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Auxiliary splice factor U2AF26 and transcription factor Gfi1 cooperate directly in regulating CD45 alternative splicing

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By alternative splicing, different isoforms of the transmembrane tyrosine phosphatase CD45 are generated that either enhance or limit T cell receptor signaling. We report here that CD45 alternative splicing is regulated by cooperative action of the splice factor U2AF26 and the transcription factor Gfi1. U2AF26 promoted formation of the less-active CD45RO by facilitating exon exclusion. Gfi1 antagonized that process by directly interacting with U2AF26, identifying a previously unknown link between a transcription factor and alternative splicing. The presence of Gfi1 led to formation of the more-active CD45RB, whereas loss of Gfi1 favored CD45RO production. We propose that the relative abundance of U2AF26 and Gfi1 determines the ratio of CD45 isoforms, thereby regulating T cell activation.

By means of alternative splicing, different mature transcripts with different protein-encoding potential can evolve from one pre-mRNA molecule. It is estimated that up to 60% of all human genes are alternatively spliced[1]. The potentially very large number of protein isoforms that can be generated by alternative splicing represents a substantial source of proteome diversity that can vary with the developmental or differentiation stage of a cell or can depend on external stimuli[1–3]. During the regular splicing process, the 3′ and 5′ splice sites that flank exons must be recognized by the 'spliceosome', a multiprotein-RNA complex that catalyzes the splice reaction. In addition to the 3′ and 5′ splice sites, there are sequences in exons that enhance or inhibit 'spliceosomal' recruitment[4]; these are called exonic splicing enhancers or silencers. Furthermore, a tight link has been established between transcription and splicing in terms of promoter and enhancer activity or chromatin structure, but direct interactions between transcription factors and components of the splicing machinery have also been shown[5].

U2af1l4 encodes the splice factor U2AF26, a 26-kilodalton protein with 76% sequence similarity to the related U2 small nuclear ribonucleoprotein auxiliary factor 35 (U2AF35)[6], which is encoded by *U2af1*. U2AF35 and U2AF26 contain two zinc fingers and a noncanonical RNA-recognition motif of the ribonucleoprotein domain type in the N-terminal part of the protein, but only U2AF35 bears an arginine-serine–rich domain and a glycine stretch at the C terminus. Both U2AF26 and U2AF35 can associate with U2AF65 and participate in constitutive and enhancer-dependent splicing[6,7], in part through binding to the AG dinucleotide at the 3′ splice site, thereby facilitating formation of the spliceosome[8].

CD45 is a transmembrane tyrosine phosphatase with a heavily glycosylated extracellular domain that is found on all nucleated hematopoietic cells[9]. The CD45 pre-mRNA contains three variable exons, called exons 4, 5 and 6 or exons A, B and C, that are alternatively spliced to produce eight different isoforms in mouse T cells depending on their developmental stage and activation status[10]. Many experiments in mutant cell lines and CD45-deficient mice have established involvement of this protein in antigen-dependent B cell and T cell activation[11–13]. In particular, CD45 has been recognized as a critical regulator of T cell receptor (TCR)–mediated signaling. After antigen-induced crosslinking of the TCR, CD45 removes an inhibitory phosphate group from kinases such as Src or Lck, thereby enabling the activation of 'downstream' mediators such as Zap70 by tyrosine phosphorylation[14]. During T cell activation, there is a shift in expression from the CD45 isoforms containing exons A, B and C to the CD45RO isoform lacking all three exons[15,16]. This 'activation-induced alternative splicing' may be part of a negative feedback regulation, as CD45RO seems to limit T cell proliferation[17]. How the shift in CD45 isoform expression is controlled and which factors are involved is only poorly understood.

T cell activation is controlled by many factors, including those regulating transcription. One transcription factor induced from basal expression after antigen recognition is the nuclear zinc finger transcriptional repressor Gfi1 (refs. 18–20). Studies using transgenic mice have shown accelerated entry of T cells into S phase after antigenic stimulation in the presence of high constitutive expression of Gfi1, whereas Gfi1-deficient T cells respond less well to TCR stimulation[18] (unpublished data). While attempting to elucidate the

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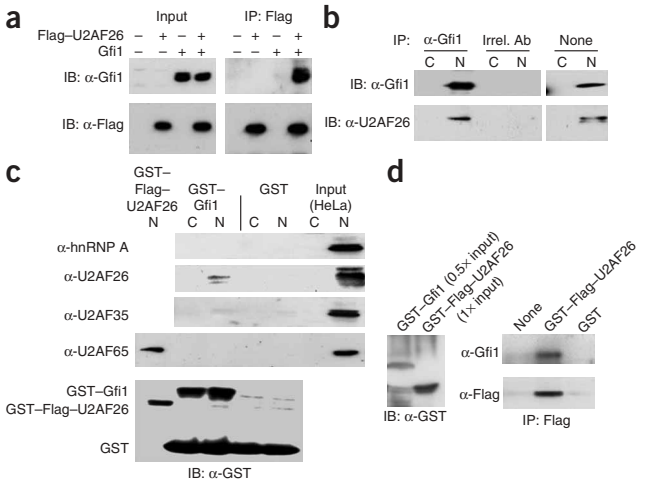

Figure 1 Gfi1 interacts with U2AF26. (**a**) Immunoblot (left) or immunoprecipitation and immunoblot (right) of Cos7 cells transfected with full-length cDNA constructs encoding Gfi1 and Flag-tagged U2AF26 (Flag–U2AF26). Right, anti-Flag was used for immunoprecipitation (IP) and immunoblot (IB) was used to analyze bound proteins. α, antibody to (**b**) Immunoprecipitation and immunoblot of Jurkat cytoplasmic (C) or nuclear (N) extracts. Anti-Gfi1 (α-Gif1) or an isotype-matched control antibody (Irrel Ab.) was used for immunoprecipitation. None, no immunoprecipitation (input). (**c**) Immunoblot of GST, GST–Gfi1 and GST–Flag-tagged U2AF26. Proteins were bacterially expressed, purified and immobilized, then were incubated with HeLa cytoplasmic (C) or nuclear (N) extracts; bound proteins were analyzed after extensive washing (antibodies, left margin). (**d**) Left, immunoblot of GST–Gfi1 and GST–Flag-tagged U2AF26 eluted from glutathione beads with reduced glutathione. Right, immunoprecipitation and immunoblot: GST–Gfi1 was mixed with GST or GST–Flag-tagged U2AF26, followed by immunoprecipitation with anti-Flag in the presence of RNase T1 and immunoblot of precipitated proteins (antibodies, left margin). Results are all representative of three independent experiments.

function of Gfi1 in T cell activation, we found that the splice factor U2AF26 bound to Gfi1 and that both proteins seemed to cooperate in regulating alternative splicing of the gene encoding CD45 (*Ptprc*). In particular, U2AF26 induced exon skipping in CD45 pre-mRNA, thereby inducing formation of the CD45RO isoform that limits T cell activation, whereas Gfi1 counteracted that effect and promoted formation of the higher-molecular-weight CD45 isoforms associated with T cell activation.

RESULTS

Gfi1 interacts with U2AF26

To identify Gfi1-binding-partner candidates, we did yeast two-hybrid screens with full-length Gfi1 or a truncated Gfi1 lacking the zinc finger domains (**Supplementary Fig. 1** online). Several clones containing inserts encoding a C-terminal part of U2AF26 emerged from those screens (**Supplementary Fig. 1**). To confirm the interaction, we transfected full-length cDNA constructs together into Cos7 cells (**Fig. 1a**, left) and noted precipitation of Gfi1 together with Flag-tagged U2AF26 (**Fig. 1a**, right), whereas Flag-tagged U2AF26 did not interact with the unrelated transcription factor p53 (data not shown). We further confirmed interaction between Gfi1 and U2AF26 by coimmunoprecipitation of endogenous proteins from Jurkat nuclear extracts (**Fig. 1b**). Precipitation experiments showed interaction of a glutathione *S*-transferase (GST)–Gfi1 fusion protein with U2AF26 but not with U2AF35, U2AF65 or heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) from HeLa nuclear extracts, demonstrating the specificity of the interaction. We used the previously described[6] interaction between U2AF26 and U2AF65 as positive control (**Fig. 1c**). We confirmed direct physical interaction between Gfi1 and U2AF26 by using bacterially expressed, purified GST-Gfi1 and GST-U2AF26, which showed interaction in the presence of RNase excluding an indirect, RNA-mediated complex (**Fig. 1d**). In addition, immunofluorescence experiments demonstrated colocalization of both proteins in the nuclei of transfected cells (data not shown).

Only full-length Gfi1 protein and a truncated version lacking amino acids 1–189 were efficiently retained by a GST-U2AF26 fusion protein in a precipitation assay (**Supplementary Fig. 1**), indicating that a region adjacent to the zinc finger domain and the zinc finger in Gfi1 were necessary for the interaction with U2AF26. We furthermore mixed wild-type and two Flag-tagged U2AF26 mutants, one corresponding to a naturally occurring splice variant lacking amino acids 162–194, with radiolabeled Gfi1 protein translated *in vitro*. We then immunoprecipitated these with antibody to Flag and found that a region including the second zinc finger of U2AF26 was needed for interaction with Gfi1 (**Supplementary Fig. 1**).

U2AF26 affects alternative splicing of *Ptprc*

As Gfi1 has an established function in T cell development and activation[18,19,21], we hypothesized that the Gfi1-U2AF26 interaction might affect CD45 pre-mRNA splicing, given that CD45 is one of the main regulators of T cell activation. After transfecting RK13 kidney epithelial cells (which we used because they lack endogenous CD45, U2AF26 and Gfi1; data not shown) with a *PTPRC* 'minigene' construct[10] (containing the genomic PTPRC region shown in **Fig. 2a** in an expression vector) plus a vector directing the expression of Flag-tagged U2AF26, we analyzed isoform expression by RT-PCR (**Fig. 2b**). Production of the CD45RBC and CD45RB isoforms was reduced substantially with increasing amounts of U2AF26 (**Fig. 2b**, top, lanes 2–5), and the ratio of CD45RB to CD45RO was altered substantially in favor of the CD45RO isoform (**Fig. 2c**, left). In parallel with the U2AF26-mediated skipping of exon B, expression of the CD45RAB isoform was induced (**Fig. 2b**, top), excluding the possibility of nonspecific downregulation of higher-molecular-weight isoforms. However, the CD45RB/CD45RO ratio remained unaffected when the nonfunctional mutant of U2AF26 lacking the second zinc finger was transfected (**Fig. 2d**).

To investigate whether the downregulation of higher-molecular-weight CD45 isoforms was unique to U2AF26, we tested the activity of the closely related U2AF35 in the same assay. U2AF35 also induced CD45 exon skipping, but much less so than U2AF26 (**Fig. 2b**, top, and **c**, left). This occurred despite similar protein abundance (**Fig. 2b**, bottom) and a similar efficiency in inducing the CD45RAB isoform (**Fig. 2c**, right). To quantify the different effects of U2AF26 and U2AF35 on alternative splicing of *Ptprc*, we established a quantitative real-time PCR assay specific for either CD45RO or CD45RB and CD45RBC using probes spanning exon-exon junctions (**Supplementary Fig. 2** online). With this assay we again found U2AF26 to be far more potent than U2AF35 in inducing formation of the CD45RO isoform (**Supplementary Fig. 2**), confirming the results obtained by standard RT-PCR (**Fig. 2b**,**c**).

To test whether ablation of U2AF26 expression would have the reverse effect, we transfected the *PTPRC* minigene and a U2AF26-specific small interfering RNA (siRNA) into mouse 3T3 fibroblasts, in which the CD45RO form is predominant[22] (**Fig. 2e**). That could be

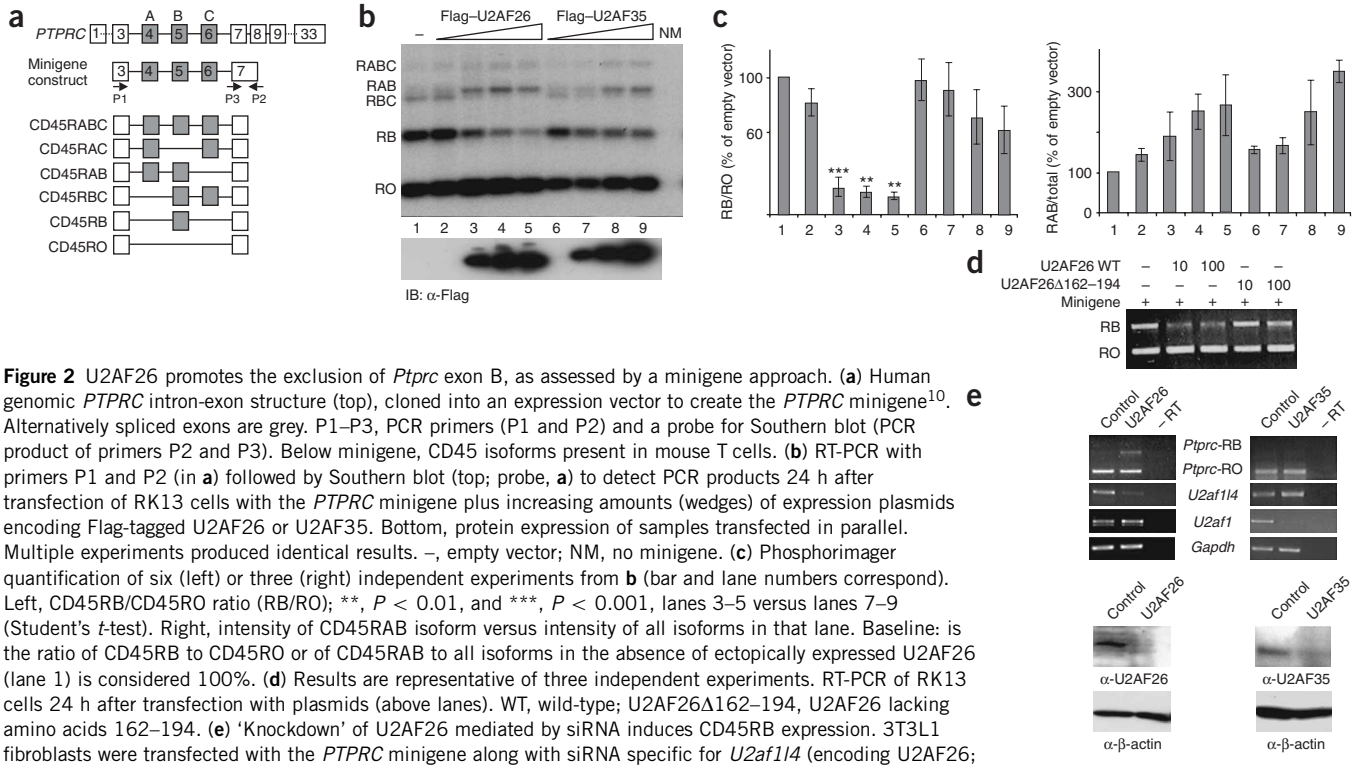

Figure 2 U2AF26 promotes the exclusion of *Ptprc* exon B, as assessed by a minigene approach. (**a**) Human genomic *PTPRC* intron-exon structure (top), cloned into an expression vector to create the *PTPRC* minigene[10]. Alternatively spliced exons are grey. P1–P3, PCR primers (P1 and P2) and a probe for Southern blot (PCR product of primers P2 and P3). Below minigene, CD45 isoforms present in mouse T cells. (**b**) RT-PCR with primers P1 and P2 (in **a**) followed by Southern blot (top; probe, **a**) to detect PCR products 24 h after transfection of RK13 cells with the *PTPRC* minigene plus increasing amounts (wedges) of expression plasmids encoding Flag-tagged U2AF26 or U2AF35. Bottom, protein expression of samples transfected in parallel. Multiple experiments produced identical results. –, empty vector; NM, no minigene. (**c**) Phosphorimager quantification of six (left) or three (right) independent experiments from **b** (bar and lane numbers correspond). Left, CD45RB/CD45RO ratio (RB/RO); **, $P < 0.01$, and ***, $P < 0.001$, lanes 3–5 versus lanes 7–9 (Student's *t*-test). Right, intensity of CD45RAB isoform versus intensity of all isoforms in that lane. Baseline: is the ratio of CD45RB to CD45RO or of CD45RAB to all isoforms in the absence of ectopically expressed U2AF26 (lane 1) is considered 100%. (**d**) Results are representative of three independent experiments. RT-PCR of RK13 cells 24 h after transfection with plasmids (above lanes). WT, wild-type; U2AF26Δ162–194, U2AF26 lacking amino acids 162–194. (**e**) 'Knockdown' of U2AF26 mediated by siRNA induces CD45RB expression. 3T3L1 fibroblasts were transfected with the *PTPRC* minigene along with siRNA specific for *U2af1l4* (encoding U2AF26; left) or *U2af1* (encoding U2AF35; right) or a nonsilencing control siRNA (Control), and 'knockdown' of *U2af1l4* mRNA (left) or *U2af1* mRNA (right) and U2AF26 protein (left) or U2AF35 protein (right) was analyzed by RT-PCR and immunoblot, respectively. Primers P1 and P2 (**Fig. 1a**) were used for *Ptprc*-specific PCR; *Gapdh* encodes glyceraldehyde 3-phosphate dehydrogenase (loading control); –RT, no reverse transcriptase. For immunoblot analysis, membranes were incubated with anti-U2AF26 (left) or anti-U2AF35 (right) and then with anti-β-actin (loading control). Results are representative of three independent experiments.

due to higher endogenous expression of U2AF26 in 3T3 fibroblasts than in RK13 cells (data not shown). The silencing proved to be efficient and specific, as U2AF26 mRNA and protein expression were decreased, whereas transcript abundance of the close homolog *U2af1* (encoding U2AF35) was not detectably altered (**Fig. 2e**, left). CD45-specific PCR showed that in the absence of U2AF26, production of CD45RB mRNA was enhanced (**Fig. 2e**, top left). As a control, we also 'knocked down' expression of U2AF35, which did not affect the *Ptprc* splicing pattern in our minigene system (**Fig. 2e**, right). We concluded from these experiments that U2AF26 was far more potent than U2AF35 in inducing *Ptprc* exon skipping and we therefore focused on U2AF26 and its function in T cell activation.

U2AF26 overexpression in T cells alters *Ptprc* splicing

To test whether U2AF26 was able to induce formation of the CD45RO isoform *in vivo*, we generated transgenic mice expressing Flag-tagged U2AF26 in the T cell compartment by use of the well established proximal *Lck* promoter (**Fig. 3a**). Analysis of extracts from thymus and spleen from several transgenic lines with antibodies to both Flag and U2AF26 demonstrated robust transgene expression (**Fig. 3b**), with the highest expression in lines TG1 and TG7 (**Fig. 3c**). To more closely analyze the expression pattern in peripheral T cells, we used real-time PCR to compare transgene expression in resting versus stimulated purified splenic T cells. We detected expression of the transgene encoding the Flag-tagged U2AF26 in naive T cells, but found it was downregulated after T cell activation, whereas U2AF26 expression was still higher than that in wild-type cells (**Supplementary Fig. 3** online and data not shown). We therefore used either naive T cells or a time point early after stimulation for further analysis.

RT-PCR analysis of splenic T cells from the TG7 line with high expression of the transgene, purified by magnetic-activated cell sorting, showed that in both $CD4^+$ and $CD8^+$ T cells the CD45RBC and CD45RABC isoforms were no longer detectable and the CD45RB/CD45RO ratio was shifted toward the CD45RO isoform in the transgenic mice (**Fig. 3d**). In addition, $CD4^+$ transgenic T cells showed upregulation of a CD45 isoform lacking exons 4–7 (ref. 23; **Fig. 3d**). Real-time PCR confirmed a change in CD45 isoform expression toward the CD45RO isoform for $CD4^+$ and $CD8^+$ purified splenic T cells as well as for thymocytes from mice transgenic for the Flag-tagged U2AF26 (**Supplementary Fig. 2**).

Flow cytometry confirmed a shift to lower CD45RB expression in transgenic compared with wild-type thymocytes in a U2AF26 dose–dependent way. The effect was most noticeable in the TG1 and TG7 lines with high transgene expression, was less prominent in lines with intermediate transgene expression and was barely detectable in the TG5 line with low transgene expression (**Fig. 4a** and data not shown). The downregulation of CD45RB was isoform specific, as total CD45 protein abundance remained unaltered (**Fig. 4a**, right). For peripheral T cells from the spleen, flow cytometry confirmed a U2AF26 dose–dependent downregulation of CD45RB expression (**Fig. 4b**).

Impaired TCR signaling in mice transgenic for Flag-U2AF26

To test whether transgenic expression of the Flag-tagged U2AF26 had functional consequences on T cell activity, we assessed thymidine

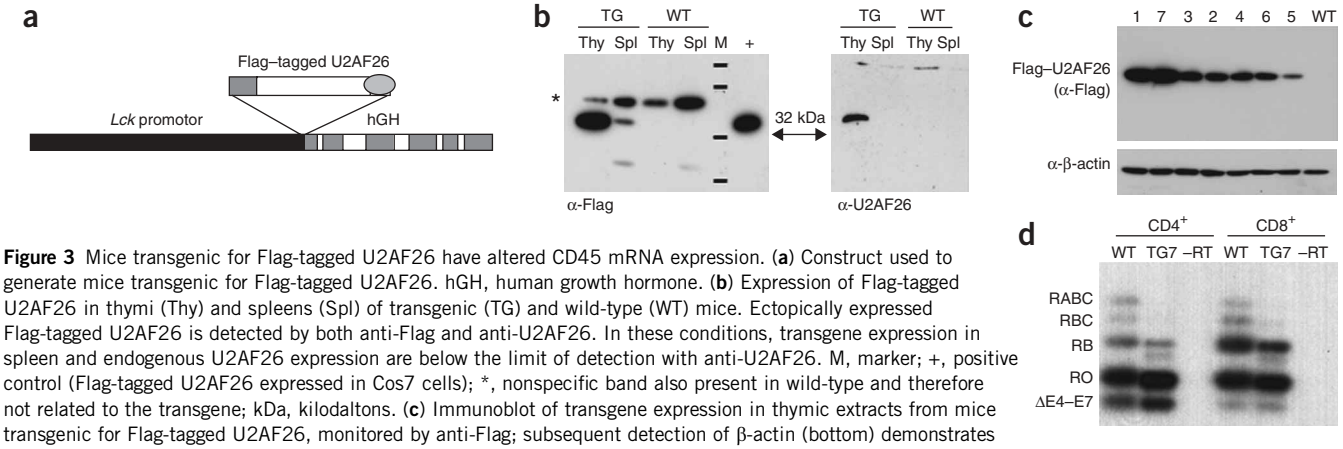

Figure 3 Mice transgenic for Flag-tagged U2AF26 have altered CD45 mRNA expression. (**a**) Construct used to generate mice transgenic for Flag-tagged U2AF26. hGH, human growth hormone. (**b**) Expression of Flag-tagged U2AF26 in thymi (Thy) and spleens (Spl) of transgenic (TG) and wild-type (WT) mice. Ectopically expressed Flag-tagged U2AF26 is detected by both anti-Flag and anti-U2AF26. In these conditions, transgene expression in spleen and endogenous U2AF26 expression are below the limit of detection with anti-U2AF26. M, marker; +, positive control (Flag-tagged U2AF26 expressed in Cos7 cells); *, nonspecific band also present in wild-type and therefore not related to the transgene; kDa, kilodaltons. (**c**) Immunoblot of transgene expression in thymic extracts from mice transgenic for Flag-tagged U2AF26, monitored by anti-Flag; subsequent detection of β-actin (bottom) demonstrates equal loading. Each transgenic line (lane numbers indicate transgenic lines TG1–TG7) was assayed in at least three independent experiments. (**d**) Reduced CD45RB expression in peripheral T cells of mice transgenic for *Lck*-driven Flag-tagged U2AF26 (TG7 line). $CD4^+$ or $CD8^+$ splenic T cells from mice of the TG7 line or wild-type mice were purified by magnetic-activated cell sorting and were assayed for *Ptprc* expression by RT-PCR; PCR products were detected by Southern blot with an internal ^{32}P-labeled probe. The identity of cDNA encoding the CD45 isoforms (left margin) was confirmed by sequencing and corresponds to the published pattern in wild-type cells[38]. ΔE4–E7, Ptprc lacking exons 4–7. Results are representative of three pairs of mice.

incorporation 24 h after stimulation. An early time point such as that should demonstrate differences in the ability of naive T cells to become activated and was therefore suited for investigation of the effect of the *Lck*-driven transgene encoding Flag-tagged U2AF26, despite its down-regulation after T cell stimulation (**Supplementary Fig. 3**). After being stimulated with antibody to CD3 (anti-CD3) and the phorbol ester phorbol 12-myristate 13-acetate (PMA), T cells from the TG1 and TG7 lines had a considerably impaired proliferative response (**Fig. 4c** and data not shown). The effect was less prominent in the TG2 line, which correlated with lower transgene expression (**Fig. 4c**). Notably, when we used a mitogenic combination that acts independently of TCR signaling (PMA plus ionomycin), the difference in the proliferative response of transgenic and wild-type T cells was smaller (**Fig. 4c**). To establish a direct link among U2AF26 expression, disturbed CD45 isoform expression and the inability to respond properly to TCR stimulation, we investigated a TCR-proximal signaling event: activation of the 'downstream' mediator Zap70 by phosphorylation of its tyrosine residue at position 319 (Y319). Whereas splenic T cells or thymocytes from wild-type mice that had been purified by magnetic-activated cell sorting showed robust Zap70 Y319 phosphorylation after CD3 crosslinking, cell populations from the TG1 and TG7 lines showed downregulation and absence, respectively, of Zap70 phosphorylation (**Fig. 4d**). Those data indicated a severe signaling defect that could have been due to the prevalence of the less-active CD45RO isoform in

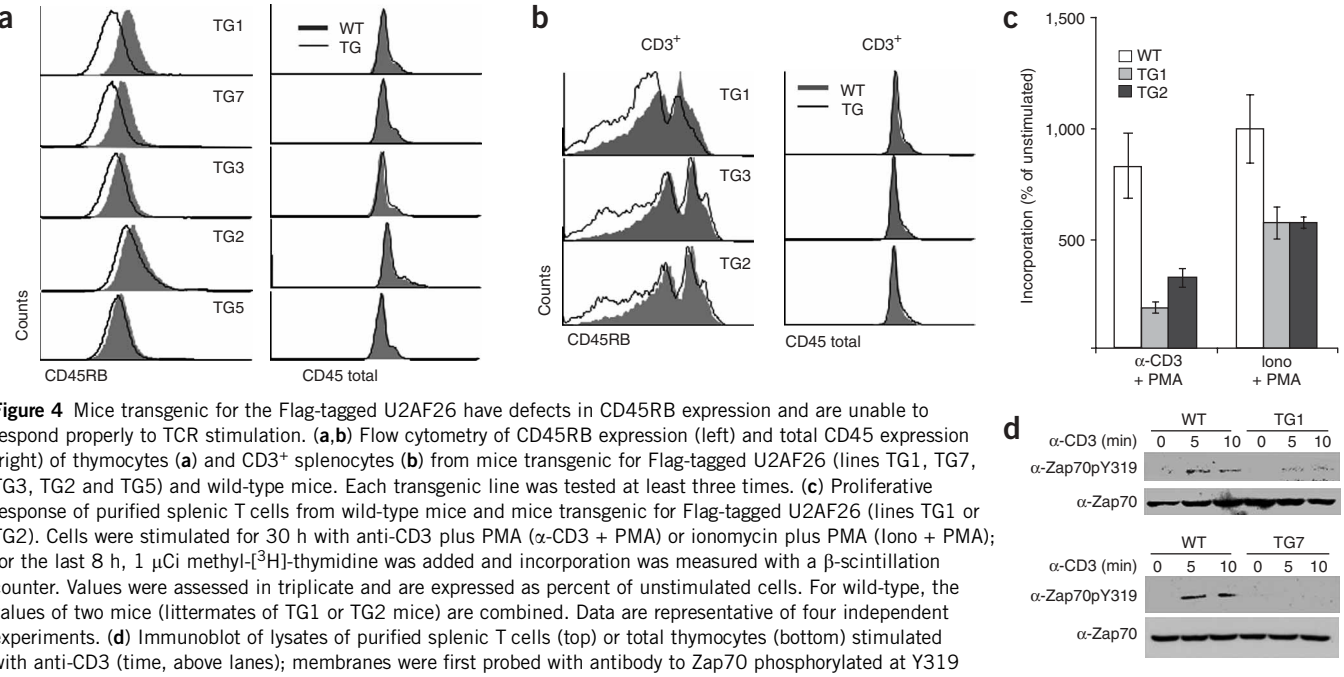

Figure 4 Mice transgenic for the Flag-tagged U2AF26 have defects in CD45RB expression and are unable to respond properly to TCR stimulation. (**a**,**b**) Flow cytometry of CD45RB expression (left) and total CD45 expression (right) of thymocytes (**a**) and $CD3^+$ splenocytes (**b**) from mice transgenic for Flag-tagged U2AF26 (lines TG1, TG7, TG3, TG2 and TG5) and wild-type mice. Each transgenic line was tested at least three times. (**c**) Proliferative response of purified splenic T cells from wild-type mice and mice transgenic for Flag-tagged U2AF26 (lines TG1 or TG2). Cells were stimulated for 30 h with anti-CD3 plus PMA (α-CD3 + PMA) or ionomycin plus PMA (Iono + PMA); for the last 8 h, 1 μCi methyl-[^{3}H]-thymidine was added and incorporation was measured with a β-scintillation counter. Values were assessed in triplicate and are expressed as percent of unstimulated cells. For wild-type, the values of two mice (littermates of TG1 or TG2 mice) are combined. Data are representative of four independent experiments. (**d**) Immunoblot of lysates of purified splenic T cells (top) or total thymocytes (bottom) stimulated with anti-CD3 (time, above lanes); membranes were first probed with antibody to Zap70 phosphorylated at Y319 (α-Zap70pY319), then were washed thoroughly and reprobed with anti-Zap70 (α-Zap70). Data are representative results of three independent experiments.

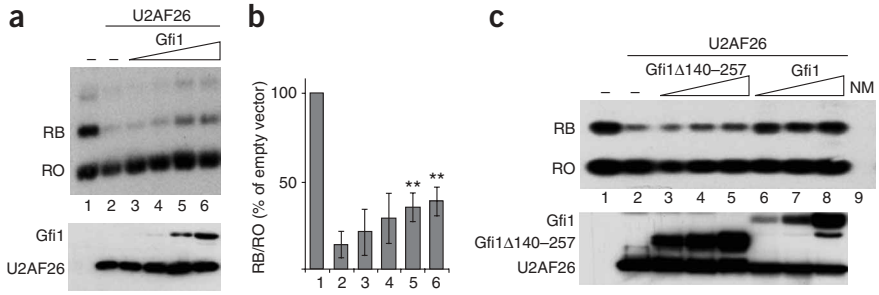

Figure 5 Gfi1 reverses the U2AF26-mediated reduction in CD45RB expression. (**a**) Gfi1 counteracts the effect of overexpressed U2AF26. RK13 cells were transfected with expression plasmids encoding Flag-tagged proteins (above blots; wedge indicates increasing amounts), along with the *PTPRC* minigene; 24 h later, RT-PCR was done as described for **Figure 2** (top). Bottom, anti-Flag immunoblot of protein expression by cells transfected in parallel. Data are representative of three experiments. (**b**) Phosphorimager quantification of the results in **a**. The ratio of CD45RB to CD45RO in the absence of ectopically expressed U2AF26 is considered 100% (baseline). **, $P < 0.01$, lanes 3–6 versus lane 2 (no Gfi1 expression). Results are representative of three independent experiments. (**c**) A Gfi1 deletion mutant lacking amino acids 140–257 (Gfi1Δ140–257) does not interfere with U2AF26-mediated exon skipping. RK13 cells were transfected with expression plasmids, encoding Flag-tagged proteins along with the *PTPRC* minigene, and CD45 isoform expression (top) and protein expression (bottom) were analyzed as described in **a**. Similar results were obtained in three experiments. NM, no minigene.

transgenic T cells. The differences between wild-type and transgenic T cells were not due to altered expression of costimulatory molecules or altered presence of T cell subtypes, as we did not find any substantial changes in the expression of CD3, CD4, CD8, CD44 or CD62L (**Supplementary Fig. 4** online and data not shown).

Gfi1 antagonizes the activity of U2AF26

As Gfi1 was able to bind U2AF26, we hypothesized that Gfi1 could influence the effect of U2AF26 on the alternative splicing of *Ptprc*. In the *PTPRC* minigene assay, Gfi1 was able to reverse the effect of U2AF26 and to restore formation of the CD45RB isoform (**Fig. 5a**,**b**). In contrast, a mutant Gfi1 lacking amino acids 140–257 that did not bind U2AF26 in a GST precipitation assay (**Supplementary Fig. 1**) also did not reverse the effect of U2AF26 on the alternative splicing of *Ptprc* (**Fig. 5c**, top), despite higher expression of the mutant protein (**Fig. 5c**, bottom). We quantified the effect of Gfi1 and the mutant Gfi1 lacking amino acids 140–257 on the alternative splicing of *Ptprc* using real-time PCR. In this assay, large amounts of Gfi1 were able to reverse the U2AF26-mediated loss of CD45RB, whereas the mutant Gfi1 showed only weak activity (**Supplementary Fig. 2**), which might have been due to residual binding of the mutant protein to U2AF26.

Ptprc splicing and TCR response in *Gfi1*$^{-/-}$ mice

To further investigate the function of Gfi1 on the alternative splicing of *Ptprc*, we analyzed Gfi1-deficient mice[24]. Splenic T cells purified from *Gfi1*$^{-/-}$ mice had a significantly lower CD45RB/CD45RO ratio than did wild-type T cells ($P < 0.001$; **Fig. 6a**), which we also noted by real-time PCR (**Supplementary Fig. 2**). Comparison of the abundance

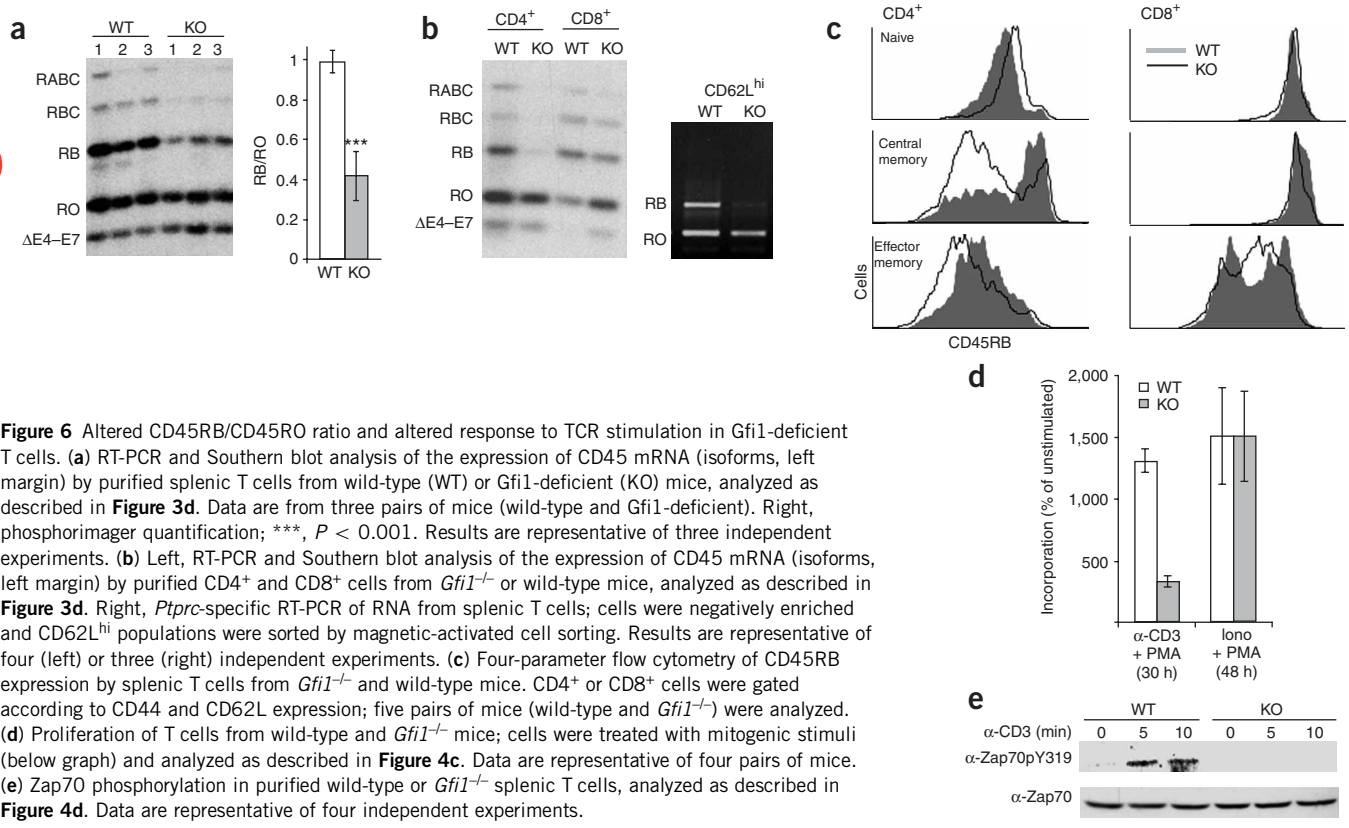

Figure 6 Altered CD45RB/CD45RO ratio and altered response to TCR stimulation in Gfi1-deficient T cells. (**a**) RT-PCR and Southern blot analysis of the expression of CD45 mRNA (isoforms, left margin) by purified splenic T cells from wild-type (WT) or Gfi1-deficient (KO) mice, analyzed as described in **Figure 3d**. Data are from three pairs of mice (wild-type and Gfi1-deficient). Right, phosphorimager quantification; ***, $P < 0.001$. Results are representative of three independent experiments. (**b**) Left, RT-PCR and Southern blot analysis of the expression of CD45 mRNA (isoforms, left margin) by purified CD4$^+$ and CD8$^+$ cells from *Gfi1*$^{-/-}$ or wild-type mice, analyzed as described in **Figure 3d**. Right, *Ptprc*-specific RT-PCR of RNA from splenic T cells; cells were negatively enriched and CD62L^{hi} populations were sorted by magnetic-activated cell sorting. Results are representative of four (left) or three (right) independent experiments. (**c**) Four-parameter flow cytometry of CD45RB expression by splenic T cells from *Gfi1*$^{-/-}$ and wild-type mice. CD4$^+$ or CD8$^+$ cells were gated according to CD44 and CD62L expression; five pairs of mice (wild-type and *Gfi1*$^{-/-}$) were analyzed. (**d**) Proliferation of T cells from wild-type and *Gfi1*$^{-/-}$ mice; cells were treated with mitogenic stimuli (below graph) and analyzed as described in **Figure 4c**. Data are representative of four pairs of mice. (**e**) Zap70 phosphorylation in purified wild-type or *Gfi1*$^{-/-}$ splenic T cells, analyzed as described in **Figure 4d**. Data are representative of four independent experiments.

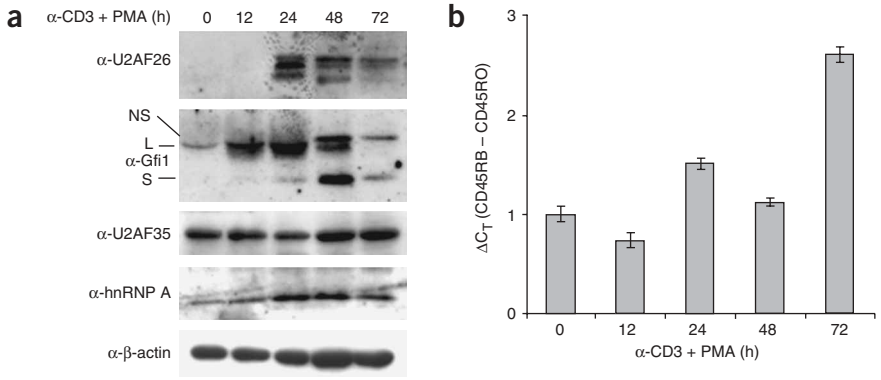

Figure 7 U2AF26 and Gfi1 are induced with different kinetics after T cell activation. (**a**) Immunoblot analysis of lysates of splenic T cells negatively enriched by magnetic-activated cell sorting and stimulated with 0.5 μg/ml of anti-CD3 plus 250 ng/ml of PMA (times), blotted sequentially (antibodies, left margin). The several U2AF26-specific signals detected could be the result of alternative splicing or post-translational modifications such as phosphorylation. The appearance of Gfi1 isoforms after T cell activation (S, small; L, large) has been reported before[19]; NS, nonspecific band. Four independent experiments produced similar results. (**b**) Real-time PCR of cDNA generated from T cells purified and stimulated as described in **a**. PCR was specific for CD45RO or CD45RB (**Supplementary Fig. 2**). Cycling threshold (ΔC_T) values (CD45RB – CD45RO) were calculated and were set to 1 for unstimulated cells. Samples were assessed in triplicate; data are representative of four mice.

of CD45RB and CD45RO relative to that of glyceraldehyde 3-phosphate dehydrogenase by real-time PCR showed that the altered CD45RB/CD45RO ratio was mediated mainly by a loss of CD45RB and not by a gain of CD45RO (**Supplementary Fig. 2**). Instead, the *Ptprc* isoform lacking exons 3–7 seemed to be upregulated in *Gfi1*$^{-/-}$ mice (**Fig. 6a**). The loss of Gfi1 therefore seemed to also interfere with alternative splicing of *Ptprc* exon 7, as noted above in CD4$^+$ T cells and thymocytes of mice transgenic for the Flag-tagged U2AF26 (**Fig. 3d** and data not shown). CD4$^+$ or CD8$^+$ subpopulations from Gfi1-deficient mice had the same loss of CD45RB (**Fig. 6b**), as did the CD62L^{hi} subset of splenic T cells (**Fig. 6b**), indicating that Gfi1 is needed to maintain the correct CD45RB/CD45RO ratio in T cells.

Next we used flow cytometry to compare CD45RB expression in wild-type and *Gfi1*$^{-/-}$ naive T cells (CD44^{lo}CD62L^{hi}), central memory T cells (CD44^{hi}CD62L^{hi}) and effector memory T cells (CD44^{hi}CD62L^{lo}) from the CD4$^+$ and the CD8$^+$ subsets. CD4$^+$ central and effector memory cells and CD8$^+$ effector memory cells from *Gfi1*$^{-/-}$ mice showed the most noticeable shift toward lower CD45RB expression, whereas this effect was at best marginal in the other subpopulations, and total CD45 expression was not altered (**Fig. 6c** and data not shown). Concomitant with reduced expression of the CD45RB isoform, *Gfi1*$^{-/-}$ T cells responded much less to the antigenic stimulation of treatment with anti-CD3 and PMA, whereas we found no difference in wild-type versus *Gfi1*$^{-/-}$ cells after treatment with PMA and ionomycin (**Fig. 6d**). As with our results using cells from mice transgenic for the Flag-tagged U2AF26, we found that *Gfi1*$^{-/-}$ T cells were unable to respond with phosphorylation of Zap70 Y319 after TCR stimulation (**Fig. 6e**).

Gfi1 and U2AF26 are induced after T cell activation

To investigate whether Gfi1 and U2AF26 were involved in regulating the activation-induced alternative splicing *in vivo*, we analyzed the expression of both proteins in primary T cells after antigenic activation. Using immunoblot analysis, we detected substantial upregulation of U2AF26 expression at 24, 48 and 72 h after stimulation, whereas we found only small changes in U2AF35 and hnRNP A expression (**Fig. 7a**). Consistent with early and transient upregulation of Gfi1 mRNA after T cell activation[19], we detected induction of Gfi1 protein after 12 h of stimulation and the appearance of different Gfi1 isoforms, as described before[19], as well as a nearly complete loss of Gfi1 expression 3 d after activation (**Fig. 7a**). To correlate the expression of Gfi1 and U2AF26 with the changes in CD45 isoform expression after T cell activation, we used real-time PCR to quantify CD45RB and CD45RO isoforms at different time points after stimulation. In this assay, we detected an early increase in *Ptprc* isoforms containing exon B and an accumulation of CD45RO mRNA 3 days after activation, which correlated with increased Gfi1 expression 12 h after stimulation and increased U2AF26 expression 3 d after activation (**Fig. 7b**). This expression pattern again suggests involvement of Gfi1 and U2AF26 in regulating alternative splicing of *Ptprc* during T cell activation.

DISCUSSION

In this report, we have proposed a previously unknown mechanism for the regulation of alternative splicing of *Ptprc*. In our model, the splice factor U2AF26 and the transcription factor Gfi1 act antagonistically in the *Ptprc* alternative splicing process and either induce or inhibit formation of the CD45RO isoform, respectively, and as a consequence regulate antigen-dependent T cell activation. Such a regulatory mechanism of *Ptprc* splicing is of critical importance, as malfunction of the CD45 phosphatase or inappropriate splicing of *Ptprc* pre-mRNA has dire consequences for T cell function and could lead to immunodeficiency syndromes[25] or autoimmune disease[26].

We have presented evidence that U2AF26 regulates the formation of CD45 mRNA isoforms through the exclusion of alternatively spliced exons, in particular exon B (exon 5). Minigene assays of transfected cells, constitutive expression in T cells of transgenic mice and siRNA-mediated ablation of U2AF26 expression in mouse fibroblasts unambiguously demonstrated that higher U2AF26 expression correlated well with 'favored' expression of CD45RO mRNA. Those results were unexpected, as U2AF26 and its close homolog U2AF35 have been reported so far to support only exon inclusion[6,8,27]. However, our data have provided strong support for the idea of an additional function for U2AF26, although the biochemical mechanism remains to be elucidated. The exonic splicing silencer sequences of all three alternatively spliced *Ptprc* exons are bound by hnRNP L, which then induces exon skipping[28,29]. As expression of hnRNP L is not induced after T cell activation[28], whereas expression of U2AF26 is, cooperation of U2AF26 and hnRNP L represents an interesting model for investigation. The finding that the U2AF26 transgenic lines TG1 and TG2 had only a modest difference in T cell proliferation despite a large difference in transgene expression is also consistent with the idea that other factors in addition to U2AF26 are involved in regulating *Ptprc* exon skipping. Transgenic expression of U2AF26 could saturate other components that then become the limiting factors in a regulatory complex, and higher transgene expression would not elicit a further increase of the effect in a linear way.

Yeast two-hybrid data, coimmunoprecipitation and biochemical assays strongly supported the idea of complex formation between Gfi1 and U2AF26. The amount of proteins in precipitates from Jurkat nuclear extracts and from Cos7 cells overexpressing Flag-tagged U2AF26 and Gfi1 suggested a 1:1 stoichiometry between the two proteins. Experiments with the *PTPRC* minigene showed that 'titration' of Gfi1 into cells in which the CD45RO isoform prevailed because of U2AF26 expression restored CD45RB production in a dose-dependent way. As a Gfi1 mutant lacking amino acids 140–257 that was no longer able to bind U2AF26 had only a weak effect on alternative splicing of *Ptprc*, we conclude that direct protein-protein interaction is needed for Gfi1 to counteract U2AF26-mediated *Ptprc* exon skipping. The remaining effect of the mutant Gfi1 lacking amino acids 140–257 could be explained by residual binding to U2AF26, which would be consistent with the finding that the zinc finger domain in Gfi1 contributes to U2AF26 binding and was not detected in our GST precipitation assay because of stringent washing conditions. As we have no evidence that Gfi1 is or becomes part of the spliceosome or binds to RNA itself or that its activity is regulated by U2AF26, our results can best be explained by simple physical binding of U2AF26 to Gfi1, which would remove U2AF26 from the spliceosome, thereby altering the outcome of splicing processes. The finding that Gfi1 did not interact with U2AF65, as would be expected if U2AF26 could simultaneously bind to Gfi1 and U2AF65, raises the possibility that the U2AF26-Gfi1 and U2AF26-U2AF65 interactions are mutually exclusive. That finding is consistent with a model in which binding to Gfi1 prevents U2AF26 from regulating the splicing process.

In addition, we have shown that Gfi1-deficient mice and mice transgenic for the Flag-tagged U2AF26 had the same defect in *Ptprc* splicing: increased CD45RO production. That supports the idea of an antagonistic function for both proteins in regulating alternative splicing *in vivo*. In *Gfi1*$^{-/-}$ mice, the most notable alteration was in CD4^{+} central and effector memory T cells and CD8^{+} effector memory T cells, which could have been due to differential expression of Gfi1 and U2AF26 in different T cell subpopulations.

The idea that U2AF26 is involved in regulating alternative splicing of *Ptprc* during T cell activation *in vivo* was further supported by the upregulation of U2AF26 expression at 24, 48 and 72 h after activation, which coincides with gradual downmodulation of larger CD45 isoforms and accumulation of CD45RO[16]. Notably, Gfi1 is rapidly and transiently induced after T cell activation, with a peak in mRNA expression 6 h after stimulation[19]. Published reports have shown transient expression of CD45 mRNA molecules encoding higher-molecular-weight isoforms in the early phase of T cell activation[30], an observation consistent with our model in which Gfi1 counteracts the effect of U2AF26 on *Ptprc* exon skipping, thereby favoring the expression of higher-molecular-weight isoforms. Although the situation is complicated by the existence of different Gfi1 and U2AF26 protein isoforms, which warrants further investigation, our data suggest that the relative concentrations of both proteins and their ratio is critical for the production of specific CD45 isoforms in T cells.

As CD45 proteins containing regions encoded by the alternatively spliced exons are glycosylated, a model has been suggested in which the size of the carbohydrate groups parallels CD45 activity because of reduced formation of inactive dimers[9,31]. Alternatively, different abilities of CD45 isoforms to localize to glycosphingolipid-enriched membrane domains have been suggested[32]. In contrast, other transfection-based studies have shown that CD45RO can be integrated more efficiently into the TCR signaling complex and is therefore more active than larger CD45 isoforms in triggering the TCR response[33,34]. In experiments with CD45-deficient mice reconstituted with various *Ptprc* transgenes, no difference was noted between CD45RO and CD45RABC in triggering TCR signaling[35,36]. However, on a wild-type background, the larger CD45RABC protein was able to enhance T cell activation after certain stimuli, whereas CD45RO was not[37]. Our experiments left the *Ptprc* locus unaltered, but showed that mice with enhanced expression of the CD45RO isoform had a defect in T cell proliferation and TCR signaling.

Although we cannot exclude the possibility that defects other than disturbed *Ptprc* splicing were responsible for the lack of a proper T cell response in our experiments, several findings supported the contention that CD45RO limits T cell activation. The inability of *Gfi1*$^{-/-}$ and mice transgenic for the Flag-tagged U2AF26 to respond to CD3 crosslinking was not due to a general defect in proliferation, as activation 'downstream' of the TCR seemed to be less affected in *Gfi1*$^{-/-}$ mice and mice transgenic for the Flag-tagged U2AF26. Moreover, by monitoring proximal events 'downstream' of TCR signaling, we showed a defect in Zap70 Y319 phosphorylation in both mouse models. After TCR stimulation, the kinases Lck and Fyn are activated by CD45-mediated removal of an inhibitory phosphate group and Lck in turn phosphorylates Zap70. The degree of Zap70 phosphorylation can therefore be used to measure CD45 activity. As we do not have evidence of any other defects that could influence proximal TCR signaling in either one of our mouse models, we suggest that the observed defect in the TCR response in Gfi1-deficient and mice transgenic for the Flag-tagged U2AF26 is at least in part due to 'favored' expression of the less-active CD45RO isoform.

METHODS

Yeast two-hybrid assay. *Gfi1* or *Gfi1*ΔZF (encoding Gfi1 lacking the zinc finger domains) was cloned into pGBKT7 (Clontech) in frame with the yeast transcription factor Gal4–DNA-binding domain. Those constructs were used as 'baits' to screen a mouse lymphoma cDNA library (Matchmaker Gal4 cDNA library; Clontech). The screening was done as described by the manufacturer. The bait vector and library plasmid were transfected together into yeast strain AH109 by the lithium acetate method and yeast were plated on selective plates containing 5 mM 3-aminotriazole. The β-galactosidase activity of colonies that grew on plates lacking histidine, adenine, tryptophan and leucine was assessed by a filter-based assay. Interactions were confirmed by retransfection of isolated library plasmids into yeast cells containing the *Gfi1* or *Gfi1*ΔZF bait vector.

Immunoprecipitation, immunoblots and flow cytometry. Cos7 cells were transfected with various plasmids and were lysed 24 h later in lysis buffer containing 0.5 mM phenylmethylsulfonyl fluoride, 1 mg/ml of aprotinin, 1 mg/ml of pepstatin A, 1 mg/ml of leupeptin and 1 mM Na_3VO_4. For immunoprecipitation, a lysate volume equivalent to 200 μg protein was dissolved in 0.7 ml lysis buffer containing 3% BSA and protease inhibitors as described above. Precipitation was done overnight at 4 °C with 400 ng anti-Gfi1 (N20; Santa Cruz) and pre-equilibrated protein G–Sepharose or M2 affinity gel suspension. Beads were washed three times in lysis buffer and samples were then separated by SDS-PAGE. Proteins were transferred to Hybond-C membranes (Amersham), which were blocked with 0.05% (volume/volume) Tween 20 and 1% (weight/volume) BSA in PBS. Incubation with primary antibody was done overnight at 4 °C in blocking solution. After being washed, membranes were incubated for 1 h at 22 °C with horseradish peroxidase–coupled secondary antibody (Dianova) in 0.05% Tween 20 and 2% milk powder in PBS and were developed with the SuperSignal ECL system (Pierce). The following primary antibodies were used: anti-Gfi1 (559680), anti-Zap70 (610239) and antibody to Zap70 phosphorylated at Y319 (612574; all from BD Bioscience); anti-Flag (200471; Stratagene); and anti-β-actin (1616), anti-hnRNP A1 (10032), anti-U2AF35 (19961), anti-U2AF65 (19958), anti-Gfi1 (8558) and anti-GST (138; all from Santa Cruz Biotechnology). For detection of U2AF26, an antibody was raised against an epitope corresponding to amino acids 192–208 of U2AF26, a region with only weak homology to U2AF35. Before being used, the serum was purified with GST-U2AF26. Flow cytometry

was done as described[19]. The following antibodies were used (all from BD Bioscience): phycoerythrin-conjugated anti-CD4, anti-CD44 and anti-CD45RB; fluorescein isothiocyanate–conjugated anti-CD8, anti-CD44 and anti–total CD45; peridinine chlorophyll protein complex–indodicarbocyanine (Cy5.5)–conjugated anti-CD3, anti-CD4 and anti-CD8; and allophycocyanin-conjugated anti-CD44 and anti-CD62L.

Generation and manipulation of proteins. *In vitro* translation was done in a single-step transcription-translation reticulocyte lysate system (Promega) according to the supplier's information. For the generation of GST fusion proteins, the appropriate DNA fragments were subcloned into the bacterial expression vector pGEX-3X and were expressed by isopropyl-D-thiogalactopyranoside induction. The fusion protein was isolated by affinity chromatography with glutathione-coupled Sepharose beads according to the supplier's instructions (Amersham). For analysis of protein-protein interactions, GST fusion proteins were incubated overnight at 4 °C with 100 μg of lysates of Cos7 cells overexpressing various proteins or with 150 μg of HeLa nuclear or cytoplasmic extracts in lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$ and 1% (volume/volume) Triton-X-100) containing 3% BSA and protease inhibitors as described above. Beads were 'spun down' at 1,700*g* at 4 °C and were washed extensively with lysis buffer. Bound proteins were eluted from the beads by the addition of SDS sample buffer and were separated by SDS-PAGE. For demonstration of direct interaction between GST-Gfi1 and GST–Flag-tagged U2AF26, GST proteins were eluted from GST beads for 5 min at 22 °C with reduced glutathione (10 mM in 50 mM Tris-HCl, pH 8.0, containing protease inhibitors as described above). GST beads were 'spun down' as described above, the elution step was repeated twice and the combined supernatants were assayed by immunoblot for the presence of eluted proteins. This blot was used to estimate the relative amounts of eluted proteins, and equal amounts were used in coimmonoprecipitation experiments as described above in the presence of 2 μg RNase T1.

Minigene splicing assay, RT-PCR and real-time PCR. RK13 cells (rabbit kidney epithelial cells) were seeded in 12-well plates at a density of 5×10^4 cells/well. Cells were transfected 24 h later with 100 ng human *PTPRC* minigene[22] per transfection and various expression plasmids using Rotifect (Roth) according to the manufacturer's instructions. The total amount of DNA in each transfection was kept constant by the addition of empty vector where necessary. RNA was prepared 24 h later by the addition of peqGOLD RNAPure (Peqlab) as recommended by the manufacturer. After DNase I digestion, cDNA was prepared with random hexamers and SuperScriptII reverse transcriptase (Invitrogen). Samples were then analyzed by PCR, agarose gel electrophoresis and Southern blot. Identity of the products was confirmed by sequencing. For PCR of mouse tissue, single-cell suspensions from various organs were prepared, red blood cells were lysed and RNA was prepared as described above. For quantification, PCR products were transferred to nylon membranes, hybridized to an internal ^{32}P-labeled PCR fragment and quantified with a phosphorimager (FujiFilm Bas-1500). Significance was calculated by Student's *t*-test. The identity of mouse CD45 isoforms was confirmed by sequencing. Real-time PCR with cDNA generated as described above was done with TaqMan and primers and probes designed by Applied Biosystems according to the manufacturer's instructions. All samples were assessed in triplicate.

Transfection of siRNA. The 3T3 fibroblasts were seeded in 24-well plates at a density of 5×10^4 cells/well and were transfected 10 h later using Lipofectamine 2000. Cells were incubated overnight with transfection mixture containing 20 pmol siRNA. After transfection, cells were maintained in growth medium for 36 h and then were transfected overnight with 100 ng *PTPRC* minigene using Lipofectamine 2000. Cells were collected 24 h later and cDNA was prepared as described above. For immunoblot analysis, cells transfected in parallel were lysed in boiling SDS sample buffer and were separated by 12% SDS-PAGE. The following siRNA constructs were used: U2AF26, 5′-UCAGC CAGACCAUAGUCCUGCUCAA-3′ (Invitrogen); and U2AF35, 5′-GCAACUU CAUGCAUUUGAA-3′ (Qiagen).

Generation of transgenic mice. The construct used to generate mice transgenic for *Lck*-driven, Flag-tagged U2AF26 was obtained by PCR with the primers U2AF26startKozakBglII (5′-GAAGATCTGCCACCATGGCTGAATATTTAGCT TCG-3′) and FlagrevBglII (5′-CTAGATCTCTATTTATCGTCATCGTCTTT-3′) with pCMV-U2AF26-Flag as the template. The PCR product was cloned into TopoTA and the *Bgl*II fragment was inserted into a vector cut with *Bam*H1 that contained the proximal *Lck* promoter in Bluescript (**Fig. 3a**). The construct was freed from backbone sequences, was purified and was injected into fertilized mouse oocytes using standard methods. Fertilized mouse oocytes were derived from mating of (C57BL/6 × C3H) F_1 mice. Successful integration of the injected DNA was monitored by Southern blot analysis of DNA obtained from tail-tip tissue. All transgenic mouse lines were maintained by breeding of the founders with inbred C57BL/6 mice. All animal experiments were according to German Animal Protection Law and were done with license granted by the Bezirksregierung (Duesseldorf, Germany; G022/02Z).

T cell proliferation and Zap70 phosphorylation assay. Splenic T cells from 6- to 8-week-old mice were negatively enriched with a 'pan–T cell' isolation kit and an autoMACS (Miltenyi). Purified cells were routinely checked for purity by flow cytometry. For methyl-[^{3}H]thymidine incorporation assay, 5×10^4 purified splenic T cells were cultured for 30 h in 96-well plates (Nunc) in RPMI 1640 medium supplemented with 10% FBS, L-glutamine, penicillin G, streptomycin and 2-mercaptoethanol in the presence of 0.05 μg anti-CD3 (500A2; Pharmingen) and 25 ng/ml of PMA (Sigma) or in the presence of 250 ng/ml of PMA and 1 μM ionomycin (Sigma). For the last 8 h, 1 μCi of methyl-[^{3}H]thymidine was added as a single pulse. After cells were collected, incorporated methyl-[^{3}H]thymidine was measured with a β-scintillation counter (Wallac). For the Zap70 phosphorylation assay, 1×10^6 purified T cells or 2.5×10^6 total thymocytes in medium as described above were stimulated for various times with 0.05 μg anti-CD3 (500A2; Pharmingen). Cells were than transferred to ice, were 'spun down' and were lysed for 20 min on ice in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet-P40 and protease inhibitors. Cell debris were removed by centrifugation at 4 °C and supernatants were analyzed by 8% SDS-PAGE. Membranes were first probed with antibody to Zap70 phosphorylated at Y319, were washed thoroughly and then were reprobed with anti-Zap70.

Primer sequences. These are available in the **Supplementary Methods** online.

Statistical methods. Bar graphs represent mean ± s.d. Significance was calculated with the two-tailed Student's *t*-Test.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

G.t.D. provided essential reagents and helped to write the paper; F.H. did the experimental work; F.H. and T.M. designed the experiments, analyzed the data and wrote the paper; and T.M. supervised the work.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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