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11. *TAD2* and *TAD3* were disrupted in the diploid *S. cerevisiae* strain BMA41 [N. Amrani, M.-E. Dufour, N. Bonneaud, F. Lacroute, *Mol. Gen. Genet.* **252**, 552 (1996)]. Cells were transformed with PCR-amplified fragments bearing the *TRP1* marker gene flanked by *TAD2* or *TAD3* sequences, respectively. Tryptophan prototrophs were selected and correct genomic integration of the *TRP1* gene was verified by PCR. In *tad2Δ* cells the coding sequence from amino acids 74 to 210 of *TAD2* was replaced by the *TRP1* marker, and in *tad3Δ* cells, amino acids 152 to 233 were replaced.
12. The primers YJR1 (5′-CGCGGATCCTTGAGACTACTCTTGGGGAC-3′) annealing 300 base pairs (bp) upstream and YJR2 (5′-ATCGAATTCTACGAAAGGATAC-3′) annealing 209 bp downstream of the ORF YJL035c were used to amplify *TAD2* from genomic DNA by PCR and cloned into pCR2.1 (Invitrogen). The Eco RI fragment containing *TAD2* was subcloned into plasmid pFL38 (*27*), resulting in pFL38-Tad2. The ORF of YJL035c was amplified with primers YJ1 (5′-GGACTAGTGCAGCATATTAAACATATGAGG-3′) and YJ2 (5′-CGACTAGTTTAGATTTCTTATGTACATTAAAC-3′) and cloned by means of Spe I (underlined) into pGalΔTrp-FLIS$_6$-Tad1 (*7*) to generate pGal-FLIS$_6$-Tad2.
13. A library of mutant *TAD2* alleles was made by PCR-mediated mutagenesis and transformed into *tad2Δ::TRP1*[pFL38-Tad2] yeast cells. Replica plating on FOA and selection of ts strains resulted in the isolation of the *tad2-1* allele in which codons for Cys$^{126}$ (TGT) and Leu$^{153}$ (CTG) are changed to Ser (AGT) and Pro (CCG), respectively.
14. A. P. Gerber and W. Keller, data not shown.
15. Cells were cultured in 10 liters of rich medium containing 2% galactose. The cell pellet (165 g) was washed with buffer A [50 mM tris-HCl (pH 7.9), 100 mM KCl, 10% glycerol, 1 mM β-mercaptoethanol, 0.01% NP-40, 0.5 mM phenylmethylsulfonyl fluoride, pepstatin (0.7 μg/ml), and leupeptin (0.4 μg/ml)], and extracts were prepared in liquid nitrogen. After centrifugation, the cleared supernatant was passed over $Ni^{2+}$-nitrilotriacetic acid (NTA) agarose (Qiagen), and bound proteins were eluted with 250 mM imidazole. Fractions containing FLAG-Tad2p-His$_6$ were passed twice over 400 μl of an anti–M2-FLAG affinity matrix (Kodak) and eluted with buffer B [buffer A containing 25 mM KCl, 0.1 mM EDTA, and 0.1 mM dithiothreitol (DTT) instead of β-mercaptoethanol] supplemented with FLAG-peptide (50 μg/ml) (Kodak). Fractions containing tagged Tad2p were loaded on a MonoQ column (PC 1.6/5; Smart System, Pharmacia) that had been equilibrated in buffer C (buffer B containing 0.2 mM EDTA and 1 mM DTT) and developed with a 2-ml gradient from 25 to 300 mM KCl. Fifty-microliter fractions were collected. Fractions with tRNA editing activity eluted at 145 mM KCl (~4 μg).
16. The comparison of theoretical with experimentally determined peptide masses indicated that the putative ATG in S53395 (ATG$_2$) is not the correct start codon but part of a second intron. To confirm this, we amplified the *TAD3* ORF with primers YL8 (5′-GGACTAGTTAAGAAAGTTAATAATCCGC-3′) and YL2 (5′-CGACTAGTCCGCAGCAGACATCCCGGTCAAC-3′) on *S. cerevisiae* cDNA. Spe I sites for subcloning are underlined. *TAD3* with introns was obtained by PCR on genomic DNA with primers YLreg1 (5′-GGTCTGTAGATCAATGTCAAGC-3′) annealing 237 bp upstream and YLreg2 (5′-GTTCAAGCAGCAACTACAGTCG-3′) hybridizing 386 bp downstream of the *TAD3* ORF. The fragment was further cloned into pFL38 and pFL36, respectively (*27*).
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18. In pGal-FL-Tad3, the ORF of *TAD3* with a NH$_2$-terminal FLAG epitope is on a plasmid containing *ADE2* and the Gal promoter. pGal-FL-ΔNTad3 was generated with primer YL1 (5′-GGACTAGTGAGATCTAACAGAAATCAGGATC-3′) annealing at ATG$_2$ (*15*).
19. A Nco I–Eco RI fragment from pGal-FLIS$_6$-Tad2 coding for the ORF of *TAD2* with an NH$_2$-terminal FLAG epitope and a COOH-terminal 6xHis-tag was subcloned into pTrcHisB (Invitrogen), resulting in pTrc-FLIS$_6$-Tad2. The ORF of *TAD3* (*16*) was subcloned by means of Spe I, generating pTrc-FLIS$_6$-Tad3. Plasmids were transformed into BL21 (DE3). *Escherichia coli* cells and protein expression were induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 5 hours at 23°C. Cells were lysed by sonication and the extract was fractionated on a Ni-NTA column and on anti–FLAG-M2 affinity matrix. rTad3p was further purified on a 1-ml HiTrap Q column (Pharmacia).
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22. Site-directed mutagenesis was carried out as described [I. Mikaelian and A. Sergeant, *Nucleic Acids Res.* **20**, 376 (1992)] with primers YJ-E56A (5′-GTAGCACATGCTGCGTTCATGGGGATC-3′) to generate pTrc-FLIS6-Tad2E56A and YL-V218E (5′-CATCGACCACAGTGAAATGGTGGGATC-3′) for pTrc-FLIS6-Tad3V218E.
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28. We thank H. Langen (Hoffmann La-Roche) for the mass spectrometry analysis; H. Grosjean, J. Atkins, and H. Himeno for the tRNA templates; and members of our laboratory and M. A. O'Connell for critical reading of the manuscript. Supported by Boehringer Ingelheim Fonds (A.P.G.), the University of Basel, the Swiss National Science Foundation, the Human Frontiers Science Program, and the Louis-Jeantet Foundation for Medicine.

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# Regulation of Lineage Commitment Distinct from Positive Selection

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Developing αβ T cells diverge into the CD4 and CD8 lineages as they mature in the thymus. It is unclear whether lineage commitment is mechanistically distinct from the process that selects for the survival of T cells with useful T cell receptor (TCR) specificities (positive selection). In HD mice, which lack mature CD4$^+$ T cells, major histocompatibility complex (MHC) class II–restricted T cells are redirected to the CD8 lineage independent of MHC class I expression. However, neither TCR-mediated signaling nor positive selection is impaired. Thus, the HD mutation provides genetic evidence that lineage commitment may be mechanistically distinct from positive selection.

Developing αβ thymocytes go through three major phenotypic stages, first expressing neither CD4 nor CD8 (double negative; DN), then expressing both (double positive; DP), and finally expressing only one or the other (CD4$^+$8$^-$ or CD4$^-$8$^+$) (SP). At the DP stage thymocytes are selected to undergo the alternative outcomes of negative selection, positive selection, or death by neglect depending on the interaction of the αβTCR complex with intrathymic major histocompatibility complex ligands (*1, 2*). Coincident with positive selection, thymocytes undergo lineage commitment, a process that ensures the correlation of TCR specificity toward class I or II MHC with the cell's functional phenotype as a CD8$^+$ killer or a CD4$^+$ helper T cell. Various mechanisms have been proposed to explain how this correlation is achieved (*3–6*). At the molecular level CD4 and CD8 coreceptors (*7, 8*) as well as the Ras-MAPK (*9*) and Notch (*10*) pathways are likely to play a role. The mutant HD mouse (*11*) is deficient in generation of peripheral CD4$^+$ T cells because of a specific defect in thymic development not affecting antigen presentation or CD4 function, distinct from other spontaneous and induced mouse mutants with similar phenotypes (*12*). Although the HD defect was shown to be intrinsic to the hematopoietic lineage (*11*), it is unclear whether it maps to thymocytes or to bone marrow–derived nonthymocytes, which could cause aberrant negative selection of class II–restricted thymocytes.

To test this, we cotransferred bone marrow from *HD*$^{-/-}$ and *HD*$^{+/+}$ mice into the same *RAG*$^{-/-}$ recipients (*13*). If another cell type were acting in trans to prevent thymocytes from

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maturing to the CD4 lineage, then in a mixed chimera thymocytes of both $HD^{-/-}$ and $HD^{+/+}$ origins should behave similarly. On the other hand, if the defect were intrinsic to developing thymocytes, $HD^{-/-}$ and $HD^{+/+}$ thymocytes should behave distinctly. To distinguish between thymocytes of $HD^{-/-}$ and $HD^{+/+}$ origin, we obtained $HD^{+/+}$ bone marrow from B6.SJL congenic mice that carry the *CD45.1* allele on an otherwise C57BL/6 background, in contrast to $HD^{-/-}$ mice that carry the *CD45.2* allele. Thymocytes of different origins behaved autonomously—that is, $HD^{-/-}$ ($CD45.2^+$) cells matured exclusively to the CD8 lineage, whereas $HD^{+/+}$ ($CD45.1^+$) cells gave rise to $CD4^+$ and $CD8^+$ SP T cells in normal proportions (Fig. 1). In addition, thymocytes of $HD^{-/-}$ origin give rise to a population of $CD4^+8^{lo}$ cells, an intermediate stage that in normal mice includes precursors of both SP $CD4^+$ and $CD8^+$ mature T cell subsets (*5, 14*) and that is increased in $HD^{-/-}$ mice (*11*). In agreement with the thymic subset composition, peripheral T cell populations of $HD^{+/+}$ and $HD^{-/-}$ origin consist, respectively, of both SP $CD4^+$ and $CD8^+$ cells or only SP $CD8^+$ cells. In addition, $HD^{-/-}$ peripheral T cells include a minor population of $CD4^+8^+$ cells, which is a typical feature of $HD^{-/-}$ mice (*11*). Thus, abnormal development of $HD^{-/-}$ thymocytes is not mediated by another hematopoietic cell type but is inherent to developing thymocytes. It can be further deduced that the defective HD product is unlikely to be a soluble factor.

Thymocyte development depends on signaling via the TCR, coreceptors, and associated tyrosine kinases $p56^{lck}$ and ZAP-70. Initially, $p56^{lck}$ phosphorylates components of the TCR complex, thereby leading to the recruitment of additional signaling factors including ZAP-70, which in turn is phosphorylated by $p56^{lck}$. We have examined these early signaling events in $HD^{-/-}$ mice by an in vitro immune complex kinase assay (*15, 16*). $HD^{-/-}$ and $HD^{+/+}$ thymocytes exhibit equivalent phosphorylation of CD3ζ, CD3ε, ZAP-70, $p56^{lck}$, and the exogenous substrate enolase, showing that $p56^{lck}$ is activated normally and that ZAP-70 associates with the TCR complex and is phosphorylated normally (Fig. 2A). In agreement, immunoblot analysis of TCR complexes from unstimulated thymocytes or from TCRβ/CD4 antibody (anti-TCRβ/CD4) cross-linked $HD^{-/-}$ thy-

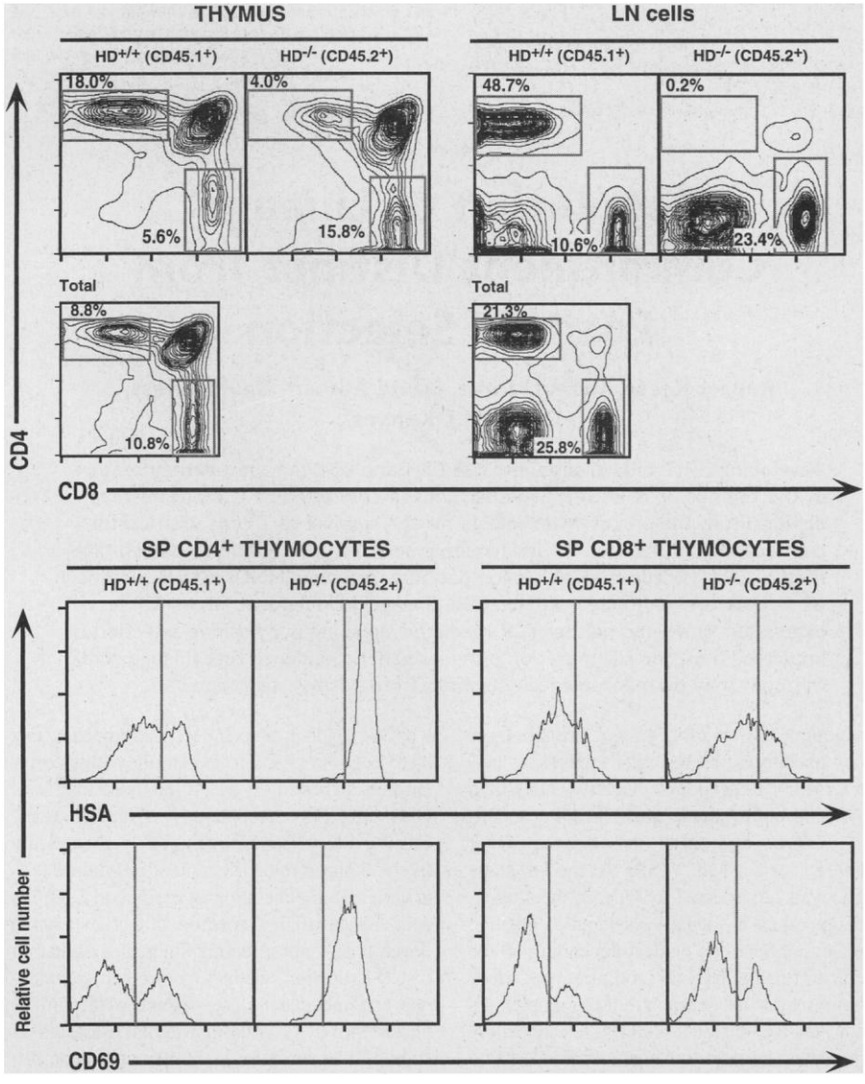


**Fig. 1.** Coreconstitution of $RAG^{-/-}$ hosts with $HD^{-/-}$ and $HD^{+/+}$ bone marrow. (**Upper**) Thymocytes and lymph node (LN) cells from an irradiated $RAG^{-/-}$ mouse reconstituted with bone marrow from both $HD^{-/-}$ ($CD45.2^+$) and $HD^{+/+}$ ($CD45.1^+$) mice were stained with antibodies specific for CD4 and CD8, as well as CD45.1 or CD45.2, and CD69 or HSA. Samples were gated on the basis of CD45.1/CD45.2 expression, to distinguish cells of $HD^{-/-}$ and $HD^{+/+}$ origin, as indicated. CD4:CD8 expression profiles are also shown for total cell samples. (**Lower**) HSA and CD69 staining profiles of gated SP $CD4^+$ and SP $CD8^+$ thymocytes. Mature cells bear the $HSA^{lo}CD69^{lo}$ phenotype. Note the absence of $HSA^{lo}$ and $CD69^{lo}$ cells in the $HD^{-/-}$ SP $CD4^+$ subset.

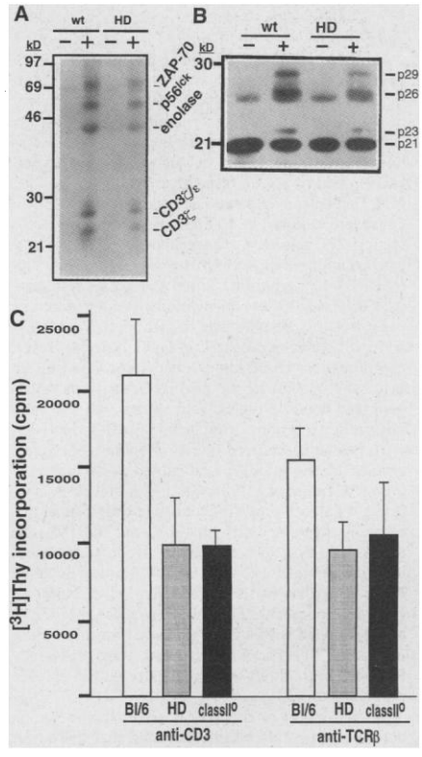


**Fig. 2.** Assays of T cell function in $HD^{-/-}$ mice. (**A**) In vitro kinase activity of anti-CD3ζ immunoprecipitates from anti-TCRβ/CD4 stimulated (lanes +) or unstimulated (lanes −) total thymocytes of $HD^{-/-}$ or WT mice. (**B**) Anti-phosphotyrosine immunoblot analysis of CD3ζ isoforms from $HD^{-/-}$ or WT thymocytes stimulated (lanes +) or unstimulated (lanes −) with anti-TCRβ/CD4. (**C**) Proliferation of total splenocytes from $HD^{-/-}$, $I\text{-}A^{b}_{\beta\,-/-}$, and WT mice in response to anti-CD3ε or anti-TCRβ treatment. Background proliferation in response to phosphate-buffered saline alone has been subtracted.

mocytes show normal constitutive phosphorylation of CD3ζ (p21 isoform) and the normal appearance of higher molecular weight phosphorylated isoforms of CD3ζ and ZAP-70 after cross-linking (Fig. 2B) (*17*). To measure the function of TCR-mediated signaling in mature T cells, we assayed the proliferative response of $HD^{-/-}$ splenocytes to cross-linking with plate-bound anti-CD3ε or anti-TCRβ (*18*). The response of $HD^{-/-}$ splenocytes, although less than that of wild-type (WT) animals, is equivalent to that of $I\text{-}A^{b-/-}_{\beta}$ mice, which similarly lack peripheral $CD4^+$ T cells (Fig. 2C). Consistent with normal TCR expression and signaling in $HD^{-/-}$ mice, breeding experiments showed that the HD defect is unlinked to genes encoding proximal components of the TCR-mediated signaling cascade—that is CD3δ, TCRαβ, and $p56^{lck}$—and DNA sequencing showed that the ZAP-70 mRNA from $HD^{-/-}$ mice is normal (*19*).

The absence of mature SP $CD4^+$ T cells in $HD^{-/-}$ mice indicates that class II–restricted thymocytes undergo an abnormal developmental fate—that is, either their development is blocked or they undergo alternative development, specifically, to the CD8 lineage. The latter possibility is suggested by the increased representation of SP $CD8^+$ thymocytes in $HD^{-/-}$ relative to WT mice (*11*). To evaluate these two possibilities, we crossed the AND TCR transgene onto the $HD^{-/-}$ background, thereby limiting the repertoire of developing thymocytes to a single class II–restricted specificity (*20, 21*). $HD^{-/-}$ mice bear the $H\text{-}2^b$ haplotype and express the $I\text{-}A^b$ product, which mediates efficient positive selection of $AND^+$ thymocytes (*22*). To exclude the possibility of rearrangement and expression of endogenous TCR products, we did these experiments on a $RAG^{-/-}$ background. A comparison of peripheral T cells from $AND^+$ $HD^{-/-}$ $RAG^{-/-}$ $H\text{-}2^b$ mice with $AND^+$ $HD^{+/-}$ $RAG^{-/-}$ $H\text{-}2^b$ littermates revealed different subset distributions (Fig. 3). Although, $AND^+$ $HD^{+/-}$ mice show the expected predominance of SP $CD4^+$ T cells, $AND^+$ $HD^{-/-}$ mice give rise exclusively to SP $CD8^+$ T cells. Analysis of thymocytes from $AND^+$ $HD^{-/-}$ $RAG^{-/-}$ mice directly shows the occurrence of redirection of class II–restricted $AND^+$ thymocytes to the CD8 lineage. Thus, $AND^+$ $HD^{-/-}$ mice contain 10 times more SP $CD8^+$ thymocytes than their $AND^+$ $HD^{+/-}$ littermates. As usual, in $HD^{-/-}$ mice, a substantial population of $CD4^+8^{lo}$ intermediate cells is also observed (Fig. 3). In normal mice, thymocytes that are undergoing positive selection show high surface expression of the activation marker CD69 (*23*), whereas the most mature SP thymocytes that are ready to exit to the periphery exhibit low levels of CD69 and heat stable antigen (HSA) but high levels of CD62L (*24*). To compare thymocyte maturation routes in $AND^+$ $HD^{+/-}$ versus $HD^{-/-}$ mice, we determined the CD4/CD8 expression profiles of cells contained within the $CD69^{hi}$ and $CD62L^{hi}$ $HSA^{lo}$ populations. In $AND^+$ $HD^{+/-}$ mice both $CD69^{hi}$ and $CD62L^{hi}$ $HSA^{lo}$ populations consist primarily of $CD4^+8^-$ thymocytes (Fig. 3). In contrast, in $AND^+$ $HD^{-/-}$ mice, the $CD69^{hi}$ population

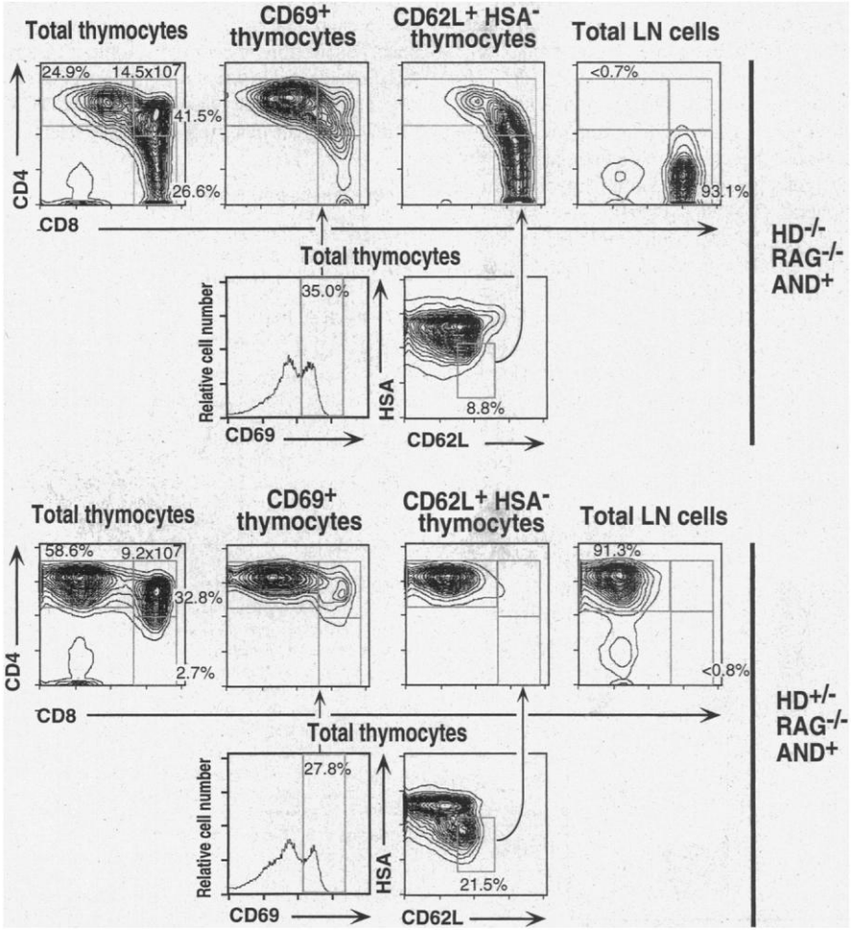


**Fig. 3.** T cell development in $HD^{-/-}$ and $HD^{+/-}$ mice carrying the class II–restricted AND TCR transgene. Thymocytes and lymph node (LN) cells from $AND^+$ $HD^{-/-}$ $RAG^{-/-}$ and $AND^+$ $HD^{+/-}$ $RAG^{-/-}$ mice were stained with antibodies specific for CD4, CD8, CD69, HSA, and CD62L and analyzed by flow cytometry. Histograms represent total cell samples or specific gated subsets, as indicated. $CD69^+$ thymocytes are thought to be undergoing positive selection, and $CD62L^{hi}$ $HSA^{lo}$ thymocytes are considered fully mature. Note the absence of fully mature SP $CD4^+$ and SP $CD8^+$ subsets in $AND^+$ $HD^{-/-}$ $RAG^{-/-}$ and $AND^+$ $HD^{+/-}$ $RAG^{-/-}$ mice, respectively.

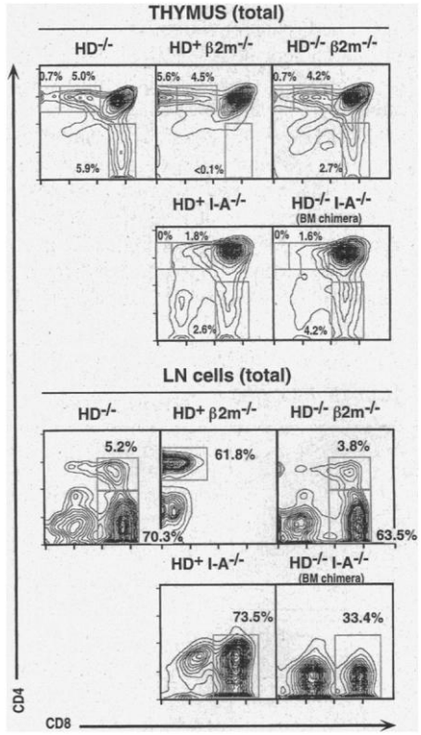


**Fig. 4.** T cell development in $HD^{-/-}$ mice in the absence of MHC class I expression. Thymocytes and lymph node (LN) cells from $HD^{-/-}$ $\beta_2M^+$ $I\text{-}A^{b+}_{\beta}$, $HD^+$ $\beta_2M^{-/-}$, $HD^{-/-}$ $\beta_2M^{-/-}$, $HD^+$ $I\text{-}A^{b-/-}_{\beta}$ and $HD^{-/-}$ $I\text{-}A^{b-/-}_{\beta}$ mice were stained with antibodies specific for CD4 and CD8 and analyzed by flow cytometry. Note the presence of substantial numbers of SP $CD8^+$ thymocytes and mature T cells in $HD^{-/-}$ $\beta_2M^{-/-}$ mice.

consists almost entirely of $CD4^+8^{lo}$ cells, whereas the $CD62L^{hi}$ $HSA^{lo}$ population is composed predominantly of $CD4^-8^+$ cells. Thus class II–restricted $CD4^-8^+$ cells in $AND^+$ $HD^{-/-}$ mice are mature and probably pass through the intermediate $CD4^+8^{lo}$ stage, consistent with the proposed maturation route of class I–restricted $CD4^-8^+$ thymocytes in normal mice (*5, 14*).

Some class II–restricted TCRs can support inefficient differentiation to the CD8 lineage in normal mice or efficient maturation in $CD4^{-/-}$ mice (*6, 25*). In both cases, alternative development requires MHC class I expression by thymic epithelial cells. To test whether there is a similar requirement for class I expression in the redirection of class II–restricted thymocytes in $HD^{-/-}$ mice, we crossed the *HD* mutation onto a $\beta_2M^{-/-}$ background in which class I expression is blocked (*21, 26*). Doubly deficient $HD^{-/-}$ $\beta_2M^{-/-}$ mice arising from this cross were identified phenotypically by the simultaneous absence of MHC class I expression and peripheral $CD4^+$ T cells. These mice still generate SP $CD8^+$ thymocytes, which are mature based on expression of αβTCR, CD69, HSA, and CD62L (Fig. 4) (*27*). In agreement, peripheral T cells consist exclusively of $CD8^+$ cells. To confirm this result, we generated radiation chimeras by transferring hematopoietic cells of $HD^{-/-}$ origin into $HD^{+/+}$ $\beta_2M^{-/-}$ hosts. Because positively selected thymocytes upregulate class I expression (*28*), $HD^{-/-}$ thymocytes undergoing positive selection in these chimeras can be readily distinguished from residual host-derived thymocytes on the basis of their expression of the MHC class I product $H\text{-}2K^b$. Consistent with our previous result, $HD^{-/-}$ precursors give rise only to mature cells of the CD8 lineage as well as to $CD4^+8^{lo}$ intermediate cells (*29*).

To similarly test the dependence of thymic development in $HD^{-/-}$ mice on MHC class II expression, we transferred $HD^{-/-}$ bone marrow into irradiated $I\text{-}A^{b\,-/-}_{\beta}$ $RAG^{-/-}$ hosts. SP $CD8^+$ thymocytes and peripheral T cells are still generated in these chimeras, indicating that MHC class I–dependent maturation to the CD8 lineage is unaffected by the *HD* defect (Fig. 4). However, the $CD4^+8^{lo}$ subset that is so abundant in $HD^{-/-}$ mice is substantially reduced, confirming that most of these cells in $HD^{-/-}$ mice are class II restricted.

Finally, to assess the MHC requirements for development of a particular class II–restricted TCR in the $HD^{-/-}$ background, we transferred $AND^+$ $HD^{-/-}$ $RAG^{-/-}$ bone marrow into both $\beta_2M^{-/-}$ $RAG^{-/-}$ $H\text{-}2^b$ and $I\text{-}A^{b\,-/-}_{\beta}$ $RAG^{-/-}$ $H\text{-}2^b$ recipients. Consistent with our preceding results, SP $CD8^+$ thymocytes of the mature $CD69^{lo}CD62L^{hi}HSA^{lo}$ phenotype and peripheral SP $CD8^+$ T cells are still generated in the absence of MHC class I, as are intermediate $CD4^+8^{lo}$ thymocytes expressing the $CD69^{hi}$ $CD62L^{lo}HSA^{hi}$ phenotype (Fig. 5). In contrast, in the MHC class II–deficient background neither intermediate nor fully mature subsets are detected, nor are there any peripheral T cells [except for a minor population of peripheral $CD4^-8^-$ T cells, also found in $AND^+$ $I\text{-}A^{b\,-/-}_{\beta}$ $HD^+$ mice and thus unrelated to the HD phenotype (*29*); similar DN cells arise in other TCR transgenics in the absence of positive selection (*30*)]. Thus maturation of $AND^+$ $CD8^+$ T cells in the $HD^{-/-}$ background depends on expression of a selecting class II ligand and is independent of MHC class I.

Redirection and complete maturation of class II–restricted thymocytes to the CD8 lineage favors the instructive model and is inconsistent with the stochastic/selective model of lineage commitment, because the latter predicts that thymocytes that adopt the incorrect lineage would be incapable of surviving the subsequent selection step. Of further mechanistic importance is the fact that redirection in $HD^{-/-}$ mice is independent of class I MHC. In contrast, when such redirection occurs in $HD^+$ mice it is strictly dependent on class I MHC, suggesting that CD8 engagement is normally required for CD8 lineage commitment (*6, 25*). The abolition of this requirement in $HD^{-/-}$ mice suggests that a CD8 lineage-determining pathway is constitutively activated. It has been proposed that lineage commitment is regulated by TCR signal strength, so that stronger and weaker signals give rise to $CD4^+$ and $CD8^+$ T cells, respectively (*6*). It seems unlikely, however, that redirection of class II–restricted thymocytes in $HD^{-/-}$ mice could be due to a weakened TCR signal, because all other TCR-mediated processes examined in $HD^{-/-}$ mice, in particular positive selection, appear to be normal. Instead these results argue that lineage-specifying signals are qualitatively distinct from the TCR-mediated signals that support positive selection.

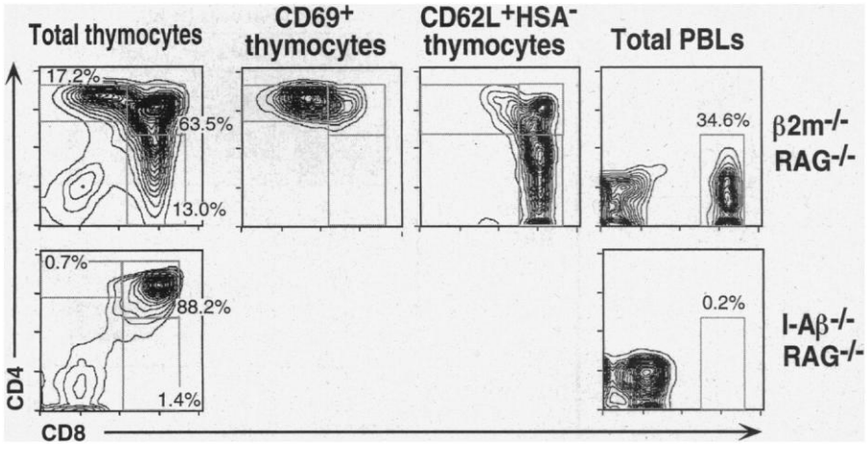


**Fig. 5.** MHC dependence of AND TCR-mediated development in $HD^{-/-}$ mice. Thymocytes and lymph node cells (PBLs) from irradiated $RAG^{-/-}$ $\beta_2M^{-/-}$ (**top row**) or $RAG^{-/-}$ $I\text{-}A^{b\,-/-}_{\beta}$ (**bottom row**) recipients reconstituted 5 weeks earlier with bone marrow from $AND^+$ $HD^{-/-}$ $RAG^{-/-}$ mice were stained with antibodies specific for CD4 and CD8 as well as CD69, CD62L, and HSA. Some samples were gated on the basis of CD69, HSA, and CD62L expression, as in Fig. 3, to distinguish cells that have undergone positive selection ($CD69^+$) or completed maturation ($HSA^{lo}CD62L^{hi}$). These subsets were absent in the case of chimeras generated in $RAG^{-/-}$ $I\text{-}A^{b\,-/-}_{\beta}$ hosts.

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13. Bone marrow was isolated from the rear leg bones of $HD^{-/-}$ ($CD45.2^+$) and $HD^{+/+}$ ($CD45.1^+$) donors and depleted of $\alpha\beta TCR^+$ cells by fluorescence-activated cell sorting. We combined $5 \times 10^5$ T cell–depleted bone marrow cells from each donor in 0.2 ml of RPMI-1640 medium and injected them intravenously into each of five $RAG^{-/-}$ $H\text{-}2^b$ recipients that had been irradiated (900 rad) 24 hours previously. Peripheral blood and thymus samples were obtained 5 weeks after bone marrow transfer. All five mice showed similar reconstitution of both B and T cell compartments.
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16. Total $HD^{-/-}$ or WT thymocytes were stimulated with anti-TCRβ and -CD4, lysed, and subjected to immunoprecipitation with anti-CD3ζ. This leads to the isolation also of CD4-associated $p56^{lck}$, due to the presence of cross-linking anti-CD4 antibody in the lysate. To measure in vitro kinase activity (Fig. 2A), immune complexes were incubated with adenosine [γ-$^{32}$P]triphosphate and the exogenous kinase substrate enolase, separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), blotted, and exposed to x-ray film. To detect CD3ζ phospho-isoforms (Fig. 2B), immune complexes were separated by SDS-PAGE, blotted, and probed with phosphotyrosine antibody 4G10 (Upstate Biotech).
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18. We coated 96-well U-bottom tissue culture plates overnight with anti-CD3ε (2C11) or anti-TCRβ (H57) (Pharmingen) (10 mg/ml) or with phosphate-buffered saline for unstimulated controls. Total $HD^{-/-}$, $I\text{-}A\beta^{-/-}$, or C57BL/6 splenocytes depleted of red blood cells by density gradient centrifugation over Lympholyte M (Cedarlane, ON) were plated ($5 \times 10^6$ cells per well) in 200 ml of RPMI-1640 medium supplemented with 10% fetal calf serum. Proliferation was assayed after 3 days by addition of 1 mCi of [$^3$H]thymidine per well followed by incubation for an additional 16 hours. All assays were carried out in triplicate. Data are shown with background values from unstimulated controls already subtracted.
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# A Biochemical Genomics Approach for Identifying Genes by the Activity of Their Products

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For the identification of yeast genes specifying biochemical activities, a genomic strategy that is rapid, sensitive, and widely applicable was developed with an array of 6144 individual yeast strains, each containing a different yeast open reading frame (ORF) fused to glutathione *S*-transferase (GST). For the identification of ORF-associated activities, strains were grown in defined pools, and GST-ORFs were purified. Then, pools were assayed for activities, and active pools were deconvoluted to identify the source strains. Three previously unknown ORF-associated activities were identified with this strategy: a cyclic phosphodiesterase that acts on adenosine diphosphate–ribose 1″-2″ cyclic phosphate (Appr>p), an Appr-1″-p–processing activity, and a cytochrome c methyltransferase.

A major task in the analysis of any biochemical activity is the purification and identification of the polypeptide or polypeptides responsible for that activity. Purification is often difficult, time consuming, and expensive, yet it is often a necessary prerequisite for cloning of the gene and subsequent detailed biochemical and genetic study. An alternative to purification is expression cloning: the introduction of cDNA pools into various host cells, followed by screening for activity and identifying the responsible cDNA (*1*). This method is inherently limited to those proteins that are easily detectable in the background of host cell proteins. Yet, given the accumulation of complete genome sequences, such as that of the yeast *Saccharomyces cerevisiae*, the sequences of genes encoding every biochemical activity of these organisms are already available. The challenge is how to use this information to connect biochemical activity with a specific gene.

We developed a rapid and sensitive genomic method for identifying yeast genes encoding biochemical activities, which is applicable for almost any detectable activity. We first constructed an array of 6144 strains, each of which bears a plasmid expressing a different GST-ORF fusion under control of the $P_{CUP1}$ promoter (*2*). To identify genes encoding particular biochemical activities, we purified this genomic set of GST-ORFs in 64 pools of 96 fusions each (*3*). Then, we assayed the pools for a particular activity and deconvoluted active pools to identify the strain and ORF responsible for the activity. Assay of the GST-ORF pools demonstrates that each of two previously known tRNA splicing activities is detected only in the pools that contain their respective GST fusions: tRNA ligase (*4*) in pool 35 (Fig. 1A) and 2′-phosphotransferase (*5*) in pool 46 (Fig. 1B).

The GST-ORFs were used to identify three previously unknown genes by biochemical assay of their products. A highly specific cyclic phosphodiesterase (CPDase) (*6*) that could convert Appr>p, produced during tRNA splicing (*7*), to Appr-1″-p was localized to pool 4 (Fig. 2A), and an otherwise uncharacterized Appr-1″-p–processing activity was found in pool 6 (Fig. 2B). We further explored the usefulness of the pools by searching for a protein-modifying enzyme. Yeast cytochrome c is known to have a trimethyllysine (*8*), and pool 23 has a methyltransferase that is active with horse cytochrome c, but not with bovine serum albumin (Fig. 2C).

To determine the strain responsible for each activity, we prepared and assayed the GST-ORFs from each of the 8 rows and 12 columns of strains from the corresponding microtiter plates. In this way, CPDase activity was associated with strain MRM 319 (expressing YGR247w) in row C and column 7 (position C7) of plate 4 (Fig. 3A), Appr-1″-p processing was associated with MRM 546 (expressing YBR022w) at position F6 of pool 6, and cytochrome c methyltransferase activity was associated with MRM 2122 (expressing YHR109w) at position A10 of plate 23.

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