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Negative regulation of CD8 expression via CD8 enhancer-mediated recruitment of the zinc finger protein MAZR

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Abstract

Coreceptor expression is tightly regulated during thymocyte development. Deletion of specific *Cd8* enhancers leads to variegated expression of CD8 $\alpha\beta$ heterodimers in double-positive (DP) thymocytes. Here we show CD8 variegation was correlated with an epigenetic “off” state, linking *Cd8* enhancer function with chromatin remodeling of the adjacent genes *Cd8a* and *Cd8b1* (*Cd8*). We show the zinc finger protein MAZR, encoded by the *Zfp278* gene, bound the *Cd8* enhancer and interacted with the corepressor N-CoR complex in double-negative thymocytes. MAZR was down-regulated in DP and single-positive CD8⁺ thymocytes. Enforced expression of MAZR led to impaired *Cd8* activation and variegated CD8 expression. Our results demonstrate epigenetic control of the *Cd8* gene loci and identify MAZR as an important regulator of *Cd8* gene expression.

Cell fate specifications during T lymphocyte differentiation are linked with spatial and temporal control of the expression of developmentally regulated genes¹. Previous work has shown that epigenetic modifications are an important regulatory level to control gene expression^{2,3}. *Cd4* and *Cd8* (i.e. *Cd8a* and *Cd8b1*) coreceptor gene expression have also been suggested to be epigenetically regulated⁴. For CD4, a T cell-specific enhancer located 13 kb upstream of the *Cd4* promoter directs expression in both helper and cytotoxic T cells⁵, while a silencer element located in the first intron of *Cd4* provides subset specificity by silencing the transcription of *Cd4* in double-negative (DN) thymocytes and mature CD8⁺ T cells⁶⁻⁸. Conditional gene targeting in mice showed that the silencer element is required for establishment but not maintenance of *Cd4* silencing, suggesting that the silencing of CD4 expression in mature CD8⁺ T cells is regulated at the epigenetic level^{9,10}.

For CD8 (usually consisting of CD8 α and CD8 β heterodimers on thymic-derived T cells) at least five distinct *Cd8 cis*-acting elements (E8_I – E8_V) were identified in transgenic reporter expression assays that individually or in combination directed expression in the T cell lineage¹¹. These studies indicate a complex regulatory network of developmental stage- and subset-specific *cis*-regulatory elements to achieve proper expression of the linked *Cd8a* and *Cd8b1* genes during T cell development. To analyze the various *cis*-elements in more detail, mice with single and combinatorial germline deletion analysis of enhancers were generated^{12,15}. Individual deletions of either enhancer E8_I or E8_{II} did not alter the

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interest.

expression pattern of CD8 during T cell development, while deletion of both enhancers impaired the expression of CD8 in double-positive (DP) thymocytes¹⁴. A population of “CD8-negative” DP thymocytes appeared that was indistinguishable from DP thymocytes by the expression of other surface markers and functional phenotype. The concurrent appearance of “CD8-negative” DP thymocytes in addition to bona fide DP cells indicated variegation of CD8 expression in the absence of E8_I and E8_{II}. Additional studies have shown that a similar phenotype is observed upon deletion of the *Cd8 cis*-element designated cluster II (E8_V), by the combined deletion of E8_{II} and E8_{III}, or by altering the activity of either the BAF (Brahma-related gene/Brahma-associated factor) chromatin remodeling complex, or of members of the Ikaros family¹⁵⁻¹⁸. Collectively, these results suggest that *Cd8a* and *Cd8b1* gene expression is epigenetically regulated and that some of the *Cd8 cis*-regulatory elements may function as recruitment sites for factors involved in chromatin remodeling to facilitate activation of the *Cd8a* and *Cd8b1* gene loci during the DN to DP transition of T cell development.

To investigate whether deletion of E8_I and E8_{II} leads to alteration of chromatin remodeling during T cell development, we have compared the epigenetic state of the *Cd8a* and *Cd8b1* gene loci in sorted “CD8-negative” and bona fide DP thymocytes isolated from E8_I,E8_{II} doubly deficient. Chromatin immunoprecipitation (ChIP) assays and DNA methylation analysis revealed that “CD8-negative” DP thymocytes have epigenetic modifications at the *Cd8a* and *Cd8b1* gene complex that correspond to an “off” state of chromatin. Variegation of CD8 expression in DP thymocytes could be partially reverted by intercrossing E8_I,E8_{II} doubly deficient mice with conditional DNA methyltransferase 1 (Dnmt1)-deficient mice¹⁹, further indicating a partial “epigenetic block” of CD8 expression due to the absence of *cis*-regulatory elements. To study the molecular basis of the epigenetic regulation of CD8 expression in more detail, we searched for factors that bind to enhancer E8_{II}. Yeast one-hybrid screening approaches led to the isolation of the zinc finger protein MAZR (Myc-associated Zn finger related factor²⁰). MAZR was highly expressed in DN thymocytes and its expression was down-modulated with progressive thymocyte differentiation. We could show that *Cd8* enhancers recruit MAZR to the *Cd8* gene loci. MAZR interacts via its N-terminal domain, which contains a BTB-POZ motif (bric-a-bric, tramtrack, broad complex; poxvirus zinc finger) with nuclear receptor corepressor (N-CoR) complexes in DN thymocytes. Retroviral-mediated constitutive expression of MAZR during T cell development unexpectedly resulted in variegated expression of CD8 in DP thymocytes, suggesting that MAZR negatively regulates chromatin modification at the *Cd8* gene loci required for the activation of CD8 expression. Thus, our results show that CD8 expression is regulated at the epigenetic level and imply that CD8 expression is actively repressed at the DN stage. Furthermore, they indicate that *Cd8* enhancers have the potential to function both as negative and positive *cis*-acting elements by recruiting factors that block or facilitate chromatin modifications required for a transcriptional “on” state, respectively.

RESULTS

Epigenetic changes in E8_I,E8_{II} doubly deficient mice

Germline deletion of E8_I and E8_{II} leads to the appearance of a population of CD4 SP cells with low to intermediate CD3 (CD3^{lo}) and intermediate CD5 (CD5^{int}) surface expression phenotype characteristic of DP thymocytes¹⁴. These cells represent DP thymocytes that fail to express CD8 and therefore we refer to them as “CD8-negative” DP cells. In parallel with the appearance of “CD8-negative” DP cells the percentage of bona fide DP cells was reduced. These results demonstrate variegated expression of CD8 in DP thymocytes and point towards epigenetic regulation of the *Cd8* gene complex. They also suggest that E8_I and E8_{II} may serve as recruitment sites for chromatin modifying factors during the DN to DP transition of thymocyte development. To investigate whether E8_I and E8_{II} function as

recruitment elements, we analyzed epigenetic modifications at the *Cd8a* and *Cd8b1* gene complex in CD8-expressing and “CD8-negative” DP thymocytes in E8_I,E8_{II} doubly deficient mice. To obtain a pure population of “CD8-negative” DP thymocytes devoid of any mature CD4 SP thymocytes for ChIP experiments, thymocytes from E8_I,E8_{II} doubly deficient mice backcrossed onto a *Tcra*^{-/-} background were used (Fig. 1a, as previously shown¹⁴). “CD8-negative” and bona fide DP thymocytes were isolated and analyzed by ChIP experiments for differences in histone modifications, including H3 and H4 acetylation and H3 lysine 4 (H3K4) tri-methylation content at several regions within *Cd8* gene loci. The non-expressing *Cd8* alleles showed decreased H3 and H4 acetylation at all amplicons analyzed as compared to the expressing *Cd8* alleles (Fig. 1b). In contrast, the abundance of acetylated H3 and H4 was not reduced at the *Cd4* promoter or *Gapdh* gene regions. A similar correlation was also observed for the *Cd8a* and *Cd8b1* promoter loci with respect to H3K4 tri-methylation, a modification associated with promoters of transcriptionally active genes²¹. We also performed ChIP assays with antibodies against di- or tri-methylated histone H3K9, a histone modification associated with transcriptionally inactive chromatin²². However, we found that no *Cd8a* and *Cd8b1* gene complex specific fragments could be precipitated, indicating that H3K9 methylation is not involved in the regulation of CD8 expression during the DN to DP transition (data not shown).

As thymocytes develop from the DN to the DP stage progressive CpG demethylation of DNA at the *Cd8* gene loci occurs concomitantly with the initiation of co-receptor expression^{23,24}. This finding suggests that CpG demethylation is one of the epigenetic changes required for (or correlating with) the proper initiation of CD8 expression. To determine whether expressing and non-expressing *Cd8* alleles display differences in CpG methylation, DNA from “CD8-negative” and bona fide DP thymocytes from E8_I,E8_{II} × *Tcra*^{-/-} mice was isolated. DNA methylation was assayed by treating the DNA with sodium bisulfite followed by PCR amplification as described previously¹⁹. While CD8-expressing DP thymocytes contained clusters of non-methylated CpG dinucleotides upstream of the *Cd8a* and *Cd8b1* promoter proximal regions, the respective CpG clusters were almost completely methylated in “CD8-negative” DP thymocytes (Fig. 2).

Loss of Dnmt1 leads to partial reactivation of CD8

To investigate whether the failure to activate the *Cd8* gene loci properly in E8_I,E8_{II} doubly deficient mice in “CD8-negative” DP thymocytes was caused by the sustained CpG methylation of the *Cd8a* and *Cd8b1* gene complex, E8_I,E8_{II} doubly deficient mice were intercrossed with conditional Dnmt1-deficient mice. These mice have a “floxed” *Dnmt1* gene and express Cre-recombinase under the tissue-specific *Lck* proximal promoter, which becomes active early at DN2 stage of thymocyte development¹⁹. Deletion of Dnmt1 in DN thymocytes could partially rescue the variegated expression phenotype of E8_I,E8_{II} doubly deficient mice as revealed by the reduction of “CD8-negative” DP cells (Supplementary Fig. 1 online). This result highlights the role of *Cd8* enhancers in mediating *Cd8* gene loci-specific chromatin modifications.

MAZR binds to E8_{II} *in vivo*

The results described above indicated that E8_I and E8_{II} may serve as recruitment sites for factors that regulate chromatin. By using a combination of bioinformatics approaches and luciferase reporter gene transactivation assays, a 154 bp E8_{II} core enhancer containing two regulatory elements (RE) was identified within the 4.4 kb genomic E8_{II} fragment (I.B. and W.E., manuscript in preparation). Deletion of RE-1 (nucleotides 123-154) resulted in a complete loss of enhancer activity (data not shown). Yeast one-hybrid screens (YOH) using RE-1 as bait and a GAL4-activation domain (AD) 1200M murine thymoma cell line cDNA fusion library as prey led to the isolation of a cDNA fragment that encoded the murine zinc

finger protein MAZR. MAZR is a 641 amino acid long transcription factor that is encoded by the *Zfp278* gene. In addition to the N-terminal BTB-domain it contains seven C₂H₂ zinc fingers²⁰. The YOH clone insert encoded the first 409 aa including the BTB-domain and three complete zinc finger domains (Supplementary Fig. 2a online). MAZR binds to guanine-rich sequences²⁰, which were also present within RE-1 (Supplementary Fig. 2b online). Binding of MAZR to RE-1 was confirmed *in vitro* by electrophoretic mobility shift assays (EMSA) using a 5' Myc-tagged version of MAZR (Fig. 3a).

To determine whether MAZR binds also to E8_{II} *in vivo*, ChIP experiments were performed. Therefore, polyclonal rabbit antibodies were generated against a glutathione S-transferase (GST)-MAZR fusion protein (aa 146-293) (see Supplementary Fig. 3 online for anti-MAZR serum specificity). Since MAZR expression was highest in DN thymocytes (Fig. 3c and Fig. 3d), anti-MAZR serum was used to immunoprecipitate MAZR–DNA complexes from formaldehyde-treated DN thymocytes. MAZR–DNA complex precipitations with the anti-MAZR serum led to a selective enrichment of E8_{II} sequences compared to mock precipitations (pre-immune serum), indicating *in vivo* binding of MAZR to E8_{II} (Fig. 3b).

Downregulation of MAZR during T cell development

MAZR is expressed in multiple tissues including fetal liver, bone marrow and thymus²⁰. To determine the expression pattern of MAZR in various thymocyte subsets, real-time quantitative PCR (qPCR) analysis and immunoblot analysis was performed on sorted DN, DP, CD8 SP and CD4 SP thymocyte populations. MAZR was expressed more abundantly in DN thymocytes as compared to DP cells (Fig. 3c and Fig. 3d). MAZR expression was further reduced in CD8 SP thymocytes. Almost no MAZR expression was detected in peripheral T cells (Fig. 3d).

MAZR induces variegation of CD8 expression

MAZR was originally isolated as an interacting protein of the B cell and neuronal transcriptional repressor Bach2. MAZR can also transactivate the *Myc* and *Fgf4* promoter in transient reporter expression assays²⁰. Since MAZR interacted with E8_{II}, we investigated whether it regulates *Cd8a* and *Cd8b1* gene expression. Therefore, MAZR was ectopically expressed in thymocytes using retroviral-mediated gene transduction of hematopoietic stem cells followed by bone marrow (BM) transplantation into irradiated recipients (see Supplementary Fig. 4a online for the constructs used). Enforced expression of MAZR resulted in a small increase in the percentage of CD4 SP thymocytes leading to an approximately two-fold increase of the CD4/CD8 ratios in GFP⁺ thymocytes as compared to GFP[−] thymocytes (2.0 ± 0.7 , $n = 5$) (Fig. 4b and data not shown). In contrast, GFP⁺ thymocytes transduced with the parental MIG-R vector had no increase in the percentage of CD4 SP thymocytes, resulting in similar CD4/CD8 ratios in GFP⁺ and GFP[−] thymocytes (1.1 ± 0.1 , $n = 5$).

To investigate the increase in the percentage of CD4 SP thymocytes in more detail, the developmental stage of MAZR-transduced CD4 SP cells was determined. Mature CD4 SP thymocytes have high surface expression of CD3 and CD5, while immature DP thymocytes are CD3^{lo}CD5^{int}²⁵, a phenotype that was also observed in MIG-R transduced CD4 SP and DP thymocytes, respectively (Fig. 4b). In contrast, the GFP⁺ CD4 SP population displayed two distinct cell subsets in thymocytes expressing MAZR constitutively. In addition to mature CD3^{hi}CD5^{hi} CD4 SP cells, a CD3^{lo}CD5^{int} CD4⁺CD8[−] subset was detected in all mice reconstituted with the MAZR-transduced BM (Fig. 4b and Fig. 5a, left panel). Furthermore, CD4⁺CD3^{lo} cells developed in chimeric *Rag2*^{−/−} mice reconstituted with MAZR-transduced *Tcra*^{−/−} BM (Supplementary Fig. 5 online). This finding suggests that these cells develop before the onset of positive selection. The appearance of the “CD8-

negative” DP subset was more pronounced when gated only on cells expressing high amounts of GFP, which correlated with higher MAZR expression (Fig. 5a). This result demonstrated a dose-dependent influence of MAZR on the expression of CD8. The phenotype in MAZR expressing thymocytes is thus reminiscent of the appearance of “CD8-negative” DP thymocytes and the CD8 variegation phenotype observed in E8_I,E8_{II} doubly deficient mice or in cluster II (E8_V)-deficient mice, thus linking MAZR to the regulation of CD8 expression.

Variegation of CD8 in the absence of E8_I or E8_{II}

Enforced expression of MAZR might negatively regulate *Cd8a* and *Cd8b1* gene expression through interaction with E8_{II}. In contrast to combined deletions, deletion of either E8_I or E8_{II} alone had only a marginal effect on the expression of CD8 during thymocyte development^{12,14}. Thus, a potential repressive role of MAZR acting at E8_{II} could be counterbalanced by compensatory factors acting at E8_I. To test this possibility, E8_I-deficient BM cells were transduced with MAZR-expressing retrovirus and transplanted into irradiated E8_I-deficient mice. The percentage of “CD8-negative” DP thymocytes (CD4⁺CD3^{lo}CD5^{int}) upon forced MAZR expression increased substantially in E8_I-deficient thymocytes as compared to wild-type thymocytes (Fig. 5a, middle). To confirm that the increased CD4⁺CD3^{lo} cells in MAZR-transduced E8_I-deficient BM chimeric mice had a DP phenotype (and were not mature CD4 SP thymocytes that for unknown reasons are CD3^{lo} and CD5^{int}), CD4⁺CD3^{lo}, DP and CD4⁺CD3^{hi} cells were sorted and the expression of Th-POK (also known as cKrox) was determined. Th-POK is induced in developing CD4 SP thymocytes upon T cell receptor stimulation of DP cells and is a master regulator for CD4 lineage differentiation^{26,27}. While Th-POK was expressed in CD4⁺CD3^{hi} cells, it was not expressed in DP or in CD4⁺CD3^{lo} cells (Fig. 5b). This finding indicated that the CD4⁺CD3^{lo} cells are not mature CD4 SP cells and thus showed again the presence of “CD8-negative” DP cells upon forced expression of MAZR.

To determine whether E8_{II} is the only recruitment site and therefore required for MAZR function, E8_{II}-deficient BM cells were transduced with MAZR retrovirus and transplanted into irradiated E8_{II}-deficient mice. Increased variegation was also observed upon forced expression of MAZR in E8_{II}-deficient thymocytes (Fig. 5a, right). This finding indicates that in addition to E8_{II} other *Cd8 cis*-regulatory elements were able to recruit MAZR to the *Cd8a* and *Cd8b1* gene complex.

Multiple MAZR binding sites at the *Cd8* gene loci

Nucleotide sequence analysis based on the MAZR binding motif within E8_{II} RE-1 and the published MAZR consensus binding site revealed at least 11 additional potential binding sites within the five known *Cd8 cis*-regulatory elements. ChIP experiments were performed to determine whether MAZR interacts with these sites. MAZR–DNA complex precipitations from DN thymocytes with the polyclonal rabbit anti-mouse MAZR serum led to a selective enrichment of at least 7 of the 11 PCR fragments containing the binding sites compared to fragments without MAZR binding sites or to mock (pre-immune serum) precipitations (Fig. 6a). Thus, MAZR is recruited to multiple sites within the *Cd8* gene loci in DN thymocytes. To test whether MAZR was recruited to these sites in DP cells, comparative ChIP experiments between DN and DP thymocytes were performed. At least 4 of the 7 amplicons were less amplified in DP thymocytes as compared to DN cells, indicating reduced MAZR binding at the *Cd8a* and *Cd8b1* gene complex in DP cells (Fig. 6b).

MAZR interacts with the N-CoR corepressor

The BTB domains of most known transcription factors are able to interact with N-CoR and/or SMRT (silencing mediator of retinoic acid and thyroid hormone receptor)

corepressors^{28,31}. This interaction is dependent on critical conserved amino acid residues^{28,31} that are also present within the BTB domain of MAZR (Supplementary Fig. 4c online). To test whether the BTB domain of MAZR interacts with N-CoR as well, co-immunoprecipitation experiments were performed. HEK293T cells were transfected with N-CoR and either wild-type MAZR or a mutant form of MAZR (MAZR^{mut}) containing point mutations within the conserved amino acid residues of the BTB domain required for corepressor interaction²⁸. MAZR interacted with N-CoR in a BTB domain-dependent manner, since only wild-type but not MAZR^{mut} co-immunoprecipitated with N-CoR (Fig. 7a). More importantly, interaction between MAZR and N-CoR was also observed in primary DN thymocytes (Fig. 7b). Thus, these findings point to a potential molecular mechanism of how MAZR negatively regulates the expression of CD8.

The BTB domain alone does not induce CD8 variegation

MAZR binds to E8_I and other *Cd8* *cis*-regulatory elements, suggesting a direct regulation of CD8 expression. However, forced expression of MAZR could also sequester endogenous factors from the *Cd8a* and *Cd8b1* gene complex, resulting in an indirect influence of MAZR on CD8 expression. Thus, additional BM transplantation experiments with truncated forms of MAZR (see Supplementary Fig. 4b online for MAZR truncations) were performed to discriminate whether CD8 expression is directly or indirectly regulated by MAZR. None of the C-terminal truncations of MAZR gave variegated expression of CD8 (Fig. 8), even though the truncated molecules retained their capacity to localize to the nucleus and were still able to bind N-CoR (data not shown). These data indicate that MAZR regulates CD8 expression directly by binding to the *Cd8* gene loci.

DISCUSSION

It has been shown previously that the combined deletion of *Cd8* enhancers E8_I and E8_{II} leads to variegated expression of CD8 in DP thymocytes¹⁴. Here we demonstrate that CD8 variegation correlated with an epigenetic “off” state of the *Cd8a* and *Cd8b1* gene complex in cells that failed to express CD8, thus linking *Cd8* enhancers to *Cd8* gene loci-specific chromatin modification pathways. We further show that the zinc finger protein MAZR interacts in DN thymocytes with CD8 enhancers and with N-CoR corepressor complexes. Expression of MAZR was down-regulated during thymocyte development, and forced expression of MAZR caused impaired activation and variegated expression of CD8 in DP thymocytes. Our results demonstrate epigenetic control of the *Cd8* gene loci and identify MAZR as an important regulator of *Cd8* gene expression. The results also imply that CD8 is actively repressed at the DN stage, and indicate that CD8 enhancers can function either as negative or positive *cis*-acting elements by recruiting factors that block or facilitate chromatin modifications required for an epigenetic “on” stage.

Combined deletion of E8_I and E8_{II} causes variegation of CD8 expression, resulting in the development of a subset of DP thymocytes that does not express CD8 (ref. ¹⁴). These “CD8-negative” DP thymocytes showed lower histone H3 and H4 acetylation over the whole *Cd8* gene loci. Furthermore, these cells showed lower H3K4 tri-methylation and sustained DNA methylation at the *Cd8a* and *Cd8b1* promoter region compared to bona fide DP thymocytes. Therefore, all epigenetic patterns analyzed indicate that the *Cd8a* and *Cd8b1* gene complex is locked in an epigenetic “off” state in “CD8-negative” DP thymocytes. However, thymocytes that failed to activate the *Cd8* gene loci due to deletion of *cis*-regulatory elements have not completely lost the potential to express CD8. Conditional deletion of Dnmt1 at the DN2 stage leads to a partial re-activation of CD8 expression and therefore to a reduction of CD8 variegation. It has been shown that deletion of Dnmt1 at the DN2 stage is accompanied by demethylation of the *Cd8* gene loci (but not induction of CD8 expression in TCRαβ-lineage DN cells)¹⁹. Thus, removal of negative epigenetic marks at the *Cd8* gene

loci at the proper developmental stage can at least partially rescue impaired CD8 expression in DP thymocytes. This observation highlights the need for *Cd8 cis*-regulatory elements to recruit factors that facilitate epigenetic modifications required for initiating CD8 expression.

Differences in the epigenetic pattern resulting from deletion of *cis*-regulatory elements at the *Cd8* gene loci imply that E8_I and E8_{II} (and probably other *cis*-regions) recruit factors that induce activating chromatin modifications. These modifications alter the repressive state present at early stage DN thymocytes and therefore it was expected that factors that bind to *Cd8* enhancers positively influence the expression of CD8. However the observation that forced expression of MAZR causes variegation and therefore negatively influences CD8 expression indicates a more complex regulatory activity of *Cd8 cis*-acting elements. Depending on the developmental stage, *Cd8* enhancers recruit either negative or positive regulators of CD8 expression. Members of the BAF chromatin remodelling complex and the Ikaros gene family have been shown to act as positive regulators^{16,18}. Recently it has been suggested that Runx3 (recruited by E8_I) is another positive acting factor required for CD8 expression after positive selection in CD8 SP thymocytes³². In contrast, MAZR appears to function as a negative regulator of CD8 expression in DN thymocytes. It is not known whether the same or distinct sites within these enhancers are required for the recruitment of positive or negative acting factors.

ChIP assays have shown that MAZR interacts *in vivo* with several CD8 enhancers in DN thymocytes. Multi-site recruitment has also been observed for members of the Ikaros gene family. Several functional binding sites for Ikaros were shown to overlap with hypersensitivity sites within E8_I and E8_V¹⁶. Thus, recruitment of either negative or positive acting regulatory factors to multiple sites with the *Cd8a* and *Cd8b1* gene complex seems to ensure the proper and coordinated regulation of CD8 expression. To understand the complex regulatory pattern, it will be important to determine the developmental order of recruitment of MAZR and other factors by using ChIP assays combined with *Cd8* gene loci-specific DNA microarray analysis ("ChIP on CHIP").

We could demonstrate that MAZR interacted in a BTB domain dependent manner with N-CoR upon overexpression in cell lines. More importantly, this interaction was also observed in primary DN thymocytes. N-CoR and the related SMRT corepressor molecules are components of complexes containing proteins involved in epigenetic modifications of chromatin templates (like Sin3A, NURD, SWI/SNF-like BAF and histone deacetylases) and it has been shown that N-CoR is recruited by several transcription factors^{33,34}. The association with histone deacetylases has been suggested as part of the mechanism of how N-CoR interacting transcription factors are able to repress transcription. Thus, enhancer-mediated recruitment of MAZR and MAZR–N-CoR interactions may provide a molecular mechanism by which epigenetic modifications corresponding to a repressive state are introduced at the *Cd8* gene loci in DN thymocytes. However, N-CoR-deficient mice have a block at the DN stage of thymocyte development³³, indicating that the absence of N-CoR alone is not sufficient for allowing CD8 expression in DN thymocytes. Progression to the DP stage correlates with a down-modulation of both N-CoR and MAZR, leading to a reduction of MAZR at the *Cd8a* and *Cd8b1* gene complex, and thus allowing positive regulators to overcome the repressive state and to activate CD8. If high concentrations of MAZR due to forced expression are maintained, the balance between repressing and activating complexes at the *Cd8* gene loci is disturbed, thus leading to variegated expression of CD8 and the appearance of "CD8-negative" DP thymocytes. This hypothesis is supported by the observation that the degree of MAZR-induced CD8 variegation is increased in the absence of E8_I or E8_{II} that serve also as recruitment sites for positive acting factors. Nevertheless, one could argue that due to the high amounts of MAZR upon forced expression endogenous factors are sequestered from the *Cd8* gene loci, thereby leading to

CD8 variegation. However, we consider this scenario unlikely since forced expression of a form of MAZR that lacks the C-terminal zinc fingers (MAZR Δ ZF) did not induce variegation. This truncated version retained a properly folded BTB domain that could bind N-CoR, localized to the nucleus, and should therefore be able, like the full-length form, to sequester endogenous factors from the *Cd8* gene loci. Thus, these data together with result from ChIP assays strongly indicate that MAZR acts directly at the *Cd8a* and *Cd8b1* gene complex.

Finally, our results imply active repression of CD8 expression in DN thymocytes. Recruitment of MAZR and N-CoR corepressor complexes via *Cd8* enhancers may therefore prevent CD8 expression in DN cells. Since it has been shown that premature transgenic expression of CD8 in DN2 and DN3 cells causes a partial developmental block leading to reduced numbers of DP thymocytes³⁵, active repression of CD8 expression by MAZR in DN thymocytes may be essential for β -selection and proper T cell development.

Taken together, we propose that CD8 enhancers regulate CD8 expression by controlling the balance between closed and open state of chromatin at the *Cd8* gene loci. Dependent on the developmental stage, they recruit either negative- or positive-acting regulators of CD8 expression. MAZR appears to function as a negative regulator of CD8 expression in DN thymocytes. Therefore, the gradual down-regulation of endogenous MAZR expression during thymocyte development may be a prerequisite for the proper initiation of CD8 expression.

METHODS

Cell lines and Culture

HEK293T and Phoenix-E cell lines were grown in Dulbecco's modified Eagle's medium, both supplemented with 10% fetal calf serum (FSC) and antibiotics.

Mice

C57BL/6J were purchased from Harlan. Conditional LckCre-Tg $\times$ Dnmt1^{fl/fl} mice, E8_I-deficient and E8_{II}-deficient mice, and *Tcra*^{-/-} $\times$ E8_I,E8_{II} doubly deficient mice have been described^{12,14,19}. All mice were bred and maintained in the animal facility of the Medical University of Vienna. All animal experiments were done according to protocols approved by the Federal Ministry for Education, Science and Art.

Bisulfite sequencing analysis

The bisulfite-based methylation analysis was performed as described¹⁹. The sample was amplified by two sequential PCR reactions. The primers used were described previously¹⁹ or are listed in Supplementary Table 1 online. The gel-purified PCR products were cloned into the pCR2.1-TOPO TA cloning vector (Invitrogen), and 12 individual clones were sequenced for each group.

Chromatin immunoprecipitation (ChIP)

10⁷ DP, "CD8-negative" DP, or DN thymocytes (purity of each population >90%) were treated with 0.3% formaldehyde in PBS for 5 min at 37 °C, followed by addition of glycine (0.25 M final concentration). After washing twice with PBS, cells were lysed on ice and sonicated to obtain 0.5-1 kb sheared chromatin fragments. Subsequent ChIP steps were performed according the protocol from Upstate Biotechnology. 5 μ l of anti-AcH3 (#06-599; Upstate Biotechnology), 10 μ l of anti-AcH4 (#06-866; Upstate Biotechnology), 8 μ l of anti-(Me)₃H3K4 (#07-473; Upstate Biotechnology), 3 μ l anti-tri- or anti-di-methyl-H3K9 (kindly provided by T. Jenuwein, Research Institute of Molecular Pathology, Vienna,

Austria) 10 μ l of anti-MAZR rabbit serum or rabbit pre-immune serum were used per reaction. The presence of DNA target sequences was assessed by PCR. The PCR was carried out for 3 min at 95 °C, followed by 40 cycles of 30 sec at 95 °C, 45 sec at 58 °C, and 1 min at 72 °C. PCR products were resolved on a 2% agarose gel and quantified using LumiImager analyzer and Lumianalyst software package (Roche). Signal intensities at 1:3 input dilutions were used to calculate relative signal intensities for each amplicon. The relative values (arbitrary units) for each amplicon are shown below the PCR blots and were calculated according to ref. ³² : $[(\text{intensity antibody precipitates}) - (\text{intensity mock precipitates})]/(\text{intensity input})$. The primers used for the various PCR reactions are listed in Supplementary Table 2 online.

Retroviral constructs and virus production

Full-length or truncated forms of murine MAZR were cloned into the MIG-R retroviral vector (kindly provided by H. Singh, University of Chicago, IL). High-titer viral preparations were generated by transient transfection of retroviral constructs by standard calcium-phosphate precipitation onto the ecotropic producer cell line Phoenix-E. Viral supernatants were harvested 2 and 3 days post-transfection, filtered through 0.45 μ m filters and directly used for the infection of hematopoietic stem cells.

Retroviral infection of hematopoietic stem cells (HSC)

Transduction and transplantation of BM cells was done according to published protocols³⁶ with minor modifications. Briefly, C57BL/6J donor mice were injected intraperitoneally with 5-fluorouracil (5-FU; Sigma) at 10 mg/ml 4 days prior to BM harvest. After red blood cell lysis, BM cells were pre-stimulated for 24h in DMEM (supplemented with 10% FCS, penicillin/streptomycin and L-Glutamine) in the presence of recombinant murine SCF (5 U/ml), IL-6 (10⁴ U/ml) and IL-3 (6 U/ml) (Peprotech).

For retroviral transductions, 1 - 2 $\times$ 10⁷ activated BM cells were transferred to a non-TC 10 cm plate (Sterilin) pre-coated with RetroNectin® (Takara INC). Infection was done according to the manufacturers instructions by incubation with 7 ml of viral supernatant supplemented with SCF, IL-3 and IL-6 at concentrations indicated above for 2-3 rounds. BM cells were harvested 48 h post infection, washed three times with PBS and injected at 0.5 - 3 $\times$ 10⁶ cells/ mouse into the tail veins of lethally irradiated C57BL/6J recipient mice. γ -irradiation was performed at 8.25 Gy using a Hille TH-150 instrument. Mice were given acidified drinking water supplemented with 25 μ g/ml neomycin and 25,000 U/ml polymyxin B sulfate for 1 week.

Isolation of nuclear protein extracts

Nuclear protein extracts were prepared as described³⁷. Briefly, HEK293T cells (total of 1 $\times$ 10⁷ cells) were harvested and washed once with PBS. The cells were pelleted (280 $\times$ g for 5 min) and resuspended in 1 ml of cold buffer A (10 mM HEPES; pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitors; Roche). Following a 10 min incubation on ice, 50 μ l of 10% NP-40 was added, and the cells were lysed for additional 10 min on ice. The nuclei were pelleted (280 $\times$ g for 4 min) and resuspended in 100 μ l buffer C (20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 420 mM NaCl, 25 % glycerol, 0.2 mM EDTA, 0.5 mM DTT and 0.2 mM PMSF) for the extraction of nuclear proteins. After 30 min incubation on ice, the extract was pelleted at 16,000 $\times$ g in a microcentrifuge for 3 min at 4 °C. The supernatant was snap frozen in liquid N₂ and stored at -80 °C until use.

Electrophoretic mobility shift assay (EMSA)

Duplex oligonucleotides were labeled with T4 polynucleotide kinase and γ -[^{32}P]-ATP. Labeled oligonucleotides (2.5×10^4 c.p.m.) were incubated in the binding reaction buffer (10 mM Tris-HCl; pH 7.5, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 1 mM MgCl_2 , 4 % glycerol, 1 μg bovine serum albumin (BSA), 1 μg poly dI:dC) with 10 μg of nuclear protein extract HEK293T cell line transfected with Myc-tagged MAZR construct. MAZR cDNA was cloned in-frame into *EcoRI* restriction site of pCDNA3-Myc plasmid (kindly provided by R. de Martin, Medical University of Vienna, Austria). The binding reaction was carried out in a total volume of 15 μl for 30 min at 30°C. For supershift experiments, 0.1, 0.5, 1 or 3 μg of anti-Myc monoclonal antibody (OP10L, Calbiochem) was added to the reactions, respectively. Protein-DNA complexes were separated on a 4% non-denaturing polyacrylamide/bisacrylamide (19:1) gel ($0.5 \times \text{TBE}$) at 10 mA for 2-3 h at 4 °C. Afterwards the gel was dried and exposed to a Storage PhosphorImager Screen (Molecular Dynamics). The following radiolabeled duplex oligonucleotide was used for EMSA (only the sequence of one oligonucleotide strand is indicated: 5'-AGGTGTGCTGCCCCAGGTCCACCCGCAGGAGGAGAGGGGGCT-3'.

Generation of a rabbit anti-MAZR serum

A MAZR fragment (corresponding to aa 146-293) was cloned into the pGEX-5X-1 vector and the GST-MAZR fusion plasmid was transformed into *Escherichia coli* strain BL-21. 800 μg of GST-MAZR¹⁴⁶⁻²⁹³ fusion protein was batch purified using glutathione sepharose 4B according to manufacturer's instructions (Amersham Biosciences). The GST fusion protein was used for rabbit immunizations.

Flow cytometric analysis and antibodies

Thymus was removed from euthanized animals and placed into 60 mm tissue culture dishes containing staining buffer (phosphate-buffered saline supplemented with 2% FCS and 0.1% sodium azide). Single cell suspensions were made by passing the tissue through a 70 μm nylon cell strainer. The cell suspensions were washed once with staining buffer and $0.5 - 5 \times 10^5$ cells were incubated on ice with Fc-block (Pharmingen) for 5 min and subsequently with the appropriate antibodies for 30 min. Afterwards, the cells were washed once with staining buffer and analyzed or incubated with secondary antibodies on ice for 30 min. The following antibodies were used for the stainings: PE-anti-mCD8 α (CT-CD8a), TC-anti-mCD4 (CT-CD4), Cy5-anti-mCD3 ϵ (500A2) and Cy5-anti-mCD8 α (CT-CD8a) from Caltag, PE-anti-mCD5 (53-7.3) from Pharmingen. Cells were analyzed using Becton Dickinson FACSCalibur flow cytometer and Cellquest Pro software (Becton Dickinson).

Cell sorting

Thymocytes were stained with FITC-anti-CD8, TC-anti-CD4, and PE-anti-CD5 antibodies to sort CD4⁻CD8⁻ (DN), CD4⁺CD8⁺ (DP), CD4⁺ (CD4 SP), and CD8⁺CD5^{hi} (CD8 SP) thymocyte subpopulations using FACSARIA cell sorter (Becton Dickinson). For the isolation of peripheral CD4⁺ and CD8⁺ T cells, splenocytes were briefly treated with red blood cell lysis buffer, washed, and stained with FITC-anti-CD4 and FITC-anti-CD8, respectively. Subsequently, the cells were incubated with anti-FITC magnetic beads (Miltenyi) and purified over a MACS LS Column to >97% purity according to the manufacturer's instructions.

Yeast one-hybrid screen

The screen was carried out using the Matchmaker One-Hybrid System according to the manufacturer's instructions (Clontech Laboratories). Three tandem repeats of the E8_{II} RE-1

were inserted into the pHISi-1 vector and used as bait to screen for the interacting proteins. The cDNA expression library was cloned into pACT2 vector.

cDNA synthesis, RT-PCR and real-time qPCR

Total RNA was isolated from sorted thymocyte subsets using TRI reagent (Sigma) and cDNA synthesis was performed using random hexamer and oligo-dT primers and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Cycling conditions for RT-PCR were 2 min at 95 °C, followed by 35 cycles of 30 sec at 95 °C, 45 sec at 60 °C, and 1 min at 72 °C. Real-time qPCR was carried out using SYBR green and the LightCycler detection system (Roche). The real-time qPCR conditions were 5 sec at 95 °C, 5 sec at 65 °C and 15 sec at 72 °C over a total interval of 55 cycles. The following primers were used for MAZR: 5'-GCAGGTGCACACTTCTGAGCGAC-3', 5'-GCATTTCTGGCCTTCTCGGTTACA-3'; for Hprt1: 5'-GATACAGGCCAGACTTTGTTG-3', 5'-GGTAGGCTGGCCTATAGGCT-3'; for Th-POK: 5'-CACCTTCCACCTCTCTGGCTCGA-3', 5'-TTGCTTGCGGTGGTGTCCCA-3'.

Generation of full-length MAZR and site-directed mutagenesis

Full-length *Zfp278* cDNA (encoding for 641 aa) was generated by combining the *Zfp278* cDNA sequence from the YOH clone (encoding for 409aa) with a cDNA fragment encoding the C-terminal MAZR sequence. The *Zfp278* 3' cDNA sequence was PCR amplified from thymocyte cDNA using 5'-GCAGGTGCACACTTCTGAGCGAC-3' and 5'-ACAGGTACCGAATTTCGTCGACGGGACACAGCATGTCTCACTTC-3' as primers and inserted as *Xho*I fragment into the *Zfp278* YOH clone. BTB^{MAZR} and MAZR Δ ZF truncated forms of MAZR were amplified as *Xho*I fragments by PCR using Myc-tagged *Zfp278* cDNA as template. The following primers were used: 5'-AGACTCGAGGCTGCCACCATGGAACAAAAG-3' with either 5'-AGACTCGAGTCATTAAGATCTCGATGACCGAC-3' for BTB^{MAZR} or 5'-AGACTCGAGTCATTATGGAAGGATGCCTGC-3' for MAZR Δ ZF. Mutations into the BTB domain of MAZR (MAZR^{mut}) were introduced by site directed mutagenesis using two-step overlap PCR. The following primers were used: for 1st step PCR (1) 5'-AGACTCGAGGCTGCCACCATGGAACAAAAG-3', (2) 5'-GAGTGATTGGTTCAGGTTGTGCGACATCTCCGTAC-3' and (3) 5'-TCGCACAACCTGAACCAATCACTCAAAAACGGCGG-3', (4) 5'-ACAGGTACCGAATTTCGTCGACGGGACACAGCATGTCTCACTTC-3'; for 2nd step PCR products from the 1st step and primers (1) and (4). Mutated sequences are underlined. The final PCR product was digested with *Xho*I restriction enzyme and inserted instead of the wild-type sequence.

Transfection, immunoprecipitation and immunoblot analysis

HEK293T cells were seeded on 10 cm tissue culture plates and transfected with 30 μ g FLAG-tagged N-CoR (kind gift of K. Jepsen and M. Rosenfeld, University of California, San Diego, CA) and 20 μ g of the various MAZR expression plasmids. 48 h post-transfection, the cells were harvested, washed with PBS, and lysed in lysis buffer (20 mM Tris-HCl, pH 8.0, 138 mM NaCl, 10 mM EDTA, 100 mM NaF, 1% NP-40, 10% glycerol) containing protease inhibitors (Roche). Lysates were snap-frozen in liquid N₂ and stored at -80 °C until use. After thawing cell debris was removed by 10 min centrifugation, and lysates were incubated with anti-N-CoR with gentle rotation over night at 4 °C. Upon addition of 30% (Vol/Vol) protein G beads (Roche) and 1 h rotation at 4 °C, the immunocomplexes were pelleted, washed, and the beads were resuspended in Laemli buffer. Polyacrylamide gel electrophoresis and immunoblotting was performed according to standard procedures³⁸. N-CoR immunoprecipitations and immunoblots were performed with

polyclonal anti-N-CoR (C-20; Santa Cruz). Immunoblot analysis for MAZR and actin expression was performed with rabbit anti-MAZR serum or rabbit anti-actin (A2066, Sigma), respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Epigenetic changes at the *Cd8a* and *Cd8b1* gene complex in E8_I,E8_{II} doubly deficient ($\Delta 1\Delta 2/\Delta 1\Delta 2$) thymocytes. **(a)** Flow cytometric analysis of CD4 and CD8 expression on thymocytes isolated from either $+/\Delta 1\Delta 2 \times Tcr\alpha^{-/-}$ or $\Delta 1\Delta 2/\Delta 1\Delta 2 \times Tcr\alpha^{-/-}$ mice as reported previously¹⁴. The numbers in the quadrants indicate the percentage of the corresponding thymocyte subpopulations (of total gated thymocytes). Sorting areas for “CD8-negative” (CD8-neg) and bona fide DP thymocytes for ChIP (Fig. 1b) and DNA methylation (Fig. 2) assays are indicated by rectangles. Data are representative of more than 10 mice analyzed. **(b)** Top: Map of the *Cd8* gene loci after the deletion of E8_I and E8_{II}. Horizontal arrows indicate the transcriptional orientation of the *Cd8* genes. Vertical arrows indicate the localization of DH sites that constitute clusters II, III, and IV³⁹. The horizontal bars indicate genomic fragments used to define enhancers E8_{III}, E8_{IV}, and E8_V as previously reported¹¹, and *Cd8a* (P8a) and *Cd8b1* (P8b) promoter regions. The triangles represent loxP sites left after the deletion of E8_I and E8_{II}. Only relevant *Bam*HI (B) and *Eco*RI (E) sites are shown.

Bottom: PCR analysis of chromatin-immunoprecipitated DNA from sorted “CD8-negative” DP (-) and DP (+) thymocytes. Six amplicons denoted by the bars spanning the *Cd8* gene cluster are shown. The *Cd4* promoter region (P4) and the *Gapdh* locus were amplified as controls. Input DNA was used undiluted, or at 1:3 and 1:10 dilutions to ensure PCR quantification within a non-saturated amplification range. Relative abundance of histone H3 or H4 acetylation, and H3K4 trimethylation ((Me)₃-H3K4) at the corresponding gene loci in CD8 expressing DP thymocytes were set to 1 (=100%). ChIP assays for AcH3 and AcH4 were repeated at least three times with three independent samples, while assays for (Me)₃H3K4 were repeated twice.

Fig. 2. DNA methylation of the promoter, exon, and intron regions of the *Cd8a* and *Cd8b1* genes. Genomic DNA from DP and “CD8-negative” DP thymocytes was treated with sodium bisulfite and amplified by PCR as indicated by black thin lines (after ref. ^{23,24}). Positions are indicated relative to the initiation codon. Individual PCR products were cloned and sequenced, each line representing one clone. Four clones of each of three independently prepared thymocytes for each group were sequenced. Unmethylated CpG sites are indicated by open circles, whereas methylated CpG sites are indicated by filled circles.

Fig. 3.

Enhancer E8_{II} binding properties and expression pattern of MAZR. (a) Electrophoretic mobility shift assay (EMSA) showing *in vitro* binding of MAZR or Myc-MAZR to E8_{II} RE-1 (Supplementary Fig. 2b online). Open wedge indicates addition of increasing amounts of anti-Myc. Data are representative of two independent experiments. (b) Chromatin immunoprecipitation (ChIP) assay showing *in vivo* interaction of MAZR with RE-1 in DN thymocytes. Primer pairs used for PCR correspond to amplicon 6 (Supplementary Table 2 online). Input DNA was used undiluted, or at 1:3 and 1:10 dilutions. Data are representative of seven independent experiments (c) Real-time qPCR quantification of MAZR expression in various thymocyte subsets. *MAZR* (*Zfp278*) transcript abundance was normalized to that of *Hprt1* transcripts. (d) Immunoblot analysis showing MAZR and N-CoR expression levels in sorted thymocyte subpopulations and peripheral splenic CD8⁺ and CD4⁺ T cells. Whole cell lysates from 3×10^6 cells were used for each population. Control, protein lysate generated from thymocytes that were transduced with MAZR retrovirus (see Methods). The arrow indicates the position of MAZR. Actin protein abundance was used to confirm equal loading. Data in **c** and **d** are representative of two independent experiments.

Fig. 4.

Forced expression of MAZR in thymocytes induces variegation of CD8 expression. **(a)** Two-color flow cytometry of MAZR transduced wild-type thymocytes. Representative dot plots show CD4 versus CD8 expression. Numbers in the dot plot quadrants indicate the percentage of the corresponding thymocyte subpopulations of total gated GFP⁻ (top) and GFP⁺ thymocytes (bottom), respectively. **(b)** Expression of CD3 and CD5 on CD4 SP (left), DP (middle) and CD8 SP (right) thymocytes (as defined by the gates in Fig. 4a). Top, expression of CD3 (left) and CD5 (right) in MIG-R-transduced thymocytes. Bottom, expression of CD3 (left) and CD5 (right) in MAZR-transduced thymocytes. Expression patterns for GFP⁺ and GFP⁻ populations are indicated. Numbers in the CD4 SP-gated histograms indicate the percentage of cells within the indicated regions (i.e. CD3^{lo} and CD5^{int}, respectively) of total gated thymocytes. Data are representative of at least 5 experiments.

Fig. 5. MAZR-induced variegation in wild-type, E8_I- and E8_{II}-deficient thymocytes **(a)** Representative GFP expression profile of thymocytes transduced with MIG-R and MAZR. Gating areas for GFP⁻ (-) and GFP⁺ (+) cells (thin line rectangles), and high (hi) GFP-expressing cells (thick line rectangles) are shown. Scatter plots show the percentage of CD3^{lo} (top) and CD5^{int} (bottom) cells within the CD4 SP subset of MIG-R and MAZR transduced wild-type (wt; left), E8_I-deficient ($\Delta 1/\Delta 1$; middle) or E8_{II}-deficient ($\Delta 2/\Delta 2$; right) thymocytes, respectively. Each circle represents one mouse. Horizontal bars indicate average value. **(b)** Left, representative histograms of GFP⁺ thymocytes indicating gating areas for CD4⁺CD8⁻CD3^{lo}, CD4⁺CD8⁻CD3^{hi} and DP thymocytes, respectively, that were used to determine expression as shown in the right panel. Right, expression of the zinc finger transcription factor Th-POK in MAZR-transduced thymocytes. Semi-quantitative RT-PCR of *Zbtb7b* (*Th-POK*) mRNA levels in CD4⁺CD3^{lo}, DP, and CD4⁺CD3^{hi} populations of MAZR-transduced E8_I-deficient thymocytes. Open wedges indicate undiluted sample, 1:3, and 1:10 dilutions, respectively. M, marker; -, RT(-) control. *Hprt1* mRNA was used as quantification control.

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Fig. 7.

BTB domain-dependent interaction of MAZR and N-CoR. **(a)** HEK293T cells overexpressing N-CoR and either MAZR^{mut} (lane 1) or MAZR (lane 2) were lysed and N-CoR was immunoprecipitated using anti-N-CoR. Immunoblots were performed using either anti-MAZR serum or anti-N-CoR. Input shows anti-MAZR immunoblot on total cell lysates. **(b)** Lysates of 10^7 sorted DN thymocytes (lane 1), 10^7 total thymocytes (lane 2 and 3) were immunoprecipitated with anti-N-CoR (lanes 1 and 2). For mock IP (lane 3) thymocyte extracts were incubated with protein G beads only. Immunoblots were performed using either anti-MAZR serum or anti-N-CoR. Immunoprecipitation data shown with DN and total thymocyte populations are representative of three independent experiments each.

Fig. 8. The BTB domain alone is not sufficient to induce variegated expression of CD8 in DP thymocytes. Scatter plot showing percentage of CD3^{lo} cells within GFP⁻, GFP⁺, and GFP^{hi} CD4 SP thymocytes of MIG-R, BTB^{MAZR}, and MAZR Δ ZF transduced wild-type thymocytes. Representative gating areas for GFP expression are as in Fig. 5a. Each circle represents one mouse. Horizontal bars indicate average value.