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

Herbert G. Kasler,∗1 Bryan D. Young,∗,1 Denis Mottet,∗,2 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 autoregulatory 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: 4782–4793.

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-TCRs 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 them. The class IIa histone deacetylases (i.e., HDAC 4, 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 hybridomas 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 loxp site-flanked HDAC7 allele, as well as an HDAC7-VP16 fusion protein.
transgene that reverses 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 4 (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 euthanasia 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 °C/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 shown 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 3 and 6 wk of age, and controls were sex-matched animals from the same litter. All experimental strains were either made on a C57BL/6 genetic background or backcrossed into C57BL/6 for at least six generations. C57BL/6, AND, OT-1, OT-2, and p56kKkk proximal promoter-cre (lck-cre) mice were obtained from The Jackson Laboratory. CD4-cre mice bearing HDAC7 null and floxed cre (lck-cre) mice were obtained from The Gladstone Institutes Transgenics Core Facility by pronuclear injection of linearized p1013 locus-controlling HDAC7 transgenes were prepared at the Gladstone Institutes Transgenics Core Facility by pronuclear injection of linearized p1013 locus-controlling region (LCR) constructs (see Plasmids below) into C57BL/6 recipients according to standard protocols. Primers for detecting the HDAC7-VP16 transgene, homologous to the lck proximal promoter and the N-terminal region of the HDAC7, respectively, were as follows: forward primer, 5'-CTG-TGGCGGTGGTGGCATTCCAGGGTG-3'; reverse primer, 5'-GAGAAC-CTGCGCCAGCTTGTCTTGCTGG-3'. Homozygosity of HDAC7-VP16 transgenics was determined based on relative copy number, using quantitative PCR. All PCR experiments were repeated at least twice with DNA from two independent PCR amplifications. The resulting cDNA was then amplified using specific primers and SuperScript II reverse transcriptase. The resulting cDNA was used as a template to amplify the HDAC7 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.

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, events 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 gating on cells with no expression of CD3, CD4, CD8, NK1.1, Mac-1, Gr-1, or Ter119. Analytical flow cytometry was performed using a FACSComp dye flow cytometer (BD Biosciences). Subsequent data processing and preparation for presentation were done using the FlowJo 7.0 data analysis package (Tree Star). Cell sorting was performed using the BD FACS Diva 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 Na3VO4). 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 (A mer 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 the LI-COR Odyssey scanning system. 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 53-67, conjugated to PE (BD Biosciences); CD3ε, clone 145-2C11, conjugated to FITC (UCSF Core Facility) or clone CAMD5, conjugated to 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-6-G (Gr-1), RB6-8C5, allophycocyanin-conjugated (eBioscience); Ter-119, clone TER-119, allophycocyanin-conjugated (eBioscience); NK1.1, clone PK1.36, 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. 8 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)15 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 Hot Start polymerase mix (MCLAB) and primers specific to the genes of interest. Primers for quantitative PCR were selected from published results and were verified to produce a single peak amplification product consistent with the predicted melting temperature. The resulting cDNA was used in a primer set for each gene to analyze RT-PCR amplification. The resulting cDNA was used as a template to amplify the HDAC7 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.

Plasmids

The HDAC7-VP16 mutant insert was prepared as described elsewhere (9). It was cloned by blunt-ended ligation into the multi-cloning site of the p1013 LCR plasmid vector. The p1013 LCR vector was constructed by cutting a 5-kb 5′ fragment of the CD2 LCR from the human CD2 mini-locus construct (obtained from Dimitris Kioussis) and inserting it down-stream of the transcriptional cassette of the p1013 lck proximal promoter-driven transgenic expression construct (obtained from Roger Pomerlatter). Further details are available upon request. The resultant constructs were linearized and bacterial vector sequences removed before injection into mouse embryos.

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Eg2, 14318992a1; Enrifl, 19526962a1; Gapdh, 6679937a1; Gata3, 6679591a1; Hdc5, 6996294a1; Il17a, 6608389a1; Il2t, 27330250a1; Nr4a1, 6753241a1; Nr4a3, 767397f1; Pck1, 6679229a1; Pik3cd, 6679319a1; Pik3cg, 9937984a1; Ppm1n, 30520153a1; Ppca3, 309129a1; Ptkc, 6679937a1; Ptk2h, 27369678a1; Pttn4, 6679495a1; Ror, 6573341a1; Sell, 6654542a2; Slap2, 26262849a3; Socs3, 667178s1; Stat4, 6755670a1; Tgb2r, 2736347a1; Trib2, 3154201a1; Vav3, 6793032a1. Threshold cycle (Ct) data for each sample were normalized to corresponding values for GAPDH before comparison, and the relationship between Ct and relative template concentration was determined empirically for each primer pair.

**Northern blotting**

Total RNA was prepared from thymocytes of HDAC7-deficient and littermate control animals using TRIzol (Invitrogen). Fifteen micrograms RNA/lane was resolved by formaldehyde agarose electrophoresis, and RNA was transferred to Hybond-XL (Amer sham Biosciences)—charged nylon membranes. Membranes were hybridized with specific end-labeled 40-mer DNA oligonucleotide probes overnight at 42°C in ULTRahyb-Oligo hybridization buffer (Ambion), washed, exposed to BAS IIII storage phosphor screens (Fuji), and imaged using a Molecular Imager FX scanner (Bio-Rad). Each membrane was probed for three Jb segments as well as Co, with the second band in each set of three probes. Image data were acquired and analyzed using Quantity One 1.0 analysis software (Bio-Rad). Raw 16-bit TIFF image data were used as is for quantitative analysis. Jo/Co ratios were determined or each band, and fold representation for the KOs was calculated as the mean of three experimental/littermate control pairs, expressed as the Jo/Co ratios of KO/WT. For visualization, data were linearly and uniformly rescaled and mapped to 8-bit grayscale to facilitate visual comparison of the images. A minimal uniform background tone was also additively incorporated into each image to define its boundary.

**Results**

**HDAC7 is exported from the cell nucleus during positive selection of thymocytes**

Previous experiments 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 b-selection occurs, to mature CD4+ and CD8-positive 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- or 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, since 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 the HDAC7 function on thymocyte maturation (Fig. 2). The observation that introducing a positively selecting TCR transgene reduced rather than exacerbated the effect of 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 Ick-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 led us to hypothesize that the phenotype might be mitigated by the expression of a positively selecting TCR.

The observation that introducing a positively selecting TCR could partially rescue the defect in positive selection in HDAC7 KO thymocytes led us to hypothesize that the phenotype might be related to a deficiency in the generation of Ag receptors. Among other possible explanations, defective TCR generation by HDAC7-deficient thymocytes might result from a defect at the pre-TCR signal and is sufficient to mediate progression through β-selection, HDAC7-deficient thymocytes led us to hypothesize that the phenotype might be related to a deficiency in the generation of Ag receptors. Among other possible explanations, defective TCR generation by HDAC7-deficient thymocytes might result from a defect at the pre-TCR signal and is sufficient to mediate progression through β-selection.
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 progress 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, second 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 either CD4 cre-mediated HDAC7 deletion (Fig. 3A, second panel from top) or 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
undergo repeated Va-Ja 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 Va3 or Va10 families of V segments and the Ca segment, 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 Va3 (left) or Va10 (right) segment families and a reverse primer specific to Ca, 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/Ca 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 Va3 (left) or Va10 (right) segment families and a reverse primer specific to Ca, 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/Ca 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 5’ 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 (Ick−/−) thymocytes, and HDAC7-VP16 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 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, red numbers) and repressed (Fig. 4A, green 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-VP16 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-VP16 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-VP16 expression (Fig. 4C, top block and third block from top), and >35% are induced both during positive selection and due to HDAC7-VP16 expression (Fig. 4C, top block). In contrast, <17% of the most induced genes in the HDAC7 KO overlap with neither positive selection nor HDAC7-VP16 (Fig. 4C, bottom block), and only two of these genes (2.5%) are discordant between HDAC7 KO thymocytes and either positive selection or HDAC7-VP16 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-VP16 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 autoimmunity, 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-VP16 transgenics (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-VP16 transgenics (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 co-stimulation, homing, and differentiation. Among these were Cdt5, Cdt2, and Cd62l, three maturation markers that are upregulated during or after positive thymic selection (26–28), as well as Ctl4 and Bta. Cdt4 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, Pi3kδ/γ, Pkc0, 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 (NRA4A3). Surprisingly, we also did not observe upregulation of either of these molecules in HDAC7-VP16 transgenic thymocytes. However, we did note the upregulation of two positive modulators of cell death, Ndr1-1 (38) and Dap (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. Hdc5, another previously identified direct target of HDAC7 (11), was however upregulated both in HDAC7-deficient and HDAC7-VP16 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 real-time 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 Pkc0, 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 autoregulatory loop that will result in constitutive activation of pathways that mediate its own nuclear exclusion. We therefore examined the status and abundance of this serine residue in unstimulated 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.
Thymocytes that lack HDAC7 exhibit gene expression changes normally associated with positive selection in the absence of any TCR signal. These include changes in many known modulators of TCR signaling, in a pattern that suggests a broad potentiation of associated pathways. The resultant altered activity of multiple effectors of TCR signaling, particularly p38 MAPKs, causes accelerated apoptosis of DP thymocytes, producing defects in both T cell numbers and the T cell repertoire. HDAC7 thus appears to act downstream of HDAC7 targets in a prior study done by our group, involving microarray analysis of T cell hybridomas with altered HDAC7 function (11). In this study, we show that HDAC7 plays an essential role in regulating cell survival and TCR signaling in DP thymocytes.

**Discussion**

In this study, we show that HDAC7 plays an essential role in regulating cell survival and TCR signaling in DP thymocytes. Thymocytes that lack HDAC7 exhibit gene expression changes normally associated with positive selection in the absence of any TCR signal. These include changes in many known modulators of TCR signaling, in a pattern that suggests a broad potentiation of associated pathways. The resultant altered activity of multiple effectors of TCR signaling, particularly p38 MAPKs, causes accelerated apoptosis of DP thymocytes, producing defects in both T cell numbers and the T cell repertoire. HDAC7 thus appears to act downstream of HDAC7 targets in a prior study done by our group, involving microarray analysis of T cell hybridomas with altered HDAC7 function (11). These include Camk2d, Cd5, Dap, Dusp2/4, Egr1 and Egr2, Hda5, IFN-γ receptor, Ndrg-1, Pd-1, Plik3kd, l-selectin, Socs1, and TGF-β receptor.

Whereas the role of HDAC7 in regulating differentiation in endothelial cells and apoptosis in T cells is established, its apparent role in maintaining the tonic signaling state of the cell was previously unknown and represents a novel mechanism of action for class IIa HDACs. Prior studies have identified the orphan steroid receptor Nur77 as a direct (via MEF2) HDAC7 target mediating cell death. Unexpectedly, we did not find that expression of Nur77 or its functionally redundant homolog Nor-1 is increased in either HDAC7-deficient or HDAC7-VP16 transgenic thymocytes. We did, however, establish that Hda5, another direct target of HDAC7 in thymocytes (11), is increased in expression when HDAC7 function is absent or reversed by expression of HDAC7-VP16 (Fig. 5C, Supplemental Table I). Our finding with respect to Nur77 is puzzling, given that in T cell hybridomas, HDAC7-VP16 expression does induce expression of Nur77 (9, 11). One possible 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-VP16 transgenic mice, and among these there is clearly observable induction of at least one other direct target of HDAC7, Hdac5 (see 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 null mice show a marked loss of viability in DP thymocytes, a phenotype similar to what we observe in HDAC7 KOs (24). We have not however 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). Members of the Bcl-2 family are also known to make important contributions to thymocyte apoptosis (45–47), but we found that the expression levels of 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 by 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 serve to stably maintain its cytoplasmic localization (Fig. 7F). Such a model would readily explain the homogeneous distribution of HDAC7 subcellular localization observed in unstimulated and positively selected thymocytes (Fig. 1B) and the apparent persistence of HDAC7 nuclear exclusion until its expression is lost in the periphery. In this model, the change in tonic TCR signaling (putatively downstream of the transcriptional activation of MEF2) that is caused by HDAC7 nuclear export increases the activity of Rho family GTPases and diacylglycerol, which activate p38 and PKD (49). Activation of PKD in turn increases the phosphorylation of HDAC7, keeping it out of the nucleus. Activation of p38 will meanwhile result in the phosphorylation and further activation of MEF2 (50), reinforcing the upregulation of TCR signaling. HDAC7 nuclear exclusion could thus function as a stable developmental switch that, once triggered, mediates a stable change in gene expression regardless of continued signaling input, a notion that is consistent with the way that HDAC7 and other class IIa HDACs function in other developmental contexts (4–7).

Finally, the large number of molecules associated with helper T cell differentiation identified as HDAC7 targets suggests a role for HDAC7 in that process. Helper T cell differentiation is a highly overrepresented canonical pathway among HDAC7 targets according to Ingenuity Pathways Analysis (Fig. 5B). Important mediators of Th differentiation such as Gata3, Stat4, and the Irf4, Il18, and Tgfβ receptors, are all induced in HDAC7 KO thymocytes, and multiple Socs molecules, which suppress differentiating cytokine signals, are repressed. Indeed, 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.
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Disclosures

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