The Journal of Immunology
RESEARCH ARTICLE | DECEMBER 20 2023

The Cyclin D3 Protein Enforces Monogenic TCRβ Expression by Mediating TCRβ Protein–Signaled Feedback Inhibition of Vβ Recombination

Erica J. Culberson; ... et. al

J Immunol (2024) 212 (4): 534–540.
https://doi.org/10.4049/jimmunol.2300623

The Cyclin D3 Protein Enforces Monogenic TCRβ Expression by Mediating TCRβ Protein–Signaled Feedback Inhibition of Vβ Recombination

Erica J. Culberson,*,[1] Kymberle C. Shields,*,[1] Rebecca A. Glynn,*,† Brittney M. Allyn,*,‡ Katharina E. Hayer,*,§,¶ and Craig H. Bassing*,†,‡

In jawed vertebrates, adaptive immunity depends on the process of V(D)J recombination creating vast numbers of T and B lymphocytes that each expresses unique Ag receptors of uniform specificity. The asynchronous initiation of V-to-(D)J rearrangement between alleles and the resulting protein from one allele signaling feedback inhibition of V recombination on the other allele ensures homogeneous receptor specificity of individual cells. Upon productive Vβ-to-DβJβ rearrangements in noncycling double-negative thymocytes, TCRβ protein signals induction of the cyclin D3 protein to accelerate cell cycle entry, thereby driving proliferative expansion of developing αβ T cells. Through undetermined mechanisms, the inactivation of cyclin D3 in mice causes an increased frequency of αβ T cells that express TCRβ proteins from both alleles, producing lymphocytes of heterogeneous specificities. To determine how cyclin D3 enforces monogenic TCRβ expression, we used our mouse lines with enhanced rearrangement of specific Vβ segments due to replacement of their poor-quality recombination signal sequence (RSS) DNA elements with a better RSS. We show that cyclin D3 inactivation in these mice elevates the frequencies of αβ T cells that display proteins from RSS-augmented Vβ segments on both alleles. By assaying mature αβ T cells, we find that cyclin D3 deficiency increases the levels of Vβ rearrangements that occur within developing thymocytes. Our data demonstrate that a component of the cell cycle machinery mediates TCRβ protein–signaled feedback inhibition in thymocytes to achieve monogenic TCRβ expression and resulting uniform specificity of individual αβ T cells. ***The Journal of Immunology*****, 2024, 212: 534–540.**

The ability of jawed vertebrates to produce enormous numbers of T and B lymphocytes that each expresses distinct Ag receptors (AgRs) of unique specificity provides adaptive immunity against a broad range of foreign pathogens and malignant host cells. These protein receptors consist of heterodimeric αβ or γδ TCRs or heterotetrameric IgH/Igκ or IgH/Igλ BCRs with unique Ag-binding V regions and shared effector C regions. Germline TCR and Ig loci are comprised of many V, J, and in some cases D gene segments located upstream of C region exons. In developing T and B lymphocytes, the RAG1/RAG2 (RAG) endonuclease recombines gene segments to assemble the second exons of TCR or Ig genes, respectively (1). Gene segments are flanked by recombination signal sequence (RSS) elements consisting of heptamers and nonamers separated by 12 or 23 nt (2). To conduct V(D)J recombination, RAG binds one RSS, captures a second RSS of different length (synapsis), and simultaneously cleaves between each RSS and the coding segment (2). Subsequently, RAG functions with DNA double-strand break repair proteins to ligate RSS ends into a signal join and process/ligate-coding ends into a coding join (2). The combination of joining events and imprecision of coding join formation cooperate to generate a vast repertoire of different AgR genes distributed across T and B cells. The importance of AgR gene assembly is demonstrated by the fact that genetic mutations that diminish RAG endonuclease activity result in reduced numbers of T and B cells, diminished AgR diversity, and SCID (3).

The lymphocyte lineage–, developmental stage–, and allele-specific regulation of the assembly and expression of AgR genes ensures that most individual αβ T and IgH/Igκ B cells exhibit homogeneous specificity. Common lymphoid progenitors differentiate into double-negative thymocytes or pro–B cells that arrest within the G_1 cell cycle phase, induce RAG expression, and transcriptionally activate TCRβ or IgH loci, respectively (4–8). This allows RAG to bind TCRβ or IgH D-J segments and form a recombination center that mediates D-to-J recombination (9). Independent changes in three-dimensional structures of TCRβ or IgH loci juxtapose their recombination center and V segments across large linear genomic distances to drive V-to-DJ recombination, which occurs asynchronously between alleles of each participating locus (6, 10, 11). The assembly of in-frame VDJ coding joins generates expression of TCRβ or IgH proteins that activate intracellular signaling pathways to silence RAG expression and promote cellular survival, proliferation, and differentiation into G_1-arrested double-positive (DP) thymocytes or pre–B cells, respectively (5–7). These AgR protein signals also feedback inhibit V rearrangements to ensure the assembly and expression of TCRβ or IgH genes from one allele, a phenomenon referred to as allelic exclusion (6, 10, 11). In DP thymocytes and pre–B cells, re-expression of RAG and activation

*Department of Pathology and Laboratory Medicine, Children's Hospital of Philadelphia, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA; †Cell and Molecular Biology Graduate Group, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA; ‡Immunology Graduate Group, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA; §Biomedical Engineering Doctoral Degree Program, School of Biomedical Engineering, Science and Health Systems, Drexel University, Philadelphia, PA; and ¶Department of Biomedical and Health Informatics, Children's Hospital of Philadelphia, Perelman School of Medicine, Philadelphia, PA

[1]Cofirst authors.

ORCIDs: 0000-0002-1463-3111 (K.E.H.); 0000-0003-1703-0786 (C.H.B.).

Received for publication September 19, 2023. Accepted for publication November 27, 2023.

This work was supported by Division of Intramural Research, National Institute of Allergy and Infectious Diseases Grant RO1 AI143661 (to C.H.B.).

Address correspondence and reprint requests to Dr. Craig H. Bassing, Children's Hospital of Philadelphia, 4054 Colket Translational Research Building, 3501 Civic Center Boulevard, Philadelphia, PA 19104. E-mail address: bassing@email.chop.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: AgR, Ag receptor; DN, double-negative; DP, double-positive; RAG, RAG1/RAG2; RSS, recombination signal sequence; SP, single-positive.

https://doi.org/10.4049/jimmunol.2300623

of TCRα or Igκ loci promote V-to-J recombination, which for Igκ happens on one allele at a time (12, 13). RAG cleavage on one Igκ allele activates intracellular signals that feedback inhibit Igκ recombination on the other allele to help orchestrate Igκ allelic exclusion (14, 15). The assembly of in-frame VJ coding joins leads to expression of TCRα or Igκ proteins that form TCRs or BCRs, respectively, which are subject to selection based on their Ag specificity (7). The positive selection of these receptors signals feedback inhibition of V rearrangements and maturation of αβ T or κ^+ B cells. Although TCRα V-to-J recombination is not regulated between alleles, positive selection often enforces TCRα allelic exclusion via posttranslational mechanisms (16). The interdependent regulation of lymphocyte development and V(D)J recombination produces αβ T and κ^+ B cell populations wherein 90–95% of cells express AgRs of one type and uniform specificity (6).

Despite being investigated since 1965, the molecular mechanisms that govern AgR allelic exclusion are largely undetermined but thought to involve epigenetic changes that silence chromatin over germline V gene segments and spatially segregate these elements from distal (D)J gene segments (6). To elucidate precise mechanisms underlying this fundamental aspect of adaptive immunity, we study the mouse TCRβ locus (*Tcrb*) that has 22 Vβ segments 250–735 kb upstream of two Dβ-Jβ-Cβ clusters (Dβ1-Jβ1-Cβ1 and Dβ2-Jβ2-Cβ2) and the immediately downstream *V31* Vβ that lies in the opposite genomic orientation of all other *Tcrb* coding segments (Fig. 1A) (5). Any Vβ segment can rearrange to DβJβ complexes assembled in or between each Dβ-Jβ-Cβ cluster, and each *Tcrb* allele can support as many as two different Vβ-to-DβJβ rearrangements. Following rearrangement of an upstream Vβ segment to a Dβ1Jβ1 complex, one of the remaining upstream Vβ segments can recombine to a Dβ2Jβ2 complex, deleting the VβDβ1Jβ1 coding join to prevent the expression of two different TCRβ proteins from the same allele (17). However, after the rearrangement of *V31* to a Dβ1Jβ1 or Dβ2Jβ2 complex by inversion, an upstream Vβ segment can rearrange through inversion to a Dβ2Jβ2 complex or deletion to a Dβ1Jβ1 complex, generating a *Tcrb* allele capable of expressing two distinct TCRβ proteins (18–20). Thus, to ensure expression of only one type of TCRβ protein on an individual αβ T cell, mechanisms must inhibit assembly of two in-frame *Tcrb* genes on the same allele and on both alleles. The intrinsic poor qualities of Vβ RSSs stochastically curtail the frequency of initiation of Vβ segment rearrangements to inhibit the assembly and expression of two different TCRβ proteins from a single allele (18, 19). This genetic mechanism for inefficient Vβ recombination also limits the fraction of DN thymocytes that initiates Vβ recombination on both alleles before TCRβ protein emanating from one allele can enforce permanent feedback inhibition of Vβ rearrangement on the other allele (19).

Although permanent feedback inhibition almost certainly is mediated through both silencing of Vβ chromatin and changes in *Tcrb* topology that spatially segregate Vβ and DβJβ segments in DP thymocytes (21–25), the potential contributions of changes in DN thymocytes remain elusive (26). One consequence of TCRβ signaling in DN thymocytes is transcriptional activation of cyclin D3 protein expression, which accelerates cells through the G_1 and into the S phase to drive proliferation and expansion as cells differentiate into DP thymocytes concomitant with silencing of cyclin D3 and arrest in the G_1 phase of the cell cycle (27). The genetic inactivation of cyclin D3 in mice increases the frequency of mature αβ T cells that express TCRβ proteins from both alleles and thus display Ag receptors of nonuniform specificity (28, 29). This cyclin D3 deficiency has no discernable effects on silencing of Vβ chromatin or spatial segregation of Vβ and DβJβ segments in DP thymocytes (21, 30), implying that the inability of TCRβ-selected DN thymocytes to express cyclin D3 leads to increased levels of Vβ rearrangements before these cells become DP thymocytes. However, an alternative possibility is that cyclin D3 deficiency favors the selection of DN thymocytes that express TCRβ proteins from in-frame rearrangements on both alleles. Although cyclin D3 inactivation elevates the frequency of αβ T cells displaying biallelic TCRβ protein expression, the rare incidence of these dual-TCRβ cells precluded determining whether they arise from an increased level of Vβ rearrangement in DN thymocytes, greater survival, proliferation, and differentiation of dual-TCRβ DN thymocytes, or both means.

We previously reported that cyclin D3 deficiency in mice increases the frequency of individual αβ T cells that express $V2^+$ and $V31^+$ TCRβ proteins (28). Thus, to determine whether TCRβ protein–signaled induction of cyclin D3 expression mediates feedback inhibition of Vβ recombination, we leveraged our mouse strains with enhanced frequency of rearrangement of the *V2* and/or *V31* Vβ segments. These mice carry replacement of the poor-quality RSS of these Vβ segments with the same stronger RSS (18, 19, 31). This genetic engineering raises the incidences that *V2* and *V31* initiate recombination in DN thymocytes before enforcement of TCRβ protein–signaled feedback inhibition, thereby increasing the percentages of αβ T cells that express $V2^+$, $V31^+$, or both $V2^+$ and $V31^+$ TCRβ proteins (18, 19, 31). We now show in this study that genetic inactivation of cyclin D3 in our RSS-enhanced mice further raises the frequencies of both $V2^+$ and $V2^+V31^+$ naive αβ T cells, but unexpectedly lowers the fraction of $V31^+$ naive αβ T cells. By quantifying nonselected, out-of-frame TCRβ rearrangements in bulk populations of naive αβ T cells, we find that cyclin D3 deficiency increases the level of *V2* rearrangement that occurs within developing thymocytes. Our data indicate that a component of the cell cycle machinery mediates TCRβ protein–signaled feedback inhibition to help mice achieve monogenic TCRβ expression and resulting uniform specificity of individual αβ T cells.

Materials and Methods

Mice

All mice assayed were 4–6 wk old, on the C57BL/6 background, of each sex, and housed under specific pathogen-free conditions. All husbandry, breeding, and studies were performed in accordance with national guidelines and regulations and approved by the Institutional Animal Care and Use Committee of the Children's Hospital of Philadelphia. Mice carrying the $V2^R$, $V31^R$, $V2^RV31^R$, $V31^{NT}$, or $Ccnd3^{-/-}$ alleles and genotyping strategies for these alleles were reported previously (18, 19, 27, 32).

Flow cytometry

Single-cell suspensions were prepared from the thymuses and spleens of mice, depleted of RBCs, and Fc receptors were blocked using anti-CD16/CD32. Cells were stained in PBS containing 2% FCS and 1 mM EDTA with the following Abs: anti-CD4 allophycocyanin–eFluor 780 (clone RM4-5, Invitrogen, catalog no. 47-0042-82), anti-CD8a Pacific Blue (clone 53-6.7, BD Pharmingen, catalog no. 558106), anti-TCRβ allophycocyanin (clone H57-597, BD Pharmingen, catalog no. 553174), anti-Trbv2 PE (clone KT4, BD Pharmingen, catalog no. 553366), and anti-Trbv31 FITC (clone 14-2, BD Pharmingen, catalog no. 553258). Single cells were gated based on forward and side scatter and assayed for expression of other proteins using indicated gates. Data were collected on an LSRFortessa and analyzed with FlowJo software (Tree Star).

Adaptive immunosequencing

We performed Adaptive immunosequencing on sort-purified $V2^+V31^-$ or $V2^-V31^+$ single-positive (SP) thymocytes. For each of the two experiments, we pooled equal numbers of cells from 2 $Ccnd3^{+/+}$ or 10 $Ccnd3^{-/-}$ background mice. Genomic DNA was isolated using a DNeasy blood and tissue kit (Qiagen, catalog no. 69506) and submitted to Adaptive Biotechnologies for their mouse TCRβ assay at the survey resolution. For statistical analyses, we performed multiple unpaired *t* tests.

Statistical analysis

Data are reported as mean ± SD. Statistical analyses were conducted as indicated in the figure legends using Prism 9.

Results

Cyclin D3 deficiency changes the representation and dual expression of RSS-augmented V2 *and* V31 *segments on αβ T cells*

We first assayed effects of cyclin D3 deficiency on TCRβ protein expression in our mice containing *V2* and *V31* RSSs replaced with the better 3′Dβ1 RSS on both *Tcrb* alleles (Fig. 1) (18). In these $V2^RV31^R/V2^RV31^R$ mice, the fractions of αβ T cells that express $V2^+$, $V31^+$, or both $V2^+$ and $V31^+$ TCRβ proteins within their AgRs are greater than normal due to the increased frequency of initiation of *V2* and *V31* recombination before TCRβ protein-mediated feedback inhibition (19). We reasoned that the higher frequencies of biallelic *Tcrb* gene assembly and dual-TCRβ–expressing cells would provide a more sensitive in vivo system to determine the consequences of cyclin D3 deficiency on Vβ recombination. Therefore, we bred $V2^RV31^R/V2^RV31^R$ mice with mice carrying inactivation of the *Ccnd3* gene that encodes cyclin D3 protein to ultimately create and study $V2^RV31^R/V2^RV31^R$ and $V2^RV31^R/V2^RV31^R:Ccnd3^{-/-}$ mice. We first analyzed these mouse lines by flow cytometry to determine their fractions of naive mature αβ T cells that express $V2^+$, $V31^+$, or $V2^+$ and $V31^+$ TCRβ proteins. We studied thymic αβ T cells to avoid influences from potential differential expansion and/or localization of peripheral cells with an altered TCRβ repertoire, bigenic TCRβ expression, or both. This approach also assists comparison between genotypes because cyclin D3 loss lowers the numbers of αβ T lineage cells beyond the DN thymocyte stage due to decreased cellular expansion during DN-to-DP thymocyte differentiation (27). Reflecting our prior study (18), we found $V2^+$ or $V31^+$ TCRβ proteins on 26 or 64%, respectively, of thymic αβ T cells (SP thymocytes) in $V2^RV31^R/V2^RV31^R$ mice (Fig. 2; gating strategy shown in Supplemental Fig. 1). However, in $V2^RV31^R/V2^RV31^R:Ccnd3^{-/-}$ mice, we detected $V2^+$ or $V31^+$ TCRβ proteins on 32 or 58%, respectively, of thymic αβ T cells (Fig. 2), revealing that cyclin D3 deficiency increases representation of *V2* and decreases representation of *V31* on αβ T cells in the $V2^RV31^R/V2^RV31^R$ genetic background. Notably, the magnitudes in the loss of $V2^+$ cells and gain of $V31^+$ cells were equal, with each ∼6% of αβ T cells. Despite cyclin D3 inactivation reducing the fraction of $V31^+$ cells, we detected a greater percentage of αβ T cells expressing both $V2^+$ and $V31^+$ TCRβ proteins in $V2^RV31^R/V2^RV31^R:Ccnd3^{-/-}$ mice (2.9%) relative to $V2^RV31^R/V2^RV31^R$ mice (2.3%) (Fig. 2). We compared the fractions of $V2^+$ or $V31^+$ cells that also express V31 or V2, respectively, between $V2^RV31^R/V2^RV31^R:Ccnd3^{-/-}$ and $V2^RV31^R/V2^RV31^R$ mice (Supplemental Fig. 2). These calculations demonstrate that cyclin D3 inactivation has no effect on the fraction of $V2^+$ cells that express V31 but causes a 1.4-fold increase in the percentage of $V31^+$ cells that express V2 (Supplemental Fig. 2). Collectively, our flow cytometry analysis indicates that cyclin D3 deficiency changes the representation of *V2* and *V31* and the frequency that both Vβ segments are expressed on individual αβ T cells when a strong RSS controls their rearrangement.

There are several potential reasons for why cyclin D3 deficiency in the $V2^RV31^R/V2^RV31^R$ background elevates the frequencies of $V2^+$ and $V2^+V31^+$ cells and diminishes the frequency of $V31^+$ cells. One is that cyclin D3 loss elevates the incidence of *V2* rearrangement while TCRβ protein expressed from another Vβ rearrangement is driving G_1-arrested DN thymocytes into the S phase. Another is that the lack of cyclin D3 expression alters the survival, expansion, and differentiation of DN thymocytes by bolstering $V2^+$ cells and/or hindering $V31^+$ cells, which could occur on top of cyclin D3 loss increasing rearrangement of *V2* and/or *V31* as signals from TCRβ proteins are initially driving DN cells into the S phase. An additional explanation for the diminished frequency of $V31^+$ αβ T cells is a higher incidence of *V2* recombination with Dβ2Jβ2 complexes on *Tcrb* alleles that harbor in-frame *V31*-to-Dβ1Jβ1 rearrangement as $V31^+$ TCRβ proteins expressed from the latter are pushing DN cells into the S phase. Such dual inversional Vβ rearrangements would position the *Tcrb* enhancer (Eβ) within a 10-kb linear genomic distance of the $V2^+$ gene, but ∼500 kb away from the $V31^+$ gene (20). As Eβ is required for expression of an assembled *Tcrb* transgene beyond the DN thymocyte stage (33), alleles with in-frame inversional recombination of both *V2* and *V31* might express only $V2^+$ TCRβ protein. Any combination of the above-mentioned scenarios could account for the altered repertoire and elevated bigenic expression of TCRβ proteins on αβ T cells of $V2^RV31^R/V2^RV31^R:Ccnd3^{-/-}$ mice as compared with $V2^RV31^R/V2^RV31^R$ mice.

To gain insights into which possibilities contribute to the phenotypic differences between $V2^RV31^R/V2^RV31^R$ and $V2^RV31^R/V2^RV31^R:Ccnd3^{-/-}$ mice, we leveraged our mice carrying *Tcrb* alleles with only the weak *V2* or *V31* RSS replaced with the better 3′Dβ1 RSS (19). In $V2^R/V31^R$ mice where the RSS-augmented Vβ segments are on opposite alleles, the frequency for inversional rearrangement of both *V31* and *V2* on the same allele is much lower than in $V2^RV31^R/V2^RV31^R$ mice (18, 19). Thus, we reasoned that if cyclin D3 deficiency lowers the representation of $V31^+$ αβ T cells

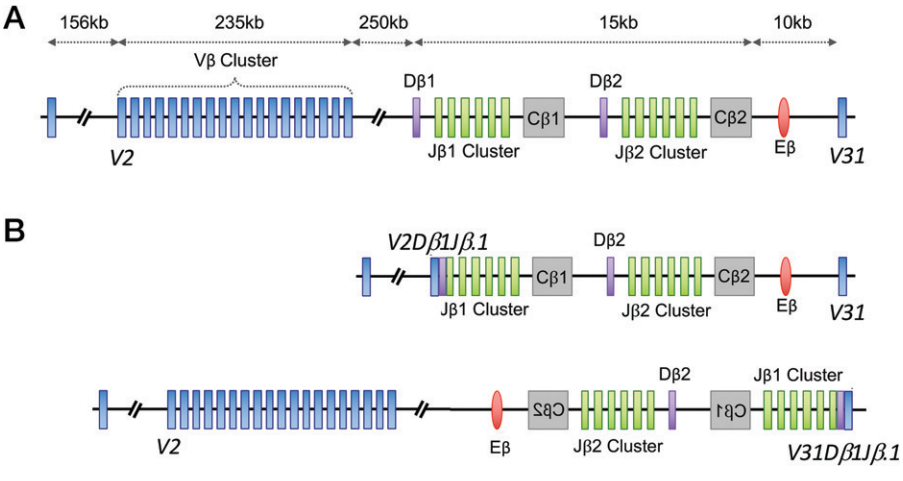

FIGURE 1. Schematic representations of germline, *V2*-rearranged, or *V31*-rearranged *Tcrb* alleles. (**A**) Schematic of the *Tcrb* locus and relative positions of V, D, and J gene segments, C exons, and the Eβ enhancer. The locations of the *Trbv2* (*V2*) and *Trbv31* (*V31*) segments are indicated. (**B**) Schematics of the *Tcrb* locus carrying rearrangement of *V2* (top) or *V31* (bottom) to a Dβ1Jβ1.1 complex.

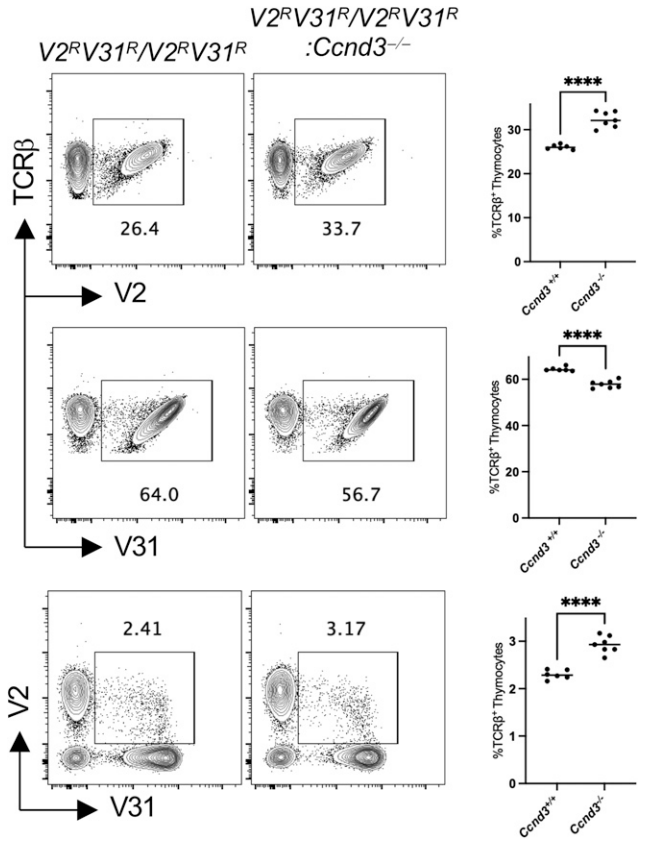

FIGURE 2. Cyclin D3 deficiency alters the representation and dual expression of *V2* and *V31* on T cells in $V2^R V31^R/V2^R V31^R$ mice. Flow cytometry analysis for effects of cyclin D3 deficiency on the representation of RSS-enhanced Vβ segments in TCRβ protein on thymic αβ T cells. Shown are representative and quantified data for the frequency of $V2^+$, $V31^+$, or $V2^+V31^+$ cells in $V2^R V31^R/V2^R V31^R$ ($n = 6$) or $V2^R V31^R/V2^R V31^R$:*Ccnd3*$^{-/-}$ ($n = 7$) mice. Graphed data are from three or more independent experiments, each with at least one mouse of each genotype, showing the mean. One-way ANOVA with Tukey multiple posttests of each Vβ RSS-enhanced genotype compared with the wild-type. $^{****}p < 0.0001$.

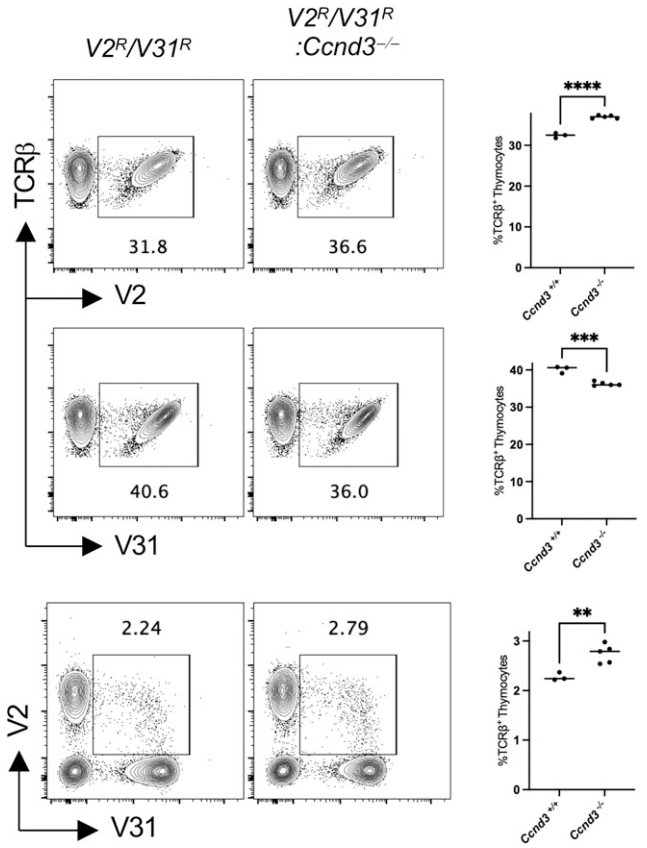

FIGURE 3. Cyclin D3 deficiency alters the representation and dual expression of *V2* and *V31* on T cells in $V2^R/V31^R$ mice. Flow cytometry analysis for effects of cyclin D3 deficiency on the representation of RSS-enhanced Vβ segments in TCRβ protein on thymic αβ T cells. Shown are representative and quantified data for the frequency of $V2^+$, $V31^+$, or $V2^+V31^+$ cells in $V2^R/V31^R$ ($n = 3$) or $V2^R/V31^R$:*Ccnd3*$^{-/-}$ ($n = 5$) mice. Graphed data are from three or more independent experiments, each with at least one mouse of each genotype, showing the mean. One-way ANOVA with Tukey multiple posttests of each Vβ RSS-enhanced genotype compared with the wild-type. $^{**}p < 0.01$, $^{****}p < 0.0001$.

by elevating the incidence of upstream inversional Vβ rearrangement on $V31^+$ alleles, cyclin D3 inactivation in $V2^R/V31^R$ mice would decrease the frequency of $V31^+$ cells to a lesser extent than in $V2^R V31^R/V2^R V31^R$ mice. To test this, we bred our $V2^R/V2^R$ and $V31^R/V31^R$ mice with *Ccnd3*$^{-/-}$ mice to ultimately create and analyze $V2^R/V31^R$ and $V2^R/V31^R$:*Ccnd3*$^{-/-}$ mice. Mirroring our previous reports (18, 19), we observed $V2^+$ or $V31^+$ TCRβ proteins on 32.4 or 40.2%, respectively, of thymic αβ T cells in $V2^R/V31^R$ mice (Fig. 3). However, in $V2^R/V31^R$:*Ccnd3*$^{-/-}$ mice, we found $V2^+$ or $V31^+$ TCRβ proteins on 37 or 36.3%, respectively, of thymic αβ T cells (Fig. 3). Importantly, the magnitudes in the loss of $V2^+$ cells and gain of $V31^+$ cells were similar, with each ~4% of αβ T cells, providing evidence against the scenario that cyclin D3 deficiency elevates upstream Vβ rearrangement through inversion on $V31^+$ alleles. We also found that cyclin D3 inactivation on the $V2^R/V31^R$ background increases the frequency of αβ T cells that express both $V2^+$ and $V31^+$ TCRβ proteins, from 2.3 to 2.7% (Fig. 3). As the RSS-enhanced *V2* and *V31* segments are on opposite alleles, this observation provides support that cyclin D3 inhibits its biallelic assembly and expression of *Tcrb* genes. In this context, cyclin D3 expression resulting from TCRβ signaling following an in-frame Vβ rearrangement on one allele might mediate feedback inhibition of Vβ rearrangement on the other allele.

Cyclin D3 deficiency increases rearrangements of the RSS-augmented V2 *segment*

We next sought to determine the consequences of cyclin D3 inactivation on the incidences of rearrangement of the RSS-augmented Vβ segments. Measuring rearrangement levels of *V2* or *V31* in $V31^+V2^-$ or $V2^+V31^-$ DN thymocytes, respectively, would be the ideal and most direct means to determine whether cyclin D3 deficiency raises the incidence that *V2* or *V31* rearrange on nonexpressing alleles in these cells before their proliferation and further differentiation. However, the tiny numbers of DN thymocytes that express TCRβ protein and have not entered the S phase proved an insurmountable technical obstacle for isolating enough DNA to measure levels of *V2* or *V31* rearrangements in these cells. We considered single-cell analyses but determined that this was neither feasible nor affordable because of the very large numbers of cells needed for PCR/sequencing to yield statistical meaningful analysis of the small difference in the fractions of dual-TCRβ cells between mice capable or incapable of expressing cyclin D3 protein. We also did not conduct intracellular Vβ staining, as this does not provide a rigorous analysis of Vβ rearrangement because of posttranscriptional silencing of in-frame TCRβ rearrangements (34). Therefore, we assayed out-of-frame Vβ rearrangements in thymic αβ T cells because such nonfunctional *Tcrb*

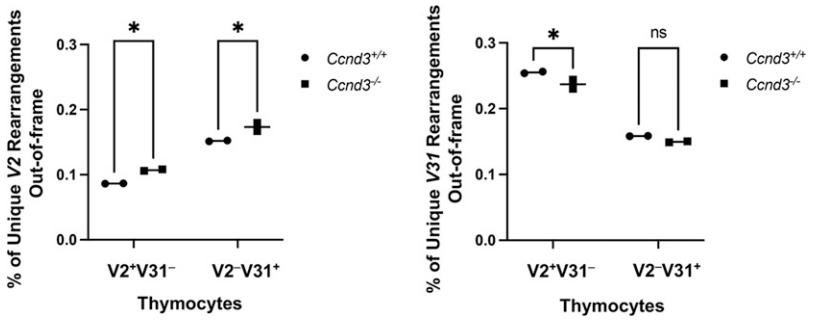

FIGURE 4. Cyclin D3 deficiency in $V2^RV31^R/V2^RV31^R$ mice increases rearrangements of *V2* but not *V31*. Graphical quantification of the percentage of total unique *Tcrb* genes involving out-of-frame rearrangements of *V2* or *V31* from Adaptive immunosequencing performed on genomic DNA isolated from $V2^+V31^-$ or $V2^-V31^+$ SP thymocytes of $V2^RV31^R/V2^RV31^R$ ($n = 2$) or $V2^RV31^R/V2^RV31^R$:$Ccnd3^{-/-}$ mice. This experiment involved analysis of DNA isolated from two different mice of each genotype. $^*p < 0.05$ by two-way ANOVA followed by a Šídák multiple comparison test. ns, not significant.

genes are not selected for or against in cells expressing TCRβ protein from an in-frame Vβ rearrangement. We employed Adaptive's immunosequencing platform, a next-generation sequencing approached that provides an accurate and comprehensive analysis of assembled *Tcrb* genes. We conducted our experiment on $V2^+V31^-$ or $V2^-V31^+$ thymocytes sorted from $V2^RV31^R/V2^RV31^R$ or $V2^RV31^R/V2^RV31^R$:$Ccnd3^{-/-}$ mice because they have greater incidences and numbers of $V2^+$, $V31^+$, and $V2^+V31^+$ αβ T cells than do $V2^R/V31^R$ or $V2^R/V31^R$:$Ccnd3^{-/-}$ mice. We determined the frequencies of unique out-of-frame *V2* or *V31* rearrangements within the total population of unique *Tcrb* gene sequences from each genotype of SP thymocytes. For $V2^+V31^-$ thymocytes, we detected a reduced frequency of out-of-frame *V31* rearrangements in $V2^RV31^R/V2^RV31^R$:$Ccnd3^{-/-}$ mice (23.73%) versus $V2^RV31^R/V2^RV31^R$ mice (25.5%) and a greater frequency of out-of-frame *V2* rearrangements in $V2^RV31^R/V2^RV31^R$:$Ccnd3^{-/-}$ mice (10.7%) relative to $V2^RV31^R/V2^RV31^R$ mice (8.64%) (Fig. 4). For $V2^-V31^+$ thymocytes, we observed similar frequencies of out-of-frame *V31* rearrangements in $V2^RV31^R/V2^RV31^R$ mice (15.84%) and $V2^RV31^R/V2^RV31^R$:$Ccnd3^{-/-}$ mice (14.96%) and an elevated frequency of out-of-frame *V2* rearrangements in $V2^RV31^R/V2^RV31^R$:$Ccnd3^{-/-}$ mice (18.34%) as compared with $V2^RV31^R/V2^RV31^R$ mice (15.2%) (Fig. 4). Collectively, these data demonstrate that cyclin D3 deficiency in $V2^RV31^R/V2^RV31^R$ mice increases the incidence for rearrangements of *V2* but not *V31*, providing a molecular basis for how cyclin D3 normally suppresses the development of αβ T cells expressing both $V2^+$ and $V31^+$ TCRβ proteins and thus AgRs of heterogeneous specificity.

Discussion

Our results offer novel mechanistic insights into how jawed vertebrates generate vast numbers of T and B cells that individually display unique AgRs of distinct uniform specificity, which is the elemental basis for adaptive immunity. We previously reported that inactivation of the cyclin D3 protein in mice elevates the percentage of individual αβ T cells that express two types of TCRβ proteins, and thus AgRs of heterogeneous specificity. However, we were unable to determine whether cyclin D3 deficiency increased Vβ rearrangement levels in DN thymocytes because of the tiny numbers of these cells that rearrange and express two Vβ segments. In the current study, we employed a genetic approach with Vβ RSS-enhanced mice that assemble both $V2^+$ and $V31^+$ genes in a greater-than-normal number of DN thymocytes, producing a much larger-than-normal fraction of αβ T cells that display both $V2^+$ and $V31^+$ TCRβ proteins within their AgRs. The substantially increased efficiency of recombination and representation of *V2* and/or *V31* in αβ T lineage cells of these mice facilitates experimental approaches to determine whether cyclin D3 deficiency raises the incidence of dual-$TCR\beta^+$ cells through increasing levels of Vβ rearrangements in thymocytes. By analyzing these mice when they can or cannot express cyclin D3 to stimulate proliferation of DN thymocytes that assemble and express an in-frame *Tcrb* gene, we show that cyclin D3 loss raises the fractions of $V2^+$ and $V2^+V31^+$ αβ T cells and lowers the fraction of $V31^+$ αβ T cells. We also show that the inactivation of cyclin D3 increases levels of rearrangement of *V2* but not *V31*, indicating a mechanism for how cyclin D3 deficiency raises the frequencies of $V2^+$ and $V2^+V31^+$ αβ T cells. The fact that cyclin D3 loss does not change the level of *V31* rearrangement but lowers the frequency of $V31^+$ αβ T cells shows that cyclin D3 deficiency biases against development of thymocytes that assemble $V31^+$ *Tcrb* genes, at least when the 3′Dβ1 RSS controls *V31* rearrangement. This strong RSS targets Vβ rearrangements directly to Jβ gene segments (35, 36), which generates shorter-than-normal TCRβ proteins (37). As Adaptive immunosequencing reveals the lack of Dβ nucleotides in ∼50% of *Tcrb* genes assembled by *V31* rearrangement in $V2^RV31^R/V2^RV31^R$ mice, it possible that cyclin D3–driven cellular proliferation masks that this pool of truncated $V31^+$ TCRβ proteins is less efficient than normal for signaling survival and maturation of thymocytes. Moreover, as discussed below, differences between the mechanisms or regulation of *V2* and *V31* rearrangements could explain why cyclin D3 deficiency differentially influences recombination of these two Vβ segments.

In normal developing αβ T cells, Vβ rearrangements occur only in G_1 phase DN thymocytes due to genetic and epigenetic regulation of RAG accessibility and RAG-mediated synapsis of Vβ and DβJβ segments, whereas cyclin D3 protein is detectable in thymocytes only during TCRβ-dependent DN-to-DP development (5, 27). In DP thymocytes, the downregulation of Vβ chromatin accessibility and spatial segregation of Vβ and DβJβ gene segments correlate with feedback inhibition of Vβ rearrangements (21, 22, 24, 38). The inability of mice to express cyclin D3 protein has no effect on the epigenetic mechanisms thought to silence Vβ rearrangements in DP thymocytes (21, 30). Accordingly, our finding that cyclin D3 deficiency causes a higher level of nonselected out-of-frame *V2* rearrangements in αβ T cells provides strong evidence that cyclin D3 enforces TCRβ allelic exclusion by inhibiting Vβ rearrangements in DN thymocytes. However, our data do not rule out an unexpected contribution for cyclin D3 protein-mediated feedback inhibition in DP thymocytes. Nevertheless, we conclude that cyclin D3 mediates TCRβ protein–signaled feedback inhibition of Vβ rearrangement in DN thymocytes to help achieve monogenic TCRβ expression and resulting uniform specificity of individual αβ T cells.

Cyclin D3 could mediate TCRβ-mediated feedback inhibition of Vβ recombination in DN thymocytes through its documented roles in promoting cellular proliferation, repressing gene transcription, or both. This cell cycle protein stimulates cellular proliferation by binding and activating the Cdk4/Ckd6 cyclin-dependent kinases to accelerate cells through the G_1 phase (39). Following in-frame *Tcrb* gene assembly on one allele in G_1 phase–arrested DN thymocytes, TCRβ protein–signaled activation of cyclin D3 moves these cells into the

S phase (27), where RAG2 proteolysis inactivates RAG expression and V(D)J recombination (40, 41). Accordingly, cyclin D3 might enforce feedback inhibition of Vβ recombination in DN thymocytes by shortening the time window for further Vβ rearrangement on the second allele. Developing B cells, but not fibroblasts, harbor a pool of cyclin D3 protein that associates with the nuclear matrix and inhibits transcription of some germline V segments in Ig loci and numerous monoallelically expressed genes (42, 43). This fraction of cyclin D3 is thought to coordinate monoallelic gene activation and cellular proliferation (43). We previously suggested the notion that cyclin D3 could mediate IgH protein–signaled feedback inhibition of V_H recombination in pro–B cells by both driving S phase entry and repressing RAG endonuclease accessibility of V_H segments (28). Likewise, cyclin D3 might facilitate TCRβ feedback inhibition in DN thymocytes by suppressing transcription and RAG accessibility of germline Vβ segments.

Our data are consistent with cyclin D3 protein mediating feedback inhibition of *V2* but not *V31* rearrangement in DN thymocytes. In the germline genomic configuration, *V2* resides ∼500 kb upstream of Dβ-Jβ segments and rearranges via deletion of intervening sequences, whereas *V31* lies ∼10–15 kb downstream of Dβ-Jβ segments and rearranges by inversion (Fig. 1B). Additional differences between these two Vβ segments include that rearrangement of *V2* but not *V31* requires locus contraction through genome folding for synapsis with Dβ-Jβ segments, and downregulation of chromatin accessibility in DP thymocytes is more dramatic for *V2* compared with *V31* (38). Accordingly, there are several reasons for why *V31* inversion rearrangements and *V2* deletion rearrangements are differentially influenced by cyclin D3 deficiency. One is that cyclin D3 protein binds over *V2* but not *V31* to repress transcription and chromatin accessibility and thus rearrangement. Another is that the chromosome folding mechanisms that direct *V2* rearrangement across large genomic distances might gain an advantage over diffusion-based collisions that mediate short-range *V31* recombination during the longer time that DN cells remain in the G_1 phase when cyclin D3 cannot be expressed.

The assembly of *Tcrb* genes must be coordinated to not only ensure uniform Ag specificity of individual αβ T cells, but also to suppress *Tcrb* locus translocations and resulting T lineage lymphoid malignancies. We propose the following model for regulation of *Tcrb* recombination during αβ T cell development. In G_1 phase–arrested DN thymocytes, both *Tcrb* alleles become transcriptionally active, simultaneously in some cells (44), but epigenetic mechanisms including stochastic association of *Tcrb* alleles with the nuclear lamina repress biallelic Vβ recombination (45). Moreover, poor-quality Vβ RSSs stochastically restrict initiation of Vβ recombination to a single Vβ segment on either allele (18, 19). RAG-mediated DNA cleavage activates the ATM protein kinase to signal rapid transient feedback inhibition of Vβ recombination, likely involving transcriptional repression of RAG expression (46), providing time for the initial *Tcrb* gene to be transcribed and tested for making protein. If this gene is assembled out of frame, the DN thymocyte stays in the G_1 phase and can reinitiate Vβ recombination after cessation of ATM signaling and re-expression of RAG. In these cells, epigenetic mechanisms, and weak Vβ RSSs, again cooperate to ensure that only one Vβ segment on either allele starts recombination, triggering another round of transient feedback inhibition. Whenever an in-frame *Tcrb* gene is assembled, the resulting TCRβ proteins signal transcriptional activation of cyclin D3 to accelerate cells into the S phase (27), prompting RAG2 proteolysis (40). As cells are first moving into the S phase, weak Vβ RSSs and epigenetic mechanisms, possibly including cyclin D3-mediated repression of RAG accessibility of Vβ segments, collaborate to decrease the opportunity for additional Vβ recombination. However, if another Vβ segment initiates recombination during this time interval, the resulting activation of ATM would signal posttranscriptional inactivation of cyclin D3 to inhibit cells with a broken *Tcrb* locus from entering the S phase, where DNA breaks are prone to generate translocations (47). Although this halting of the cell cycle would increase the chance for assembly of two in-frame *Tcrb* genes within the same cell, the inherent risk of bigenic TCRβ protein expression before αβ TCR selection of DP thymocytes likely would be less than of forming a *Tcrb* locus translocation during the proliferative expansion and differentiation of DN thymocytes. Finally, TCRβ-mediated signals that promote DN-to-DP thymocyte development activate genetic changes and epigenetic mechanisms that prevent Vβ rearrangement during *Tcra* gene assembly (21–23, 25). Importantly, most aspects of this model could apply to controlling *Igh* gene assembly during B cell development to ensure IgH allelic exclusion and suppress oncogenic *Igh* locus translocations.

Acknowledgments

We thank Adele Harman of the Children's Hospital of Philadelphia Transgenic Core for creating mice carrying each Vβ RSS replacement allele.

Disclosures

The authors have no financial conflicts of interest.

References

1. Bassing, C. H., W. Swat, and F. W. Alt. 2002. The mechanism and regulation of chromosomal V(D)J recombination. *Cell* 109(Suppl.): S45–S55.
2. Schatz, D. G., and P. C. Swanson. 2011. V(D)J recombination: mechanisms of initiation. *Annu. Rev. Genet.* 45: 167–202.
3. Delmonte, O. M., C. Schuetz, and L. D. Notarangelo. 2018. RAG deficiency: two genes, many diseases. *J. Clin. Immunol.* 38: 646–655.
4. Kumari, G., and R. Sen. 2015. Chromatin interactions in the control of immunoglobulin heavy chain gene assembly. *Adv. Immunol.* 128: 41–92.
5. Majumder, K., C. H. Bassing, and E. M. Oltz. 2015. Regulation of *Tcrb* gene assembly by genetic, epigenetic, and topological mechanisms. *Adv. Immunol.* 128: 273–306.
6. Brady, B. L., N. C. Steinel, and C. H. Bassing. 2010. Antigen receptor allelic exclusion: an update and reappraisal. *J. Immunol.* 185: 3801–3808.
7. von Boehmer, H., and F. Melchers. 2010. Checkpoints in lymphocyte development and autoimmune disease. *Nat. Immunol.* 11: 14–20.
8. Jackson, A. M., and M. S. Krangel. 2006. Turning T-cell receptor beta recombination on and off: more questions than answers. *Immunol. Rev.* 209: 129–141.
9. Ji, Y., W. Resch, E. Corbett, A. Yamane, R. Casellas, and D. G. Schatz. 2010. The in vivo pattern of binding of RAG1 and RAG2 to antigen receptor loci. [Published erratum appears in 2010 *Cell* 143: 170.] *Cell* 141: 419–431.
10. Mostoslavsky, R., F. W. Alt, and K. Rajewsky. 2004. The lingering enigma of the allelic exclusion mechanism. *Cell* 118: 539–544.
11. Vettermann, C., and M. S. Schlissel. 2010. Allelic exclusion of immunoglobulin genes: models and mechanisms. *Immunol. Rev.* 237: 22–42.
12. Levin-Klein, R., and Y. Bergman. 2014. Epigenetic regulation of monoallelic rearrangement (allelic exclusion) of antigen receptor genes. *Front. Immunol.* 5: 625.
13. Carico, Z., and M. S. Krangel. 2015. Chromatin dynamics and the development of the TCRα and TCRδ repertoires. *Adv. Immunol.* 128: 307–361.
14. Steinel, N. C., B. S. Lee, A. T. Tubbs, J. J. Bednarski, E. Schulte, K. S. Yang-Iott, D. G. Schatz, B. P. Sleckman, and C. H. Bassing. 2013. The ataxia telangiectasia mutated kinase controls Igκ allelic exclusion by inhibiting secondary Vκ-to-Jκ rearrangements. *J. Exp. Med.* 210: 233–239.
15. Glynn, R. A., and C. H. Bassing. 2022. Nemo-dependent, ATM-mediated signals from RAG DNA breaks at *Igk* feedback inhibit *Vκ* recombination to enforce Igκ allelic exclusion. *J. Immunol.* 208: 371–383.
16. Schuldt, N. J., and B. A. Binstadt. 2019. Dual TCR T cells: identity crisis or multitaskers? *J. Immunol.* 202: 637–644.
17. Brady, B. L., M. A. Oropallo, K. S. Yang-Iott, T. Serwold, K. Hochedlinger, R. Jaenisch, I. L. Weissman, and C. H. Bassing. 2010. Position-dependent silencing of germline Vβ segments on TCRβ alleles containing preassembled VβDJβCβ1 genes. *J. Immunol.* 185: 3564–3573.
18. Wu, G. S., and C. H. Bassing. 2020. Inefficient V(D)J recombination underlies monogenic T cell receptor β expression. *Proc. Natl. Acad. Sci. USA* 117: 18172–18174.
19. Wu, G. S., K. S. Yang-Iott, M. A. Klink, K. E. Hayer, K. D. Lee, and C. H. Bassing. 2020. Poor quality Vβ recombination signal sequences stochastically enforce TCRβ allelic exclusion. *J. Exp. Med.* 217: e20200412.
20. Lee, K. D., and C. H. Bassing. 2020. Two successive inversional Vβ rearrangements on a single *Tcrb* allele can contribute to the TCRβ repertoire. *J. Immunol.* 204: 78–86.
21. Majumder, K., L. J. Rupp, K. S. Yang-Iott, O. I. Koues, K. E. Kyle, C. H. Bassing, and E. M. Oltz. 2015. Domain-specific and stage-intrinsic changes in tcrb conformation during thymocyte development. *J. Immunol.* 195: 1262–1272.

22. Skok, J. A., R. Gisler, M. Novatchkova, D. Farmer, W. de Laat, and M. Busslinger. 2007. Reversible contraction by looping of the *Tcra* and *Tcrb* loci in rearranging thymocytes. *Nat. Immunol.* 8: 378–387.
23. Agata, Y., N. Tamaki, S. Sakamoto, T. Ikawa, K. Masuda, H. Kawamoto, and C. Murre. 2007. Regulation of T cell receptor β gene rearrangements and allelic exclusion by the helix-loop-helix protein, E47. *Immunity* 27: 871–884.
24. Tripathi, R., A. Jackson, and M. S. Krangel. 2002. A change in the structure of Vβ chromatin associated with TCR β allelic exclusion. *J. Immunol.* 168: 2316–2324.
25. Jackson, A., H. D. Kondilis, B. Khor, B. P. Sleckman, and M. S. Krangel. 2005. Regulation of T cell receptor β allelic exclusion at a level beyond accessibility. *Nat. Immunol.* 6: 189–197.
26. Jackson, A. M., and M. S. Krangel. 2006. A role for MAPK in feedback inhibition of *Tcrb* recombination. *J. Immunol.* 176: 6824–6830.
27. Sicinska, E., I. Aifantis, L. Le Cam, W. Swat, C. Borowski, Q. Yu, A. A. Ferrando, S. D. Levin, Y. Geng, H. von Boehmer, and P. Sicinski. 2003. Requirement for cyclin D3 in lymphocyte development and T cell leukemias. *Cancer Cell* 4: 451–461.
28. Steinel, N. C., M. R. Fisher, K. S. Yang-Iott, and C. H. Bassing. 2014. The ataxia telangiectasia mutated and cyclin D3 proteins cooperate to help enforce TCRβ and IgH allelic exclusion. *J. Immunol.* 193: 2881–2890.
29. Ehrlich, L. A., K. Yang-Iott, A. DeMicco, and C. H. Bassing. 2015. Somatic inactivation of ATM in hematopoietic cells predisposes mice to cyclin D3 dependent T cell acute lymphoblastic leukemia. *Cell Cycle* 14: 388–398.
30. Brady, B. L., and C. H. Bassing. 2011. Differential regulation of proximal and distal Vβ segments upstream of a functional VDJβ1 rearrangement upon beta-selection. *J. Immunol.* 187: 3277–3285.
31. Wu, G. S., E. J. Culberson, B. M. Allyn, and C. H. Bassing. 2022. Poor-quality Vβ recombination signal sequences and the DNA damage response ATM kinase collaborate to establish TCRβ gene repertoire and allelic exclusion. *J. Immunol.* 208: 2583–2592.
32. Yang-Iott, K. S., A. C. Carpenter, M. A. Rowh, N. Steinel, B. L. Brady, K. Hochedlinger, R. Jaenisch, and C. H. Bassing. 2010. TCRβ feedback signals inhibit the coupling of recombinationally accessible Vβ14 segments with DJβ complexes. *J. Immunol.* 184: 1369–1378.
33. Busse, C. E., A. Krotkova, and K. Eichmann. 2005. The TCRβ enhancer is dispensable for the expression of rearranged TCRβ genes in thymic DN2/DN3 populations but not at later stages. *J. Immunol.* 175: 3067–3074.
34. Steinel, N. C., B. L. Brady, A. C. Carpenter, K. S. Yang-Iott, and C. H. Bassing. 2010. Posttranscriptional silencing of VbetaDJbetaCbeta genes contributes to TCRbeta allelic exclusion in mammalian lymphocytes. *J. Immunol.* 185: 1055–1062.
35. Wu, C., C. H. Bassing, D. Jung, B. B. Woodman, D. Foy, and F. W. Alt. 2003. Dramatically increased rearrangement and peripheral representation of Vβ14 driven by the 3′Dβ1 recombination signal sequence. *Immunity* 18: 75–85.
36. Wu, C., S. Ranganath, M. Gleason, B. B. Woodman, T. M. Borjeson, F. W. Alt, and C. H. Bassing. 2007. Restriction of endogenous T cell antigen receptor beta rearrangements to Vβ14 through selective recombination signal sequence modifications. *Proc. Natl. Acad. Sci. USA* 104: 4002–4007.
37. Hughes, M. M., M. Yassai, J. R. Sedy, T. D. Wehrly, C. Y. Huang, O. Kanagawa, J. Gorski, and B. P. Sleckman. 2003. T cell receptor CDR3 loop length repertoire is determined primarily by features of the V(D)J recombination reaction. *Eur. J. Immunol.* 33: 1568–1575.
38. Chattopadhyay, S., C. E. Whitehurst, F. Schwenk, and J. Chen. 1998. Biochemical and functional analyses of chromatin changes at the TCR-β gene locus during $CD4^-CD8^-$ to $CD4^+CD8^+$ thymocyte differentiation. *J. Immunol.* 160: 1256–1267.
39. Diehl, J. A. 2004. The cyclin D3 knockout: a pound of redundancy with a dash of tissue specificity. *Cancer Biol. Ther.* 3: 162–164.
40. Lin, W. C., and S. Desiderio. 1994. Cell cycle regulation of V(D)J recombination-activating protein RAG-2. *Proc. Natl. Acad. Sci. USA* 91: 2733–2737.
41. Zhang, L., T. L. Reynolds, X. Shan, and S. Desiderio. 2011. Coupling of V(D)J recombination to the cell cycle suppresses genomic instability and lymphoid tumorigenesis. *Immunity* 34: 163–174.
42. Powers, S. E., M. Mandal, S. Matsuda, A. V. Miletic, M. H. Cato, A. Tanaka, R. C. Rickert, S. Koyasu, and M. R. Clark. 2012. Subnuclear cyclin D3 compartments and the coordinated regulation of proliferation and immunoglobulin variable gene repression. *J. Exp. Med.* 209: 2199–2213.
43. Karki, S., D. E. Kennedy, K. Mclean, A. T. Grzybowski, M. Maienschein-Cline, S. Banerjee, H. Xu, E. Davis, M. Mandal, C. Labno, et al. 2018. Regulated capture of Vκ gene topologically associating domains by transcription factories. *Cell Rep.* 24: 2443–2456.
44. Jia, J., M. Kondo, and Y. Zhuang. 2007. Germline transcription from T-cell receptor Vβ gene is uncoupled from allelic exclusion. *EMBO J.* 26: 2387–2399.
45. Schlimgen, R. J., K. L. Reddy, H. Singh, and M. S. Krangel. 2008. Initiation of allelic exclusion by stochastic interaction of *Tcrb* alleles with repressive nuclear compartments. *Nat. Immunol.* 9: 802–809.
46. Fisher, M. R., A. Rivera-Reyes, N. B. Bloch, D. G. Schatz, and C. H. Bassing. 2017. Immature lymphocytes inhibit *Rag1* and *Rag2* transcription and V(D)J recombination in response to DNA double-strand breaks. *J. Immunol.* 198: 2943–2956.
47. DeMicco, A., T. Reich, R. Arya, A. Rivera-Reyes, M. R. Fisher, and C. H. Bassing. 2016. Lymphocyte lineage-specific and developmental stage specific mechanisms suppress cyclin D3 expression in response to DNA double strand breaks. *Cell Cycle* 15: 2882–2894.