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Host-Derived Nitrate Boosts Growth of *E. coli* in the Inflamed Gut

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Changes in the microbial community structure are observed in individuals with intestinal inflammatory disorders. These changes are often characterized by a depletion of obligate anaerobic bacteria, whereas the relative abundance of facultative anaerobic Enterobacteriaceae increases. The mechanisms by which the host response shapes the microbial community structure, however, remain unknown. We show that nitrate generated as a by-product of the inflammatory response conferred a growth advantage to the commensal bacterium *Escherichia coli* in the large intestine of mice. Mice deficient in inducible nitric oxide synthase did not support the growth of *E. coli* by nitrate respiration, suggesting that the nitrate generated during inflammation was host-derived. Thus, the inflammatory host response selectively enhances the growth of commensal Enterobacteriaceae by generating electron acceptors for anaerobic respiration.

Over 90% of the cells in the human body are microbes, the majority of which reside in the large intestine, where they provide benefit to the host by stimulating the development of the immune system, supplying nutrients, and providing niche protection. The lumen of the large bowel is thought to be primarily anaerobic, with traces of oxygen being consumed by facultative anaerobic bacteria (such as Enterobacteriaceae), which constitute a small fraction (approximately 0.1%) of the microbial community (microbiota) (*1*). The vast majority of microbes in the large intestine belong to the phyla Bacteroidetes (class Bacteroidia) and Firmicutes (class Clostridia), two groups of obligate anaerobic bacteria that lack the ability to respire and instead rely on the fermentation of amino acids and complex polysaccharides for growth. On the phylum level, this bacterial community structure is conserved between humans and mice (*1*, *2*). Conditions of inflammation in the large bowel are accompanied by a microbial imbalance (dysbiosis), however, which is characterized by a marked decrease in the representation of obligate anaerobic bacteria and an increased relative abundance of facultative anaerobic bacteria belonging to the family Enterobacteriaceae (*3*–*12*) (fig. S1, A and B).

An important component of the host inflammatory response is the generation of reactive nitrogen species (RNS) and reactive oxygen species (ROS) (fig. S1C). For example, inducible nitric oxide synthase (iNOS) is expressed at high levels during intestinal inflammation, and elevated nitric oxide (NO·) concentrations are detected in the colonic luminal gas of individuals with inflammatory bowel disease (*13*–*15*). The reaction of nitric oxide radicals (NO·) with superoxide radicals ($O_2^{\cdot-}$) yields peroxynitrite ($ONOO^-$), which can either generate nitrate (NO_3^-) (*16*) or oxidize organic sulfides and tertiary amines to S-oxides and N-oxides (*17*, *18*). Similarly, inflammation-derived ROS can generate S-oxides and N-oxides (*17*, *18*). Unlike obligate anaerobic members of the gut microbiota, the facultative anaerobic Enterobacteriaceae can use nitrate, S-oxides, and N-oxides as terminal electron acceptors for anaerobic respiration. We thus hypothesized that colitis produces dysbiosis because highly oxidized by-products of intestinal inflammation (such as nitrate, S-oxides, and N-oxides) might enable commensal Enterobacteriaceae to edge out fermenting microbes in the gut lumen by using anaerobic respiration for energy production (fig. S1C).

Escherichia coli, a prototypic member of the Enterobacteriaceae, possesses three nitrate reductases, two S-oxide reductases, and three N-oxide reductases encoded by the *narGHJI*, *narZYWV*, *napFDAGHBC*, *dmsABC*, *ynfDEFGH*, *torCAD*, *torYZ*, and *yedYZ* operons, respectively (*19*). One common feature shared by these terminal reductases is the incorporation of an

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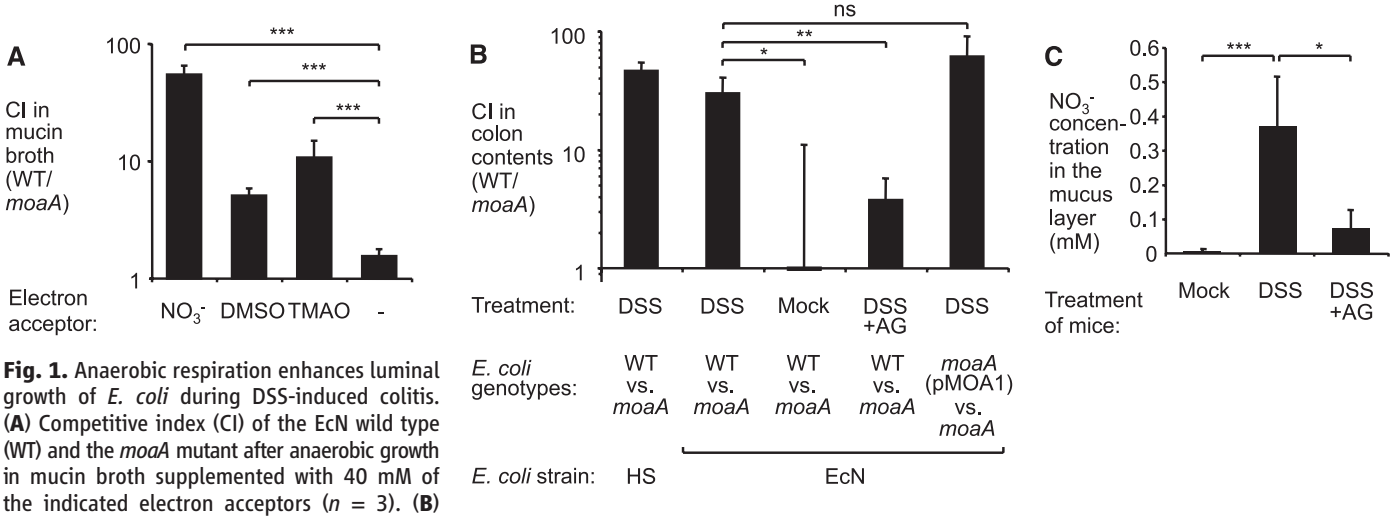

Fig. 1. Anaerobic respiration enhances luminal growth of *E. coli* during DSS-induced colitis. (**A**) Competitive index (CI) of the EcN wild type (WT) and the *moaA* mutant after anaerobic growth in mucin broth supplemented with 40 mM of the indicated electron acceptors (n = 3). (**B**) Mock-treated mice (Mock), DSS-treated mice (DSS), or mice treated with DSS and AG (DSS+AG) were inoculated with the indicated mixtures of *E. coli* strains, and the CI in colon contents was determined 5 days after inoculation. A plasmid (pMOA1) carrying the cloned *moaA* gene was used to complement the *moaA* mutant (*moaA*). The number of animals (n) is given in fig. S3C. (**C**) Concentration of nitrate (NO_3^-) determined in the cecal mucus layer of mock-treated mice (n = 4), DSS-treated mice (DSS, n = 3), or mice treated with DSS+AG (n = 4). Bars represent geometric means ± SE. *P < 0.05, **P < 0.01, ***P < 0.001; ns, not statistically significant (Student's t test).

essential molybdenum cofactor into the active site. To test the idea that anaerobic respiration provides a growth benefit in the inflamed intestine, we generated mutants deficient for the biosynthesis of the molybdenum cofactor (*moaA* mutants) in the *Escherichia coli* strains HS and Nissle 1917 (EcN) (fig. S2, A and B). These *moaA* mutants were anaerobically cocultured with the respective wild-type strains in mucin broth in the presence or absence of nitrate, DMSO (dimethyl S-oxide), or TMAO (trimethylamine N-oxide) (Fig. 1A and fig. S2C). Enrichment for the *E. coli* wild-type strains occurred in the presence of nitrate, DMSO, and TMAO, suggesting that anaerobic respiration can provide a growth benefit during the anaerobic growth conditions encountered in the intestinal mucus layer.

We next inoculated untreated mice (C57BL/6 mice) or mice with chemically induced colitis [induced by treatment with dextran sulfate sodium (DSS)] intragastrically with an equal mixture of EcN and its isogenic *moaA* mutant (fig. S1D). Both the wild-type strain and the *moaA* mutant colonized the intestine of mock-treated mice poorly, but similar numbers of each strain were recovered from colon contents 5 days after inoculation (Fig. 1B). This result suggested that in the absence of intestinal inflammation, anaerobic respiration did not provide a growth benefit for *E. coli*. DSS treatment induced inflammation in the colon and increased mRNA levels of proinflammatory markers in wild-type mice (fig. S3). In contrast to mock-treated mice, the EcN wild-type strain was recovered from colon contents of DSS-treated mice in significantly higher numbers than the *moaA* mutant 5 days after inoculation. Similar results were observed when DSS-treated mice were inoculated with an equal mixture of the human commensal *E. coli* strain HS and an isogenic *moaA* mutant. Expression of *moaA* from a low–copy-number plasmid (pMOA1) in the EcN *moaA* mutant fully restored the phenotype to wild-type levels. Outgrowth of the EcN wild-type strain over the *moaA* mutant was also observed in the DSS colitis model when mice were precolonized with *E. coli* (fig. S4). These findings supported the idea that anaerobic respiration provided a growth advantage to commensal *E. coli* during intestinal inflammation.

Although ROS can be generated by several NADPH (reduced nicotinamide adenine dinucleotide phosphate) oxidases, the sole source of NO during inflammation is iNOS. To determine the contribution of RNS to the growth advantage mediated by anaerobic respiration, DSS-treated mice were treated with the iNOS inhibitor aminoguanidine hydrochloride (AG) and inoculated with a mixture of the EcN wild type and the *moaA* mutant. Consistent with the idea that RNS are a significant source for the production of terminal electron acceptors during inflammation, the growth advantage of the EcN wild type over the *moaA* mutant in the DSS-colitis model was significantly ($P < 0.01$) blunted after AG treatment (Fig. 1B). The nitrate/nitrite redox couple has a greater redox potential than the DMSO/DMS or the TMAO/TMA redox couples, which makes nitrate the preferred respiratory electron acceptor for the growth of *E. coli* under anaerobic conditions (*19*). Therefore, we next determined whether nitrate becomes available in the lumen of the inflamed intestine. To accomplish this objective, the concentration of nitrate was determined in the cecal mucus layer of mock-treated mice or mice with DSS-induced colitis (Fig. 1C). Whereas nitrate levels were at the limit of detection in mock-treated control mice, a significant ($P < 0.001$) increase in nitrate levels was observed in DSS-treated animals. AG treatment of mice with DSS-induced colitis significantly ($P < 0.05$) dampened nitrate production, thus supporting the hypothesis that nitrate is generated in the intestinal lumen as part of the host inflammatory response.

We next tested whether nitrate respiration bestows a growth advantage on *E. coli* wild-type isolates. To this end, we inactivated the *narG*, *napA,* and *narZ* genes, which encode nitrate reductases, in the probiotic EcN. In contrast to the wild-type strain, the nitrate respiration–deficient *narG napA narZ* triple mutant lacked nitrate reductase activity and was outcompeted by the wild-type strain during competitive anaerobic growth in mucin broth in the presence of nitrate (fig. S5). To determine whether nitrate respiration provides a colonization advantage in the intestine, mock-treated and DSS-treated wild-type (C57BL/6) mice were inoculated intragastrically with an equal mixture of the EcN

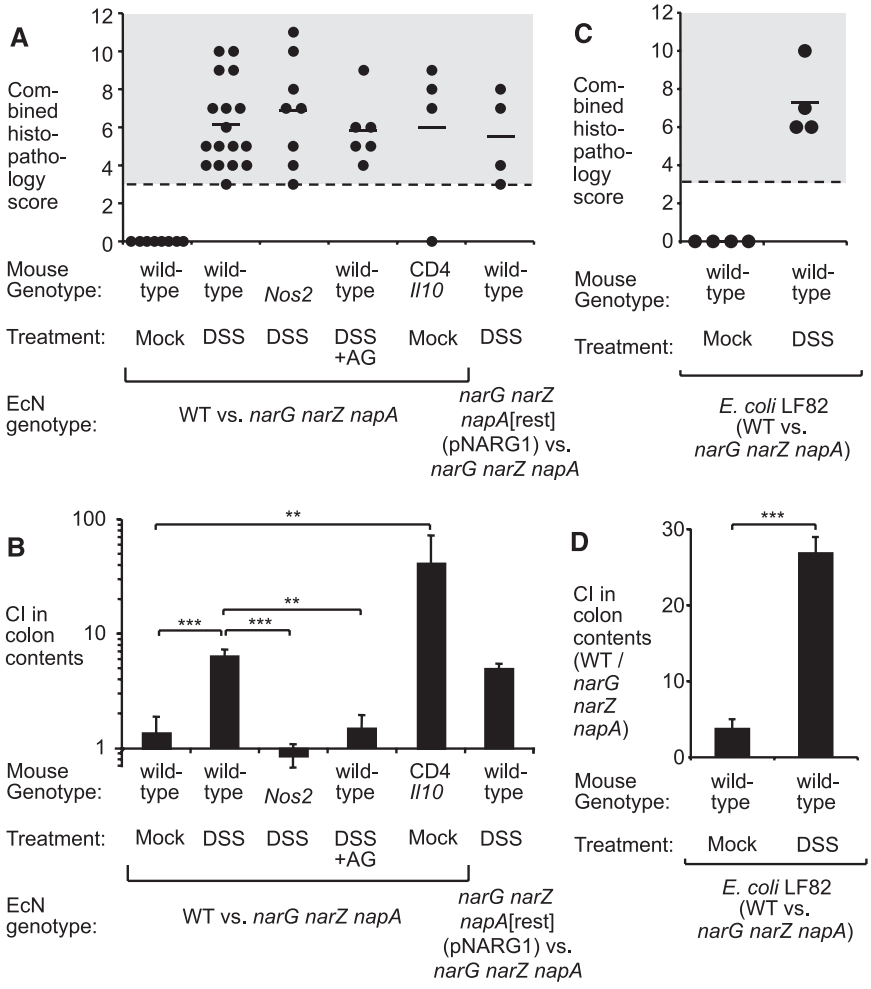

Fig. 2. Wild-type *E. coli* (WT) outcompetes a nitrate respiration–deficient mutant during colitis. Mock-treated (Mock), DSS-treated (DSS), or DSS+AG-treated wild-type mice; *Nos2*-deficient mice (*Nos2*); or mice harboring T cells deficient for the production of IL-10 (*Cd4 Il10* mice) were inoculated with the indicated mixtures of *E. coli* strains. *narG narZ napA*, *E. coli* nitrate respiration–deficient mutant. The *narG narZ napA* mutant was complemented by introducing a functional chromosomal *napA* allele and a plasmid (pNARG1) carrying the cloned *narG* gene [*narG narZ napA*(rest)(pNARG1)]. Pathological changes in the colon (**A** and **C**) and the CI recovered from colon contents (**B** and **D**) were determined 5 days after inoculation. (A) and (C) Combined histopathology score in the colon. Each dot represents data from an individual animal. Experiments were performed with EcN [(A) and (B)] or *E. coli* LF82 [(C) and (D)]. (B) and (D) Bars represent geometric means ± SE. $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t* test). *n* is given in (A) and (C).

wild type and a *narG napA narZ* triple mutant (Fig. 2). In the absence of inflammation (mock treatment, Fig. 2A and figs. S6 and S7), both the EcN wild type and the *narG napA narZ* triple mutant were recovered in similar numbers from colonic (Fig. 2B) and cecal contents (fig. S8A). In contrast, the EcN wild-type strain was more abundant than the *narG napA narZ* triple mutant when colitis was induced by administration of DSS. Similar results were obtained using an adherent invasive *E. coli* (AIEC) isolate (LF82) that was isolated from an inflammatory bowel disease patient (Fig. 2, C and D, and fig. S5, C and D). To test whether nitrate respiration provides a growth benefit in the absence of iNOS-dependent nitrate production, the competitive colonization experiment was repeated in DSS-treated iNOS-deficient mice (i.e., mice carrying a mutation in the *Nos2* gene) and DSS-treated wild-type mice (C57BL/6) that received AG. The severity of the colitis induced by the DSS treatment was similar among all treatment groups (Fig. 2A and fig. S7) 5 days after inoculation with *E. coli*. The EcN wild type and its nitrate respiration–deficient mutant were recovered in equal numbers from DSS-treated iNOS-deficient mice or from DSS+AG-treated wild-type mice. Similar results were obtained using varying concentrations of DSS (fig. S9) as well as with *E. coli* strain K-12 (fig. S10). Concomitant expression of *narG* from a low–copy-number plasmid (pNARG1) and restoration of the *napA* mutation to its wild-type allele *[napA(restored)]* in the *narG narZ napA* mutant reestablished fitness in the inflamed gut to similar levels as observed with the wild-type strain (Fig. 2B and fig. S8A). Collectively, these data suggested that the reduction of host-derived nitrate by *E. coli* confers a growth advantage during gut inflammation.

To validate our findings in a second murine model of colitis, we generated mice that harbored T cells deficient for the production of the anti-inflammatory cytokine interleukin-10 (IL-10) [*Cd4 Il10* mice ($Il10^{flox/flox}$ *Cd4-cre*)], a mouse strain that developed spontaneous colitis (Fig. 2A and S7) (*20*). After the onset of intestinal inflammation, mice were inoculated intragastrically with an equal mixture of the EcN wild type and the *narG napA narZ* triple mutant (Fig. 2B). The nitrate respiration–proficient wild-type strain outcompeted the *narG napA narZ* mutant in the colon contents of *Cd4 Il10* mice 5 days after inoculation ($P < 0.05$). To investigate whether the growth of *E. coli* by nitrate respiration can also be observed in an unrelated animal model of intestinal inflammation, bovine ligated ileal loops were inoculated with thapsigargin, a proinflammatory compound, or mock-treated (vehicle control) (Fig. 3, A to C). At 8 hours after the inoculation of thapsigargin-treated loops with a mixture of EcN and the *narG napA narZ* mutant, significantly ($P < 0.05$) greater numbers of wild-type EcN were recovered from luminal fluid and mucus (Fig. 3D).

To determine whether nitrate respiration increases bacterial recovery from the inflamed intestine when mice are inoculated with a single *E. coli* strain, DSS-treated mice were inoculated either with the EcN wild-type strain or with the *narG napA narZ* mutant. Mice inoculated with EcN or the *narG napA narZ* mutant exhibited a similar severity of colonic inflammation (Fig. 3, E to G). The EcN wild-type strain was recovered in significantly ($P < 0.01$)

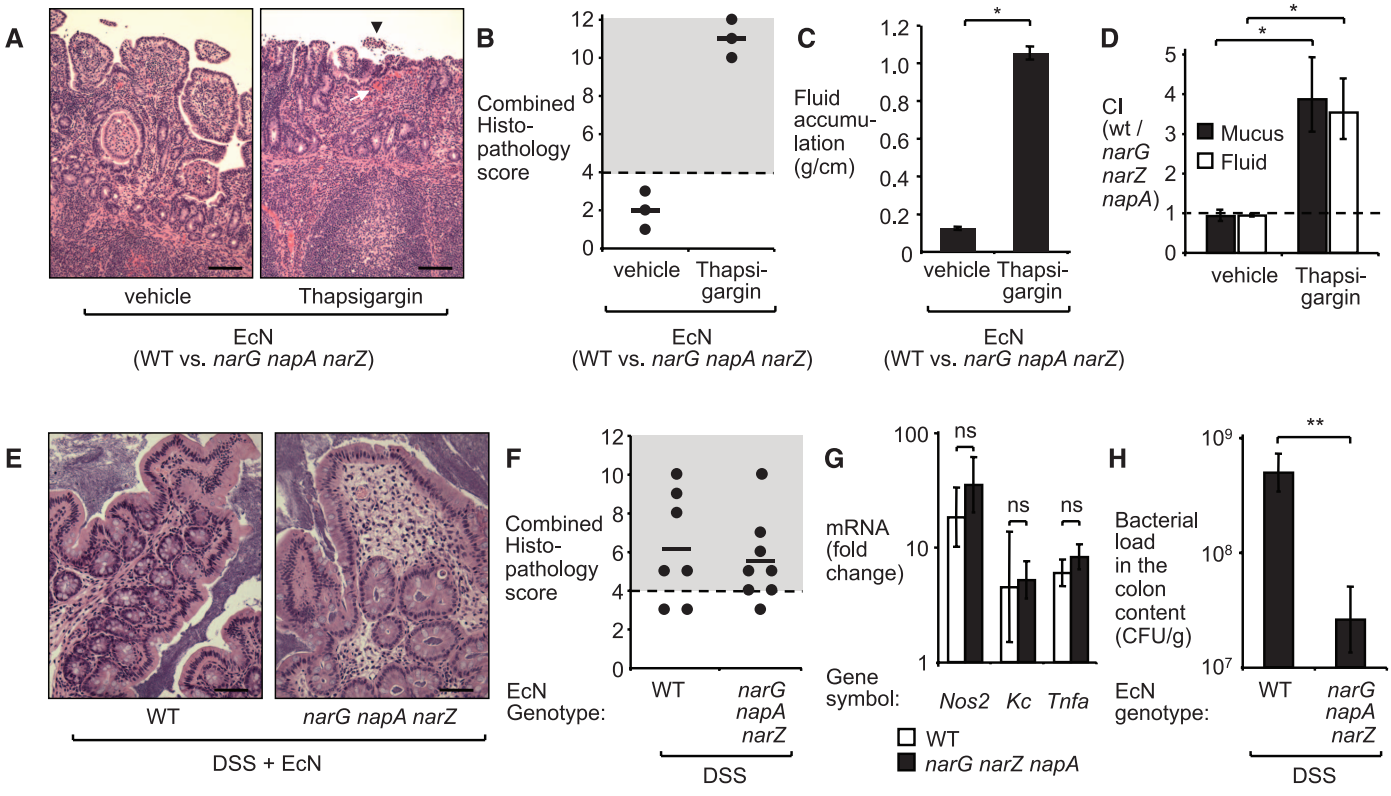

Fig. 3. Nitrate respiration enhances luminal growth of EcN during inflammation. (**A** to **D**) Bovine ligated ileal loops treated with thapsigargin or mock-treated (vehicle) were inoculated with a mixture of EcN (WT) and a nitrate respiration–deficient mutant (*narG napA narZ*). Samples were collected 8 hours after inoculation. (A) Representative hematoxylin and eosin (H&E)–stained ileal sections. Scale bar, 200 μm. (B) Combined histopathology score in the ileum. Each dot represents data from an individual animal. (C) Fluid accumulation in ligated ileal loops. (D) CIs recovered from the luminal fluid (open bars) or mucus scrapings (solid bars). (**E** to **H**) DSS-treated mice were inoculated either with EcN (WT) or with the *narG napA narZ* mutant. Inflammation in the colon (E to G) and bacterial numbers recovered from colon contents (E) were determined 5 days after inoculation. (E) Representative H&E-stained colonic sections. Scale bar, 100 μm. (F) Combined histopathology score in the colon. (G) Expression of *Nos2*, *Kc*, and *Tnfa* in colonic RNA samples analyzed by quantitative real-time polymerase chain reaction (fold increases over mock-treated mice). (H) Bacterial numbers [colony-forming units (CFU)] recovered from colon contents. In (C), (D), (G), and (H), bars represent geometric means ± SE. *$P < 0.05$, **$P < 0.01$; ns, not statistically significant (Student's *t* test). *n* is given in (B) and (F).

higher numbers from colon contents than was the nitrate respiration–deficient mutant (Fig. 3H and fig. S8B). Collectively, these data suggested that nitrate respiration conferred a marked growth advantage on commensal *E. coli* in the lumen of the inflamed gut.

The picture emerging from this study is that nitrate generated as a by-product of the host inflammatory response can be used by *E. coli*, and likely by other commensal Enterobacteriaceae, to edge out competing microbes that rely on fermentation to generate energy for growth. Obligate anaerobic microbes in the intestine compete for nutrients that are available for fermentation but cannot use nonfermentable nutrients (such as fermentation end products). The ability to degrade nonfermentable substrates probably enables *E. coli* to sidestep this competition, which explains the fitness advantage conferred by nitrate respiration in the inflamed gut. Through this mechanism, inflammation contributes to a bloom of nitrate-respiration–proficient Enterobacteriaceae, providing a plausible explanation for the dysbiosis associated with intestinal inflammation (*3*–*12*). This general principle might also influence the dynamics of host-associated bacterial communities outside the large bowel, as nitrate respiration confers a fitness advantage in the oxygen-poor and nitrate-rich environment of the cystic fibrosis airway (*21*).

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Supplementary Materials

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Materials and Methods
Figs. S1 to S11
Tables S1 and S2
References (*22*–*39*)

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Rif1 Prevents Resection of DNA Breaks and Promotes Immunoglobulin Class Switching

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DNA double-strand breaks (DSBs) represent a threat to the genome because they can lead to the loss of genetic information and chromosome rearrangements. The DNA repair protein p53 binding protein 1 (53BP1) protects the genome by limiting nucleolytic processing of DSBs by a mechanism that requires its phosphorylation, but whether 53BP1 does so directly is not known. Here, we identify Rap1-interacting factor 1 (Rif1) as an ATM (ataxia-telangiectasia mutated) phosphorylation-dependent interactor of 53BP1 and show that absence of Rif1 results in 5′-3′ DNA-end resection in mice. Consistent with enhanced DNA resection, Rif1 deficiency impairs DNA repair in the G_1 and S phases of the cell cycle, interferes with class switch recombination in B lymphocytes, and leads to accumulation of chromosome DSBs.

The DNA damage response factor p53 binding protein 1 (53BP1) is a multidomain protein containing a chromatin-binding tudor domain, an oligomerization domain, tandem breast cancer 1 (BRCA1) C-terminal (BRCT) domains, and an N-terminal domain with 28 SQ/TQ potential phosphorylation sites for phosphatidylinositol 3-kinase–related kinases [PIKKs, ataxia-telangiectasia mutated (ATM)/ATM and Rad3-related/DNA-dependent protein kinase catalytic subunit (DNA-PKcs)] (*1*–*3*). 53BP1 contributes to DNA repair in several ways: This protein facilitates joining between intrachromosomal double-strand breaks (DSBs) at a distance (synapsis) (*4*–*7*), it enables heterochromatic DNA repair through relaxation of nucleosome compaction (*2*, *3*), and it protects DNA ends from resection and thereby favors repair of DSBs that occur in G_1 phase by nonhomologous end joining (NHEJ) (*4*, *5*, *8*). Consistent with its role in DNA-end protection, 53BP1 is essential for class switch recombination (CSR) in B lymphocytes (*9*, *10*).

Structure-function studies indicate that, besides the recruitment of 53BP1 to DNA ends, protection requires 53BP1 phosphorylation (*4*), but how this protective effect is mediated is unknown. To identify phosphorylation-dependent interactors of 53BP1, we applied stable isotope labeling by amino acids in cell culture (SILAC). *Trp53bp1*$^{-/-}$ (*Trp53bp1* encodes 53BP1) B cells were infected with retroviruses encoding a C-terminal deleted version of 53BP1 (53BP1^{DB}) or a phosphomutant in which all 28 N-terminal potential PIKK phosphorylation sites were mutated to alanine (53BP1^{DB28A}) (*4*), in media containing isotopically heavy (53BP1^{DB}) or light (53BP1^{DB28A}) lysine and arginine (fig. S1, A to C) (*11*).

Most proteins coprecipitating with 53BP1^{DB} and 53BP1^{DB28A} displayed a $H/(H+L)$ ratio of ~0.5 (H, heavy; L, light), which is characteristic of phospho-independent association (average of 0.57 ± 0.09, peptide count: at least four) (Fig. 1 and table S1). Many of these proteins are nonspecific contaminants, but others such as KRAB-associated protein 1 (KAP-1), dynein light chain LC8-type 1 (Dynll1), Nijmegen breakage syndrome 1 (Nbs1), and H2AX represent authentic phospho-independent 53BP1-interacting proteins (fig. S1D). Three proteins displayed an abundance ratio that was more than four standard deviations (SDs) above the mean, indicating that these proteins interact specifically

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