Immunity, Vol. 19, 169–182, August, 2003, Copyright ©2003 by Cell Press

# Sequential Roles of Brg, the ATPase Subunit of BAF Chromatin Remodeling Complexes, in Thymocyte Development

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## Summary

**T cells develop through distinct stages directed by a series of signals. We explored the roles of SWI/SNF-like BAF chromatin remodeling complexes in this process by progressive deletion of the ATPase subunit, Brg, through successive stages of early T cell development. Brg-deficient cells were blocked at each of the developmental transitions examined. Bcl-xL overexpression suppressed cell death without relieving the developmental blockades, leading to the accumulation of Brg-deleted cells that were unexpectedly cell cycle arrested. These defects resulted partly from the disruptions of pre-TCR and potentially Wnt signaling pathways controlling the expression of genes such as *c-Kit* and c-*Myc* critical for continued development. Our studies indicate that BAF complexes dynamically remodel chromatin to propel sequential developmental transitions in response to external signals.**

## Introduction

Development of T lymphocytes bearing αβ antigen receptors (TCRαβ) is a multistep process that depends on a series of external signals given in a specific sequence (Figure 1A). Beginning at about day 14 of embryogenesis, lymphocyte precursors colonize the thymic anlage. These cells express neither CD4 nor CD8 and hence are called double-negative (DN) cells. DN cells pass through four successive stages (DN1–4) that are recognizable based on the differential expression of the IL-2 receptor CD25 and the adhesion molecule CD44. IL-7R and c-Kit signaling are required for the survival and/or proliferation of DN1 and DN2 cells (Akashi et al., 1998; Di Santo and Rodewald, 1998; Maraskovsky et al., 1997). Cells at the subsequent DN3 stage undergo Rag-catalyzed somatic recombination at the TCRβ locus and express a pre-TCR complex that signals the transition to the DN4 stage (Groettrup and von Boehmer, 1993). DN4 cells activate CD8 and subsequently derepress CD4 genes to become double-positive (DP) cells. Recombination at the *TCR*α locus then leads to the expression of *TCR*αβ, which directs the differentiation of DP cells into CD4 or CD8 single-positive (SP) cells (von Boehmer, 2000). Wnt signaling regulates the expansion of DN2, DN4, and ISP cells, the survival of DP cells, the ISP→DP, and perhaps also DN3→DN4 and DP→SP development (Gounari et al., 2001; Ioannidis et al., 2001; Okamura et al., 1998; Schilham et al., 1998; Verbeek et al., 1995).

External signals must be able to oppose energically stable chromatin structures to activate transcription. Eukaryotic cells have evolved multiple ATP-dependent chromatin remodeling complexes, the molecular motors that use energy derived from ATP hydrolysis to disrupt chromatin structure (Peterson, 2002). These complexes are attractive candidates for overcoming the energetic barriers produced by chromatin assembly, thereby promoting signal controlled chromatin reorganization and gene induction. Indeed, the first chromatin remodeling complex, the yeast Swi/Snf complex, was discovered for its roles in mating type switching and sucrose fermentation in response to environmental signals (hence the name switch/sucrose non-fermenting) (Winston and Carlson, 1992). That the yeast Swi/Snf complex plays a critical role in signaling inspired us to isolate the related mammalian BAF chromatin remodeling complexes and to study their roles in T cells (Chi et al., 2002; Khavari et al., 1993; Wang et al., 1996a, 1996b; Zhao et al., 1998). BAF complexes may be mechanistically distinct from the yeast SWI/SNF complex since they have multiple DNA binding domains, contain actin, and are bound to the insoluble matrix fraction of the nucleus (Zhao et al., 1998). These observations suggest that they probably have a distinct mechanism of recruitment to their sites of action. Thus, although we originally referred to them as mSWI/SNF (Khavari et al. 1993), this designation may be misleading. BAF complexes contribute to T cell sublineage differentiation by reciprocally regulating CD4/8 (Chi et al., 2002). In mature T cells, TCR stimulation rapidly stabilizes the binding of BAF complexes to chromatin, highlighting the roles of BAF complexes in signaling (Zhao et al., 1998). In contrast to the yeast complex, which is monomorphic, several subunits of BAF complexes are encoded by gene families that are coexpressed in the same cell, thus leading to their combinatorial assembly and the generation of perhaps hundreds of complexes (Wang et al., 1996a, 1996b). The significance of the heterogeneity of BAF complexes is unclear. For example, although mutations in Brg and its homolog Brm cause different phenotypes in mice, with the former producing early embryonic lethality (Bultman et al., 2000) and the latter little defects (Reyes et al., 1998), the lack of strong phenotypes in the Brm mutants appears to result from the ability of Brg to compensate for Brm (Reyes et al., 1998). Indeed, Brm and Brg are often coordinately downregulated in human tumors, and Brm can function interchangeably with Brg to suppress transformed phenotypes (Reisman et al., 2002).

While homozygous germline Brg deletion is early em-

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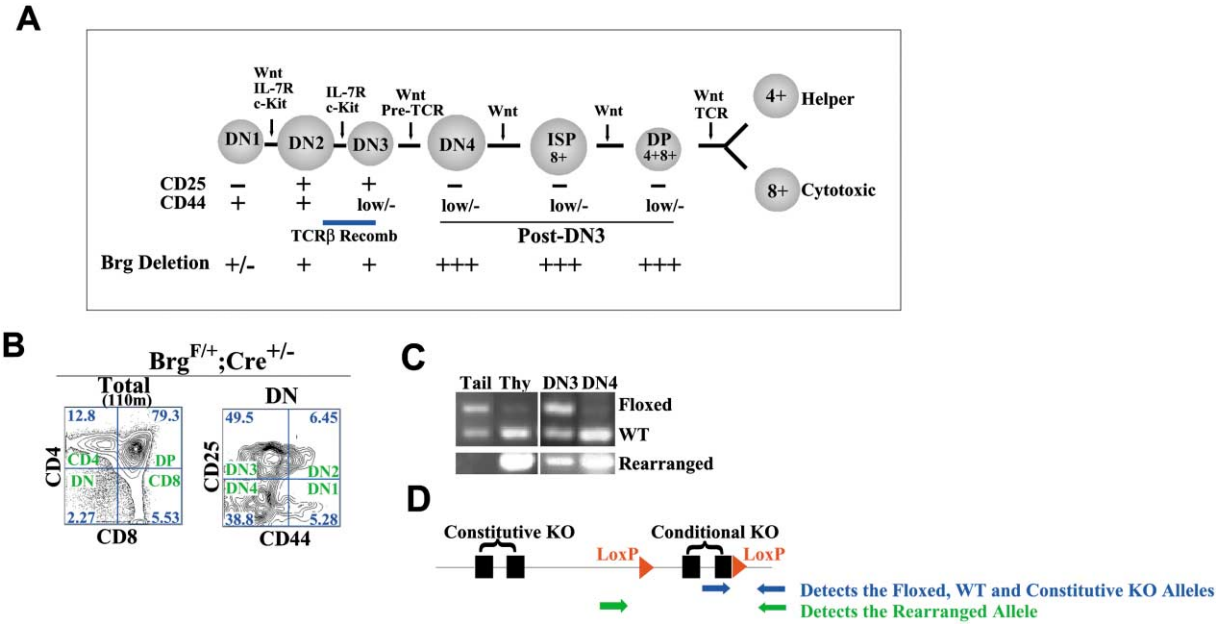



Figure 1. Cre-Mediated Deletion of a Floxed Brg Allele in Thymocytes

(A) Thymocyte development proceeds via DN→ISP→DP→SP stages as defined by CD4, CD8, CD25, and CD44 expression (Godfrey et al., 1993). ISP and DP cells are identical to DN4 cells in being $CD25^{-}CD44^{-/low}$, and the three cell types are collectively termed post-DN3 cells (Chi et al., 2002). DN2, DN4, ISP cells are proliferating blasts, as denoted by their large sizes. Also indicated is the extent of the deletion of the floxed Brg gene as described in the text.

(B–D) Brg deletion was partial in DN3 but complete in DN4 cells in $Brg^{F/+}Cre^{+/-}$ mice. Thymocytes from a $Brg^{F/+}Cre^{+/-}$ mouse were stained with CD4, CD8, and TCR to determine the CD4/8 expression pattern of total thymocytes (left panel). To determine the composition of DN cells, thymocytes were stained with CD4, CD8, CD3, CD25, and CD44, and the $CD4^{-}CD8^{-}CD3^{-}$ cells were resolved into four subsets based on CD25/44 expression (right panel). DN3 and DN4 cells were sorted and the efficiencies of deletion of the floxed Brg allele determined by PCR (Figure 1C) using primers (Sumi-Ichinose et al., 1997) that detected the floxed or rearranged allele, the latter becoming detectable as a result of Cre-mediated excision of two exons encoding part of the ATPase domain (Figure 1D). The current study also used a constitutive (germline) knockout allele (Bultman et al., 2000) lacking two upstream exons and encoding a truncated protein missing the entire C terminus of Brg (Figure 1D).

bryonic lethal (Bultman et al., 2000), heterozygous Brg deletion impairs CD8 activation, suggesting a limiting and specific role of Brg in T cell development (Chi et al., 2002). To further examine the function of Brg, we deleted Brg specifically in thymocytes. Fortuitously, Brg was deleted gradually through several stages of signal-directed early T cell development, which revealed the essential roles of Brg at all these stages.

## Results

### A Lck-Cre Transgene Produces Progressive Deletion of a Lox P-Flanked Brg Gene during Early Thymic Development

We used Cre-mediated excision to delete Brg specifically in T cells. Cre was expressed under the control of the Lck proximal promoter, which is active in immature T cells (Lee et al., 2001). Two exons essential for ATPase activity of the Brg gene were floxed as previously described (Sumi-Ichinose et al., 1997). We first determined the efficiency of deletion in $Brg^{F/+};Cre^{+/-}$ mice bearing one floxed and one wild-type Brg allele. These mice displayed normal numbers of total thymocytes and DN subsets (Figure 1B), supporting our previous findings that heterozygous germline Brg deletion does not grossly perturb thymocyte development (Chi et al., 2002). Using the PCR primers flanking the 3′ loxP site and thus detecting both WT and the floxed alleles (Figure 1D), we found that the floxed allele was virtually completely deleted in total thymocytes (Figure 1C, top panel, lane 2) and in DN4 cells (lane 4), as judged by the loss of the amplicon representing the floxed allele. In contrast, deletion was undetectable in DN3 cells by this assay (lane3). However, a more sensitive PCR assay that detects the rearrangement of the loxP sites (Figure 1D) indicates the floxed Brg was partially deleted in DN3 (Figure 1C, bottom panel lane 3) and DN2 cells (not shown). The inefficient deletion at DN3 is unexpected, as the same LCK-Cre transgene completely excised a floxed Notch-1 allele by the DN3 stage (Wolfer et al., 2002). It is possible that the loxP sites in the floxed Brg mice are not readily accessible or rearranged. Neither DN2, DN3, nor DN4 cells showed any defect in CD4 silencing in these mice (not shown), as predicted from the fact that one copy of Brg is sufficient to silence CD4 (Chi et al., 2002), and demonstrated that the deleted gene does not produce a dominant-negative protein.

### Homozygous Brg Deletion Derepresses CD4 in a Fraction of DN Cells and Blocks the DN4→DP Transition

We analyzed the effect of homozygous Brg deletion in $Brg^{F/-}$; Cre mice bearing the floxed Brg allele together

with the germline deleted allele of Brg. We found that the thymi of the Brg $^{F/-}$;Cre$^{+/-}$ mice were as small as those seen in Rag$^{-/-}$ mice, with 40- to 100-fold reduction in cell numbers. DP and SP cells were completely absent, as revealed by the CD4/8 expression profile of the total thymocytes (Figure 2A, column 1, row B). The remaining thymocytes contained a few CD4$^+$ cells, consistent with the role of Brg in CD4 silencing. The CD4$^-$CD8$^-$ cells contained normal numbers of DN1-DN3 cells, as expected from the fact that Brg was minimally deleted from these cells. In contrast, the numbers of the DN4 cells were reduced about 6-fold (column 2, row B; Figure 2B), and the remaining DN4 cells were completely arrested in development, based on the complete absence of DP cells described before (column 1, row B). A caveat is that the CD4$^-$CD8$^-$ cells in the DN4 compartment may not be T cells, but rather contaminants from other lineages. Thus, the apparent lack of DP cells could have resulted from the complete depletion of DN4 cells, as opposed to the block in the differentiation of the remaining DN4 cells. We ruled out this possibility by showing that the DN cells in the DN4 compartment were predominantly Thy1.2$^+$ (see Supplemental Figure S1 at http://www.immunity.com/cgi/content/full/19/2/169/DC1).

To pinpoint which subsets of DN cells derepressed CD4, we first used CD25/44 to resolve immature (CD3$^{-/lo}$) thymocytes into DN1, DN2, DN3, and post-DN3 cells (Figure 1A; Figure 2A, column 3). We then determined the CD4/8 expression profile for these subsets (columns 4–6; Figure 2B). The results revealed marginal CD4 derepression at both DN2 and DN3 stages, and suggested that the CD4$^+$ cells may represent those lacking Brg, given that Brg is required for CD4 silencing (Chi et al., 2002). The post-DN3 cells in the mutant were predominantly comprised of CD4$^-$CD8$^-$DN4 cells, but also contained a small number of CD4$^+$CD8$^-$ cells that were presumably DN4 cells prematurely expressing CD4 (column 6, row B).

We then analyzed Brg deletion in various subsets of cells sorted from Brg$^{F/-}$;Cre$^{+/-}$ mice (Figure 2C, top panel). Although there was no significant deletion of the floxed Brg allele in CD4$^-$DN3 cells, Brg was completely deleted in the CD4$^+$ counterpart (lane 3), indicating that Brg deletion indeed led to CD4 derepression. Brg was also completely deleted in DN4 cells including CD4$^-$DN4 cells, as expected. Most of the DN4 cells, however, retained the ability to silence CD4, because the CD4$^+$ cells only constituted a small fraction of total DN4 cells (Figure 2A, column 6, row B). It is not clear why Brg deletion did not uniformly affect the DN4 cells as do mutations in BAF57, Runx1, or the CD4 silencer (Chi et al., 2002; Taniuchi et al., 2002a, 2002b; Zou et al., 2001). Perhaps these CD4$^-$DN4 cells represented those in which Brg was only recently deleted and that residual levels of Brg protein were still present, which sufficed to repress CD4. In any case, the presence of Brg deleted DN4 cells lacking CD4 expression suggested that the CD4$^-$DN3 population also harbored cells lacking the floxed Brg allele. This is indeed the case, as demonstrated by the more sensitive PCR assay detecting the rearranged floxed Brg allele (data not shown).

We conclude that Brg deletion derepressed CD4 in a subset of DN cells, significantly depleted DN4 cells, and completely blocked the DN4→DP transition.

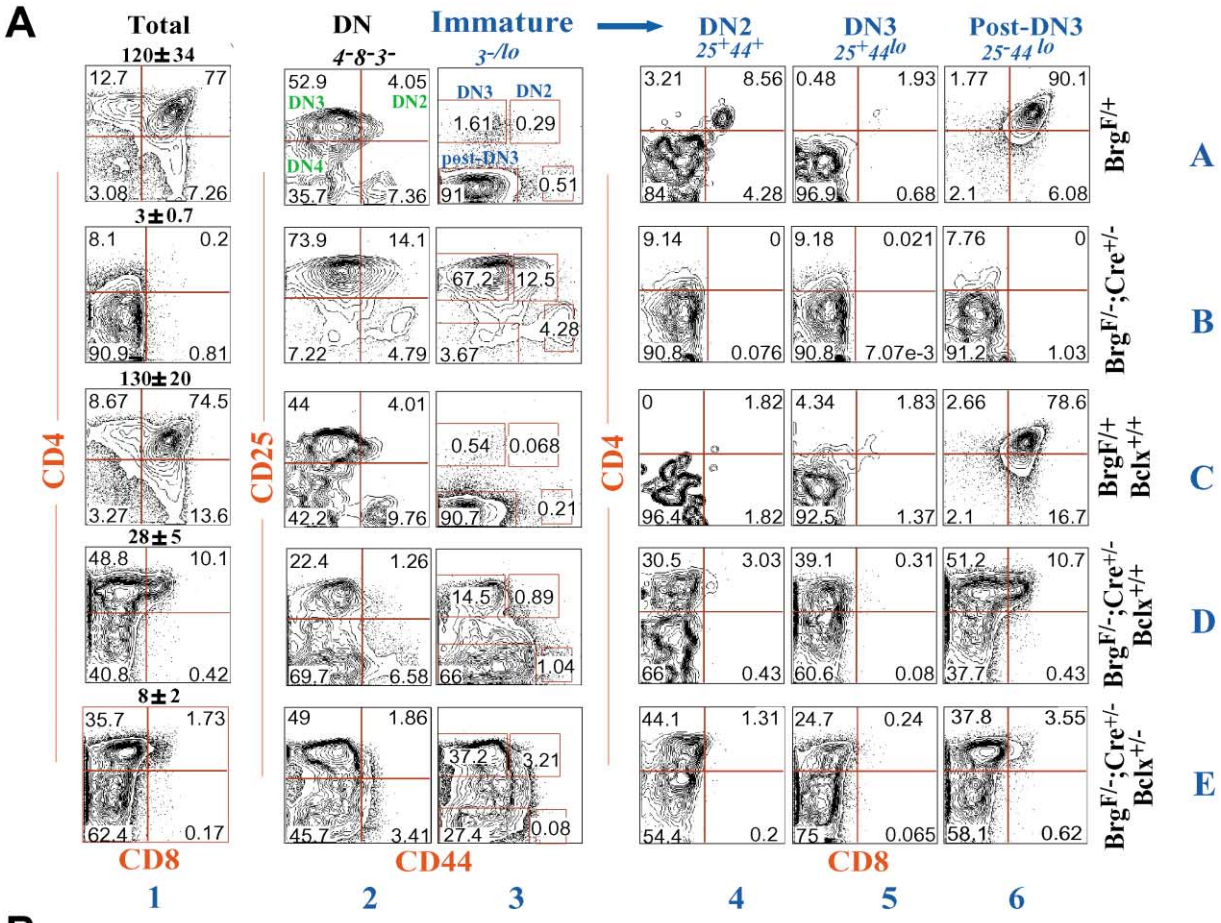
### Brm Expression Is Independent of Brg

In addition to Brg, mammalian cells also express the Brg homolog Brm. As Brm and Brg are often coordinately downregulated in human tumors (Reisman et al., 2002), the striking defects in thymocyte development resulting from Brg deletion prompted us to examine whether Brm expression was abolished in Brg-deficient cells. Semiquantitative RT-PCR using Brg-deficient DN4 cells indicated that Brm transcription was independent of Brg in T cells (Figure 2D). Thus Brm was unable to compensate for the loss of Brg during thymic development. Consistent with these data, Brm is dispensable for thymic development (M. Yaniv and M. Malissen, personal communication), indicating that the functions of Brg- and Brm-containing BAF complexes are distinct during T cell development.

### Brg Deletion Blocks the Differentiation of Most, if Not All, DN Subsets

The primary cause of the block in the DN4→DP transition might be the death of DN4 cells, the failure of the DN4 cells to differentiate, or both. To distinguish between these possibilities, we introduced a Bcl-xL transgene, under the control of the LCK proximal promoter, into Brg$^{F/-}$;Cre$^{+/-}$ mice. Bcl-xL alone did not grossly alter thymocyte development except that it increased the abundance of CD8SP cells as reported previously (Figure 2A, column 1, row C) (Chao and Korsmeyer 1997), and moderately decreased the cellularity of DN cells (Figure 2B). In contrast, the Bcl-xL transgene led to a dramatic, dose-dependent increase in the cell numbers in Brg-deficient mice (up to 10-fold, from 3 million in Brg$^{F/-}$;Cre to 8 million in Brg$^{F/-}$;Cre;Bclx$^{+/-}$ to 28 million in Brg$^{F/-}$;Cre;Bclx$^{+/+}$ mice, Figure 2A, column 1, compare rows A, D, E), indicating that Brg deletion indeed caused cell death, and that the form of death could be suppressed by Bcl-xL overexpression. This increase in cellularity, as compared with that in Brg$^{F/-}$;Cre mice, was largely due to an increase in post-DN3 cells (column 3, compare row B with D), with an averaged 50-fold increase in CD4$^-$ DN4 cells (from 0.15 million to 7 million), and a far more dramatic increase in CD4$^+$CD8$^-$cells (from barely detectable to 8.5 million, column 6, compare row B with D; see Figure 2B for the quantification of cell numbers relative to WT DN subsets). However, the post-DN3 cells contained few DP cells, indicating that Bcl-xL could not relieve the block in the DN4→DP transition. Thus Brg is specifically required for the differentiation of DN4 cells into DP cells. This is further supported by the fact that the Bcl-xL rescued CD4$^-$DN4 cells were about 14-fold more abundant than those in Bcl-xL control mice (Figure 2A, column 2, compare row C with row D; Figure 2B). Both the CD4$^-$ and the CD4$^+$ DN4 cells were completely cell cycle arrested (see next section), and thus their accumulation must have resulted from a blockade in their exiting the DN4 compartment and differentiating into the DP compartment.

Bcl-xL overexpression also caused a preferential accumulation of CD4$^+$DN2 and CD4$^+$DN3 cells (Figure 2A, columns 4 and 5, row B versus D; Figure 2B). This dem-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DN
Immature
DN2
DN3
Post-DN3
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3-/lo
25+44+
25+44lo
25-44lo
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3±0.7
130±20
28±5
8±2
CD4
CD25
CD44
CD8
1
2
3
4
5
6
BrgF/+
BrgF/-;Cre+/-
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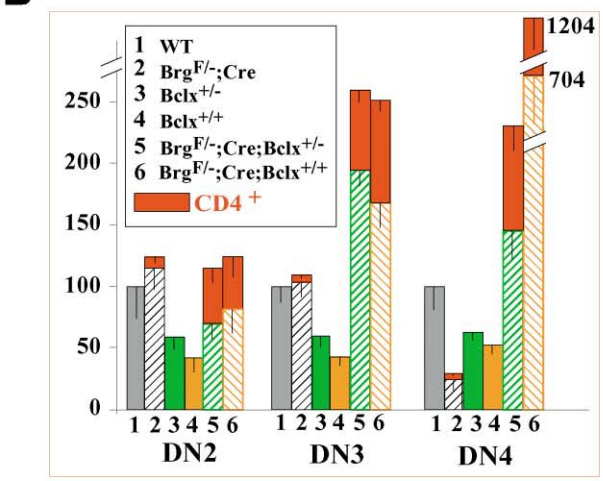
B
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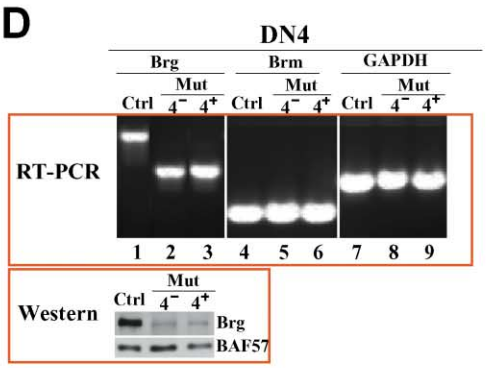
2 BrgF/-;Cre
3 Bclx+/-
4 Bclx+/+
5 BrgF/-;Cre;Bclx+/-
6 BrgF/-;Cre;Bclx+/+
CD4 +
1204
704
250
200
150
100
50
0
1 2 3 4 5 6
DN2
DN3
DN4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Brm
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4-
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RT-PCR
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Western
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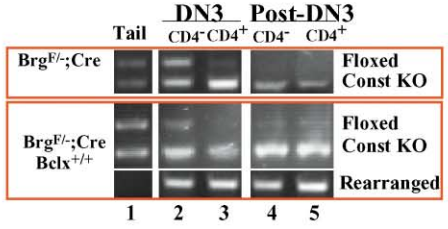
BAF57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Post-DN3
Tail
CD4-CD4+
CD4-
CD4+
BrgF/-;Cre
Floxed
Const KO
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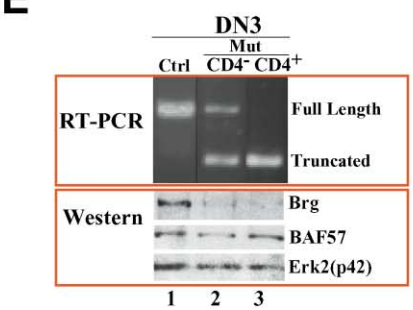
Floxed
Const KO
Rearranged
1 2 3 4 5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DN3
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Western
Brg
BAF57
Erk2(p42)
1 2 3

onstrates that as in the case of DN4, Brg deletion also derepressed CD4, blocked differentiation, and caused death at the DN2-3 stages; once the cell death was suppressed by Bcl-xL, these $CD4^+$cells accumulated due to the failure in differentiation. Indeed, even the Brg-deleted $CD4^-$DN3 cells in the $Brg^{F/-}$;$Cre^{+/-}$;$Bclx^{+/+}$ mice were enriched relative to the $CD4^-$DN3 cells with the intact floxed allele, based on the following facts. First, PCR assay revealed a significant enrichment of cells lacking the floxed Brg allele in $CD4^-$DN3 pool from $Brg^{F/-}$;$Cre^{+/-}$;Bcl-$xL^{+/+}$ mice (compare upper and lower panels in Figure 2C, lane 2). Second, this pool contained a substantial amount of truncated transcript derived from the rearranged Brg allele (Figure 2E, upper panel), concomitant with a substantial reduction in Brg protein level (Figure 2E, lower panel); the truncated transcript was barely detectable in the $CD4^-$DN3 cells from the $Brg^{F/-}$;Cre mice (data not shown). These data indicate that the $CD4^-$DN3 cells lacking Brg were also impaired in survival and differentiation, even though they still retained the ability to silence CD4. Interestingly, although the Bcl-$xL^{+/+}$ transgenes were much more effective than Bcl-$xL^{+/-}$ in causing the accumulation to Brg-deficient DN4 cells, no difference was seen in the extents of accumulation of DN2 and DN3 cells in Bcl-$xL^{+/+}$ versus Bcl-$xL^{+/-}$ rescued Brg-deficient cells (Figure 2B). This suggests that the "niches" for DN2 and DN3 cells are much smaller than that for DN4 cells, and that the moderate increase in the DN2 and DN3 cellularity resulting from Bcl-$xL^{+/-}$ rescue sufficed to fill the niches, preventing further accumulation of the DN2 and DN3 cells in Bcl-$xL^{+/+}$; $Brg^{F/-}$; Cre mice.

The effects of Bcl-xL on DN1 cells lacking Brg, however, were inconclusive (not shown), apparently because the Lck-Cre-mediated deletion is essentially undetectable in DN1 cells (Wolfer et al., 2002).

Together with the fact that the Bcl-xL rescued, Brg-deficient DN2, DN3, and DN4 cells were all blocked in proliferation (see below), the accumulation of these cells in the thymus demonstrates that Brg deletion blocked the differentiation of most if not all subsets of DN cells.

### Cell Cycle Arrest of the Bcl-xL Rescued, Brg-Deficient Cells

To analyze the proliferation of Brg-deficient thymocytes, BrdU was injected i.p. and mice sacrificed 15 min later before BrdU incorporation was measured (Figure 3A; see Figure 3B for the quantification of BrdU incorporation of each DN subset relative to the WT). As expected, $CD4^-$DN2-3 cells in $Brg^{F/-}$;Cre mice incorporated BrdU with efficiency comparable to the wild-type cells; about 30% and 15% of DN2 and DN3 cells were $BrdU^+$, respectively, in both wild-type and mutant mice (Figure 3A, rows 1 and 2), while their $CD4^+$ counterparts and DN4 cells showed marginal, if any, alterations in proliferation (Figure 3A, row 3). Remarkably, each of the Bcl-xL rescued $CD4^+$ DN subsets was cell cycle arrested (Figure 3A, rows 7 and 9). This arrest is not an artifact resulting from Bcl-xL overexpression for two reasons. First, although Bcl-$xL^{+/+}$ transgenes moderately impaired the proliferation of DN4 cells, the transgenes had little effect on DN2 and DN3 proliferation (Figure 3A, row 5), in agreement with the report that a Bcl-2 transgene selectively impairs proliferation of DN4 while sparing other DN subsets (O'Reilly et al., 1997). Second, the Bcl-$xL^{+/-}$ transgene had little effect even on DN4 cells, let alone on DN2 and DN3 cells (row 4), and yet Bcl-$xL^{+/-}$ rescued Brg-deficient $CD4^+$ cells were cell cycle arrested. These data together suggest that Brg deletion tended to block the proliferation of T cells, but it also led to rapid apoptosis of Brg-deleted cells, and thus a proliferation defect could emerge only after the suppression of cell death. Interestingly, the $CD4^-$ DN3 and $CD4^-$DN4 cells were also severely affected, consistent with the fact that these cells largely comprised Brg-deficient cells as did their $CD4^+$ counterparts (Figures 2C–2E), despite the fact that they retained the ability to silence CD4.

To exclude the possibility that BrdU incorporation did not reflect cell proliferation, we sorted out the DN4 cells from the BrdU-labeled mice and determined their DNA contents (Figure 3C). The data demonstrate that Bcl-xL rescued Brg-deficient cells were arrested at the G1 phase of cell cycle. Finally, we found that the intracellular PCNA levels of Brg-deleted cells were reduced, with the mean fluorescence intensity (MFI) precisely paralleling the efficiency of BrdU incorporation (compare Figure 3D with 3A).

We conclude that the Bcl-xL rescued cells were arrested at the G1 phase of cell cycle, and that the accumulation of these cells resulted therefore from retarded differentiation as opposed to excessive proliferation (see Supplemental Figure S2 at http://www.immunity.com/cgi/content/full/19/2/169/DC1). We next sought to determine the molecular mechanisms underlying the developmental defects of Brg-deficient cells.

### Defect in *c-Kit* Expression

The survival and/or proliferation of early T cells are dependent on IL-7R and c-Kit signaling. Surface c-Kit ex-

Figure 2. Defects in the Survival and Differentiation of Brg-Deficient Thymocytes

(A) Developmental blocks in Brg-deficient cells that could not be relieved by overexpressing Bcl-xL. Thymocytes were stained and CD4/8 and CD25/44 expressions determined for total and DN cells as described in Figure 1B (columns 1 and 2). To analyze CD4 derepression, the immature, $CD3^{lo/-}$ T cells containing DN, ISP, and DP cells were first resolved into various subsets based on CD25/44 expression (column 3) before their CD4/8 expression determined (columns 4–6).

(B) Histograms comparing the abundance of DN subsets relative to those in the WT mice that are set as 100. The $CD4^+$ DN cells in Brg mutants (red bars) are placed on top of the $CD4^-$ counterparts for clarity. The data are averaged from three mice for each genotype.

(C) Analysis of Cre-mediated deletion by PCR.

(D) RT-PCR measuring Brg and Brm transcripts in $CD4^-$ ($4^-$) and $CD4^+$ ($4^+$) DN4 cells from $Brg^{F/-}$;$Cre^{+/-}$;Bcl-$xL^{+/+}$ mice (Mut). DN4 cells from Bcl-$xL^{+/+}$ mice (Ctrl) were used as a control. The Western blot (bottom panel) demonstrated the loss of Brg protein in both $CD4^-$ and $CD4^+$ DN4 cells in the mutant.

(E) Analysis of Cre-mediated deletion of DN3 cells at RNA (top panel) and protein (bottom panel) levels by RT-PCR and Western blot, respectively.

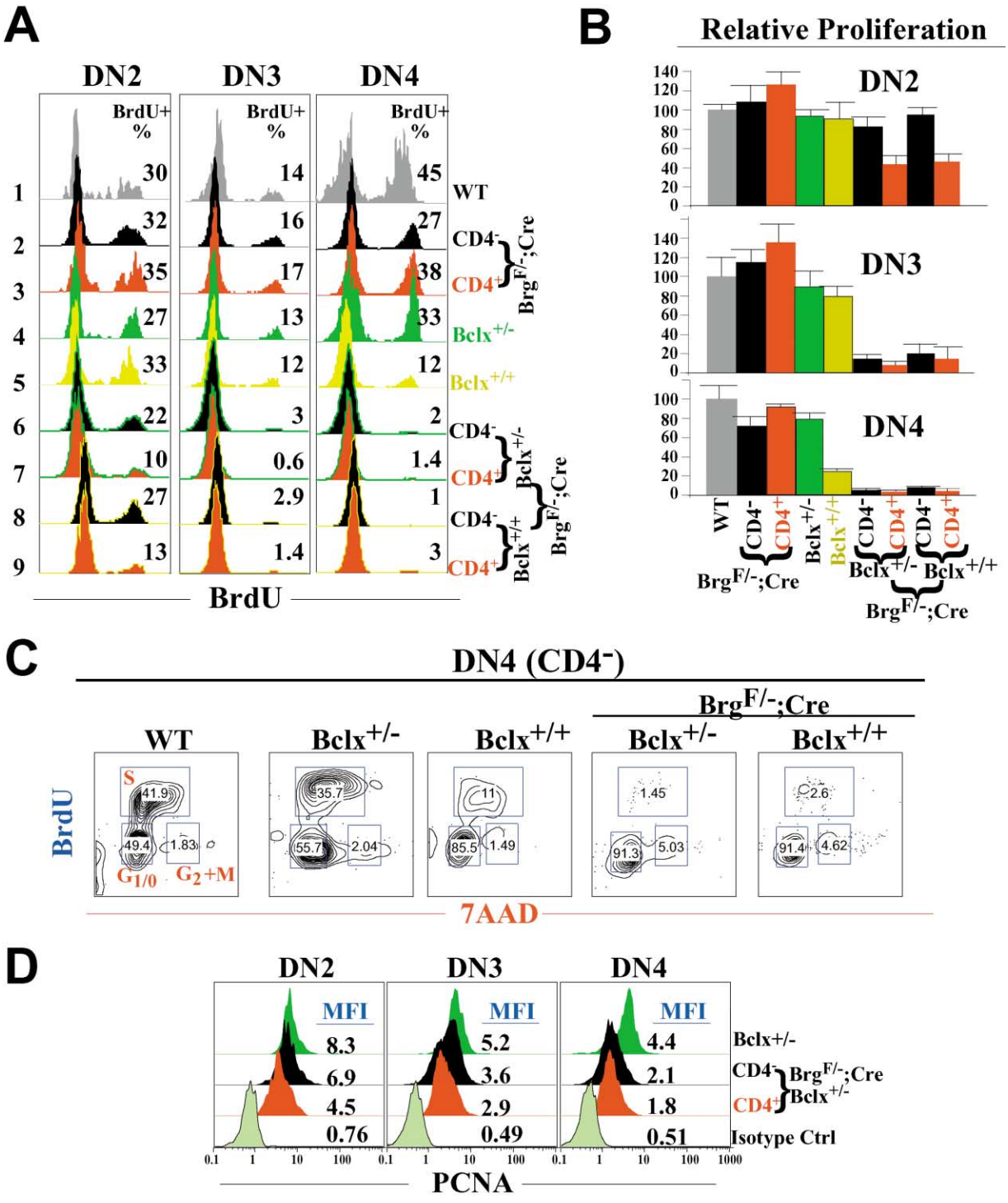



Figure 3. Cell Cycle Arrest of Brg-Deficient Cells

(A) Histograms comparing BrdU incorporation of various DN subsets. The genotypes of the mice are indicated at the right.

(B) Quantification of BrdU incorporation efficiency of various DN subsets relative to the efficiency in the WT mice. The data were averaged from three mice.

(C) DN4 cells from Figure 3A were sorted and stained with 7AAD to determine their cell cycle status in conjunction of BrdU staining. The sorted cells were 95% pure (data not shown).

(D) PCNA expression of various DN subsets. Cells were stained and analyzed as in (A) except that PCNA antibody was used instead of anti-BrdU.

pression was almost completely lost on the Brg-deficient, Bcl-xL rescued DN2 cells (Figure 4A). Bcl-xL rescued DN3 and DN4 cells similarly showed significant reduction in *c-Kit* expression, as measured by RT-PCR (Figure 4B); c-Kit was barely detectable on these cells by FACS (not shown). The defect is specific, because

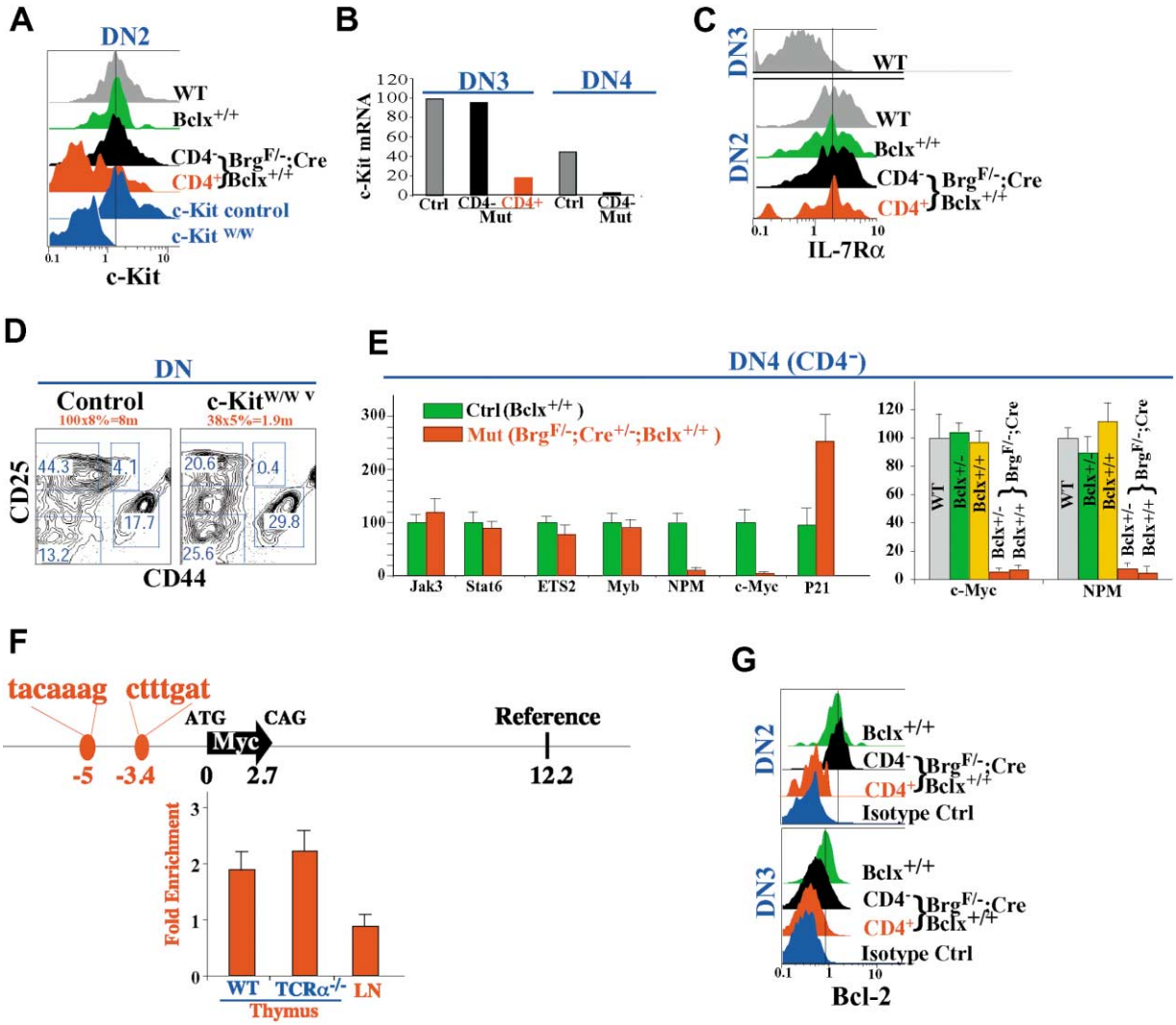



Figure 4. Specific Roles of Brg in *c-Kit*, *Myc*, and *NPM* Expression

(A and B) c-Kit expression in DN2 as measured by FACS analysis and in DN3 or DN4 cells measured by real-time RT-PCR. For FACS analysis, thymocytes from a c-Kit$^{W/Wv}$ mouse and a WT littermate (c-Kit control) were used as negative and positive controls, respectively. For RT-PCR, the mRNA level in DN3 cells from Bcl-xL$^{+/+}$ (Ctrl) mice was set as 100.

(C) FACS analysis of IL-7Rα expression.

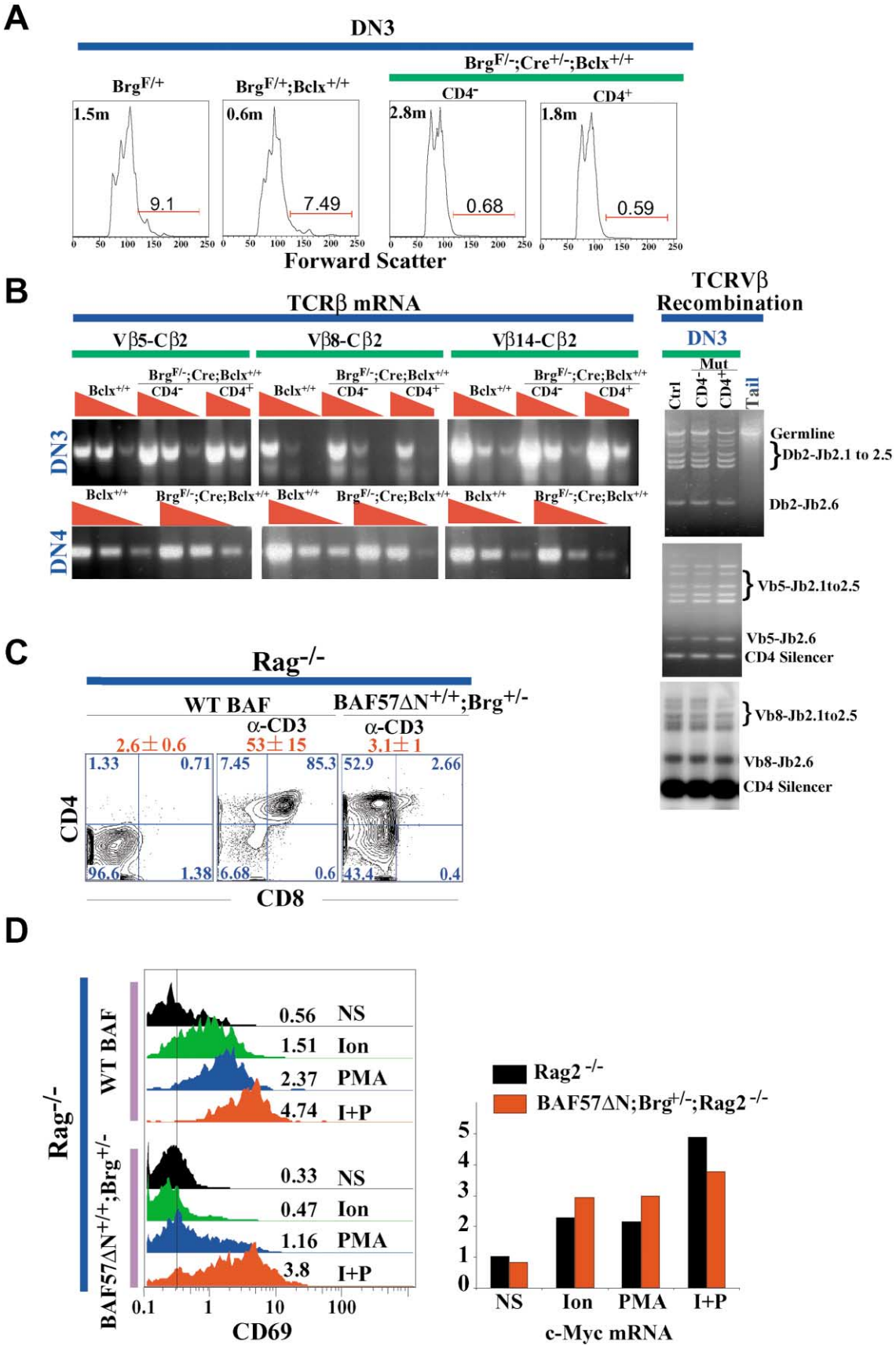
(D) Preferential depletion of DN2 cells in c-Kit$^{w/wv}$ mice.

(E) Real-time RT-PCR assays measuring gene expression in DN4 cells. The mRNA levels of the control mice were set as 100. The right panel, measuring *Myc* and *NPM* expression using RNA from mice different from those used in the left panel, highlights the reproducibility and specificity of *Myc* and *NPM* repression resulting from Brg deletion. A representative real-time PCR analysis was presented in Supplemental Figure S3 at http://www.immunity.com/cgi/content/full/19/2/169/DC1.

(F) Real-time PCR quantifying the binding of Brg to the proximal *TCF* site at the *Myc* promoter. A region 12.2 kb downstream of the *Myc* translation start codon was used as a reference for nonspecific binding (top panel). We first determined the abundance of the *TCF* site relative to the reference in the J1 and in preimmune serum precipitated materials. We then divided the *TCF* abundance in the J1 precipitated materials by that in the preimmune control to calculate the "fold enrichment" (bottom panel). A representative experiment was shown in Supplemental Figure S4.

(G) FACS analysis of Bcl-2 expression.

signaling molecules such as IL-7R (Figure 4C), Jak3, or Stat6 (Figure 4E) were unaffected by Brg deletion. To directly compare the phenotypes of Brg mutants with that of c-Kit-deficient adult mice, we examined the DN subsets in 4- week-old c-Kit-deficient mice bearing a null allele Kit$^{W}$ together with a hypomorphic allele Kit$^{Wv}$ that allows the mice to survive to adulthood. In the Kit$^{W}$/Kit$^{Wv}$ mice, there was a 2.6-fold reduction in total thymocyte number and 1.6-fold decrease in the percentage of DN cells, resulting in a net 4-fold drop in the number of DN cells (Figure 4D). DN2 cells were virtually completely depleted, but there were substantial numbers of DN3 and DN4 cells. These DN3 and DN4 cells were presumably produced by the few remaining DN2 cells that had escaped c-Kit mutation. Indeed, the remaining DN2 cells did not have any defects in survival or proliferation (not


A
DN3
BrgF/+
BrgF/+;Bclx+/+
BrgF/-;Cre+/-;Bclx+/+
CD4-
CD4+
1.5m
9.1
0.6m
7.49
2.8m
0.68
1.8m
0.59
Forward Scatter
B
TCRβ mRNA
Vβ5-Cβ2
Vβ8-Cβ2
Vβ14-Cβ2
Bclx+/+
BrgF/-;Cre;Bclx+/+
CD4-
CD4+
DN3
DN4
TCRVβ Recombination
DN3
Mut
Ctrl
CD4-
CD4+
Tail
Germline
Db2-Jb2.1 to 2.5
Db2-Jb2.6
Vb5-Jb2.1to2.5
Vb5-Jb2.6
CD4 Silencer
Vb8-Jb2.1to2.5
Vb8-Jb2.6
CD4 Silencer
C
Rag-/-
WT BAF
BAF57ΔN+/+;Brg+/-
α-CD3
2.6± 0.6
53± 15
3.1± 1
1.33
0.71
96.6
1.38
7.45
85.3
6.68
0.6
52.9
2.66
43.4
0.4
CD4
CD8
D
Rag-/-
WT BAF
BAF57ΔN+/+;Brg+/-
0.56 NS
1.51 Ion
2.37 PMA
4.74 I+P
0.33 NS
0.47 Ion
1.16 PMA
3.8 I+P
0.1
1
10
100
CD69
Rag2 -/-
BAF57ΔN;Brg+/-;Rag2 -/-
5
4
3
2
1
0
NS
Ion
PMA
I+P
c-Myc mRNA

shown), consistent with their being escapees. These data suggest that the effect of c-Kit mutation was restricted mainly to DN2 cells, which enabled the DN2 escapees to proliferate, differentiate, and repopulate the DN3 and DN4 compartments via homeostatic compensatory mechanisms. The loss of c-Kit expression thus contributed to the death/cell cycle arrest of Brg-deficient DN2 cells, but could not account for the defects in DN3 or DN4 cells in these mutants. In fact, the defects in c-Kit expression and DN2 expansion appeared to be secondary to the disruption in Wnt signaling essential for T cell development at multiple stages (Figure 1A), as demonstrated below.

### Potential Disruption of the Wnt Signaling Pathway in Brg-Deficient Cells

*c-Kit* is regulated by Wnt signaling in human colon cancer cells in a Brg-dependent manner (Barker et al., 2001). Specifically, Brg directly binds β-catenin and a dominant-negative mutant of Brg impairs the expression of the TCF dependent gene *c-Kit*, in addition to three other TCF targets *c-Myc*, *c-Myb*, and *ETS2*. Furthermore, *Brg* also genetically interacts with *TCF* in *Drosophila*. To determine if the loss of *c-Kit* expression in Brg-deficient cells might result from a defect in Wnt signaling, we examined additional Wnt target genes. *Myc* was indeed severely repressed in the Brg deleted cells, concomitant with an elevation in the mRNA level of CDK inhibitor $P21^{CIP1/WAF1}$, a direct target of *Myc* (van de Wetering et al., 2002) (Figure 4E, left panel). In addition to *Myc*, *Nucleophosmin* (*NPM*), a Wnt-activated gene in human embryonic cancer cells (Willert et al., 2002), was also repressed by Brg deletion (Figure 4E). These alterations were not nonspecific consequences of cell cycle arrest seen in the Brg- deficient, Bcl-xL rescued DN4 cells for two reasons. First, *Myb* and *ETS2*, the two Wnt-activated genes in colon cancers and often coexpressed in proliferating cells, were not repressed in Brg-deficient T cells. Second, the Bcl-$xL^{+/+}$ transgene, though moderately impairing proliferation of DN4 cells, could not affect *Myc* or *NPM* expression in the DN4 cells (Figure 4E, right panel).

Paradoxically, expression of CD44, another Wnt-activated gene in colon cells (Wielenga et al., 1999), was moderately increased in Brg-deficient post-DN3 cells (Figure 2A, column 3, compare row C with row D). However, CD44 expression is also increased in *TCF-1*-deficient DN1 and DN4 cells in aged mice (Schilham et al., 1998, Figure 2). Taken together, these data suggest that Brg mutation blocked Wnt signaling in T cells, leading to *c-Kit, Myc*, and *NPM* repression, $P21^{CIP1/WAF}$ induction, and cell cycle arrest.

### Brg Appears to Directly Regulate *Myc*

A prediction from the hypothesis that Wnt signaling might use Brg to activate *Myc* is that Brg binds the Myc promoter, a direct target of Wnt signaling (He et al., 1998). There are two perfect TCF binding sites in the promoter region located within 4 kb upstream of the translation start codon of the mouse *Myc* gene (Figure 4F, top panel). We performed chromatin immunoprecipitation assay on total thymocytes from WT mice. The J1 anti-Brg antibody, but not the preimmune serum, selectively enriched the proximal TCF site relative to a reference sequence downstream of the *Myc* gene in thymocytes (Figure 4F). Similar results were obtained with thymocytes from *TCR*α null mice lacking SP cells, indicating the Brg bound the *Myc* promoter in immature T cells. The binding is specific because Brg did not bind the *Myc* promoter in peripheral, resting T cells (Figure 4F). These results suggest that Brg directly activates *Myc* expression in response to Wnt signaling, leading to cell proliferation.

### Loss of Bcl-2 Expression in Brg-Deleted Cells

In addition to the well-established role of Wnt signaling in cell proliferation, Wnt may also promote cell survival, because *TCF-1* mutation reduces the Bcl-xL protein level and impairs the viability of DP cells (Ioannidis et al., 2001). In DN cells, Bcl-2 is the major antiapoptotic protein, and its expression was indeed severely reduced in Brg-deficient cells (Figure 4G). However, it remains to be determined if the loss of Bcl-2 expression was caused by the disruption of the Wnt pathway.

### Disruption of the Pre-TCR Signaling Pathway in Brg-Deficient DN3 Cells

The block in DN3→DN4 development in Brg-deficient cells is characteristic of defects in pre-TCR signaling, as deletions of a variety of components of the pre-TCR signaling pathway also block this transition (Fischer and Malissen, 1998). An early consequence of pre-TCR signaling is the enlargement and proliferation of DN3 cells. DN3 blasts, marked by large cell sizes, were depleted in Bcl-xL rescued, Brg-deficient DN3 cells (Figure 5A), suggesting that Brg deletion blocked pre-TCR directed proliferation of DN3 cells. In contrast, the fact that Bcl-xL effectively suppressed the death of Brg-deficient DN3 cells indicates that pre-TCR signaling retained the ability to promote the survival of DN3 cells. This is be-

Figure 5. Defects in Pre-TCR Signaling

(A) Lack of DN3 blasts in Bcl-xL rescued, Brg-deficient DN3 cells.

(B) RT-PCR measuring mRNA levels of mature TCRβ genes in DN3 and $CD4^{-}$DN4 cells (left panel), and PCR assessing VDJ recombination in DN3 cells (right panel). The mutant mice used in both cases were $Brg^{F/-}$;Cre; Bcl-$xL^{+/+}$. The PCR products were separated on agarose gels and visualized by ethidium bromide staining, except for the Vβ8 to Jβ2 recombination that was detected by Southern blot for more accurate quantitation of the bands (bottom figure in the right panel).

(C) Adult mice were injected i.p. with 300 μl anti-CD3ε supernatant. 4 days after injection, thymocytes were stained with CD4, CD8, and CD25 antibodies before FACS analysis. The cell numbers (in million) were averaged from three independent experiments.

(D) DN cells were stimulated in vitro for 3 hr with various drugs as indicated before the cells were stained with CD25 and CD69 to analyze the CD69 expression on $CD25^{+}$ cells (left panel). Alternatively, RNA was extracted and *Myc* induction quantified by real-time RT-PCR (right panel).

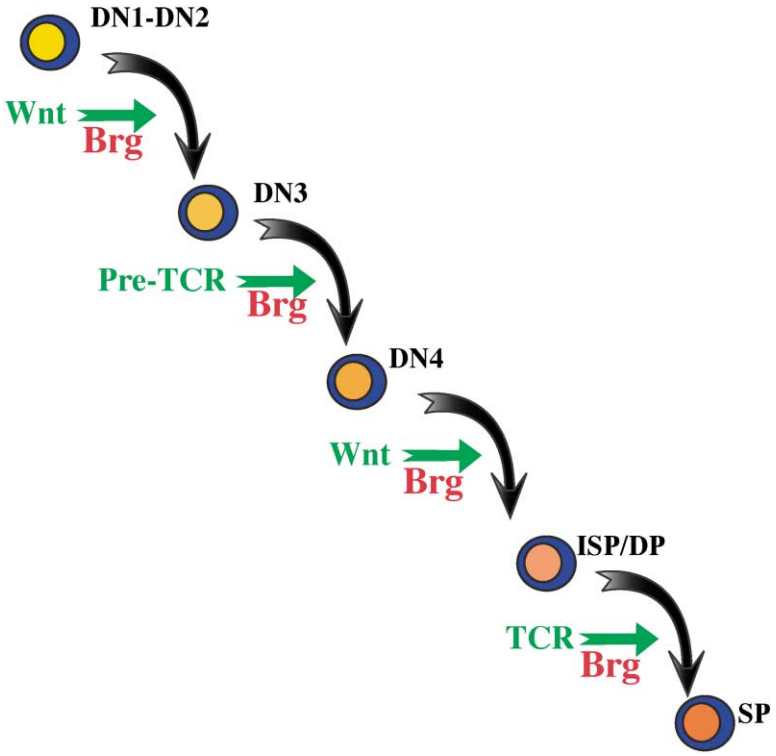



Figure 6. A Model for the Sequential Functions of BAF Complexes in Thymocyte Development

T cell development proceeds through successive stages directed by external signals such as Wnt, pre-TCR, and TCR. These and perhaps other unidentified signals use BAF complexes to remodel chromatin to activate target genes and progressively shape chromatin structure. This drives the survival, proliferation, and developmental transitions of the developing cells, leading ultimately to the formation of chromatin pattern characteristic of the mature T cells. External signals may regulate BAF complexes indirectly via other transcription factors such as β-catenin, or directly via inositol derivatives. The role of Brg in DP→SP development is inferred from the fact that mutations in BAF complexes preferentially deplete SP cells (Chi et al., 2002), and that BAF complexes act downstream of TCR signaling (Zhao et al., 1998). The changing colors of the nuclei denote the distinct chromatin patterns of differentiating cells.

cause antiapoptotic proteins in the Bcl-2 family cannot effectively oppose the death resulting from defects in pre-TCR signaling (Strasser et al., 2000). That Brg was dispensable for pre-TCR stimulated cell survival predicts that Brg deletion did not affect *TCR*β recombination or expression, as the lack of *TCR*β expression would have eliminated the entire spectrum of pre-TCR-dependent processes. Indeed, neither VDJ recombination nor the expression of the recombined *TCR*β genes was impaired in Bcl-xL rescued, Brg-deficient cells (Figure 5B). Furthermore, intracellular CD3ϵ levels were not reduced (not shown), suggesting that Brg deletion impaired pre-TCR signaling downstream of CD3.

To verify the roles of Brg in pre-TCR signaling, we took advantage of the fact that injection of an anti-CD3ϵ antibody into *Rag*-deficient mice, in which thymocyte development is arrested at the DN3 stage, mimics pre-TCR signaling and drives the synchronized development of DN cells into DP cells. We thus crossed *BAF57*Δ*N*$^{+/+}$*;Brg*$^{+/-}$ mice (Chi et al., 2002) onto a *Rag2* null background and determined if the *BAF* mutations impaired the anti-CD3- induced development. We used the *BAF57*Δ*N*$^{+/+}$*;Brg*$^{+/-}$ mice because the T cells in these mice had normal viability (not shown) and were thus free from the complications of the homozygous Brg null cells. We found that in the *Rag2*$^{-/-}$ control mice, antibody injection led to a 20-fold increase in thymocyte cellularity as a result of DP cell production (Figure 5C). In contrast, in the *BAF* mutant, anti-CD3 injection failed to increase total cell numbers or produce DP cells, but rather led to a relative increase in the CD4$^{+}$CD8$^{-}$ cells that constituted 53% of total thymocytes. The CD4$^{+}$CD8$^{-}$ cells normally comprise only 14% in these mice and are produced by premature CD4 expression on DN3 cells (Chi et al. 2002, Figure 3b). 70% of the CD4$^{+}$CD8$^{-}$ cells in the *Rag2*$^{-/-}$*;BAF57*Δ*N*$^{+/+}$*;Brg*$^{+/-}$ mice treated with an anti-CD3 antibody, however, were CD25$^{+}$, indicating that they were still at the DN3 stage (not shown). Thus the major effect of anti-CD3 stimulation on DN3 cells was to exacerbate premature CD4 derepression without propelling the developmental transition of the DN3 cells. These data confirmed that BAF mutations block pre-TCR directed events downstream of CD3.

Interestingly, the *BAF57*Δ*N*$^{+/+}$*;Brg*$^{+/-}$ mice are still able to produce DP cells, though the numbers of DP cells are reduced 3-fold (Chi et al., 2002). It is not clear why the effect of BAF mutations is more pronounced when the DN→DP transition was promoted with the anti-CD3 antibody as opposed to physiological pre-TCR. Perhaps antibody crosslinking of CD3 only produced a suboptimal signal, which synergized with the partial defect in the pre-TCR signaling pathway due to impaired BAF function to completely block DN→DP development.

The signals emanating from CD3 are transmitted through the Calcium/Calcineurin and Ras/Map kinase pathways, which can be activated by the pharmacological agents ionomycin and PMA, respectively. Ionomycin and PMA rapidly and additively induced the expression of CD69 and *Myc* in *Rag2*-deficient DN cells in vitro (Figure 5D). BAF mutations impaired CD69 but not *Myc* induction by ionomycin or PMA, indicating that both the calcium and Ras pathways use BAF complexes to regulate a subset of target genes. This conclusion supports the notion that homozygous Brg deletion only blocked a subset of pre-TCR-dependent events as described above.

## Discussion

### Brg Is Essential for Signal-Directed T Cell Development

T cell development strictly depends on the external signals received from thymic stromal cells. Brg deletion completely blocked the development of each of the DN subsets examined due, in part, to the disruptions of pre-TCR and perhaps Wnt signaling pathways. Furthermore, we previously found that SP cells are preferentially depleted in *BAF57ΔN*$^{+/-}$*; Brg*$^{+/-}$ mice, suggesting a role of Brg in DP→SP development (Chi et al., 2002). Thus, Brg is generally required for signal-directed T cell development (Figure 6). This observation suggests a conserved role of Swi/Snf-like chromatin remodeling complexes, since the yeast Swi/Snf complex is required for mating pheromones and nutrient sources (Winston and Carlson, 1992). Unlike the yeast complex, however, BAF complexes contain actin, have multiple DNA-binding subunits and are associated with the nuclear matrix (Zhao et al., 1998), which suggests important differences between these complexes.

One may argue that BAF complexes are nonspecific gene activators or components of the general transcription machinery, and the developmental defects in Brg-deficient cells are simply a consequence of global gene repression. However, Brg is entirely dispensable for serum-induced, Ras/Mapk pathway-mediated survival and proliferation of murine embryo fibroblasts (Bultman et al., 2000) and both Brg and Brm are dispensible for serum-dependent proliferation of SW13 cells (Dunaief et al. 1994). The ability of Bcl-xL to rescue Brg-deficient T cells also argues against nonspecific deterioration of transcription in these cells. Indeed, Brg deletion did not impair the expression of CD25, IL-7R, *Brm*, *Jak3*, *Stat6, ETS2*, *Myb*, or *TCR*β, and instead increased the expression of CD4, CD44, and *P21*$^{CIP1/WAF1}$. Microarray analysis further demonstrates that Brg deletion altered the expression of less than 5% of genes, many of which seem to reflect the cell cycle and growth defects secondary to the disruption of the Wnt pathway (not shown). This suggests that direct targets of Brg are very limited. Indeed, transient transfection of Brg into a Brg-deficient tumor cell line acutely affects the expression of only 82 genes (Liu et al., 2001). These data indicate that Brg mutations blocked specific signaling pathways essential for T cell development.

External signals function to promote cell survival, proliferation, and differentiation during T cell development. Brg-deficient cells died of apoptosis as a result of a defect in Bcl-2 expression, as the death was suppressible by Bcl-xL. Bcl-xL rescued cells were cell cycle arrested, indicating that Brg mutation impaired both the survival and proliferation of early thymocytes. The cell cycle arrest of the DN4 cells, and presumably DN2 cells as well, was secondary, at least in part, to *Myc* repression, perhaps as a result of the disruption of the Wnt pathway. The death/cell cycle arrest of DN2 cells additionally resulted from the loss of Wnt-dependent c-Kit expression. Indeed, the selective DN2 but not DN3 depletion in the *c-Kit* mutant (Figure 4D) bears striking resemblance to the FACS profile in the *TCF-1* null mice (Schilham et al., 1998), suggesting that *TCF-1* mutation blocks c-Kit expression. Interestingly, Brg is essential for Wnt-regulated *Myb* and *ETS2* expression in human colon cells, but not in T cells. In addition, we showed that Wnt/Brg activates CD44 in colon but not in T cells. These data thus highlight the tissue specificity of the Wnt/Brg signaling pathway. On the other hand, the conserved role of Brg in *Myc* induction in both colon and thymus helps solve the puzzle that while Brg is essential for Rb to suppress cell cycle (Dunaief et al., 1994; Zhang et al., 2000), Brg mutations only produce tumors in very restricted tissues (Bultman et al., 2000; Reisman et al., 2002). As Wnt signaling is functional in many cell types, deletion of Brg might block their proliferation via *Myc* repression, which should override the hyperproliferative tendency resulting from Rb inactivation. Together with the finding that Brg directly binds the TCF site in the *Myc* promoter, our data support the previous observations that Brg interacts with β-catenin and regulates Wnt target genes in colon cancers (Barker et al., 2001). However, a conclusive demonstration of Brg acting downstream of Wnt in T cells needs additional evidence such as genetic interactions between *Brg* and *TCF*.

BAF mutations also impaired pre-TCR signaling, as revealed by the failure of DN3 cells to express CD69 or develop into DP cells upon pre-TCR signaling in *BAF* mutants. The defect in pre-TCR signaling may account for the cell cycle arrest of Brg-deficient DN3 cells. However, BAF complexes were not essential for pre-TCR induced *Myc* expression, and *Myc* mRNA levels in Brg-deficient DN3 cells did not appear to be decreased (data not shown). Thus pre-TCR might use BAF complexes to regulate other molecules to effect proliferation. The death of Brg-deficient DN3 cells was not due a defect in pre-TCR signaling, as Bcl-xL effectively suppressed the death. Neither is it likely to be caused by the defect in Wnt signaling, because *TCF* mutations do not significantly deplete DN3 cells. These data point to additional signaling pathways controlling the survival of DN3 cells. That Brg was dispensable for pre-TCR to promote cell survival or induce *Myc* demonstrates the specificity of Brg function.

### Role of Brg in *Myc* Induction via DNA Melting

An unusual feature in human *Myc* induction is that it requires single stranded DNA binding proteins such as FBP, which binds the far upstream sequence (*FUSE*) (He et al., 2000; Levens et al., 1997; Michelotti et al., 1996). However, FBP cannot bind *FUSE* unless *FUSE* is first melted, but *FUSE* only melts as *Myc* is expressed. That Brg directly binds the *Myc* promoter suggests one solution to this dilemma: TCF first recruits BAF complexes, which subsequently denature neighboring DNA sequences by producing torsional stress (Havas et al., 2000). Indeed, the *FUSE* in the human *Myc* promoter is located 451 bp upstream of a functional TCF site (He et al., 1998), and a putative mouse FUSE is located only 182 bp upstream of the proximal TCF site that binds Brg in vivo (D. Levens, personal communication; see Supplemental Figure S5 at http://www.immunity.com/cgi/content/full/19/2/169/DC1).

## Sequential Roles of Brg in Developmental Transitions

Bcl-xL overexpression, though able to rescue the death of Brg-deficient cells, failed to restore T cell developmental progression. Thus Brg has a specific role in T cell differentiation independent of cell survival or proliferation. A parsimonious explanation is that the disruptions of the Wnt and pre-TCR pathways contribute to or even account for the defects in the differentiation of Brg-deficient cells. However, it has been argued that Wnt and pre-TCR propel DN cell development largely by promoting DN cell survival and/or proliferation as opposed to bona fide differentiation (Jiang et al., 1996; Newton et al., 2000; Petrie et al., 2000; Wu and Strasser, 2001). Therefore, additional or even distinct signals may use Brg to drive DN cell differentiation. Recent observations indicate that chromatin remodeling complexes can be regulated by inositol derivatives. Thus PIP2 induces the rapid association of the complexes with chromatin in vitro (Zhao et al., 1998) apparently via a direct interaction with Brg (Rando et al., 2002). Similarly, inositol polyphosphates directly regulate nucleosome mobilization by multiple yeast chromatin remodeling complexes in vitro, and influence chromatin remodeling in vivo (Shen et al., 2003; Steger et al., 2003). Thus BAF complexes might act as inline signaling molecules in inositol signaling. Inositol signaling is thus a good candidate for the additional control of Brg-dependent developmental transitions.

The effect of chromatin remodeling in response to transient signals is not restricted to immediate gene regulation. Chromatin alteration is often initiated and inherited without concomitant or sustained changes in gene expression, but functions to pattern the chromatin and poise the genes for later induction (Agarwal et al., 1999). A classical example is that the *IL-2* locus is partially open in T cell lineage well before actual gene expression, but is completely closed in nonhematopoietic cells (Siebenlist et al., 1986). Thus, external signals may use Brg to pattern chromatin in developing thymocytes (Figure 6, indicated by the changing colors of the nuclei). The stably altered chromatin at one stage may influence gene induction at subsequent stages. By this means a prior signal such as Wnt could program cellular responses to a subsequent signal such as the pre-TCR (Figure 6). Progressive chromatin patterning through successive developmental stages may culminate in the establishment of chromatin configurations compatible with the functional potential of mature T cells. We speculate that defects in chromatin patterning may trigger a checkpoint mechanism leading to the arrest of the developing cells, even though the aberrant chromatin does not directly perturb transcription. Such a mechanism may explain the multiple developmental arrests in Brg-deficient cells despite the limited change in gene expression.

### Experimental Procedures

#### Mice

The floxed Brg mice will be described elsewhere. The Lck-Cre (Lee et al., 2001), Bcl-xL (Chao and Korsmeyer, 1997) transgenic mice and germline Brg knockout mice (Bultman et al., 2000) were published. All these mice were maintained on mixed genetic background at Stanford Animal Facility. c-Kit null mice (WBB6F1/J-Kit$^{W}$/Kit$^{Wv}$) were purchased from Jackson lab.

#### Flow Cytometry

Single-cell suspensions were prepared from 3-7 week mice and typically stained with antibodies conjugated to APC, Texas Red, FITC, PE and Cy5; propidium iodide (PI) was used to eliminate dead cells. Specifically, to analyze CD4/CD8 expression in various subsets of DN cells, cells were stained with anti-CD4-APC, anti-CD8-Texas-Red, anti-CD25-FITC, anti-CD44-PE, anti-CD3/B220-Cy5/PI, and the Cy5/PI$^{-/lo}$ populations, representing live immature thymocytes, were resolved into DN1-3 and post-DN3 cells as described (Chi et al., 2002). To analyze c-Kit expression, cells were stained with anti-CD4-APC, anti-CD25-biotin, anti-CD44-PE, anti-CD3/8/TCR$\beta$/B220-Cy5/PI, anti-c-Kit –FITC, washed, and stained with Straptavidin-Texas Red. IL-7R$\alpha$ expression was determined by first staining the cells with anti-CD4-APC, anti-CD25-FITC, anti-CD44-PE, anti-IL7R$\alpha$-biotin, anti-CD3/8/TCR$\beta$/B220-Cy5/PI before incubation in Straptavidin-Texas Red. To assay cell proliferation, BrdU (10ug/ul) was injected intraperitoneally at 50ug/g weight and mice sacrificed 15 min later. Cell surface markers were stained with the same antibodies as in the case of c-Kit analysis except that that PI and anti-c-Kit were omitted. Cells were then fixed, permeabilized and subsequently stained with anti-BrdU-FITC using BrdU-Flow Kit (Pharmingen) following the manufacturer's protocol. Bcl-2 and PCNA expression was determined as in the case of BrdU, except that anti-Bcl-2-FITC and anti-PCNA-FITC, respectively, were used instead of anti-BrdU-FITC.

#### PCR, RT-PCR and Chromatin IP

Primers for detecting the floxed Brg allele (Sumi-Ichinose et al., 1997), Brg and Brm transcripts (Bultman et al., 2000) have been described. To measure the target genes of various signaling pathways, we performed real-time RT-PCR on Icycler (Biorad) using Quantitect One-Step RT-PCR mix (Qiagen). Mature V$\beta$ transcription and VDJ recombination were measured based on published PCR strategy (Anderson et al., 1992). ChIP was performed as described (Chi et al.,2002) except that real time PCR was used to quantify precipitated DNA. Primer sequences and PCR conditions are available upon request.

#### Acknowledgments

We thank Stan Korsmeyer for Bcl-xL mice, Terry Magnuson for Brg null mice, Andreas Strasser, Hans Clevers and David Levens for advice. TC is supported by a fellowship from Leukemia and Lymphoma Society of America and HHMI, CBW by a grant from the NIH HD39454. GCR is an investigator of HHMI. Additional support was from the NIH CA39612.

Received: April 10, 2003
Accepted: April 30, 2003
Published: August 19, 2003

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