Chromatin remodeling complex NURF regulates thymocyte maturation

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The maturation of T cells requires signaling from both cytokine and T-cell receptors to gene targets in chromatin, but how chromatin architecture influences this process is largely unknown. Here we show that thymocyte maturation post-positive selection is dependent on the nucleosome remodeling factor (NURF). Depletion of Bptf (bromodomain PHD finger transcription factor), the largest NURF subunit, in conditional mouse mutants results in developmental arrest beyond the CD4⁺ CD8^{int} stage without affecting cellular proliferation, cellular apoptosis, or coreceptor gene expression. In the *Bptf* mutant, specific subsets of genes important for thymocyte development show aberrant expression. We also observed defects in DNase I-hypersensitive chromatin structures at *Egr1*, a prototypical Bptf-dependent gene that is required for efficient thymocyte development. Moreover, chromatin binding of the sequence-specific factor Srf (serum response factor) to *Egr1* regulatory sites is dependent on Bptf function. Physical interactions between NURF and Srf suggest a model in which Srf recruits NURF to facilitate transcription factor binding at Bptf-dependent genes. These findings provide evidence for causal connections between NURF, transcription factor occupancy, and gene regulation during thymocyte development.

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Epigenetic regulators of transcription include histone-modifying enzymes, ATP-dependent chromatin remodeling complexes, DNA methylation, and the incorporation of histone variants (Goldberg et al. 2007). ATP-dependent chromatin remodeling complexes are classified into four major subfamilies (SWI/SNF, ISWI, CHD, and INO80) and are distinguished by the presence of a common SWI2/SNF2-related catalytic ATPase subunit (Clapier and Cairns 2009). These enzymes catalyze specific activities, including nucleosome sliding on a DNA template in *cis*, histone eviction, and the incorporation of variant histones, each with dramatically different effects on chromatin structure.

The founding member of ISWI remodeling complexes is the nucleosome remodeling factor (NURF) (Tsukiyama and Wu 1995). Mammalian NURF is composed of BPTF (bromodomain PHD finger transcription factor); SNF2L,

the ATPase subunit, and pRBAP46/48 (Barak et al. 2003). While the biochemical functions of remodeling complexes like NURF have been studied extensively (Choudhary and Varga-Weisz 2007), their biological functions in mammals are just beginning to be elucidated (Ho and Crabtree 2010). The NURF remodeling complex is essential for specific stages of metazoan development, functioning in prominent signaling pathways to the nucleus, including heat shock, TGFβ/Smad, JAK/STAT, WNT/β-catenin, RB, and nuclear hormone receptors (Xiao et al. 2001; Badenhorst et al. 2005; Andersen et al. 2006; Li et al. 2006; Goller et al. 2008; Kwon et al. 2008; Landry et al. 2008; Song et al. 2009). To gain a better understanding of the biological functions of the mammalian NURF complex, we depleted the Bptf subunit during mouse T-cell development—a well-studied model of cellular differentiation (Starr et al. 2003).

Peripheral T cells bearing functional antigen-specific receptors are essential components of the mammalian immune system. The selection for T cells expressing functional T-cell antigen receptors (TCRs) occurs in the thymus during positive selection (Starr et al. 2003). Positively selecting CD4⁺ CD8⁺ double-positive (DP) thymocytes receive survival signals from the TCR pathway and commit to becoming a CD4 or CD8 single-positive (SP) thymocyte

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by integrating signals from both TCR and cytokine receptors (Brugnera et al. 2000). After lineage commitment, SP thymocytes complete the maturation process and exit the thymus to populate the periphery as a functional, naïve T cell.

How signals from both TCRs and cytokine receptors regulate thymocyte maturation has been a topic of intensive study. More than 100 genes have been identified, including a number that have important functions in the maturation of thymocytes and are regulated by the TCR pathway (Huang et al. 2004; Mick et al. 2004). These genes include cell surface receptors *CD5*, *CD69*, *Ccr7*, *Il7Ra*, *CD2*, *CD6*, and *PD-1*, and transcription factors *Egr1*, *Egr2*, and *Id2*. In addition, lineage-specific transcription factors like *Tox*, *Gata3*, *Th-Pok*, and *Runx3* are essential for the specific development of either CD4 or CD8 lineages (Rothenberg and Taghon 2005). In addition to genetic regulatory elements, epigenetic factors are emerging as major regulators of these gene targets (Merkenschlager and Wilson 2008).

Chromatin remodeling complexes have essential roles in many aspects of mammalian development, including T-cell development. Mutations in the Brg1 ATPase or accessory BAF subunits of the SWI/SNF family of complexes result in proliferation defects at multiple stages and defects in CD4 and CD8 coreceptor expression (Chi et al. 2002, 2003; Gebuhr et al. 2003). Mutations in the Chd4 ATPase subunit of the NuRD remodeling complex results in defective β selection, CD4 coreceptor expression, and reduced cellular proliferation (Williams et al. 2004). These studies clearly demonstrate that complexes that regulate chromatin structure are essential for the development of thymocytes. However, little is known of the effects of the ISWI class of chromatin remodeling enzymes, whose biochemical actions, unlike that of Brg1 and related enzymes, are exclusively involved in repositioning nucleosomes without histone loss or exchange (Choudhary and Varga-Weisz 2007).

Here, we report that the differentiation of positively selected thymocytes into phenotypically and functionally mature T cells is strictly dependent on NURF. We show that NURF regulates the chromatin structure and

expression of genes important for T cell development. Functions for NURF as a specific regulator of thymocyte maturation are distinct from those of the SWI/SNF and NuRD remodeling complexes, suggesting that ATP-dependent remodeling complexes have specific and non-overlapping roles during regulated development. Analysis of transcription factor binding at a specific gene also provides evidence for a causal relationship between NURF and transcription factor occupancy in vivo.

Results

Bptf is required for thymocyte development

To investigate NURF function during thymocyte development, we depleted *Bptf* using *LCKproximal-Cre* to excise the *Bptf^{Floxed}* allele (Fig. 1A; Supplemental Fig. 1A,B; Hennet et al. 1995; Landry et al. 2008). In this system, *Bptf^{Floxed}* deletion begins during the CD4⁺ CD8⁺ double-negative (DN) stages and is complete by the CD4⁺ CD8⁺ DP stage of thymocyte development (Fig. 1B). *LCKproximal-Cre* excision of *Bptf^{Floxed}* results in exon 1–3 and exon 1–4 splice events, producing an out-of-frame transcript (data not shown) as reported previously for Cre excision of the *Bptf^{Floxed}* allele (Landry et al. 2008). *Bptf* deletion was confirmed by the absence of Bptf protein in total thymocyte extracts (Fig. 1C). Analysis of thymocytes by flow cytometry showed reductions in TCR β^{high} (Fig. 1D), CD4⁺ CD25⁺, and NKT cell populations (Supplemental Fig. 1C) but no significant differences in TCR $\gamma\delta$ or B220⁺ populations (data not shown). We further observed dramatic reductions in both CD4⁺ and CD8⁺ SP thymocytes and peripheral T cells and slight defects at the DN-to-DP transition during development, without a significant reduction in thymus cellularity in both adults and embryos (Fig. 1E; Supplemental Fig. 1D,E). Equivalent BrdU incorporation into thymocyte populations in vivo suggests that cellular proliferation is normal in the absence of Bptf (Supplemental Fig. 2A,B). Analysis of residual peripheral T cells in Bptf knockout (KO) mice showed the genomic *Bptf^{Floxed}* allele to be intact, suggesting that these few mature cells have escaped gene deletion (Supplemental

Figure 1. *Bptf* is required for thymocyte development. (A) Schematic of the NURF complex and the *Bptf^{Floxed}* and *Bptf^{ΔExon2}* alleles. Positions of PCR primers are shown. (B) PCR analysis on genomic DNA of electronically sorted thymocytes showing *LCKproximal-Cre* recombination efficiency. (C) Analysis of Bptf levels by Western blotting total thymocytes (two mice per group). (Control) *Bptf^{+/Floxed}*; (KO) *LCKproximal-Cre*, *Bptf^{-/-Floxed}* throughout manuscript. (D) Flow cytometry analysis of total live thymocytes for TCR β expression in Bptf KO and control animals. Numbers represent percent in gated population. (Gray) Isotype control; (black) control; (red) KO. (E) Flow cytometry analysis of CD4 and CD8 expression on thymocytes and splenocytes to identify DN, DP, and SP populations. Values above the dot plot represent total cellularity of thymus. (F) H&E-stained paraffin sections of KO and control thymus. (M) Medulla; (C) cortex. Bar, 500 μm .

Fig. 2C). A histological examination showed the adult thymus lacks a medulla, a region populated by post-selection $CD4^+$ and $CD8^+$ SP thymocytes (Fig. 1F). We observed normal rates of apoptosis by staining for annexin V⁺ or TUNEL⁺ cells in vivo, or by culturing DP thymocytes in medium or glucocorticoids or following anti-CD3 ϵ /CD28 cross-linking (Supplemental Fig. 2D–H). From these results, we conclude that Bptf is essential for the development of mature thymocytes and peripheral T cells.

Bptf is required for thymocyte maturation post-positive selection

To further characterize the block in thymocyte differentiation, we fractionated thymocytes by expression of cell surface markers TCR β , HSA, CD69, and CD5 (Starr et al. 2003). We observed equivalent populations of selecting thymocytes with a TCR^{int} CD5^{int}, CD69^{int} phenotype, demonstrating that positive selection is quantitatively efficient. In contrast, Bptf deficiency results in an almost complete loss of mature thymocytes that appear phenotypically as TCR β ^{high} HSA^{low}, CD69^{negative}, and CD5^{high} (Fig. 2A,B, red arrows). The few $CD4^+$ SP thymocytes that remain are immature cells that are phenotypically CD69^{high}, HSA^{high}, Ly6-D^{high}, Qa-2^{negative}, H-2K^b low, and Ccr7^{low} (Fig. 2C; Starr et al. 2003). We expressed TCR transgenes (OT1, OT2, and HY) or the anti-apoptotic human *BCL2* transgene in vivo in an attempt to rescue the maturation defect. These transgenes were unable to rescue the thymocyte maturation defects, suggesting that Bptf functions independently of apoptotic pathways and downstream from the TCR complex (Fig. 3A,B [red arrows], D; Supplemental Fig. 3A–C). Rescue of cells with the human *BCL2* transgene resulted only in $CD4^+$ SP CD69^{high}, but no mature $CD4^+$ SP CD69^{negative} thymocytes (Fig. 3E). In addition, we observed normal activation of T-cell and cytokine receptor signaling with Bptf deletion, further confirming that Bptf functions downstream from these pathways (Supplemental Fig. 3D–F).

These results demonstrate that Bptf is a new regulator of thymocyte maturation after positive selection. Because Bptf has been documented previously to be a coregulator of transcription (Barak et al. 2003), we propose that the defects are a consequence of deregulated transcription of genes important for thymocyte maturation.

Bptf regulates gene targets of receptor-dependent thymocyte development

To test this hypothesis, we monitored genome-wide transcription in equivalent cell populations of KO and control thymocytes by microarray analysis. In one approach, we stimulated electronically sorted, unsorted CD69^{low} DP thymocytes in culture with PMA + ionomycin (P+I) to mimic TCR signaling. In a second approach, we purified thymocytes from mice expressing TCR transgenes by magnetic separation to obtain immature (OT2: ~95% DP) and positively selected (OT1: ~45% $CD4^+$ $CD8^{\text{int}}$, ~45% DP) populations (Supplemental Fig. 4A,B). Bptf dependency was found for only 533 genes deregulated ≥ 1.5 -fold between KO and control (adjusted *P*-value ≤ 0.05), with

Figure 2. Bptf is required for thymocyte maturation. (A) Flow cytometry analysis of TCR β and CD5, CD69, or HSA on total live thymocytes. (B) The bar graph represents total live thymocytes as a percent of control in each gate. (*) *P*-value < 0.01 ; *N* = 4. (C) Analysis of cell surface markers on $CD4^+$ SP thymocytes by flow cytometry. (Gray) Isotype control; (black) control; (red) KO. Representative *N* = 5.

152 genes showing lower expression and 154 genes showing higher expression than control (Supplemental Data Set S1). The remaining 224 genes have a more complex Bptf-dependent expression pattern, depending on cell type or culture condition (Fig. 4A). Of the 533 genes, we found 426 genes in the less mature DP and OT2 populations, demonstrating that a major fraction of affected genes requires Bptf prior to receptor-dependent thymocyte maturation. This implies that the effects of Bptf depletion on expression of these genes occurs well before the first observable block in thymocyte development. We also observed 107 of 533 genes exclusive to the OT1 or P+I-stimulated thymocytes, indicating that Bptf also regulates genes during receptor-dependent maturation. Gene ontology analysis of the data sets identified

Figure 3. Bptf is required for positive selection. (A) Total live *Tg-HY-TCR* (TCR Vβ8⁺ population) thymocytes were analyzed in male and female mice by flow cytometry to identify DP and SP populations. Values above the dot plot represent total cellularity ($N = 5$). (B) Flow cytometry analysis of live *Tg-OT1-TCR* and *Tg-OT2-TCR* thymocytes (TCR Vα2⁺) to identify thymocyte populations. Values above the dot plot represent total cellularity of thymus ($N = 5$). (C) Flow cytometry analysis of live *Tg-hBCL2* thymocytes to identify DP and CD8⁺ and CD4⁺ SP populations. Values above the dot plot represent total cellularity of thymus ($N = 5$). (D) Ratio of KO to control CD4⁺ and CD8⁺ SP thymocytes on nontransgenic and transgenic backgrounds ($N = 5$). (E) Expression of CD69 on live CD4⁺ SP thymocytes from *Tg-OT2-TCR* (TCR Vα2⁺) and *Tg-hBCL2* thymocytes. (Gray) Isotype control; (black) control; (red) KO. Representative $N = 3$.

clusters annotated as “immune system” and related processes (P -value < 0.01) (Supplemental Fig. 5A).

Several laboratories have identified >100 genes during genome-wide investigations of transcriptional regulation during positive selection (Huang et al. 2004; Mick et al. 2004). Ten of these genes are found in our data sets (*Ck2l2*, *Tpm4*, *Sqrdl*, *Mela*, *Id2*, *Il2*, *Socs3*, *Ly6d*, *Ets2*, and *CD53*), and four of them show Bptf dependence during positive selection while six are Bptf-dependent even before positive selection (Fig. 4A,B; Supplemental Fig. 5B). To complement our microarray expression analysis, we also monitored the expression of eight well-characterized TCR-dependent genes (*Egr1*, *Egr2*, *Il2*, *Nur77*, *Ikaros*, *Rel B*, *JunB*, and

Bcl-X_L) in DP thymocytes before and after stimulation with P+I. We found two genes (*Egr1* and *RelB*) that showed clearly increased expression in unstimulated Bptf KO cells compared with controls (Fig. 4C). Upon stimulation, seven out of eight genes, except for *JunB*, showed decreased expression for mutant cells, indicating that Bptf has a positive role in regulating these genes, which include *Egr1* (Fig. 4D). We also observed consistent defects in the expression of TCR-regulated cell surface proteins Ccr7 and Il7Rα in vivo during the transition from DP to CD4⁺ CD8^{int} thymocytes (Fig. 4E; Park et al. 2010). In contrast, we observed normal regulation of CD4 and CD8 coreceptors using an in vitro coreceptor reversal assay (Supplemental Fig. 6A; Brugnera et al. 2000). From these results, we conclude that Bptf regulates a restricted set of gene targets prior to and during receptor-dependent thymocyte maturation, including a number of genes regulated by the TCR pathway. Interestingly, the regulated transcription of Bptf-dependent genes such as *Egr1*, *Egr2*, *Ikaros*, *Hes-1*, *Bcl-X_L*, and *Il7Rα* occurs at the earlier β-selection stage (Taghon et al. 2006). So, therefore, Bptf could also regulate the transcription of these genes many days before the observed block in thymocyte development (see below).

The phenotype of Bptf mutants exhibits an intriguing similarity to that of mutants for Themis, which has been speculated to be a component of the TCR signaling apparatus (Allen 2009). The similarity prompted us to investigate for overlap in gene expression data sets for *Bptf* and *Themis* mutants (Lesourne et al. 2009). We found normal *Themis* expression during Bptf KO thymocyte development, demonstrating that Bptf does not regulate *Themis* expression (Fig. 4B). Furthermore, a comparison of data sets reveals limited overlap in gene expression profiles (10 genes with a P -value of 5.9×10^{-6}), making connections between Themis and Bptf unclear at present (Supplemental Fig. 5C).

Effects of Bptf on chromatin structure prior to positive selection

The NURF complex is an ATP-dependent chromatin remodeling enzyme of the ISWI subfamily that repositions nucleosomes in vitro by histone octamer sliding without eviction (Tsukiyama and Wu 1995; Hamiche et al. 1999; Barak et al. 2003). Although ISWI enzymes have been reported to locally change nucleosome positioning at many hundreds of gene promoters to suppress antisense transcription in vivo (Fazzio and Tsukiyama 2003; Whitehouse et al. 2007), the physiological effects of ISWI-containing NURF on chromatin remains unclear in metazoans. As a first step toward addressing this question, we used quantitative chromatin profiling (Dorschner et al. 2004) to examine DNase I-hypersensitive chromatin structures at *Egr1*, chosen as a representative Bptf-dependent gene that is also functionally important for thymocyte development (Bettini et al. 2002). DNase I-hypersensitive sites are generally considered to be a consequence of several related phenomena: transcription factor binding and changes in DNA conformation, changes in nucleosome positioning or occupancy, and disruption of higher-order

Figure 4. *Bptf* is required for regulated transcription during thymocyte development. (A) Microarray analysis of *Bptf* KO and control thymocytes followed by a hierarchical clustering of genes deregulated in unstimulated thymocytes (DP), stimulated thymocytes (DP P+I), OT2 transgene-expressing thymocytes (OT2), and OT1 transgene-expressing thymocytes (OT1). (B) Microarray analysis of *Bptf* KO and control thymocytes followed by a manual clustering of genes regulated by positive selection. (C) Gene expression analysis by qPCR of unstimulated panned DP thymocytes. (D) Gene expression analysis by qPCR in panned DP thymocytes after stimulation with P+I. Representative $N = 3$. (E) Analysis of cell surface markers *Ccr7* and *Il7Rα* on DP and CD4⁺CD8^{int} thymocytes by flow cytometry. (Gray) Isotype control; (black) control; (red) KO. Representative $N = 5$.

nucleosome packing (Gross and Garrard 1988). As shown in Figure 5A, we identified four DNase I-hypersensitive sites (black arrows, green highlights) over a 15-kb region that showed clearly increased hypersensitivity (ratio, <0.70 ; P -value, <0.01) when *Bptf* KO thymocytes at the DP stage ($\sim 95\%$ DP) were compared with controls (Fig. 5A). Thus, *Bptf* is required for making DNase I sites less hypersensitive at a restricted number of sites across the *Egr1* gene. To assess the earliest developmental period when *Bptf*-dependent chromatin remodeling occurs, we performed quantitative chromatin profiling on *Bptf* KO thymocytes from a *Rag*^{-/-} background ($\sim 95\%$ DN3) (Supplemental Fig. 6B,C; Mombaerts et al. 1992). Many DNase I-hypersensitive sites were observed in control cells, but the same sites were also observed in *Bptf* KO *Rag*^{-/-} DN thymocytes at the current level of resolution (Fig. 5A). These results indicate that *Bptf* is apparently not required for the establishment or maintenance of the DNase I-hypersensitive sites at *Egr1* at or before the DN3 stage, but it is necessary for the closure of DNase I-hypersensitive sites as thymocytes develop from the DN3 to the DP stage.

As a second example of *Bptf*-dependent changes in DNase I hypersensitivity, we examined *Il2*, which is not important for thymocyte maturation (Reya et al. 1998) but whose chromatin structure is known to undergo specific changes during the commitment to the thymocyte lineage (Siebenlist et al. 1986; Yui et al. 2001). In DP thymocytes, we observed three DNase I-hypersensitive sites in *Bptf* KO

DP thymocytes (Fig. 5B, black arrows, green highlights) that were absent in controls, indicating that *Bptf* is required to render these sites less hypersensitive at or before the DP stage (Fig. 5B). Two of these three sites (-4.5 kb and $+6.6$ kb) were found to be hypersensitive in *Rag*^{-/-} DN thymocytes for both control and *Bptf* KO, indicating that remodelers other than *Bptf* are responsible for the DNase I hypersensitivity (Fig. 5B, white arrows). However, one site ($+2.1$ kb) was observed to be hypersensitive in *Bptf* KO but not control thymocytes (Fig. 5B, black arrow, green highlight) at this stage of development (Fig. 5B), suggesting that *Bptf* can be required even before β selection. Interestingly, these *Bptf*-dependent DNase I-hypersensitive sites were identified previously as being specific to the thymocyte lineage, including sites at -4.5 kb, -0.6 kb, and $+2.5$ kb (Siebenlist et al. 1986; Yui et al. 2001). Moreover, we observed *Bptf*-dependent changes of DNase I-hypersensitive sites at distal promoter-enhancer elements (~ 300 base pairs [bp] to -700 bp) of *Il2* in both DP and DN thymocytes (Supplemental Fig. 6D, black arrows); chromatin remodeling at these elements has been implicated in the transcriptional response to TCR signaling (Ward et al. 1998; Attema et al. 2002). We also examined the *CD4* silencer, a regulatory element that requires Brg1 to regulate its chromatin structure and function during thymocyte development, and found no *Bptf*-dependent effects on its chromatin structure (Supplemental Fig. 6E; Wan et al. 2009). Taken together, the results indicate that *Bptf* is a selective regulator of chromatin structure as early as the DN stages,

Figure 5. Bptf maintains chromatin structure during thymocyte development. DNase I hypersensitivity mapping by quantitative chromatin profiling at the *Egr1* (A) and *Il2* (B) loci in Bptf KO and control panned DP and *Rag*^{−/−} DN thymocytes. Bptf-dependent (black arrows, green highlights) and Bptf-independent (open arrows) DNase I hypersensitivity sites were identified as decreased amplification of a PCR product from a DNase I-treated sample relative to an undigested control. Ratio, <0.7; *P*-value, <0.01; *N* = 3.

prior to receptor-dependent thymocyte maturation for the two TCR-regulated genes examined.

Bptf is required for DNA binding of transcription factors important for expression of *Egr1*

Regulated transcription of *Egr1* occurs as early as the β -selection stage of thymocyte development and continues during positive selection and thymocyte maturation through signaling pathways activated by the pre-TCR and TCR, respectively (Miyazaki 1997; Shao et al. 1997). Accordingly, we examined *Egr1* and *Egr2* transcription in DN (*Rag1*^{−/−}) cells and, like DP cells, found significant Bptf dependence for *Egr1* and *Egr2*, but not *JunB*, transcription upon stimulation with P+I (Fig. 6A). P+I activates TCR signaling pathways in *Rag*^{−/−} DN thymocytes, mimicking the activation phase of β selection. Given the early requirement of Bptf in establishing chromatin structure and regulated transcription in *Rag*^{−/−} DN cells, we examined the effects of NURF on transcription factor binding at these early stages to avoid complications due to indirect effects on later developmental stages (DP). We first determined whether the NURF complex is localized to *Egr1* regulatory regions in DN thymocytes. Although chromatin immunoprecipitation (ChIP)-quality antibodies are not available for Bptf,

we were able to observe the Snf2 subunit of the NURF complex at the upstream region and, albeit, at lower occupancy at the *Egr1* promoter (*P*-value < 0.05) (Fig. 6B; Barak et al. 2003). Activation of *Egr1* transcription has been shown to be regulated primarily by the ternary complex (SRF-TC) containing the serum response factor (Srf) and partners Elk-1 and Elk-4, and, to a minor extent, the transcription factors of the AP-1 and NF κ B families (DeFranco et al. 1993; Aicher et al. 1999; Xi and Kersh 2003). Accordingly, we examined whether Bptf could interact with one or more of these factors. Toward this end, we performed coimmunoprecipitation experiments of endogenous proteins from native thymocyte extracts and found robust pull-down of Bptf with Srf and AP-1 (~10% of input) and no detectable pull-down with NF κ B and NFAT (Fig. 6C).

Previously, the NURF complex has been shown to remodel nucleosomes in an ATP-dependent manner to regulate the binding of transcription factors in vitro (Tsukiyama and Wu 1995; Kang et al. 2002). ChIP experiments have shown that Srf is constitutively bound to serum response elements (SREs) at the *Egr1* promoter that are essential for the activation of *Egr1* by TCR signaling (Xi and Kersh 2003; Tur et al. 2010). To determine whether Srf binding to *Egr1* is dependent on Bptf, we performed ChIP on DN thymocytes and observed significant Bptf dependence for the occupancy of Srf at the promoter and the −5.9 kb upstream region (*P*-value < 0.05) (Fig. 6D). A Western analysis shows equivalent levels of Srf protein expression in Bptf KO and control thymocytes, suggesting that defective chromatin binding is due to transcription factor access to the DNA and not to Srf expression (Supplemental Fig. 7A). Hence, Bptf-dependent binding of Srf at *Egr1* provides a plausible mechanism for the observed defects in *Egr1* transcription upon TCR signaling in Bptf mutants. Furthermore, the interactions between NURF and Srf are likely to be direct because of specific pull-down of a recombinant NURF complex with GST-Srf in vitro (Supplemental Fig. 7B). Taken together, these results suggest that the NURF complex is recruited to the *Egr1* locus through interactions with Srf, and its recruitment is in turn important for Srf binding.

Discussion

Unique phenotypes for Bptf mutants during thymocyte development

In this study, we identified Bptf—and thereby the NURF complex in which it uniquely resides—as a novel regulator of thymocyte maturation after positive selection. Functions for Bptf as a regulator of post-selection thymocyte maturation are different from those documented for other chromatin remodeling complexes. Mutations in the Brg1 ATPase and BAF subunits of the SWI/SNF complexes and the Chd4 subunit of the NURD complex result in reduced cell survival, proliferation defects, and defective CD4 and/or CD8 coreceptor expression (Chi et al. 2002, 2003; Gebuhr et al. 2003; Williams et al. 2004). Distinct from these phenotypes, Bptf does not appear to

Figure 6. Physical and functional interactions between Bptf and Srf in thymocytes. (A) Gene expression analysis of *Egr1*, *Egr2*, and *JunB* by qPCR in *Rag*^{-/-} DN thymocytes after stimulation with P+I. Representative *N* = 3. (B) Snf2 ChIP in KO and control *Rag*^{-/-} DN thymocytes at upstream (-5.9 kb) and promoter regions of *Egr1*. (*) *P*-value < 0.05. (C) Pull-down followed by quantitative Western blotting of Bptf by Srf and AP-1 from stimulated native thymocytes. (D) Srf ChIP in KO and control *Rag*^{-/-} DN thymocytes at upstream (-5.9 kb) and promoter regions of *Egr1*. (*) *P*-value < 0.05.

regulate apoptosis, cellular proliferation, or the expression of the CD4/CD8 coreceptors, but rather Bptf specifically regulates thymocyte maturation after positive selection. We observe a Bptf requirement for the development of all mature thymocyte populations tested, including TCRβ^{high}, NKT, and CD4⁺ CD25⁺ regulatory thymocytes. Functional TCR signaling is required for each of these thymocyte populations, strongly suggesting that Bptf regulates one or more steps of this pathway.

Bptf is required for maturation of CD4/CD8 TCRβ⁺ thymocytes

We began a characterization of Bptf function in CD4/CD8 TCRβ⁺ thymocytes because they represent the predominant thymocyte lineage in the mouse and humans. Our initial results suggested that Bptf regulates one or more steps of thymocyte maturation. The use of three TCR transgenes (OT1, OT2, and HY) fails to complement the maturation defects *in vivo*, indicating that NURF functions downstream from the TCR complex. These results, in combination with results showing normal T-cell and cytokine receptor activation in Bptf mutants, suggest that the chromatin remodeling protein functions downstream from these pathways. A more detailed analysis of thymocyte development shows quantitatively efficient positive selection but severe defects in the development of the most mature TCRβ^{high} HSA^{low}, CD69^{low}, and CD5^{high} thymocytes. These results document that Bptf is essential for thymocyte maturation after positive selection (Supplemental Fig. 8A). Identifying specific regulators of thymocyte maturation after positive selection is of great interest to developmental immunologists. To date, only Themis has been documented to specifically regulate thymocyte development at this period. Evidence suggests that Themis is a component of the plasma membrane-associated TCR signaling apparatus (Allen 2009). The minimal overlap between microarray data sets from Themis and Bptf mutant thymocytes suggests they may function in different pathways during thymocyte development.

To gain mechanistic insight into Bptf function, we used a microarray and quantitative RT-PCR (qRT-PCR) approach to comprehensively identify potential gene targets prior to and during positive selection. We identified many gene targets important for thymocyte development, in-

cluding transcription factors (*RelB*, *Ets2*, *Id2*, *Egr1*, *Egr2*, *Nur77*, and *Ikaros*), adhesion and motility receptors (*Ccr6*, *Ccr8*, and *Cxcr3*), cytokine signaling components (*Il2* and *Socs3*), regulators of T-cell signaling (*CD6*, *CD52*, *Icos*, and *Ctla4*), and regulators of apoptosis (*Bcl-X_L*, *Gadd45β*, *Bcl2A1B*, and *Bcl2A1D*). For example, *Egr1* and *Egr2* are known to have important functions regulating β selection and positive selection (Bettini et al. 2002; Lawson et al. 2010). The *Ets2* and *Id2* transcription factors have been shown to regulate thymocyte development at β selection (Engel and Murre 2001). *Socs3* is important for β selection and the activation of mature thymocytes (Croom et al. 2008). In addition, we observed decreased levels of cell surface expression of Ccr7 and Il7Rα protein on CD4⁺ CD8^{int} intermediates of positive selection. These markers are targets of TCR signaling and are essential for thymocyte development after positive selection, suggesting that their combined deregulation also contributes to the observed defects in thymocyte maturation (Ueno et al. 2004; Park et al. 2007, 2010). Hence, the cumulative effects of relatively small changes in transcription of multiple Bptf-dependent genes could well suffice to create a “synthetic” defect in thymocyte maturation.

Bptf as a regulator of chromatin structure prior to positive selection

The NURF complex in mammals and flies is a biochemically well-characterized ATP-dependent chromatin remodeling complex that has been shown to influence nucleosome positioning and nuclease hypersensitivity sites *in vitro* (Tsukiyama and Wu 1995; Hamiche et al. 1999; Barak et al. 2003). However, despite the genome-wide identification of DNase I-hypersensitive sites and nucleosome positioning correlating with *cis*-regulatory elements and transcription factor-binding sites (Gross and Garrard 1988; Rando and Chang 2009), causal relationships between chromatin remodeling and gene regulation remain poorly understood. We found that *Egr1* and *Il2* are two useful examples that show Bptf-dependent changes in chromatin structure and gene expression. *Egr1* is a well-documented target of receptor-dependent thymocyte maturation that is important for thymocyte development; however, until this study, its chromatin structure had not been studied in thymocytes (Huang et al. 2004; Mick et al. 2004). In contrast to *Egr1*, *Il2* is not

required for receptor-dependent thymocyte development, but provides well-documented thymocyte-specific chromatin structures at its regulatory elements (Siebenlist et al. 1986; Chen and Rothenberg 1993; Yui et al. 2001). By quantitative chromatin profiling (Dorschner et al. 2004), we observed significant changes in DNase I hypersensitivity at *Egr1* and *Il2* in DP thymocytes. These results suggest that Bptf, and thereby the NURF complex, has remodeling activities at these genes prior to the DP stage, most likely during the DN-to-DP transition (Supplemental Fig. 8A). At *Il2*, we observed Bptf to have essential roles in establishing previously documented thymocyte-specific DNase I sites at -4.5 kb, -0.6 kb, and $+2.1$ kb prior to or during the DN-to-DP transition (Siebenlist et al. 1986; Chen and Rothenberg 1993; Yui et al. 2001). Upstream regulatory elements (including the -4.5 kb site) and the promoter-enhancer (-0.7 kb to $+0.1$ kb) have been shown previously to be important for *Il2* regulation by TCR signals (Siebenlist et al. 1986; Chen and Rothenberg 1993; Yui et al. 2001). Like *Il2*, we observed Bptf-dependent changes in chromatin structure at *Egr1*; however, distal *cis*-regulatory elements have not been characterized. Because of the known correlation between DNase I-hypersensitive sites and regulatory elements, it will be of interest to use the four Bptf-dependent hypersensitive sites observed at *Egr1* (-5.9 kb, -2.9 kb, $+3.6$ kb, and $+6.1$ kb) as a guide for the future identification of functional regulatory elements.

Bptf as a regulator of Srf binding to chromatin

The pre-TCR and the TCR and its coreceptors play an essential role in β selection and positive selection, respectively (Starr et al. 2003). These receptors activate many transcription factors—including those of the AP-1, NF κ B, NFAT, and Srf—to regulate gene targets (Wang et al. 2010). In many cases, the binding of these transcription factors to gene targets is essential for TCR-dependent regulation. Pull-downs of endogenous factors from native thymocytes reveals interactions between Bptf and Srf and AP-1 but not NF κ B and NFAT. We further explored a functional connection between Bptf and Srf because mouse mutants of these factors have similar phenotypes. KO of SRF-TC components Elk-4 and Srf have defects in positive selection, with a complete loss in mature thymocytes with Srf mutation (Costello et al. 2004, 2010; Fleige et al. 2007). Known Srf gene targets *Egr1*, *Egr2*, *Nurr1*, and *Nurr77* were observed to be Bptf-dependent at one or more stages of thymocyte development, suggesting a functional connection at some gene targets. Consistent with this connection, we observe physical interactions between NURF and Srf in vitro, suggesting a direct interaction between the factors in vivo. More importantly, we found that Srf binding to *Egr1*, as revealed by ChIP, is dependent on Bptf. A similar requirement for SWI/SNF in the binding of RunX1 to the CD4 silencer in chromatin has been shown (Wan et al. 2009). It is possible that initial Srf binding to partially accessible SREs, the DNA-binding elements for Srf, recruits NURF to reposition nucleosomes, thereby allowing increased accessibility of SREs to additional Srf. The consequence of

deficient Srf binding is likely decreased TCR-dependent activation of *Egr1*, as SREs have been shown to be essential for receptor-dependent activation (Xi and Kersh 2003). (The observed pull-down between NURF and AP-1 does not appear to reflect functional interactions at *Egr1*, as AP-1 binding to *Egr1* chromatin is not reproducibly affected in Bptf KO DN thymocytes; however, ChIP shows AP-1 binding to *Il2* chromatin is Bptf-dependent in thymocytes [JW Landry, unpubl.].) The decreased binding of Srf at the SREs of *Egr1* does not appear to change DNase I accessibility at the promoter in DN cells. It is possible that the resolution of the DNase I assay is not sufficient, and that, at higher resolution, differences might be observed. Alternatively, NURF function at the *Egr1* promoter may not be as a chromatin remodeling enzyme, but rather as a structural component assisting Srf binding.

Using *Egr1* as a model Bptf-dependent gene, we propose that NURF repositions nucleosomes as early as the DN-to-DP transition, when receptor-dependent transcription is first observed. The recruitment of NURF could be through physical interactions with the SRF-TC at the upstream and promoter regions of *Egr1*, as an example, and the altered nucleosome positioning could facilitate Srf binding (Supplemental Fig. 8B, panels i–ii). These two events, in turn, are likely required for the recruitment of other transcription factors and the general transcription machinery, leading to receptor-dependent activation of transcription (Supplemental Fig. 8B, panel ii). Given that SWI/SNF has been shown to possess novel activities distinct from classical remodeling, a similar role for NURF is not excluded (Jani et al. 2008). After β selection, NURF plays an additional role in remodeling chromatin to eliminate DNase I-hypersensitive sites, perhaps directly as a chromatin remodeler, or indirectly through the regulation of transcription at β selection. These changes in chromatin structure would also be important for the binding of transcription factors for gene reactivation in response to a second wave of TCR signaling during thymocyte maturation. Further investigation of NURF function at *Egr1* during subsequent stages of receptor-dependent gene activation will allow us to define the precise repositioning of nucleosomes, binding of transcription factors, and underlying biochemical mechanisms necessary to achieve proper gene regulation in development.

Materials and methods

Animal husbandry

The *Bptf*^{Floxed} allele was developed in C.W.'s laboratory and now is available through The Jackson Laboratories as B6.129S1-*Bptf*^{tm1Cwu}/J (stock no. 009367). All other alleles were purchased from Jackson Laboratories or Taconic Farms. Animals were handled in compliance with guidelines established under the National Institutes of Health Animal Care and Use Committee under Animal Study Proposal LMCB001 and its modifications.

Flow cytometry

Thymocytes were stained with combinations of the antibodies as demanded by the experiment (see Supplemental Data Set S2

for antibodies used in this study). Following staining, cells were washed and suspended in buffer with a viability dye. Data were collected using a LSRII flow cytometry machine (BD Biosciences) and were analyzed using Flow-Jo software.

Coreceptor reversal assay

Coreceptor reversal assays were performed as described previously (Brugnera et al. 2000).

Chromatin structure assays

DNase I nuclease accessibility analysis was performed on total *Rag*^{-/-} thymocytes or panned DP thymocytes. Following isolation, thymocytes were suspended in T-cell growth medium (RPMI, 10% charcoal-stripped FCS, supplemented with glutamine, nonessential amino acids, β -mercaptoethanol, penicillin, streptomycin) and incubated for 2 h at 37°C and 5% CO₂ in a tissue culture incubator. Following resting, the cells were then fixed in 1% PFA in PBS for 15 min. Following fixation, nuclei were prepared and digested with 4.6 U/mL DNase I (Sigma) for 5 min at 37°C as described previously (Dorschner et al. 2004). DNA was then phenol-extracted, ethanol-precipitated, and analyzed by qPCR.

qPCR was performed using 2 $\times$ DyNAmo Syprogreen qPCR kit (New England Biolabs) according to manufacturer's procedures. Briefly, reactions were composed of 5 μ L of 1.2 μ M forward and reverse primers, 5 μ L of DNA at 20 ng/ μ L, and 10 μ L of 2 $\times$ qPCR mix. Reactions were heated for 3 min at 94°C, then cycled for 40 cycles at 20 sec at 94°C, 20 sec at 60°C, and 30 sec at 72°C prior to reading sample fluorescence. DNase I hypersensitivity was quantified relative to an amplicon at the Rhodopsin gene and an uncut DNA control as described previously (see Supplemental Data Set S2 for primer sequences) (Dorschner et al. 2004).

Analysis of LCKproximal-Cre recombination

Cell populations were sorted and DNA was extracted using standard techniques. DNA concentration was first normalized to GAPDH to ensure the linear amplification of the *Bptf*^{Floxed} or the *Bptf* delete exon 2 alleles (see Landry et al. 2008 for primer sequences). The deletion of exon 2 from *LCKproximal-Cre*, -/Floxed conditional KO mice was confirmed by sequencing the exon 1–8 junction in the *Bptf* transcripts as published previously (Landry et al. 2008).

For Western analysis, 50 μ g of total protein was separated by SDS-PAGE and transferred to PVDF using CAPS (Landry et al. 2008) or Tris-glycine transfer buffer at 20 V and 20-mA limits for 17 h. Following transfer, blots were blocked in 5% NFD, 0.1% Tween 20, and PBS and probed with anti-Bptf/BPTF (Landry et al. 2008), Snf2 (Millipore, no. 05-698), anti-pRbAp48 (Santa Cruz Biotechnology, sc-33170), and anti-cyclophilin B (Abcam, ab3565-100).

TCR and IL7R stimulation

For TCR stimulation assays, electronically sorted CD4⁺, CD8⁺ thymocytes were stimulated with anti-CD3 ϵ and anti-CD28-coupled magnetic beads as described previously. Cells and beads were mixed well and spun down at maximum speed in a micro-centrifuge for 8 sec, then placed in a water bath for 5 sec (p-p65, p-Elk1, and p-Jnk analysis) or 15 min (Nfat1p dephosphorylation) at 37°C. Following stimulation, proteins were extracted with Triazol (Sigma) according to the manufacturer's instructions. Extracted proteins were resolved by SDS-PAGE and analyzed by

Western blotting using antibodies to p-p65, p-Elk1, p-Jnk/SapK (Cell Signaling Technologies, nos. 3039S, 4671S, and 9181S), and Nfat1p (Novus Biologicals, no. 300-504).

IL7R stimulation assays were performed on freshly isolated total thymocytes using a previously published protocol (Brugnera et al. 2000).

Apoptosis assays

Total thymocytes were isolated as described above. Cells (5.0×10^6) were plated in six-well plates with 2 mL of T-cell growth medium containing untreated heat-inactivated FBS or charcoal-stripped/heat-inactivated FBS. At 24 h, 48 h, and 72 h, cells were stained with antibodies, annexin V, and viability dye and processed on a LSRII flow cytometer.

Negative selection was simulated in vitro using plate-bound antibody stimulation of the TCR. Ninety-six-well plates were coated with anti-CD3 ϵ -Biotin (clone 145-2C11) and anti-CD28-Biotin (clone 37.51) overnight at a ratio of 1:5 over a concentration series. Total thymocytes were added to each well in T-cell growth medium and incubated overnight, then stained with antibodies, annexin V, and viability dye and processed on a LSRII flow cytometer.

Glucocorticoid-induced apoptosis was simulated by exposure to dexamethasone. Freshly isolated total thymocytes (5.0×10^5) were plated into 24-well plates in 250- μ L volume of T-cell growth medium. Following the exposure to dexamethasone overnight, cells were stained with antibodies, annexin V, and viability dye and processed on a LSRII flow cytometer.

ChIP

Total thymocytes (50×10^6) were harvested from *Rag*^{-/-} mice or panned DP thymocytes in T-cell growth medium and incubated for 2 h at 37°C and 5% CO₂. Cells were then washed with PBS and fixed in 2 mM EGS (Pierce) in 25% DMSO/75% PBS for 30 min followed by 1% PFA in PBS for 30 min for Snf2 ChIP. For SRF ChIP, cells were fixed in 1% PFA in PBS for 15 min. Following fixation, cells were washed three times in PBS and processed using the ChIP procedure published by Upstate Biologicals. Antibodies used for pull-down were immunoprecipitation-grade Snf2 (Millipore, no. 05-698) and SRF (Santa Cruz Biotechnology, sc-335).

Quantification of pull-down was performed by real-time PCR using 2 $\times$ DyNAmo Syprogreen qPCR kit (New England Biolabs) according to manufacturer's procedures. Briefly, reactions were composed of 5 μ L of 1.2 μ M forward and reverse primers, 5 μ L of diluted cDNA template, and 10 μ L of 2 $\times$ qPCR mix (see Supplemental Table S2 for primer sequences). Reactions were heated for 10 min at 94°C, then cycled for 40 cycles at 20 sec at 94°C, 20 sec at 60°C, and 30 sec at 72°C. After each cycle, the sample was heated for 3 sec to 78°C prior to reading sample fluorescence. Pull-downs were quantified as a percentage of input using a dilution series of input as a standard curve.

Microarray and qPCR analysis

Microarray analysis was performed on purified or semipurified thymocyte populations. For in vitro stimulated data sets, electronic cell sorting was used to purify $\sim 30 \times 10^6$ cells as live CD4⁺, CD8⁺, CD69^{low} from total thymocytes. Sorted cells were then collected and suspended in T-cell growth medium. Following purification, cells were allowed to recover in T-cell growth medium for 3 h at 37°C in a tissue culture incubator. Following recovery, half of the cells were stimulated with 10 ng/mL PMA and 250 ng/mL ionomycin for 1 h. After stimulation, cells were

collected and total RNA was extracted by Triazol (Sigma) according to the manufacturer's protocol.

OT1 TCR and OT2 TCR thymocytes were purified using MACS separation technology according to the manufacturer's procedures (Miltenyi Biotec). An anti-CD8 separation was used for purifying OT2 TCR thymocytes and an anti-CD4 separation was used for the purification of OT1 TCR thymocytes. Positively selected thymocytes were collected by 500g centrifugation and total RNA was extracted by Triazol (Sigma) according to the manufacturer's protocol.

Total RNA fraction was further extracted with acid phenol, precipitated with ethanol, and resolved by electrophoresis to monitor integrity. RNA (100 ng) was labeled using the total RNA amplification kit (Ambion), hybridized to Whole-Mouse Genome Expression arrays version 2.0 (Illumina), and scanned using a Bead Station scanner (Illumina). Data sets were imported into Gene Spring for normalization and statistical analysis. Significantly deregulated genes in Mut/wild type were scored as having false-discovery rate (FDR) values of <0.05 and fold changes of expression at >1.5.

For qPCR analysis of TCR-responsive genes over a time course, panned DP thymocytes (95% pure) or total thymocytes were harvested from *Rag*^{-/-} mice, purified, and suspended in T-cell growth medium. Following purification, cells were allowed to recover in T-cell growth medium for 2 h at 37°C in a tissue culture incubator. Following recovery, cells were stimulated with 10 ng/mL PMA and 250 ng/mL ionomycin, and samples were removed at designated time points (every 30 min). After removal, cells were collected and total RNA was extracted by Triazol (Sigma) according to the manufacturer's protocol. RNA was reverse-transcribed using SuperScript II (Invitrogen) according to the manufacturer's protocol.

Quantification of gene expression was performed by real-time PCR using 2× DyNAmo Syprogreen qPCR kit (New England Biolabs) according to the manufacturer's procedures. Briefly, reactions were composed of 5 µL of 1.2 µM forward and reverse primers, 5 µL of diluted cDNA template, and 10 µL of 2× qPCR mix (see Supplemental Table S3 for primer sequences). Reactions were heated for 10 min at 94°C, then cycled for 40 cycles at 20 sec at 94°C, 20 sec at 60°C, and 30 sec at 72°C. After each cycle, the sample was heated for 10 sec to 78°C prior to reading sample fluorescence. Expression was quantified using the ddCT method using GAPDH as a control. Expression data are representative of three KO and control mice. Error bars are the standard deviation of four measurements for each time point.

In vivo and in vitro pull-downs

Total thymocytes (500×10^6) were harvested from C57BL6 mice and suspended in 10 mL of T-cell growth medium. Cells were induced overnight with 3 ng/mL PMA and 300 ng/mL ionomycin. Following induction, cells were centrifuged, resuspended in 1 mL of lysis buffer (25 mM HEPES at pH 7.6, 100 mM NaCl, 0.2% NP-40, 5 mM MgCl₂, 10% glycerol, 2× protease inhibitor cocktail (Roche, no. 05 056 489 001) and 1× phosphatase inhibitor cocktail (Sigma, P2850), and sonicated for 30 min in a Bioruptor sonicator. Following sonication, the lysate was centrifuged at 15,000g for 15 min and the soluble fraction was removed. Pull-downs were performed on 150 µL of lysate with 5 µg of antibody coupled to protein G Dynabeads overnight at 4°C with rotation. The following day, beads were washed three times with lysis buffer with 0.5× protease inhibitors and eluted with 1% SDS at 65°C. Eluted proteins were separated by 4% PAGE, blotted to PVDF, and Western blotted for Bptf using antibodies described previously (Landry et al. 2008). Antibodies used for pull-down were immunoprecipitation-grade RelA (Santa Cruz

Biotechnology, sc-109), c-Jun (Santa Cruz Biotechnology, sc-44), SRF (Santa Cruz Biotechnology, sc-335), NFATc1 (Santa Cruz Biotechnology, sc-13033), and control (Santa Cruz Biotechnology, sc-805). Quantitative Western blotting was performed with a Fujifilm LAS-3000 camera system.

GST fusions were made by subcloning SRF into pGEX4T-1 using standard cloning techniques. Approximately 5 µg of resin-bound fusion proteins were washed in *in vitro* binding buffer (25 mM HEPES at pH 7.6, 100 mM NaCl, 0.01% NP-40, 0.5 mM MgCl₂, 10% glycerol), and Flag affinity-purified human NURF complex was added to each pull-down reaction. Samples were incubated for 1 h on ice and washed three times with binding buffer. Bound proteins were eluted from resin with protein loading buffer, resolved by SDS-PAGE, and transferred to PVDF. Flag-tagged BPTF from the NURF complex was detected by Western blotting with anti-Flag antibodies (Sigma, F1804) used to detect bound NURF complex. Quantitative Western blotting was performed with a Fujifilm LAS-3000 camera system.

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