

Myb proteins regulate expression of histone variant H2A.Z during thymocyte development

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Summary

The *c-myb* gene encodes a transcription factor required for the normal development of T cells in the thymus, and for subsequent peripheral T-cell activation and survival. However, the profile of genes known to be transcriptionally regulated by c-Myb in T cells does not adequately explain the pleiotropic nature of the effects of c-Myb. We present here a detailed molecular characterization of the regulation of a novel target gene, the histone variant *H2A.Z*. We show that c-Myb is able to bind to and activate the *H2A.Z* promoter in T cells both *in vitro* and *in vivo*, and present evidence that perturbation of Myb activity during T-cell development results in reduced *H2A.Z* expression. As *H2A.Z* is absolutely required for the early stages of mammalian development, and plays essential roles in the regulation of chromatin structure in gene promoters in yeast, its regulation by c-Myb is likely to be of some importance during T-cell development.

Keywords: Myb; H2A.Z; T cells; transcription

Introduction

c-Myb is an essential transcription factor required for normal haematopoiesis. The function of c-Myb is well characterized in T-cell development where it is required at four distinct points during thymocyte maturation.^{1,2} Immature thymocytes can be broadly classified as double negative (DN) thymocytes, which lack expression of the CD4 and CD8 coreceptors, and double positive (DP) thymocytes, which express both coreceptors.³ c-Myb is required for two distinct transitions in DN thymocytes: for the earliest committed thymocytes to progress beyond the DN1 stage (CD44⁺ CD25⁻),¹ then for selection and expansion of thymocytes with successful rearrangement of the T-cell receptor β chain (TCR- β) at the DN3 stage (CD44⁻ CD25⁺).^{2,4–6} Later, at the double positive (DP) stage, c-Myb is required for cell survival and may contribute to *TCR- α* gene rearrangement.^{2,6} c-Myb is further required for differentiation from the late DP precursor to the final CD4 single positive stage, but not for the corresponding DP to CD8 single positive transition.^{2,6,7} In mature T cells, specific deletion of *c-myb* or inhibition of c-Myb by expression of a dominant negative transgene reduces the activation-induced proliferative response.^{4,6}

c-Myb activates transcription through the hexameric consensus Myb-binding site (MBS), defined as YAACT/

GG. However, molecular studies into the transcriptional targets of c-Myb have yet to explain its profound effects on haematopoietic development. c-Myb has been proposed to regulate transcription of the *CD4* and recombination activating gene (*RAG-2*) genes.^{8–10} However, while specific deletion of c-Myb in thymocytes *in vivo* resulted in impaired rearrangement of both chains of the TCR and a block in differentiation of CD4 T cells, no decrease in *RAG-2* or *CD4* expression was detected.^{2,7} Apart from the *Bcl2* family members whose products mediate the antiapoptotic function of c-Myb,^{11–13} few other promising target genes exist that could be responsible for the essential functions of c-Myb in T cells. To address this issue, we have recently published a subtraction cloning-based screen identifying 29 novel targets in a model system of haematopoiesis.¹⁴ To extend this screen to a T-cell system, we immobilized cDNA molecules isolated from the initial screen onto nylon filters and probed filters with labelled total cDNA produced from mouse splenic T cells activated *ex vivo* by interleukin (IL)-2 treatment. The screen identified 81 putative Myb-responsive genes, including the histone variant *H2A.Z*, which was isolated a total of three times.

H2A.Z is highly conserved throughout eukaryotic evolution and recent data implicate *H2A.Z* in a wide range of chromatin-related processes including transcriptional

regulation, positioning of heterochromatin boundaries, chromosomal stability and cell cycle progression.^{15–29} The *H2A.Z* gene differs markedly from the major *H2A* and other core histone genes in that it is not associated with the replication-dependent gene clusters and possesses introns, a conserved TATA box and a polyadenylation signal sequence.^{30–32} In addition, the structure of the *H2A.Z* promoter appears to be complex, with basal expression linked to Sp1 association with the proximal promoter³³ and upstream regions mediating increased transcription in a cell type- and differentiation-dependent manner.³⁴

The identification of *H2A.Z* as a potential c-Myb target was of great interest, as c-Myb has not previously been implicated as a regulator of genes involved in epigenetic regulation. Therefore, we chose to investigate the relationship between c-Myb and *H2A.Z* in detail. Here we show that Myb proteins bind directly to MBSs upstream of the *H2A.Z* gene *in vitro* and in chromatin immunoprecipitation (ChIP) assays. Expression of v-Myb, the activated form of c-Myb, induces *H2A.Z* in CD4 T cells *in vivo*. Conversely, blocking or knocking out Myb activity reduces *H2A.Z* expression in T-cell lines and in specific thymocyte subsets *in vivo*.

Materials and methods

Mouse lines

The T-cell specific vMyb4 transgenic mouse line, the floxed *c-myb* mouse line (Myb^{F/F}) and RAG-2^{-/-} mice have been described previously.^{4,35–37} T-cell specific deletion of c-Myb was induced by breeding Myb^{F/F} mice to CD2Cre or CD4Cre transgenic mice.^{38,39}

Cell culture

HD3 macrophages were routinely cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 8% fetal calf serum (FCS) and 2% chicken serum (Invitrogen, Paisley, UK). EL4 cells stably transfected with the *MERT* dominant negative transgene have been described.¹² *MERT* was activated by addition of 1–2 µM 4-hydroxytamoxifen (Sigma-Aldrich, Cambridge, UK).

Electrophoretic mobility shift assays (EMSAs)

EMSA was performed as described previously⁴⁰ using as probe 0.1 pmol of MBSwt, an IRD700-labelled double-stranded oligonucleotide of sequence 5'-CTAGGACATTA TAACGGTTTTAGTCTAG-3', and, as a source of Myb DNA-binding activity, 2 µl of rabbit reticulocyte lysate (Promega, Southampton, UK) either unprogrammed or programmed with plasmid pT7βMT.⁴ Where appropriate,

1 pmol (10×), 10 pmol (100×) or 100 pmol (1000×) of unlabelled competitors was added. Unlabelled oligonucleotide competitors were: MBSwt as above; H2AZ1, 5'-CAGA AGCCTCAGTTGTTTTTCGGCAT-3'; H2AZ2, 5'-GTACAT CTACTAACTGCACGTCTAA-3'; H2AZ3, 5'-CCAGCAT TTGTAAGTATTACATGA-3'; H2AZ4, 5'-GAACCATCT GTAAGTCTTGTGAAT-3'; H2AZ5, 5'-GAATCATGTTA CAGTTACCCACTG-3'. All mutant MBSs contained a single base pair change in the YAAC core to YCAC. Bands were visualized on a Licor Odyssey scanner (LI-COR Biosciences UK Ltd, Cambridge, UK).

Chloramphenicol acetyl transferase (CAT) reporter assays

For CAT assays, 5 × 10⁵ HD-3 cells were transfected using DMRIE-C transfection reagent (Invitrogen) in 24-well plates according to the manufacturer's instructions. Cells were harvested after 48 hr, washed in cold phosphate-buffered saline (PBS), resuspended in 200 µl of 0.25 M Tris-HCl (pH 7.8) and lysed in three freeze-thaw cycles. Protein concentrations were determined (BCA protein assay; Pierce, Cramlington, UK) and CAT activity was assayed as described previously.⁴¹ Chloramphenicol products were separated by thin layer chromatography in 5% methanol and 95% chloroform and autoradiographed. The percentage of acetylated chloramphenicol products was calculated and corrected for transfection efficiency using a β-galactosidase internal control.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed as described previously⁷ using extracts from primary thymocytes. Primers were: H2AZMBS1, 5'-GTAGAGTTGACTAGCATTTCTGC-3' and 5'-CTTATTGGAACTAAGGAATTG-3'; H2AMB2, 5'-AT GCGAAATTCGCAAGACTCAG-3' and 5'-ATAGACTTGT ACACACGGTAC-3'; H2AZ MBS33-5, 5'-GGACAGTACC GTGTGTACAAG-3' and 5'-TAAAGCTATTATGTGTCCAG C-3'.

Flow cytometry and cell sorting

Single cell suspensions were prepared from whole thymus and spleen. In some experiments, CD69^{lo} and CD69^{hi} thymocyte populations were separated by positively selecting CD69^{hi} cells by magnetic antibody cell sorting (MACS) using LS columns (Miltenyi Biotech, Surrey, UK), followed by flow sorting of individual populations. Cells in all experiments were stained at 4° in PBS containing 2% FCS, 5 mM ethylenediaminetetraacetic acid (EDTA) and 0.1% sodium azide. Antibodies were: CD69-biotin, CD4-PE, Thy1.2-PE, CD8a-FITC, CD4-APC, CD25-PE, CD44-FITC and streptavidin-PE-Cy7 (BD Biosciences, Oxford, UK). Fluorescence-activated cell sorting (FACS) was performed on a

FACSVantage electronic cell sorter (BD Biosciences) or a MoFlo (DakoCytomation, Glostrup, Denmark). DN thymocytes were sorted by FACS after depletion of CD4- and CD8-expressing thymocytes by MACS. Data were analysed using FLOWJO (Tree Star, Ashland, WA).

Northern blotting

RNA was processed using standard protocols. The mouse *H2A.Z* probe was a purified, 262-base polymerase chain reaction (PCR) product labelled with [³²P]-dCTP (PCR primer sequences: H2AZF, ATCTAGGACAACCAGC CACG; H2AZR, CTGTTGTCCTTTCTTCCCGATC). The *H2A.Z* probe was hybridized to nylon membranes in QuikHyb hybridization solution (Stratagene, La Jolla, CA) in accordance with the manufacturer's instructions, and visualized by autoradiography.

Quantitative reverse transcription–polymerase chain reaction (qRT-PCR)

Total RNA was extracted from between 5×10^4 and 5×10^5 sorted T cells using TriZol reagent (Invitrogen). RNA was reverse-transcribed using the Omniscript kit (Qiagen, Crawley, UK). qRT-PCR reactions were performed on a Taqman 7900HT analyser (Applied Biosystems, Foster City, CA). All qRT-PCR assays were obtained from Applied Biosystems. Where indicated, qRT-PCR data were subjected to the paired Student's *t*-test to determine significance of differences in *H2A.Z* mRNA expression between the transgenic and littermate control groups.

Results

Consensus MBSs upstream of the *H2A.Z* gene

We identified five conserved, putative MBSs (defined as YAACT/GG) in the 1.5 kb upstream of the *H2A.Z* proximal promoter, and a further 11 sequence elements containing the conserved MBS core (YAAC) that diverge from the conserved hexamer at only one of the last two positions. Four of the completely conserved hexamers cluster to a short, 300-bp region approximately 1 kb upstream of the first exon (Fig. 1a). This 300-bp region is highly conserved between mouse and human with conservation of three of the four MBSs. At least one MBS is also present upstream of the chicken *H2A.Z* gene (*h2af*), although there is little sequence homology overall.

Myb interacts with *H2A.Z* MBSs *in vitro* and *in vivo* and is functionally important for *H2A.Z* transcription

We performed EMSAs to determine whether a 26-kDa Myb DNA-binding domain (DBD) fragment, MT, translated in rabbit reticulocyte lysate, could be competitively

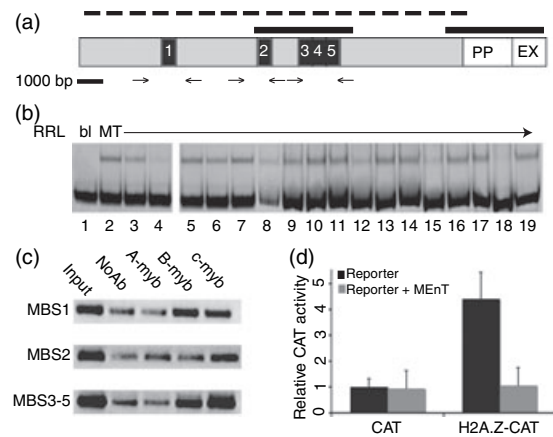


Figure 1. Myb proteins interact directly with Myb-binding sites (MBSs) immediately upstream of the histone variant *H2A.Z* proximal promoter. (a) Schematic of the genomic DNA sequence up to 2000 bases upstream of the murine *H2A.Z* cDNA. Regions of homology with the human gene are indicated by solid black bars. MBS1–5 are indicated by black boxes. Approximate positions of chromatin immunoprecipitation (ChIP) polymerase chain reaction (PCR) primers are represented by arrows and the broken line indicates the region cloned upstream of the chloramphenicol acetyl transferase (CAT) gene in CAT reporter assays. (b) Electrophoretic mobility shift assays (EMSA) showing that *in vitro* translated Myb DNA-binding domain (DBD) fragment MT recognizes *H2A.Z* Myb-binding sites. Binding reactions all contained 0.1 pmol labelled Mybswt probe: lane 1, unprogrammed reticulocyte lysate; lanes 2–19, MT-programmed lysate. Lanes 4–19 contained added unlabelled oligonucleotide competitors as described in the text. (c) ChIP assays showing B- and c-Myb proteins binding the *H2A.Z* promoter in primary thymocytes. (d) Quantification of CAT assays showing activation of parental CAT plasmid (CAT) and an *H2A.Z* promoter-linked CAT gene (H2AZ-CAT) in HD3 cells, in the absence (black bars) or presence (grey bars) of the MEnT dominant negative protein. CAT assays were performed three times. Bars represent mean values with the standard deviations indicated as error bars. bl, blank; EX, Exon; NoAb, no antibody; PP, proximal promoter; RRL, red reticulocyte lysate.

prevented from binding to a strong canonical MBS probe (MBSwt) by an excess of unlabelled *H2A.Z* MBSs. Figure 1(b), lane 2 shows the band-shift obtained with the complex between MBSwt probe and MT, and lanes 3 and 4 demonstrate competition with a 10-fold and 100-fold molar excess of unlabelled MBSwt. None of the *H2A.Z* MBSs was able to compete when added at either a 10-fold (data not shown) or 100-fold molar excess (Fig. 1b, lanes 5, 8, 11, 14 and 17). However, when added in a 1000-fold molar excess, *H2A.Z* MBSs 3, 4 and 5 were able to compete well (lanes 12, 15 and 18), and MBS1 and MBS2 competed extremely weakly (lanes 6 and 9). As controls, no competition was observed with a 1000-fold excess of any of the *H2A.Z* MBSs when they were mutated to destroy the core AAC of their Myb-binding sites (Fig. 1b, lanes 7, 10, 13, 16 and 19), indicating the specificity of the interaction, and no retarded band was observed in unprogrammed reticulocyte lysate (Fig. 1b, lane 1), demonstrat-

ing that the interaction was with the Myb DNA-binding domain fragment.

We used ChIP to determine whether the weak interactions observed *in vitro* were relevant to c-Myb activity in T cells. Cross-linked DNA–protein complexes from primary murine thymocytes were immunoprecipitated with antibodies against A-, B- and c-Myb and purified DNA was tested for enrichment of the *H2A.Z* upstream sequence by PCR with specific primers (Fig. 1a, arrows). The c-Myb antibody immunoprecipitated three fragments containing MBS1, MBS2 and MBS3–5, respectively (Fig. 1c), indicating that c-Myb occupies this region of the *H2A.Z* upstream sequence in primary T cells. The closely related Myb family member B-Myb was also able to bind to the regions encompassing MBS1 and MBS3–5 but appeared absent from the central MBS2 fragment (Fig. 1c). As control, an antibody against A-Myb, which is not expressed in T cells, did not immunoprecipitate any fragments. Therefore, B- and c-Myb are bound to the *H2A.Z* promoter region in primary thymocytes.

Transient transfection assays were used to test whether the interactions between c-Myb and the *H2A.Z* upstream region had functional importance for *H2A.Z* transcription. Transfection of a reporter gene comprising approximately 2 kb of the *H2A.Z* upstream region (Fig. 1a, broken line) driving expression of the chloramphenicol acetyl transferase (*H2AZ-CAT*) gene into HD3 macrophages, a cell line expressing high levels of endogenous c-Myb protein, resulted in greater than 4-fold activation of CAT activity compared with a control reporter gene lacking the *H2A.Z* upstream region. This activation was blocked by cotransfection of the MEN1 protein (Fig. 1d), indicating that activation of the *H2A.Z* promoter was Myb dependent. MEN1 is an active repressor of Myb transcriptional activity consisting of the c-Myb DBD fused to the *Drosophila* Engrailed repressor domain which has been used previously for interrogation of c-Myb function in thymocytes.⁴

H2A.Z transcripts are elevated in specific T-cell subsets in a transgenic mouse model of Myb function *in vivo*

We used the previously developed vMyb4 transgenic mouse line³⁵ to test whether increasing Myb transcriptional activity in thymocytes and splenic T cells *in vivo* would lead to increases in *H2A.Z* mRNA levels. Thymocyte subsets and splenic T cells were isolated by FACS from vMyb4 transgenic mice and wild-type littermate controls; CD8 lineage T cells were not compared as this lineage is absent in vMyb4 mice (Fig. 2a). We used the activation marker CD69 to distinguish DP thymocytes that had yet to rearrange their TCR- α chains (CD69^{lo}) from post-rearrangement DPs (CD69^{hi}). We observed no significant difference in *H2A.Z* transcript levels between vMyb4 transgenic mice and non-transgenic controls in

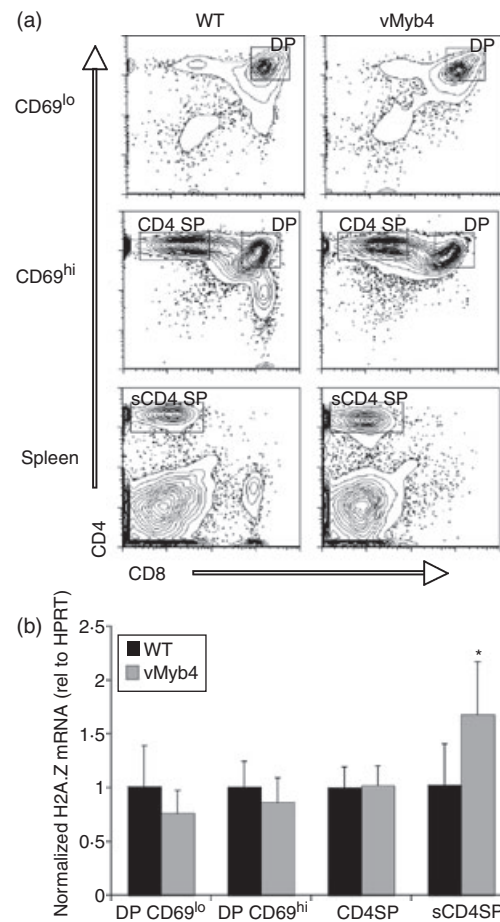


Figure 2. Elevated histone variant *H2A.Z* mRNA in vMyb4 T-cell populations *in vivo*. (a) Flow cytometry of thymocytes (top and centre panels) and splenocytes (bottom panels) from vMyb4 transgenic mice and wild-type littermate controls (WT), stained with anti-CD4 and anti-CD8 antibodies. (b) Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis of *H2A.Z* mRNA expression using RNA from sorted T lymphocytes. Data are presented as means from triplicate qRT-PCR reactions performed from each of three independent sorts. Error bars represent standard error. DP, double positive; HPRT, hypoxanthine-guanine phosphoribosyl-transferase.

CD69^{lo} DP, CD69^{hi} DP or CD4SP thymocytes (Fig. 2b; $P = 0.273$, 0.418 and 0.424 , respectively) or DN thymocytes (data not shown). However, in mature, splenic CD4 T cells, *H2A.Z* transcript levels were elevated by 68% compared with wild-type littermate controls (Fig. 2b). This difference was significant ($P = 0.032$), indicating that increased Myb transcriptional activity results in elevated *H2A.Z* mRNA in CD4 T cells *in vivo*.

Active repressors of Myb transcription reduce *H2A.Z* transcripts

EL4 thymoma cells stably transfected with the MERT inducible active repressor of Myb transcription, termed

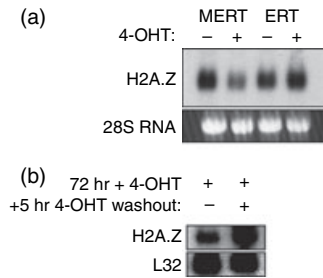


Figure 3. Northern blot analysis of histone variant *H2A.Z* RNA in EL4 cells stably transfected with the MERT active repressor. (a) MERT was induced with 4-hydroxytamoxifen (4-OHT) (+) or mock induced with vehicle (-) for 24 hr. EL4-ERT cells (an inactive version of MERT lacking the Myb DNA-binding domain) were included as a control. Data are representative of five independent experiments. (b) Rapid re-induction of *H2A.Z* following MERT switch-off. MERT was induced for 72 hr, and then 4-OHT was either washed out of the cultures (+) or not (-). RNA was prepared 5 hr after washout.

EL4-MERT cells, have been used previously to investigate regulation of the *c-Myb* target gene *Bcl2*.¹² MERT comprises the *c-Myb* DBD fused to the *Drosophila* Engrailed repressor domain and a modified version of the oestrogen-receptor hormone-binding domain that is activated only by 4-hydroxytamoxifen (4-OHT). Activation of the MERT protein by 4-OHT for 24 hr efficiently reduced steady-state *H2A.Z* transcripts (Fig. 3a). This reduction was dependent on the Myb DBD, as a control protein, ERT, which lacks the DBD, had no effect on *H2A.Z* transcripts when induced by 4-OHT. Next, to determine whether the loss of *H2A.Z* mRNA was likely to be a result of MERT directly repressing the *H2A.Z* gene, MERT protein was activated by 4-OHT for 72 hr, 4-OHT was washed out of the cell culture for 5 hr, and the level of *H2A.Z* mRNA was assessed by northern blot. *H2A.Z* mRNA was efficiently re-induced at the 5-hr time-point (see Fig. 5b below; '+' lane), suggesting that a direct interaction might be occurring.

Specific deletion of *c-Myb* leads to a reduction in *H2A.Z* mRNA

To determine whether loss of *c-Myb* in primary T cells could affect *H2A.Z* transcription, mice with floxed *c-myb* alleles (*Myb*^{F/F})³⁶ were crossed to mice carrying either a T-cell specific *CD2Cre* or a *CD4Cre* transgene.^{38,39} In *Myb*^{F/F} *CD2Cre* crosses, *c-myb* is deleted during the DN2 stage of T-cell development, and in *Myb*^{F/F} *CD4Cre* mice, *c-myb* is lost at the DN4 stage. These two crosses can therefore be used to test for the effects of loss of *c-myb* at two crucial control points for which it has previously been shown to be important:^{5,7} the DN3 (*CD25*⁺ *CD44*⁻) stage, at which thymocytes with a productively rearranged TCR- β chain are signalled to proliferate and differentiate

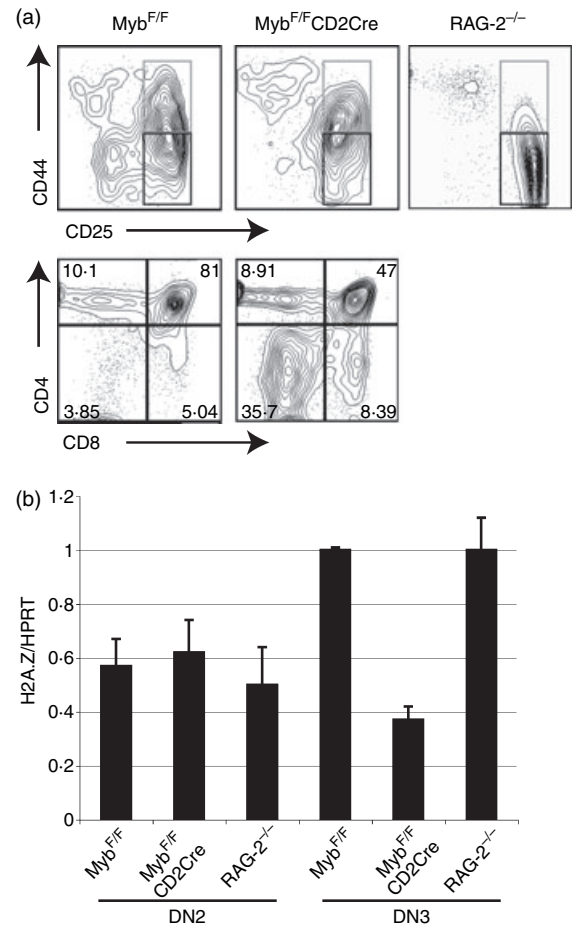


Figure 4. Deletion of *c-myb* at the double negative 2 (DN2) stage reduces histone variant *H2A.Z* expression in DN3 thymocytes. (a) Fluorescence-activated cell sorting (FACS) profiles of thymocytes. Upper panel: DN thymocytes stained with antibodies against CD44 (y-axis) and CD25 (x-axis) from *Myb*^{F/F} mice in the absence or presence of the *CD2Cre* transgene. A recombination activating gene (*RAG*)^{-2/-} profile is also shown. DN2 (grey boxes) and DN3 (black boxes) populations used for mRNA preparation are indicated. Lower panel: FACS profiles of total thymocytes stained with antibodies against CD4 (y-axis) and CD8 (x-axis) from *Myb*^{F/F} mice in the absence or presence of the *CD2Cre* transgene. Percentages of cells in each quadrant are shown. (b) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of *H2A.Z* mRNA expression using RNA isolated from sorted DN2 and DN3 cells of the indicated genotypes. Data are presented as means from triplicate qRT-PCR reactions. Error bars represent standard deviation.

to become DP, and the DP stage itself, where the process of positive selection occurs.

First, to examine the DN3 control point, DN2 and DN3 thymocyte subsets were purified by flow sorting from *Myb*^{F/F} *CD2Cre* mice and *Myb*^{F/F} littermate controls. In the former, thymuses are very small, and the percentage of DP thymocytes is roughly halved (Fig. 4a, lower right panel), as thymocytes are blocked at the DN3 stage (Fig. 4a, upper panel; compare left and centre plots). As a

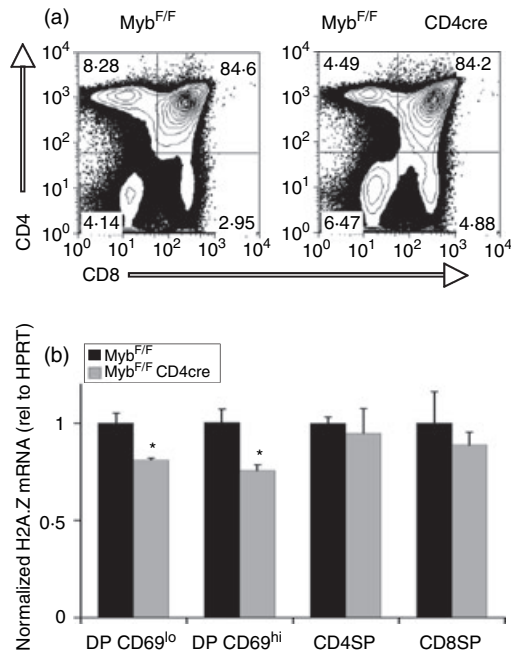


Figure 5. Deletion of *c-myb* at the double negative 4 (DN4) stage reduces histone variant *H2A.Z* expression in thymic double positive (DP) subsets. (a) Fluorescence-activated cell sorting (FACS) profiles of thymocytes stained with antibodies against CD4 (*y*-axis) and CD8 (*x*-axis) from Myb^{F/F} mice in the absence or presence of the *CD4Cre* transgene. (b) Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis of *H2A.Z* mRNA expression using RNA isolated from sorted T lymphocytes of Myb^{F/F} CD4Cre mice. Data are presented as means from triplicate qRT-PCR reactions performed from each of three independent sorts. Error bars represent standard error. HPRT, hypoxanthine guanine phosphoribosyltransferase.

control for any non-specific effects of a DN3 block, DN2 and DN3 thymocytes from RAG-2^{-/-} mice, which have an absolute block at the DN3 stage because of their inability to rearrange the TCR- β locus, were also purified (Fig. 4a, upper right panel). mRNA from the sorted populations was then extracted and *H2A.Z* transcript levels assayed by qRT-PCR. Figure 4(b) shows that, relative to both the RAG-2 and the control Myb^{F/F} subsets, *H2A.Z* mRNA was substantially decreased in the Myb^{F/F} CD2Cre DN3 postdeletion population, but not in DN2 cells, which still contain c-Myb.

Next, we purified DP thymocytes by flow sorting from Myb^{F/F} CD4Cre animals or control Myb^{F/F} littermates. These mice contain similar numbers and percentages of DP cells, but have a defect in progression to the CD4 SP lineage⁷ (Fig. 5a). Similar to the situation in DN3 cells, we observed small but consistent reductions in *H2A.Z* expression, evident in a 19% reduction in CD69^{lo} DPs and a 25% reduction in CD69^{hi} DPs (Fig. 5b). Both of these differences were significant ($P = 0.017$ and $P = 0.012$, for CD69^{lo} and CD69^{hi} DPs, respectively). No significant differences were observed in SP T cells, either in

the thymus (Fig. 5b) or in the spleen (data not shown), consistent with low levels of endogenous c-Myb expressed in these cells.^{2,7} Taken together, these data suggest that c-Myb contributes to *H2A.Z* expression at both the DN3 and DP stages of thymocyte development *in vivo*, such that loss of c-Myb results in a significant drop in levels of *H2A.Z* mRNA.

Discussion

Despite the essential role of c-Myb at multiple points in T-cell development, transcriptional targets that mediate this role have proved difficult to identify. Here we describe the regulation by c-Myb of the histone variant *H2A.Z* in T-cell lines and in thymocytes *in vivo*. c-Myb and its close relative B-Myb interact with conserved MBSs upstream of the *H2A.Z* promoter, and endogenous c-Myb activates the upstream region in reporter assays. Repression of Myb activity using the MERT protein also led to repression of endogenous *H2A.Z* in EL4 T cells, and the gene is rapidly re-induced following inactivation of MERT. Mouse models of Myb overexpression and deletion were used to characterize the regulation of *H2A.Z* by c-Myb in progressive stages of thymocyte differentiation. Expression of v-Myb in transgenic mice led to elevated expression of *H2A.Z* transcripts in mature splenic CD4 T cells, whereas deletion of *c-myb* in the thymocytes of conditional knockout mice reduced *H2A.Z* expression specifically in DN3 and DP thymocytes.

Transcriptional regulation of *H2A.Z* is clearly multifaceted. Ubiquitous transcription factors such as Sp1 may be important in the basal expression of *H2A.Z*.³³ However, evidence exists that different regions of the *H2A.Z* upstream sequence are required for maximal expression depending on differentiation status.³⁴ *H2A.Z* may have a basal cellular function that requires a certain level of ubiquitous expression in addition to specialized functions that require higher levels of *H2A.Z* during differentiation. As c-Myb expression is highly restricted to immature and activated cells of the haematopoietic system and some immature epithelial cell types, it is likely to contribute to *H2A.Z* transcription in a highly restricted context, and thus may stimulate a specialized function of *H2A.Z* in differentiating cells, such as the thymocytes reported here.

The developmental stage-specific regulation of *H2A.Z* expression we observed here may reflect differences in the endogenous c-Myb activity between thymocyte subsets. For example, increasing Myb activity (by expressing v-Myb) has no effect in DP thymocytes where endogenous c-Myb activity is high, whereas strong effects are observed in resting splenic CD4SP T cells, which have low c-Myb activity. The opposite situation is observed when *c-myb* is deleted: *H2A.Z* expression is reduced in DN3 and DP thymocytes where c-Myb activity is high, but there is no detectable difference in mature SP T cells, where c-Myb

activity is low. The residual *H2A.Z* expression observed even after deletion of *c-Myb* suggests that *c-Myb* is one member of a group of transcriptional activators that contribute to *H2A.Z* expression, and furthermore that there is probably some functional redundancy between *c-Myb* and *B-Myb*, both of which bind the *H2A.Z* promoter in thymocytes. The recent development of a conditional *B-Myb* knockout mouse model⁴² will make it possible to test this latter idea directly.

What *c-Myb*-specific functions might *H2A.Z* mediate during T-cell development? *H2A.Z* is an interesting target gene for a number of reasons. Deposition of *H2A.Z* into nucleosomes decreases the stability of inter-nucleosomal interactions,^{43,44} leading to a state apparently poised for activation dependent on the activity of other regulatory proteins.^{22,23} As *H2A.Z* nucleosomes are distributed widely throughout the genome, the consequences of activation of *H2A.Z* by *c-Myb* may be the indirect transcriptional regulation of a large number of genes. This can be interpreted in the context of a recent report that *c-Myb* regulates the acetylation of histone H3,⁴⁵ perhaps establishing a new general function of *c-Myb* as a regulator of histones, by both transcriptional and post-translational mechanisms. It would also be interesting to determine whether *H2A.Z* has a role in mediating TCR rearrangement, as deletion of *c-myb* has been reported to affect this process.² There exists some evidence that histone modification events regulate accessibility and selection of *TCR* gene segments in V(D)J recombination,^{46–49} and it is tempting to speculate that histone variants such as *H2A.Z* could also be important in regulating chromatin structure during this process. However, as yet, the function of *H2A.Z* in mammals is largely unknown. As the physiological role of *c-Myb* in T cells is well characterized, the cell- and developmental stage-specific regulation of *H2A.Z* expression by *c-Myb* may provide important clues as to the function of *H2A.Z* itself.

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References

- Allen RD 3rd, Bender TP, Siu G. *c-Myb* is essential for early T cell development. *Genes Dev* 1999; **13**:1073–8.
- Bender TP, Kremer CS, Kraus M, Buch T, Rajewsky K. Critical functions for *c-Myb* at three checkpoints during thymocyte development. *Nat Immunol* 2004; **5**:721–9.
- Anderson G, Jenkinson EJ. Lymphostromal interactions in thymic development and function. *Nat Rev Immunol* 2001; **1**:31.
- Badiani P, Corbella P, Kioussis D, Marvel J, Weston K. Dominant interfering alleles define a role for *c-Myb* in T cell development. *Genes Dev* 1994; **8**:770–82.
- Pearson R, Weston K. *c-Myb* regulates the proliferation of immature thymocytes following beta-selection. *EMBO J* 2000; **19**:6112–20.
- Lieu YK, Kumar A, Pajeroski AG, Rogers TJ, Reddy EP. Requirement of *c-myb* in T cell development and in mature T cell function. *Proc Natl Acad Sci USA* 2004; **101**:14853–60.
- Maurice D, Hooper J, Lang G, Weston K. *c-Myb* regulates lineage choice in developing thymocytes via its target gene *Gata3*. *EMBO J*, in press.
- Siu G, Wurster AL, Lipsick JS, Hedrick SM. Expression of the *CD4* gene requires a *Myb* transcription factor. *Mol Cell Biol* 1992; **12**:1592–60.
- Nakayama K, Yamamoto R, Ishii S, Nakauchi H. Binding of *c-Myb* to the core sequence of the *CD4* promoter. *Int Immunol* 1993; **5**:817–24.
- Wang QF, Lauring J, Schlissel MS. *c-Myb* binds to a sequence in the proximal region of the *RAG-2* promoter and is essential for promoter activity in T-lineage cells. *Mol Cell Biol* 2000; **20**:9203.
- Frampton J, Ramqvist T, Graf T. *v-Myb* of E26 leukemia virus up-regulates *bcl-2* and suppresses apoptosis in myeloid cells. *Genes Dev* 1996; **10**:2720–31.
- Taylor D, Badiani P, Weston K. A dominant interfering *Myb* mutant causes apoptosis in T cells. *Genes Dev* 1996; **10**:2732–44.
- Salomoni P, Perrotti D, Martinez R, Franceschi C, Calabretta B. Resistance to apoptosis in *CTLL-2* cells constitutively expressing *c-Myb* is associated with induction of *BCL-2* expression and *Myb*-dependent regulation of *bcl-2* promoter activity. *Proc Natl Acad Sci USA* 1997; **94**:3296–301.
- Lang G, White JR, Argent-Katwala MJ, Allinson CG, Weston K. *Myb* proteins regulate the expression of diverse target genes. *Oncogene* 2005; **24**:1375–84.
- Santisteban MS, Kalashnikova T, Smith MM. Histone *H2A.Z* regulates transcription and is partially redundant with nucleosome remodeling complexes. *Cell* 2000; **103**:411–22.
- Adam M, Robert F, Larochelle M, Gaudreau L. *H2A.Z* is required for global chromatin integrity and for recruitment of RNA polymerase II under specific conditions. *Mol Cell Biol* 2001; **21**:6270–9.
- Meneghini MD, Wu M, Madhani HD. Conserved histone variant *H2A.Z* protects euchromatin from the ectopic spread of silent heterochromatin. *Cell* 2003; **112**:725–36.
- Krogan NJ, Baetz K, Keogh MC *et al.* Regulation of chromosome stability by the histone *H2A* variant *Htz1*, the *Swr1* chromatin remodeling complex, and the histone acetyltransferase *NuA4*. *Proc Natl Acad Sci USA* 2004; **101**:13513–8.
- Rangasamy D, Greaves I, Tremethick DJ. RNA interference demonstrates a novel role for *H2A.Z* in chromosome segregation. *Nat Struct Mol Biol* 2004; **11**:650–8.
- Guillemette B, Bataille AR, Gevry N, Adam M, Blanchette M, Robert F, Gaudreau L. Variant histone *H2A.Z* is globally localized to the promoters of inactive yeast genes and regulates nucleosome positioning. *PLoS Biol* 2005; **3**:e384.
- Li B, Pattenden SG, Lee D, Gutierrez J, Chen J, Seidel C, Gerton J, Workman JL. Preferential occupancy of histone variant *H2AZ* at inactive promoters influences local histone modifications

- and chromatin remodeling. *Proc Natl Acad Sci USA* 2005; **102**:18385–90.
- 22 Raisner RM, Hartley PD, Meneghini MD, Bao MZ, Liu CL, Schreiber SL, Rando OJ, Madhani HD. Histone variant H2A.Z marks the 5' ends of both active and inactive genes in euchromatin. *Cell* 2005; **123**:233–48.
 - 23 Zhang H, Roberts DN, Cairns BR. Genome-wide dynamics of Htz1, a histone H2A variant that poises repressed/basal promoters for activation through histone loss. *Cell* 2005; **123**:219–31.
 - 24 Babiarz JE, Halley JE, Rine J. Telomeric heterochromatin boundaries require NuA4-dependent acetylation of histone variant H2A.Z in *Saccharomyces cerevisiae*. *Genes Dev* 2006; **20**:700–10.
 - 25 Dhillon N, Oki M, Szyjka SJ, Aparicio OM, Kamakaka RT. H2A.Z functions to regulate progression through the cell cycle. *Mol Cell Biol* 2006; **26**:489–501.
 - 26 Greaves IK, Rangasamy D, Devoy M, Marshall Graves JA, Tremethick DJ. The X and Y chromosomes assemble into H2A.Z, containing facultative heterochromatin, following meiosis. *Mol Cell Biol* 2006; **26**:5394–405.
 - 27 Keogh MC, Mennella TA, Sawa C *et al.* The *Saccharomyces cerevisiae* histone H2A variant Htz1 is acetylated by NuA4. *Genes Dev* 2006; **20**:660–72.
 - 28 Millar CB, Xu F, Zhang K, Grunstein M. Acetylation of H2AZ Lys 14 is associated with genome-wide gene activity in yeast. *Genes Dev* 2006; **20**:711–22.
 - 29 Jin C, Felsenfeld G. Nucleosome stability mediated by histone variants H3.3 and H2A.Z. *Genes Dev* 2007; **21**:1519–29.
 - 30 Dalton S, Robins AJ, Harvey RP, Wells JR. Transcription from the intron-containing chicken histone H2A.F gene is not S-phase regulated. *Nucl Acids Res* 1989; **17**:1745–50.
 - 31 Hatch CL, Bonner WM. The human histone H2A.Z gene. Sequence and regulation. *J Biol Chem* 1990; **265**:15211–220.
 - 32 Harvey RP, Whiting JA, Coles LS, Krieg PA, Wells JR. H2A.F: an extremely variant histone H2A sequence expressed in the chicken embryo. *Proc Natl Acad Sci USA* 1983; **80**:2819–28.
 - 33 Hatch CL, Bonner WM. Characterization of the proximal promoter of the human histone H2A.Z. *Gene DNA Cell Biol* 1995; **14**:257–66.
 - 34 Hatch CL, Bonner WM. An upstream region of the H2AZ gene promoter modulates promoter activity in different cell types. *Biochim Biophys Acta* 1996; **1305**:59–62.
 - 35 Badiani P, Kioussis D, Swirsky D, Lampert I, Weston K. T-cell lymphomas in v-Myb transgenic mice. *Oncogene* 1996; **13**:2205.
 - 36 Emambokus N, Vegiopoulos A, Harman B, Jenkinson E, Anderson G, Frampton J. Progression through key stages of haemopoiesis is dependent on distinct threshold levels of c-Myb. *EMBO J* 2003; **22**:4478–85.
 - 37 Shinkai Y, Koyasu S, Nakayama K, Murphy KM, Loh DY, Reinherz EL, Alt FW. Restoration of T cell development in RAG-2-deficient mice by functional TCR transgenes. *Science* 1993; **259**:822–8.
 - 38 Lee PP, Fitzpatrick DR, Beard C *et al.* A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. *Immunity* 2001; **15**:763–9.
 - 39 de Boer J, Williams A, Skavdis G *et al.* Transgenic mice with hematopoietic and lymphoid specific expression of Cre. *Eur J Immunol* 2003; **33**:314–8.
 - 40 Weston K. Extension of the DNA binding consensus of the chicken c-Myb and v-Myb proteins. *Nucl Acids Res* 1992; **20**:3043–9.
 - 41 Miller JH. *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1972.
 - 42 Garcia P, Berlanga O, Watson R, Frampton J. Generation of a conditional allele of the B-myb gene. *Genesis* 2005; **43**:189–93.
 - 43 Suto RK, Clarkson MJ, Tremethick DJ, Luger K. Crystal structure of a nucleosome core particle containing the variant histone H2A.Z. *Nat Struct Biol* 2000; **7**:1121–8.
 - 44 Abbott DW, Ivanova VS, Wang X, Bonner WM, Ausio J. Characterization of the stability and folding of H2A.Z chromatin particles: implications for transcriptional activation. *J Biol Chem* 2001; **276**:41945–54.
 - 45 Mo X, Kowenz-Leutz E, Laumonier Y, Xu H, Leutz A. Histone H3 tail positioning and acetylation by the c-Myb but not the v-Myb DNA-binding SANT domain. *Genes Dev* 2005; **19**:2447–54.
 - 46 Stanhope-Baker P, Hudson KM, Shaffer AL, Constantinescu A, Schlissel MS. Cell type-specific chromatin structure determines the targeting of V(D)J recombinase activity in vitro. *Cell* 1996; **85**:887–97.
 - 47 Mathieu N, Hempel WM, Spicuglia S, Verthuy C, Ferrier P. Chromatin remodeling by the T cell receptor (TCR)-beta gene enhancer during early T cell development. Implications for the control of TCR-beta locus recombination. *J Exp Med* 2000; **192**:625–35.
 - 48 McMurry MT, Krangel MS. A role for histone acetylation in the developmental regulation of VDJ recombination. *Science* 2000; **287**:495–511.
 - 49 Mauvieux L, Villey I, de Villartay JP. TEA regulates local TCR-Jalpha accessibility through histone acetylation. *Eur J Immunol* 2003; **33**:2216–22.