The Chromatin Remodeler Mi-2β Is Required for CD4 Expression and T Cell Development

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Summary
Changes in chromatin structure underlie the activation or silencing of genes during development. The chromatin remodeler Mi-2β is highly expressed in thymocytes and is presumed to be a transcriptional repressor because of its presence in the nucleosome remodeling deacetylase (NuRD) complex. Using conditional inactivation, we show that Mi-2β is required at several steps during T cell development: for differentiation of β selected immature thymocytes, for developmental expression of CD4, and for cell divisions in mature T cells. We further show that Mi-2β plays a direct role in promoting CD4 gene expression. Mi-2β associates with the CD4 enhancer as well as the E box binding protein HEB and the histone acetyltransferase (HAT) p300, enabling their recruitment to the CD4 enhancer and causing histone H3-hyperacetylation to this regulatory region. These findings provide important insights into the regulation of CD4 expression during T cell development and define a role for Mi-2β in gene activation.

Introduction
The precise regulation of gene expression is fundamental to cell fate decisions and lineage specification during all developmental processes. Heritable changes in chromatin structure are increasingly recognized as critical events in shaping the gene expression pattern specific to each cell lineage. T cell development in the immune system is one of the best-studied paradigms of lineage specification. During the process, the coordinated acquisition of CD4 and CD8 coreceptors in maturing βT cells follows a complex pattern of gene expression, whereby these molecules function both as stage-specific markers and as functional signaling complexes that direct cell fate toward the helper and cytotoxic T cell lineages, respectively.

The most immature thymic precursor cells to seed the thymus from the bone marrow retain multipotency and are capable of differentiating into T cells, B cells, or NK cells (Shortman and Wu, 1996). Early T lineage-restricted precursors express neither CD4 nor CD8 coreceptors and are designated double-negative (DN) cells. In-frame rearrangement of the T cell receptor β (TCRβ) locus and cell surface expression of TCRβ protein within the pre-TCR complex (termed “β selection”) signals cells to proliferate and acquire both CD4 and CD8 coreceptors, with CD8 usually being expressed first in most mouse strains (von Boehmer et al., 1998). The resulting cells are termed double-positive (DP) thymocytes. Productive recombination at the TCRβ locus results in the expression of intermediate levels of the TCRαβ complex on the surface of DP cells. Most DP thymocytes (95%) die, either because their T cell receptors interact poorly with peptide major histocompatibility complexes (MHC) or because they bind too efficiently to self ligands and autoimmunity is risked (Robey and Fowlkes, 1994). A small number of DP thymocytes expressing TCRs that recognize self MHC-ligand complexes with the appropriate signal intensity are “positively selected” through a multistep process to become mature single-positive (SP) cells (Guidos, 1996). SP thymocytes maintain expression of one coreceptor while the other coreceptor is silenced. These are referred to as lineage-specified CD4+ or CD8+ mature T cells, which exit the thymus to circulate to the periphery.

The genetic elements that define the putative control regions in the CD4 and CD8 genes have been extensively characterized (Kioussis and Ellmeier, 2002). Activation of the CD4 locus relies on a 5’ proximal enhancer that is active throughout T cell development (Killeen et al., 1993; Sawada and Littman, 1991; Sawada et al., 1994) and possibly a 3’ enhancer/LCR that functions coordinately with the 5’ enhancer at the DP stage (Adlam and Siu, 2003). Expression of CD4 during T cell differentiation is restricted to DP and CD4 SP cells by a silencer that overrides the activity of the 5’ enhancer in DN and CD8 SP cells, thus conferring a lineage-specific expression pattern (Sawada et al., 1994; Siu et al., 1994). Recent genetic studies have implied DNA binding and chromatin remodeling factors in the regulation of CD4 and CD8 gene expression during T cell differentiation (Kioussis and Ellmeier, 2002). Studies on CD4 regulation have focused on factors governing its lineage restriction through silencer activity and have shown a dependence on the Runt domain-containing DNA binding factors Runx1 and Runx3 and the SWI/SNF chromatin remodeling complex (Chi et al., 2002; Taniuchi et al., 2002). The 2 MDa nucleosome remodeling histone deacetylase (NuRD) complex (Tong et al., 1998; Xue et al., 1998; Zhang et al., 1998) is highly expressed in the thymus and associates with the Ikaros family of lymphoid-lineage regulating factors in differentiating and mature T cells, suggesting that this chromatin remodeling complex may be important for T cell development (Kim et al., 1999). The NuRD complex is comprised of at least eight polypeptides, including Mi-2, a SNF2-like ATP-dependent chromatin remodeler. Genetic studies in C. elegans and Drosophila and biochemical purification of vertebrate
Figure 1. Lck<sup>αc</sup>-Cre-Induced Deletion of Mi-2β in Developing T Cells

(A) For conditional inactivation strategy, exons 12–21 of Mi-2β were flanked by LoxP sites (black triangles); the Neo marker was flanked by Frt sites (white triangles). Flp recombinase excises the Neo gene from the targeting construct and generates the Mi-2β<sup>+/LoxP<sup> allele. Lck<sup>αc</sup>-Cre recombinase induced deletion of the Mi-2β ATPase domain in thymocytes, generating the Mi-2β<sup>+/H9004</sup>F<sup> allele, or if introduced before Flp, the Mi-2β<sup>+/H11001</sup> allele.

(B) Southern blot analysis of Mi-2β deletion efficiency. Genomic DNA from tail, thymus, lymph nodes (LN), and LN T cells (LN T) was extracted from wt (Mi-2β<sup>+/+</sup>/), heterozygous (Lck<sup>αc</sup>-Cre/Mi-2β<sup>+/LoxP<sup>, or Mi-2β<sup>-deficient (Lck<sup>αc</sup>-Cre/Mi-2β<sup>LoxP<sup> mice. EcoRI-digested DNA was probed as indicated in (A). Digestion fragments from both the + and LoxP<sup> alleles migrated at 8.5 kb; the ∆ allele (Neo<sup>) migrated at 5 kb. The 8.5 kb LoxP<sup> fragment was reduced to 3.5 kb after Cre-mediated excision of exons 12–21 of Mi-2β<sup>(∆) allele. Deletion efficiency was >95% in thymus and varied from 13%–45% in total LN and 15%–65% in LN T cells.
Mi-2 with HDACs have implied that Mi-2 is a negative regulator of gene expression.

To investigate the importance of the Mi-2 chromatin remodeling complex in T cell development, we conditionally inactivated Mi-2β (CHD4), the most abundantly expressed of the two Mi-2 homologs, in thymocytes. We show that Mi-2β functions as a positive regulator of the DN to DP transition and of TCR-mediated proliferative responses in mature T cells and provide unexpected evidence that Mi-2β is involved in the positive regulation of CD4 expression in differentiating DP thymocytes. Mi-2β is found associated with the CD4 enhancer in CD4-expressing cells, and recruitment of the p300 histone acetyl transferase and the HEB transcription factor to the enhancer is Mi-2β dependent.

Results

Conditional Inactivation Strategy for the Mi-2β Locus

To examine the role of Mi-2β during development, we generated mice that carry Cre-recombinase recognition signals (LoxP sites) upstream of exon 12 and downstream of exon 21 (Figure 1A). Deletion of exons 12–21 from the Mi-2β locus removes the ATnPase domain, which is required for its remodeling activity. Splicing of exon 11 to exon 22 would be expected to generate an out of frame mRNA, possibly resulting in a truncated protein. The originally modified Mi-2β locus also contained the pgk-neomycin expression cassette, flanked by FLP recombine signals (Frt sites; Figure 1A, Mi-2βLoxPNeo). To avoid interference effects on expression of Mi-2β, the pgk-neo gene was deleted in the mouse germine by crossing onto an actin-FLP transgenic line (Dymecki, 1995). The flipped Mi-2β locus was designated as Mi-2βLoxPF (Figure 1A). To inactivate Mi-2β during T cell development, mice homozygous for the Mi-2βLoxPF allele and carrying an LckαLck proximal promoter-Cre transgene were generated (Figure 1A, Mi-2βLckCre). This Lckα-Cre transgene is active from the double-negative (DN) stage of T cell differentiation to mature T cells (Lee et al., 2001). We also generated a germline deletion of the ATnPase domain designated as Mi-2βδ-1 that was combined with the Mi-2βLoxPF allele and the Lckα-Cre transgene. In both Mi-2βLoxPF and Mi-2βLoxPFLoxPF strains, Lckα-Cre-mediated deletion in thymic T cells was calculated to occur at >95% frequency (Figure 1B). In the periphery, although a significant number of T cells had undergone deletion, an undeleted fraction was also observed (Figure 1B).

RNA analysis of thymocyte subsets in which Mi-2β was conditionally inactivated indicated that the production of full-length Mi-2β mRNA was dramatically reduced in DN and DP thymocytes and was absent in CD8+ CD4- and SP (Figure 1C, E11–13). A mutant variant was detected that lacked exons 12–21 but which contained downstream sequences (Figure 1C, E30–34). The presence of Mi-2β protein was examined using a monoclonal antibody that detects an epitope in exon 11, which lies upstream of the deleted region. A dramatic reduction in the amount of full-length Mi-2β protein was seen in the thymus (Figure 1D). A truncated Mi-2β protein was not detected (data not shown), suggesting that it was either not produced by the mutant mRNA or that it was not stable. Further examination of purified thymocyte subsets revealed that Mi-2β protein was significantly reduced in the Mi-2β−/−/− DN population, was further reduced in DP cells, and was virtually absent in the CD8−/− and CD4−/− SP populations (Figure 1E).

We also examined expression of the second Mi-2 family member, Mi-2α, which shares strong homology with Mi-2β. Whereas the level of Mi-2α is very low in the wild-type (wt) thymus, it was upregulated in the KO thymus (Figure 1D). The increase in Mi-2α protein levels reflected an upregulation of Mi-2α mRNA that was detected in all thymocyte subsets from the DN through the DP to the SP stages of differentiation (Figure 1C, Mi-2α).

In contrast to Mi-2α, the levels of other components of the NuRD complex, i.e., HDAC2 and RbAp48, as well as Ikaros and Aiolos, which constitute components of a lymphoid version of the NuRD complex, were not altered by the Mi-2β deletion (Figure 1D and data not shown). Brg-1, the ATnPase subunit of the SWI/SNF remodeling complex, and the nuclear factor YY1 were also present at normal levels.

Mi-2β Inactivation Interferes with CD4 Expression in DP Thymocytes

Analysis of Mi-2β KO mice revealed a consistent reduction in thymus cellularity compared to wt or heterozygous littermates, regardless of whether the Mi-2βLoxPF target was biallelic or monoallelic. In all KO mice analyzed (n > 40), a similar change in thymocyte profiles was detected relative to wt (Figure 2B). The percentage of DP thymocytes was severely reduced, whereas CD8+ cells were greatly increased in a “compensatory” fashion. DP and CD4+ SP thymocytes expressed lower levels of CD4 (Figures 2B and 2C), and CD8+ cells ranged from CD4+ to CD4−. As DN thymocytes mature to become DP, they progress through a transient immature intermediate single-positive (ISP) stage during which CD8 is expressed without CD4 (Figure 2A). ISPs express little or no TCR or CD5 and express high levels of HSA. The CD8− populations that accumulate in the KO could be arrested ISPs, DP cells that lack CD4, or mature SP CD8+ cells. Markers that do not rely on CD4 expression and are
differentially expressed during the DN to SP progression were used to distinguish these possibilities (Figure 2A).

Thymocytes upregulate TCR expression as they progress from the DN (TCR<hi>) through the DP (TCR<interd>) to the SP (TCR<lo>) stages of their differentiation (Figures 2A and 2B, Mi-2<−/−>). Despite an increase in CD8-expressing cells, the overall T cell receptor (TCR) expression profile was not dramatically altered in the Mi-2β-deficient thymus (Figure 2B), suggesting that developmental progression was largely normal. To assess the maturity of the overrepresented CD8<hi> population in the KO, thymocytes were subgated based on CD4 and CD8 expression. Within the KO subsets, the expected increase in TCR was detected from DN to DP to CD4<lo> SP subsets (Figure 2B, TCR). Strikingly, the majority of the CD8-expressing KO thymocytes expressed TCR levels comparable to the wt DP population (Figure 2B and Supplemental Figure S1 at http://www.immunity.com/cgi/content/full/20/6/719/DC1). Very few of the KO CD8<hi> cells were TCR<lo>, suggesting that the majority were not ISP (Supplemental Figure S1 on Immunity’s website). A small number of CD8<lo>/TCR<lo> SP were also detected (Supplemental Figure S1, TCR<lo> gate), but most of the aberrant CD8-expressing thymocytes were not mature SP cells. Intermediate “DP-like” levels of the CD5 marker, which parallels TCR expression during thymocyte differentiation but is not dependent on rearrangement events, were also detected on the Mi-2β-depleted CD8<hi> CD4<−/−> populations (data not shown).

The CD8-expressing thymocytes that accumulated in the KO thymus were therefore DP-like in their intermediate expression levels of TCR and CD5.

Thymocyte populations were also evaluated using the HSA marker (Figure 2B, HSA). HSA is normally expressed at highest levels within the highest CD8<lo> populations (DN3 and DN4). It is then progressively downregulated through thymocyte differentiation (Figures 2A and 2B, wt). Among the KO thymocyte subsets, the expected decrease in HSA levels was detected from DN to DP to CD4<lo> SP subsets (Figure 2B, KO). However, the majority of the CD8-expressing populations in the KO thymus expressed HSA at a level similar to that of the DP and not the CD8<lo> ISP or CD8<−/−> SP cells (Figure 2B), suggesting that, despite their lack of CD4 expression, they otherwise resemble wt DP.

The “identity” of the CD8<hi>CD4<−/−> populations was further examined by RT-PCR analysis of Fas and Bcl-2 (Figure 2D). Expression of the apoptotic factor Fas is normally low in the immature DN and mature SP but is upregulated in DP to allow for appropriate selection of the TCR repertoire at this stage of differentiation (Figure 2A) (Linette et al., 1994; Strasser et al., 1995). The anti-apoptotic factor Bcl-2 displays a complementary expression profile to Fas (Figure 2A). The DN, DP, and CD4 SP thymocyte subsets in the KO thymus exhibited similar Fas and Bcl-2 expression to their wt counterparts (Figure 2C). In KO CD8<hi> CD4<−/−> cells, Fas expression was higher and Bcl-2 expression was lower than normally detected at this stage of differentiation, and both were comparable to wt DP levels (Figure 2C). Collectively, phenotypic analysis of thymocytes strongly supports the hypothesis that the majority of CD8<hi>CD4<−/−> cells that accumulate upon Mi-2β deletion are DP that have failed to express appropriate levels of CD4.

Thymocyte Apoptotic Properties Remain Unaltered in the Absence of Mi-2β

In the KO thymus, the percentage of DP and DP-like (CD8<lo>CD4<−/−>, CD8<−/−>CD4<−/−>) populations sum to 79%, which is within the expected range for DP in the wt (Figure 2B). Since thymus cellularity was reduced ~3-fold in the KO, the absolute number of DP cells was also reduced ~3-fold. Thus, we examined whether there was an increase in apoptosis within the KO thymocytes that could account for the decrease in thymus cellularity.

Sorted thymocyte subpopulations were stained with Annexin V and propidium iodide (PI). No significant difference in apoptotic cells (Annexin V<+>) or dead cells (Annexin V<+>/PI) was detected within the DP subpopulations of the wt and KO thymus (Supplemental Figure S2A, available on Immunity’s website, DP). A small increase in apoptotic cells was detected within the CD8<hi>CD4− and CD8<−/−>CD4<−/−> subsets upon Mi-2β deletion (Supplemental Figure S2A on Immunity’s website). The higher apoptotic index of these populations in the KO compared to the wt in part correlates with their proposed DP nature. Nonetheless, the majority of these CD8<hi> cells were not apoptotic. Apoptosis was also examined using the TUNEL assay on thymic sections. A similar number of apoptotic cells was seen in wt and in KO thymic sections (data not shown). Thus, loss of Mi-2β and the reduction of CD4 expression on DP and SP thymocytes has no significant effect on their ability to die by apoptosis.
The DN to DP Transition Is Impaired upon Mi-2β Deficiency

Despite the significant decrease in thymocyte cellularity caused by Mi-2β deletion, the absolute number of DN thymocytes was unaltered if not elevated (Figure 3A). To further examine the effect of the Mi-2β mutation at the DN stage of differentiation, thymocytes were gated for Lin−/Thy1+, and their distribution into the DN1-DN4 populations was examined. A decrease in the DN3 and an increase in the DN4 population were seen reproducibly among KO thymocytes (Figure 3B). Expression of TORβ as an intracellular (IC) protein can be detected between the DN3 and DN4 stages of development, and signaling through the pre-TCR complex is responsible for triggering this developmental transition. The majority of DN4 cells in both the wt and KO thymi expressed IC-TCRβ although a larger population of negative cells was reproducibly seen in the KO (Figure 3C). At the DN3 stage, a small population of IC-TCRβ+ cells was detected in wt, which was again reduced in the KO (Figure 3C). Evaluation of mRNA expression of pre-TCR signaling components like p70s and p5kg transcripts did not reveal any significant difference between the wt and KO DN3 and DN4 populations (data not shown). The percentage of cycling cells and their profiles were similar between the DN populations in KO and wt (Figure 3C). More dead cells were detected within the KO DN3 and DN4 although the majority of cells in these populations were viable (Figure 3C).

Developmental transitions can be better evaluated in the late fetal thymus where the differentiation of one rather than of several asynchronously entering waves of precursors is detected. In the late fetal/neonatal wt thymus the majority of precursors have developed to the DP stage of differentiation (Figure 3D, Mi-2β−/−). In sharp contrast, in the KO thymus, the majority of cells remain at the DN stage (Figure 3D, Mi-2β−/−) and are blasting (Figure 3D, FSC), consistent with being immature cycling cells. Further analysis of the mutant DN populations revealed an accumulation of DN4 cells (Figure 3D) similar to what was seen in the adult.

Mi-2β is thus required for normal transition from the late DN (DN4) to the DP stage of differentiation subsequent to β-selection. In the absence of Mi-2β, differentiating thymocytes appear to stall at the DN4 stage, but some of them eventually make the transition to DP. This inefficient precursor transition is likely responsible for the reduction in total thymocyte cellularity. Upregulation of other homologous factors at the DN4 stage, such as Mi-2α (Figure 3C), may partially rescue the DN-DP transition.

The Role of Mi-2β in TCR-Mediated Proliferative Responses

The ability of KO thymocytes to mature to the SP stage was evaluated. Gating on TCRβ+ cells in the thymus revealed a relative decrease in CD4+ SP and, consequently, a relative increase in CD8+ SP thymocytes, although this could be due to an overflow of TCRβ+ DP-like cells in the TCRβ1 gate (Supplemental Figure S1 on Immunity’s website, TCRβ1 gate). The ability of Mi-2β-deficient CD4+ and CD8+ SP cells to exit the thymus and accumulate in the periphery was examined. A 2- to 5-fold reduction in T cells was noted in the spleen and lymph nodes, with a similar decrease evident in both the CD4+ and CD8+ populations (Figure 4A). The mutant DP thymocytes were therefore able to differentiate into CD4+ and CD8+ T cells and seed the periphery, suggesting that a fraction of these cells undergo maturation and selection events. Similar to the thymus, mutant CD4+ T cells in the periphery expressed lower levels of CD4+ (Figure 4A).

T cells respond to engagement of their antigen receptors by proliferating. To assess the proliferative capacity of KO T cells, the Lck-Cre/Mi-2β+ strain was bred onto a GFP reporter of Cre activity (Novak et al., 2000), and GFP+ cells were sorted and used for in vitro proliferation assays. PCR analysis confirmed that >95% of the GFP+ cells had deleted Mi-2β (Figure 4B). A 5- to 7-fold decrease in 3H-thymidine incorporation was observed for KO T cells relative to wt 48 hr after TCR/CD3 stimulation, indicating that fewer KO T cells were in S phase (Figure 4B). KO T cells expressed normal levels of the TCR/CD3 complex (data not shown). A proliferation defect was also seen with PMA and calcium ionophore, which bypass proximal TCR signaling events and directly activate protein kinase C signaling. A relatively small increase in apoptotic and dead cells was seen 48 hr after activation with KO T cells (Supplemental Figure S2 on Immunity’s website), but the majority of cells were alive. The ability of KO- and wt-activated T cells to go through consecutive cell divisions was also evaluated by CFSE labeling. A progressive decrease in cycling T cells was seen from the second to the third cell division and is consistent with the lower 3H-thymidine incorporation (Figure 4C). In addition, a larger number of nonblast T cells that retained high levels of CFSE, suggesting failure to enter the cell cycle, were observed. TCR engagement of KO T cells induced expression of activation markers, like CD25 and CD69, but an increased number of negative cells were detected (Figure 4D).

Thus, Mi-2β deficiency has two effects on T cell proliferation: it causes both a reduction on the number of T cells that become activated and of those that can proceed through successive cell divisions efficiently.

A CD4-Based Transgene Relies on Mi-2β for Expression

During T cell development, CD4 expression is coordinately regulated by positive and negative elements surrounding the CD4 locus, namely a proximal enhancer (Sawada and Littman, 1991), silencer (Sawada et al., 1994; Siu et al., 1994), and LCR/3′ DP enhancer working in combination with the proximal enhancer (Adlam et al., 1997; Adlam and Siu, 2003). Collectively, our data indicate that Mi-2β may be acting through these regulatory elements to activate the CD4 locus.

We have begun to examine the effect of Mi-2β mutation on the activity of these regulatory elements using a GFP transgenic reporter. In the absence of the silencer, a CD4 enhancer-promoter-GFP cassette (Manjunath et al., 1999) expresses at high levels in all thymocyte subsets, including DP, but not in non-T cells (Figure 5, wt). Mi-2β depletion caused a reduced GFP expression in all subpopulations compared to wt (Figure 5, KO). Importantly, GFP levels were most reduced in CD8+CD4− and
Activation of CD4 Requires Mi-2β

Figure 3. Effects of Mi-2β Deficiency on the DN to DP Transition

(A) Bar graph summary of cell numbers and percentages of total thymocytes and DN populations in KO (n = 24) versus wt (n = 26).

(B) DN1-DN4 profiles in the adult wt and KO thymus. Lin−/Thy1+ thymocytes were examined for expression of CD44 and CD25, revealing an increase in the mutant DN4 population.

(C) DN3 (CD44+/CD25−) and DN4 (CD44+/CD25+) populations were sorted and analyzed for expression of IC-TCRβ, cell cycle profile (propidium iodide), and apoptosis (AnnexinV/propidium iodide). mRNA was prepared from the same sorted subsets, and expression of Mi-2β (exons 11-13), Mi-2α, and β-actin was determined.

(D) Temporal block at the DN stage of differentiation in neonatal thymus of Mi-2β KO. In contrast to wt, the majority of mutant thymocytes show a high FSC reflecting an immature blasting state. Further analysis of DN thymocytes (as in [B]) revealed an accumulation of DN4 (CD44+ /CD25−) cycling cells in the KO.

CD8+CD4+ cells, mirroring the progressive loss of Mi-2β (Figures 1C and 1E) and decrease in endogenous CD4 expression (Figures 2B and 2C) in these subsets. The effect of Mi-2β depletion was also examined on the Rosa26-YFP transgene to determine whether it affected other unrelated regulatory regions in a similar fashion.
Immunity

Figure 4. T Cell Maturation in the Absence of Mi-2β

(A) Mi-2β-deficient mice exhibit a reduction in peripheral T cells. CD4 and CD8 staining on peripheral LN from 6-week-old wt and KO littermates is shown. The total LN cell count for each animal and the percentage of cells in each quadrant is shown. An arrowhead points to the MFI of CD4 expression in each animal. Arrowheads indicating that CD8 fluorescence is unchanged are included as a control.

(B) Mi-2β-deficient T cells are hypoproliferative. Since Mi-2β deletion was 100% in peripheral T cells, Lckpr-Cre/Mi2βLoxPF/LoxPF mice were crossed to the LacZ/EG indicator strain in which cells actively expressing Cre recombinase are marked by GFP expression. GFP LN cells were then sorted that were Lckpr-Cre/Mi2β/LoxPF/LoxPF (wt) or Lckpr-Cre/Mi2βLoxPF/LoxPF (KO). Mi-2β deletion efficiency in Lckpr-Cre/Mi2βLoxPF/LoxPF GFP T cells (95%) is displayed in the PCR panel on the right. The GFP T cell pool also contains a fraction of Mi-2β+/− T cells, suggesting that GFP expression in the indicator line lags behind Cre activity. LN or GFP T cells were activated in vitro with either α-CD3 or PMA and ionomycin. Bar graphs of the scintillation counts represent the relative proliferation index for the wt or KO cells.

(C) Inability of activated Mi-2β+/− T cells to go through normal cell divisions is revealed by FACS analysis of a combination of CFSE labeling and cell size (FSC). The overlay of CFSE staining of wt versus KO T cells at 48 hr after activation shows a difference in the fraction of cycling cells.

(D) Induction (0 to 48 hr) of activation markers (CD25 and CD69) in Mi-2β+/− T cell activation cultures.

The Rosa26-YFP transgene is expressed throughout T cell differentiation with its levels showing an increase from the DP to the SP stage (Figure 5). wt and mutant DN, DP, and CD4+ DP thymocytes expressed similar levels of the Rosa26-YFP reporter. Mutant CD8−/CD4+ and CD8−/CD4− thymocytes expressed lower levels of the reporter consistent with their DP state of differentiation. Therefore, in the context of a transgenic reporter, Mi-2β is specifically affecting the activity of the CD4 enhancer-promoter elements.

A Direct and Specific Association between Mi-2β and the CD4 Enhancer

To test the hypothesis that Mi-2β directly regulates CD4 expression through its association with CD4 regulatory elements, we performed chromatin immunoprecipitations (ChIP) on wt thymus, as 85% of the cells express CD4. After ChIP with anti-Mi-2β antibodies (Abs), a multiplex PCR was performed to evaluate the specific enrichment of each regulatory element (Figures 6B and 6C; Mi-2β+/−). Anti-Mi-2β Abs reproducibly gave strong enrichment at the proximal enhancer compared to control Abs (Figure 6B and Supplemental Figure S3A on Immunity’s website, Mi-2β+/−). Only low levels of enrichment were detected over the promoter and LCR/3’ enhancer regions. The strong proximal enhancer enrichment was lost in the KO thymus (Figures 6B and 6C, Mi-2β+/−).

These findings provide evidence for a direct and specific association between Mi-2β and the CD4 proximal enhancer that is required for CD4 gene regulation during T cell differentiation. They suggest that the changes observed in thymocyte profiles in the KO reflect direct involvement of Mi-2β in modulating the chromatin state of CD4 regulatory elements.

Recruitment of HAT Activities to the CD4 Regulatory Elements Requires Mi-2β

Next, we evaluated the histone acetylation state of the CD4 regulatory regions in the presence and absence
Activation of CD4 Requires Mi-2β

Figure 5. Mi-2β Is Required for Developmentally Regulated Expression of a CD4 Transgenic Reporter

Decreased expression of an exogenous GFP-expressing CD4 transgene in Mi-2β-deficient thymocytes. Five thymocyte subpopulations were identified based on CD4 and CD8 expression in the wt and KO thymus (see gates, top). Expression levels of the T-GFP-CD4 transgene (Manjunath et al., 1999) are shown as overlaid histograms within these subsets (wt, green; KO, red). Expression of a Rosa26-YFP transgene in KO thymocyte subpopulations is provided as a control for specificity of the Mi-2β deficiency effect on expression of the CD4-transgene. Dotted lines indicate the MFI in each of the subpopulations in the T-GFP-CD4 and Rosa26-YFP transgenes.

of Mi-2β. High levels of histone H3-acetylation (AcH3), which are generally associated with gene activation, were detected at the proximal enhancer in the wt thymus (Figures 6B and 6D). Significant AcH3 was also detected at the CD4 promoter (Figures 6B and 6D), possibly reflecting recruitment of HAT-containing transcription initiation complexes (Mizzen et al., 1996). AcH3 was present at much lower levels in other regions of the gene (i.e., exon 10 and 3′ LCR/enhancer) (Figures 6B and 6D). A great reduction in AcH3 at the proximal enhancer and promoter was seen in the KO thymus (Figures 6B and 6D). We thus hypothesize that Mi-2β specifically associating with the CD4 proximal enhancer changes chromatin structure that allows recruitment of HATs.

We examined which of the previously described HATs was responsible for CD4 enhancer hyperacetylation. Anti-p300 Abs showed very strong enrichment for the CD4 enhancer, which was drastically reduced upon Mi-2β depletion (Figure 6D). CBP, a protein closely related to p300, and other tested HATs (PCAF, Gcn5, and Tip60) showed little or no discernible enrichment (data not shown). Although we cannot exclude the possibility that the detection of some of these factors with ChIP is limited by the antibody used, these studies identify a specific Mi-2β-dependent association of p300 with the CD4 enhancer in thymocytes that is likely responsible for the histone hyperacetylation at this region.

Mi-2β Promotes HEB Recruitment to the CD4 Enhancer

The CD4 enhancer has binding sites for E box proteins, which are critical for its activity (Sawada and Littman, 1991) and are bound by HEB and E2A-related transcription factors in vitro (Sawada and Littman, 1993). Similar to the Mi-2β−/− phenotype, HEB knockout leads to accumulation of CD8-expressing immature thymocytes, a significant fraction of which resemble DP cells (Barndt et al., 1999). We therefore investigated whether HEB bound to the CD4 enhancer in vivo and whether this binding was affected by lack of Mi-2β. Abs to HEB showed very strong enrichment for the CD4 enhancer, which was drastically reduced upon Mi-2β depletion (Figure 6B). Since Mi-2β deficiency did not alter HEB transcription (data not shown), these findings indicate Mi-2β remodeling activity at the CD4 enhancer enables strong HEB binding.

A Physical Association between Mi-2β, p300, and HEB

The physical interactions between Mi-2β, p300, and HEB were evaluated. Immunoprecipitation of p300 from thymocytes, whether in the absence or presence of ethidium bromide, revealed a DNA-independent association
Figure 6. Mi-2β Association and Histone H3 Acetylation of CD4 Regulatory Elements

(A) Schematic representation of the CD4 locus. Exons around the CD4 locus are shown in the upper half, and the known CD4 regulatory elements are indicated in the bottom half of the gene.

(B) Results of α-Mi-2β and α-AcH3 ChIP. Chromatin samples from wt (upper panels) or Mi-2β KO (lower panels) thymocytes were immunoprecipitated using control mouse IgG, α-Mi-2β, or α-AcH3. Coprecipitated DNA was analyzed by multiplex PCR. In each panel, the upper bands are from the region of interest, whereas the bottom bands (asterisks) are internal controls from an irrelevant genomic region. Fold enrichment relative to IgG ChIP was calculated as described (Harker et al., 2002). Relatively strong enrichment of the proximal enhancer and to a lesser extent, the promoter, was obtained with α-Mi-2β and α-AcH3. Enrichment was diminished in the KO sample, indicating dependence on the presence of Mi-2β.

(C) Graphical presentation of the calculated fold enrichment for α-Mi-2β ChIP from (B). The results for the wt and KO are plotted with black and gray boxes, respectively.

(D) Fold enrichment for α-AcH3 ChIP from (B) is plotted as (C).

of p300 with Mi-2β and HEB but not with HDAC2 (Figure 7C). Immunopurification of Flag-Mi-2β from thymocytes also revealed its association with p300 and HEB (Figure 7C). Finally, immunoprecipitations with anti-HEB Abs confirmed HEB’s association with Mi-2β and p300 (Figure 7C). Thus, the ability of Mi-2β to physically associate with p300 and HEB is likely important for their recruitment to the CD4 enhancer, a necessary step for subsequent hyperacetylation and transcriptional activity of this locus.
Activation of CD4 Requires Mi-2β

Figure 7. Mi-2β Promotes Recruitment of HEB and p300 to the CD4 Enhancer

(A) p300 HAT association with the CD4 regulatory regions. ChIP was performed using α-p300. The results of the multiplex-PCR analyses for the proximal enhancer, promoter, exon 10, LCR, and 3' enhancer are shown. Asterisks indicate the internal control. Calculated fold enrichment of p300 association with the CD4 regulatory region is shown on the right as a bar graph. α-p300 gave strong enrichment at the first half of the proximal enhancer, which was abolished in the KO.

(B) HEB association with the CD4 enhancer is Mi-2β dependent. ChIP analysis with α-HEB was performed as described in (A). Given the very strong association of HEB with the enhancer, PCR reactions for the target and internal control were performed separately and a 1:9 dilution of the template was used for target amplification.

(C) Immunoprecipitations of thymocyte nuclear extracts with α-p300 (and ethidium bromide), α-HEB, or IgG control and of thymocyte nuclear extracts expressing flag-tagged Mi-2β with α-flag followed by elution and Western blotting with α-p300, α-Mi-2β, α-HEB, and α-HDAC2 reveal a physical association between HEB, p300, and Mi-2β but not HDAC2.

Discussion

In this study, we examine the role of the chromatin remodeler Mi-2β during T cell differentiation by conditionally inactivating the gene in mice. We show that Mi-2β activity is required in at least three distinct stages of T cell development: in late DN to support their transition to the DP stage, in DP for normal expression of the CD4 coreceptor, and finally in mature T cells for their proliferative expansion (Figure 8A). Using independent genetic approaches and biochemical studies, we provide unexpected evidence that Mi-2β is involved in the positive regulation of the CD4 gene during differentiation in the thymus through its direct interactions with the CD4 enhancer as well as the HAT p300 and the E box binding protein HEB.

Mi-2β Is Required for Normal Expression of CD4 during T Cell Development

Conditional inactivation of Mi-2β during T cell development produced a dramatic decrease of DP thymocytes, which represent the majority in the wt thymus. The decrease was accompanied by a compensatory increase in CD8-expressing thymocytes, which were equally distributed between the CD8+CD4− and CD8+CD4+ subpopulations. Further characterization of the Mi-2β-
The lack of Mi-2β results in the recruitment of basal transcription factors resulting in activation of enhancer and the expression of CD4 chromatin in an open configuration. Interactions between the activated expression on CD4 SP T cells. deficiency manifests downstream of expression on DP and a downward arrow lower levels of CD4 expression. DN4 cells express IC-TCR αβ and p300 interacting with HEB are recruited to HEB cognate sites (E box sites) on the CD4 enhancer. Mi-2β promotes chromatin fluidity in an ATP-dependent fashion and stabilizes HEB binding. The presence of p300 in complex with either HEB or Mi-2β or both induces local histone hyperacetylation (flags) that locks chromatin in an open configuration. Interactions between the activated enhancer and the CD4 promoter (broad arrow) stabilize recruitment of basal transcription factors resulting in CD4 gene expression.

Fig. 8. The Role of Mi-2β in T Cell Development and a Model on CD4 Gene Regulation

(A) A summary of the effects of Mi-2β depletion on T cell development. X indicates a block in DN differentiation, defect in CD4 expression, and a defect in antigen receptor-mediated proliferative expansion of SP T cells. “+/−” shows severe reduction in CD4 expression on DP and a downward arrow lower levels of CD4 expression on CD4 SP T cells.

(B) Mi-2β and p300 interacting with HEB are recruited to HEB cognate sites (E box sites) on the CD4 enhancer. Mi-2β promotes chromatin fluidity in an ATP-dependent fashion and stabilizes HEB binding. The presence of p300 in complex with either HEB or Mi-2β or both induces local histone hyperacetylation (flags) that locks chromatin in an open configuration. Interactions between the activated enhancer and the CD4 promoter (broad arrow) stabilize recruitment of basal transcription factors resulting in CD4 gene expression.

depleted thymocytes using a battery of T cell developmental markers indicated that the majority of CD8+ cells were DP thymocytes that failed to express appropriate levels of CD4. Mi-2β-depleted CD8+ thymocytes expressed intermediate levels of TCR, CD5, and HSA; high levels of Fas; and low levels of Bcl-2, a gene expression profile normally associated with DP. A decrease in CD4 surface expression was detected in Mi-2β-deficient DP and to a smaller extent in CD4+ SP T cells in the thymus and in the periphery, which correlated with a reduction in CD4 mRNA. Thus, the reduction in Mi-2β levels affects the ability of DP thymocytes to activate or maintain expression of CD4, and they range phenotypically from CD8+CD4bright to CD8+CD4dim to CD8-CD4+. Taken together, these studies support the hypothesis that during T cell differentiation, CD4 expression relies on Mi-2β activity. Alternative mechanisms operating in DP and CD4+ SP T cells, i.e., an increase in expression of the family member Mi-2α, may provide compensation for the lack of Mi-2β and rescue CD4 expression, albeit to lower levels.

Functional Dependence of the CD4 Enhancer on Mi-2β

A proximal enhancer in the CD4 locus acts upon its promoter to confer a high level of gene expression at all stages of T cell development (Sawada and Littman, 1991). The dependency of these positive CD4 regulatory elements on Mi-2β was studied. An Mi-2β dependence of the CD4 proximal enhancer was shown in the context of a transgenic reporter. Reporter expression was most dramatically reduced in the DP-like populations, paralleling the observations made with the endogenous CD4 locus. The effect was specific for the CD4-driven GFP, as a GFP reporter under the control of distinct regulatory elements exhibited no change in expression upon Mi-2β depletion. These studies support the hypothesis that the CD4 enhancer depends on Mi-2β for its activity, whether in the context of the endogenous CD4 locus or as a transgene.

Mi-2β Controls the Transition from DN to DP Subsequent to β Selection

Mi-2β is also required for the transition of DN thymocytes to the DP stage of differentiation. Although DP thymocytes and their SP progenies are consistently reduced relative to wt, the number of DN thymocytes is if anything elevated. There appears to be an increase in both the relative but also absolute representation of DN4 cells both in the adult and in the late fetal stage. In the late fetal thymus, a severe block between DN and DP is detected, with very few cells progressing to DP and the majority of cells poised at DN4. As these mutant DN4 cells express IC-TCRβ and pTα, the effect of Mi-2β deficiency manifests downstream of β selection. The lack of Mi-2β therefore has a dramatic effect in the development of fetal thymocytes, which postnaturally appears to be partially alleviated. Upregulation of the second family member Mi-2α at the DN4 stage may in part compensate for Mi-2β deficiency and rescue some but not all of the DN4 precursors to the DP stage.

Given prevailing models of T cell differentiation, which support that β selected thymocytes transit to DP without any further restriction, these studies on Mi-2β-deficient thymocytes provide us with insight into regulatory events that take place at the DN4 stage and are required for further differentiation. Mutations in the TCF/LEF nuclear effectors of the Wnt signaling pathway, in the E box gene HEB, and in the chromatin remodeler Brg-1 also affect the late DN to DP transition (Barndt et al., 1999; Chi et al., 2003; Schilham et al., 1998). Biochemical studies described here provide evidence that Mi-2β and HEB work together to regulate CD4 gene expression during T cell differentiation. It will therefore be important to examine further which of these factors belong to the same regulatory pathway operating in late DN and to identify their gene targets that control this developmental process.

Mi-2β Is Required for Normal T Cell Proliferation

Mi-2β-depleted peripheral T cells are unable to undergo normal proliferation, implicating Mi-2β as a positive regulator in this process. Although antigen receptor engagement in these mutant T cells upregulated activation (G1) markers like CD25 and CD69, the number of cells...
in S phase, measured by \[^{3}H\]-thymidine incorporation, was reduced. CFSE labeling also revealed a progressive reduction in mutant T cells in successive cell divisions. Mi-2\(\beta\) may play a role in chromosome propagation, for example as part of the cohesin complex (Hakimi et al., 2002), and its absence may result in chromosome aberrations. This may cause cycling cells to abort their division, causing their progressive decrease at later cell cycles. The increase in apoptotic cells seen in the mutant activation cultures may represent such aborted cell cycle events consistent with this working hypothesis.

**Recruitment of HEB, p300 HAT to the CD4 Enhancer and Enhancer Hyperacetylation Is Dependent on Mi-2\(\beta\)**

The E box transcription factor HEB has been implicated as an activator of CD4 expression working through the CD4 enhancer (Sawada and Littman, 1991). Interestingly, HEB deficiency, like Mi-2\(\beta\) inactivation, partially impairs the DN to DP transition and causes the accumulation of CD8\(^{+}\)expressing cells with DP characteristics (Barndt et al., 1999). Our ChIP experiments show a strong and specific association between HEB and the CD4 enhancer that is dependent on Mi-2\(\beta\).

Further chromatin studies have revealed the existence of islands of histone hyperacetylation on the enhancer and to a lesser extent on the promoter but not throughout the CD4 locus. This suggests that hyperacetylation is a specific regulatory event and not a mere reflection of active transcription. Mi-2\(\beta\) deficiency caused a reduction in histone hyperacetylation of the CD4 enhancer and promoter. Other regions of the CD4 locus that were modestly acetylated remained unaffected upon Mi-2\(\beta\) depletion. Thus, the Mi-2\(\beta\) effect on hyperacetylation of the CD4 enhancer and promoter is specific for these regions and not a global effect on chromatin.

In search of the HAT that hyperacetylates the CD4 regulatory regions, we have established a specific association between p300 and the CD4 enhancer. Mi-2\(\beta\) deficiency caused a reduction in the association between p300 and the CD4 enhancer, consistent with the reduction in histone hyperacetylation of this region. p300 is therefore likely responsible for the hyperacetylated state of chromatin at the enhancer, although it is also possible that other HATs are also recruited to this region.

A direct and specific association between Mi-2\(\beta\) and the proximal CD4 enhancer in CD4-expressing thymocytes was established by ChIP. These studies provide support for a direct role of Mi-2\(\beta\) in modulating the activity of the proximal CD4 enhancer, either as part of the NuRD complex or independently. Mi-2\(\beta\) binding to the CD4 enhancer may attenuate its activity in CD4-expressing thymocytes through the HDAC activity of the NuRD complex. In the absence of Mi-2\(\beta\), an increase in Mi-2\(\alpha\) expression (normally present at very low levels) that may function as a more potent attenuator in the context of the NuRD complex, may shut down CD4 expression. However, the direct association between Mi-2\(\beta\) and the activators of gene expression, HAT p300 and HEB in the absence of HDAC, combined with the fact that Mi-2\(\beta\) provides efficient recruitment of these factors to the CD4 enhancer, precludes this possibility. Rather, these data support a model by which direct binding of Mi-2\(\beta\):HAT:HEB complexes to the enhancer promotes positive rather than negative events in gene expression.

Recently, a 3' enhancer/LCR was described in the CD4 locus. These regulatory regions together with the proximal enhancer were shown to support CD4 expression in DP thymocytes (Adlam et al., 1997). No significant association between Mi-2\(\beta\) and this regulatory region was detected. The islands of histone H3 hyperacetylation, caused by Mi-2\(\beta\) recruitment to the CD4 regulatory regions, were also not observed over the 3' enhancer/LCR. Taken together, these results support that the Mi-2\(\beta\) effects on CD4 expression are mediated through its interactions with the proximal enhancer and not through the 3' enhancer/LCR.

**A Model of CD4 Regulation by Mi-2\(\beta\)**

Based on the above findings, we propose the following working model on how Mi-2\(\beta\) activates CD4 gene expression (Figure 8B). First, Mi-2\(\beta\) is recruited to the CD4 enhancer, presumably through its HEB interactions. Mi-2\(\beta\) then provides ATP-dependent chromatin fluidity and stabilizes HEB binding. The HAT p300 either through its association with Mi-2\(\beta\) or HEB is also recruited at the CD4 enhancer and locks the chromatin into an open configuration by increasing the local histone acetylation. Activation of the CD4 enhancer precipitates binding of basal transcription factors and their associated HATs to the promoter, leading ultimately to CD4 expression. Inactivation of Mi-2\(\beta\) decreases the frequency with which these regulatory events occur, causing variable and reduced expression of the CD4 locus.

As a chromatin remodeler, Mi-2\(\beta\) functions as a catalyst of chromatin fluidity. Because of its association with HDACs, Mi-2\(\beta\) has been presumed to be involved in establishing a repressive chromatin structure. However, Mi-2\(\beta\) may also function outside the NuRD complex. Recent studies have shown that Mi-2 may be involved in a variety of molecular events associated with gene expression. Studies in yeast have shown that Mi-2 homologs exist in at least two separate complexes, one involved in transcriptional termination (Allen et al., 2002) and a second in transcriptional elongation (Krogan et al., 2002). Studies of Drosophila salivary glands have shown that dMi-2 localizes to chromatin puffs, implying a role in transcriptional activation (Hirose et al., 2002). Recently, vertebrate Mi-2\(\beta\) protein subdomains have been shown to have both transcriptional repressing and activating properties through their association with distinct chromatin-modifying enzymes, including Brg-1 (Shimon et al., 2003). The existence of a multiprotein supercomplex that includes ALL-1, Mi-2, and Brahma as well as HAT, HDAC, and methyl transferase activities that is bound to the promoter of an active ALL-1-dependent Hox A9 gene was recently reported (Nakamura et al., 2002). Finally, coimmunoprecipitation studies performed under this investigation provide strong evidence that Mi-2\(\beta\) functions in a complex with p300 HAT and HEB in the absence of HDAC.

Our findings reveal interactions in developing T cells between Mi-2\(\beta\) and HAT-bearing transcription factors that are critical for CD4 expression. The CD4 gene is identified as a specific target of Mi-2\(\beta\) during T cell development, and a function for Mi-2\(\beta\) in the positive
regulation of gene expression in cooperation with HAT is established. We anticipate that future studies will uncover additional scenarios in which Mi-2β plays a role in the activation of specific genes during development.

Experimental Procedures

Recombination Constructs and Targeting of ES Cells

The recombination vector described in Figure 1A was constructed with Mi-2β genomic fragments, a FRT site-flanked neomycin resistance cassette, and a LoxP recognition site cassette and was targeted into J1 embryonic stem cells as previously described (Georgopoulos et al., 1994; Li et al., 1992). DNA was prepared, digested with EcoRI, and analyzed by Southern blotting using a 2 kb EcoRI/BglII digestion fragment DNA probe situated outside the homologous recombination site (Figure 1A, probe). Mutant ES cells were injected into blastocysts from C57Bl/6 mice. The genotypes of the offspring were determined by EcoRI digestion/Southern blot and by polymerase chain reaction (PCR) of tail DNA using the primers and conditions shown in the Supplemental Table S1, available on Immunity’s website.

Mice

All mice were bred and housed in specific pathogen-free conditions. Animals carrying the Lckpr-Cre transgene (Lee et al., 2001) were identified by PCR genotyping of tail DNA using the primers and conditions shown in Supplemental Table S1 (available on Immunity’s website). Transgenic mice expressing Flp recombinase were used as previously described (Dymecki, 1995). The Mi-2β-deficient strain was maintained as intercrosses of Mi-2β+/+; Lckpr-Cre tg1, or Mi-2β+/−; Lckpr-Cre tgX Mi-2β+/+tgFlomut, and the offspring were genotyped by PCR and/or Southern blotting of tail DNA.

Antibodies

All the antibodies used in this study are listed in Supplemental Table 2 available on Immunity’s website.

Flow Cytometry

Phenotypic analysis of thymocytes for expression of lineage and developmental markers was performed as described previously (Molnar and Georgopoulos, 1994; Winandy et al., 1995) using a FACSScalibur flow cytometer (Becton Dickinson) and FlowJo 4.1 software (Tree Star, Inc.). Purification of DN thymocytes was performed by staining unfractionated thymocytes with a cocktail of FITC-labeled lineage-specific antibodies (CD4, CD8, B220, Mac-1/CD11b, GR-1/Ly6, NK1.1, TCRβ/γ, Ter119/Ly-76, biotin/Avdin-PE-TR Thy1/CD90, APC-CD44, and Avidin-PE-Cys CD25, DN3 cells (FITC, Thy-1−, CD25−, CD4−) and DN4 cells (FITC, Thy-1+, CD25+, CD4+) were purified using a Mo-Flo cell sorter (DakoCytomation). LN T cells were purified using antibody staining and sheep anti-rat Ig Dyna beads (DynaBiotech Inc.) according to the manufacturer’s protocol. Apoptotic cells were identified using an AnnexinV-FITC apoptosis detection kit (BD-Pharmingen).

T Cell Proliferative Responses

Flat-bottomed 96-well tissue culture plates were coated with anti-CD3ε (10 μg/mL). Unfractionated LN or purified T cells were plated in triplicate at 1 x 10⁶ cells/well in RPMI medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 10 mM HEPES, 50 μM β-mercaptoethanol, and 100 μg/mL penicillin/streptomycin. For calcium ionophore activations, media were supplemented with 0.01 μg/mL PMA and 0.7 μM monomycin. After 48 hr of culture, cells were pulsed for 12 hr with 3H-methyl thymidine (2 μCi/well), harvested (Harvester 96, TomTec), and counted (Betaplate Counter, Wallac).

CFSE Labeling

For 10 min at 37°C, 1 x 10⁶ sorted LN T cells of each indicated genotype were incubated in 5 μM CFSE/1X HBSS solution. The labeling reaction was stopped with the addition of 0.5 ml fetal bovine serum. Cells were washed through 1 x HBSS, centrifuged, and the pellet resuspended in 1 ml RPMI medium, supplemented as described above.

RT-PCR

T cells were purified through cell sorting techniques as described above. RNA was extracted from specified populations using Trizol (Gibco BRL). Reverse transcription was carried out using random primers (Georgopoulos et al., 1994). The cDNAs were amplified by PCR using the primers and conditions shown in Supplemental Table S1 on Immunity’s website. PCR reactions were done in the presence of 1 μCi α-32P-dCTP, and PCR products were run on 8% acrylamide gels and visualized by X-ray films.

Immunoprecipitations and Western Analysis

Whole-cell lysates and nuclear extracts were prepared from wt and KO thymocytes and their subsets or thymocytes expressing an Mi-2βtransgene as previously described (Kim et al., 1999). For immunoprecipitation experiments, nuclear extracts were precleared with protein A agarose beads in the presence or absence of 10 μg/mL ethidium bromide to disrupt DNA-protein interactions. Precleared extracts were immunoprecipitated on protein-A agarose beads with either rabbit IgG, conjugated α-p300, conjugated α-flag, or α-HEB. A mock purification of nuclear lysates from wt thymus on anti-flag agarose beads was also performed as a control. Immunoprecipitates on α-p300-agarose or α-flag-agarose beads were eluted with 100 μg/ml of the N-15 peptide (sc-584 P, Santa Cruz Biotechnology) or 300 μg/ml of the flag peptide for 1 hr. Eluted proteins were separated in SDS-6% polyacrylamide gel electrophoresis and Western blotted.

Chromatin Immunoprecipitation Assays

ChIP and the subsequent PCR analyses were performed as described (Harker et al., 2002). The antibodies used are listed in Supplemental Table S2 on Immunity’s website. All the PCR amplicons for regulatory elements were designed to cover human-mouse conserved sequences. The primer sequences used in the multiplex PCR are available upon request.

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