The Ig heavy chain protein but not its message controls early B cell development

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Development of progenitor B cells (ProB cells) into precursor B cells (PreB cells) is dictated by immunoglobulin heavy chain checkpoint (IgHCC), where the IgHC encoded by a productively rearranged Igh allele assembles into a PreB cell receptor complex (PreBCR) to generate signals to initiate this transition and suppressing antigen receptor gene recombination, ensuring that only one productive Igh allele is expressed, a phenomenon known as Igh allelic exclusion. In contrast to a productively rearranged Igh allele, the Igh messenger RNA (mRNA) (IghHR) from a nonproductively rearranged Igh allele is degraded by nonsense-mediated decay (NMD). This fact prohibited firm conclusions regarding the contribution of stable IghHR to the molecular and developmental changes associated with the IgHCC. This point was addressed by generating the IghΔStaTM allele model from IghΔSta mice having a premature termination codon at position +5 in leader exon of IghTer5H allele. This prohibited NMD, and the lack of a transmembrane region (ATM) prevented the formation of any signaling-competent PreBCR complexes that may arise as a result of read-through translation across premature Ter5 stop codon. A highly sensitive sandwich Western blot revealed read-through translation of IghΔStaH message, indicating that previous conclusions regarding a role of IghHR in establishing allelic exclusion requires further exploration. As determined by RNA sequencing (RNA-Seq), this low amount of IgHC sufficed to initiate PreB cell markers normally associated with PreBCR signaling. In contrast, the IghTer5HΔStaTM knock-in allele, which generated stable IghHR but no detectable IgHC, failed to induce PreB development. Our data indicate that the IgHCC is controlled at the level of IgHC and not IghHR expression.

Significance

Immunoglobulin heavy chain checkpoint (IgHCC) is a critical step during early B cell development. The role of immunoglobulin heavy chain (Igh) at this step is well established. However, with the expanding knowledge of RNA in regulating central biological processes, there could be a noncoding contribution of IgHC mRNA (IghHR) in controlling the IgHCC. Here, we generated a novel mouse model that enabled us to determine a potential role of IghHR in the IgHCC, independent of Igh signaling. Our data indicate that IghHR has no role in IgHCC and the latter is predominantly controlled by IghC, as proposed earlier. Furthermore, this study highlights the sensitivity of progenitor B cells to low amounts of IgHC.

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on the presence of signaling-competent PreBCR and is well supported by the several mouse models that either lack the transmembrane (TM) region essential for PreBCR assembly and signaling, lack components of PreBCR itself such as lambda 5, or are deficient in PreBCR-associated downstream signaling molecules Syk and ZAP-70 (24–26). Furthermore, mice carrying mutations in Igκ and Igλ, which either block their association with IgHCC or interfere with intracellular signaling cascades, also support this model (27–30). Accordingly, formation of a PreBCR is a critical IgH checkpoint (IgHCC) that is followed by clonal expansion, survival, and differentiation into PreB cells (31).

Regarding transcriptional rate, both productively and nonproductively rearranged IgH loci are transcribed at a similar rate (27). However, only the transcripts from a productively rearranged allele are stable and accumulate, whereas the messenger RNA (mRNA) from a nonproductively rearranged allele carrying multiple translation stop codons is subjected to nonsense-mediated mRNA decay (NMD) and thus rapidly degraded (32–34). This led us to propose an additional feedback inhibition model in which accumulation of stable coding IgHR is sensed by the ProB cell as a product of a productively rearranged IgH allele to inhibit further IgH rearrangements (35). In this regard, IgHC allelic exclusion could relate to XCI, which starts with the expression of a long noncoding RNA, Xist, from one of the two X chromosomes that will be silenced (16). Initiation of XCI is genetically controlled by the X inactivation center (Xic) that harbors Xist, which acts as a master regulator of XCI (36–39). In somatic cells, the three-dimensional (3D) distribution of Xist RNA domain coincides with part of the 3D space occupied by inactive X chromosome (Xi) territory (39). Xist accumulates in cis along the entire X chromosome and triggers a series of events, including chromosome-wide gene silencing, global chromatin modifications, and chromosome reorganization (40, 41). Accumulation of Xist on an X chromosome leads to the formation of a silent nuclear compartment that lacks RNA polymerase II and associated transcription factors (42). Gene silencing of X-linked genes by Xist is also determined by its ability to recruit multiple factors to Xi. Recruitment of these factors leads to the formation of facultative heterochromatin conformation (40). In mammalian nuclei, transcription rate, both productively and nonproductively rearranged IgH loci are transcribed at a similar rate (27). However, only the transcripts from a productively rearranged allele are stable and accumulate, whereas the messenger RNA (mRNA) from a nonproductively rearranged allele carrying multiple translation stop codons is subjected to nonsense-mediated mRNA decay (NMD) and thus rapidly degraded (32–34). This led us to propose an additional feedback inhibition model in which accumulation of stable coding IgHR is sensed by the ProB cell as a product of a productively rearranged IgH allele to inhibit further IgH rearrangements (35). In this regard, IgHC allelic exclusion could relate to XCI, which starts with the expression of a long noncoding RNA, Xist, from one of the two X chromosomes that will be silenced (16). Initiation of XCI is genetically controlled by the X inactivation center (Xic) that harbors Xist, which acts as a master regulator of XCI (36–39). In somatic cells, the three-dimensional (3D) distribution of Xist RNA domain coincides with part of the 3D space occupied by inactive X chromosome (Xi) territory (39). Xist accumulates in cis along the entire X chromosome and triggers a series of events, including chromosome-wide gene silencing, global chromatin modifications, and chromosome reorganization (40, 41). Accumulation of Xist on an X chromosome leads to the formation of a silent nuclear compartment that lacks RNA polymerase II and associated transcription factors (42). Gene silencing of X-linked genes by Xist is also determined by its ability to recruit multiple factors to Xi. Recruitment of these factors leads to the formation of facultative heterochromatin conformation (40). In mammalian nuclei, nuclear periphery correlates with gene silencing. Lamin B receptor (LBR) interacts with Xist RNA and influences localization of Xi to nuclear lamina to facilitate its ability to influence Xi localization toward the edge of the nucleolus (46). During XCI, Xi undergoes 3D architectural changes. Circularized chromosome conformation capture (4C) analysis of transcriptionally active genes on the active X chromosome and the same silent genes on Xi show a lack of specific chromosomal interaction on Xi (47, 48). Interestingly, 3D reorganization of Xi is also dictated by Xist (49, 50).

To address experimentally whether the feedback inhibition of gene rearrangements in the control of IgH allelic exclusion is mediated only by the IgHCC, or if IgHR also has a role in this process, we used a previously established mouse model that expresses an untranslatable form of IgHR (43, 44). In addition, Xi undergoes 3D architectural changes. Circularized chromosome conformation capture (4C) analysis of transcriptionally active genes on the active X chromosome and the same silent genes on Xi show a lack of specific chromosomal interaction on Xi (47, 48). Interestingly, 3D reorganization of Xi is also dictated by Xist (49, 50).

To address experimentally whether the feedback inhibition of gene rearrangements in the control of IgH allelic exclusion is mediated only by the IgHCC, or if IgHR also has a role in this process, we used a previously established mouse model that expresses an untranslatable form of IgHR by placing a premature translation termination codon at codon position +5, hence called IgHRΔTM (51). This approach kept untranslated IgHR relatively stable, as an early premature stop codon is very inefficient in triggering NMD (51, 52). This strategy was employed to dissect the potential impact of IgHR from IgHCC on allelic exclusion.

In our initial study (35), failure to identify any detectable IgHC from the IgHR allele supported the suitability of the IgHR allele. Furthermore, in order to explore the effect of accumulation of IgHR on VDJ recombination in IgHR model, recombination efficiency as measured for V to DJ rearrangements of the wild-type (WT) allele in PreB cells was quantified by two independent TagMan PCR assays. The first assay quantified the relative frequency of the V to DJ rearrangements of the endogenous IgHR allele (product level) and the second the relative frequency of the remaining germ line Dij element (substrate level). Both assays indicated impaired recombination efficiency of IgHR WT allele in the IgHR WT system. These results closely correlated with the reduced frequency of PreB cells in the IgHR WT system mouse model. In addition, independent experiments using an exogenous recombination substrate showed that impaired recombination observed in PreB cells from the IgHR model was not due to reduced recombination activity. Since the initial Western blots were relatively insensitive in detecting minute amounts of IgHC encoded from the IgHR allele, the results summarized above misled us in implying a contribution of IgHR in establishing allelic exclusion, suggesting a model in which IgHR might exert a noncoding function in establishing allelic exclusion, in analogy to the role of Xist in XCI.

The fact that IgH allelic exclusion is associated with ProB to PreB cell transition led us to hypothesize that the accumulation of a stable IgHR may contribute to initiate ProB cell differentiation. Indeed, transcriptome and IgH loci conformation analyses revealed that ProB cells expressing IgHR mRNA acquired PreB cell features. However, detailed analyses of RNA-sequencing (RNA-Seq) data suggested the existence of a signaling-competent PreBCR in the IgHR model system that was confirmed by a highly sensitive Western blot. Consequently, an optimized mouse model expressing stable IgHR mRNA, lacking the TM region IgHRΔTM mouse model was generated. This would render IgHC unable to signal and thus ultimately degraded. Transcriptomic and IgH locus conformation analyses of IgHRΔTM showed that despite high expression of IgHRΔTM mRNA, the ProB cells failed to acquire PreB cell features in this setting.

In conclusion, here we provide a mouse model, IgHRΔTM that finally enabled us to determine the contribution of the IgHR in the IgHCC, independent of IgHCC signaling potential. Our analysis showed that, apparently, IgHR has no obvious role in allelic exclusion and PreB cell development, which are, as proposed previously, at least not exclusively associated with IgHCC (8, 24). Furthermore, our results led us to conclude that locus conformation of IgH alleles is not influenced by IgH transcription but is mainly governed by PreB cell development.

**Results**

*The IgHR ΔTM Knock-in Allele Triggers PreB Cell Development.* Allelic exclusion is intimately linked to PreB cell differentiation, which in turn is controlled by the IgHCC, raising the question of whether early B cell differentiation can be triggered by the expression of stable IgHR mRNA alone. To determine if a stable IgHRΔTM mRNA (35) can kick-start some aspects of early B cell development, we followed an unbiased RNA-Seq approach taking advantage of the IgHR ΔTM knock-in mice. We first established a PreB cell gene signature by defining all differentially expressed genes (false discovery rate [FDR] < 0.01) from WT (CD19+, CD20+, IgM−, c-Kit−, CD25−) PreB cells compared with Rag1−/− PreB cells (Fig. 1C). Having established this PreB cell signature, we compared the mRNA expression profiles of differentiation-arrested PreB cells from IgHRΔTM/Ter5H with those of Rag1−/− mice. This comparison indicated clear features of advanced differentiation in the IgHRΔTM setting (Fig. 1B). Strikingly, among differentially regulated genes, we identified IκBα, IκBα (CD25), CD2, and CD22 (Fig. 1C). Importantly, these genes are normally associated with a signaling-competent PreB cell receptor (53). In order to exclude any contribution of RAG, we compared IgHRΔTM/Rag1−/− with Rag1−/− mice. This comparison showed that the developmentally
**Fig. 1.** Induction of PreB cell markers in the Igh$^{Ter5H}$ knock-in system is triggered by minute traces of IgH chain protein. (A) MA plot generated from RNA-Seq data showing differential gene expression (FDR < 0.01) between WT PreB and Rag1$^{ko/ko}$ PreB cells to establish PreB cell gene signature. (B) MA plot showing relative enrichment of PreB cell gene signature in Igh$^{Ter5H/Ter5H}$ compared with Rag1$^{ko/ko}$. (C) The average log$_2$ counts per million after trimmed mean of M-values normalization and removing the batch effect using voom function under the limma and edgeR package shows the mRNA expression of Ikzf3, Il2ra, cd2, and cd22. The genetic background of PreB cells (c-Kit+, CD25$^-$) of the indicated genotypes and wild-type PreB cells (c-Kit$^-$, CD25$^+$) are indicated. The FDR shows the statistical significance. Differences with an FDR < 0.05 are considered significant. (D) MA plot showing relative enrichment of PreB cell gene signature in Igh$^{Ter5H/Ter5H}$ and Igh$^{Ter5H/WT;Rag1^{ko/ko}}$ compared with Rag1$^{ko/ko}$. (E) Western blot showing the presence of trace amounts of IgHC in Igh$^{Ter5H/Ter5H}$ system is indicated on the right. Actin is shown as a loading control on the left. The reduction in IgHC in Igh$^{Ter5H/Ter5H}$ is shown as fold change after normalization to WT.
advanced features shown in Igh\textsuperscript{TerSH} arise independently of RAG (Fig. 1D).

**PreB Cell Development in Igh\textsuperscript{TerSH} Knock-in System Does Not Exclude IgHC Contribution.** A substantial fraction of PreB cell markers was found induced in the Igh\textsuperscript{TerSH} knock-in system, which led us to consider two possibilities: Either stable IgHR expression initiates differentiation, or, given the existence of read-through translation, previously undetected minute amounts of IgHC might be generated that suffice to induce developmental progression (35).

To distinguish between these possibilities, we established a highly sensitive sandwich Western blot system. Using a polyclonal donkey anti-goat to detect a polyclonal goat anti-mouse IgM antibody, we found detectable amounts of IgHC in B cell progenitors from the Igh\textsuperscript{TerSH/TerSH} model, which were 147-fold reduced compared with WT (Fig. 1E). Apparently, while a premature stop codon at position +5 allows stable mRNA expression, it appeared insufficient in preventing translation. The failure to dissect the contribution of IgHR and IgHC in PreB cell development required further optimization of the Igh\textsuperscript{TerSH/TerSH} system.

**Fig. 2.** Igh\textsuperscript{TerSH}/TerSH system shows stable Igh\textsuperscript{TerSH} mRNA expression but no detectable trace of IgH chain protein. (A) FACS identification of ProB (c-Kit\textsuperscript{+}, CD25\textsuperscript{−}) and PreB cells (c-Kit\textsuperscript{−}, CD25\textsuperscript{+}) from bone marrow of the respective mice genotypes. (B) The average log\textsubscript{2} counts per million after trimmed mean of M-values normalization and removing the batch effect using voom function under the limma and edgeR package shows the mRNA expression of the targeted VHB1.8 knock-in allele. The FDR indicates the statistical significance. The difference with FDR less than 0.05 is considered significant. (C) Western blot confirming the degradation of IgHC in the Igh\textsuperscript{TerSH/TerSH} system. n.s., nonsignificant.
Generation of \(\text{Igh}^{\text{TerSH/TM/TerSH/TM}}\) Knock-in Mice from the \(\text{Igh}^{\text{TerSH/TerSH}}\) Model and Its Validation. Knowing that the TM region of membrane-bound IgHC is essential for PreBCR assembly and signaling (54), we deleted the TM exon in the \(\text{Igh}^{\text{TerSH}}\) locus (SI Appendix, Fig. S1 A and B). To accomplish this goal, we first derived mouse embryonic stem cells from blastocysts isolated from super ovulated \(\text{Igh}^{\text{TerSH/TerSH}}\) mice. Subsequently, an \(\text{Igh}^{\text{TerSH/TM}}\) knock-in allele was derived from the \(\text{Igh}^{\text{TerSH/TerSH}}\) embryonic stem cell lines.

Fig. 3. ProB cells from \(\text{Igh}^{\text{TerSH/TerSH}}\), \(\text{Igh}^{\text{TerSH/TM/TerSH/TM}}\), and \(\text{Igh}^{\text{TerSH/TM/TM/TM}},\text{Rag1}^{\text{ko/ko}}\) predominately arrest at Fraction C. The violin plot represents the absolute numbers of total nucleated cells of B cell subsets from bone marrow (Fractions A, B, C, C', D, E, and F, according to Philadelphia staining) for each genotype. Each data point represents the value from an individual mouse. ROUT (robust regression and outlier removal) method in GraphPad Prism under default settings is used to identify outliers from the data, which are removed for the subsequent analysis. A one-way ANOVA test with Tukey’s multiple comparison test was applied to calculate the \(P\) value to determine the statistical significance. \(P < 0.05\) is considered statistically significant, and only the significant values are shown. Fr, Fraction.
cells using Crispr-Cas9 and specific guide RNAs (gRNAs) targeting the flanking region of the TM exon. Ig\textsubscript{H}\textsubscript{Ter5H} knock-in clones were injected into C57B/J6 blastocytes to generate chimeric mice and introduce the mutation into the germ line (SI Appendix, Fig. S1C).

This strategy ensured that the open reading frame (ORF) and thus the stability of the IgHR remain intact, but any residual read-through translation product is incapable of PreBCR assembly and signaling. RNA-Seq data confirmed an abundant expression of Ig\textsubscript{H}TM mRNA in the (CD19\textsuperscript{+}, B220\textsuperscript{+}, IgM\textsuperscript{+}, c-Kit\textsuperscript{+}, CD25\textsuperscript{+}) ProB cells from Ig\textsubscript{H}Ter5HTM mice (Fig. 2B). To confirm the absence of IgH in ProB cells in a homozygous Ig\textsubscript{H}Ter5HTM setting (54), we repeated the sandwich Western blot on lysates prepared from ProB cells from Ig\textsubscript{H}Ter5HTM knock-in mice. The absence of detectable levels of IgH validated our system (Fig. 2C). Having excluded IgH expression and at the same time confirmed the stability of the Ig\textsubscript{H}Ter5HTM mRNA, we now had a system in hand to study the role of Ig\textsubscript{H}Ter5HTM mRNA in controlling PreB cell development in the absence of IgH.

PreB Cell Development is Predominantly Controlled by the IgH and Not IgHR. To determine in more detail the developmental stage in which B cell precursors become arrested, we performed Philadelphia staining (55), which enables a detailed characterization of PreB cell subsets, specifically a separation into Pre–Pro B cells (Fraction A), ProB cells (Fraction B and C), and the PreB cell fractions comprising large, early ProB cells (Fraction C) and small late ProB cells (Fraction D). These analyses revealed that at the cellular level, B cell precursors from Ig\textsubscript{H}Ter5HTM and Ig\textsubscript{H}Ter5HTM\textsubscript{Rag1\textsuperscript{loko}} mice behaved like those from Rag\textsuperscript{loko} mice, where B cell progenitors arrest at Fraction C (Fig. 3 and SI Appendix, Fig. S2 A and B).

To provide an in-depth analysis of the potential contribution of IgHR in controlling early onset of PreB cell development, we compared the transcriptomes between PreB cells from Ig\textsubscript{H}Ter5HTM and Rag\textsuperscript{loko} mice. We observed that most PreB cell markers identified in the Ig\textsubscript{H}Ter5HTM setting were no longer differentially expressed in Ig\textsubscript{H}Ter5HTM mice (Fig. 4A). To further exclude any confounding issue related to RAG expression, the Ig\textsubscript{H}Ter5HTM knock-in allele was introduced into the Rag\textsuperscript{loko} background to generate Ig\textsubscript{H}Ter5HTM\textsubscript{Rag1\textsuperscript{loko}} mice (Fig. 4B). In order to determine the contribution of PreB cell signature genes found in differentially up-regulated genes in arrested ProB cells from Ig\textsubscript{H}Ter5HTM and Ig\textsubscript{H}Ter5HTM\textsubscript{Rag1\textsuperscript{loko}} mice, we compared those to ProB cells from Rag\textsuperscript{loko} mice. To provide the relative overlap of ProB cells from different genotypes with the WT PreB cell, we normalized those according to the actual number of genes in the PreB cell signature (Fig. 4C). This analysis clearly indicated that among all of the genotypes, ProB cells from Ig\textsubscript{H}Ter5HTM\textsubscript{Rag1\textsuperscript{loko}} have the least overlap with PreB cells. Only 9 of the 1,247 genes (0.7%) that defined the PreB cell signature were differentially expressed in Ig\textsubscript{H}Ter5HTM\textsubscript{Rag1\textsuperscript{loko}}. The same analysis revealed that 10% of the genes that were differentially up-regulated in Ig\textsubscript{H}Ter5HTM\textsubscript{Rag1\textsuperscript{loko}} compared with Rag\textsuperscript{loko} belong to PreB cell signature (Fig. 4D). Most relevant regarding a potential role for IgHR in the IgHCC, a nonsignificant difference in the steady-state level of IgHR between Ig\textsubscript{H}Ter5HTM\textsubscript{Rag1\textsuperscript{loko}} and Ig\textsubscript{H}Ter5HTM\textsubscript{Rag1\textsuperscript{loko}} was found (Fig. 3B). These results led us to conclude that irrespective of its high expression, the Ig\textsubscript{H}Ter5HTM message does not contribute to PreB cell differentiation. In order to determine the magnitude of similarity among different genotypes, Pearson correlation coefficient was calculated based on the expression of protein coding genes (SI Appendix, Table S1). Hierarchical clustering revealed that both Ig\textsubscript{H}Ter5HTM and Ig\textsubscript{H}Ter5HTM\textsubscript{Rag1\textsuperscript{loko}} clustered closer to WT PreB cells irrespective of batches. On the contrary, Ig\textsubscript{H}Ter5HTM\textsubscript{Rag1\textsuperscript{loko}} clustered together with Rag\textsuperscript{loko}, again irrespective of different batches (Fig. 4E). This analysis further strengthened our conclusion about the role of IgH message in PreB cell differentiation.

Igh Locus: Chromatin Conformation Is Predominantly Ruled by Differentiation and Not Transcription. The Ig\textsubscript{H}Ter5HTM mRNA expression failed to induce transcriptional changes associated with PreB cell differentiation. However, apart from transcriptional changes, PreB cell differentiation is associated with defined topological changes at the Igh locus. During the transition from ProB to PreB cell differentiation, the Igh locus changes from a contracted to a decontracted configuration in ProB and PreB cells, respectively (17, 18, 21). In order to determine if differentiation or transcription is responsible for the local conformational changes in Igh locus, the distance between two probes located at the two ends of Igh locus was measured using fluorescence in situ hybridization. Of note, except for the deleted 332-base-pair fragment containing the TM exon, the transcriptional units of both Ig\textsubscript{H}Ter5HTM and Ig\textsubscript{H}Ter5HTM loci were kept identical. This excluded any confounding issues related to regulatory elements of the modified Igh locus that may influence the results. Our analyses revealed that despite high Ig\textsubscript{H}Ter5HTM mRNA expression, the Igh locus remained relatively contracted compared with the Igh\textsubscript{Ter5H} (Fig. 5 A and B). This suggests that Igh locus decontraction is associated with the PreB cell stage and not dictated by IgH transcription or the Igh transcript.

Discussion

The IgHCC represents a critical, tightly regulated step in early B cell development. The developmental transition from a ProB to a PreB cell strictly depends on the somatic generation of a productive rearrangement of a V gene segment to one of the two preexisting DJ-rearranged Igh alleles in ProB cells (56). The ProB cell becomes developmentally arrested if rearrangement is unsuccessful, leading to apoptosis (27). Successful rearrangement leads to PreB cell differentiation in which further rearrangements at the Igh alleles are prohibited, ensuring “allelic exclusion.” This phenomenon provides the exquisite antigen specificity of B cell–mediated immune responses. According to the feedback model, the IgH gene encoded by the productively rearranged VDJ join, that is, the IgHCC is sensed by the cell and prohibits further rearrangements at both Igh alleles (8, 24).

Our previous studies with Ig\textsubscript{H}Ter5HTM transgenic and Ig\textsubscript{H}Ter5HTM knock-in mouse models implicated a contribution of the Igh message in establishing allelic exclusion. This led us to propose an alternate feedback inhibition model in which the accumulation of a stable mRNA from a productive VDJ rearrangement is sensed by the cells to prevent further rearrangement (35). In contrast, transcripts from a nonproductively rearranged allele are subject to NMD and cannot initiate this feedback inhibition.

Based on the tight coordination between early B cell development and allelic exclusion, we aimed to explore the developmental changes that might be governed by stable IgHR in the absence of IgHCC. However, detailed transcriptional analyses and highly sensitive Western blot analyses revealed minute amounts of IgHR in IgHCC Knockouts (Ig\textsubscript{H}Ter5HTM). Apparently, an IgHR with a premature translational stop codon can be processed via read-through translation. This observation indicated that previous conclusions required further exploration, necessitating the generation of a new mouse model that could dissect the role of IgHR from IgHCC during early B cell development.

Extensive transcriptional analyses of the new model revealed that the IgHCC is not controlled by IgHR. Furthermore, our data strongly suggest that conformational changes at the Igh locus are regulated by developmental rather than transcriptional circuits. Our results indicate that ProB cells are apparently highly effective in sensing minute amounts of IgHCC that arise as a result of
Fig. 4. The Igh<sup>Ter5HΔTM/Ter5HΔTM</sup> knock-in fails to induce PreB cell markers. (A and B) MA plots displaying a minor fraction of PreB cell signatures differentially expressed in Igh<sup>Ter5HΔTM/Ter5HΔTM</sup> and Igh<sup>Ter5HΔTM/Ter5HΔTM;Rag1<sup>ko/ko</sup></sup> system compared with Rag1<sup>ko/ko</sup>. (C) Relative percentage of PreB cell signature genes differentially expressed in ProB cells from different models when compared with Rag1<sup>ko/ko</sup> ProB cells. (D) Venn diagrams showing the number of common genes among the mentioned genotypes. (E) Hierarchical clustering analysis based on Pearson correlation coefficient values calculated from the expression values of protein coding genes showing the magnitude of similarity among different genotypes.
read-through translation. Apparently, this level of translation can initiate but not complete the developmental progression toward PreB cells. In addition, the very low amounts of IgHC in the Ter5H system may also be due to the active degradation of IgHC associated with PreB cell development (57–60), which may further contribute to its low expression (35). Compared with signaling-deficient models that may still be able to provide some signal, we present an efficient system in which trace amounts of signaling-incompetent, PreBCR assembly–deficient IgHC initially produced by read-through translation becomes rapidly degraded, and thus only the effect of stable IgHR could be addressed.

Finally, these insights strongly support the initial feedback model in which the IgHC is required to control allelic exclusion and drive early B cell development (4, 8, 24). These data are in line with previous observations made in the T cell lineage, in which the TCR-β chain but not a frame-shifted message controls the developmental progression toward the α chain but not a frame-shifted message controls the developmental progression toward the α
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Methods

**Generation of IgH<sup>ΔTM/Ter5H</sup> Mouse Model.** To generate IgH<sup>ΔTM/Ter5H</sup> mouse model, we first derived mouse embryonic stem cells from blastocyst isolated from super ovulated IgH<sup>ΔTM/Ter5H</sup> mice. Region flankin g TM at Ter5H locus was targeted by Crispr-Cas9. Embryonic stem (ES) cells were transfected with a px330 plasmid encoding specific gRNAs (CCGTCTAGCTTGAGCTATT and ACAAGTGACAGCAATTCA) and Cas9. gRNAs were designed using the https://lab.bio/guide-design-resources tool. Subsequently, clones with the desired deletion of the TM region were selected by PCR and injected into C57/6J blastocysts. Chimeric mice were crossed to C57/6J, and the offspring were tested for germ line transmission of the Ig<sup>ΔTM/Ter5H</sup> knock-in allele. IgH<sup>ΔTM/Ter5H</sup> knock-in mice were maintained on a C57/6J background to exclude confounders related to the genetic background. All mice used for this were maintained under specific pathogen-free conditions at the animal laboratory facility of the Netherlands Cancer Institute (NKI; Amsterdam, Netherlands). Mice used for experiments were between 6 and 8 wk old and of both genders. All experiments were approved by the Animal Ethics Committee of the NKI and performed in accordance with the Dutch Experiments on Animals Act and the Council of Europe.

**Genotyping PCR.** Mice were genotyped for the deletion of the TM region using the forward primer (Igh<sup>ΔTM/Ter5H</sup>-FWD: GTTAGAGCAAGCAAGCAGCAGG-G) and reverse primer (Igh<sup>ΔTM/Ter5H</sup>-REV: CCTTGGCGCCGCCCCATG TGGACTTGGTTTACAGC). The WT allele was identified as a PCR product of 960 base pairs, while IgH<sup>ΔTM/Ter5H</sup>-allele produced a 628-base-pair DNA fragment. Rag1 status was detected by using the combination of the forward primer 1 (Rag1-FWD1: GGCTTAGACACTTCTGCCGCATCTGTGG), reverse primer 1 (Rag1-REV1: CTGACCGCAGTTCTGGCGCAG), reverse primer 2 (Rag1-REV2: CCAC CACTGTGGAGGGACACTTCACTTAG), and reverse primer 3 (Rag1-REV3: CTACC GGTGAGTCGAAGGGACATTCACTTAG).

**Flow Cytometry and Sorting.** For flow cytometry experiments, two femurs and two tibiae from each mouse were used to isolate bone marrow, but for sorting, both the hip bones were also used. Single-cell suspensions were made from bone marrow, and the cells were subjected to erythrocye lysis for 1 min on ice. Following erythrocyte lysis, the cells were stained with a mixture of fluorescently labeled antibodies for 30 min on ice in the dark to identify distinct cellular populations. The 7-AAD– or Zombie NIR–positive cells were identified as dead cells and were excluded from the analysis. Zombie NIR stock was prepared in dimethyl sulfoxide according to the manufacturer’s instructions. For staining with Zombie NIR, the cells were washed with phosphate-buffered saline (PBS) and then stained for 20 min on ice in the dark with Zombie NIR diluted in PBS. All monoclonal antibodies used for flow cytometry experiments and sorting were shown along with their respective clone, conjugated

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**Fig. 5.** IgH<sup>ΔTM/Ter5H</sup> system shows contracted conformation of IgH loci. (A) Scatter dot plot shows the distribution of distances determined between two oligo probes on distal ends of IgH locus as measured by fluorescence in situ hybridization for PreB cells (c-Kit+, CD25<sup>+</sup>) of the indicated genotypes and wild-type PreB cells (c-Kit<sup>+</sup>, CD25<sup>+</sup>). Data are presented as mean ± SD. Statistical significance is determined by the P value calculated by unpaired Student’s t test with two-tailed distributions. P < 0.01 is considered statistically significant. (B) The graph displays the cumulative frequency percentage of all of the data points for a given distance. n.s., nonsignificant.
fluorochrome, dilution, and vendor (SI Appendix, Table S2). For cell sorting and the analysis for Fig. 2A and SI Appendix, Fig. S3, the antibodies mix was prepared in PBS carrying 0.3% bovine serum albumin, 2 mM ethylenediaminetetraacetic acid, and 0.02% Azide. For the analysis for SI Appendix, Fig. S2, the antibody mix was prepared in a brilliant stain buffer (catalog number 566349) that was purchased from BD Bioscience. The 7-AAD was added to prestained cells just before fluorescence-activated cell sorting (FACS) measurement. The specific cell population was sorted by FACSAria Fusion (BD Biosciences), or MoFlo Astrios (Beckman Coulter) in fetal calf serum (FCS) precoated tubes. Flow cytometry was performed using the LSR Fortessa (BD Biosciences), and data were analyzed with FlowJo software (Tree Star Inc. and BD Biosciences).

**Fluorescence In Situ Hybridization.** Bacterial artificial chromosome (BAC) probes CT7-34H6 (3′ Igh) and RPZ4-3617F (5′ Igh) were labeled by nick translation with ChromaTide Alexa Fluor 488 or 594-5-UTP (Molecular Probes). For one coverslip, 0.5 μg of oligo probes were precipitated and resuspended in 10 μL of hybridization buffer (50% formamide/20% dextran sulfate/5 × Denhardt's solution), denatured for 5 min at 95 °C, and preannealed for 45 min at 37 °C before overnight hybridization with cells. The 3D images were acquired by confocal microscopy on a Leica SP8 Acousto-Optical Beam Splitter system. Optical sections separated by 0.3 μm were collected, and stacks were analyzed using ImageJ software.

**RNA-Seq Sample Preparation.** Sorted cells were resuspended in TRIzol (Ambion Life Technologies), and total RNA was extracted according to the manufacturer's protocol. Quality and quantity of the total RNA was assessed by the 2100 Bioanalyzer using a nano chip (Agilent). RNA libraries were generated using the TruSeq Stranded mRNA sample preparation kit (Illumina) according to the manufacturer's protocol. The libraries were analyzed for size and quantity of cDNAs on a 2100 Bioanalyzer using a DNA 7500 chip (Agilent), diluted, and pooled in multiplex sequencing pools. The libraries were sequenced as 65 base single reads on a HiSeq2500 (Illumina).

**RNA-Seq Library Preparation.** Strand-specific complementary DNA (cDNA) libraries were generated using the TruSeq Stranded mRNA sample preparation kit (Illumina) according to the manufacturer's protocol. The libraries were analyzed for size and quantity of cDNAs on a 2100 Bioanalyzer using a DNA 7500 chip (Agilent), diluted, and pooled in multiplex sequencing pools. The libraries were sequenced as 65 base single reads on a HiSeq2500 (Illumina).

**RNA-Seq Preprocessing.** Strand-specific RNA reads (11 to 33 million reads per sample), 65-base-pair single-end, were aligned against the mouse reference genome (Ensembl build 38) using TopHat (version 2.1, bowtie version 1.1). Tophat was supplied with the following parameters: `-prefilter-multithreads -no-coverage-search –bowtie1-library-type fr-firststrand`. In order to count the number of reads per gene, a custom script which is based on the same ideas as HTSeq-count was used. A list of the total number of uniquely mapped reads for each gene that is present in the provided GTF file was generated.

**Gene Expression Analysis.** Differential expression analysis was performed in R language (version 3.5.1) using edgeR package. Default arguments were used with the design set to specific genotypes. Genes that have no expression across all samples within the dataset were removed. Analysis was restricted to genes that have at least a 2 counts per million (cpm) value in all samples in specific contrasts to exclude very-low-abundance genes. Immunoglobulin heavy variable (Ighv) genes were excluded to avoid any confounding issue. The FDR was determined after the Benjamini-Hochberg multiple testing correction. Genes with an FDR below 0.01 were considered to be differentially expressed. Sets of differentially expressed genes in indicated conditions were called gene signatures. MA (ratio intensity) plots were generated after carrying differential expression analysis done by the edgeR package (63, 64). Counts were shown as the average log2 cpm after trimmed mean of M-values normalization and removing the batch effect. Batch effects were corrected by voom function under the limma (3.44.3) and edgeR package. For calculating Pearson correlation, only protein coding genes with a cpm value greater than 2 in all of the samples were taken. After correcting for the batch effect and library normalization, Pearson correlation was calculated using cor function in R with the default parameters. The correlation value was used as input to consensus clustering analysis. Hierarchical consensus clustering analysis was done by the hclust function in R, and the dendrogram was visualized using by the dendrosort package (0.3.3). The RNA-Seq datasets reported in this article have been deposited at the National Center for Biotechnology Information under the accession number GSE144275 (Token number: ijwuxsygdwvhbqh).

**Statistics.** Statistical analyses for Figs. 3 and 5A were performed using GraphPad Prism (version 8.0.0).

**Data Availability.** All study data are included in the article and supporting information.

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