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Molecular basis of CD4 repression by the Swi/Snf-like BAF chromatin remodeling complex

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Abstract

The BAF chromatin remodeling complex directly binds the CD4 silencer and is essential for CD4 repression during T cell development, because deletion of the ATPase subunit Brg1 or a dominant negative mutant of BAF57 each impairs CD4 repression in early thymocytes. Paradoxically, BAF57 is dispensable for remodeling nucleosomes *in vitro* or for binding of the BAF complex to the CD4 silencer *in vivo*. Thus, it is unclear whether BAF57-dependent CD4 repression involves chromatin remodeling, and if so, how the remodeling translates into CD4 repression. Here we show that nucleosomes at the CD4 silencer occupy multiple translational frames. BAF57 dominant negative mutant does not alter these frames, but reduces the accessibility of the entire silencer without affecting the flanking regions, concomitant with localized accumulation of linker histone H1 and eviction of Runx1, a key repressor of CD4 transcription that directly binds the CD4 silencer. Our data indicate that precise nucleosome positioning is not critical for the CD4 silencer function and that BAF57 participates in remodeling H1-containing chromatin at the CD4 silencer, which enables Runx1 to access the silencer and repress CD4.

Keywords

BAF57; CD4; chromatin

Introduction

Chromatin remodeling complexes (CRC) are capable of using energy derived from ATP hydrolysis to physically disrupt DNA-histone contacts and facilitate the binding of transcription factors to nucleosomal DNA [for recent reviews, see references [1,2]]. There are multiple types of CRCs in mammals, the best-known is the Swi/Snf-like BAF complexes that plays diverse but tissue-specific roles in gene regulation [3–5]. The BAF complex consists of about 8–15 subunits including the catalytic ATPase subunit Brg1 or its homolog Brm. Surprisingly, the majority of these 15 subunits are dispensable for chromatin remodeling *in vitro*. The first clue came from the observation that the N-terminal HMG domain of BAF57, an HMG-box protein, is dispensable for the BAF complex to remodel

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mononucleosomes *in vitro* [6]. Subsequently, it was found that only 4 of the BAF subunits (Brg1, BAF45, BAF155 and BAF170) are required to reconstitute a core complex possessing full remodeling activity on mononucleosomes and nucleosomal arrays [7]. The *in vivo* roles of the remaining 11 "dispensable" BAF subunits remain unclear, except that some of these subunits are thought to function by helping recruit the BAF complex to target genes via interactions with sequence-specific transcription factors or chromatin, thus indirectly contributing to chromatin remodeling. For example, BAF250 contains an ARID DNA-binding domain and a C-terminal domain capable of interacting directly with the glucocorticoid receptor *in vitro*, thus leading to the hypothesis that BAF250 may act by recruiting the complex to its targets through either protein-DNA or protein-protein interaction [8]. Similarly, BAF60a interacts with p53 while its homolog BAF60c binds cardiac transcription factors, and the two subunits are thought to act by recruiting the BAF complex to p53 target genes and heart-specific enhancers, respectively [9,10]. Finally, BAF53b is hypothesized to act as a molecular bridge between the BAF complex and histones, thus helping tether the BAF complex to specific target genes [11]. However, it is possible that the dispensable subunits might function at the step after the BAF complex is recruited to the target genes, *i.e.* they directly participate in disrupting endogenous chromatin templates *in vivo*, even though they are dispensable for remodeling reconstituted chromatin templates *in vitro*. In addition, these subunits might have functions in gene regulation unrelated to Brg1/Brm-catalyzed chromatin remodeling. Indeed, evidence is mounting that the BAF complex has novel, ATPase-independent functions in gene regulation. Thus, an ATPase-dead mutant of Brg1 retains the ability to effectively stimulate a transiently transfected reporter gene and partially activate at least one endogenous gene in a Brg1-deficient cell lines[12,13]. Furthermore, the Brg1 homolog Brm (though not Brg1) is found to be associated with splicesomes and regulate alternative splicing. Importantly, this function is independent of the Brm ATPase activity[14].

The developmental stages of thymocytes are marked by regulated expression of the antigen coreceptor CD4 and CD8. The earliest thymocytes are "double negative" (DN) cells expressing neither CD4 nor CD8. These cells subsequently express CD4 and CD8 to become "double positive" (DP) cells. DP cells then differentiate into either CD4 or CD8 cells with mutually exclusive CD4/CD8 expression. Temporal-specific CD4 expression is controlled by a 434-bp transcription silencer that acts dominantly over the constitutively active CD4 promoter/enhancer, since deletion of the silencer leads to constitutive CD4 expression throughout T cell development [15]. Thus, the silencer is active in DN and CD8 cells, which leads to CD4 repression, but inactive in DP and CD4 cells, which leads to CD4 expression. The BAF complex binds the CD4 silencer and is essential for CD4 repression in DN cells, since deleting Brg1 or overexpresssing a dominant negative mutant of BAF57 lacking the HMG domain each leads to CD4 derepression in DN cells [16–18]. Importantly, in contrast to the mode of action known for multiple other dispensable subunits, the BAF57 HMG domain is not required for targeting the BAF complex to the silencer. How then do BAF57 and the BAF complex repress CD4? Several scenarios have previously been proposed [4]. The BAF complex may act by remodeling the CD4 silencer and facilitating the binding of transcription repressors, which somehow requires BAF57. Alternatively, the BAF complex may repress CD4 via a BAF57-dependent mechanism unrelated to remodeling, which is possible because the CD4 silencer function is not strictly correlated with its accessibility[19], and because BAF complex possesses novel, ATPase-independent functions in gene regulation, as described above. Of note, this possibility is not incompatible with the fact that Brg1 deletion leads to CD4 derepression, as Brg1 is required for the BAF complex to bind chromatin[20], and furthermore, Brg1 itself may harbor a novel, ATPase-independent activity outside the ATPase domain. Brg1 deletion will thus eliminate both the classical remodeling activity and the novel functions of the BAF complex indiscriminately.

To address how BAF57 and the BAF complex repress CD4, we examined the effects of BAF57 dominant negative mutation and Brg1 deletion on the nucleoprotein structure of the CD4 silencer. Our results indicate that the BAF complex uses BAF57 to remodel the endogenous, H1-containing chromatin at the CD4 silencer, which facilitates the binding of Runx1, a key repressor of CD4 transcription.

Results

Effects of BAF57 dominant negative mutation on CD4 silencer chromatin structure

We first asked whether BAF57 mutation alters the chromatin structure of the silencer. We focused our analysis on DN cells, because in DN cells, the silencer is active and its activity depends on the BAF complex. DN cells develop via four linear stages, DN1–4. DN3 and DN4 cells each constitute >45% of total DN cells, the remaining being DN1 and DN2 cells. DN cells constitute <5 % of total thymocyte population in wild-type mice or mice expressing dominant negative BAF57 lacking the HMG domain, which would require extensive purification of DN cells before biochemical analysis. To circumvent this problem, we took advantage of the fact that Rag2-deficiency, which prevents VDJ recombination, completely blocks T cell development at the DN3 stage. Thus, to determine the effect of BAF57 mutation on the CD4 silencer, we introduced the transgene expressing BAF57 dominant negative mutant into Rag2 KO mice, and compared total thymocytes from these mice with that from Rag2 KO mice (Figure 1). Thymocytes in both lines are arrested at the same (i.e., the DN3) developmental stage, and are thus directly comparable. For convenience, Rag KO mice are termed “WT” DN cells, and the transgenic mice expressing the BAF57 deletion mutant on Rag KO background termed “57ΔN” DN cells, even though 57ΔN “DN” cells express CD4 and are thus technically $CD4^+$ cells (Figure 1). In addition, we examined DN cells lacking Brg1 (“Brg1 KO”), although these cells are not directly comparable with WT and 57ΔN cells because of differences in strain background, cellular composition and severity of developmental defects (see Materials and Methods). Finally, B cells, where the CD4 silencer is inactive, are included as a control.

We first analyzed the translational position of the nucleosomes at the silecer, using micrococcal nuclease (MNase) that preferentially cleaves linker regions between nucleosomes. Chromatin was exhaustively digested with MNase and the cleavages mapped by ligation-mediated (LM)-PCR using a primer set located near the center of the silencer. We focused on this segment of the silencer because it shows the most severe reduction in restriction enzyme accessibility (see next paragraph). Both the forward and reverse primers detected multiple bands in the MNase-digested chromatin in DN and B cells, which suggests the presence of at least five potential nucleosome frames (Figure 2). Although we were unable to unambiguously define nucleosome positions using this assay (see Material and Methods), it is obvious that the nucleosomes at the CD4 silencer occupy multiple frames in both DN and B cells, and that these frames are indistinguishable between two cell types (compare columns 2 vs. 5 and 7 vs. 10), which is unexpected because the silencer is active in DN but inactive in B cells. Thus, the nucleosome frames at the silencer do not correlate with its function. Not surprisingly, neither BAF57 nor Brg1 mutation alters the frames (columns 3–4 and 8–9).

In parallel with the MNase digestion assay, we used restriction enzymes to probe the CD4 silencer accessibility. We selected enzymes whose recognition sites consist of at least 5 base pairs; this can presumably make the assay more sensitive. A total of 5 enzymes were used, with their recognition sites depicted in Figure 3A. Nuclei were partially digested with each of these 5 “first restriction enzymes” before the DNA was purified and digested to completion with the corresponding “second restriction enzymes” that cut in the vicinity of the first enzymes (Figure 3A). LM-PCR was used to detect cleavages by both the first and

the second enzymes, the latter serving as normalization controls. The digestion patterns of three representative enzymes are shown in Figure 3B, and the accessibility in the BAF57 mutant relative to WT cells was quantified and plotted in Figure 3C. BAF57 mutation significantly reduces the CD4 silencer accessibility, with the reduction observed for all the enzymes tested and across the entire length of the silencer. The most severe reduction is seen at BseR1 (275), where the accessibility in the mutant is less than 15% of the WT level (Figure 3B, top central panel, lanes 1–2; Figure 3C). Remarkably, the reduction in accessibility is restricted to the CD4 silencer but not the flanking regions, with two notable exceptions. First, the accessibility of Dde I (−30) in BAF57 mutant is reduced to 30% of the WT level (Figure 3B, lower image in the middle left panel, lane 1–2; Figure 3C). However, Dde I (−30) is only 30-bp upstream of the CD4 silencer, and so its chromatin structure may also be subject to regulation by the BAF complex just as the CD4 silencer proper. The second exception is that the accessibility of BseR I (1130), located 696-bp downstream of the CD4 silencer, is reduced to 36% of the WT level (Figure 3B, top right panel, lane 1–2; Figure 3C). The underlying mechanism is unclear, but the BAF complex is undetectable at this region (data not shown), suggesting that the reduction in accessibility at BseR I (1130) is an indirect consequence of BAF57 mutation or even a result of some nonspecific effects unrelated to BAF57 mutations. The accessibility is similarly reduced in Brg1 KO cells as compared with WT cells (Figure 3B and C). This reduction can not be explained by the fact that Brg1 KO cells are mostly at a later developmental stage than the “WT” cells (see Materials and Methods), because in the presence of Brg1, the silencer is constitutively open in all developing thymocytes [19]. Thus, BAF57 mutation or Brg1 deletion each causes localized reduction in the restriction enzyme accessibility along the entire 434-bp CD4 silencer. Of note, the silencer in DN cells from BAF mutant mice is more accessible than that in B cells (Figure 3B, middle panels, lane 2–3 vs. 4). This shows that the silencer is not completely closed in the BAF mutants, which may result partly from the fact that the silencer is located downstream of the promoter and thus its chromatin is subject to disruption by elongating Pol II in the mutant DN cells where CD4 is being expressed. Furthermore, in BAF57ΔN cells, the expression level of the dominant negative BAF57 mutant is rather low (see Discussion), which is expected to only moderately impair BAF57-dependent remodeling.

The decrease in the restriction enzyme accessibility in BAF57 mutant and Brg1 KO cells indicate that BAF57 and Brg1 mutation each impair chromatin-remodeling at the CD4 silencer. To corroborate this conclusion, we examined the effect of BAF mutations on histone abundance at the silencer. Chromatin remodeling is known to be associated with losses of core and/or linker histones. For example, remodeling of the MMTV nucleosome template, which is catalyzed by the BAF complex, leads to a ~2-fold reduction in linker histone H1 abundance without significant depletion of core histone H2B [21], while remodeling of the cis-acting element at the rat tyrosine aminotransferase gene is associated with a ~2-fold reduction in the abundance of both H3 and H1[22]. We thus compared the H3 and H1 abundance in WT and mutant DN cells. B cells, where the silencer is inactive, were again included as a control. We found that the abundance of the core histone H3 at the CD4 silencer does not differ between DN and B cells, where the silencer is accessible and inaccessible, respectively (Figure 4A). Not surprisingly, BAF57 or Brg1 mutation does not alter H3 abundance in DN cells. In contrast, H1 abundance is increased 2.2-fold in B cells, indicating that H1 abundance reflects CD4 silencer accessibility. In BAF57ΔN cells, H1 is similarly enriched at the CD4 silencer, although to a less extent than in B cells (1.5-fold enrichment as opposed to 2.2-fold), which is consistent with the fact that the silencer remains partially accessible in BAF57ΔN cells but completely closed down in B cells. The H1 enrichment in BAF57ΔN cells is not an artifact, because the enrichment is restricted to the silencer and not observed at the flanking regions (Figure 4B). As expected, Brg1 deletion also increases H1 abundance as compared with WT cells (Figure 4A). These data

reinforce the notion that the BAF57 dominant negative mutant impairs chromatin remodeling at the CD4 silencer.

BAF57 mutation prevents Runx1 from accessing the CD4 silencer

We next explored the mechanism by which BAF57-dependent remodeling at the CD4 silencer is translated into CD4 repression. One possibility is that the BAF complex opens up the CD4 silencer, allowing repressors to access the silencer and subsequently inhibit CD4 transcription. A key repressor of CD4 transcription is Runx1 [23], which binds the silencer in DN cells [19]. It has been speculated that Runx1 recruits the BAF complex to the CD4 silencer [23]. However, it is equally possible that the BAF complex remodels the CD4 silencer prior to the entry of Runx1. We first used western blot to determine whether Runx1 expression is affected in BAF mutant cells. Runx1 consists of 56 kD and 58 kD isoforms whose relative abundance appears to remain constant throughout T cell development [24]. Interestingly, we found that the relative abundance of the two isoforms is cell-type specific: in total thymocytes consisting predominantly of DP cells isolated from C57/B6 mice, the 58kD isoform predominates, while the opposite is true in “WT” cells consisting mostly of DN3 cells (Materials and Methods) (Figure 5A, lane 1–2). This discrepancy between the current and the previous work might result from the use of different Runx1 antibodies and/or different mouse strains. Importantly, BAF57 mutation does not significantly alter the relative or absolute abundance of the two Runx1 isoforms (compare lanes 2–3). However, in Brg1 KO cells, the 58 kD isoform becomes slightly more abundant (lane 4), which may reflect the fact that Brg1 KO cells consist of a mixture of DN3 and DN4 cells (Materials and Methods), although we can not exclude the possibility that Brg1 deletion differentially affects the expression of the two isoforms. We next performed ChIP assay and found that both BAF57 and Brg1 mutations abolish Runx1 binding to the CD4 silencer (Figure 5B, row 3; Figure 5C). As a control, we examined HEB binding to the CD4 enhancer in BAF57ΔN and Brg1 KO cells; HEB is a E-box binding transcription activator that directly binds the CD4 enhancer to stimulate CD4 expression and is preloaded at the CD4 enhancer in DN cells [19,25,26]. HEB binding is indeed unaffected in the mutant cells (Figure 5), confirming that BAF mutations do not generally block transcription factor binding to the CD4 locus. As another control, we examined the effect of BAF mutations on Runx1 binding to the Th-POK locus. Th-POK is a master regulatory transcription factor that drives DP→CD4 differentiation. Runx1 binds the Th-POK silencer, a 674 bp fragment within the Th-POK locus, and is essential for Th-POK repression and subsequent development of CD8 cells[27]. Runx1 binds the Th-POK silencer constitutively in DP, CD4 and CD8 cells, suggesting that it also binds the silencer in DN cells [27]. Runx1 was indeed detectable at the Th-POK silencer in WT DN cells, and neither BAF57 or Brg1 mutation affects this binding, demonstrating that BAF mutations do not generally block Runx1 binding to its target genes (Figure 5C). Collectively, these data indicate the BAF mutations selectively reduce CD4 silencer accessibility, thus preventing Runx1 from binding the CD4 silencer and repressing CD4.

Discussion

In this study, we analyzed the effects of BAF mutations on the nucleoprotein structure of the CD4 silencer to gain insights into how the BAF complex represses CD4. A major finding is that the CD4 silencer function is regulated independently of nucleosome positioning. This is unexpected, given that nucleosome positioning is a key determinant of chromatin function [28–32]. However, a similar observation has been made on a cis-regulatory element controlling the expression of the rat tyrosine aminotransferase gene, where the nucleosomes occupy multiple frames, and these frames remain unchanged regardless of the functional

states of the regulatory element [22]. Future studies are needed to address why nucleosome positioning can sometimes be unimportant for chromatin accessibility.

We also found that BAF57 is essential for the BAF complex to remodel to the CD4 silencer *in vivo*, even though BAF57 is totally dispensable for remodeling *in vitro*. Together with the previous finding that the BAF57 HMG domain is dispensable for targeting the BAF complex to the CD4 silencer, the data conclusively establish that the BAF57 HMG domain participates in the actual chromatin remodeling process at a step after the recruitment of the BAF complex. To our knowledge, BAF57 is the first "dispensable" subunit shown to function in this manner. How does BAF57 promote remodeling *in vivo*? BAF57 mutation leads to H1 accumulation, indicating that the chromatin at the CD4 silencer prior to BAF57-dependent remodeling contains more H1 than that after remodeling. H1 stabilizes chromatin, and its removal has been hypothesized to be a prerequisite for chromatin remodeling [33], raising the possibility that BAF57 might be necessary for removing H1, thus facilitating subsequent remodeling by Brg1. However, on a mononucleosomal template containing H1, the BAF complex is able to slide the nucleosome in the continuous presence of H1, indicating that H1 removal is not a prerequisite of remodeling in this *in vitro* system. This raises the possibility that remodeling at the CD4 silencer might occur without large-scale H1 displacement, which is consistent with the fact that H1 abundance is only slightly increased at the CD4 silencer in the BAF mutants. Therefore, it is possible H1 is not a target of BAF57. In this scenario, the accumulation of H1 and reduction in accessibility at the CD4 silencer in the BAF57 mutant cells may reflect a BAF57-dependent process distinct from the hypothetical H1 removal.

The final finding from this study is that BAF57 mutation prevents the access of Runx1 to the CD4 silencer, which explains how BAF-dependent chromatin remodeling leads to CD4 repression. It has been suggested, based on the fact that both Runx1 and the BAF complex are essential for CD4 repression, that Runx1 acts to recruit the BAF complex to the CD4 silencer [23]. It is now clear that repressors distinct from Runx1 must be responsible for recruiting the BAF complex to the silencer. Consistent with this idea, the silencer contains binding sites for multiple potential transcription factors. The factor that recruits the BAF complex must be able to bind the silencer in the absence of the BAF-dependent remodeling. *In vivo* footprinting assays might reveal the identity of such factors.

In all the experiments described above, the effects of BAF57 dominant negative mutation on nucleoprotein structure of the CD4 silencer are indistinguishable from that of Brg1 deletion. However, the data must be interpreted with caution, given the multiple differences between BAF57Δ and Brg1 KO cells beyond the shared defect in CD4 repression (see Materials and Methods). We also note that the expression level of the deletion mutant of BAF57 in BAF57ΔN cells is fairly low, reaching only that of the endogenous protein (Figure 5A). We expect that higher-level expression of this mutant, or deletion of the BAF57 gene, will produce stronger phenotype, including more pronounced H1 accumulation at the CD4 silencer. Finally, we wish to stress that our previous paper on a related subject does not detract from the novelty of the current work: the former study defines the nucleoprotein structure of the CD4 locus throughout T cell development in WT mice [19], whereas the current study is focused on the effects of BAF mutations on this structure in DN cells. The previous paper shows that the accessibility, histone modifications and transcription factor binding at the CD4 locus undergo complex changes during T cell development. However, the data provide no insights into how BAF57 represses CD4 in DN cells. Neither does that study address nucleosome positioning and linker histone abundance at the silencer.

Materials and Methods

Mice

We have previously generated the Rag2 KO mice expressing BAF57 dominant negative mutant, but the mice were on a mixed genetic background [17], which may obscure subtle effects of the BAF57 mutation. To address this caveat, we backcrossed the mice via 6 successive generations onto C57/B6 background, which interestingly exacerbates the defect in CD4 repression (compare Figure 1 with Figure 4B in reference # [17]). Total thymocytes from C57/B6 Rag2 KO mice and C57/B6 Rag2 KO mice expressing the BAF57 dominant negative mutant were directly used for biochemical analysis. DN cells lacking Brg1 ("Brg1 KO") were total thymocytes harvested from $Brg1^{Flox/-}$; LCK-Cre; $Bcl\text{-}xL^{+/+}$ mice bearing a floxed Brg1 allele, a germline Brg1 KO allele, a transgene expressing Cre from the LCK-proximal promoter and a transgene expressing Bcl-xL from the same promoter [18]; the Bcl-xL transgene is necessary because Brg1-deleted DN cells rapidly die of apoptosis in the absence of ectopic Bcl-xL expression. Of note, Brg1 KO cells are not directly comparable with BAF57ΔN cells: although each partially derepresses CD4, Brg1 KO cells are on a mixed genetic background, are mostly at the DN4 stage and have multiple defects, whereas BAF57ΔN cells are now on the C57/B6 background and are mostly DN3 cells whose only detectable defect is CD4 derepression [[17,18] and Figure 1]. B cells in Figure 2 and Figure 4 were magnetically purified from C57/B6 mice whereas B cells used for the restriction enzyme accessibility assay (Figure 3) and Runx1 ChIP (Figure 5) were from CD1 mice; silencer accessibility and Runx1 binding in CD1 mice are indistinguishable from that in C57/B6 mice [19]. All animal experiments were performed according to the institutional guidelines.

MNase digestion assays were done as described [34] with the following modifications. Specifically, cells were first fixed with 1% paraformaldehyde for 5 min to prevent nucleosome sliding *in vitro*. Cells were washed and extensively digested with MNase to reduce chromatin to nucleosome core particles, incubated overnight at 60°C in the presence of proteinase K. DNA was then purified, phosphorylated and ligated to a small linker annealed from oligonucleotides DR19 and DR20. The ligated mononucleosomal-length DNA (~170 bp) was then gel-purified and analyzed by PCR using two sets of primer pairs: DR20/TC642 and DR20/TC639 for amplifying fragments terminating at the 5′ and 3′ cleavages, respectively. The two groups of amplicons were then detected by primer extension using corresponding nested primers: TC642N and TC639N for fragments generated by DR20/TC642 and DR20/TC639, respectively. The sequences are: Dr19, CACGAATTCCC; DR20, GCTATGTACTACCCGGGAATTCGTG; TC642, CACCCTCACATGGCCCCCAGAGATG; TC642N, TCACATGGCCCCCAGAGATGGGAGA; TC639, ATCTCTGGGGGCCATGTGAGGGTG; TC639N, GGGGGCCATGTGAGGGTGGCAGG. Ideally, a nucleosome would protect ~147 bp DNA, and the intensity of the band representing the 5′ border would be comparable to that representing the 3′ border. In reality, the situation can be more complex, in part because PCR amplification is biased toward shorter amplicons while the efficiencies of linker ligation and even of MNase-digestion can be sequence-dependent (T. Grange, personal communications). Also, an atypical nucleosome may incorporate less or more than 147 bp DNA. Given these caveats, we can only tentatively assign nucleosome borders to the cleavages detected by LM-PCR in Figure 2. Of note, the MNase digestion pattern can not reveal linker histone footprint, given that the chromatin was digested to core particles lacking linker DNA.

Restriction enzyme accessibility assay was performed as described [19]. Briefly, 0.4 million lymphocytes were digested with restriction enzymes in 10 ul 1x NEB digestion buffer II in

the presence of 0.1% NP40 which permeabilizes the membrane. After the digestion, the reaction was heated to 80°C for 20 min to inactivate the enzyme. One ul proteinase K (10 ug) was added and the sample digested overnight at 50°C. The sample was then heated to 80°C for 20 min, and PMSF added to 0.1mM to completely inactivate proteinase K. DNA was then directly digested for 3 hours with a second enzyme (0.3 ul) that cut in the vicinity of the first enzyme, and a mixture of linkers recognizing the first and second cuts were added together with NEB T4 DNA ligase (0.3 ul). The reaction was incubated at room temperature for 12 hours before PCR analysis using 1 ul of ligated DNA. The amplicons were detected by primer extension using radiolabeled, nested primers. The extension started at the nested primers but was terminated sequentially at the first and then the second cuts. The amplicons were resolved on 6% sequencing gels and the intensity of the short relative to the long amplicons was taken as a measure of accessibility. The accessibility in BAF mutants relative to WT cells was then plotted against the positions of the restriction enzyme sites. Restriction enzyme cleavage sites nd oligonucleotide sequences are provided in Supporting Information Tables 1 and 2.

Chromatin immunoprecipitation (ChIP) assays

Chromatin was fixed, sonicated, precleared, immunoprecipitated and quantified essentially as we described [19,35]. To quantify subtle changes in H1 abundance, we first determined the abundance of the target sequences (the CD4 silencer or its flanking sequences) and a control sequence (a fragment from the GAPDH gene) precipitated by histone antibodies or rabbit IgG, each normalized to that in chromatin input. The DNA precipitated by IgG was subtracted to determine the DNA amounts specifically precipitated by histone antibodies. The abundance of the CD4 sequences in the BAF mutants was then normalized to GAPDH abundance and displayed as % of the abundance in B cells (Figure 4A) or as % of the abundance in DN cells expressing WT BAF complex (Figure 4B). The semi-quantitative multiplex PCR assay measuring the effects of BAF mutations on Runx1 and HEB binding have been described, as well as the corresponding antibodies [Runx1, Calbiochem (PC824); HEB, Santa Cruz Biotech (sc-357X)][19]. Anti-histone H1 monoclonal antibody (AE-4) is from Santa Cruz Biotech (sc-8030) and has been used for ChIP assays [36]. ChIP-grade H3 (ab1791) antibody is from Abcam.

Western blot

0.3 million cells were loaded onto a NuPAGE 4–12% Bis-Tris gel (Invitrogen), and probed with anti-Brg1 (Millipore 07-478), anti-Runx1 (Calbiochem PC824) and anti-BAF57 antibodies. The molecular weights of Runx1 and BAF57 proteins are similar, which would require membrane stripping if the same membrane were used for sequential detection of both proteins. To avoid this, the sample set in Figure 5 A was loaded in duplicate and probed in parallel with Runx1 and BAF57 antibodies. The membrane used for BAF57 western blot was striped and reprobed with an anti-tubulin antibody to generate a loading control for the experiment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

LM-PCR ligation-mediated PCR

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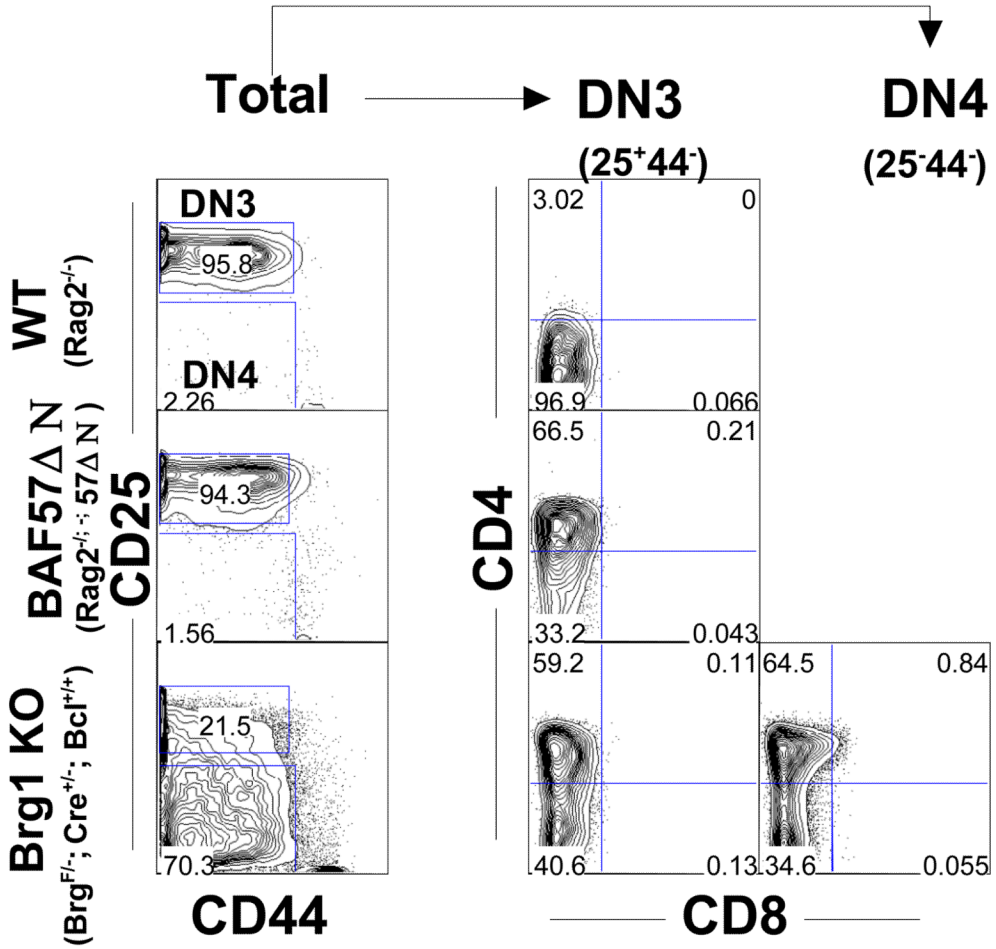

Figure 1.

FACS analysis of thymocytes. Total thymocytes, stained with antibodies against CD4, CD8, CD25 and CD44, were first resolved into two major subsets, DN3 and DN4, based on CD25/44 expression (column 1) before each subset was analyzed for CD4/8 expression (column 2–3). The two minor subsets of DN cells, namely DN1 ($CD25^{-}$ $CD44^{-}$) and DN2 ($CD25^{+}CD44^{+}$) cells, are not shown. Thymocytes in $Rag2^{-/-}$ (WT) and $Rag2^{-/-}$; $BAF57\Delta N^{+/+}$ (BAF57ΔN) mice are almost exclusively at the DN3 stage, while that in Brg1 KO mice are predominantly at the DN4 stage. CD4 is derepressed to similar extents in BAF57ΔN and Brg1 KO mice. The experiment was repeated at least 3 times with similar results.

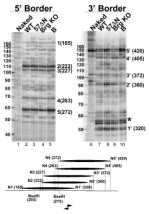

Figure 2.

BAF mutations do not alter nucleosome positioning at the CD4 silencer. Chromatin was exhaustively digested. Naked DNA (*Naked*) was partially digested with MNase and used for comparison. PCR was done in duplicates; only the bands seen in both reactions were considered true cleavages. The numbers in the brackets denote the positions of the cleavages and hence the borders of nucleosomes. Unambiguous determination of nucleosome frames is difficult (see the *Materials and methods*), but there are 5 potential nucleosome frames (N1–5) located between nt 165 and 420 of the CD4 silencer as depicted at the bottom diagram, with N4 and N5 being close to the "ideal" situation because the intensities of the bands representing the 5′ and 3′ borders are comparable and the lengths of nucleosomal DNA are close to 147 bp (142 and 148 bp for N4 and N5, respectively). The bottom diagram also shows the positions of the two BseR1 sites whose accessibility, together with that of multiple other restriction enzymes, was determined as described in Fig. 3. Note that some cleavages, including the prominent bands between the 3′ borders of N1 and N2 (lanes 7–10, asterisks), cannot be assigned nucleosome borders; such "orphan" bands have also been observed at the rat tyrosine aminotransferase gene [22]. The experiment was done twice with similar results.

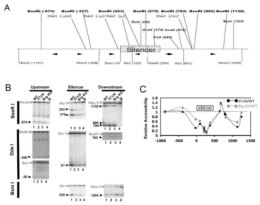

Figure 3.

BAF mutations reduce the accessibility of the CD4 silencer to restriction enzymes. **(A)** Restriction enzyme map of the CD4 silencer and flanking regions. The position of the first nucleotide of the CD4 silencer sequence is set as +1, while that of the upstream sequences are given negative numbers. The enzymes for probing accessibility (BseR1, Dde I, Bsm I, Ava II and Pst I) are indicated above the DNA; for clarity, distinct character fonts/styles are used for the names of different enzymes. Enzymes for generating normalization controls are indicated below the DNA, except that BseR1 (−327) and BseR I (784), used to probe the accessibility and thus indicated above the DNA, are also used to control for Dde I (386) and Dde I (782), respectively. The arrowheads underneath the DNA denote primers used in the primer extension steps of LM-PCR. **(B)** Accessibility of the CD4 silencer and flanking regions to BseR1, Dde I and Bsm I. The arrowheads denote cleavage products generated by these three “first restriction enzymes”, with the adjacent numbers referring to the positions of the enzyme recognition sites. The asterisks mark the cleavage products generated by the “second restriction enzymes” (HincII, BseRI, SacI and AluI). **(C)** The intensities of the primer extension products in Figure 3B were quantified, and the accessibility, defined as the intensities of the short relative to the long extension products, was calculated. Ava II and PstI digestions were similarly quantified (not shown). The accessibility in BAF57 mutant (57 ΔN) and Brg1 KO relative to WT cells was then plotted against the positions of the restriction enzyme sites. The numbers on the x-axis are the nucleotide numbers as defined in Figure 3A. All the restriction sites depicted in Figure 3A are included (dots/triangles) except for BseR (−327) that is resistant to digestion (not shown). Of note, for the most part, the accessibility of the CD4 silencer and that of the flanking regions to all the restriction enzymes in WT (i.e., $Rag2^{-/-}$ cells), BAF mutants and B cells shown in Figure 3B was analyzed in parallel with the accessibility in CD4, DP and CD8 cells shown in Figure 4 of a previous paper [19], and thus the same WT and B lanes, used as controls in both studies, were duplicated between the two papers.

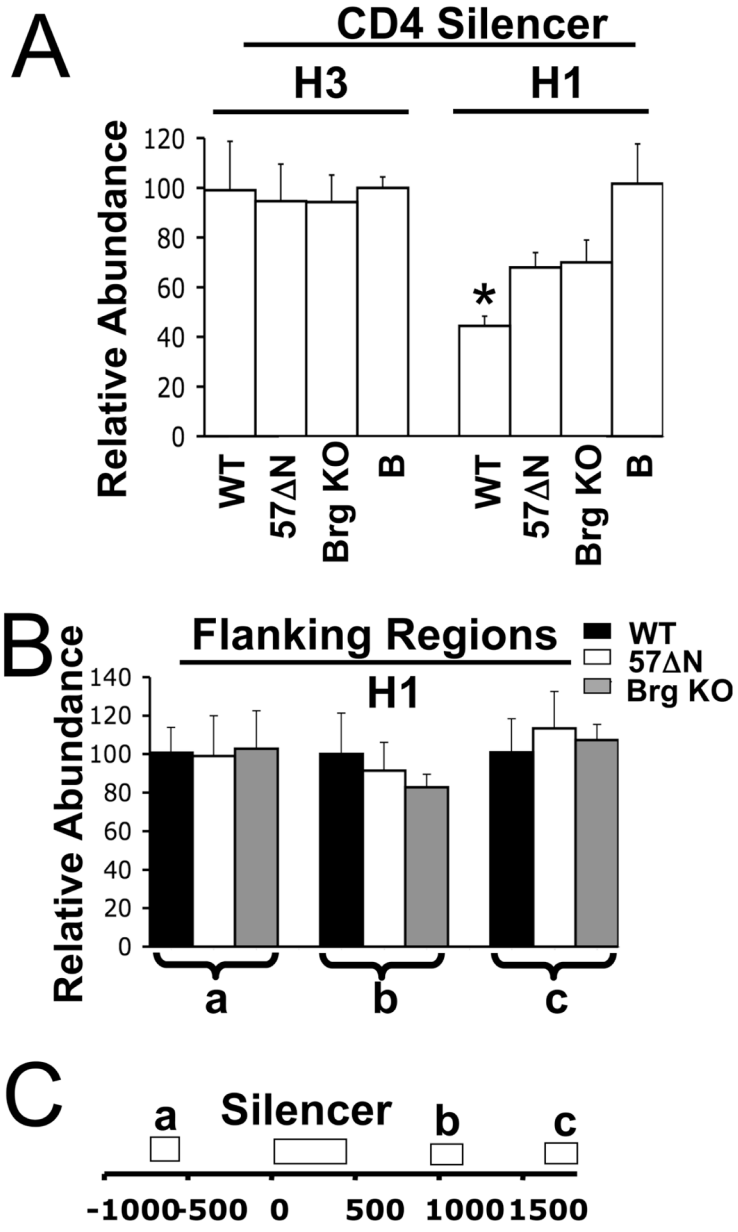

Figure 4.

Quantitative PCR assays measuring the effects of BAF mutations on histone abundance. **(A)** BAF mutations lead to an accumulation of linker histone H1 at the CD4 silencer without altering H3 abundance. The abundance (H1 or H3) in B cells is arbitrarily set as 100. The values were averaged from 3 independent experiments and displayed as mean ± SD. The asterisk indicates a significant difference ($p = 0.015$) in the H1 abundance between WT and the BAF mutants (BAF57ΔN or Brg KO) as determined by two-tailed Student's t-test. **(B)** BAF57 mutation does not alter H1 abundance in the regions (*a*-c) flanking the CD4 silencer. The abundance of H1 in WT cells is set at 100. Data in Figure 4A and B were averaged from three independent experiments. **(C)** Diagram depicting the positions of the CD4 silencer and two flanking regions analyzed.

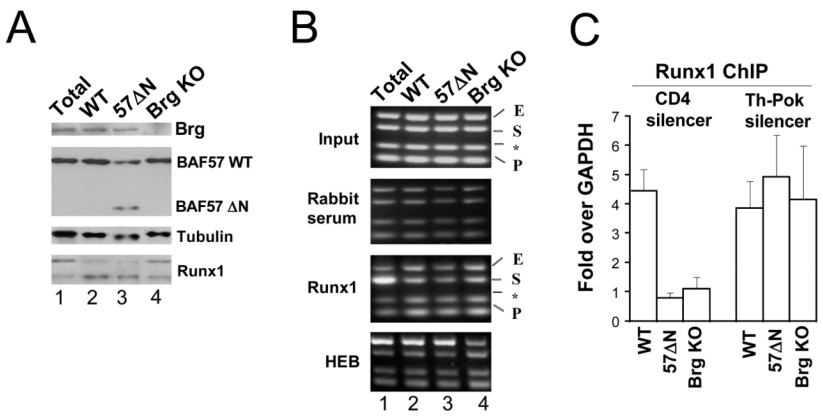

Figure 5.

Effects of BAF mutations on transcription factor binding to the CD4 locus. **(A)** Western blot showing that BAF57ΔN expression does not affect Runx1 expression. Note that BAF57 ΔN abundance does not exceed that of the endogenous BAF57 (lane 3). β-Tubulin was used as a loading control. **(B)** ChIP assays showing that BAF57 and Brg1 mutations block binding of Runx1 to the CD4 silencer without impairing HEB binding to the enhancer. DNA was analyzed by multiplex PCR detecting sequences from the CD4 enhancer (E), silencer (S), promoter (P) and the GADPH gene (*). The experiment was repeated with an independent chromatin preparation, yielding comparable results (not shown). (C) DNA from three independent Runx1 ChIP experiments, including the two experiments in Figure 5B, was analyzed by q-PCR measuring Runx1 binding to the CD4 silencer (left) or the Th-POK silencer (right) relative to that of the GAPDH gene in WT and BAF mutants. The values were averaged and displayed as mean ± SD.