ily activate ATM through the oxidation pathway. This may be due to trace amounts of Mn$^{3+}$ in preparations of MnCl$_2$ (30).

To further investigate the functional effects of the C2991L mutation, we used lymphoblasts from an A-T patient lacking functional ATM and stably complemented these cells with either wild-type or C2991L alleles of ATM under the control of an inducible promoter (fig. S7). After induction, cells were exposed to a low concentration of H$_2$O$_2$ or to camptothecin, a topoisomerase poison that induces DNA breaks. Consistent with the results with purified proteins in vitro, wild-type ATM responded to both H$_2$O$_2$ and camptothecin, whereas the mutant allele only responded to camptothecin treatment (Fig. 4, A and B). To determine whether these phosphorylation events affected cell survival, we monitored both groups of cells for apoptosis, which is induced by ROS or DNA damage in lymphocytes (31). The cells expressing wild-type ATM showed a strong apoptotic response to both H$_2$O$_2$ and camptothecin (as measured by a fluorescent caspase 3 substrate assay), whereas cells expressing the mutant allele only underwent caspase activation in response to camptothecin (Fig. 4, C and D). Confirmation of these results was also obtained by using propidium iodide staining and annexin V to measure cleavage of nuclear DNA and loss of membrane integrity during apoptosis (figs. S8 and S9).

Considering that C2991L and wild-type heterodimers are inactive in vitro (Fig. 3C), we also overexpressed either the C2991L or a wild-type allele of ATM in cells expressing endogenous wild-type ATM (fig. S10). The cells were treated with bleomycin or H$_2$O$_2$, and phosphorylation of p53 on Ser$^{15}$ and Chk2 on Thr$^{68}$ was quantified. Cells overexpressing wild-type ATM showed higher phosphorylation of both p53 and Chk2 in response to H$_2$O$_2$ compared with cells overexpressing C2991L ATM; thus, ectopic expression of the C2991L mutant acted as a dominant negative and inhibited the oxidative activation of wild-type ATM in human cells.

Immortalized lymphoblasts derived from an A-T patient expressing the R3047X ATM allele were also analyzed for responses to H$_2$O$_2$ and DNA damage, which showed that R3047X ATM failed to autophosphorylate ATM or phosphorylate Chk2 after exposure to H$_2$O$_2$ but showed a response to camptothecin that was similar to that seen in cells expressing wild-type ATM (Fig. 4 and fig. S10). It is difficult to make quantitative comparisons because the WT and mutant cells were derived from different individuals. The small decrease in responsiveness of the mutant cells to camptothecin could mean that the reduced response to H$_2$O$_2$ also reflects a deficit in response to DSBs, but we interpret the result to show a specific deficit in the oxidative response of the R3047X mutant.

We identified and characterized a pathway of ATM activation that is separate from the previously defined pathway that depends on MRN and DNA ends. ATM appears to act as a redox sensor in human cells, and, given the large number of substrates identified as ATM targets after DNA damage (32), ATM may similarly regulate global cellular responses to oxidative stress. The observation that the R3047X mutation generates an ataxia phenotype in A-T patients but retains normal activation in response to DNA damage suggests that most of the clinical manifestations of A-T may result from an inability to effectively regulate ROS, an observation that has important consequences for A-T treatment strategies.

**References and Notes**

2. A. Barzilai, G. Rotman, Y. Shiloh, DNA Repair (Amst.) 1, 3 (2002).
11. Materials and methods are available as supporting material on Science Online.

The Ligase PIAS1 Restricts Natural Regulatory T Cell Differentiation by Epigenetic Repression

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CD4$^+$Foxp3$^+$ regulatory T ($\text{Foxp3}^+$) cells are important for maintaining immune tolerance. Understanding the molecular mechanism that regulates $\text{Foxp3}^+$ differentiation will facilitate the development of effective therapeutic strategies against autoimmune diseases. We report here that the SUMO E3 ligase PIAS1 restricts the differentiation of natural $\text{Foxp3}^+$ cells by maintaining a repressive chromatin state of the $\text{Foxp3}$ promoter. PIAS1 acts by binding to the $\text{Foxp3}$ promoter to recruit DNA methyltransferases and heterochromatin protein 1 for epigenetic modifications. PIAS1 deletion caused promoter demethylation, reduced histone H3 methylation at Lys$^3$, and enhanced promoter accessibility. Consistently, $\text{Foxp3}^+$ mice displayed an increased natural $\text{Foxp3}^+$ cell population and were resistant to the development of experimental autoimmune encephalomyelitis. Our studies have identified an epigenetic mechanism that negatively regulates the differentiation of natural $\text{Foxp3}^+$ cells.

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11. Materials and methods are available as supporting material on Science Online.
Fig. 1. Enhanced CD4+Foxp3+ Treg differentiation in Pias1−/− mice. (A) Western blot analysis of whole-cell extracts from freshly isolated thymocytes and splenocytes with an antibody specific for Ser90-phosphorylated Pias1, total Pias1, or tubulin. (B) Cells from thymus or spleen of male (n = 7) wild-type and Pias1−/− littermates were analyzed by flow cytometry to determine the percentage and the absolute cell numbers of Foxp3+CD4+ cells (gated on a CD4+CD8+ population). Similar results were obtained with female mice. (C) Bone marrow was isolated from wild-type and Pias1−/− littermates and injected into the sublethally irradiated Rag1−/− recipient mice (n = 8). The thymic CD4+Foxp3+ population was analyzed 4 weeks after reconstitution by flow cytometry. (D) Same as in (C) except that Pias1−/− or wild-type bone marrow (CD45.2) was mixed with wild-type C57SJL bone marrow (CD45.1) and injected into the Rag1−/− mice (n = 3 for wild-type and n = 4 for Pias1−/−). Experiments in (A) to (D) were performed at least three times (n = 3 to 8 for each experiment). P value was determined by unpaired t test.

Fig. 2. Pias1−/− mice are resistant to MOG-induced EAE. (A) The percentage of CD4+Foxp3+ cells in wild-type (LN+/+) and Pias1−/− (LN−−) lymph node cells 10 days after a single MOG35-55 injection emulsified in Freund’s complete adjuvant (n = 7). (B) Lymphocytes from Pias1−/− mice and wild-type littermates 10 days after MOG35-55 injection as in (A) were either untreated or treated with 4 μg/ml of MOG35-55 for 3 days in the presence of brefeldin A during the last 5 hours of culture. IFN-γ- or IL-17-producing CD4+ cells were assayed by intracellular staining followed by flow cytometry. (C) Same as in (B) except that cells were either untreated or treated with MOG35-55 or CD3-specific antibody for 3 days. Cell proliferation was measured by one-color cell proliferation kit. (D) Pias1−/− female mice and their wild-type littermates were immunized with MOG35-55 and pertussis toxin to induce EAE as described in (L7). The development of EAE was scored (n = 4). (E) Wild-type and Pias1−/− littermates were immunized as in (D), and lymphocytes were isolated 21 days after the first MOG35-55 injection and cultured for 3 days in vitro (n = 4). Cytokine production in the cell supernatant was measured by enzyme-linked immunosorbent assay (ELISA). Shown in (A) to (E) is a representative of three independent experiments (n = 3 to 7 for each experiment). Error bars represent SEM. P value was determined by unpaired t test.
increase in the frequency of thymic CD4+Foxp3+ cells (Fig. 1C). Transplantation experiments were also performed by injecting Rag1−/− mice with a mixture of bone marrow from wild-type CD45.1+ C57SJL mice and either wild-type or Pias1−/− mice (CD45.2). The percentage of thymic CD4+Foxp3+ from Rag1−/− mice reconstituted with Pias1−/− bone marrow (CD45.2) was significantly higher than that of the wild-type controls, whereas no difference was observed in thymic CD4+Foxp3+ differentiation of the CD45.1+ control T cells (Fig. 1D). These studies suggest that Pias1 negatively regulates iTreg differentiation.

Peripheral naïve CD4+ T cells can be differentiated into so-called induced T reg (iTreg) cells from various sorted T cell populations with freshly sorted CD4+CD8+ thymocytes from male Pias1−/− or Pias1−/− mice (n = 4), using Pias1-specific antibody or IgG. Bound DNA was quantified by quantitative real-time fluorescence polymerase chain reaction (QPCR), with specific primers against the various regions of the Foxp3 promoter or CNS2 region to digest a nondigestible CNS1 region of the Foxp3 locus. Shown in (A) to (D) is a representative of three independent experiments (n = 4 to 6 for each experiment). Error bars represent SEM. P value was determined by unpaired t test.

Fig. 3. Pias1 maintains a repressive chromatin state of the Foxp3 promoter. (A) ChIP assays were performed with freshly sorted CD4+CD8+ thymocytes from male Pias1−/+ or Pias1−/− mice (n = 4), using Pias1-specific antibody or IgG. Bound DNA was quantified by quantitative real-time fluorescence polymerase chain reaction (QPCR), with specific primers against the various regions of the Foxp3 locus, and normalized with the input DNA. (B) Methylation analysis of the Foxp3 promoter was performed by bisulfite conversion of genomic DNA from various sorted T cell populations of wild-type and Pias1−/− male mice (n = 4). The x axis represents the positions of the CpG sites relative to the transcription start site (+1) in the Foxp3 gene; the y axis represents the percentage. (C) ChIP assays were performed the same way as in (A) except that antibodies against trimethylated H3K9, histone H3 trimethylated at Lys4 (H3K27), or acetylated histone H3 (AcH3) were used, and the primers against the Foxp3 promoter region were used. (D) REA assays were performed with thymic CD4+CD8+ or splenic CD4+CD25+ T cells. The data were quantified by QPCR and expressed as a ratio of digest at the Foxp3 promoter or CNS2 region to digestion at a nondigestible CNS1 region of the Foxp3 locus. Shown in (A) to (D) is a representative of three independent experiments (n = 4 to 6 for each experiment). Error bars represent SEM. P value was determined by unpaired t test.
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dS1 disruption caused a significant reduction of DNA methylation of the Foxp3 promoter (Fig. 3B and fig. S8A). In con-
dition (fig. S11A). The amount of STAT5 protein or STAT5
osphorylation was not affected by Pias1 disruption (fig. S11A). A significant increase of nuclear factor of activated T cells (NFAT) binding to
the Foxp3 promoter was also observed in Pias1−/−
T cells. Furthermore, Pias1 disruption resulted in the increased frequency of Foxp3+ CD4+CD8+ thymocytes (fig. S12).

We examined whether Pias1 may maintain a repressive chromatin structure through the re-
cruitment of DNA methyltransferases (DNMTs), which promote DNA methylation of chromatin (26). Pias1 was shown to interact with DNMT3B in a yeast two-hybrid screen and in 293T cells upon overexpression (27, 28). DNMT3A and
DNMT3B, but not DNMT1, were present in Pias1 immunoprecipitates from thymocytes or splenic CD4+CD25+ cells (Fig. 4A). Furthermore, sequential ChIP studies indicated that Pias1 forms a complex with DNMT3A and DNMT3B on the Foxp3 gene promoter (Fig. 4B). DNMT3B and DNMT3A bound to both the Foxp3 promoter and the CNS2 region, but not the CNS1 element, in the wild-type CD4+CD8+ thymocytes (Fig. 4C). Pias1 deletion completely abolished the binding of both DNMT3A and DNMT3B to the Foxp3 promoter (Fig. 4C), but not the CNS2 region. The levels of DNMT expression were not affected by Pias1 disruption (fig. S13). In addition, a modest binding of DNMT1 to the Foxp3 promoter was observed, which was inhibited by

**Fig. 4.** Pias1 is required for the promoter recruitment of DNMT3A and DNMT3B. (A) Coimmunoprecipitation assays. Protein extracts from wild-type thymocytes or splenic CD4+CD8+ T cells were subjected to immunoprecipitation with Pias1-specific antibody or IgG, followed by immunoblotting with the indicated antibodies. (B) ChIP assays were performed with wild-type thymocytes by using Pias1-specific antibody or IgG. The presence of the Foxp3 promoter region in the precipitates was quantified by QPCR. In the re-ChIP experiments, Pias1-specific antibody precipitates were released, reimmunoprecipitated with an antibody against DNMT3A or DNMT3B, and analyzed for the presence of the Foxp3 promoter sequence. (C) ChIP assays were performed with freshly sorted CD4+CD8+ thymocytes from male Pias1−/− or Pias1+/− mice (n = 4), using an antibody against DNMT3A or DNMT3B. Bound DNA was quantified by QPCR with the specific primers against Foxp3 promoter, CNS1 or CNS2 region. (D) Methylation analysis of the Foxp3 promoter was performed by bisulfite conversion of genomic DNA from thymocytes of Rag1−/− mice reconstituted with either control GFP (Con) or Cre-GFP (Cre) retrovirus-infected Dnmt3a2lox/2lox/Dnmt3b2lox/2lox bone marrow. The x axis represents the positions of the CpG sites relative to the transcription start site (+1) in the Foxp3 gene; the y axis represents the percentage. Shown in (A) to (D) is a representative of three independent experiments (n = 4 to 6 for each experiment). Error bars represent SEM. P value was determined by unpaired t test.
Pias1 disruption (fig. S14). The Pias1-dependent recruitment of DNMTs to the Foxp3 promoter was also observed in splenic CD4+CD25+ cells (fig. S15), but not in CD4+CD25− Treg cells (fig. S16). Collectively, these results indicate that Pias1 is associated with DNMT3A and DNMT3B in T cells and is required for their recruitment to the Foxp3 gene promoter.

To test if DNMT3A and DNMT3B play a role in the Foxp3 promoter methylation, we performed transplantation experiments using mice in which the functional domains of both Dnmt3a and Dnmt3b genes are flanked by two loxP sites (Dnmt3a<sub>2lox/2lox</sub>Dnmt3b<sub>2lox/2lox</sub>) (29, 30). Bone marrow cells from Dnmt3a<sub>2lox/2lox</sub>Dnmt3b<sub>2lox/2lox</sub> mice were infected with green fluorescent protein (GFP) or Cre-GFP retrovirus, and the deletion of Dnmt3a<sub>2lox/2lox</sub>Dnmt3b<sub>2lox/2lox</sub> was confirmed (fig. S17A). Sorted GFP<sup>+</sup> bone marrow was transplanted into sublethally irradiated Rag1<sup>−/−</sup> mice. Decreased Foxp3 promoter methylation was observed in thymocytes from the Rag1<sup>−/−</sup> mice reconstituted with the Cre retrovirus–transduced bone marrow (fig. 4D). ChIP assays indicated that the decreased DNMT3A and DNMT3B expression by the Cre transduction had no substantial effect on the binding of Pias1 to the Foxp3 promoter (fig. S17B).

Heterochromatin protein 1 (HP1) plays an important role in promoting H3K9 methylation and is known to interact with DNMTs (31). HP1γ was strongly associated with the Foxp3 promoter in wild-type, but not Pias1<sup>−/−</sup>, thymocytes (fig. S18). The Pias1-mediated epigenetic gene regulation is selective, because the chromatin status of genes such as Clna4 was not affected by Pias1 disruption (fig. S19).

Our studies have identified an epigenetic control mechanism in the negative regulation of Foxp3<sup>+</sup> T<sub>reg</sub> differentiation. Pias1 acts by maintaining a repressive chromatin state of the Foxp3 promoter, at least partly through the recruitment of DNMTs and HP1 to promote epigenetic modifications (fig. S20). Pias1 disruption results in the formation of a permissive chromatin structure of the Foxp3 promoter and enhanced promoter accessibility to transcription factors such as STAT3 and NFAT, which lead to the increased probability that precursor cells will differentiate into Foxp3<sup>+</sup> T<sub>reg</sub> cells (fig. S20). The physiological role of Pias1 in the regulation of Foxp3 gene is supported by the observed increase of the Foxp3<sup>+</sup> nt<sub>reg</sub> population in Pias1<sup>−/−</sup> mice and the resistance of Pias1<sup>−/−</sup> mice toward the development of EAE. Thus, the Pias1 pathway may represent a therapeutic target for the treatment of autoimmune diseases. DNMTs have no intrinsic sequence specificity (26). Our finding that Pias1 regulates the binding of DNMTs to the Foxp3 promoter, but not the CNS2 element, suggests that Pias1 may be an important cofactor that confers specificity in the DNMT-mediated chromatin methylation. The Pias1-mediated DNA methylation and histone modifications may serve as a fine-tuning mechanism in the control of epigenetic modifications during T cell differentiation.

References and Notes
17. Materials and methods are available as supporting material on Science Online.
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Materials and Methods
Figs. S1 to S21
References
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