labelling was 2.3 Cy5 molecules per HMGB1 molecule. After 30 min at room temperature, sample cells were mixed and mounted on slides using Vectashield (Vector Laboratories) containing 1.5 μg ml^{-1} 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), and observed on an Axiophot microscope with a TRITC filter (Carl Zeiss). The two pools of cells incubated with unlabelled HMGB1 were layered onto discontinuous gradients formed by 5 ml of 1.16 M sucrose in PBS and a 6-ml cushion of 2 M sucrose in PBS, and centrifuged at 30,000*g* for 90 min in a SW27 Beckman rotor. We recovered apoptotic and non-apoptotic chromatin that was free of membrane debris from the bottom of the tubes and applied it to a 12% SDS–PAGE gel. The amount of recombinant HMGB1 bound to apoptotic and non-apoptotic chromatin was determined by immunoblotting using an antibody to HMGB1 (Pharmingen) at 1:3,000 dilution. Aliquots of apoptotic and non-apoptotic chromatin were also probed with antibodies to acetyl-histone H4 (R10, a gift from B. Turner), to acetyl-histone H3 (Lys 9, Biolabs) and to acetyl-lysine (Biolabs).

Inflammation assays

To measure TNF-α production *in vitro*, bone marrow was recovered from the hind legs of female C56Bl6 mice, diluted to 5×10^6 cells per ml in Optimem and dispensed in 96-well microtitre plates (120 μl per well). Necrotic cells (lysed by three cycles of freeze-thawing) or apoptotic cells were added to the indicated final concentration into the wells and incubated at 37 °C for 18 h. We assayed TNF-α in the supernatant by enzyme-linked immunoabsorbent assay (Quantikine M, R&D Systems). TSA was added at 200 ng ml^{-1} together with TNF-α where indicated, and was washed away before mixing the apoptotic cells with bone marrow cells.

To measure inflammation *in vivo*, 1-day-old mice (weighing 1.1 ± 0.1 g) were injected intraperitoneally with 20 μl of PBS containing 320 μg of acetaminophen (Sigma) and 320 μg of antibodies (Pharmingen BD) where indicated. After 9 h, the mice were analysed for serum ALT activity with the GP-Transaminase kit (Sigma) and for MPO activity in liver extracts as described[24]. We used the non-parametric Mann–Whitney test for statistical analysis of MPO/ALT ratios. Similar results were obtained using Student's *t*-test on the MPO amounts of mice that were paired to minimize the difference in ALT amounts.

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Competing interests statement

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Reciprocal regulation of CD4/CD8 expression by SWI/SNF-like BAF complexes

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Thymic development produces two sub-lineages of T cells expressing either CD4 or CD8 co-receptors that assist antibody production and mediate cell killing, respectively. The mechanisms for mutually exclusive co-receptor expression remain poorly defined[1,2]. We find that mutations in the high mobility group (HMG) domain of BAF57—a DNA-binding subunit of the mammalian SWI/SNF-like chromatin-remodelling BAF complexes—or in the BAF complex ATPase subunit Brg, impair both CD4 silencing and CD8 activation. Brg is haploinsufficient for CD8 activation, but not for CD4 silencing, whereas BAF57 mutations preferentially impair CD4 silencing, pointing to target- and subunit-specific mechanisms of chromatin remodelling. BAF complexes directly bind the CD4 silencer, but the BAF57 HMG domain is dispensable for tethering BAF complexes to the CD4 silencer or other chromatin loci *in vivo*, or for remodelling reconstituted templates *in vitro*[3,4], suggesting that chromatin remodelling *in vivo* requires HMG-dependent DNA bending. These results indicate that BAF complexes contribute to lineage bifurcation by reciprocally regulating lineage-specific genes, reminiscent of the role of the yeast SWI/SNF complex in mediating mating-type switching[5,6].

As germline deletion of several subunits of BAF complexes lead to either early embryonic lethality[7–9] or produce little phenotype[10], we studied the developmental roles of the complexes in the T-cell lineage by inactivating the HMG protein BAF57. The Lck proximal promoter was used to direct expression in transgenic mice of dominant negative mutants of BAF57 lacking the entire amino terminus (BAF57ΔN) or bearing a point mutation (K112I) that disrupts DNA binding[3] (Fig. 1a). Expression of either mutant suppressed endogenous BAF57 expression by up to tenfold apparently through autoregulation (Fig. 1b), indicating that the

BAF57ΔN transgene potentially inactivates 90% of BAF complexes. Immunoprecipitation of BAF complexes from thymic nuclear extracts using the J1 anti-BRG antibody revealed that the BAF57ΔN protein was assembled into BAF complexes, because the abundance of BAF57ΔN relative to the endogenous BAF57 and BRG in the immunopurified complexes (bound) was comparable to that in the crude nuclear extract (Fig. 1c, compare lane 2 with 4). The immunopurified BAF complexes were stable: when challenged with 2 M NaCl, the endogenous subunits remained stoichiometrically bound to BRG, although most of the mutant BAF57 protein dissociated from the complexes (Fig. 1c, lanes 5 and 6). We conclude that T-cell-specific expression of BAF57 mutants gave rise to complexes specifically deficient in HMG-mediated functions.

Adult *BAF57* transgenic mice showed only a mild (less than twofold) reduction in total thymic cellularity, but the CD4 and CD8 expression pattern was significantly altered, which was best revealed in T-cell antigen receptor (TCR)$\beta^{-/lo}$ cells (Fig. 2, upper panel). Specifically, in *BAF57* transgenic mice, there appeared a TCR$\beta^{-/lo}$ $CD4^+$ $CD8^-$ population that merged into the $CD4^+$ $CD8^+$ (double positive) population, the latter showing reduced CD8 but enhanced CD4 expression (compare columns 1 and 3). These changes were synthetically enhanced by a *Brg* null allele[7] (compare columns 3 and 4); $Brg^{+/-}$ mice were viable, but also contained TCR$\beta^{-/lo}$ $CD4^+$ $CD8^-$ cells whose abundance was similar to that in *BAF57ΔN* heterozygous mice ($BAF57\Delta N^{+/-}$; not shown). The synthetic effect indicates that BAF57 mutations specifically impaired the function of BAF complexes. Altered CD4 and CD8 expression was not a consequence of global developmental defects, because there was little change in the expression of CD25 (Fig. 3a), TCRβ, CD69 (Supplementary Information Fig. 1), CD3, HSA, interleukin (IL)-7R or Bcl2 (not shown). However, mature TCR^+ thymocytes were significantly reduced in number (up to 30- fold, bottom panel), and CD8 T cells showed a mild and variable increase in CD4 expression (middle panel).

Thymic development begins with $CD4^-$ $CD8^-$ double negative (DN) T cells that can be subdivided into four stages (DN1–4) (see Methods). Rearrangement of the *TCRβ* gene at the DN3 stage leads to CD8 expression (at the $CD4^-$ $CD8^+$ immature single positive stage) and subsequently CD4 derepression (at the double positive stage) before the double positive cells undergo positive selection to become single positive cells with mutually exclusive co-receptor expression. We thus analysed CD4 and CD8 expression at each of these stages and found that BAF mutations compromised both CD4 silencing and CD8 expression (see below).

The defect in CD4 silencing was first evident as premature CD4 expression at the DN3 stage, with 19% and 49% of DN3 cells expressing CD4 in $BAF57\Delta N^{+/-}$ and *BAF57ΔN* homozygous ($BAF57\Delta N^{+/+}$) mice, respectively (Fig. 3a, column 2). No defect was observed in DN1 or DN2 cells, apparently because of lower transgene expression in these cells (not shown). Indeed, CD4 was derepressed in DN1–2 cells when both copies of the *Brg* gene were deleted from these cells using Cre-mediated excision (T.H.C. *et al.*, manuscript in preparation). The $Brg^{+/-}$ allele, which did not by itself derepress CD4 (Fig. 3c), enhanced the CD4 level on the $BAF57\Delta N^{+/-}$ background such that CD4 was expressed in 25% of DN3 cells in $BAF57\Delta N^{+/-}$; $Brg^{+/-}$ mice (Fig. 3a, column 2). CD4 was further derepressed in the mutants as the cells progressed beyond the DN3 stage, as indicated by disappearance of the DN4 cells in the post-DN3 pool. The DN4 cells presumably expressed CD4 prematurely and were thus converted to the $CD3^-$ $CD4^+$ $CD8^-$ cells in the BAF mutants (column 3). However, the $CD3^-$ $CD4^+$ $CD8^-$ cells were far more abundant than the DN4 cells,

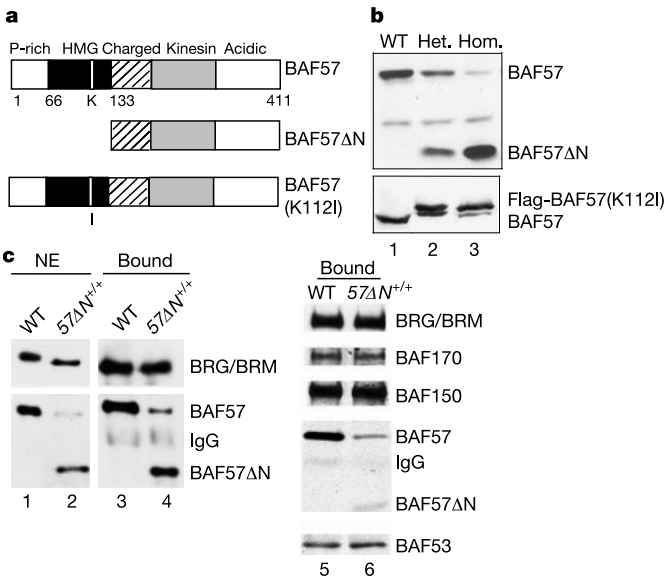

Figure 1 The *BAF57* transgenes suppressed endogenous BAF57 expression and produce BAF complexes deficient in HMG functions. **a**, The BAF57 deletion and point mutants. **b**, Western blot analysing BAF57 expression in thymocytes from wild-type (WT), heterozygous (Het.) or homozygous (Hom.) *BAF57ΔN* (top panel) or *BAF57(K112I)* (bottom panel) transgenic mice. The transgenes were Flag-tagged. **c**, Western blot analysing the BAF complexes in thymic nuclear extracts (NE) from wild-type or homozygous *BAF57ΔN* ($57\Delta N^{+/+}$) mice before (lanes 1–2) or after (lane 3–6) immunoprecipitation with the J1 anti-BRG antibody. The beads were washed with RIPA buffer containing either 0.3 M NaCl (lanes 3–4) or 2 M NaCl (lanes 5–6). IgG, immunoglobulin-γ.

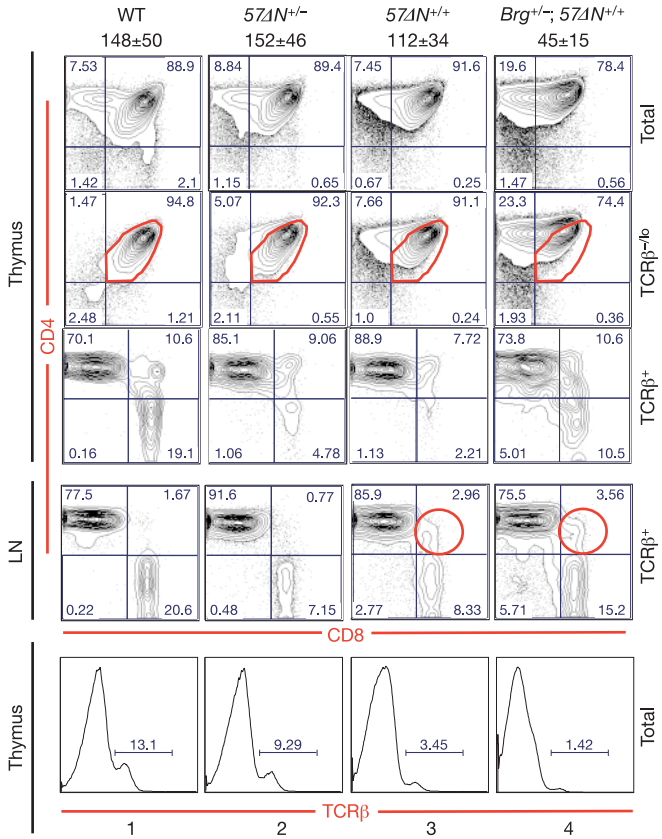

Figure 2 BAF complexes are required for thymic development. Co-receptor (upper and middle panels) and TCRβ (bottom panel) expression of various mutant mice. Genotypes of the mice and the total thymocyte numbers are indicated on top of each column. Cells were stained with antibodies against CD4, CD8 and TCRβ. The red contours highlight reduced CD8 and increased CD4 expression, whereas the red circles denote the $CD4^+$ $CD8^+$ lymph node (LN) T cells.

constituting up to 53% of the post-DN3 blast population and 23% of total immature thymocytes in *BAF57ΔN*$^{+/+}$; *Brg*$^{+/-}$ mice (Figs 3a (column 3) and 2 (column 4)), suggesting an additional defect in BAF mutants (see below). As expected, CD4 was also expressed in DN3 cells from the *BAF57ΔN*$^{+/+}$; *Brg*$^{+/-}$; *Rag2*$^{-/-}$ mice, where thymocytes did not develop beyond the double negative stage (Fig. 3b).

In contrast to enhanced CD4 expression, CD8 expression was impaired in BAF mutants. This was first suggested by the fact that the double positive cells from BAF mutants expressed lower levels of CD8 and merged into the novel CD3$^-$ CD4$^+$ CD8$^-$ population, which appeared to be produced by a further decline in CD8 expression to below detectable levels (Figs 2 and 3a). If these changes reflect a specific defect in CD8 expression, one would predict that they might be synthetically aggravated in combination with loss of one CD8α allele[11]. This was indeed the case (Fig. 3e), confirming that both the reduction in CD8 levels in double positive cells and the concomitant accumulation of CD3$^-$ CD4$^+$ CD8$^-$ cells originated from a CD8 defect, and suggesting that the CD3$^-$ CD4$^+$ CD8$^-$ cells had accumulated owing to impaired or delayed CD8 expression in developing double positive cells. Indeed, the CD3$^-$ CD4$^+$ CD8$^-$ cells expressed CD8 to become double positive when cultured *in vitro*, but the efficiency was compromised by BAF mutations in a dose-dependent manner (Fig. 3d), consistent with the CD3$^-$ CD4$^+$ CD8$^-$ cells comprising mainly (early) double positive cells lacking detectable CD8 expression. Although CD4 derepression can potentially kill CD8^{hi} double positive cells by inhibiting TCR signalling[12], impaired CD8 expression in double positive cells was unlikely to be

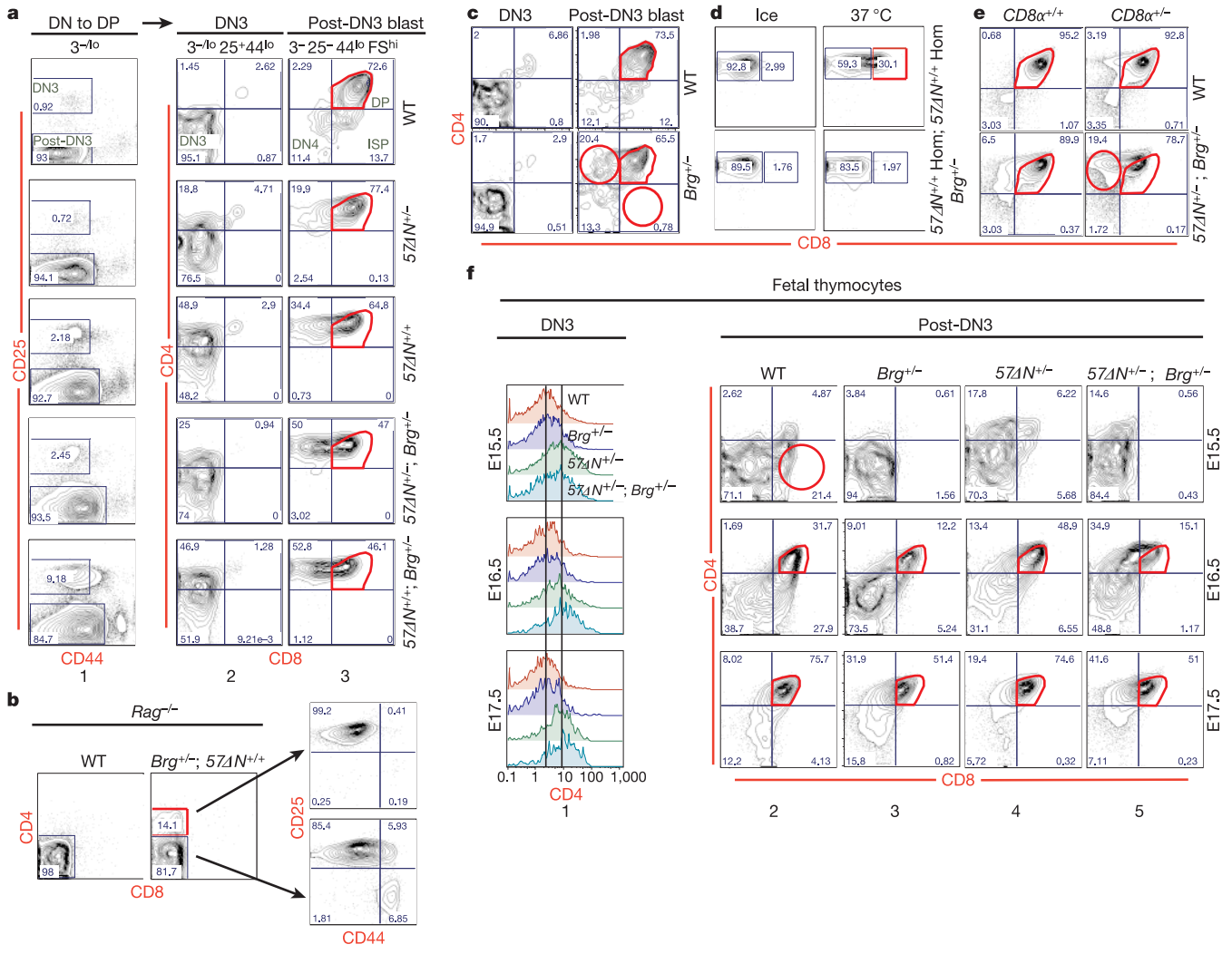

Figure 3 Reciprocal CD4 and CD8 misregulation in the BAF mutants. **a**, CD4 and CD8 expression on DN3 and post-DN3 blasts as defined by CD3 (3), CD25 (25), CD44 (44) and forward scatter (FS) (see Methods for details). DN, double negative; DP, double positive; ISP, immature single positive. **b**, CD4 derepression in *BAF57ΔN*$^{+/+}$; *Brg*$^{+/-}$ mice crossed onto the *Rag2*$^{-/-}$ background. The CD4$^+$ and CD4$^-$ thymocytes in the BAF mutant were further analysed for CD25 versus CD44 expression, which revealed that the CD4$^+$ cells were exclusively at the DN3 stage (right panel). **c**, The *Brg*$^{+/-}$ allele selectively suppressed CD8 without derepressing CD4. The circles denote the disappearance and appearance of immature single positive and TCR$^-$ CD4$^+$ CD8$^-$ cells, respectively, and the contours indicate reduced CD8 expression on double positive cells. **d**, *In vitro* differentiation of the TCR$^-$ CD4$^+$ CD8$^-$ cells. Sorted thymocytes from mutant mice were cultured at 37 °C or kept on ice overnight, restained with CD4 and CD8 antibodies and analysed. **e**, Genetic interaction between the BAF complexes and the CD8α gene. *BAF57ΔN*$^{+/+}$; *Brg*$^{+/-}$ and *CD8α*$^{-/-}$ mice were crossed to each other and to wild-type mice to produce mice with the indicated genotypes. The cells were stained with CD4, CD8α and TCRβ antibodies, and CD4 and CD8 expression on immature T cells were compared. The circle denotes synthetic increase in the abundance of TCR$^-$ CD4$^+$ CD8$^-$ cells in the *BAF*; *CD8α* double mutant, and the contours highlight the synthetic CD8 expression defect in double positive cells. **f**, Co-receptor expression during fetal development. *BAF57ΔN*$^{+/-}$; *Brg*$^{+/-}$ males were crossed to wild-type female mice that were killed at various times as indicated. Fetal thymi were assessed as in Fig. 3a except that the post-DN3 cells included the entire CD3$^{lo/-}$ population, as opposed to the CD3$^-$ FS^{hi} blastic subset in adult mice. The circle denotes immature single positive cells.

an artefact resulting from death of $CD8^{hi}$ double positive cells, because there was no significant defect in the viability of double positive cells in BAF mutants (not shown), and CD4 misregulation does not influence CD8 expression[13–16] (see also Fig. 4, column 2). Consistent with these data, the heterozygous *Brg* deletion did not impair CD4 silencing (Fig. 3c) and yet produced the $CD3^-$ $CD4^+$ $CD8^-$ population while eliminating the $CD8^{hi}$ double positive cells as effectively as the *BAF57ΔN* heterozygous allele (Fig. 3a). These data indicate that BAF mutations impair CD8 expression at the double positive stage independently of CD4 derepression.

CD8 expression was even more susceptible to BAF mutations at the earlier immature single positive ($CD3^-$ $CD4^-$ $CD8^+$) stage, as demonstrated by the complete and paradoxical loss of immature single positive cells—a critical developmental intermediate—in every BAF mutant examined (Fig. 3a, column 3). The loss of immature single positive cells is not due to premature CD4 expression in these $CD4^-$ $CD8^+$ cells, which could have turned them into 'double positive' cells, because the heterozygous Brg deletion completely eliminated the immature single positive cells without derepressing CD4 (Fig. 3c), whereas a CD4 silencer mutation (I.T. and D.R.L., manuscript in preparation) partially depressed CD4 without eliminating the immature single positive cells (Fig. 4a, column 2). Indeed, the triad of concurrent thymic developmental defects—that is, the reduction in CD8 expression in the double positive cells, emergence of the $CD3^-$ $CD4^+$ $CD8^-$ population and loss of immature single positive cells—were each exquisitely sensitive to BAF mutations and were observed in $Brg^{+/-}$ mice lacking any other detectable phenotypes including CD4 derepression. Together, these defects demonstrate that the BAF mutations specifically impaired CD8 expression at both immature single positive and double positive stages, and that Brg is haploinsufficient for CD8 activation. By these criteria, Brg is a major regulator of CD8 expression.

A prediction from the analysis of co-receptor expression in adult mice is that BAF mutations should alter the timing of CD4 and CD8 expression during fetal development. In wild-type controls (Fig. 3f, column 2), CD4 became detectable at embryonic day (E)16.5 as double positive cells emerged, whereas immature single positive cells expressing CD8 (red circle) had already appeared by E15.5, comprising 20% of the total thymic population. Notably, in $BAF57\Delta N^{+/-}$ and $BAF57\Delta N^{+/-}$; $Brg^{+/-}$ littermates, CD4 was prematurely derepressed at both DN3 (column 1) and post-DN3 (column 4–5) stages, the effect reaching a plateau by E15.5 in DN3 cells. Immature single positive cells, however, were virtually missing throughout development in $Brg^{+/-}$ and $BAF57\Delta N^{+/-}$; $Brg^{+/-}$ mice, which consequently lacked CD8 expression until E16.5 when $CD8^{int/lo}$ double positive cells emerged (columns 3 and 5). These data demonstrate premature CD4 but delayed CD8 expression in BAF mutants. A second prediction is that the $CD3^-$ $CD4^+$ $CD8^-$ cells, representing double positive cells lacking CD8, should develop with comparable kinetics as the $CD4^+$ $CD8^+$ double positive cells. Indeed, in $Brg^{+/-}$ mice where the $CD3^-$ $CD4^+$ $CD8^-$ pool was free of DN4/immature single positive cells prematurely expressing CD4, these cells emerged at E16.5 and further accumulated at E17.5, concurrent with double positive cells (column 3). Note that although heterozygous Brg deletion effectively impaired CD8 expression without derepressing CD4 (column 3), the *BAF57ΔN* transgene preferentially derepressed CD4 while only mildly repressing CD8 during fetal development (column 4). The differential requirement of BAF subunits in gene regulation suggests that chromatin remodelling at distinct loci involves different mechanisms.

In contrast to significantly altered CD4 and CD8 expression during thymic development, co-receptor expression in mature T cells was only slightly affected (Fig. 2, middle panel), apparently because BAF mutations blocked the double positive to single

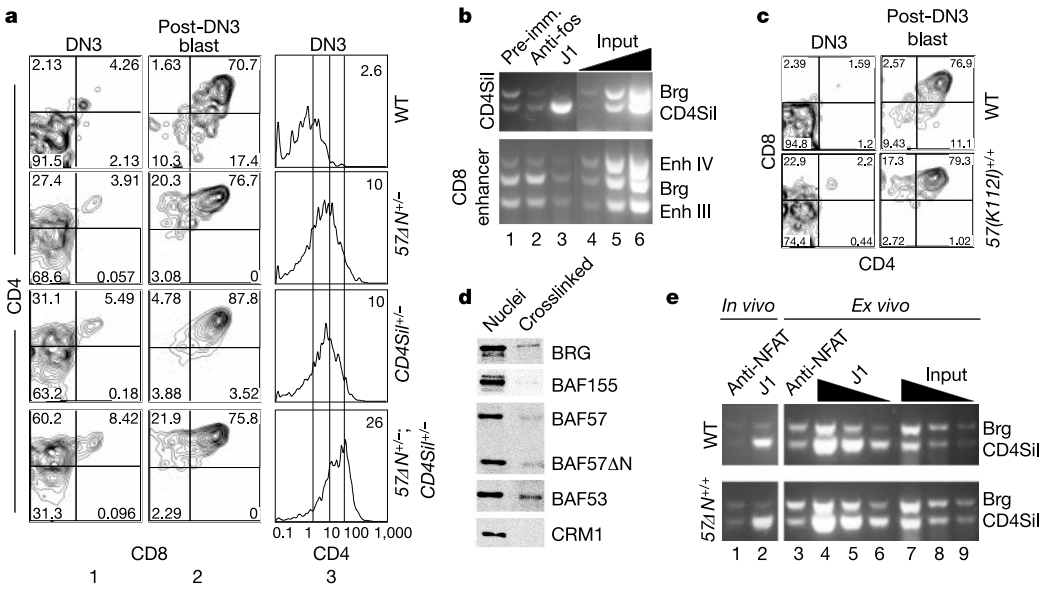

Figure 4 BAF complexes bind the CD4 silencer independently of the BAF57 HMG domain. **a**, Synthetic CD4 derepression in mice carrying mutations in both BAF complexes and the CD4 silencer (CD4Sil). The histograms quantify the mean fluorescence levels of the DN3 populations. **b**, BAF complexes bound the CD4 silencer but not the CD8 enhancers in the M1200 thymoma line[23]. Shown are multiplex PCRs measuring the abundance of CD4 silencer (top) or CD8 enhancers (bottom) in crosslinked chromatin before (input) or after immunoprecipitation with various antibodies. A region in the Brg genomic sequence was co-amplified to control for DNA yields. The amplification was in the linear range of PCR as shown by a threefold titration of immunoprecipitated input (lanes 4–6). **c**, The BAF57(K112I) point mutation produced the same defects as the BAF57 deletion mutant. **d**, Western blot analysing the BAF subunits in the nuclei or crosslinked chromatin from $BAF57\Delta N^{+/-}$ mice. The crosslinking was specific, as CRM1, an abundant nuclear protein, was not detected. The amount of the nuclei loaded on the gel was equivalent to one-tenth of the chromatin sample in terms of DNA quantity. **e**, Chromatin immunoprecipitation showing that the BAF57 HMG domain is dispensable for binding the CD4 silencer. Thymocytes were crosslinked either by perfusing the anaesthetized mice with 2% formaldehyde (*in vivo*) or as single cell suspensions (*ex vivo*) before immunoprecipitation. The amplification was in the linear range of PCR as shown by a threefold titration of immunoprecipitated input (lanes 8 and 9) and precipitated chromatin (lanes 4–6).

positive transition (Fig. 2, bottom panel), thus providing selective pressure for a mature T-cell population dominated by relatively normal T cells.

The CD4 gene is actively suppressed by a 434-base pair silencer[1,2,13]. To determine whether BAF complexes controlled CD4 expression through the CD4 silencer, we crossed *BAF57ΔN*$^{+/-}$ mice with mice bearing point mutations in the endogenous CD4 silencer (I.T. and D.R.L., manuscript in preparation). The CD4 silencer and *BAF57ΔN*$^{+/-}$ mutations each partially derepressed CD4 on DN3 cells (Fig. 4a, column 1), leading to a 3.8-fold enhancement in the mean fluorescence intensity in CD4 expression (from 2.6 U in the wild type to 10 U in the mutants, column 3), but in combination, they produced a tenfold increase (from 2.6 to 26 U). This suggests a genetic interaction between BAF complexes and the CD4 silencer, and raises the possibility that BAF complexes directly bind the CD4 silencer. Indeed, J1 anti-BRG or anti-BAF57 antibodies, but not irrelevant antibodies (pre-immune, anti-Fos or anti-NFATc2), selectively precipitated the CD4 silencer in a thymoma line (Fig. 4b, upper panel; data not shown). This binding was specific, because neither CD8 enhancer III nor IV (ref. 1) bound BAF complexes (Fig. 4b, lower panel).

The BAF57 deletion mutant lacked the entire N terminus including the HMG and the proline-rich domains. Mice transgenic for the point mutant BAF57(K112I) also showed reciprocal CD4 and CD8 misregulation (Figs 1a and 4c), demonstrating a specific requirement for HMG function *in vivo*. The HMG domain is dispensable for tethering BAF complexes to chromatin in general, because BAF57ΔN was crosslinked to chromatin as effectively as the endogenous BAF57 (Fig. 4d). It was also dispensable for targeting BAF complexes to the CD4 silencer (Fig. 4e). These results are consistent with the presence of multiple DNA/chromatin binding domains in BAF complexes[17,18], and suggest that it is the DNA bending activity unique to the HMG domain that is required for chromatin remodelling *in vivo*.

Studies of the developmental roles of the SWI/SNF-like complexes in multicellular organisms have been hampered by the early lethality in *Drosophila*[19] and mammals[7–9]. Our results reveal that a conserved function of yeast and mammalian complexes is to influence lineage differentiation. Furthermore, the fact that BAF complexes can reciprocally regulate co-receptor expression provides a new entry point for studying the mechanisms of positive selection, as BAF complexes are known to respond to TCR stimulation in peripheral T cells[20]. The fact that the Brg homologue Brm is dispensable for thymic development (M. Yaniv and M. Malissen, personal communication) reinforces the idea that combinatorial assembly of BAF complexes gives rise to divergent biological functions. □

Methods

Mice

BAF57 transgenic mice were produced by standard methods and maintained on the CD1 background.

Flow cytometry

For co-receptor expression in adult mice, single-cell suspensions were prepared and typically subjected to five-colour analysis using anti-CD4-APC, anti-CD8-Texas red, Anti-CD25-fluorescein isothiocyanate (FITC), anti-CD44-phycoerythrin (PE), anti-CD3-Cy5, anti-B220-Cy5 and propidium iodide. Normal thymic development before the single positive stage proceeds from CD3$^-$ CD25$^-$ CD44$^+$ (DN1) to CD3$^-$ CD25$^+$ CD44$^+$ (DN2) to CD3$^-$ CD25$^+$ CD44^{low} (DN3) to CD3$^{-/low}$ CD25$^-$ CD44^{low} (post-DN3); the post-DN3 cells comprise DN4, immature single positive (CD3$^-$ CD4$^-$ CD8$^+$) and double positive cells[21]. We resolved immature thymocytes—marked by no to low CD3 expression and thus including all the cells from the double negative to double positive stage—into DN1–3 and post-DN3 subsets (Fig. 3a, column 1) based on CD25 and CD44 expression, and we determined CD4 and CD8 expression in each of these subsets (Fig. 3a, columns 2 and 3). For post-DN3 cells, we analysed post-DN3 blasts, the most immature cells in the post-DN3 pool that are marked by high forward scatter (FS) and no CD3 expression; this was necessary to enrich for and reveal the rare DN4 and immature single positive cells. Note the elevation in CD44 expression in post-DN3 cells and correspondingly enlarged post-DN3 gates in the mutants (Fig. 3a, column 1); this adjustment did not alter flow cytometric profiles of post-DN3 cells. Co-receptor expression during fetal development was analysed exactly as in adult mice, except that the gate for post-DN3 cells was set on the entire CD3$^{-/lo/int}$ cells, as opposed to the blastic subset in this population.

Chromatin immunoprecipitation and analysis of crosslinked proteins

Chromatin immunoprecipitation (ChIP) was performed as described[22], except that the crosslinked chromatin was purified using a CsCl gradient, the chromatin was precleared with protein A pre-immune antibody complex, and that BSA and salmon sperm DNA were added during immunoprecipitation. To analyse the proteins in the chromatin fraction purified with the $CsCl_2$ gradient, a portion of the chromatin was precipitated with acetone, the pellet resuspended in 2 × SDS-gel loading buffer and incubated at 65 °C overnight to reverse crosslinking and solubilize the proteins before analysis.

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Competing interests statement

The authors declare that they have no competing financial interests.

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