Groucho/transducin-like Enhancer-of-split (TLE)-dependent and -independent transcriptional regulation by Runx3

Merav Yarmus, Eilon Woolf, Yael Bernstein, Ofer Fainaru, Varda Negreanu, Ditsa Levanon, and Yoram Groner*

Department of Molecular Genetics, Weizmann Institute of Science, Rehovot 76100, Israel

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Regulation of gene expression by tissue-specific transcription factors involves both turning on and turning off transcription of target genes. Runx3, a runt-domain transcription factor, regulates cell-intrinsic functions by activating and repressing gene expression in sensory neurons, dendritic cells (DC), and T cells. To investigate the mechanism of Runx3-mediated repression in an in vivo context, we generated mice expressing a mutant Runx3 lacking the C-terminal VWRPY, a motif required for Runx3 interaction with the corepressor Groucho/transducin-like Enhancer-of-split (TLE). In contrast with Runx3+/−/+ mice, which displayed ataxia due to the death of dorsal root ganglia TrkC neurons, Runx3VWRPY+/−/− mice were not ataxic and had intact dorsal root ganglia neurons, indicating that ability of Runx3 to tether Groucho/TLE is not essential for neurogenesis. In the DC compartment, the mutant protein Runx3VWRPY− had normally developed skin Langerhans cells but failed to restrain DC spontaneous maturation, indicating that this latter process involves Runx3-mediated repression through recruitment of Groucho/TLE. Moreover, in CD8+ thymocytes, Runx3VWRPY− up-regulated eE/CD103-like WT Runx3, whereas unlike wild type, it failed to repress eE/CD103 in CD8+ splenocytes. Thus, in CD8-lineage T cells, Runx3 regulates eE/CD103 in opposing regulatory modes and recruits Groucho/TLE to facilitate the transition from activation to repression. Runx3VWRPY− also failed to mediate the epigenetic silencing of CD4 gene in CD8+ T cells, but normally regulated other pan-CD8+ T cell genes. These data provide evidence for the requirement of Groucho/TLE for Runx3-mediated epigenetic silencing of CD4 and pertain to the mechanism through which other Runx3-regulated genes are epigenetically silenced.

Mammalian Runx3 is one of three transcription factors that comprise the RUNX family (1–4). RUNX proteins regulate lineage-specific gene expression in developmental pathways (2, 3) and also could be involved in autoimmune diseases (5). Loss of Runx3 function is associated with defects in neurogenesis and thymopoiesis, and with the development of colitis, gastritis, and asthma-like features (6–13). Regulation of gene expression by tissue-specific transcription factors involves both turning on and turning off transcription of target genes. Runx3 acts as a bifunctional regulator, which up-regulates but also down-regulates, gene expression (14). How does Runx3 act in vivo both as an activator and a repressor of target genes?

It is believed that a DNA-bound Runx3 elicits repression by tethering corepressors such as the transducin-like Enhancer-of-split (TLE) (15), the mammalian homolog of Drosophila Groucho (Gro) (16), to a subset of target promoters (14, 17, 18). However, the biological significance and in vivo targets of Runx3-mediated transcriptional repression are largely unknown. We (19) and others (20) have shown that both Runx1 and Runx3 interact with the corepressor Gro/TLE through a conserved motif of five amino acids VWRPY, located at the C terminus of RUNX proteins. Gene knockouts of Runx1 and Runx3 demonstrated that during thymopoiesis, they act as transcriptional repressors of CD4 and as growth regulators of CD8-lineage T lymphocytes (11, 12, 21). Rescue experiments by using in vitro-cultured fetal liver cells and knock-in chimera mice have indicated that the VWRPY motif of Runx1 plays a role during early T cell development in regulation of CD4 expression and T cell homeostasis (22, 23). On the other hand, using enforced expression by a retroviral system in organ cultures indicated that the VWRPY motif was not required for either Runx1- or Runx3-mediated repression of CD4 (24). To resolve this discrepancy and study the mechanism of Runx3-mediated repression of negatively regulated target genes in vivo, we generated mice expressing a mutant Runx3 lacking the VWRPY motif (Runx3VWRPY−).

Using mice homozygous for the mutant allele (Runx3VWRPY−) mice in comparison with WT and null (Runx3+/−) mice, we derived previously unavailable information on the positive and negative functions of Runx3 in the in vivo context of the animal. Gro/TLE-dependent and -independent functions of Runx3 now are demonstrated in dorsal root ganglia (DRG) TrkC sensory neurons, dendritic cells (DC), and CD8-lineage T lymphocytes, and CD8+ T cell-specific target genes, whose regulation by Runx3 requires the recruitment of Gro/TLE, are identified.

We show that in contrast to Runx3+/− mice that display ataxia and growth retardation (9), Runx3VWRPY− mice grew normally and were not ataxic, indicating that the ability of Runx3 to recruit Gro/TLE is not essential for these processes. Runx3 plays an important role in DC development (7). DC are bone marrow-derived cells specialized in uptake, processing, and presentation of antigens to T cells (25) and play an important role in maintenance of self-tolerance (26). Tissue-resident DC are normally maintained at an immature state by immunosuppressive cytokines such as TGF-β, secreted by the surrounding cellular environment (26). In DC compartment, Runx3 functions as a component in the TGF-β signaling pathway and has a dual role: It promotes development of epidermal Langerhans cells (LC), a distinct skin DC population, and restrains maturation of tissue-resident DC (7). Runx3VWRPY− was able to promote LC development but failed to restrain DC maturation, indicating that this latter function involves interactions of Runx3 with Gro/TLE.

During T cell development, the mutant Runx3VWRPY− positively regulated eE/CD103 expression in CD8+ thymocytes, as was previously reported for WT Runx3 (27). However, unlike WT Runx3, the mutant failed to repress eE/CD103 in peripheral CD8+ T cells. These data demonstrate that within the same cell lineage, Runx3 regulates the same gene in opposing regulatory modes, and that the mechanism underlying the transition from activation to repression requires recruitment of Gro/TLE. Moreover, in developing CD8+ T cells, Runx3VWRPY− was unable to repress the CD4

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Abbreviations: DC, dendritic cells; DRG, dorsal root ganglia; Gro, Groucho; LC, Langerhans cells; SP, single protein; TLE, transducin-like Enhancer-of-split.

*To whom correspondence should be addressed. E-mail yoram.groner@weizmann.ac.il.

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gene, which is otherwise epigenetically silenced (11, 28–30) but regulated normally the expression of other pan-CD8+ T-cell genes. These data provide evidence for Gro/TLE requirement for Runx3-mediated epigenetic silencing of CD4 and pertain to the mechanism through which other Runx3-regulated genes are epigenetically silenced.

Results

Expression of Runx3VWRPY+/− Protein and Phenotypic Features of Runx3VWRPY+/− Mice. To obtain Runx3VWRPY+/− mice, we first generated a mutant allele Runx3VWRPY−/−, in which the codons of the five C-terminal amino acids VWRPY, were changed to the stop codon UAG and to codons encoding substituted amino acids designed to create a new NotI site (Fig. 1A; see also Fig. 5, which is published as supporting information on the PNAS web site). To facilitate selection of positive ES cells, a lox-P-flanked neomycin (neo) cassette was inserted into intron no. 5 (Fig. 1A). Chimeric mice were generated and used to pass on the mutant Runx3VWRPY−/− through the germ line. To eliminate potential phenotypic effects caused by the neo gene, it was removed by crossing heterozygous Runx3VWRPY−/− mice onto a PGK-Cre transgenic mice (ref. 32; Fig. 1A). RT-PCR and Western blot analyses were used to demonstrate expression of Runx3VWRPY− mRNA and protein (Fig. 1). The absence of VWRPY motif in Runx3 protein, derived from the mutant allele, was confirmed by using antibodies specific to the VWRPY pentapeptide (Fig. 1).

Further analysis showed that contrary to Runx3−/− mice, Runx3VWRPY−/− mice have indistinguishable phenotypes. Previous studies showed that newborn Runx3−/− mice had sensory ataxia, due to the death of TrkC+ neurons in DRG, and that mice displayed a reduced growth rate (8, 9). In contrast, Runx3VWRPY−/− mice had an apparent phenotype indistinguishable from WT mice; they were not ataxic and displayed normal growth (Fig. 1E). Further analysis showed that contrary to Runx3−/− mice, which lack expression of Runx3 (9), expression of Runx3VWRPY−...
protein in DRG of Runx3<sup>VWRPY</sup>−/− mice was similar to that in WT mice (Fig. 1F), indicating that TrkC<sup>+</sup> neurons in Runx3<sup>VWRPY</sup>−/− mice are viable. More convincingly, immunostaining of the calcium-binding protein parvalbumin, a marker of TrkC<sup>+</sup> neurons (9, 33), gave a similar pattern in DRG from WT and Runx3<sup>VWRPY</sup>−/− mice (Fig. 1F). We conclude that Runx3<sup>VWRPY</sup>− is capable of supporting neurogenesis of TrkC<sup>+</sup> neurons when expressed from its endogenous locus and that Runx3 capacity to recruit Gro/TLE is not essential for this process.

Enhanced Spontaneous Maturation of Runx3<sup>VWRPY</sup>−/− DC. In addition to its function in fate determination of DRG sensory neurons, Runx3 plays a role in development and maturation of skin LC and tissue DC, where it functions as a component in TGF-β signaling pathway (7). TGF-β plays a dual role in the LC/DC compartment: it promotes development of epidermal LC and inhibits maturation of DC. When Runx3 function was lost, Runx3<sup>−/−</sup> mice lacked epidermal LC, and their DC did not respond to TGF-β-induced maturation inhibition and spontaneously matured (7). Unlike Runx3<sup>−/−</sup>, skin epidermis of Runx3<sup>VWRPY</sup>−/− mice contained abundant LC similar to WT littermate mice (Fig. 2A). But in contrast to WT DC, which exhibited a low level of spontaneous maturation, a substantial proportion (50%) of Runx3<sup>VWRPY</sup>−/− DC spontaneously matured, resembling the Runx3<sup>−/−</sup> phenotype (Fig. 2B). Even exogenously added TGF-β did not completely inhibit the spontaneous maturation of Runx3<sup>VWRPY</sup>−/− DC (Fig. 2C). Thus, Runx3<sup>VWRPY</sup>− activity was sufficient to promote TGF-β-dependent development of skin LC but was insufficient to promote TGF-β-induced maturation inhibition of DC. These data show that during LC/DC development, Runx3 mediates both positive and negative cues of TGF-β, some of which require engagement of Gro/TLE.

Runx3-Mediated Silencing of the CD4 Gene Requires Recruitment of Gro/TLE. During thymopoiesis, CD4<sup>+</sup> CD8<sup>+</sup> double-positive thymocytes differentiate into mature single-positive (SP) CD4<sup>+</sup> or SP CD8<sup>+</sup> cells (34). In SP CD8<sup>+</sup> cells, the expression of CD4 is transcriptionally repressed (30, 35). A region of 430 bp, known as the CD4 silencer, is required for this process (29, 30, 36–38). This silencer encompasses two functional Runx-binding sites, which are essential for the irreversible epigenetic silencing of CD4 in mature SP CD8<sup>+</sup> cells (11, 28–30). Runx3 is highly expressed in SP CD8<sup>+</sup> and, when lost, transcriptional silencing of CD4 is impaired, leading to accumulation of an abnormal population of mature CD8<sup>+</sup> T cells in which expression of CD4 is not repressed (11, 12, 21).

We assessed the distribution of CD4/CD8 among mature (TCR<sup>hi</sup>HSA<sup>−low</sup>) T cells in thymus and spleen of Runx3<sup>VWRPY</sup>−/− mice in comparison with that of WT and Runx3<sup>−/−</sup> mice. A profound increase in the proportion of mature CD8<sup>+</sup> T cells that also expressed CD4 was observed in both thymus and spleen of Runx3<sup>VWRPY</sup>−/− mice compared with WT mice (Fig. 3A). The distribution of mature CD4<sup>+</sup>/CD8<sup>+</sup> thymocytes in Runx3<sup>VWRPY</sup>−/− mice was similar to that in Runx3<sup>−/−</sup> mice and markedly different from that in WT mice (Fig. 3A Upper; refs. 11 and 12). Interestingly, among splenocytes, the frequency of CD4<sup>+</sup>/CD8<sup>+</sup> in Runx3<sup>VWRPY</sup>−/− mice was even higher than that in Runx3<sup>−/−</sup> mice (Fig. 3A Lower). The average (n = 5) frequency of CD4<sup>+</sup>/CD8<sup>+</sup> splenic T cells in Runx3<sup>VWRPY</sup>−/− and Runx3<sup>−/−</sup> mice was 12.0 ± 4.4% and 6.4 ± 2.4%, respectively, compared with 0.67 ± 0.2% in WT mice (Fig. 3B). These data unequivocally show that silencing of CD4 by Runx3 requires the recruitment of the corepressor Gro/TLE. It also indicates that CD4 expression was significantly less repressed in CD8<sup>+</sup> splenocytes of Runx3<sup>VWRPY</sup>−/− mice as compared with splenocytes of Runx3<sup>−/−</sup> mice.

Using transfection assays, we also assessed the ability of Runx3 to negatively regulate the CD4 silencer in conjunction with Gro/TLE. Reporter constructs, in which human elongation factor promoter, with or without CD4 silencer, regulate luciferase (luc) transcription, were cotransfected into two cell lines (COS-7 and HEK-293) with constructs expressing TLE1 (CMV-TLE1) and constructs expressing either WT Runx3 (CMV-Runx3) or mutant Runx3 (CMV-Runx3<sup>VWRPY</sup>−) (Fig. 6, which is published as supporting information on the PNAS web site). WT Runx3 was significantly more active than Runx3<sup>VWRPY</sup>− in reducing CD4 silencer-derived luc activity (Fig. 6), indicating that in the context of the CD4 silencer, Runx3-mediated transcriptional repression largely depends on its ability to tether Gro/TLE.

 Besides silencing of CD4, Runx3 also regulates other CD8/T cell-specific genes (39, 40). Hence, in the absence of Runx3 function, proliferative capacity of peripheral Runx3<sup>−/−</sup>/CD8<sup>+</sup> T cells was impaired, resulting in a profound reduction in the proportion of splenic CD8<sup>+</sup> T cells (Fig. 3A; refs. 11 and 12). In contrast, no significant difference in proportion of CD8<sup>+</sup> T cells (Fig. 3A) or in cell proliferation ability (Fig. 3D) was noted between Runx3<sup>VWRPY</sup>−/− and WT. These data show that in developing
CD8\(^+\) T cells, silencing of CD4 and cell proliferative capacity are two independent Runx3-mediated processes. When Runx3 ability to recruit Gro/TLE is lost, repression of CD4 is impaired, but cell proliferation remains intact, underscoring the fact that Runx3 can function as a positive regulator in one context and as a negative regulator in another.

**Runx3 in Conjunction with Gro/TLE Down-Regulates αE/CD103 in Peripheral CD8\(^+\) T Cells.** Grueter et al. (27) have recently shown that Runx3 positively regulates the expression of the αE/CD103 integrin during T cell development and that knockdown of Runx3 markedly reduced the frequency of CD4\(^-\)CD8\(^+\) T cells. αE/CD103 mediates the interaction with E-cadherin on epithelial cells and is normally expressed on 80–90% of mature CD8\(^+\) thymocytes and on a much lower proportion (40–50%) of CD8\(^+\) splenocytes (27, 41).

We assessed the ability of Runx3 to positively regulate expression of αE/CD103. More than 90% of SP CD8\(^+\) thymocytes in WT and Runx3\(^{-/-}\) mice expressed αE/CD103 compared with <10% in Runx3\(^{-/-}\) mice (Fig. 4A). This finding is not only consistent with data obtained by Grueter et al. (27), but it also implies that similarly to WT, Runx3\(^{-/-}\) can positively regulate the expression of αE/CD103. Compared with thymocytes, there was a lower proportion of CD8\(^+\)CD103\(^+\) splenocytes in WT mice and even lower proportion in Runx3\(^{-/-}\) mice (Fig. 4B), in full agreement with the published data (27). Strikingly, in Runx3\(^{-/-}\) mice, the proportion of CD8\(^+\)CD103\(^+\) splenocytes was not reduced compared with thymocytes, and >90% of Runx3\(^{-/-}\) splenocytes expressed CD103 (Fig. 4B). In fact, the ratio of CD103\(^+\)/CD103\(^-\) splenocytes in Runx3\(^{-/-}\) mice was ~9-fold higher compared with WT, whereas in thymocytes, the ratio was similar (Fig. 4C). Of note, the fact that Runx3\(^{-/-}\) splenocytes express higher-than-WT levels of αE/CD103 implies that the normally reduced expression of αE/CD103 in splenic CD8\(^+\) T cells (27, 41) is not due to a pause in transcriptional activation but rather to a down-regulation of αE/CD103 expression. These data show that in peripheral CD8\(^+\) T cells, Runx3, in conjunction with Gro/TLE, down-regulates αE/CD103 expression. This conclusion is further supported by the marked decline of αE/CD103 expression that was observed in proliferating cultured splenocytes from WT but not from Runx3\(^{-/-}\) mice (Fig. 7, which is published as supporting information on the PNAS web site). Thus, during development of CD8-lineage T cells, Runx3 functions both as a positive and negative regulator of the same gene and must recruit Gro/TLE to accomplish the transition.

We next asked whether Runx3 directly regulates αE/CD103 transcription. αE/CD103 is a heterodimer composed of the αE and β7 chains. Because the β7 chain is highly expressed in T cells, the limiting factor in αE/CD103 expression is the transcription of the αE gene (42). We used RT-PCR to monitor αE transcription in thymocytes and splenocytes of WT, Runx3\(^{-/-}\), and Runx3\(^{-/-}\) mice (Fig. 4D). The loss of Runx3 did not completely abolish αE transcription in CD8\(^+\) thymocytes or splenocytes (Fig. 4D), as also evidenced by the low, yet detectable, surface expression...
remains normal, but down-regulation in peripheral CD8 lineages. Runx3 controls mice (analyzed as in transcript was similar in Runx3VWRPY/H20841 Runx3 function is lost, whereas in thymocytes, the level was similar (Fig. 4A). Anti-CD8 and anti-CD103 mAb and analyzed by FACS. CD103 expression is higher, whereas in thymocytes, the level was similar (11, 28, 30), our data indicate that Runx3 involvement in epigenetic silencing of CD4 requires recruitment of Gro/TLE. When Runx3 is lost or is unable to tether Gro/TLE, CD4 is derepressed. But why is derepression of CD4 significantly higher in Runx3VWRPY/H11005 splenic T cells as compared with Runx3+/− cells? A possible explanation could be that while in Runx3+/− cells, the loss of Runx3 is partially compensated by Runx1 activity (11, 30), in Runx3VWRPY/− cells, such compensation is less efficient. We hypothesize that this phenomenon occurs because the mutant protein, which is unable to elicit repression but can still bind to the CD4 silencer’s RUNX sites, outcompetes Runx1. As a result, Runx1 compensation diminishes, leading to the observed higher derepression of CD4 in Runx3VWRPY/− T cells compared with Runx3 cells. This hypothesis is supported by findings that in a compound mutant mouse Runx3+/−/Runx1−/−, i.e., null for Runx3 and histologically normal for Runx1, CD4 expression in CD8+ T cells is completely derepressed and all peripheral CD8+ T cells also expressed CD4 (12).

How tethering of Gro/TLE to target promoters/silencers evokes transcriptional repression is not yet fully understood. However, as Gro/TLE interacts with histones and histone deacetylases (18, 43), it is likely that recruitment of Gro/TLE by the DNA-bound Runx3 modulates local chromatin structure at the CD4 locus, resulting in a repressed transcriptional state (44). Our data provide evidence for a Runx3-Gro/TLE-mediated epigenetic silencing and pertain to the mechanism of Runx3-mediated repression and silencing of other genes (14). Interestingly, repression of both αE/CD103 and CD4 occurs in mature CD8+ T cells and may reflect a cell stage-specific availability of components, such as chromatin modifications enzymes (18) required for Runx3-Gro/TLE-mediated repression.

Previous studies have shown that the chromatin remodeling complexes BRG1-associated factor (BAF) are involved in transcriptional repression of CD4 during thymopoiesis (45, 46). The available information on molecular events that lead to silencing of CD4 during CD8-lineage differentiation was recently integrated into a hypothetical model (30). It predicts that CD4 repression in DP thymocytes is initiated by a step, which precedes the engagement of BAF and involves binding of Runx3 to the CD4 silencer and recruitment of another “as-yet-unknown” component, which facilitates histone deacetylation. Our data not only support this model but are also consistent with the possibility that Gro/TLE constitutes the missing link.

**Discussion**

Through comparisons of biogenesis and function of TrkC neurons, LC/DC and CD8+ T lymphocytes in Runx3VWRPY/−, Runx3+/−, and WT mice, we obtained unique insights into the bifunctional nature of Runx3 and into the mechanism by which it regulates target genes. Whereas Runx3 could function as either transcriptional activator or transcriptional repressor (14, 28), the phenotypic consequences of these opposing regulatory modes and how the transition from activation to repression occurs in the *in vivo* context of the animal have not been addressed. We found that in developing TrkC neurons, the ability of Runx3 to mediate transcriptional repression, in conjunction with Gro/TLE, was not essential for the development of functional TrkC neurons. In contrast, in the LC/DC compartment, where Runx3 functions as a component of TGF-β signaling cascade, recruitment of Gro/TLE is required for proper maturation of DC but not for normal development of skin LC.

Analysis of Runx3VWRPY/− function in the CD8+ T cell-lineage provided evidence that Runx3 regulates transcription of αE/CD103 in opposing regulatory modes and that repression of both αE/CD103 and CD4 requires the binding of the corepressor Gro/TLE. Given that CD4 transcription in CD8+ T cells is epigenetically repressed (11, 28, 30), our data indicate that Runx3 involvement in epigenetic silencing of CD4 requires recruitment of Gro/TLE. When Runx3 is lost or is unable to tether Gro/TLE, CD4 is derepressed. But why is derepression of CD4 significantly higher in Runx3VWRPY/− splenic T cells as compared with Runx3+/− cells? A possible explanation could be that while in Runx3+/− cells, the loss of Runx3 is partially compensated by Runx1 activity (11, 30), in Runx3VWRPY/− cells, such compensation is less efficient. We hypothesize that this phenomenon occurs because the mutant protein, which is unable to elicit repression but can still bind to the CD4 silencer’s RUNX sites, outcompetes Runx1. As a result, Runx1 compensation diminishes, leading to the observed higher derepression of CD4 in Runx3VWRPY/− T cells compared with Runx3+ cells. This hypothesis is supported by findings that in a compound mutant mouse Runx3+/−/Runx1−/−, i.e., null for Runx3 and histologically normal for Runx1, CD4 expression in CD8+ T cells is completely derepressed and all peripheral CD8+ T cells also expressed CD4 (12).

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Materials and Methods

Generation of Runx3<sup>WtTRP</sup>−/− Mice. A fragment of Runx3, spanning almost the entire intron 5 and exon 6 (Fig. 1), was cloned from a 129/Sv mouse genomic library (Stratagene). To generate the mutant WtTRP allele, the nucleotides encoding the C-terminal end WtTRP, which serves as a TLE-binding site, were modified to encode a stop codon (UAG) followed by codons for the amino acids arginine (R) and proline (P) (Fig. 5). A loxP-flanked neo cassette was integrated into intron 5 of the targeted E1 ES clones were identified (Fig. 1 and 5), taking advantage of the newly created NotI site. Several chimeric males were generated from targeted ES cells and crossed to ICR mice to establish germ-line transmission. Homozygotes then were generated from targeted R1 ES clones were identified (Fig. 1 and 5), taking advantage of the newly created Nol site. Several chimeric males were generated from targeted ES cells and crossed to ICR mice to confirm the neo cassette. Cre-mediated excision of the neo was confirmed by PCR. Mice were bred and maintained in a pathogen-free facility. Mouse experiments were approved by the Institutional Animal Care and Use Committee of The Weizmann Institute.

RT-PCR Analysis. RNA was isolated from thymocytes and splenocytes of WT, Runx3<sup>WtTRP</sup>−/−, and Runx3<sup>−/−</sup> mice, and PCR products were derived by using the following sets of primers: For Runx3, exon 2 (5′GGCAAGATGGGCGAGAACAG and 6′TCAGG-TCAGTTTTCGCTT) and at 0, 48, and 72 h of incubation time, cells were stained with anti-CD3β and anti-CD4 mAb and analyzed by FACS.

Preparation and analysis of LC, bone marrow-derived DC, and T lymphocytes was carried out as described in refs. 7 and 12. Single-cell suspensions were prepared in FACS buffer (7, 12), incubated with antibodies, and analyzed by using a FACS Calibur (Becton Dickinson) and CELLQuest software (Becton Dickinson). mAbs included CD4-biotinylated, CD8α-perc, CD8β/Perp, TCRβ-FTTC, HSA-PE, CD11c-APC, CD11b-PE, IA/IE (MHC II)-PE, CD3-biotinylated, CD103-biotinylated, and streptavidin-APC (Pharmingen). Differences between average values of WT and mutant mice (either Runx3<sup>WtTRP</sup>−/− or Runx3<sup>−/−</sup>) were evaluated by using Student’s t test.

Cell Proline Proliferation Assays. Splenic CD8+ cells were isolated by using a magnetic cell sorting separation system (Miltenyi Biotec, Auburn, CA), labeled by incubation with 5 μM 6-carboxyfluorescein diacetate, succinimidyl ester (Molecular Probes) and further processed as detailed in ref. 12. Cells were stimulated with anti-CD3 mAb [(2 μg/ml; Pharmingen) plus IL-2 (20 U/ml; PeproTech, Rocky Hill, NJ)] and at 0, 48, and 72 h of incubation time, cells were stained with anti-CD8β and anti-CD4 mAb and analyzed by FACS.

Cell Transfection and Reporter Gene Assays. Reporter gene assays were conducted with HEK-293 and COS-7 cells by using vectors described in Fig. 6. Cells were transfected by using lipofectamine (Invitrogen) (COS-7) and CaPO4 (HEK-293) and luciferase measured by the Luciferase Assay System (Promega) according to the manufacturer’s instructions. Difference between average values was evaluated statistically by using a paired Student’s t test.

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