Histone Deacetylase 7 Regulates Cell Survival and TCR Signaling in CD4/CD8 Double-Positive Thymocytes

Herbert G. Kasler, Bryan D. Young, Denis Mottet, Hyung W. Lim, Amy M. Collins, Eric N. Olson and Eric Verdin

J Immunol published online 11 March 2011
http://www.jimmunol.org/content/early/2011/03/11/jimmunol.1001179

Supplementary Material http://www.jimmunol.org/content/suppl/2011/03/11/jimmunol.1001179.DC1

Subscription Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Histone Deacetylase 7 Regulates Cell Survival and TCR Signaling in CD4/CD8 Double-Positive Thymocytes

Herbert G. Kasler,*† Bryan D. Young,‡¹ Denis Mottet,*‡ Hyung W. Lim,* Amy M. Collins,* Eric N. Olson, † and Eric Verdin*

CD4/CD8 double-positive thymocytes express the transcriptional repressor histone deacetylase (HDAC)7, a class IIa HDAC that is exported from the cell nucleus after TCR engagement. Through signal-dependent nuclear export, class IIa HDACs such as HDAC7 mediate signal-dependent changes in gene expression that are important to developmental fate decisions in multiple tissues. We report that HDAC7 is exported from the cell nucleus during positive selection in mouse thymocytes and that it regulates genes mediating the coupling between TCR engagement and downstream events that determine cell survival. Thymocytes lacking HDAC7 are inefficiently positively selected due to a severely shortened lifespan and exhibit a truncated repertoire of TCR αβ segments. The expression of multiple important mediators and modulators of the response to TCR engagement is altered in HDAC7-deficient thymocytes, resulting in increased tonic MAPK activity that contributes to the observed loss of viability. Remarkably, the activity of protein kinase D, the kinase that mediates nuclear export of HDAC7 in response to TCR signaling, is also increased in HDAC7-deficient thymocytes, suggesting that HDAC7 nuclear export governs a self-sustaining autoexcitatory loop. These experiments add to the understanding of the life/death decision in thymic T cell development, define a novel function for class IIa HDACs, and point to a novel feed-forward mechanism whereby these molecules regulate their own state and mediate stable developmental transitions. The Journal of Immunology, 2011, 186: 000–000.

T cell development involves several cell-fate decisions mediated by TCR signals. The first of these is at the β-selection checkpoint, when ligand-independent signals from the newly rearranged TCR β-chain and the pre-TCR molecule are required for developmental progression (1). After β-selection, thymocytes proliferate extensively, begin producing TCR α-chains, and enter the CD4/CD8 double-positive (DP) stage. At the DP stage, αβ TCRs are tested to determine whether they recognize self ligands sufficiently to be potentially useful. Thymocytes receiving no TCR signal at the DP stage die within a few days by neglect, whereas low-level TCR stimulation delivers a survival signal to DP thymocytes, resulting in positive selection and maturation to the CD4 or CD8 single-positive (SP) stage. During the DP and SP stages, thymocytes are also subject to death by negative selection, a distinct process triggered by strong TCR signals from self ligands (2).

The TCR signaling apparatus can distinguish between ligands spanning a very narrow range of affinities and translate those differences into categorically different developmental outcomes (3). Presumably, categorical changes in gene expression patterns must accompany these sharp thresholds, suggesting the existence of high-gain gene expression switches that can undergo stable state changes across these. The class IIa histone deacetylases (i.e., HDAC4, 5, 7, and 9) are strong candidates for such signal-dependent binary switches. They are repressors of transcription that are known to regulate cell-fate decisions in the developmental contexts of angiogenesis, bone calcification, and hypertrophic cardiac growth (4–7). They are characterized by an extensive N-terminal domain that regulates nucleocytoplasmic shuttling and mediates recruitment to target promoters via interaction with transcription factors such as myocyte enhancer-binding factor (MEF)2D. Class IIa HDACs are exported from the cell nucleus in response to signal-mediated phosphorylation at specific conserved serine residues (8), thus functioning as signal-dependent repressors of transcription.

Several lines of evidence suggest that HDAC7, which is particularly abundant in the thymus, serves as a gene expression switch in T cell development. Characterization of HDAC7 in tissue culture systems has shown that TCR signaling causes nuclear export of HDAC7 via its phosphorylation by protein kinase D (PKD) (9, 10). HDAC7 regulates the expression of the proapoptotic orphan steroid receptor Nur77, and mutation or loss of HDAC7 affects TCR-induced apoptosis (9). Although this suggests a role in negative selection, putative HDAC7 targets identified in T cell hybrids are mostly genes differentially expressed during positive selection, and mutating or silencing HDAC7 interferes with in vitro differentiation of a DP thymoma (11).

In this study, we assess the effects of loss of HDAC7 function in thymocytes in vivo, using cre-mediated deletion of a lox site-flanked HDAC7 allele, as well as an HDAC7-VP16 fusion protein.
transgene that reverses its normal function. We find that HDAC7 is essential to maintaining both the basal pattern of gene expression and cell viability in unstimulated DP thymocytes, and that nuclear export of HDAC7 establishes a transcriptional state change during positive selection that has broad effects on the tonic signaling state of the cell.

Materials and Methods

Immunofluorescence microscopy

Thymocytes were prepared by standard methods from wild-type (WT), OT-1, or AND TCR transgenic mice. Thymocytes were stained for expression of CD4 and CD8 with Abs conjugated with Pacific Blue and allophycocyanin, respectively (see Abs and Western blotting below), and sorted to isolate DP and SP populations. Sorted thymocytes were centrifuged at 750 rpm for 3 min onto adhesive slides using a Shandon centrifuge (Thermo Shandon). Approximately 175,000 cells were spun onto each slide. Cells were immediately fixed with 3.7% paraformaldehyde for 10 min at room temperature, washed with PBS, and permeabilized with 0.1% Triton X-100 in PBS for 5 min. All steps from euthanization of the animal to fixation of the cells were performed on ice and within 90 min.Slides were blocked for 1 h with PBS-1% BSA and then exposed to the primary Ab (see Abs and Western blotting below) at 4 µg/ml in PBS-1% BSA overnight at 4°C. After washing in PBS-1% BSA, cells were incubated with secondary Abs (see Abs and Western blotting below) at 2 µg/ml in PBS-1% BSA for 1 h at room temperature. Slides were then incubated with TOPRO-3 nuclear dye (Molecular Probes) at 25 µg/ml for 30 min at room temperature. After washing, slides were mounted with Mowiol mounting medium and analyzed with a Leica TCS SP5 laser-scanning confocal microscope. Negative controls, including slides incubated with an irrelevant polyclonal Ab, slides exposed to secondary Abs only, and slides processed normally with thymocytes from HDAC7-deficient animals, all yielded negligible signals (data not shown). Images show are representative of at least six fields for each slide and of at least three slides prepared in independent experiments. Images were cropped and prepared for presentation using Adobe Photoshop. Individual channel luminosities were uniformly adjusted to facilitate visual interpretation.

Mouse strains

All operations involving animals were performed in accordance with U.S. Department of Agriculture guidelines and a University of California, San Francisco (UCSF) Institutional Animal Care and Use Committee-approved protocol. Animals were housed under standard conditions in a specific pathogen-free barrier facility at UCSF. Unless otherwise stated, all animals were analyzed at between 3 and 6 wk of age, and controls were sex-matched controls from the same litter. All experimental strains were either made on C57BL/6, AND, OT-1, OT-2, or p56lck proximal promoter-driven transgenic expression construct (obtained from Roger Perlmutter). Further details are available upon request. The resultant constructs were analyzed at between 3 and 6 wk of age, and controls were sex-matched controls from the same litter. Animals from the same litter. All experimental strains were either made on C57BL/6, AND, OT-1, OT-2, or p56lck proximal promoter-driven transgenic expression construct (obtained from Roger Perlmutter).

Flow cytometry

Lymphoid cells were prepared from mouse thymus and spleen by standard techniques, stained with fluorochrome-conjugated Abs for 30 min at 4°C (see Abs and Western blotting below), and fixed with PBS containing 2% paraformaldehyde. With the exception of experiments measuring cell viability, gates in the displayed histograms were gated by forward and side scatter to exclude dead cells. For analysis of early thymocyte subsets with CD25 and CD44 (Fig. 2B, 2C, 2E), T cell precursors were identified by staining with no expression of CD3, CD4, CD8, NK1.1, Mac-1, Gr-1, or Ter119. Analytical flow cytometry was performed using a FACSCalibur flow cytometer (BD Biosciences). Subsequent data processing and presentation for preparation were done using the FlowJo 7.0 data analysis package (Tree Star). Cell sorting was performed using the BD FACSDiva platform (BD Biosciences).

Abs and Western blotting

Cell lysates for Western blotting were prepared from thymocytes using RIPA buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholate) with protease inhibitor mixture (Sigma-Aldrich) and phosphatase inhibitors (1 mM NaF, 100 µM NaVO4). For all experiments other than those shown in Fig. 7D and 7A, SDS-PAGE-resolved proteins were transferred to nitrocellulose membranes (Amer sham Biosciences) and visualized using ECL detection reagents (Amer sham Biosciences). For quantitative Western blot analysis in Fig. 7D and 7A, fluorescent dye-conjugated secondary Abs were employed (LI-COR Biosciences) and visualized using a LI-COR Odyssey infrared scanner. Raw 16-bit TIFF image data from these scans were quantitatively analyzed using ImageJ (Wayne Rasband, National Institutes of Health). Band volumes for proteins of interest were normalized first against corresponding values for β-actin and then against the mean actin-normalized values for WT control samples. Abs used for Western blotting were as follows: HDAC7, H-273 rabbit polyclonal (Santa Cruz Biotechnology); β-actin, clone C-4 (MP Biomedicals); death-associated protein (Dap), A-16 goat polyclonal (SCB); phospho-Erk, D13.14.4E rabbit monoclonal (Cell Signaling Technology); phospho-Jnk, 81E11 rabbit monoclonal (Cell Signaling Technology); phospho-p38, rabbit polyclonal (catalog no. 9211; Cell Signaling Technology); Jnk, rabbit polyclonal (catalog no. 9252; Cell Signaling Technology); p38, rabbit polyclonal (catalog no. 9212; Cell Signaling Technology). Abs used for flow cytometry and cell sorting were as follows: CD4, clone GK1.5, FITC-, PE-, or Alexa Fluor 647 (AF647)-conjugated (UCSF Hybridoma Core Facility), Pacific Blue-conjugated (BD Biosciences); CD8α, clone YTS169.4, conjugated to AF647 (UCSF Hybridoma Core Facility) or clone 53-6-7, conjugated to PE (BD Biosciences); CD3e, clone 145-2C11, conjugated to FITC (UCSF Core Facility) or allophycocyanin (BD Biosciences); CD44, clone IM7.8.1, FITC-conjugated (Invitrogen); CD25, clone 3C7, PE-conjugated (BD Biosciences); CD147, clone RL73, PE-conjugated (eBioscience); CD11b (Mac-1), clone M1/70, allophycocyanin-conjugated (eBioscience); Ly-6G (Gr-1), RB6-8C5, allophycocyanin-conjugated (eBioscience); Ter-119, clone TER-119, allophycocyanin-conjugated (eBioscience); NK1.1, clone PK136, allophycocyanin-conjugated (eBioscience). Abs used for confocal immunofluorescence microscopy were as follows: primary Ab, rabbit anti-HDAC7 H-273 (Santa Cruz Biotechnology); secondary Ab, AF488-conjugated goat anti-rabbit IgG A11008 (Invitrogen). Secondary Abs used for quantitative Western blotting were IRDye 680LT-conjugated goat anti-mouse and IRDye 800CW-conjugated goat anti-rabbit (LI-COR Biosciences).

Quantitative RT-PCR

Data shown in Fig. 5 are based on analysis of four independent WT/ HDAC7 knockout (KO) littermate pairs. Total RNA was prepared from HDAC7 KO thymocytes and corresponding WT controls using the RNeasy Mini kit (Qiagen), then reverse transcribed using oligo(dT)18 primers and SuperScript II reverse transcriptase. The resulting cDNA was then amplified in an ABI 7900HT real-time PCR system (Applied Biosystems) using SYBR Green HotStar polymerase mix (MCLAB) and primers specific to the genes of interest. Primers for quantitative PCR were selected from pairs available at the Harvard/Massachusetts General Hospital Primer Bank (http://pga.mgh.harvard.edu/primerbank/). Only reactions yielding single-peak amplification products consistent with the predicted melting temperature for use were quantified. The Primer Bank ID numbers of the pairs used for Fig. 5C are as follows: Ak3, 6753032a1; Argap24, 9080832a1; Argap29, 656383a1; Bda2, 2924395a1; Cacn8, 668082a4; Caml2d, 18158420a1; Cd2, 7304949a3; Cd4, 7304953a1; Cd5, 31829467a1; Cdf6, 675335a1; Cflar, 6753274a1; Cll4, 35198147a1; Dgke, 9506541a1; Dusp2, 6753694a2; Dusp4, 28892737a1; Dusp6, 13399314a2; Egrl, 6681285a2;
Eg2, 1431892a; Etfb1, 19526962a; Gapdh, 6679937a; Gata3, 667951a; Hdc5, 6996924a; Il1r1, 6753403a; Il2ra, 6688393a; Itla1, 27370250a; Nrd1a, 6754216a; Nrd1c, 67567397a; Pcl1, 6679292a; Pik3cd, 6679319a; Pik3cg, 9937894a; Ppp1r1m, 30520513a; Ppp3ca, 3091291a; Ptkc, 6679937a; Ptk2b, 27369678a; Ppln4, 6679495a; Rorc, 6755344a; Sell, 6755454a2; Slap2, 26326849a3; Socs3, 671758a; Stat1, 6755670a; Tgfbr2, 27363474a; Trib2, 31542100a; Vav3, 6753032a; Wslo1, 6754567a; Zhk1, 6755344a; Zhk2, 6755344a.

DNA microarray analysis
Microarray data associated with this study have been deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE26488. CD4/CD8 DP thymocytes were isolated by FACS (using Abs to CD4 and CD8) from three animals each for WT, HDAC7-VP16 transgenic, and OT-2/OT-2 injected pairs were constituted by guest on April 18, 2017 http://www.jimmunol.org/ Downloaded from

Ex vivo cell death assay
Thymi from HDAC7-deficient or transgenic animals or corresponding littermate controls were disrupted on ice in RPMI 1640 medium with 10% FBS, pelleted, and then erythrocytes were lysed with ACK buffer. Cells were resuspended in RPMI 1640 medium supplemented with 10% FBS and standard (1%) concentrations of glutamine, penicillin, streptomycin, and 2-ME (Invitrogen) and placed in culture. Cell viability was measured by staining with annexin V (BD Biosciences) and propidium iodide (Molecular Probes) according to the manufacturers’ directions, followed by flow cytometric analysis using a FACSCalibur flow cytometer (BD Biosciences) and data analysis using FlowJo (Tree Star).

JA usage analysis
CD4/CD8 DP thymocytes were isolated by FACS from three HDAC7 loxp+/lck-cre animals and corresponding littermate controls (HDAC7 loxp+, lck-cre) with normal HDAC7 expression. Total RNA was prepared from these thymocytes using RNAeasy kit reagents (Qiagen) and used to make microarray probes. Probes were prepared using Affymetrix GeneChips. Raw 16-bit TIFF image data for each sample were normalized to corresponding values for GAPDH before comparison, and the relationship between Ct and template concentration was determined empirically for each primer pair.

Results
HDAC7 is exported from the cell nucleus during positive selection of thymocytes
Previous studies show that HDAC7 is expressed at high levels in DP thymocytes, but expression in earlier populations was not assessed (9). We therefore isolated mouse thymocyte populations from the DN3 stage, when β-selection occurs, to mature CD4- and CD8-negative cells and assayed expression of HDAC7 in each population by Western blot (Fig. 1A). We detected HDAC7 expression at all stages from DN3 forward, although less was observed in SP thymocytes and splenic T cells than at earlier stages. In both cell lines and primary thymocytes ex vivo, TCR stimulation causes rapid nuclear export of HDAC7 (9), but how HDAC7 responds to physiologic TCR signals has not been established. We therefore examined the subcellular localization of HDAC7 in thymocytes receiving different types of in vivo TCR signals (Fig. 1B). In WT DP thymocytes, which mostly receive no TCR stimulation, HDAC7 was present in the nucleus of nearly all cells (Fig. 1B, top left). In CD4 SP thymocytes, which have been positively selected, HDAC7 localization was in contrast almost universally cytoplasmic (Fig. 1B, top right). We also examined DP thymocytes from AND and OT-1 TCR transgenic animals, in which most DP thymocytes present in the steady-state have received a positively selecting TCR signal (18, 19), and observed predominantly cytoplasmic localization of HDAC7 in both the DP and SP populations from both strains (Fig. 1B, middle and bottom). HDAC7 nuclear export thus appears to occur in DP cells during positive selection and is apparently sustained throughout the SP stage of thymocyte development. This in turn suggests that HDAC7 might regulate gene expression during positive selection and that nuclear exclusion of HDAC7, once established, mediates a lasting change in the state of differentiation of thymocytes.

Loss of HDAC7 function in the thymus affects T cell maturation at the DP stage
To investigate its role during thymocyte differentiation, we deleted HDAC7 conditionally in thymocytes. A loxp site-flanked HDAC7 gene (5) was deleted by cre expression using the T cell-specific p56lck proximal (lck-cre) or CD4 (CD4-cre) promoters, resulting in loss of HDAC7 in total thymocytes (Fig. 1D). Although both of these cre drivers could delete HDAC7 in DP thymocytes, we found that deletion was often more complete with lck cre, so except where otherwise stated we used this strain for our analyses of HDAC7 deletion. We also generated animals with thymic
expression of an HDAC7 fusion protein, HDAC7-VP16, in which the HDAC7 catalytic domain is replaced by the transcriptional activation domain of HSV VP16 (Fig. 1C). In the absence of TCR stimulation, this protein activates transcription at HDAC7 target genes rather than repressing it, thus at least partially reversing normal HDAC7 function. This mode of action is supported by the ability of HDAC7-VP16 to induce Nur77 expression in T cell hybridomas in the absence of Ag receptor signaling (9), as well as the ability of analogous constructs based on other class IIa HDACs to induce differentiation in other systems in the absence of normally required signals (20, 21). HDAC7-VP16 was expressed under the control of the p56<sup>LK</sup> proximal promoter, with an expression level slightly lower than that of the endogenous protein in the thymus and nearly complete loss of transgene expression in peripheral T cells (Fig. 1E).

Loss or reversal of HDAC7 function had readily apparent effects on thymic T cell development. Thymic deletion of HDAC7 via either cre driver and expression of HDAC7-VP16 all reduced the proportion of CD4 and increased the proportion of CD8 SP thymocytes (Fig. 2A). However, nearly all of these CD8 SP cells proved to be immature SP (ISP) cells, the proliferating precursors of DP thymocytes, based on expression of the maturity marker CD3 and the proliferation marker CD147 (see Supplemental Fig. 1A). When the numbers of cells present at each developmental stage were assessed, we found that deletion of HDAC7 mediated by both cre drivers increased the ISP population, moderately reduced the population of DP cells, and substantially reduced the population of mature CD4 and CD8 SP thymocytes. There was also a reduction in both CD4 and CD8 T cells in the spleen (Fig. 2B). Heterozygous expression of the HDAC7-VP16 transgene resulted in a profile of subsets very similar to what was observed for the KO animals (Fig. 2C, open columns), whereas homozygous expression resulted in a small increase in DN3 and DN4 cells, as well as in a substantial reduction in the DP population (Fig. 2C, filled columns). Loss of HDAC7 function therefore appears to lead to a significant defect in the ability of DP thymocytes to become positively selected and to mature to the SP stage, and its reversal by expression of HDAC7-VP16 seems additionally to impair either the generation or viability of DP cells.

**Loss of HDAC7 does not grossly affect the TCR affinity thresholds for positive selection, nor does it alter β-selection**

One hypothesis that could account for the defect in positive selection in HDAC7 KO thymocytes is that loss of HDAC7 function might cause large changes in the TCR activation thresholds that define the boundaries between neglect and positive selection or between positive and negative selection, thus reducing the population of TCRs that can mediate survival. For this mechanism to account for the large decline in SP thymocytes due to loss of HDAC7, most TCR specificities that normally mediate positive selection would instead have to lead to cell death in HDAC7-deficient thymocytes. To test this, we deleted HDAC7 (via the lck-cre driver) in mice transgenic for three TCR specificities that are normally positively selected (OT-1, OT-2, and AND). Unexpectedly, we found that in all cases, introduction of a TCR transgene reduced rather than exacerbated the effect of loss of HDAC7 function on thymocyte maturation (Fig. 2B versus Fig. 2A, filled columns). Loss of HDAC7 function therefore appears to lead to a significant defect in the ability of DP thymocytes to become positively selected and to mature to the SP stage, and its reversal by expression of HDAC7-VP16 seems additionally to impair either the generation or viability of DP cells.
thymocytes might be able to mature to the DP stage without producing a TCR β-chain. The resulting TCR-deficient DP thymocytes might then be incapable of progressing further. This notion is supported by the phenotype of thymocytes expressing a constitutively active mutant of PKD, the kinase that phosphorylates HDAC7 in response to TCR signals. Thymocytes expressing activated PKD can mature to the DP stage without a functional TCR β-chain, bypassing the β-selection checkpoint (22). We therefore tested whether PKD exerts this effect through HDAC7 by deleting HDAC7 in animals lacking the Rag1 recombinase, in which no TCR β-chain can be produced. Rag1 deficiency normally results in a block at the DN3 stage (23). In contrast to what was observed in Rag1-deficient PKD-transgenic mice, we saw no progression beyond the DN3 stage in HDAC7/Rag1 double KOs (Fig. 2E). Thus, thymic deletion of HDAC7 with lck-cre is not sufficient to bypass β-selection, and any defect in the generation of positively selecting TCRs in the HDAC7 KO must occur subsequent to β-selection.

**HDAC7 regulates cell survival in DP thymocytes**

Another possible explanation for impaired positive selection in HDAC7 KO thymocytes is increased apoptosis at the DP stage, a hypothesis consistent with the reduced DP population we observed in the HDAC7 KOs and especially the high-expressing HDAC7-VP16 transgenics (Fig. 2B, 2C). When investigating this hypothesis, we found that in ex vivo cell culture, lck-cre-driven HDAC7 KO thymocytes became apoptotic very rapidly, being 30% less viable than their HDAC7-expressing counterparts 3 h after isolation, and only a third as viable after 24 h (Fig. 3A, top panel). At 3 h in culture, there was already extensive cleavage of caspase-3, indicating strong activation of apoptotic pathways (Fig. 3B). Thymocyte viability was similarly reduced as a result of expression of HDAC7-VP16 (Fig. 3A, bottom two panels). HDAC7 function in DP thymocytes thus appears to be required to maintain normal thymocyte viability.

Mice deficient in retinoic acid-related orphan receptor (Ror)-γ are a well-studied example of the phenotype resulting from impaired thymocyte viability. In these animals, shortened thymocyte survival time results in a distribution of subsets very similar to what we observed for HDAC7 deletion (24). Ror-γ KO thymocytes mature inefficiently in part because their short survival window at the DP stage allows insufficient time to fully sample the available repertoire of TCR α-chains. DP thymocytes normally

**FIGURE 2.** Perturbation of HDAC7 function is associated with altered thymocyte maturation. A, Scatter plots showing altered CD4/CD8 expression in thymocytes in which HDAC7 has been deleted via the lck-cre driver (first row) or the CD4-cre driver (second row), and thymocytes transgenic for HDAC7-VP16 (third and fourth rows), together with corresponding littermate controls (left). Results shown are representative of at least six independent experiments. B, Percentage relative to littermate controls of cell numbers present in the indicated thymic developmental subsets for HDAC7-deficient thymocytes generated using the CD4-cre (open columns) or lck-cre (filled columns) drivers. DN1–4, double-negative 1–4; ISP, SP4/8, mature (TCR-ii) CD4 and CD8 SP thymocytes; spl4/spl8, splenic CD4 and CD8 T cells. C, Percentage relative to littermate controls of cell numbers present in the indicated thymic developmental subsets for HDAC7-VP16 transgenic mice that are heterozygous (open columns) or homozygous (filled columns) for the transgene. Error bars in B and C are ±SEM for six or more independent experiments. *p < 0.05, paired Student t test. D, Percentage relative to TCR transgene-expressing littermate controls of cell numbers present in the indicated thymic developmental subsets for OT-1 (white columns), OT-2 (black columns), or AND (gray columns) TCR transgenic thymocytes deficient in HDAC7. Error bars are ±SD of three to four independent experiments. *p < 0.05, paired Student t test.
undergo repeated Vα-Jα rearrangements at the TCR α-chain locus, sequentially producing multiple α-chains with different specificities. For the Jα segments, the rearrangements move down the array from the 5′ end (Jα61) to the 3′ end (Jα1) (25). If the survival time of thymocytes is shortened, as in the Rorγ KO, usage of the more 3′ Jα segments is abrogated and positive selection is markedly impaired (17).

To determine whether the loss of viability we observed in HDAC7-deficient thymocytes was of similar consequence, we assayed Jα usage in these cells by the same method used for the Rorγ KO (17). We amplified cDNA from HDAC7-expressing and HDAC7 KO DP thymocytes with primers specific to the Vα3 or Vα10 families of V segments and the Cα segment, and then resolved and blotted the resulting fragments. Probing these blots with end-labeled oligonucleotides specific to a selection of Jα segments spanning from Jα2 at the 3′ end of the locus (top) to Jα58 at the 5′ end (bottom). D, Quantification of data shown in B. The ratios of Jα/Cα signal were determined for each Jα band, and ratios for each KO were divided by ratios for each littermate control. Graph shows mean of the three KO/LMC ratios thus generated. Error bars indicate SD. In all panels, the genotype of HDAC7 KO thymocytes is loxp/null, cre+ and the genotype of littermate controls is either loxp/WT, cre+ or null/WT, cre+.

FIGURE 3. HDAC7-deficient thymocytes have a reduced lifespan in vivo. A, Accelerated apoptosis in HDAC7-deficient and HDAC7-VP16 transgenic thymocytes. Thymocytes were prepared from HDAC7 KO (top two graphs, black symbols) and HDAC7-VP16 transgenic mice (bottom two graphs, black symbols), as well as corresponding littermate controls (gray symbols), and placed in tissue culture. Apoptosis was measured by annexin V staining. Measurements were made at 0, 3, 24, 48, and 72 h of culture. Error bars represent SD of 5–10 independent experiments. B, Western blot showing cleavage of caspase-3 in HDAC7-deficient (KO) and HDAC7-expressing (‡) littermate control thymocytes after 3 h in culture. C, Truncated Jα segment usage in HDAC7 KO. RT-PCR products were generated using forward primers specific for the Vα3 (left) or Vα10 (right) segment families and a reverse primer specific to Cα, blotted and probed with primers specific to the indicated Jα segments, spanning from Jα2 at the 3′ end of the locus (top) to Jα58 at the 5′ end (bottom). D, Quantification of data shown in B. The ratios of Jα/Cα signal were determined for each Jα band, and ratios for each KO were divided by ratios for each littermate control. Graph shows mean of the three KO/LMC ratios thus generated. Error bars indicate SD. In all panels, the genotype of HDAC7 KO thymocytes is loxp/null, cre+, and the genotype of littermate controls is either loxp/WT, cre+ or null/WT, cre+.
in usage of the most S’ Jα segments (Fig. 3D). These results suggest that loss of HDAC7 during the DP stage results in a shortened thymocyte lifespan that both impairs the ability of thymocytes to become positively selected and truncates the TCR α-chain repertoire.

**HDAC7 mediates gene expression changes associated primarily with positively selecting TCR signals**

Having established that HDAC7 regulates cell death pathways in thymocytes, we set out to identify its cellular targets by assessing the effects of loss of HDAC7 function on gene expression in DP thymocytes. We generated gene expression profiles for unstimulated thymocytes with normal HDAC7 expression, HDAC7 conditional KO (lck-cre) thymocytes, and HDAC7-VIP16 transgenic (homozygous) DP thymocytes. We also profiled DP thymocytes from OT-2 transgenic mice, which receive predominantly positively selecting TCR signals, and from OT-2 mice injected with the OVA-derived agonist peptide, which receive predominantly negatively selecting TCR signals (see Fig. 4A). Profiles for each condition were generated using Affymetrix Mouse Gene 1.0 ST expression arrays, comprising ~27,000 annotated transcripts. Different binary comparisons between these gene expression changes profiles represent the effect of loss or reversal of HDAC7 function in unstimulated thymocytes, and also gene expression changes associated with positively and negatively selecting TCR signals (see Fig. 4A). Genes significantly induced (Fig. 4A, green numbers) and repressed (Fig. 4A, red numbers) in each of these comparisons were identified by analysis with the SAM algorithm (13). For full information on all genes differentially expressed in each of these comparisons, please see “Summary of Differentially Expressed Genes” at the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE26488.

Both loss of HDAC7 and expression of HDAC7-VIP16 caused gene expression changes that overlapped very significantly with positive selection (p < 10^-58 and p < 10^-41, respectively), and less so with negative selection (p < 10^-11 and p < 0.6, respectively; Fig. 4B, top two Venn diagrams; see Supplemental Fig. 2A for downregulated genes). As expected, the overlap between loss of HDAC7 and HDAC7-VIP16 expression was very significant (p < 10^-66, Fig. 4B, lower left Venn diagram; see Supplemental Fig. 2A for downregulated genes), and the union of these two sets of differentially expressed genes also overlaps much more strongly with positive than with negative selection (Fig. 4B, lower two Venn diagrams). Among the most highly induced genes in HDAC7 KO thymocytes, >60% are also induced during positive selection (Fig. 4C, top two blocks), 58% are also induced by HDAC7-VIP16 expression (Fig. 4C, top block and third block from top), and >35% are induced both during positive selection and due to HDAC7-VIP16 expression (Fig. 4C, top block). In contrast, <17% of the most induced genes in the HDAC7 KO overlap with neither positive selection nor HDAC7-VIP16 (Fig. 4C, bottom block), and only two of these genes (2.5%) are discordant between HDAC7 KO thymocytes and either positive selection or HDAC7-VIP16 expression (Fig. 4C, asterisks). There is a similarly strong relationship between the genes most strongly repressed by loss of HDAC7 and those repressed during positive selection or HDAC7-VIP16 expression (see Supplemental Fig. 2B).

**HDAC7 regulates cell survival in DP thymocytes via multiple TCR-regulated pathways**

Examination of the functions of the genes differentially expressed in HDAC7 KO thymocytes showed a pervasive theme of signal transduction. We used the Ingenuity Pathways Analysis bioinformatics suite (Ingenuity Systems) to analyze the representation of genes induced or repressed in the KO in terms of biological process ontology and canonical signaling pathways (Fig. 5A, 5B). The analysis with respect to biological processes revealed a strong correlation of HDAC7 targets with autoimmune, cell death, and lymphocyte development (Fig. 5A; see Supplemental Table I for full annotated list). Many of these categories were also overrepresented in the HDAC7-VIP16 transgensics (Fig. 5A, asterisks, Supplemental Table I). Examination of the canonical signaling pathways associated with the HDAC7 KO gene set yielded multiple Ag receptor and costimulatory pathways among the most highly overrepresented categories, as well as helper T cell differentiation (Fig. 5B), all of which were also overrepresented in the HDAC7-VIP16 transgensics (Fig. 5B, asterisks; see Supplemental Table I for full list).

Many genes identified in these analyses were cell surface receptors, including several with known functions in T cell costimulation, homing, and differentiation. Among these were Cd5, Cd4, and Cd62l, three maturation markers that are upregulated during or after positive thymic selection (26–28), as well as Ctd4 and Btla. Cd4 and the negative selection-associated Pd-1 were both downregulated in HDAC7 KO thymocytes. In addition to these molecules associated with costimulation, there was a notable cluster of molecules associated with helper T cell differentiation. The IFN-γ receptor and the transcription factor Gata3, which respectively support Th1 and Th2 development in mature T cells, were both upregulated in HDAC7-deficient thymocytes. Other factors involved in effector differentiation, such as the Tgfβ receptor, Il18 receptor, and Stat4 (29, 30), were also upregulated in the absence of HDAC7, whereas the suppressive factors Socs1, Socs3 (31), and Il10ra (32) were downregulated. Another major theme that emerged from our examination of HDAC7 targets was regulators and members of the kinase cascade downstream of TCR engagement. Several important signal transducers downstream of the TCR, including Akt3, Pyk2, Vav3, Pi3kbβ/γ, Pck0, Camk2, and calcineurin (33), were upregulated in the HDAC7 KO. Conversely, several members of the MAPK phosphatase family (34), as well as the downmodulators of TCR signaling Slap2 (35), Tribbles2 (36), and Dgke (37), were downregulated in the absence of HDAC7.

Unexpectedly, expression of the proapoptotic orphan steroid receptor Nur77 (Nr4a1), a previously identified direct transcriptional target of HDAC7 in the DO.11.10 T cell hybridoma (9, 11), was repressed rather than induced in HDAC7 KO thymocytes, as was the functionally redundant orphan steroid receptor Nor-1 (Nr4A3). Surprisingly, we also did not observe upregulation of either of these molecules in HDAC7-VIP16 transgenic thymocytes. However, we did note the upregulation of two positive modulators of cell death, Ndrg-1 (38) and Dag (39), and the downregulation of the survival and maturation-promoting molecules Egr-1 and Egr-2 (40) in HDAC7-deficient thymocytes, suggesting a proapoptotic pattern of differential expression in thymocytes lacking HDAC7, regardless of Nur77 or Nor-1. Hdac5, another previously identified direct target of HDAC7 (11), was however upregulated both in HDAC7-deficient and HDAC7-VIP16 transgenic DP thymocytes.

After identifying all of these putative HDAC7 targets of particular interest, we attempted to independently confirm their differential expression in HDAC7 KO thymocytes using quantitative PCR analysis. Of 47 genes analyzed in this fashion, 36 were confirmed to be changed in expression in the manner indicated by the microarray results (Fig. 5C), 5 were found not to be significantly changed in expression, and 6 did not yield conclusive results. Genes discussed above not found to be significantly changed by quantitative PCR included Nur77, Nor-1, and Pck0, whereas those that did not yield conclusive results by quantitative
PCR included Socs1, Dap, and Ndrg-1. Expression changes in these last three genes could however be validated using Northern and Western blotting (see Supplemental Fig. 2C). Overall, this pattern of gene expression changes suggests a potentiation of multiple signaling pathways that mediate responses associated with thymocytes that have been positively selected, including effector differentiation in response to cytokine signals, homing to peripheral lymphoid tissues, costimulation, and cell death.

Based on the set of genes confirmed as differentially expressed, we mapped the relevant HDAC7 targets to a TCR/costimulatory pathway schematic (Fig. 6). These 26 gene expression changes impinged at multiple levels upon pathways leading from the TCR to the major entry points into the nucleus (Fig. 6, purple-shaded genes), comprising NFAT, NF-κB, and the ERK, JNK, and p38 MAPks. Activating changes outnumbered inhibitory ones 17 to 7, suggesting that HDAC7 nuclear export acts in a broad and concerted way to potentiate the downstream responses to TCR engagement.

Among the changes to the TCR signaling pathway caused by loss of HDAC7, there were a particularly large number that would be predicted to potentiate the RAC-mediated activation of JNK and p38 MAPks (Fig. 6). Several of these changes were also caused by expression of HDAC7-VP16 (Fig. 6, asterisks). Because the activation of JNK and p38 is highly relevant to thymocyte apoptosis,
we examined the tonic state of these MAPKs in unstimulated thymocytes lacking HDAC7. Unstimulated WT and HDAC7 KO DP thymocytes were isolated, as well as HDAC7-VP16 and OT-2 TCR transgenic DP thymocytes. These populations were analyzed by Western blot using Abs specific for phospho-p38 and phospho-JNK. We found increased basal phosphorylation of p38 and JNK in both HDAC7-deficient and HDAC7-VP16 transgenic thymocytes (Fig. 7A,7B), consistent with what normally occurs during positive selection (Fig. 7A, OT-2). Thus, as predicted by the pathway assignment above, HDAC7 functions as a modulator of the coupling between TCR signaling and the activity of these MAPKs.

Because there is already evidence linking p38 to apoptosis of thymocytes (41), we investigated the possibility that the elevated basal activity of p38 in HDAC7 KO thymocytes might contribute to their accelerated apoptosis, using pharmacologic inhibitors of the p38 family. Immediately after isolation, both HDAC7-expressing and HDAC7 KO thymocytes were ∼8% apoptotic as assayed by annexin V binding (Fig. 7C, t = 0). After 3 h, HDAC7-expressing thymocytes were still only 11% apoptotic whereas HDAC7-deficient ones showed 35% annexin V-positive cells. This rapid apoptosis was markedly inhibited by treatment with the specific p38 inhibitor ML3403 at 10 μM, diminishing by 4-fold the difference between HDAC7-expressing and HDAC7 KO thymocytes (Fig. 7C, last two columns). In contrast, the JNK inhibitor SP 600125 provided no protection when used at 20 μM concentration in this assay (data not shown). Thus, the tonic activation of p38 caused by loss of HDAC7 appears to account for at least part of the proapoptotic phenotype in these cells, establishing a novel pathway whereby HDAC7 regulates thymocyte survival.

Finally, the finding that HDAC7 regulates modulators of TCR signaling (see Fig. 6), combined with the observation that HDAC7 nuclear export is apparently a stable change in thymocytes that occurs during positive selection (see Fig. 1B), suggested to us that nuclear export of HDAC7 might govern an autoexcitatory loop that will result in constitutive activation of pathways that mediate its own nuclear exclusion. We therefore examined the state of PKD in HDAC7-deficient thymocytes. PKD, the kinase that mediates nuclear export of HDAC7 in response to TCR signaling (10, 42), autophosphorylates at serine 916 when active (43). When we examined the status of this serine residue in unstimulated HDAC7-expressing and HDAC7 KO thymocytes by Western blot, we found that there was a substantial increase in PKD serine 916 phosphorylation when HDAC7 was absent (Fig. 7D,7E), suggesting that loss of HDAC7 is itself sufficient to activate PKD. Based on these findings, the initial loss of HDAC7 from the cell nucleus apparently mediates a change in the tonic state of the TCR signaling apparatus that can maintain its continued nuclear exclusion in the absence of further TCR engagement. HDAC7 nuclear exclusion may therefore mediate a stable change in the signaling state of the cell in response to a transient TCR signal, and thus function as a one-way developmental switch.

FIGURE 5. Bioprocess analysis and quantitative PCR confirmation of gene regulation by HDAC7. A, Top 10 Ingenuity Pathways Analysis (IPA) biofunction terms (bold type) overrepresented among genes differentially expressed in HDAC7 KO thymocytes. Nonredundant cell terms with p values ≤10−5 are listed for each category heading. Asterisks indicate significant overrepresentation of the indicated categories in HDAC7-VP16 transgenic animals as well as in the KOs. B, Top 19 IPA canonical signaling pathways overrepresented among genes differentially expressed in HDAC7 KO thymocytes. Genes were selected for bioprocess/pathway analysis using the criteria of a SAM false detection rate of ≤10% and a fold differential expression of ≥1.5. Asterisks indicate significant overrepresentation of the indicated categories in HDAC7-VP16 transgenic animals as well as in the KOs. C, Confirmation by real-time PCR of differential expression of putative HDAC7 targets of particular functional interest. Gray bars indicate fold differential expression as determined by real-time PCR; black bars indicate the corresponding microarray values. Error bars are ±SD of four independent HDAC7-expressing/KO littermate pairs. Differential expression of all genes shown in graphs was significant at p < 0.05 according to a two-tailed Student t test.
These include Camk2d, Cd5, Dap, Dusp2/4, Egr1 and Egr2, targets in a prior study done by our group, involving microarray targets of HDAC7 in this study were also identified as HDAC7 not all of the molecules we identified as particularly interesting to maintain the viability of thymocytes at the DP stage. Many but perhaps other developmental decisions, in a manner that is required Thymocytes that lack HDAC7 exhibit gene expression changes normally associated with positive selection in the absence of any Thymocytes that lack HDAC7 exhibit gene expression changes normally associated with positive selection in the absence of any consequence of loss of HDAC7 function, since a substantial induction of Nur77 expression cannot however be an immediate explanation that could account for this result would be that Nur77 expression is induced by loss of HDAC7 function or HDAC7-VP16 expression, but that this induction is “censored” due to the immediate apoptosis and clearance of any thymocytes that upregulate Nur77 beyond some critical level. Such a censored induction of Nur77 expression cannot however be an immediate consequence of loss of HDAC7 function, since a substantial number of DP thymocytes are found in HDAC7 thymic KO and HDAC7-deficient thymocytes. Ror-γ null mice show a marked loss of viability in DP thymocytes, a phenotype similar to what we observe in HDAC7 KOs (24). We have however not found any connection between Ror-γ and HDAC7 in terms of shared known targets or mutual regulation, and Ror-γ was actually found to be slightly upregulated in HDAC7-deficient thymocytes (Fig. 5C) (11). In contrast, if Nur77 is directly introduced into thymocytes as a constitutive transgene, there are almost no DP thymocytes observed (44). Further studies, perhaps using HDAC7/Nur77/Nor-1–deficient animals, will be required to determine whether these molecules make such a cryptic contribution to the cell death phenotype we have observed.

We have however investigated some known mechanisms that regulate thymocyte survival with respect to their possible contribution to the phenotype of HDAC7-deficient thymocytes. Ror-γ inhibition of perturbation of class IIa HDACs in different tissues such as endothelium, bone, and myocardium. This idea is supported the nuclear export of HDAC7 to the differential expression of these modifications of these molecules, and we cannot rule out that these changes may also be rendered cryptic by the rapid death and clearance of affected thymocytes. Future work will focus on determining how the modulators of TCR signaling and MAPK activity that we have identified as HDAC7 targets may contribute to the basal activation of p38 observed upon loss of HDAC7, on mapping the cascade of gene regulation events that leads from the nuclear export of HDAC7 to the differential expression of these factors, and on identifying what factors downstream of p38 are responsible for accelerated cell death. Another interesting possibility raised by our findings is that modulation of MAPK and other signaling pathways might mediate some of the phenotypic effects of perturbation of class IIa HDACs in different tissues such as endothelium, bone, and myocardium. This idea is supported the finding that p38 has been implicated in the development of hypertrophic cardiomyopathy in mouse models (48).

Questions remain as to the physiologic significance of nuclear exclusion of HDAC7 during positive selection. Although HDAC7 nuclear exclusion is not itself sufficient to lead to positive selection,
it appears to mediate a change in the signaling state of the cell that is part of the process, and retention of HDAC7 in the cell nucleus is required to maintain the viability of unstimulated DP thymocytes long enough to allow them to fully sample the repertoire of available TCR α-chains and thus optimize their chances of getting positively selected. HDAC7 thus appears to function as a negative regulator of the coupling between TCR engagement and the downstream events that determine cell fate. As such, it may be required to maintain the relationship between signaling inputs and outputs that defines the DP developmental stage. The increased population of ISP cells evident in the HDAC7 KO might thus represent a difficulty in establishing the DP phenotype in the absence of HDAC7. HDAC7 could also be regarded as a developmental switch that enables subsequent differentiation programs, such as negative selection or effector differentiation, once positive selection has occurred.

Interestingly, nuclear exclusion of HDAC7 also appears to affect the signaling elements that govern the behavior of HDAC7 itself. In thymocytes lacking HDAC7, PKD, the kinase that mediates HDAC7 nuclear exclusion, is phosphorylated in a manner that indicates its activation. These findings suggest that the initial nuclear export of HDAC7 triggers an autoexcitatory loop that will maintain both HDAC7 nuclear exclusion and the gene expression changes that are part of the process, and retention of HDAC7 in the cell nucleus is required to maintain the viability of unstimulated DP thymocytes. HDAC9, another class IIa HDAC, has already been shown to play a role in regulatory T cell function (51), and HDAC7 has been shown to participate in a complex with FOXP3 and TIP60 (52), suggesting it may play a similar role. Establishing the appropriate experimental systems to investigate these possibilities is an area of ongoing work in our laboratory, which we anticipate will reveal important new aspects of the extensive differentiation program regulated by HDAC7.

FIGURE 7. Changes in tonic kinase activity caused by loss of HDAC7 function. A, Western blots showing phosphorylation of p38 and JNK MAPks in DP thymocytes that are WT (first lane), HDAC7 KO (second lane), HDAC7-VP16 transgenic (third lane), or OT-2 TCR transgenic (fourth lane) for three independent sets of mice for each genotype. Expression of total p38, JNK, HDAC7, and β-actin are also shown. B, Quantification of actin-normalized fluorescent signals for p38, phospho-p38, JNK, and phospho-JNK from the Western blot shown in A. Error bars represent the SD of three different animals of the indicated genotype. Units arbitrarily represent the mean fluorescent signal of the WT bands as 1. *p < 0.05, two-tailed Student t test. C, Early wave of ex vivo apoptosis in HDAC7-deficient thymocytes is blocked by p38 inhibition. Thymocytes were harvested and cultured in medium containing either the p38 inhibitor ML3403 or vehicle. Percentage of apoptotic cells is shown for WT (first two bars) and HDAC7-deficient (second two bars) thymocytes immediately after isolation (left) and after 3 h in culture (right). Error bars represent the SD of four independent experiments; the p value is based on a two-tailed Student t test. D, Western blots showing phosphorylation of PKD1 at serine 916, as well as expression of PKD, HDAC7, and actin in WT and HDAC7 KO thymocytes from four different animals for each genotype. E, Quantification of actin-normalized signals for PKD and phospho-PKD expression from Western blots shown in D. Error bars represent SD of the four samples shown. Arbitrary units set mean fluorescence signal of WT bands to 1. F, Schematic illustrating putative autoexcitatory loop that maintains both HDAC7 nuclear exclusion and the gene expression changes it mediates after its initial nuclear export in response to a positively selecting TCR signal.
Acknowledgments
We thank John Carroll for graphics and Veronica Fonseca for administrative assistance, Dimitris Kiousis and Roger Perlmutter for transgenic expression vectors, Linda Ta for microarray hybridization and scanning, and Sid Espineda for microinjection. We thank Andy, Sara, Ken, and Meg Kurtzig and Leslie Mulholland for generous support.

Disclosures
The authors have no financial conflicts of interest.

References
Supplementary figure legends

**Figure S1.** A. Representative flow histograms showing expression of CD3ε (left) and CD147 (right) in CD8 SP thymocytes from HDAC7-deficient (right) and corresponding littermate controls (left). Low CD3ε expression and high CD147 expression define the ISP population. Plots are representative of at least six independent experiments. B. Scatter plots showing CD4 and CD8 expression in AND, OT-1, or OT-2 TCR transgenic thymocytes deficient in (top) or expressing (bottom) HDAC7. Results are representative of at least three independent experiments for each panel.

**Figure S2.** A. Venn diagrams indicating overlap of genes repressed due to HDAC7 deficiency, positive selection, HDAC7-VP16 expression, and negative selection. Numbers outside of the diagrams are the P-values (according to the binomial distribution) of the indicated numbers of overlapping genes. B. Comparison of differential expression profiles corresponding to HDAC7 deficiency, positive selection, and HDAC7-VP16 expression for 79 genes that scored in the bottom 100 for both SAM score and fold differential expression in HDAC7 knockout thymocytes. Top group: genes concordantly repressed in all three conditions; second group: genes repressed only in HDAC7 knockout thymocytes and positive selection; third group: genes repressed in HDAC7 KO and HDAC7-VP16 transgenic thymocytes but not positive selection; bottom group: genes repressed only in HDAC7 KO thymocytes. Single asterisks indicate 4 genes discordantly regulated between HDAC7 knockout thymocytes and positive selection. Double asterisks indicate 6 genes discordantly regulated between HDAC7 knockout thymocytes and HDAC7-VP16 transgenic thymocytes. C. Differential expression of Socs1 and Ndrg1 in HDAC7 knockout thymocytes shown by Northern blot (top 2 panels) and differential
expression of Dap shown by Western blot. Numbers above Northern blots indicate GAPDH-normalized expression values for the corresponding bands.