

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

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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<sup>-/-</sup> mice, which displayed ataxia due to the death of dorsal root ganglia TrkC neurons, Runx3<sup>VWRPY<sup>-/-</sup></sup> 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 Runx3<sup>VWRPY<sup>-/-</sup></sup> promoted 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<sup>+</sup> thymocytes, Runx3<sup>VWRPY<sup>-/-</sup></sup> up-regulated  $\alpha E/CD103$ -like WT Runx3, whereas unlike wild type, it failed to repress  $\alpha E/CD103$  in CD8<sup>+</sup> splenocytes. Thus, in CD8-lineage T cells, Runx3 regulates  $\alpha E/CD103$  in opposing regulatory modes and recruits Groucho/TLE to facilitate the transition from activation to repression. Runx3<sup>VWRPY<sup>-/-</sup></sup> also failed to mediate the epigenetic silencing of *CD4* gene in CD8<sup>+</sup> T cells, but normally regulated other pan-CD8<sup>+</sup> 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.

$\alpha E/CD103$  gene repression | *CD4* silencer | CD8 lineage lymphocytes | epigenetic silencing | transcriptional repression

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 thymo-

poiesis, 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 (Runx3<sup>VWRPY<sup>-/-</sup></sup>).

Using mice homozygous for the mutant allele (Runx3<sup>VWRPY<sup>-/-</sup></sup> mice) in comparison with WT and null (Runx3<sup>-/-</sup>) 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<sup>+</sup> T cell-specific target genes, whose regulation by Runx3 requires the recruitment of Gro/TLE, are identified.

We show that in contrast to Runx3<sup>-/-</sup> mice that display ataxia and growth retardation (9), Runx3<sup>VWRPY<sup>-/-</sup></sup> 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- $\beta$ , secreted by the surrounding cellular environment (26). In DC compartment, Runx3 functions as a component in the TGF- $\beta$  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). Runx3<sup>VWRPY<sup>-/-</sup></sup> was able to promote LC development but failed to restrain DC maturation, indicating that this latter function involves interactions of Runx3 with Gro/TLE.

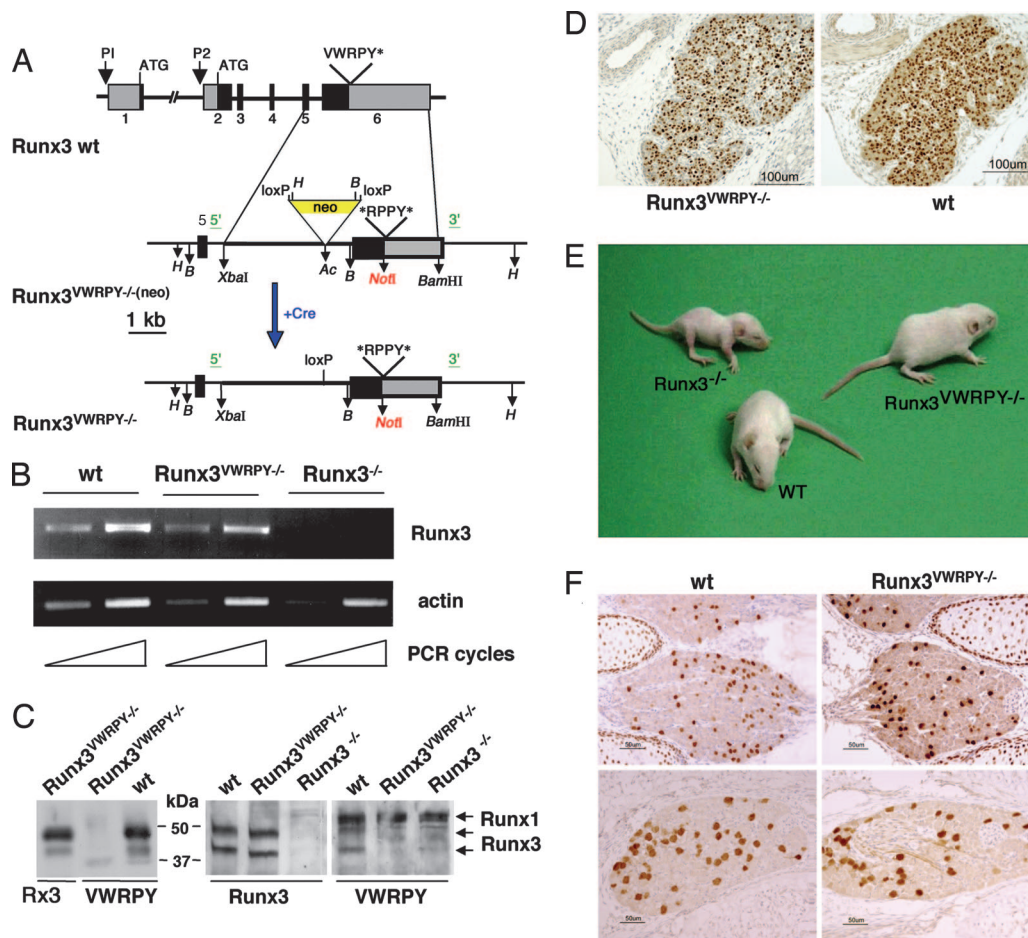
During T cell development, the mutant Runx3<sup>VWRPY<sup>-/-</sup></sup> positively regulated  $\alpha E/CD103$  expression in CD8<sup>+</sup> thymocytes, as was previously reported for WT Runx3 (27). However, unlike WT Runx3, the mutant failed to repress  $\alpha E/CD103$  in peripheral CD8<sup>+</sup> 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<sup>+</sup> T cells, Runx3<sup>VWRPY<sup>-/-</sup></sup> was unable to repress the *CD4*

Conflict of interest statement: No conflicts declared.

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

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**Fig. 1.** *Runx3<sup>VWRPY-/-</sup>* mice expressing the mutant protein display a normal outward phenotype. (A) Disruption of the Gro/TLE binding site (VWRPY) in *Runx3*. A scheme is shown of *Runx3* genomic locus, targeting vector, and mutant allele. The targeting vector spans the *Xba*I-*Bam*HI fragment of intron 5 and exon 6. Relevant restriction sites are shown (*H*, HindIII; *B*, BglIII; *Ac*, AclI). The 3' and 5' external probes used for the Southern blot analysis, shown in Fig. 5, are marked in green. The floxed *neo* gene later was removed, giving rise to *Runx3<sup>VWRPY-/-</sup>*. (B) Semiquantitative RT-PCR analysis of RNA isolated from thymocytes of WT, *Runx3<sup>-/-</sup>*, and *Runx3<sup>VWRPY-/-</sup>* mice. Representative results of three independent experiments by using RNA obtained from three different mice are shown. Similar results were obtained by using RNA isolated from spleens of these mice. (C) Western blot analysis of proteins extracted from HEK-293 cells (Left) transfected with vectors expressing either WT Runx3 or *Runx3<sup>VWRPY-/-</sup>* cDNAs (Center) (CMV-Runx3 and CMV-*Runx3<sup>VWRPY-/-</sup>* in Fig. 6) and from the spleen (Right) of WT, *Runx3<sup>-/-</sup>* and *Runx3<sup>VWRPY-/-</sup>* mice. Blots were reacted with antibodies against either Runx3 (31) or VWRPY as designated. Arrows indicate the 48- and 46-kDa Runx3 proteins and the 50-kDa Runx1 protein, which reacts with anti-VWRPY Ab. (D) Immunostaining with anti-Runx3 antibodies of thymus sections from *Runx3<sup>VWRPY-/-</sup>* and WT mice showing similar patterns of Runx3 staining. (Magnification:  $\times 10$ .) (E) Picture depicts 1-month-old WT, *Runx3<sup>-/-</sup>*, and *Runx3<sup>VWRPY-/-</sup>* mice. *Runx3<sup>-/-</sup>* mice are smaller and have a clearly recognizable phenotype, characterized by severe ataxia and posture abnormalities, whereas WT and *Runx3<sup>VWRPY-/-</sup>* mice have indistinguishable phenotypes. (F) Immunostaining of DRG sections from WT and *Runx3<sup>VWRPY-/-</sup>* embryonic day 14.5 embryos with anti-Runx3 (Upper) and embryonic day 17.5 embryos with anti-parvalbumin (Lower) showing similar patterns of immunostaining (Magnification:  $\times 20$ .)

gene, which is otherwise epigenetically silenced (11, 28–30) but regulated normally the expression of other pan-CD8<sup>+</sup> 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 *Runx3<sup>VWRPY-/-</sup>* Protein and Phenotypic Features of *Runx3<sup>VWRPY-/-</sup>* Mice.** To obtain *Runx3<sup>VWRPY-/-</sup>* mice, we first generated a mutant allele *Runx3<sup>VWRPY-/-</sup>*, 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 *Runx3<sup>VWRPY-/-</sup>*

through the germ line. To eliminate potential phenotypic effects caused by the *neo* gene, it was removed by crossing heterozygous *Runx3<sup>VWRPY-/-</sup>* mice onto a PGK-Cre transgenic mice (ref. 32; Fig. 1A). RT-PCR and Western blot analyses were used to demonstrate expression of *Runx3<sup>VWRPY-/-</sup>* mRNA and protein (Fig. 1B and C). The absence of VWRPY motif in Runx3 protein, derived from the mutant allele, was confirmed by using antibodies specific to the VWRPY pentapeptide (Fig. 1C). Immunohistochemistry on sections from *Runx3<sup>VWRPY-/-</sup>* mice, homozygous to the VWRPY minus mutant allele, revealed thymus architecture and staining pattern of *Runx3<sup>VWRPY-/-</sup>* that were similar to WT (Fig. 1D).

Previous studies showed that newborn *Runx3<sup>-/-</sup>* mice had sensory ataxia, due to the death of TrkC<sup>+</sup> neurons in DRG, and that mice displayed a reduced growth rate (8, 9). In contrast, *Runx3<sup>VWRPY-/-</sup>* 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<sup>-/-</sup>* mice, which lack expression of Runx3 (9), expression of *Runx3<sup>VWRPY-/-</sup>*

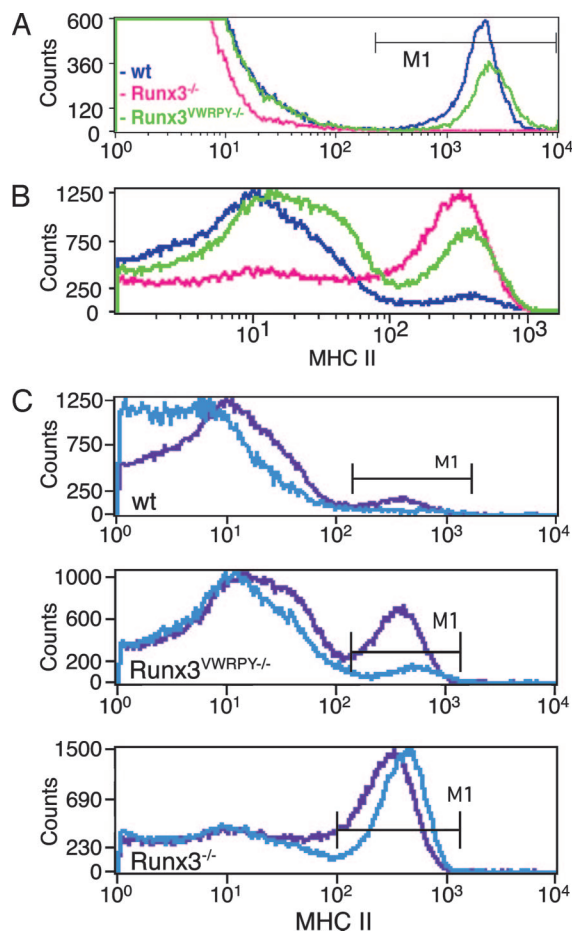
protein in DRG of  $\text{Runx3}^{\text{VWRPY-/-}}$  mice was similar to that in WT mice (Fig. 1F), indicating that  $\text{TrkC}^+$  neurons in  $\text{Runx3}^{\text{VWRPY-/-}}$  mice are viable. More convincingly, immunostaining of the calcium-binding protein parvalbumin, a marker of  $\text{TrkC}^+$  neurons (9, 33), gave a similar pattern in DRG from WT and  $\text{Runx3}^{\text{VWRPY-/-}}$  mice (Fig. 1F). We conclude that  $\text{Runx3}^{\text{VWRPY-}}$  is capable of supporting neurogenesis of  $\text{TrkC}^+$  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  $\text{Runx3}^{\text{VWRPY-/-}}$  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- $\beta$  signaling pathway (7). TGF- $\beta$  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,  $\text{Runx3}^{-/-}$  mice lacked epidermal LC, and their DC did not respond to TGF- $\beta$ -induced maturation inhibition and spontaneously matured (7). Unlike  $\text{Runx3}^{-/-}$ , skin epidermis of  $\text{Runx3}^{\text{VWRPY-/-}}$  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 ( $\approx 50\%$ ) of  $\text{Runx3}^{\text{VWRPY-/-}}$  DC spontaneously matured, resembling the  $\text{Runx3}^{-/-}$  phenotype (Fig. 2B). Even exogenously added TGF- $\beta$  did not completely inhibit the spontaneous maturation of  $\text{Runx3}^{\text{VWRPY-/-}}$  DC (Fig. 2C). Thus,  $\text{Runx3}^{\text{VWRPY-}}$  activity was sufficient to promote TGF- $\beta$ -dependent development of skin LC but was insufficient to promote TGF- $\beta$ -induced maturation inhibition of DC. These data show that during LC/DC development, Runx3 mediates both positive and negative cues of TGF- $\beta$ , some of which require engagement of Gro/TLE.

**Runx3-Mediated Silencing of the  $CD4$  Gene Requires Recruitment of Gro/TLE.** During thymopoiesis,  $CD4^+CD8^+$  double-positive thymocytes differentiate into mature single-positive (SP)  $CD4^+$  or SP  $CD8^+$  cells (34). In SP  $CD8^+$  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  $CD8^+$  cells (11, 28–30). Runx3 is highly expressed in SP  $CD8^+$  and, when lost, transcriptional silencing of  $CD4$  is impaired, leading to accumulation of an abnormal population of mature  $CD8^+$  T cells in which expression of  $CD4$  is not repressed (11, 12, 21).

We assessed the distribution of  $CD4/CD8$  among mature ( $\text{TCR}^{\text{high}}\text{HSA}^{\text{low}}$ ) T cells in thymus and spleen of  $\text{Runx3}^{\text{VWRPY-/-}}$  mice in comparison with that of WT and  $\text{Runx3}^{-/-}$  mice. A profound increase in the proportion of mature  $CD8^+$  T cells that also expressed  $CD4$  was observed in both thymus and spleen of  $\text{Runx3}^{\text{VWRPY-/-}}$  mice compared with WT mice (Fig. 3A). The distribution of mature  $CD4^+/CD8^+$  thymocytes in  $\text{Runx3}^{\text{VWRPY-/-}}$  mice was similar to that in  $\text{Runx3}^{-/-}$  mice and markedly different from that in WT mice (Fig. 3A Upper; refs. 11 and 12). Interestingly, among splenocytes, the frequency of  $CD4^+CD8^+$  in  $\text{Runx3}^{\text{VWRPY-/-}}$  mice was even higher than that in  $\text{Runx3}^{-/-}$  mice (Fig. 3A Lower). The average ( $n = 5$ ) frequency of  $CD4^+CD8^+$  splenic T cells in  $\text{Runx3}^{\text{VWRPY-/-}}$  and  $\text{Runx3}^{-/-}$  mice was  $12.0 \pm 4.4\%$  and  $6.4 \pm 2.4\%$ , respectively, compared with  $0.67 \pm 0.2\%$  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  $CD8^+$  splenocytes of  $\text{Runx3}^{\text{VWRPY-/-}}$  mice as compared with splenocytes of  $\text{Runx3}^{-/-}$  mice.

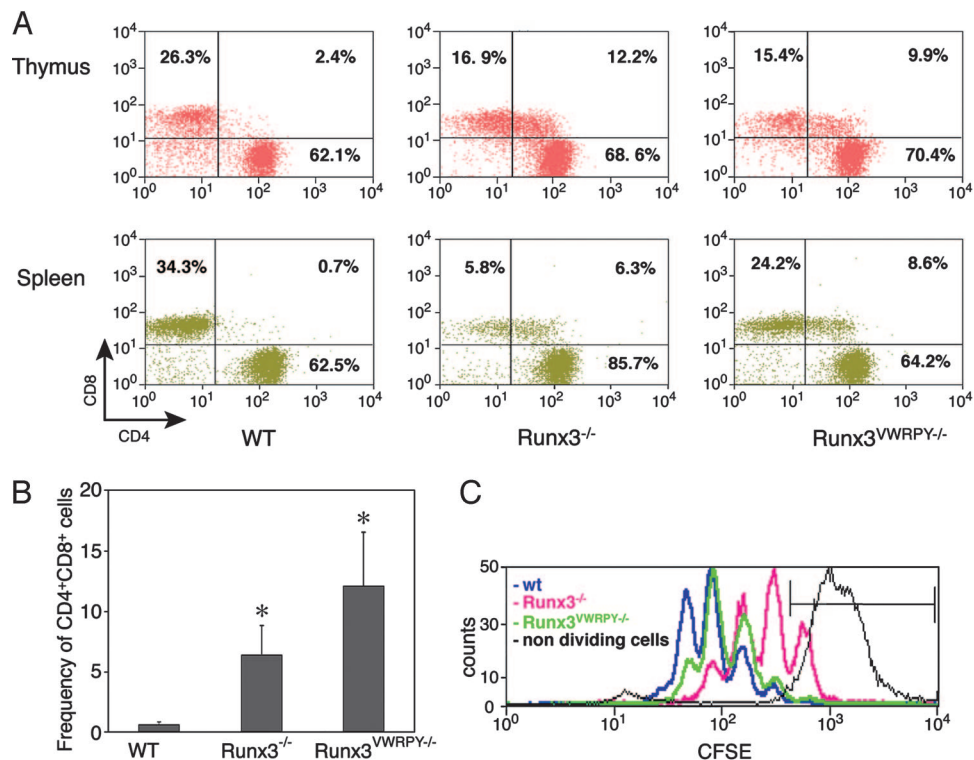
Using transfection assays, we also assessed the ability of Runx3 to negatively regulate the  $CD4$  silencer in conjunction with Gro/TLE. Reporter constructs, in which human elongation factor promoter, with or without  $CD4$  silencer, regulate luciferase (luc)



**Fig. 2.**  $\text{Runx3}^{\text{VWRPY-}}$  fully supports the development of LC but is less efficient than WT in restraining the spontaneous maturation of DC. (A) Epidermal LC highly express MHC II (7). Cells from epidermal sheaths of WT,  $\text{Runx3}^{\text{VWRPY-/-}}$ , and  $\text{Runx3}^{-/-}$  mice were isolated, stained with anti-MHC II mAb, and analyzed by FACS. LC ( $\text{MHC II}^{\text{high}}$ ) were present in WT and  $\text{Runx3}^{\text{VWRPY-/-}}$  mice (7.0% and 5.1%, respectively) but not in  $\text{Runx3}^{-/-}$  mice (0.2%). (B)  $\text{Runx3}^{\text{VWRPY-/-}}$  DC display enhanced spontaneous maturation. Bone marrow-derived DC grown for 8 days were stained with anti-CD11c and anti-MHC II mAb and analyzed by FACS. MHC II levels in  $\text{CD11c}^+$ -gated cells are shown. The average frequencies and SE of mature DC in four independent experiments were as follows:  $15.6 \pm 2.5\%$  for WT,  $47.4 \pm 7.3\%$  for  $\text{Runx3}^{\text{VWRPY-/-}}$  and  $64.8 \pm 5.5\%$  for  $\text{Runx3}^{-/-}$ . (C) Bone marrow-derived DC grown for 8 days in the presence (blue) or absence (purple) of TGF- $\beta$  (10 ng/ml) were stained with anti-CD11c and anti-MHC II mAb and analyzed by FACS. Shown are MHC II levels in  $\text{CD11c}^+$ -gated cells of WT,  $\text{Runx3}^{\text{VWRPY-/-}}$ , and  $\text{Runx3}^{-/-}$  mice.

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- $\text{Runx3}^{\text{VWRPY-}}$ ) (Fig. 6, which is published as supporting information on the PNAS web site). WT Runx3 was significantly more active than  $\text{Runx3}^{\text{VWRPY-}}$  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  $CD8^+$  cell-specific genes (39, 40). Hence, in the absence of Runx3 function, proliferative capacity of peripheral  $\text{Runx3}^{-/-}$   $CD8^+$  T cells was impaired, resulting in a profound reduction in the proportion of splenic  $CD8^+$  T cells (Fig. 3A; refs. 11 and 12). In contrast, no significant difference in proportion of  $CD8^+$  T cells (Fig. 3A) or in cell proliferation ability (Fig. 3D) was noted between  $\text{Runx3}^{\text{VWRPY-/-}}$  and WT. These data show that in developing



**Fig. 3.** Recruitment of Gro/TLE is required for Runx3-mediated CD4 silencing but not for Runx3-dependent CD8<sup>+</sup> T cell proliferation. (A) Mature thymic and splenic CD8<sup>+</sup> T cells of Runx3<sup>VWRPY-/-</sup> mice also expressed CD4. Thymocytes (Upper) and splenocytes (Lower) from WT, Runx3<sup>VWRPY-/-</sup>, and Runx3<sup>-/-</sup> mice were stained with anti-TCR- $\beta$ , anti-HSA, anti-CD8 $\alpha$ , and anti-CD4 mAb and analyzed by FACS. Mature cells were gated as TCR<sup>high</sup>HSA<sup>-low</sup>. The frequencies of SP CD8, SP CD4, and DP CD4<sup>+</sup>CD8<sup>+</sup> populations are indicated. A representative experiment of five independent analyses is shown. Note the profound reduction of splenic CD8<sup>+</sup> T cells in Runx3<sup>-/-</sup> but not in Runx3<sup>VWRPY-/-</sup> or WT mice. (B) Frequency of mature splenic CD8<sup>+</sup> cells that also expressed CD4 is significantly (\*) increased in Runx3<sup>VWRPY-/-</sup> ( $n = 5$ ;  $P < 0.0001$ ) and Runx3<sup>-/-</sup> mice ( $n = 5$ ;  $P < 0.0003$ ) compared with WT. Frequency of splenic CD4<sup>+</sup>CD8<sup>+</sup> in Runx3<sup>VWRPY-/-</sup> mice was significantly higher ( $n = 5$ ;  $P < 0.015$ ) compared with Runx3<sup>-/-</sup> mice. (C) Runx3<sup>VWRPY-/-</sup> splenic CD8<sup>+</sup> T cells display normal proliferation. 5,6-Carboxyfluorescein diacetate, succinimidyl ester (CFSE) cell division assay of CD8<sup>+</sup> splenocytes isolated from WT (blue), Runx3<sup>VWRPY-/-</sup> (green), and Runx3<sup>-/-</sup> (pink) mice. The black line represents a control experiment of cells incubated without anti-CD3 and IL2 and, thus, were nondividing. The overall cell division rate of WT or Runx3<sup>VWRPY-/-</sup> was similar, whereas that of Runx3<sup>-/-</sup> cells was much lower. Cells from WT or Runx3<sup>VWRPY-/-</sup> mice underwent four population divisions compared with only three divisions of Runx3<sup>-/-</sup> cells. After 72 h in culture, >20% of Runx3<sup>-/-</sup> cells remained undivided compared with  $\approx 1\%$  and 2% of WT and Runx3<sup>VWRPY-/-</sup>, respectively.

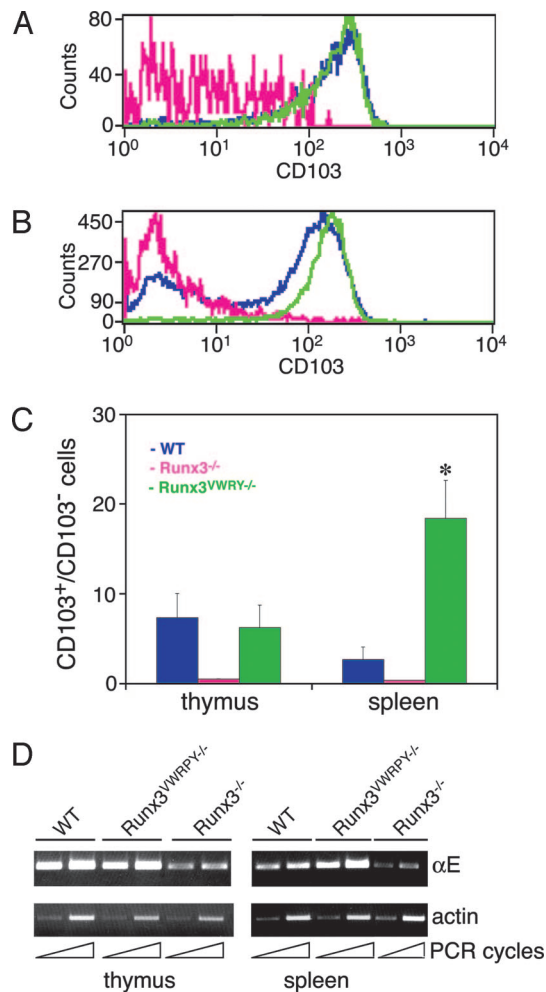
CD8<sup>+</sup> T cells, silencing of *CD4* and cell proliferative capacity are two independent Runx3-mediated processes. When Runx3 ability to recruit Gro/TLE is lost, repression of *CD4* is impaired, but cell proliferation remains intact, underscoring the fact that Runx3 can function as a positive regulator in one context and as a negative regulator in another.

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

We assessed the ability of Runx3<sup>VWRPY-/-</sup> to positively regulate expression of  $\alpha E/CD103$ . More than 90% of SP CD8<sup>+</sup> thymocytes in WT and Runx3<sup>VWRPY-/-</sup> mice expressed  $\alpha E/CD103$  compared with <10% in Runx3<sup>-/-</sup> 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<sup>VWRPY-/-</sup> can positively regulate the expression of  $\alpha E/CD103$ . Compared with thymocytes, there was a lower proportion of CD8<sup>+</sup>CD103<sup>+</sup> splenocytes in WT mice and even lower proportion in Runx3<sup>-/-</sup> mice (Fig. 4B), in full agreement with the published data (27). Strikingly, however, in Runx3<sup>VWRPY-/-</sup> mice, the proportion of CD8<sup>+</sup>CD103<sup>+</sup> spleno-

cytes was not reduced compared with thymocytes, and >90% of Runx3<sup>VWRPY-/-</sup> splenocytes expressed CD103 (Fig. 4B). In fact, the ratio of CD103<sup>+</sup>/CD103<sup>-</sup> splenocytes in Runx3<sup>VWRPY-/-</sup> mice was  $\approx 9$ -fold higher compared with WT, whereas in thymocytes, the ratio was similar (Fig. 4C). Of note, the fact that Runx3<sup>VWRPY-/-</sup> splenocytes express higher-than-WT levels of  $\alpha E/CD103$  implies that the normally reduced expression of  $\alpha E/CD103$  in splenic CD8<sup>+</sup> T cells (27, 41) is not due to a pause in transcriptional activation but rather to a down-regulation of  $\alpha E/CD103$  expression. These data show that in peripheral CD8<sup>+</sup> T cells, Runx3, in conjunction with Gro/TLE, down-regulates  $\alpha E/CD103$  expression. This conclusion is further supported by the marked decline of  $\alpha E/CD103$  expression that was observed in proliferating cultured splenocytes from WT but not from Runx3<sup>VWRPY-/-</sup> 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  $\alpha E/CD103$  transcription.  $\alpha E/CD103$  is a heterodimer composed of the  $\alpha E$  and  $\beta 7$  chains. Because the  $\beta 7$  chain is highly expressed in T cells, the limiting factor in  $\alpha E/CD103$  expression is the transcription of the  $\alpha E$  gene (42). We used RT-PCR to monitor  $\alpha E$  transcription in thymocytes and splenocytes of WT, Runx3<sup>VWRPY-/-</sup>, and Runx3<sup>-/-</sup> mice (Fig. 4D). The loss of Runx3 did not completely abolish  $\alpha E$  transcription in CD8<sup>+</sup> thymocytes or splenocytes (Fig. 4D), as also evidenced by the low, yet detectable, surface expression



**Fig. 4.** Runx3<sup>VWRPY</sup><sup>-/-</sup> activates expression of  $\alpha E$ /CD103 in CD8<sup>+</sup> thymocytes but fails to down-regulate  $\alpha E$ /CD103 expression in peripheral CD8<sup>+</sup> T cells. (A)  $\alpha E$ /CD103 expression in thymocytes from 2-month-old WT (blue), Runx3<sup>VWRPY</sup><sup>-/-</sup> (green), and Runx3<sup>-/-</sup> (pink) mice. Cells were stained with anti-CD8 and anti-CD103 mAb and analyzed by FACS. CD103 expression is shown in SP CD8 thymocytes. (B)  $\alpha E$ /CD103 expression in CD8<sup>+</sup> splenocytes from 2-month-old WT, Runx3<sup>VWRPY</sup><sup>-/-</sup>, and Runx3<sup>-/-</sup> mice, color-coded and analyzed as in A. (C) The average ratio and SE of CD103<sup>+</sup>/CD103<sup>-</sup> in CD8<sup>+</sup> thymocytes ( $n > 3$ ) and splenocytes ( $n > 5$ ) of WT (blue), Runx3<sup>VWRPY</sup><sup>-/-</sup> (green), and Runx3<sup>-/-</sup> (pink) mice. The average proportion of CD103<sup>+</sup> in CD8<sup>+</sup> splenocytes of Runx3<sup>VWRPY</sup><sup>-/-</sup> mice was significantly (\*) higher than in WT mice ( $P = 0.003$ ). (D) Runx3 regulates  $\alpha E$ /CD103 expression through transcriptional regulation of the  $\alpha E$  gene. RT-PCR (Upper) representative results of two and three independent experiments by using independently derived cDNAs of thymus and spleen RNA, respectively, are shown. In thymocytes, the level of  $\alpha E$  transcript was similar in Runx3<sup>VWRPY</sup><sup>-/-</sup> and WT, whereas in splenocytes, it was higher in Runx3<sup>VWRPY</sup><sup>-/-</sup> compared with WT.

of  $\alpha E$ /CD103 in Runx3<sup>-/-</sup> thymocytes and splenocytes (Fig. 4 B and C). However, in comparison with WT splenocytes, the  $\alpha E$  transcription level in Runx3<sup>VWRPY</sup><sup>-/-</sup> splenocytes was significantly higher, whereas in thymocytes, the level was similar (Fig. 4D). These  $\alpha E$  transcription levels correlated well with  $\alpha E$ /CD103 surface expression (Fig. 4 B and C). These results indicate that in CD8-lineage T cells, Runx3 controls  $\alpha E$ /CD103 expression through transcriptional regulation of the heterodimeric subunit  $\alpha E$ . When Runx3 function is lost,  $\alpha E$ /CD103 expression in CD8<sup>+</sup> thymocytes and splenocytes diminished. However, when only the ability to recruit Gro/TLE is lost,  $\alpha E$ /CD103 expression in thymocytes remains normal, but down-regulation in peripheral CD8<sup>+</sup> T cells is

impaired, which attests to the crucial role of Runx3-Gro/TLE in mediating the switch from transcriptional activation to repression.

## Discussion

Through comparisons of biogenesis and function of TrkC neurons, LC/DC and CD8<sup>+</sup> T lymphocytes in Runx3<sup>VWRPY</sup><sup>-/-</sup>, Runx3<sup>-/-</sup>, and WT mice, we obtained unique insights into the bifunctional nature of Runx3 and into the mechanism by which it represses 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- $\beta$  signaling cascade, recruitment of Gro/TLE is required for proper maturation of DC but not for normal development of skin LC.

Analysis of Runx3<sup>VWRPY</sup><sup>-/-</sup> function in the CD8 T cell-lineage provided evidence that Runx3 regulates transcription of  $\alpha E$ /CD103 in opposing regulatory modes and that repression of both  $\alpha E$ /CD103 and *CD4* requires the binding of the corepressor, Gro/TLE. Given that *CD4* transcription in CD8<sup>+</sup> 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 Runx3<sup>VWRPY</sup><sup>-/-</sup> splenic T cells as compared with Runx3<sup>-/-</sup> cells? A possible explanation could be that while in Runx3<sup>-/-</sup> cells, the loss of Runx3 is partially compensated by Runx1 activity (11, 30), in Runx3<sup>VWRPY</sup><sup>-/-</sup> 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 Runx3<sup>VWRPY</sup><sup>-/-</sup> T cells compared with Runx3<sup>-/-</sup> cells. This hypothesis is supported by findings that in a compound mutant mouse Runx3<sup>-/-</sup>/Runx1<sup>+/-</sup>, i.e., null for Runx3 and hemizygous for Runx1, *CD4* expression in CD8<sup>+</sup> T cells is completely derepressed and all peripheral CD8<sup>+</sup> 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  $\alpha E$ /CD103 and *CD4* occurs in mature CD8<sup>+</sup> 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.

## Materials and Methods

**Generation of Runx3<sup>VWRPY</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 VWRPY− allele, the nucleotides encoding the C-terminal end VWRPY, 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 inserted into the unique *AccI* site in intron 5 (Fig. 1). Correctly targeted R1 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 crossed onto the transgenic Cre-deleter mouse strain PGK-Cre (32) to generate Runx3<sup>VWRPY</sup>−/− mice lacking 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>VWRPY</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 exon 6 (5′CGTAGGGAAGGAGCGGTCAA), detected both P1 and P2 promoter-derived transcripts (47), yielding a 673-bp fragment. For  $\alpha E$ , 5′CCAGAAGGCCAAAATTTC (sense) and 5′TCAGCAGCGACTCCTTTCCGCTT (antisense) yielded a 197-bp fragment. mRNA levels within samples were assessed by PCR analysis of actin cDNA.

**Immunohistochemistry and Histology.** Paraffin sections from embryos at embryonic day 14.5 and 17.5 for immunostaining with anti-Runx3 and anti-PV, respectively, were processed as described in ref. 9. Primary antibodies were rabbit anti-Runx3 (1:1,000) and anti-PV (1:1,500) (Swant, Bellinzona, Switzerland). Biotinylated

secondary antibodies and the ABC complex from Vectastain kit (Vector Laboratories) were used for detection.

**Flow Cytometry.** 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 FACSCalibur (Becton Dickinson) and CELLQUEST software (Becton Dickinson). mAbs included CD4-biotinylated, CD8 $\alpha$ -Percep, CD8 $\beta$ -PE/Percep, TCR $\beta$ -FITC, 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>VWRPY</sup>−/− or Runx3<sup>−/−</sup>) were evaluated by using Student's *t* test.

**Cell Proliferation Assays.** Splenic CD8<sup>+</sup> cells were isolated by using a magnetic cell sorting separation system (Miltenyi Biotec, Auburn, CA), labeled by incubation with 5  $\mu$ M 5,6-carboxyfluorescein diacetate, succinimidyl ester (Molecular Probes) and further processed as detailed in ref. 12. Cells were stimulated with anti-CD3 mAb [(2  $\mu$ g/ml; Pharmingen) plus IL-2 (20u/ml; PeproTech, Rocky Hill, NJ) and at 0, 48, and 72 h of incubation time, cells were stained with anti-CD8 $\beta$  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 lipofectamin (Invitrogen) (COS-7) and CaPO<sub>4</sub> (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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