basal activities of complex I and III of the mitochondrial respiratory chain (Singh et al., 2015), suggesting that NLRX1 may act to tie mitochondrial oxidative...
phosphorylation (Oxphos) activity to the cellular availability of glucose. As such, NLRX1 could play a role as a tumor suppressor by inhibiting mitochondrial Oxphos-dependent generation of ATP in conditions of nutrient sufficiency. This hypothesis is supported by several lines of evidence. First, NLRX1 expression is strongly reduced by SV40- or Ras-dependent transformation in a genome-wide study (Danielsson et al., 2013), a result that we confirmed by analyzing Nlrx1 expression by qPCR and western blotting in primary versus in SV40-transformed MEFs (Soares et al., 2014). Second, we recently demonstrated that Nlrx1 deletion in the AOM/DSS model (Soares et al., 2014), but whether this effect was epithelial-intrinsic or due to increased inflammation was not addressed. Nlrx1 mice received an intraperitoneal injection of AOM (10 mg/kg) followed by three cycles of DSS (2.5% in drinking water). In both male and female cohorts of mice receiving AOM/DSS treatment, animals lost weight during DSS cycles, which they recovered during the intercycles (Figure 2B). A trend toward increased weight loss in Nlrx1 mice as compared to Nlrx1 mice was observed. Colon shortening, an indication of intestinal inflammation, was observed in both Nlrx1 mice, and Nlrx1 mice, although analysis of Muc2 expression by qPCR revealed increased expression in Nlrx1 mice (Figure 1H). Together, these results show that deletion of Nlrx1 in IECs does not significantly alter intestinal architecture.

Expression of Nlrx1 in the Intestinal Epithelium Protects against Colitis-Associated Tumorigenesis

We previously reported that whole-body deletion of Nlrx1 exacerbated colitis-associated tumorigenesis in the AOM/DSS model (Soares et al., 2014), but whether this effect was epithelial-intrinsic or due to increased inflammation was not addressed. Nlrx1 and Nlrx1 mice received an intraperitoneal injection of AOM (10 mg/kg) followed by three cycles of DSS (2.5% in drinking water). In both male and female cohorts of mice receiving AOM/DSS treatment, animals lost weight during DSS cycles, which they recovered during the intercycles (Figure 2A). A trend toward increased weight loss in Nlrx1 mice as compared to Nlrx1 mice was observed. Colon shortening, an indication of intestinal inflammation, was observed in both Nlrx1 mice and Nlrx1 mice, although it appeared to be slightly more pronounced in Nlrx1 mice (Figure 2B). Moreover, histological analysis of colon sections showed no significant difference overall between Nlrx1 mice and Nlrx1 mice with regards to inflammation-associated parameters (Figure 2C). In particular, sub-mucosal edema, tissue damage, polymorphonuclear (PMN) infiltration and hyperplasia were similar between both groups of mice. However, analysis of tumor burden showed that AOM/DSS treated Nlrx1 mice developed approximately twice as many tumors as Nlrx1 mice (Figure 2D) and histopathological scoring of these tumors revealed an increased number of adenomas in Nlrx1 mice (Figures 2E and 2F).

The above results suggest that the impact of Nlrx1 deficiency on tumorigenesis is epithelial-intrinsic and not driven by changes
in the inflammatory environment. In agreement with a cancer cell-intrinsic role of NLRX1, publically available data show that expression of NLRX1 is lower in tumors than the normal corresponding tissue in human cancers, including bladder, cervical, esophageal, colon, and skin cancers (Figure S2A). Moreover, analysis of two large data sets of colorectal cancer demonstrated that NLRX1 expression was significantly reduced in colon adenocarcinoma or colon mucinous adenocarcinoma as compared to normal colon (Figure S2B). Finally, analysis of 1,010 human cancer cell lines (from the Cancer Cell Line Encyclopedia) showed that the cell line with the lowest expression of NLRX1 was NCI-H716, which is derived from a colon adenocarcinoma (Figure S2C). Thus, available data from human cancer and cancer cell lines indicate that NLRX1 expression is reduced in multiple cancers and in particular in colon cancer.

Mutations in the Adenomatous polyposis coli (APC) gene are found in sporadic colon cancers in humans (Raskov et al., 2014) and result in uncontrolled Wnt signaling and proliferation of IECs. Mice harboring the Apc<sup>min</sup> mutation develop spontaneous tumors in the small intestine and in the colon (Su et al., 1992). Nlrx1-deficient mice were crossed with mice carrying the Apc<sup>min</sup> mutation. Interestingly, Apc<sup>min/+</sup>Nlrx1<sup>/−/−</sup> mice had a significantly reduced lifespan and had to be euthanized at age 101.1 ± 8.38 days as compared to littermate Apc<sup>min/+</sup>Nlrx1<sup>+/+</sup> mice that survived until day 132.5 ± 6.39 (Figures 3A and 3B). Moreover, Apc<sup>min/+</sup>Nlrx1<sup>/−/−</sup> mice had more colonic tumors and those were larger than the ones observed in Apc<sup>min/+</sup>Nlrx1<sup>+/+</sup> mice (Figures 3C and 3D). Thus, NLRX1 protects against tumorigenesis induced by dysregulation of the Apc/Wnt signaling axis.

Together, these results suggest that NLRX1 acts in an epithelial-intrinsic manner to suppress colorectal tumorigenesis.

### Epithelial-Specific Deletion of Nlrx1 Does Not Affect Peak Tissue Damage Induced by DSS, but Potentiates Expression of Late Tissue Healing Factors TNFα and TGFβ

In order to better understand how IEC-specific deletion of Nlrx1 contributes to tumorigenesis susceptibility in the colitis-associated AOM/DSS model, DSS-induced colitis was analyzed in Nlrx1<sup>fl/fl</sup> and Nlrx1<sup>IEC</sup> mice. Following treatment with 2.5% DSS, mice were analyzed for peak tissue damage at day 6 and late tissue healing factors at day 16 following AOM/DSS induction. In agreement with previous reports, Nlrx1<sup>IEC</sup> mice had a significantly reduced peak tissue damage as compared to littermate Nlrx1<sup>fl/fl</sup> mice (Figures 4A and 4B). However, Nlrx1<sup>IEC</sup> mice had a significantly increased expression of late tissue healing factors, including TNFα and TGFβ, as compared to Nlrx1<sup>fl/fl</sup> mice (Figures 4C and 4D). Thus, NLRX1 protects against tumorigenesis induced by dysregulation of the Apc/Wnt signaling axis.
Figure 2. Expression of Nlrx1 in the Intestinal Epithelium Protects against Colitis-Associated Tumorigenesis

Two independent studies were performed in Nlrx1<sup>fl/fl</sup> and Nlrx1<sup>IEC</sup> mice to analyze the effect of AOM/DSS on tumorigenesis. Cohorts #1 and #2 were littermate females and males, respectively, either left untreated (n ≥ 3) or receiving AOM/DSS treatment (n ≥ 7).

(A) Weight curves.
(B) Measurement of colon length.
(C) Histopathological evaluation of inflammation-associated parameters.
(D and E) Breakdown of the tumor number per size (diameter) (D) and by grade (E).
(F) Representative micrographs showing polyps in colons from AOM/DSS-treated mice (aberrant crypt foci, ACF and high grade dysplasia, HGD) (non-significant, NS) (*p < 0.05 and **p < 0.01).
DSS in drinking water for 5 days, both groups of mice lost weight similarly, up to day 7 (i.e., 2 days after removal of DSS from drinking water) (Figure 4A). From that time point, both animal groups recovered weight progressively, indicative of a healing process, but Nlrx1fl/fl mice recovered faster during the d10–d15 period (Figure 4A). Despite this difference in the recovery from DSS-induced colitis, inflammation appeared to be comparable between the two groups of mice, as evidenced by similar colon length at sacrifice (Figure 4B), histology (Figure 4C), blinded inflammatory scores on colonic sections (Figure 4D), levels of keratinocyte chemoattractant or CXCL1 in the serum (Figure S3A), and of secreted IL-1β from intestinal explants in culture (Figure S3B). Of note, this analysis further showed that the peak of intestinal erosion and inflammation was observed at day 7 in both Nlrx1fl/fl and Nlrx1ΔIEC mice, which correlates well with the weight loss data (see Figure 4A), but suggests that the faster recovery of Nlrx1ΔIEC mice is independent from inflammation. Next, we performed qPCR analysis on colon mucosa samples from DSS-treated Nlrx1fl/fl and Nlrx1ΔIEC mice at day 7 and d12. At the peak of tissue damage (day 7), inflammation-associated genes such as A20, Il10, and Il6 were transcriptionally upregulated in both groups of mice and this activation persisted, at least partially up to d12 (Figure S3F and S3G). Interestingly, while Tnf, Egf, and Tgfb1 were not transcriptionally upregulated at day 7 in either group of mice, we observed a delayed upregulation of these genes at d12, and this upregulation was significantly more robust in Nlrx1ΔIEC mice as compared to Nlrx1fl/fl mice (Figures 4E–4G). Previous work demonstrated that microbial products, in synergy with Notch signaling, could potentiate Tnf expression in the intestine (Hu et al., 2008). Interestingly, Notch signaling was activated early (day 7) during colitis as evidenced by the upregulation of the Notch target Hey1, and this induction was significantly more pronounced in Nlrx1ΔIEC mice than Nlrx1fl/fl mice (Figure S3H). At d12, Notch signaling was still active since expression of Atoh1, which is inhibited by Notch, was more

**Increased Epithelial Proliferation in Nlrx1ΔIEC Mice during Epithelial Regeneration**

Treatment with DSS results in a massive epithelial erosion and healing requires upregulation of IEC proliferation and migration. Because we observed increased expression of Tnf in Nlrx1ΔIEC mice during the regeneration phase following DSS treatment (see above), we aimed to analyze epithelial proliferation.

Immunohistochemistry (IHC) staining using anti-Ki-67, a marker of cellular proliferation, revealed that an increased number of epithelial cells were proliferative in the crypts of Nlrx1ΔIEC mice as compared to Nlrx1fl/fl mice at d12 and d20 post-DSS treatment (Figures 5A–5C). It must be noted that at the peak of DSS-induced tissue damage (day 7), the epithelium was too eroded to perform analysis of Ki-67. In the AOM/DSS-treated animals analyzed above (see Figure 2), which were sacrificed at d20 of the third DSS cycle, a similar increase in the number of
Ki-67+ cells was observed in the crypts of Nlrx1IEC mice as compared to Nlrx1fl/fl mice (Figure 5D).

In resting conditions, epithelial cell death must approximately be equal to proliferation in order to keep colonic crypt length constant. Because we previously showed that NLRX1 controls apoptotic cell death, we sought to determine if the more robust proliferation observed in DSS-treated Nlrx1IEC IECs was the result of increased apoptosis. IHC analysis of apoptosis using an antibody against the cleaved form of caspase-3 showed an increase in apoptosis in the colonic crypts of d12 DSS-treated mice as compared to unchallenged animals, but this increase was similar between Nlrx1fl/fl and Nlrx1IEC mice (Figures 5E and 5F), thus ruling out the possibility that increased proliferation in DSS-treated Nlrx1IEC mice was the result of increased apoptosis. Together, these results show that NLRX1 negatively regulates IEC proliferation during the epithelial regeneration phase following intestinal injury.

Expression of NLRX1 Inhibits TNF-Mediated Proliferation and Signaling in Primary Intestinal Organoids

In order to gain insights into the mechanism through which epithelial-intrinsic expression of NLRX1 inhibits epithelial proliferation during the regeneration phase post-DSS, we isolated intestinal crypts and cultured primary intestinal organoids from Nlrx1fl/fl and Nlrx1IEC mice in Matrigel, as previously described (Sato et al., 2009). Since we observed increased expression of TNF in the colons of Nlrx1IEC mice as compared to Nlrx1fl/fl mice (Figure 5D), we hypothesized that TNF could contribute to the hyper-proliferative response observed in Nlrx1IEC mice recovering from DSS. TNF stimulation for 24 h of freshly passed organoids from Nlrx1fl/fl mice resulted in a modest non-significant increase in the diameter of the organoids (Figures 6A and 6B). In contrast, TNF stimulation more than doubled the surface of organoids from Nlrx1IEC mice (Figures 6A and 6B), and those also had more numerous and longer digitations, showing that these structures displayed increased crypt growth. Next, TNF-stimulated organoids were pulsed with EdU, which incorporates into proliferative cells, and analyzed by flow cytometry. Interestingly, organoids from Nlrx1IEC mice displayed more EdU+ cells than those from Nlrx1fl/fl mice (Figures 6C and 6D), thus showing that the increase in organoid size following
TNF stimulation was caused, at least in part, by hyper-proliferation.

Intestinal organoids grow from rapidly dividing pluripotent intestinal stem cells (ISCs) that are found at the bottom of the crypts and express the marker \( \text{Lgr}5 \) (Barker et al., 2007). Another ISC population, likely acting as a reserve ISC, expresses \( \text{Bmi}1 \) (Sangiorgi and Capecchi, 2008; Tian et al., 2011). Cells leaving the ISC niche lose expression of the \( \text{Lgr}5 \) and \( \text{Bmi}1 \) markers and form the transit-amplifying (TA) zone, in which cells remain highly proliferative and still express numerous ISC markers, but progressively differentiate into specific IEC populations. qPCR analysis revealed that stimulation with TNF resulted in a modest increase in the expression of \( \text{Lgr}5 \) and \( \text{Bmi}1 \) in intestinal organoids from both \( Nlrx1^{+/} \) and \( Nlrx1^{IEC} \) mice; however, this increase was not significantly different between the two groups of organoids (Figures S4A and S4B). Next, we analyzed the expression of \( \text{Olfm}4 \), an ISC marker that, in contrast to \( \text{Bmi}1 \) and \( \text{Lgr}5 \), remains expressed in the TA zone (Itzkovitz et al., 2012). Interestingly, we observed stronger upregulation of \( \text{Olfm}4 \) in organoids from \( Nlrx1^{IEC} \) than those from \( Nlrx1^{+/} \) mice following TNF stimulation (Figure 6E), suggesting that NLRX1 specifically represses proliferation of cells in the TA zone, rather than cells in the ISC niche.

Uncontrolled proliferation of cells in the ISC niche or the TA zone can drive tumorigenesis. It was shown that the transcription factor \( \text{Myb} \), which is highly expressed in ISCs (Muñoz et al., 2012), was required for proliferation in colonic crypts (Malaterre et al., 2007) and \( \text{Myb} \) activation results in colon tumorigenesis in mice (Malaterre et al., 2015). Strikingly, TNF stimulation resulted in only a transient up-regulation of \( \text{Myb} \) expression in organoids from \( Nlrx1^{+/} \) mice, while this upregulation persisted in those from \( Nlrx1^{IEC} \) mice (Figure 6F). Thus, dysregulated expression of \( \text{Myb} \) in the colonic crypts of \( Nlrx1^{IEC} \) mice during epithelial restitution following injury could contribute to the exacerbated tumorigenesis observed in these mice in the AOM/DSS model.

We have demonstrated above that epithelial regeneration following DSS-induced injury resulted in exacerbated expression of \( \text{Tnf} \), \( \text{Egf} \), and \( \text{Tgfb}1 \) in \( Nlrx1^{IEC} \) mice as compared to \( Nlrx1^{+/} \) mice, suggesting that these factors could be expressed by the intestinal epithelium itself, since non-IECs in both groups of mice are WT. In support for this, we observed that stimulation of organoids from \( Nlrx1^{IEC} \) and \( Nlrx1^{+/} \) mice with TNF resulted in potent transcriptional upregulation of both \( \text{Tnf} \) and \( \text{Tgfb}1 \) (Figures 6G and 6H), as well as a more modest induction of \( \text{Egf} \) (Figure S4C). In contrast, \( \text{Il6} \) transcript was undetectable in these conditions (data not shown). Therefore, these data support the notion that \( \text{Tnf} \), \( \text{Tgfb}1 \), and \( \text{Egf} \) can be expressed through paracrine regulation by the intestinal epithelium.
In order to better understand the mechanism through which TNF triggered a hyper-proliferative response in organoids from Nlrx1^{DIEC} mice, we analyzed by western blotting the activation of Akt, a protein that plays a central role in the regulation of epithelial cell proliferation (Lee et al., 2010) and whose dysregulation is strongly associated with colorectal cancer (Agarwal et al., 2013; Leystra et al., 2012). TNF stimulation resulted in rapid upregulation of Akt phosphorylation in both groups of organoids; however, the effect was stronger and more sustained in organoids from Nlrx1^{DIEC} mice than those from Nlrx1^{fl/fl} mice (Figure 6I).

Finally, we analyzed NF-\( \kappa \)B signaling, since this pathway is also critical for the control of IEC proliferation and intestinal tumorigenesis (Myant et al., 2013; Steinbrecher et al., 2008). Similar to Akt activation, we observed that TNF stimulation triggered degradation of the inhibitory molecule I\( \kappa \)B\( \alpha \), which indicates NF-\( \kappa \)B activation, in both groups of organoids, but the effect was again more sustained in organoids from Nlrx1^{DIEC} mice than those from Nlrx1^{fl/fl} mice (Figure 6I). Together, these results indicate that TNF-mediated hyper-proliferative response in organoids from Nlrx1^{DIEC} mice was associated with exacerbated activation of the Akt and NF-\( \kappa \)B signaling pathways and upregulation of the transcription factor Myb.

**DISCUSSION**

Chronic injury of the intestinal mucosa can favor the development of colorectal cancer. This is highlighted by the fact that inflammatory bowel disease confers increased risk of developing gastrointestinal neoplasia in humans. Similarly, repeated exposure to the epithelial irritant DSS greatly accelerates tumor formation in the AOM/DSS model in mice (Rosenberg et al., 2009). It is clear that inflammation plays a critical role in this effect, and inflammation-associated cytokines such as IL-6 are known to contribute to the pathogenesis of colorectal cancer (Waldner and Neurath, 2014). However, it is less appreciated that epithelial regeneration and wound repair pathways, which are triggered as a direct consequence of mucosal injury and are amplified by inflammatory mediators, likely play central roles in colitis-associated intestinal tumorigenesis. Indeed, these important processes rely on cellular proliferation and migration and on signaling pathways dependent on NF-\( \kappa \)B, Akt/PI3K, and Wnt/\( \beta \)-catenin, all of which being critical actors of colorectal cancer pathogenesis. Because inflammation and epithelial repair pathways are tightly interconnected and are spatially and temporally coordinated, it is difficult to determine what aspects of inflammation (i.e., recruitment of immune cells, upregulation of phagocytosis, secretion of inflammatory or...
immune defense mediators, microbiota dysbiosis, and upregulation of epithelial repair) have the most important impact on tumorigenesis. One way to approach this question and to identify key molecular pathways linking specific arms of inflammation to tumorigenesis is to perform analysis of mice with tissue-specific gene deletion. Here, by using mice harboring a deletion of Nlrx1 only in IECs, we identified an epithelial-intrinsic role for this NLR molecule in the control of IEC proliferation during the epithelial regeneration phase following DSS treatment. Our data suggest that Nlrx1 deletion exacerbates cellular responses to TNF, which could generate a paracrine or autocrine amplification loop in IECs by triggering Tnf expression. This effect might also amplify the expression of other wound healing genes, such as Tgfb1 and Egf, as suggested by our results in intestinal organoids. Thus, our data suggest that, by restricting the activation of Akt and NF-κB in IECs in response to TNF stimulation, NLRX1 tones down the effect of TNF on the proliferation of TA cells in the intestinal crypt, providing a protection against colitis-associated tumorigenesis. It is possible that a similar mechanism underlies the protective role of NLRX1 against tumorigenesis in mice carrying the Apcmin mutation, since increased mucosal expression of Tnf was observed in these mice and correlated with tumor formation (McClellan et al., 2012).

Our previous results demonstrated that full-body deletion of Nlrx1 resulted in increased inflammation following DSS treatment (Soares et al., 2014), which is in line with other studies showing an inhibitory effect of NLRX1 on inflammation (Allen et al., 2011; Xia et al., 2011; Eltas et al., 2014; Kang et al., 2015). Although this effect is likely immune cell-intrinsic, the underlying mechanism is not clearly elucidated, nor are the immune cell subsets identified that are undergoing uncontrolled activation in Nlrx1−/− deficient mice. Nevertheless, these observations, together with the results of the present study, suggest that NLRX1 might mitigate colitis-associated tumorigenesis at two levels: (1) by downregulating inflammation and (2) by inhibiting pro-proliferative responses in the intestinal epithelium in response to inflammatory and pro-regeneration cues.

Our data show that, in intestinal organoids, NLRX1 diminishes TNF-induced activation of both Akt- and NF-κB-dependent pathways. Since Akt was shown to be essential for TNF-mediated activation of NF-κB (Ozes et al., 1999), it is likely that the primary effect of NLRX1 is at the level of Akt signaling. Alternatively, NLRX1 could regulate a step or an event that controls both pathways, such as mitochondrial ROS levels or expression of TNF receptors. Future studies will aim at identifying the molecular mechanism by which NLRX1 mitigates Akt and NF-κB signaling in response to TNF stimulation in IECs.

Overall, our study identifies an epithelial-intrinsic role for NLRX1 in the control of colon tumorigenesis through the modulation of epithelial proliferation following injury. Experiments in primary intestinal organoids further revealed that NLRX1 directly impacted Akt and NF-κB pathways, expression of the proliferation-associated transcription factor Myb, and proliferation of crypt TA cells in response to TNF. Identifying the mechanism through which NLRX1 controls these pathways will allow the designing of novel therapeutic strategies aimed at regulating hyper-proliferative responses in colon cancer without having to suppress inflammatory cytokine networks.

**EXPERIMENTAL PROCEDURES**

**Animals**

All mice were bred and housed at the Division of Clinical Medicine (DCM) of the University of Toronto and experiments were performed according to guidelines of the DCM and following protocols approved by the University of Toronto Committee on Use and Care of Animals. Mice were kept in specific pathogen-free conditions with periodic testing for murine norovirus and used for experiments at age 6–10 weeks except otherwise indicated. All experiments were performed using littermate animals that were separated at weaning. Nlrx1−/− mice have been described previously (Soares et al., 2013).

**Generation of IEC-Specific NLRX1-Deficient Mice**

NLRX1−/− mice (Nlrx1fl/fl) were generated by crossing Nlrx1fl/fl mice (Soares et al., 2013) with transgenic mice B6.SJL-Tg(Vil-cre)997Gum/J (Jackson Laboratories), which express Cre recombinase under the control of the mouse villin promoter. The following PCR primers (5′-3′) were used to genotype WT, floxed, and Nlrx1fl/fl mice: primer 1- (CCA TTT GCC AAT CCC ACT CAC), primer 2 (ACC AAG AAC CTA ACC CAC GGT C), primer 3 (TTG CCA GCC ATC TGT TGT TTG), oimR1878 (GTG TGT GAC AGA GAA AGG), and oimR1879 (AGA TCT TCA GGT TCT GCG GG).

**Induction of DSS-Induced Colitis**

Colitis was induced with 2.5% (w/v) DSS (MP Biomedicals) dissolved in sterile, distilled water for experimental days 5, followed by normal drinking water until the end of the experiment.

**Colitis-Associated Colorectal Cancer**

Mice were injected intraperitoneally with 10 mg/kg AOM (Sigma-Aldrich). After 5 days, 2.5% DSS was given in drinking water over 5 days, followed by regular drinking water for 2 weeks. This cycle was repeated three times, and mice were sacrificed 2 weeks after the last DSS cycle. The experiments were performed with at least 4–8 mice in each AOM-treated condition.

**Reagents and Antibodies**

AOM (Sigma-Aldrich, A5486), DSS (MP Biomedicals, 160110). The following antibodies were used for protein detection: rabbit polyclonal AKT rabbit polyclonal (Cell Signaling Technology, 9272S), rabbit polyclonal Phospho-AKT (Ser473) (Cell Signaling Technology, 9271S), rabbit polyclonal anti-Ki-67 (D3B5) (Cell Signaling Technology, 12202), mouse monoclonal anti-tubulin (Sigma-Aldrich, T9026), rabbit polyclonal anti-cleaved caspase-3 (Asp175) (Cell Signaling Technology, 9661S), rabbit anti-chromogranin A (Abcam, ab15160), rabbit anti-Muc2 (H300) (Santa Cruz, sc-15334) mouse monoclonal anti-tubulin (Sigma-Aldrich, T9028), rabbit polyclonal anti-α6 (D3B5) (Cell Signaling Technology, 12202), mouse monoclonal anti-NLRX1 (Millipore, 04-146) and rabbit monoclonal anti-NLRX1 (E130) (Abcam, ab32518), goat anti-rabbit (ThermoScientific / Molecular Probes, A-21245), AB solution (Sigma-Aldrich, B8438), Periodic Acid- Schiff (PAS) kit (Sigma-Aldrich, 395B), Alexa Fluor 647 donkey anti-rabbit (Life Technologies, A31573), and Click-IT EdU Flow Cytometry Assay Kit (Life Technologies, C10419).

**Statistical Analysis**

Prism software was used to plot data and determine statistical significance using a Student’s t test (for two groups) or ANOVA for data sets with three or more sets of data to analyze. Data are presented as means ± SEM. A p value of <0.05 was considered to be statistically significant.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.02.065.

**AUTHOR CONTRIBUTIONS**

I.T., D.J.P., and S.E.G. designed the study and analyzed data. I.T. performed most experiments of the study, S.E.G. wrote the manuscript. S.A.K. and
M.A.R. performed western blots, E.G.F. performed immunofluorescence, R.M. did EdU assays, and C.M. performed some qPCRs. S.W., D.A.W., and C.J.S. performed blinded pathological scoring.

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