Hematopoietic not systemic impairment of Roquin expression accounts for intestinal inflammation in Roquin-deficient mice

Dina Montufar-Solis, Nadarajah Vigneswaran, Niyati Nakra, Jeremy S. Schaefer & John R. Klein

Department of Diagnostic and Biomedical Sciences, School of Dentistry, The University of Texas Health Science Center at Houston, Houston, TX 77054 USA.

Roquin, an RNA binding RING finger E3 ubiquitin ligase, localizes to cytosolic RNA granules that regulate mRNA stability and translation. E3 ligases, which determine the specificity of protein degradation by associating with a substrate, are defined by their catalytic domains: Homologous to E6-AP Carboxyl Terminus (HECT) domain proteins have intrinsic ligase activities, whereas Really Interesting New Gene (RING) domain proteins function as scaffolds in the degradation process. Roquin is coded for by the Rc3h1 gene in mice. Sanroque mice (Rc3h1san/san), which have an M199R mutation in the Roquin protein, develop extensive chronic inflammation consisting of lymphadenopathy, splenomegaly, thrombocytopenia, necrotizing hepatitis, increased CD4⁺ follicular T cells and expression of the inducible costimulator (ICOS) marker of T cell activation. Additionally, we recently demonstrated that Rc3h1san/san mice have increased ICOS and OX40 expression in mesenteric lymph node T cells, and that a Crohn’s disease-like chronic small intestinal inflammatory response develops in those animals. The latter is of particular interest given the paucity of animal models of small intestine inflammation.

Studies using Rc3h1 knockout mice reported that animals had a caudal spine deformity and most animals died within six hours of birth, apparently due to a defect in lung development. Conditional knockouts targeting Rc3h1 in T cells or in the entire hematopoietic system failed to exhibit a breach in self-tolerance or changes in follicular T cell differentiation despite an increase in ICOS expression, expansion of CD8 effector cells, and the presence of short-lived effector cells (SLECs). Roquin-deficient mice generated in our laboratory using a gene trap insertion into the Rc3h1 gene (Rc3h1ervo/ervo mice) also had high post-birth mortality; however, surviving animals developed chronic intestinal inflammation similar to that of Rc3h1san/san mice.

Because mice with a disrupted Rc3h1 gene (Rc3h1ervo/ervo mice) have physiological changes that extend beyond the immune system, the intestinal inflammatory response and tissue destruction observed in those animals could be caused by non-immunological perturbations, e.g., Roquin-mediated changes in epithelial cell permeability or effects directed at other non-hematopoietic tissues. To address this, we have used bone marrow (BM) radiation chimeras generated by injecting Rc3h1ervo/ervo BM into irradiated normal (NL) mice (Rc3h1ervo/ervo → NL chimeras) to...
determine whether the intestinal inflammation in \( \text{Rc3h1}^{\text{gt/gt}} \) mice was due to a disruption of the \( \text{Rc3h1} \) gene in cells of the immune system (in which case inflammation would be evident), or whether it required systemic Roquin impairment (in which case inflammation would be absent). The findings reported here demonstrate that the inflammatory response in \( \text{Rc3h1}^{\text{gt/gt}} \) mice is driven by a direct failure of Roquin expression in cells of the immune system. Moreover, they attest to the utility of using \( \text{Rc3h1}^{\text{san/san}} \) and \( \text{Rc3h1}^{\text{gt/gt}} \) chimeras to dissect the cellular and molecular mechanisms that regulate the inflammatory response in the intestinal mucosa.

**Results**

Radiation chimeras generated from \( \text{Rc3h1}^{\text{gt/gt}} \) BM develop small intestine inflammation and secrete proinflammatory cytokines. Radiation chimeras were generated as described in the Materials and Methods. Mice were studied between 6 and 21 weeks of age. Histopathological analyses of blinded tissue sections were done for the duodenum, jejunum, ileum, cecum, ascending colon, transverse colon, descending colon, liver, kidney, lung, and spleen. The small intestine of \( \text{Rc3h1}^{\text{gt/gt}} \) mice and \( \text{Rc3h1}^{\text{gt/gt}} \) → NL chimeras exhibited variable degrees of inflammation and villus atrophy depending upon the region (Fig. 1, rows a and b, respectively). Inflammation and tissue injury of the organs were unremarkable in control NL → NL chimeras (Fig. 1, row c). The average small intestine pathology scores are shown in Fig. 1d, which indicated that inflammation was present throughout the duodenum, jejunum, and ileum of \( \text{Rc3h1}^{\text{gt/gt}} \) and \( \text{Rc3h1}^{\text{gt/gt}} \) → NL chimeras. Similar to our observation using \( \text{Rc3h1}^{\text{san/san}} \) mice, \( \text{Rc3h1}^{\text{gt/gt}} \) → NL chimeras were devoid of inflammation in the colon (data not shown). \( \text{Rc3h1}^{\text{gt/gt}} \) → NL chimeras also had significantly more lamina propria lymphocytes (LPLs) than NL → NL mice (Fig. 1e).

To understand the mechanisms that underlie the intestinal inflammatory response in \( \text{Rc3h1}^{\text{gt/gt}} \) → NL chimeras, LPLs from \( \text{Rc3h1}^{\text{gt/gt}} \) → NL chimeras and NL → NL chimeras were studied for expression of IL-17A, IFN\( \gamma \), TNF\( \alpha \), and IL-10 synthesis by intracellular staining. \( \text{Rc3h1}^{\text{gt/gt}} \) → NL chimeras (Fig. 2b and c) had increased numbers of CD44\( ^{+} \), CD62L\( ^{+} \), and ICOS\( ^{+} \) producing cells compared to NL → NL mice and NL chimeras. Similar to our observation using \( \text{Rc3h1}^{\text{san/san}} \) mice4,7; Ki67 is a marker of cell proliferation10. The majority of KLRG1\( ^{+} \) cells in all four groups of mice were ICOS\( ^{+} \)CD62L\( ^{+} \) cells (Fig. 3a–d, top histogram panel); a characteristic of intestinal leukocytes due to the low expression of CD62L needed to facilitate migration of cells from lymph nodes to the intestine. Of interest was the finding that, compared to NL mice and NL → NL chimeras, \( \text{Rc3h1}^{\text{gt/gt}} \) → NL chimeras had proportionally more CD44\( ^{+} \)CD62L\( ^{+} \) KLRG1\( ^{+} \) SLECs (Fig. 3a–d, lower histogram panel and Fig. 3e), indicating that there was an influx of SLECs into the lamina propria of both \( \text{Rc3h1}^{\text{gt/gt}} \) mice and \( \text{Rc3h1}^{\text{gt/gt}} \) → NL chimeras.

**KLRG1\(^{+}\) LPLs are non-proliferating ICOS\(^{+}\) cells that are predominantly effector/memory cells.** To further characterize the KLRG1\(^{+}\) cell population, LPLs were stained for expression of ICOS and Ki67. ICOS expression is increased in \( \text{Rc3h1}^{\text{san/san}} \) mice\(^{9}\); Ki67 is a marker of cell proliferation\(^{10}\). The majority of KLRG1\(^{+}\) cells in all four groups of mice were ICOS\(^{+}\) (Fig. 4a–d, top histogram panel); however, there was an increase in the overall proportion of ICOS\(^{+}\) cells in \( \text{Rc3h1}^{\text{gt/gt}} \) mice and \( \text{Rc3h1}^{\text{gt/gt}} \) → NL chimeras relative to NL mice and NL → NL chimeras (Fig. 4e). Interestingly, KLRG1\(^{+}\) LPLs were primarily non-proliferating cells as seen by a lack of Ki67 staining (Fig. 4a–d, lower histogram panel), and there were fewer

---

**Figure 1** | Histopathological analysis of intestinal tissue sections from (a) \( \text{Rc3h1}^{\text{gt/gt}} \) mice, (b) \( \text{Rc3h1}^{\text{gt/gt}} \) → NL chimeras, and (c) NL → NL chimeras for the duodenum, jejunum, ileum, and cecum. Note the areas of inflammation (boxed regions) in intestinal tissues of \( \text{Rc3h1}^{\text{gt/gt}} \) and \( \text{Rc3h1}^{\text{gt/gt}} \) → NL chimeras. Micrographs are 100 × original magnification; scale bars are 100 μm. (d) Mean pathology scores ± SEM of 8 \( \text{Rc3h1}^{\text{gt/gt}} \) mice, 5 \( \text{Rc3h1}^{\text{gt/gt}} \) → NL chimeras, and 4 NL → NL chimeras. *\( p < 0.05 \), †\( p < 0.01 \) compared to NL → NL chimeras. (e) Mean numbers of small intestine LPLs ± SEM of 5 \( \text{Rc3h1}^{\text{gt/gt}} \) → NL chimeras and 4 NL → NL chimeras. *\( p < 0.05 \) compared to NL → NL chimeras.
proliferating cells overall in Rc3h1<sup>1<sup>opg</sup> mice and Rc3h1<sup>1<sup>opg</sup> → NL chimeras than in normal mice (Fig. 4f). These findings collectively suggest that there is a continual influx of activated SLECs into the lamina propria of Rc3h1<sup>1<sup>opg</sup> mice and Rc3h1<sup>1<sup>opg</sup> → NL chimeras; however, those cells do not undergo appreciable levels of clonal expansion once they have reached the intestinal mucosa. This is consistent with the observation that KLRG1<sup>1<sup>+ cells experience a wave of proliferation during the inductive phase of activation but are generally proliferation non-responsive once they have entered the effector phase<sup>9</sup>.

KLRG1<sup>1<sup>+ cells have been used to differentiate central/memory and effector/memory cells based on expression of the CCR7 chemokine receptor. KLRG1<sup>1<sup>−CCR7<sup>1<sup>− cells are considered to be central/memory cells; KLRG1<sup>1<sup>−CCR7<sup>1<sup>− cells are considered to be effector/memory cells<sup>1<sup>. The ratio of effector/memory to central/memory KLRG1<sup>1<sup>+ cells in the present study was ~3-fold greater regardless of the type of animal (Table 1). Thus, the lamina propria consists of a population of effector/memory cells, which increase in numbers in mice with chronic small intestinal inflammation due to an absence of Roquin-mediated control.

**Chronic systemic inflammation is present in Rc3h1<sup>1<sup>opg and Rc3h1<sup>1<sup>opg → NL chimeras.** Chronic inflammatory responses also were evident in extra-intestinal tissues, most notably in the liver and spleen and to a lesser extent in the kidney and lung of Rc3h1<sup>1<sup>opg and Rc3h1<sup>1<sup>opg → NL chimeras (Fig. 5, rows a and b, respectively), but was absent in the liver, spleen, kidney, and lung of NL → NL chimeras (Fig. 5, row c). Liver pathology consisted of intense mononuclear leukocytic infiltrate around the central vein and portal tract. The average liver pathology score of Rc3h1<sup>1<sup>opg and Rc3h1<sup>1<sup>opg → NL chimeras is shown in Fig. 5d, which indicates that there was more severe liver pathology in Rc3h1<sup>1<sup>opg mice compared to Rc3h1<sup>1<sup>opg → NL chimeras.

Rc3h1<sup>1<sup>opg and Rc3h1<sup>1<sup>opg → NL chimeras had chronic nephritis characterized by perivascular and interstitial mononuclear inflammatory infiltrate (Fig. 5, rows a and b, respectively). Necrotic hepatocytes associated with lymphocytic infiltrates were seen within the lobules without piecemeal necrosis. Lung tissue sections revealed lymphocytic pneumonitis characterized by perivascular and interstitial lymphocytic infiltrate with focal areas of interstitial fibrosis (Fig. 5, rows a and b, respectively). Splenic follicular hyperplasia, an immunopathological feature of Rc3h1<sup>1<sup>1<sup>opg<sup>1<sup>− mice, was evident in Rc3h1<sup>1<sup>opg and Rc3h1<sup>1<sup>opg → NL chimeras (Fig. 5, rows a and b, respectively). Although overall more Rc3h1<sup>1<sup>opg mice had inflammation in the liver, kidney, spleen, and lung than Rc3h1<sup>1<sup>opg → NL chimeras, that difference was not statistically significant (Fig. 5e). In contrast, slightly more Rc3h1<sup>1<sup>opg → NL chimeras had inflammation in the ileum compared to Rc3h1<sup>1<sup>opg mice, although this also was not statistically significant (Fig. 5e).

Caspase 3, a downstream effector caspase, plays a central role mediating apoptosis in a wide variety of cells. Hence, immunodetection of activated (cleaved) caspase 3 is frequently used to identify apoptosis in situ in tissue sections. Areas of cleaved caspase-3 positive hepatocytes were noted within the lobules and around the central veins and portal tract in the liver of Rc3h1<sup>1<sup>opg and Rc3h1<sup>1<sup>opg → NL chimeras, but not NL → NL chimeras (Fig. 6a). This was the predominant area of chronic inflammatory cells infiltrate in liver sections (Fig. 5, liver panels a and b). These findings indicate that immune effector cells trigger apoptosis in adjacent hepatocytes.

Based on the above findings that Rc3h1<sup>1<sup>opg and Rc3h1<sup>1<sup>opg → NL chimeras developed spontaneous perivascular and peribronchiolar lymphoid lung hyperplasia, whereas the lungs of NL → NL chimeras
had minimal inflammation, we analyzed the immunophenotypes of the pulmonary infiltrating cells by staining for the presence of CD3+ T cells and CD20+ B cells. Perivascular and peribronchiolar hyperplastic lymphoid cells in Rc3h1gt/gt and Rc3h1gt/gt RNL chimeras were composed predominantly of T cells (≈60%) and B cells (≈40%) (Fig. 6, panels b and c). In contrast, peribronchiolar and lobular inflammatory cells in NL → NL chimeras were mostly T cells (≈90%) with a very few B-cells (≈5%) (Fig. 6, panel c). These findings indicate that the lymphocytic infiltrate in the lungs of Rc3h1gt/gt and Rc3h1gt/gt RNL chimeras consist of lymphoid hyperplasia rather than lymphoma or prelymphoma neoplasia.

Collectively, these findings demonstrate that mice generated from BM of animals with targeted disruption of the Rc3h1 gene develop extensive inflammation similar to that of Rc3h1san/san and Rc3h1gt/gt mice7. Because the Rc3h1 gene was unaltered in non-hematopoietic cells of Rc3h1gt/gt RNL chimeras, the pathology which ensued in Rc3h1gt/gt mice was attributable to the inflammatory response generated from cells of the immune system.

Oral infection of Rc3h1gt/gt → NL chimeras with L. monocytogenes results in more liver pathology and greater numbers of bacteria in the Peyer’s patches. We were interested in determining the extent to which the host response to an infectious agent, in this case L. monocytogenes, would differ in Rc3h1gt/gt → NL chimeras and NL → NL chimeras. Groups of each type of animal were infected with bacteria12 as described in the Methods. Rc3h1gt/gt → NL chimeras had increased numbers of L. monocytogenes in Peyer’s patches at day 2 post-infection (Fig. 7a), and greater liver pathology at day 4 post-infection (Fig. 7b), indicating that mice with ablated Rc3h1 gene had a compromised response to oral L. monocytogenes infection.

**Discussion**

There is rapidly growing interest in Roquin due to its role in regulating the immune response and curtailing autoimmune pathology. Our recent studies were the first to examine the intestinal inflammatory response in Rc3h1san/san mice, which have a mutation in the Roquin protein, and in Rc3h1gt/gt mice, which have a disruption of the Rc3h1 gene7. Both types of animals developed small intestinal inflammation in the ileum as well as in the duodenum and jejunum, though not in the colon7. The targeted feature of the inflammatory response to the small intestine in Roquin-mutant and Roquin-deficient mice is of particular significance given the lack of available animal models of Crohn’s disease in the small intestine. Small intestine inflammation develops in TNF−/− mice due to high levels of TNF expression13,14, and in some mouse strains following parasitic infection15. Additionally, SAMP1/Yit mice spontaneously develop ileitis by 20–30 weeks of age16,17. Those animals are limited for studies into the underlying molecular basis of disease, however, because the genetic defect...
has not been sufficiently characterized despite some progress in that area\(^\text{18}\). By contrast, the \(Rc3h1\) gene and its paralog, \(Rc3h2\), are well defined. The full gene and protein sequences are available and the chromosomal locations are known. The miR-223 microRNA has been shown to repress \(Rc3h1\) gene expression\(^\text{19}\), thereby providing additional information into the regulatory elements that control Roquin. Thus, the Roquin experimental system is ideal for studies into the molecular basis of autoimmunity in the gut and elsewhere.

The mode of action whereby Roquin represses inflammation in the intestine may involve suppression of IL-17 synthesis. Studies in our laboratory previously demonstrated an opposing relationship between Roquin expression and IL-17 production in the intestine\(^\text{19}\). We also observed that enforced expression of Roquin in IL-17-producing EL4 cells results in repressed IL-17 output (unpublished). This was confirmed in the present study by the experiments showing an increase in IL-17, IFN\(_\gamma\), and TNF\(_\alpha\) synthesis in \(Rc3h1^{gt/gt}\) mice compared to their respective controls \((p < 0.05)\); mean values \(\pm\) SEM of 2–3 samples per group. LPLs from \(Rc3h1^{gt/gt}\) mice and \(Rc3h1^{gt/gt}\) mice have proportionally fewer Ki67\(^+\) proliferating cells compared to their respective controls \((p < 0.01)\). Mean values \(\pm\) SEM of 2–3 samples per group.

There were notable differences in the degree of immunopathology in the \(Rc3h1^{gt/gt}\) mice made in our laboratory and mice with a disrupted \(Rc3h1\) gene produced by others. In an initial study, mice with \(Rc3h1\) gene ablation failed to develop autoimmunity despite high post-birth mortality\(^\text{8}\). In two subsequent studies, a \(Rc3h1^{san/san}\)-like phenotype was present in mice with combined disruption of \(Rc3h1\) and its paralog, \(Rc3h2\)\(^\text{20,21}\). Although the basis for the differences between those findings and ours remain unclear, \(Rc3h1^{gt/gt}\) mice made in our laboratory consistently and reliably developed small intestinal inflammation, particularly in the ileum where 92.3% of \(Rc3h1^{gt/gt}\) mice and \(Rc3h1^{gt/gt}\) mice developed an inflammatory response.

Because \(Rc3h1^{gt/gt}\) mice made in our laboratory were generated by a random insertion of a gene-trap into the murine genome, the likelihood of simultaneous insertion into both the \(Rc3h1\) and \(Rc3h2\) genes was extremely remote. We therefore conclude, based on the studies here and our previous work\(^\text{7}\), that ablation of only the \(Rc3h1\) gene is by itself sufficient to render an autoimmune phenotype in mice. It should be noted, however, that Roquin-1 and Roquin-2 expression were reported to be variable in mice throughout various immunological compartments\(^\text{21}\). If, in fact, Roquin-2 is capable of compensating for and mollifying the autoimmune response caused by deregulation of Roquin-1, as suggested\(^\text{20,21}\), tissues with low Roquin-2 synthesis would be particularly prone to autoimmunity while other tissues may be spared. As yet unknown tissue-specific cofactors also may be necessary for optimal Roquin-mediated repression\(^\text{21}\).

KLRG1 is a type 2 integral membrane protein of the C-type lectin family that has been linked to a number of function-related activities. In mice, KLRG1 is expressed on NK cells and also defines a set of T cells that have effector activity but lack antigen-induced proliferation\(^\text{21}\). E-cadherin, the KLRG1 ligand, is expressed on epithelial cells

| Table 1 | Ratio of KLRG1\(^+\)CCR7\(^+\) Effector/Memory:KLRG1\(^+\)CCR7\(^-\) Central/Memory LPLs |
|----------|--------------------------------------------------|
| NL       | \(Rc3h1^{gt/gt}\) | \(Rc3h1^{gt/gt}\)→NL | NL→NL |
| 3.30 ± 1.32\(^\text{a,b}\) | 3.43 ± 1.5\(^\text{a,b}\) | 3.93 ± 0.66\(^\text{a,b}\) | 3.15 ± 0.22\(^\text{a,b}\) |

\(^\text{a}\)Mean values \(\pm\) SEM.

\(^\text{b}\)\(p\) > 0.05 compared to other animal groups.
and on some classical antigen presenting cells, in particular Langerhans cells. E-cadherin is expressed at high levels on the intestinal epithelium, thus providing continual expression of the KLRG1 ligand. Blockade of E-cadherin has been shown to enhance T cell proliferation. It was recently demonstrated that treatment of mice with an agonistic antibody to the 4-1BB T cell activation marker induced a novel population of KLRG1 effector cells to infiltrate melanoma tumors. The finding reported here that proportionally…

Figure 5 | Histopathological analysis of tissue sections from the kidney, lung, liver, and spleen from (a) Rc3h1^p/e^p mice, (b) Rc3h1^p/e^p → NL chimeras, and (c) NL → NL chimeras. Inflammation was most common in the liver of Rc3h1^p/e^p and Rc3h1^p/e^p → NL chimeras, and was less common in the kidney, lung, and spleen. Inflammation was absent in the kidney, lung, and liver of NL → NL chimeras. Micrographs are 100 × original magnification; scale bars are 100 μm. (d) Mean liver scores ± SEM of 10 Rc3h1^p/e^p mice and 8 Rc3h1^p/e^p → NL chimeras. *p < 0.05 comparing Rc3h1^p/e^p mice and Rc3h1^p/e^p → NL chimeras. (e) Percent of mice with inflammation in the liver, kidney, spleen, lung, and ileum of Rc3h1^p/e^p mice compared to Rc3h1^p/e^p → NL chimeras.

Figure 6 | (a) Caspase 3 expression in the liver of Rc3h1^p/e^p mice (GT), Rc3h1^p/e^p → NL chimeras (GT → NL), and NL → NL chimeras, showing increased caspase staining in mice with ablated Rc3h1. Lung tissues in the three groups of mice stained for the presence of (b) CD20 B cells and (c) CD3 T cells. Note the abundance of B cells in mice with ablated Rc3h1 compared to lung tissue from control mice. Micrographs are 100 × original magnification; scale bars are 100 μm.
animal counterparts were Ki67
demonstrate that Roquin has the capacity to control chronic inflam-
exist for sustained Roquin suppression. The studies reported here

SCIENTIFIC

Rc3h1gt/gt

CCR7 expression11.

cells were predominantly effector/memory cells based on the lack of
is consistent with the KLRG1 phenotype. Aligned with that, KLRG1
expression of Roquin resulted in repressed ICOS expression, CD28
remains unclear; however, it could reflect the concomitant genera-
capable of mounting an antigen-reactive response. The basis for this
developed a strong intestinal inflammatory response, they were less

Figure 7 | Effect of oral infection in Rc3h1gp/gp → NL chimeras, Rc3h1gp/gp
NL chimeras and NL → NL chimeras were infected orally with L. monocytagenes as described in the Methods. Rc3h1gp/gp → NL chimeras had (a) more bacteria in the Peyer’s patches, and (b) more liver pathology than NL → NL chimeras. * p < 0.05. Mean values ± SEM of 3 mice per group.

more CD44hi CD62Llo KLRG1+ cells infiltrate the lamina propria of Rc3h1gp/gp mice and Rc3h1gp/gp → NL chimeras than normal mice is consistent with an increase in effector T cells and provides a logical explanation for the basis of small intestinal inflammation in those animals. This is further reinforced by our observation of increased ICOS expression on KLRG1− cells. That most KLRG1− cells in Rc3h1gp/gp mice and Rc3h1gp/gp → NL chimeras as well as their normal animal counterparts were K667− and thus non-proliferating cells also is consistent with the KLRG1 phenotype. Aligned with that, KLRG1− cells were predominantly effector/memory cells based on the lack of CCK7 expression11.

Rc3h1gp/gp → NL chimeras were more susceptible to oral infection with L. monocytagenes than NL → NL chimeras as determined by the presence of liver pathology and the numbers of bacteria in the Peyer’s patches, suggesting that although Rc3h1gp/gp → NL chimeras developed a strong intestinal inflammatory response, they were less capable of mounting an antigen-reactive response. The basis for this remains unclear; however, it could reflect the concomitant generation of suppressive effector cells. Additional studies will be needed to address this.

Finally, it is worth noting that the full functional role of Roquin remains to be elucidated. It is possible, for example, that the evolutionary adaptation of Roquin is primarily geared toward activating the immune system in a beneficial manner rather than suppressing it. This could occur by repressing Roquin expression during the early phase of an immune response, thus leading to increased expression of ICOS and OX40, two key molecules used during T cell activation. In that context, it was recently demonstrated that although enforced expression of Roquin resulted in repressed ICOS expression, CD28 expression and CD28-mediated immunity were concomitantly augmented. Clearly, autoimmunity could develop if sufficient stimuli exist for sustained Roquin suppression. The studies reported here demonstrate that Roquin has the capacity to control chronic inflammation in the intestine and elsewhere. Additional experimental work using mice with defective Roquin expression will be useful for addressing this Roquin paradox.

Methods

Rc3h1gp/gp mice and BM radiation chimeras. The method used to generate Rc3h1gp/gp mice has been previously described. Animals were bred to homozygosity for more than nine generations onto a C57Bl/6 background. Hematopoietic radiation chimeras were made by injecting 5 × 10⁸ 10⁹ BM cells from Rc3h1gp/gp mice into NL syngeneic gender-matched mice by retro-orbital injection within 4 hrs of 900 cGy total body irradiation (Rc3h1gp/gp → NL chimeras). Control radiation chimeras were generated using NL C57Bl/6 BM injected into syngeneic gender-matched mice (NL → NL chimeras). C57Bl/6 mice were purchased from Harlan (Indianapolis, IN). Mice were used in accord with the University of Texas Health Science Center at Houston Institutional Animal Welfare Committee. The use of animals for the experiments conducted in this study was approved by permit No. HSC-AWC-12-039 of the University of Texas Health Science Center at Houston Animal Welfare Committee.

Histopathological analyses. Representative H&E stained tissue sections from the duodenum, jejunum, ileum, cecum, ascending colon, transverse colon, descending colon, liver, kidney, lung, and spleen were examined by a board-certified pathologist blinded to the studied groups. Intestinal inflammation and associated villi injury for each section was graded by summing up the scores for inflammation and villi and crypt injury as described previously. The degree of inflammation and hepatocyte necrosis in liver sections was graded using a modified scoring system as described previously. Tissue sections of lung and kidney were examined for inflammation, tissue injury, and fibrosis.

Cell isolation and staining. Intestinal lamina propria leukocytes (LPLs) were isolated and stained as previously reported. Antibodies used were: PE-anti-CD8α (53-6-7); PE-anti-CD278 (ICOS) (7E.17G9); FITC-anti-CD44 (IM7); PE-anti-Ki67 (SolA15); Anti-KLRG1 647- anti-Ki67 (SolA15); APC-anti-KLRG1 (2F1); APC- and FITC-anti-

1. Gecheanov, A. The ubiquitin proteasome pathway: on protein death and cell life. Embo J 17, 7151–7160 (1998).
2. Lin, A. E. & Mak, T. W. The role of E3 ligases in autoimmunity and the regulation of autoreactive T cells. Curr Opin Immunol 19, 665–673 (2007).
3. Vinuesa, C. G. et al. A RING-type ubiquitin ligase family member required to

4. Yu, D. Roquin represses autoimmunity by limiting inducible T-cell co-

5. Heissmeyer, V. Molecular control of Tfh-cell differentiation by Roquin family proteins.

6. MacDonald, T. T. & Carter, P. B. Cell-mediated immunity to intestinal infection.

7. Bertossi, A.

8. Lin, A. E. & Mak, T. W. The role of E3 ligases in autoimmunity and the regulation of autoreactive T cells. Curr Opin Immunol 19, 665–673 (2007).
9. Vinuesa, C. G. et al. A RING-type ubiquitin ligase family member required to

10. Scholzen, T. & Gerdes, J. The Ki-67 protein: from the known and the unknown.

11. MacDonald, T. T. & Carter, P. B. Cell-mediated immunity to intestinal infection. Infection Immun 28 (1980).

of the University of Texas Health Science Center at Houston Animal Welfare Committee.
13. Kontoyiannis, D., Pasparakis, M., Pizarro, T. T., Cominelli, F. & Kolls, G. Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. *Immunity* **10**, 387–398 (1999).

14. Armaka, M. *et al.* Mesenchymal cell targeting by TNF as a common pathogenic principle in chronic inflammatory joint and intestinal diseases. *J Exp Med* **205**, 331–337 (2008).

15. Egan, C. E., Cohen, S. B. & Denkers, E. Y. Insights into inflammatory bowel disease using *Toxoplasma gondii* as an infectious trigger. *Immunol Cell Biol* **90**, 668–675 (2012).

16. Rivera-Nieves, J. *et al.* Emergence of perianal fistulizing disease in the SAMP1/YitFc mouse, a spontaneous model of chronic ileitis. *Gastroenterology* **124**, 972–982 (2003).

17. Matsumoto, S. *et al.* Inflammatory bowel disease-like enteritis and caecitis in a senescence accelerated mouse P1/Yit strain. *Gut* **43**, 71–78 (1998).

18. Kozaiwa, K. *et al.* Identification of a quantitative trait locus for ileitis in a spontaneous mouse model of Crohn’s disease: SAMP1/YitFc. *Gastroenterology* **125**, 477–490 (2003).

19. Schaefer, J. S., Montufar-Solis, D., Vigneswaran, N. & Klein, J. R. Selective upregulation of microRNA expression in peripheral blood leukocytes in IL-10-/- mice precedes expression in the colon. *J Immunol* **187**, 5834–5841 (2011).

20. Pratama, A. *et al.* Roquin-2 shares functions with its paralog Roquin-1 in the repression of mRNAs controlling T follicular helper cells and systemic inflammation. *Immunity* **38**, 669–680 (2013).

21. Vogel, K. U. *et al.* Roquin paralogs 1 and 2 redundantly repress the Icos and OX40 costimulator mRNAs and control follicular helper T cell differentiation. *Immunity* **38**, 655–668 (2013).

22. Hensson, S. M. & Akbar, A. N. KLRG1--more than a marker for T cell senescence. *Age* **31**, 285–291 (2009).

23. Bagratiak, E. U., Tang, M., Wang, H. C. & Klein, J. R. CD43 potentiates CD3-induced proliferation of murine intestinal intraepithelial lymphocytes. *Immunol Cell Biol* **79**, 303–307 (2001).

24. Curran, M. A. *et al.* Systemic 4-1BB activation induces a novel T cell phenotype driven by high expression of Eomesodermin. *J Exp Med* **210**, 743–755 (2013).

25. Kim, H. J. *et al.* The role of Roquin overexpression in the modulation of signaling during in vitro and ex vivo T-cell activation. *Biochem Biophys Res Commun* **417**, 280–286 (2012).

26. Yardeni, T., Eckhaus, M., Morris, H. D., Huizing, M. & Hoogstraten-Miller, S. Retro-orbital injections in mice. *Lab Anim* **40**, 155–160 (2011).

27. Gown, A. M. & Willingham, M. C. Improved detection of apoptotic cells in archival paraffin sections: immunohistochemistry using antibodies to cleaved caspase 3. *J Histochem Cytochem* **50**, 449–454 (2002).

28. Schaefer, J. S., Montufar-Solis, D., Vigneswaran, N. & Klein, J. R. ICOS promotes IL-17 synthesis in colonic intraepithelial lymphocytes in IL-10-/- mice. *J Leuk Biol* **87**, 301–308 (2010).

**Author contributions**

J.R.K. conceived and designed the experiments, D.M.-S., N.V., N.N. and J.S.S. performed the experiments. J.R.K., N.V., D.M.-S., J.S.S. and N.N. analyzed the data. J.R.K., N.V. and J.S.S. wrote the paper.

**Additional information**

This study was supported by a grant from the Crohn’s and Colitis Foundation of America and NIH Grants DK035566 and AI100159.

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Montufar-Solis, D., Vigneswaran, N., Nakra, N., Schaefer, J.S. & Klein, J.R. Hematopoietic not systemic impairment of Roquin expression accounts for intestinal inflammation in Roquin-deficient mice. *Sci. Rep.* **4**, 4920; DOI:10.1038/srep04920 (2014).

This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivs 3.0 Unported License. The images in this article are included in the article’s Creative Commons license, unless indicated otherwise in the image credit; if the image is not included under the Creative Commons license, users will need to obtain permission from the license holder in order to reproduce the image. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-nd/3.0/