# CHAPTER 4

## PRO-B CELLS SENSE PRODUCTIVE IMMUNOGLOBULIN HEAVY CHAIN REARRANGEMENT IRRESPECTIVE OF POLYPEPTIDE PRODUCTION

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## ABSTRACT

B lymphocyte development is dictated by the protein products of functionally rearranged immunoglobulin (Ig) heavy (H) and light (L) chain genes. Ig rearrangement begins in pro-B cells at the IgH locus. If pro-B cells generate a productive allele, they assemble a pre-B cell receptor complex, which signals their differentiation into pre-B cells and their clonal expansion. Pre-B cell receptor signals are also thought to contribute to allelic exclusion by preventing further IgH rearrangements. Here we show in two independent mouse models that the accumulation of a stabilized μH mRNA that does not encode μH chain protein specifically impairs pro-B cell differentiation and reduces the frequency of rearranged IgH genes in a dose-dependent manner. Because noncoding IgH mRNA is usually rapidly degraded by the nonsense-mediated mRNA decay machinery, we propose that the difference in mRNA stability allows pro-B cells to distinguish between productive and nonproductive Ig gene rearrangements and that μH mRNA may thus contribute to efficient H chain allelic exclusion.



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## INTRODUCTION

Developing B lymphoid cells generate immunoglobulin (Ig) genes by recombination of gene segments (1). This process is initiated in pro-B cells of the bone marrow with the assembly of diversity (D) and joining (J) gene segments at both IgH alleles. Subsequently, a variable (V) gene segment can be recombined to a pre-existing DJ-joint to form a VDJ exon (1). Once a functional $V_H$ exon has been generated, a heavy (H) chain is produced, which assembles with the surrogate light (L) chain and the signal molecules Igα/Igβ to form the pre-B cell receptor complex (pre-BCR). The pre-BCR provides signals for clonal expansion, survival, and differentiation into pre-B cells (2). Of the two IgH alleles, only one contributes to the BCR, a phenomenon known as allelic exclusion. This process is thought to be regulated at the level of V-to-DJ recombination (3, 4) and ensures that each B cell produces a single clonotypic antibody. Monospecificity of a B cell is important, since only a monospecific BCR allows efficient generation of self-tolerant B cells during B cell ontogeny, while at later stages in B cell development allelic exclusion contributes to efficient antigen-specific antibody responses.

B cell ontogeny is characterized by a biphasic induction of the V(D)J recombinase (RAG) and a sequential rearrangement of IgH and IgL chain alleles. RAG is turned off in B cells expressing a functional, self-tolerant immunoglobulin; while perhaps too simplistic, this by and large explains both allelic and isotypic exclusion at the L chain loci. Although it is tempting to propose analogous models for allelic exclusion of IgH and IgL chain genes, there are, in fact, great differences—not only in temporal sequence of gene assembly, but also in strictness of exclusion: While a small percentage of B cells does express two different L chains (5), only one in $10^4$ cells expresses two H chains (6).

Various competing theories on the mechanism of IgH chain allelic exclusion have been proposed, and they are not necessarily mutually exclusive (7). In a stochastic model, allelic exclusion is considered to be a statistical consequence of a low frequency of rearrangements encoding functional H chains (8, 9). In its bare-bones form, the stochastic hypothesis seems to be disproven for the IgH locus, as pro-B cells expressing signaling-defective forms of the pre-BCR have a large proportion of μH chain double producers (10). Mice with defective Ig receptor signaling support a genetic model in which the pre-BCR complex controls allelic exclusion. Firstly, only transgenes encoding the membrane but not the cytoplasmic form of the μH chain mediate allelic exclusion (11). Secondly, concomitant deletion of the (pre) BCR-associated Syk family kinases Syk and ZAP-70 resulted in allelic inclusion (12), as did mutations in Igα and Igβ (13-15), which either block their association with the μH chain or interfere with intracellular signaling cascades. Similarly, allelic inclusion occurred at the T cell receptor (TCR)-β locus in mice with disruptions of either the TCR adapter protein SLP-76 or the TCR-associated kinase p56lck (16, 17).

In the genetic regulation model of H chain allelic exclusion, μH chain protein (as part of the pre-BCR) inhibits further rearrangements at the IgH locus, so that a

second, functional IgH gene cannot be assembled (18). But how is this inhibition accomplished? Prior to the rearrangement of a V gene segment, both H chain alleles are in a DJ-rearranged configuration (19) and are indistinguishable with regard to germ line transcription (20), nuclear localization (21), and locus contraction (22). Therefore, V-to-DJ recombination must be either asynchronous to allow enough time for H-chain surface expression and pre-BCR signaling, or a productive VDJ recombination event must halt recombination until pre-BCR signals have been initiated. Because ATM is activated by recombination-induced DNA double strand breaks, it is thought to play a role in this process (23). Afterwards, both IgH loci are "decontracted" to suppress further V-to-DJ rearrangements, and the partially rearranged IgH allele is silenced by pericentromeric relocation, thereby making it inaccessible for Rag (21, 22, 24-27). Given that allelic exclusion of the IgH allele is quite effective and double producers are less frequent than predicted in most models that interfere with H chain signaling, a feedback inhibition of V-to-DJ recombination by the pre-BCR alone appears insufficient.

The recent discovery of noncoding RNA as a critical regulator of gene expression led us to consider an additional mechanism for H chain allelic exclusion, in which the mRNA that encodes a productive μH chain is sensed by the pro-B cell. With the intronic IgH enhancer in close proximity to the $V_H$ promoter, an immediate consequence of any V-to-DJ recombination is a high transcription rate of the rearranged locus and the appearance of H transcripts. While transcription rates of productively and nonproductively rearranged IgH loci are similar (28), only transcripts from a productively rearranged (coding) allele are stable and accumulate. In contrast, noncoding (nonsense) mRNA from a nonproductively rearranged allele is rapidly degraded by the nonsense-mediated mRNA decay (NMD) mechanism (29-31). Thus, stable coding μH mRNA could indicate the presence of a productive IgH allele and exclude the rearrangement of the other allele, while unstable μH mRNA encoded by a nonproductively rearranged IgH gene would be degraded and have no effect. To experimentally uncouple an effect mediated by the mRNA from any signal transmitted by its product of translation, the μH chain protein, we utilized an exception in NMD activity: When located close to the translation start site, a premature termination codon is not recognized by the NMD (32). This exception allowed us to create IgH alleles that are transcribed into stable untranslated μH mRNAs and to assay their effect on B cell development.

## RESULTS

### Mice expressing nonsense μH mRNA that is not degraded

To determine the effects of a stable μH mRNA on B cell development and/or VDJ recombination, we established three mouse lines in which stable μH mRNA production is separated from translation into μH chain protein. One line (designated Ter3) expresses mRNA from the productively rearranged H chain transgene $V_H$17.2.25,

which has been rendered nonproductive by converting codon 3 of the leader exon into a translational stop codon (Fig. 1A). In contrast to the premature termination codon found in most nonproductive H chain transcripts, the nonsense codon in Ter3 transcripts triggers only a weak NMD response, resulting in stable and abundant μH Ter3 transcripts (32). The Ter3 line presented here has approximately 10 transgene copies integrated into a genomic region of chromosome 5 without annotated features, and is representative of lines established from three independent founders. In addition we used two H chain gene knock-in lines, in which the endogenous $D_{Q52}J_H$ cluster was replaced by a $V_H$B1-8 VDJ exon that was rendered nonproductive by the introduction of a termination codon at position 5 (designated the Ter5 allele, Fig. 1B). In one Ter5 line, transcription of the targeted H chain gene is driven by its

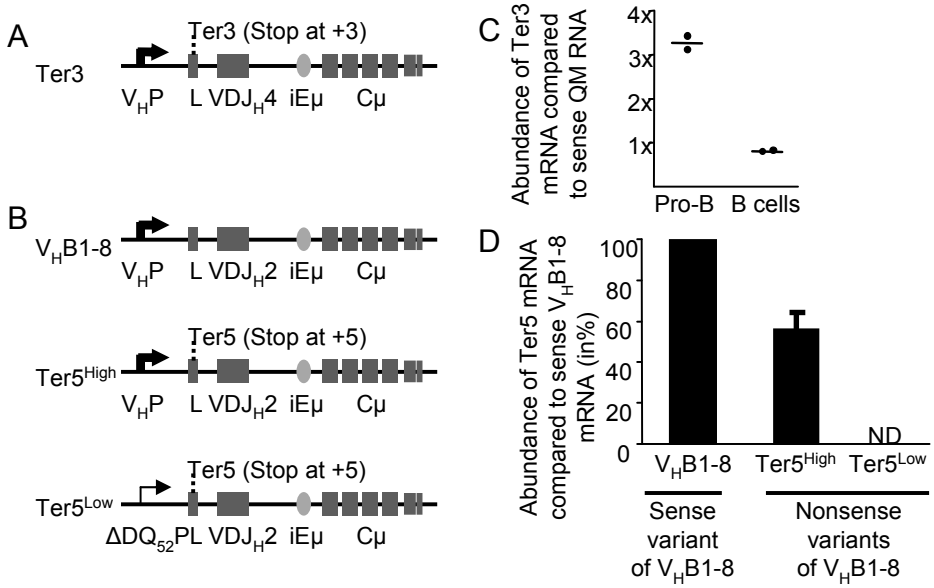



**Figure 1.** Construction of mice expressing stabilized noncoding (nonsense) μH mRNA. (A) The Ter3 transgene consists of a mutated $V_H$17.2.25 VDJ exon, in which codon +3 in the leader has been changed to a stop codon (Ter3), followed by the intronic IgH enhancer (iEμ) and the complete genomic Cμ region. (B) Schematic organization of the targeted IgH locus in three strains of knock-in mice expressing $V_H$B1-8 μH mRNA either as a sense variant ($V_H$B1-8, top row) or as nonsense variants with a translational stop codon at position 5 ($Ter5^{High}$ and $Ter5^{Low}$, middle and bottom rows) under the control of either the endogenous $V_H$ promoter ($V_H$P, top and middle rows) or a weak truncated $D_{Q52}$ promoter ($D_{Q52}$P, bottom row). (C) Ter3 mRNA abundance was determined in FACS-sorted pro-B and splenic B cells from Ter3, $IgH^{QM/wt}$ mice that express both nonsense $V_H$17.2.25 μH mRNA from the Ter3 transgene and sense $V_H$17.2.25 μH mRNA from the knock-in IgH locus of the quasi-monoclonal (QM) mouse. Both H chain transcripts were amplified in one reaction by RT-PCR, and their abundances were estimated from the fragment intensities after a Ter3 transgene-specific restriction digest (see Figure S1). (D) Quantification of $V_H$B1-8 μH mRNA in splenocytes from heterozygous $V_H$B1-8, $Ter5^{High}$, and $Ter5^{Low}$ mice by quantitative TaqMan RT-PCR using primers specific for the $V_H$B1-8 sequence. ND = not detected.

physiological H chain promoter, while in the other, transcription is driven by a weak truncated $D_{Q52}$ promoter (33). This results in high ($Ter5^{High}$ mouse) and low ($Ter5^{Low}$ mouse) amounts of noncoding H transcripts, respectively (see below).

To determine the abundance of the nonsense $V_H$17.2.25-Ter3 mRNA, we bred Ter3 mice to quasi-monoclonal (QM) mice, in which the endogenous $D_{Q52}J_H$ cluster is replaced with the functional $V_H$17.2.25 exon (34); i.e., QM mice express "Ter3" mRNA without a nonsense codon (Fig. S1A). The mice used for our determination thus had the genotype $Ter3^{Tg}$, $IgH^{QM/wt}$, and they expressed both sense and nonsense $V_H$17.2.25 μH mRNA. From these mice we FACS-sorted pro-B and splenic B cells and amplified both H chain transcripts in one reaction by RT-PCR. Although the promoter and VDJ exons are nearly identical, a codon change in the Ter3 transgene created a specific restriction site, which enabled us to estimate the respective mRNA abundances from the fragment intensities (Fig. S1B). Compared to coding QM mRNA, the amount of noncoding Ter3 mRNA in splenic B cells was almost identical (Fig. 1C and Fig. S1B); in pro-B cells, for unknown reasons the amount was three times higher.

For the Ter5 mice, we compared the abundance of μH mRNA in splenocytes from heterozygous $Ter5^{High}$ mice, $Ter5^{Low}$ mice, and mice with the same VDJ knock-in allele but no premature termination codon. In $Ter5^{Low}$ mice, we detected no transcripts; in $Ter5^{High}$ mice, the Ter5 mRNA accumulated up to 60% of the transcripts encoded by the sense allele (Fig. 1D). Therefore, the abundance of nonsense μH mRNA in the Ter3 and $Ter5^{High}$ mice was by and large within the physiological range of μH mRNA that is translated.

**Ter3 and Ter5 μH mRNAs are not translated into μH chain protein**

We also confirmed that no truncated μH chain was produced in any of these mice. Accordingly, we bred the Ter3 mouse to a Rag2-deficient mouse. In the resulting recombination-deficient Ter3 mice, the further differentiation of pro-B cells ought to be completely blocked. Indeed, this was the case: just as in the Rag2-deficient mouse, none of the ($c\text{-}kit^+$, $CD19^+$) pro-B cells produced any intracellular μ chain. In the wild-type control mouse, however, almost 40% of all cells stained with a polyclonal anti-IgM antibody (Fig. S2A). We also analyzed lysates from bone marrow cells of homozygous $Ter5^{Low}$ and $Ter5^{High}$ mice by Western blotting with a polyclonal anti-IgM serum. Again, although the wild-type produced large amounts of μH chain, there was none present in the Ter5 mice or the Rag-deficient mice (Fig. S2B). Nonsense μH mRNA from both Ter3 and Ter5 mice also lack larger out-of-frame ORFs, which could result in the accumulation of a stress-inducing polypeptide.

Because they are neither degraded nor translated, the noncoding Ter3 and Ter5 μH mRNAs serve as a valid surrogate of a μH mRNA stabilized by translation; therefore, they enable us to study their effect on B cell development in the absence of μH chain protein and pre-BCR signals. Especially the $Ter5^{High}$ mouse with its physiological IgH promoter starts with pro-B cells that closely mimic wild-type pro-B cells that have just productively rearranged their IgH locus. The two types of cells differ only in the translatability of their in-frame rearrangement.

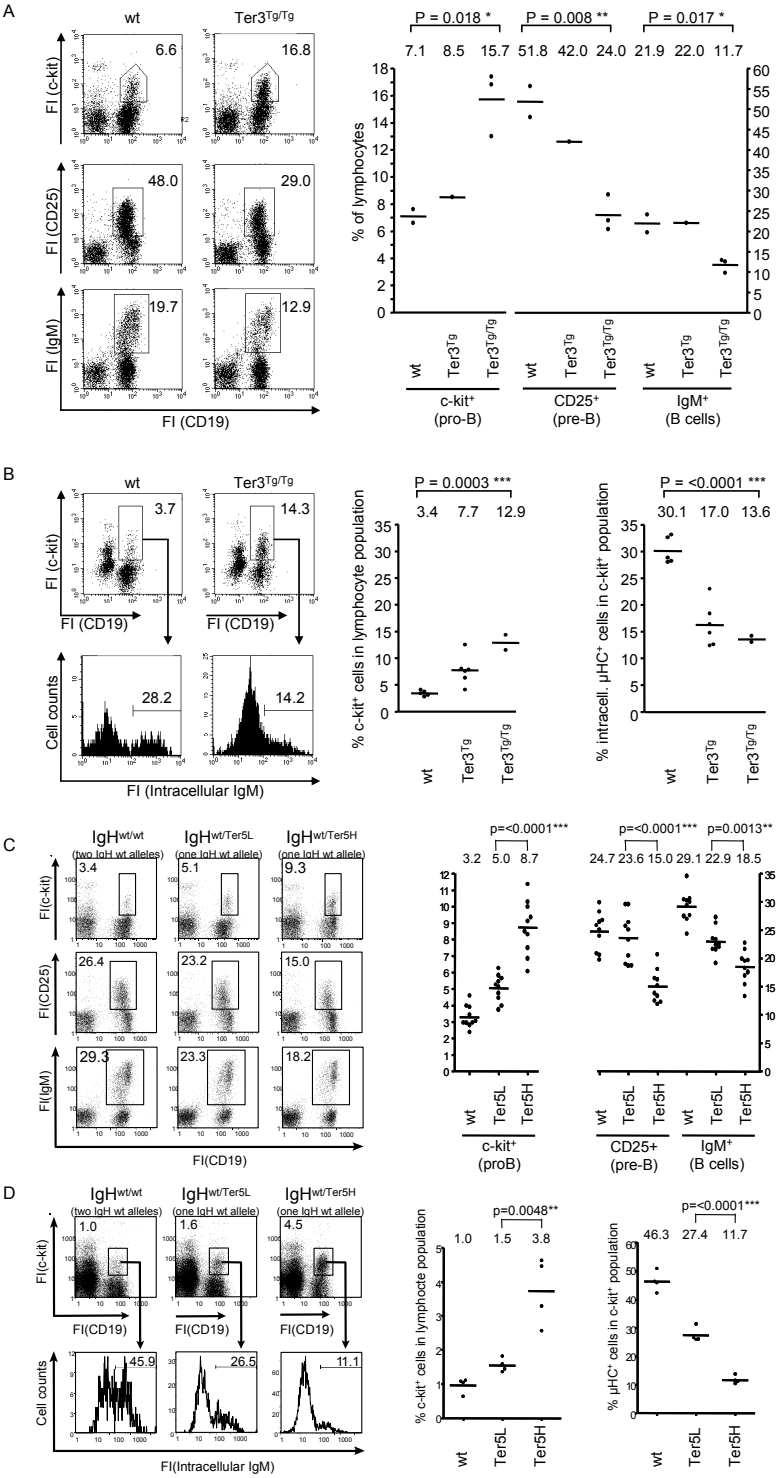
**Pro-B cell differentiation is impaired by stable noncoding μH mRNA.**

If the accumulation of a stable μH mRNA interfered with B cell differentiation and/or VDJ rearrangement, we would expect to find an impaired transition of B lymphoid precursors from the μH chain-negative pro-B to the μH chain-positive pre-B cell stage. Indeed, the number of c-kit$^{+}$ pro-B cells was increased, and the number of subsequent developmental stages was decreased in Ter3 and Ter5$^{High}$ mice, both relatively and in absolute numbers (Fig. 2A,C and Tab. S1). The impairment of early B cell development was dose-dependent; i.e., more noncoding mRNA inhibited B cell development to a greater degree (Ter3$^{Tg/Tg}$ vs. Ter3$^{Tg}$). In addition, the frequency of cells with newly synthesized intracellular μH chains in the c-kit$^{+}$ pro-B population was decreased in both Ter3$^{Tg}$ and Ter5$^{High}$ mice (Fig. 2B,D), indicating that stable noncoding μH mRNA interferes with B cell development before or at the stage of V-to-DJ rearrangement. We note that IgH rearrangement in Ter5 mice is restricted to a single IgH allele and, therefore, is only half as likely to be productive as rearrangement in wild-type mice. This may explain the phenotypic difference between Ter5$^{Low}$ and wild-type mice, but it does not account for the pronounced difference between Ter5$^{Low}$ and Ter5$^{High}$ mice. The Ter3 allele, on the other hand, was introduced as a conventional transgene; in Ter3 mice, both endogenous IgH alleles are thus unchanged and can rearrange. Therefore, the differences in pro-B cell differentiation between wild-type, Ter3$^{Tg}$, and Ter3$^{Tg/Tg}$ mice ought to be due to the amount of noncoding μH mRNA transcribed from the transgene.

**Cell specificity of stable μH mRNA effect**

The question arose as to whether the effect of stable μH mRNA on B cell development is nonspecific. However, μH mRNA itself is a transcript unique to B cells; as long as the stable μH mRNA contains a regular VDJ exon and is expressed at the same level as in wild-type cells, its activity ought to faithfully reflect that of translatable μH mRNA in wild-type B cells. Nevertheless, we ruled out some explanations for the impairment in pro-B cell differentiation. According to one hypothesis, increased competition for ribosomes or transcription factors (like Pax5) in the transgenic mice would reduce the expression of μH chain from the productively rearranged endogenous locus and/or the Pax5 target gene CD19, thereby inhibiting pro-B cell development. However, there was no difference in the expression of intracellular μH chain between pro-B cells from wild-type, Ter3$^{tg}$ (Fig. 2B, histogram), heterozygous Ter5$^{High}$ (Fig. 2D, histogram), or between total bone marrow cells from Ter3$^{Tg}$ and wild-type mice, or from IgH$^{QM/wt}$ and IgH$^{QM/wt}$/Ter3$^{Tg}$ mice (Fig. S3A). Nor was there any difference in membrane expression of CD19 between pro-B cells from $J_H^{-/-}$ and $J_H^{-/-}$/Ter3$^{Tg}$ mice (Fig. S3B), in which B cell development is arrested at the pro-B cell stage. Therefore, the expression of nonsense μH mRNA from a single endogenous locus (Ter5 mouse) or multiple transgenes (Ter3 mouse) does not reduce the availability of transcription factors involved in the expression of the endogenous IgH and CD19 locus.

In another experiment, we differentiated between the splenic B cell populations in Ter3 transgenic mice and found them unaltered, as compared to wild-type. These


A
wt
Ter3Tg/Tg
6.6
16.8
48.0
29.0
19.7
12.9
Fl (c-kit)
Fl (CD25)
Fl (IgM)
Fl (CD19)
P = 0.018 *
P = 0.008 **
P = 0.017 *
7.1 8.5 15.7 51.8 42.0 24.0 21.9 22.0 11.7
% of lymphocytes
wt
Ter3Tg
Ter3Tg/Tg
c-kit+ (pro-B)
CD25+ (pre-B)
IgM+ (B cells)
B
3.7
14.3
28.2
14.2
Cell counts
Fl (Intracellular IgM)
P = 0.0003 ***
3.4 7.7 12.9
% c-kit+ cells in lymphocyte population
P = <0.0001 ***
30.1 17.0 13.6
% intracell. µHC+ cells in c-kit+ population
C
IgHwt/wt (two IgH wt alleles)
IgHwt/Ter5L (one IgH wt allele)
IgHwt/Ter5H (one IgH wt allele)
3.4
5.1
9.3
26.4
23.2
15.0
29.3
23.3
18.2
Fl(c-kit)
Fl(CD25)
Fl(IgM)
Fl(CD19)
p=<0.0001***
p=<0.0001***
p=0.0013**
3.2 5.0 8.7
24.7 23.6 15.0 29.1 22.9 18.5
wt
Ter5L
Ter5H
c-kit+ (proB)
CD25+ (pre-B)
IgM+ (B cells)
D
IgHwt/wt (two IgH wt alleles)
IgHwt/Ter5L (one IgH wt allele)
IgHwt/Ter5H (one IgH wt allele)
1.0
1.6
4.5
45.9
26.5
11.1
Fl(c-kit)
Fl(CD19)
Cell counts
Fl(Intracellular IgM)
p=0.0048**
1.0 1.5 3.8
% c-kit+ cells in lymphocte population
p=<0.0001***
46.3 27.4 11.7
% µHC+ cells in c-kit+ population
wt
Ter5L
Ter5H

populations included recirculating naive B cells (population *a* in Fig. 3A), transitional type II B cells (population *b* in Fig. 3A), and marginal zone, immature transitional type I, B1 and memory B cells (population *c* in Fig. 3A). Finally, we looked at T cell development, which requires recombination of the TCR-β locus at the pro-T cell stage (the double-negative DN III stage). Pre-T cells of $Ter3^{Tg/Tg}$ mice express μH mRNA at levels approximately one fifth of that in pre-B cells (Fig. 3B) and two fifths of that in Ter3 heterozygous pre-B cells. VDJ recombination at the TCR-β locus occurs in the DN III population (pro-T cell stage), and impaired recombination should increase this population (35). But this and the other thymic cell populations were unaltered (Fig. 3C).

**Frequency of recombined IgH alleles is decreased by stable μH mRNA**

As far as we could determine, stable μH mRNA affected only the numbers of pro-B and pre-B cells. In pro-B cells, it also reduced the number of μ-expressing cells (Fig. 2B,D), presumably because of a reduced frequency of recombined IgH alleles. To measure this frequency directly, we isolated DNA from pro-B cells and determined the ratio of VDJ rearrangements vs. germ line configuration by quantitative TaqMan PCR. The forward primer was specific for the most abundant $V_H1$ (J558) family, and the reverse primer was specific for the $J_H3$ segment, which is used in neither the Ter3 nor the Ter5 transgene. Both homozygous $Ter3^{Tg/Tg}$ and heterozygous $Ter5^{High}$ mice had about half as many $V_H1$-$DJ_H3$ rearrangements as wild-type and heterozygous $Ter3^{Low}$ mice, respectively (Fig. 4A). These results correlate closely with the decreased frequency of μH chain-positive cells in the pro-B cell populations of the respective mice (Fig. 2B,D).

In a second TaqMan PCR, we assessed the relative frequency of $D_{Q52}$ elements that still contained their upstream recombination signal, i.e., IgH alleles that were either in the germ line configuration or had a $D_{Q52}J_H$ rearrangement (Fig. 4B). In this assay, DNA from heterozygous $Ter5^{High}$ mice gave a threefold stronger signal than DNA from heterozygous $Ter5^{Low}$ mice, indicating a higher proportion of pro-B cells with either a non-rearranged H allele or an allele with a $D_{Q52}$-to-$J_H$ rearrangement in $Ter5^{High}$ mice. Furthermore, the frequency of immature B cells with a $V_H$-replaced QM allele was lower in QM mice with a transgenic Ter3 allele than in QM mice without it (Fig. S4). These observations support our hypothesis that developmental inhibition occurs before or at the stage of V-to-DJ recombination.

◁ **Figure 2.** Noncoding μH mRNA impairs pro-B cell differentiation. (A) Bone marrow cells of 6-week-old Ter3 mice and wild-type littermates were membrane stained with the indicated antibodies, and fluorescence intensities (FI) of cells in the lymphocyte gate were determined by flow cytometry. The percentages of cells in the individual gates are indicated. (B) Pro-B cells ($c\text{-}kit^+/CD19^+$) analyzed for intracellular μH chain. Bone marrow cells were membrane stained with antibodies against c-kit and CD19, permeabilized, and re-stained with antibodies against μH chain. (C, D) Bone marrow cells of 6-week-old wild-type and heterozygous $Ter5^{Low}$ and $Ter5^{High}$ mice were stained as described above. Results of an entire litter (A, B) or of multiple litters (C, D) are summarized in the diagrams shown to the right; one dot represents one mouse.

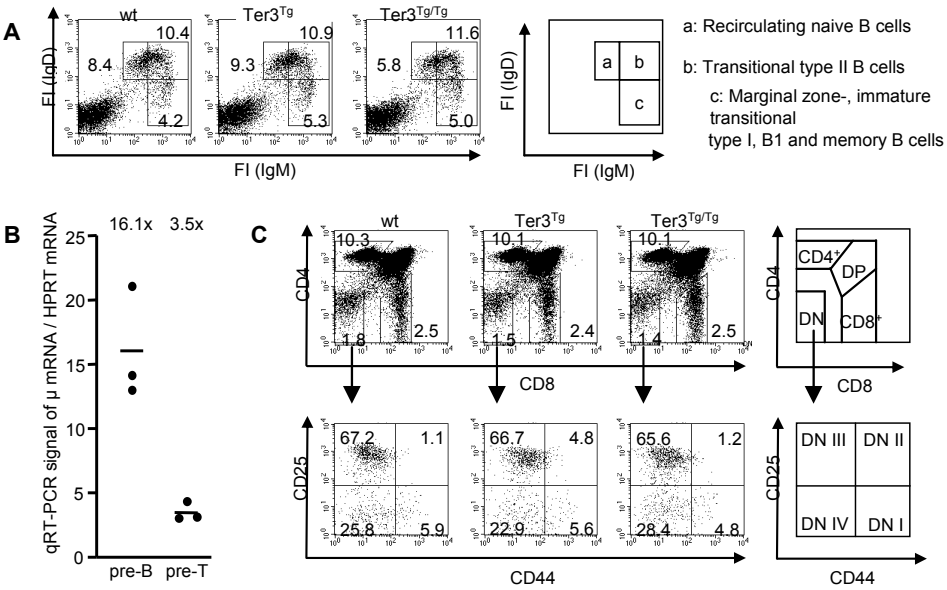



**Figure 3.** Ter3 mRNA does not affect later B cell stages and thymic development. (A) Splenocytes from 6-week-old mice membrane stained with the indicated antibodies. Fluorescence intensities (FI) of cells in the lymphocyte gate were determined. The percentages of cells in the individual gates are indicated. The following populations are marked clockwise by squares in the dot plot diagram and explained in the schematic diagram to the right: population *a*, recirculating naive B cells ($IgM^{dull}$, $IgD^{high}$); population *b*, transitional type II B cells ($IgM^{high}$, $IgD^{high}$); and population *c*, $IgM^{high}$, $IgD^{dull}$ cells including marginal zone, immature transitional type I, B1 and memory B cells. (B) Ter3 µH chain and HPRT mRNA abundances were measured by quantitative RT-PCR in sorted pre-B and pre-T cells from three $Ter3^{Tg/Tg}$ mice. (C) Thymocytes from 6-week-old mice were membrane stained with the indicated antibodies, and fluorescence intensities (FI) of cells in the lymphocyte gate were determined. The percentages of cells in the individual gates are indicated. Upper panel: Cells were analyzed for CD4 and CD8 expression. Lower panel: The double-negative (DN) population indicated in the upper diagrams was divided into stages DN I to IV, based on CD25 and CD44 expression.

### VDJ recombinase is unaffected by stable µH mRNA

To determine whether stable µH mRNA interferes with VDJ recombination by directly inhibiting the RAG recombinase, we transduced sorted pro-B cells from the bone marrow of homozygous $Ter5^{High}$ and $Ter5^{Low}$ mice with a VDJ recombination plasmid that contains an inverted EGFP gene. The EGFP cassette is flanked by recombination signal sequences (RSS) and can be activated upon recombination activating gene (RAG)-mediated reversion (35). RAG activities in pro-B cells from both wild-type and $Ter5^{High}$ mice were similar (Fig. 5). Hence, the accumulation of noncoding µH mRNA does not directly affect the VDJ recombinase, a finding consistent with the normal development of B cells beyond the pre-B cell stage.

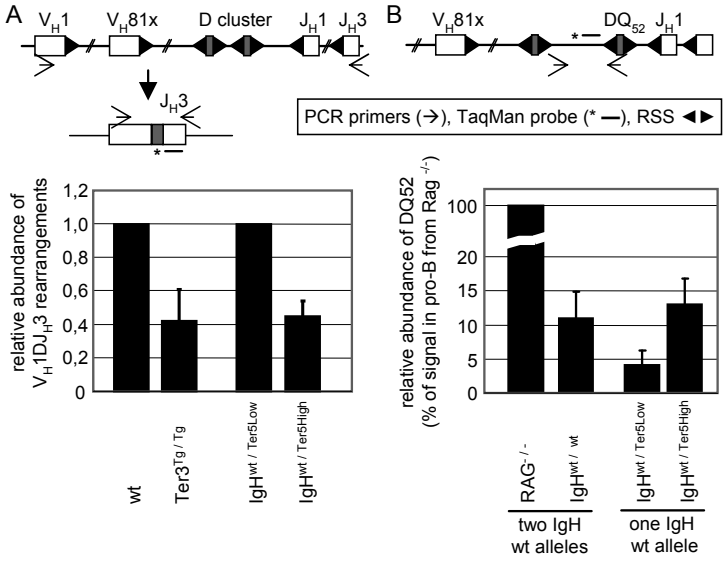



**Figure 4.** VDJ recombination frequency is decreased by stable μH mRNA. (A) Quantification of $V_H1DJ_H3$ rearrangements in pro-B cells by TaqMan PCR. Genomic DNA from FACS-sorted $CD19^+$/c-$kit^+$ pro-B cells of the indicated genotypes was analyzed using the indicated primers and probe. Signals in $Ter3^{Tg/Tg}$ and $IgH^{wt/Ter5High}$ mice were normalized to those in wild-type and $IgH^{wt/Ter5Low}$ mice, respectively. (B) Quantification of germ line or $D_{Q52}J_H$-rearranged IgH loci by TaqMan PCR. Amplification of the sequence 5' of the $D_{Q52}$ gene segment is possible only on germ line or $D_{Q52}J_H$-rearranged IgH loci; it is deleted by all other D-to-$J_H$ rearrangements or a $V_H$-to-$D_{Q52}$ rearrangement. Genomic DNA from sorted $CD19^+$/c-$kit^+$/surface $IgM^-$ pro-B cells of the indicated mice was analyzed using the indicated primers and probe. Results (mean ± SD) are from one of two independent experiments.

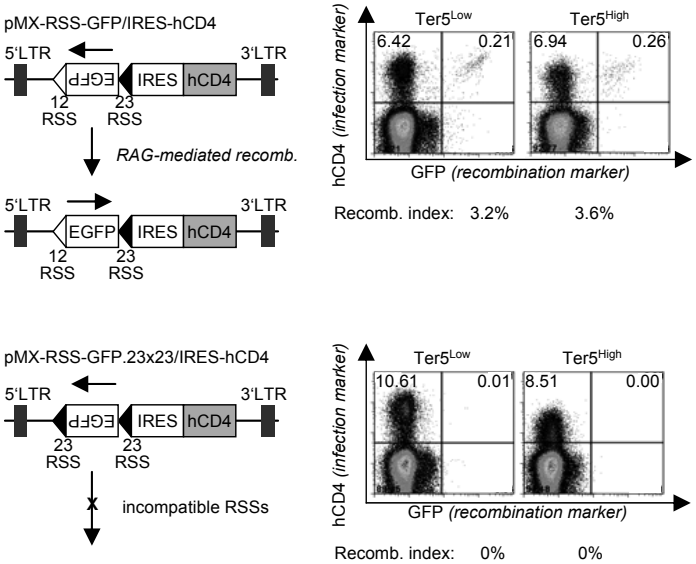



**Figure 5.** VDJ recombinase activity is unaffected by stable μH mRNA. Total bone marrow cells from homozygous Ter5Low and Ter5High mice were isolated and transduced with two viral vectors containing an inverted EGFP flanked by recombination signal sequences (RSS). The first vector contained two compatible RSS, which allows inversion and expression of the EGFP. The second variant contained two incompatible RSS and served as negative control. Frequency of recombined GFP-positive cells in the infected hCD4-positive population was measured 48 h after infection and is depicted as recombination activity index.

## DISCUSSION

Currently, allelic exclusion at the IgH locus is explained by a feedback mechanism that presumes signaling via the pre-BCR containing the μH chain encoded by a productive allele. An analysis of mice with a deletion including the μ membrane exons (μMT mice) shows that sequences spanning and flanking the membrane exons are needed for IgH allelic exclusion (10). However, from this experiment it is not clear whether these sequences are only necessary to generate the μH chain protein, or whether the (stable) μH mRNA, or even the untranslated regions of the mRNA, also contribute to allelic exclusion.

In this study, we investigated the potential role of μH mRNA in the remarkable strictness of exclusion at the IgH locus. Our data show that a stable μH mRNA impairs pro-B cell development in the absence of a μH chain signal. We cannot exclude that a non-translatable but stable mRNA other than that encoding μH chain would have the same effect. But with the many stable translated mRNAs encoding functional proteins already present in a cell, just adding any other one might not be expected to make a difference. At any rate, as we did not detect an effect on the development of pre-B and immature B cells into mature B cell subsets (Fig. 3A), or on T cell development in the thymus (Fig. 3C), the effect would have to be restricted to pro-B cells. One could also argue that a well-transcribed IgH allele will sequester transcription factors for target genes whose expression levels are critical at an early stage in B cell development, like the μH chain and CD19 gene. However, as the expression levels of μH chain and CD19 were not affected by the expression of Ter3 and Ter5 mRNA (Figs. 2B, 2D, S3), we think that the observed phenotype is not caused by transcription factor sequestration.

Our result rather fit the observations in mice with decreased nonsense-mediated mRNA degradation (NMD), which are reported in the accompanying manuscript by Frischmeyer-Guerrerio et al. Their study demonstrated a critical role for the NMD in T and B lymphocyte development. B cells may thus distinguish between productively and nonproductively rearranged IgH alleles at the RNA level, and this distinction may help in allelic exclusion. But how can μH mRNA contribute to allelic exclusion? We demonstrated the effect of a μH mRNA on B cell differentiation in mice with either a conventional IgH transgene (the Ter3 mouse) or a VDJ exon knock-in (the Ter5 mouse). One explanation for the observed phenotype could be that expression of a μH mRNA simply confers a growth disadvantage to pro-B cells. In this case, pro-B cells with a productive IgH gene, and thus expressing a stable coding μH mRNA, would grow more slowly than cells that have not yet rearranged; but cells without a productive VDJ rearrangement do not progress anyway, because they do not receive a differentiation signal from a μH chain protein. A cell with two productive VDJ rearrangements would grow even more slowly and would be at a disadvantage compared to a cell with only one productive VDJ rearrangement.

Alternatively, we propose a model in which stable μH mRNA has a suppressive effect on VDJ recombination, even in the absence of μH chain protein. Because NMD

selectively degrades noncoding transcripts from nonproductive IgH alleles, only sense μH transcripts accumulate and are capable of inhibiting, directly or indirectly, V-to-DJ rearrangements. Because we excluded a direct effect on RAG activity, μH mRNA might interfere with the opening and/or the accessibility of the second IgH allele, for example, in combination with antisense RNA transcribed during VDJ recombination from the IgH locus (20). Alternatively, abundant μH transcripts could act similarly to Xist, which mediates X chromosome inactivation (36). In this model, suppression of recombination at the other IgH allele is followed by the strong feedback signal from the pre-BCR, which would shut it down permanently. Such an adapted feedback model of allelic exclusion could bridge the time gap between a productive VDJ rearrangement and the initiation of an H-chain-dependent pre-BCR signal.

In summary, we have identified a specific μH mRNA-dependent process that - independent of a μH chain signal - distinguishes between a productive and a nonproductive IgH allele and thereby may contribute to the establishment of allelic exclusion at the IgH locus.



## MATERIALS AND METHODS

### Supplemental online procedures

Standard procedures and methods such as animal handling, flow cytometry, and RNA analyses as well as statistical methods are described in supplemental online material.

### Rag activity assay in primary pro-B cells

The viral vectors pMX-RSS-GFP/IRES-hCD4 and pMX-RSS-GFP.23x23/IRES-hCD4 (35) (kindly provided by M. Schlissel) were packaged in PlatE cells and used to infect total bone marrow cells from homozygous Ter5L and Ter5H mice. Frequency of GFP-positive cells in the hCD4-positive population was measured by flow cytometry 48 hours after infection and is depicted as recombination activity index.

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# SUPPLEMENTAL MATERIALS AND METHODS

## Mice

Ter3 transgenic animals were established using the vector pμTer3 described previously(1). Briefly, we changed codon 3 in the leader peptide of the H chain expression vector pμGPT to an in-frame TGA stop codon and replaced downstream in-frame ATG codons in the $V_H$ region sequence by Ala codons to prevent reinitiation and translation of a truncated H chain protein. To construct transgenic Ter3 mice, a 12.3kb *Sal*I/*Xho*I fragment bearing the IgH promoter, the Ter3 $V_H$ region of the hybridoma 17.2.25 and a genomic Cμ region from a Sv129 mouse (H chain allotype a) was isolated from pμTer3 and injected into the pronucleus of C57/BL6 oocytes. Three independent lines (7204, 7302, and 7307) were established by backcrossing the corresponding founders to C57/BL6 animals. Here we present data of the 7307 line. The insertion sites of the lines 7204 and 7307 were mapped by circular PCR to positions chr5: 109.975.633 and chr5:10.473.198, respectively. Offspring were screened by PCR for the presence of the Ter3 transgene. Wildtype configuration of the allele was detected in with primers flanking the insertion site. For 7204 line, we used the primers 5′ of Ter7204 fwd (CAA GAC AAC TCT CGA CTA CAT GTG AG) and 3′ of Ter7204 rev (CTG TCC TGA AAC TCA CCT TGT AGA CC) and for 7307 line the primers 5′ of Ter7307 fwd2 (AAC ATC AAG TTT CCA AGT AGT GGT GG) and 3′ of Ter7307 rev3 (GCT TCT ACT AGA TTC AGT GTA TCT GG). Presence of the transgene was detected with the respective forward primer and the

primer IgMTer113 rev (CTG TCA AAG CTA CTT GAT GAG GAT GC), which binds to a sequence in the transgene. QM(2), $V_H$B1-8(3), Ter5H (pII(4)) and Ter5L (pΔ(4)) mice have been described previously. All animal experiments were performed according to institutional and national guide lines.



**Antibodies**

Fluorochrome-conjugated mAbs against CD19 (clone 1D3, PerCP), c-kit (clone ack45, PE), CD25 (clone PC61, PE), $IgM^a$ (clone DS-1, FITC), and $IgM^b$ (clone AF6-78, PE) were purchased from BD PharMingen and Cy5-conjugated goat abs against mouse μH chain from Southern Biotechnology. The unlabeled monoclonal rat antibody against the $V_H$17.2.25 idiotype (clone R2.438.8, kind gift of T. Imanishi-Kari, Tufts University, Boston, MA) were detected with a secondary Cy5-conjugated goat-anti-rat IgG serum (Chemicon).

**Flow cytometry**

Single cell suspensions were prepared from bone marrow, spleen and thymus of 6- to 8-week-old mice. Erythrocytes were removed by incubation with 0.15 M $NH_4Cl$, 20 mM HEPES for 5min at RT, and cells were membrane-stained with respective antibodies for 60 min on ice. For intracellular staining, cells were first fixed and permeabilized using the Fix and Perm kit (An der Grub Biotechnologies). Stained cells were examined in a FACSCalibur (BD Biosciences), and data were analyzed with the Cell Quest software (BD Biosciences). Cell sorting was performed on a MoFlo cell sorter (DakoCytomation). Only events falling in the viable lymphocyte gate, as judged by forward/sideward scattering, are shown in histograms and dot plots.

**Quantitative TaqMan PCR (qPCR)**

All qPCRs were performed on Applied Biosystems 7300 or 7500 Real-Time PCR Systems. The amount of Ter5 mRNA was measured in splenocytes from heterozygous $V_H$B1-8, $Ter5^{High}$ (Ter5H) and $Ter5^{Low}$ (Ter5L) mice using primers $V_H$B1-8 fwd (GAG CTG TAT CAT CAT CCT CTT CTT G) or Ter5-VHb1-8 fwd (GAG CTG ACT CAT CAT CCT CTT CTT G) with $V_H$B1-8 rev (CAG GCT GCT GCA GTT GGA) and the fluorescently labeled $V_H$B1-8 probe (6FAM-AGC AAC AGC TAC AGG TGT CCA CTC CCA-TAM). To detect $V_H$1-$D_H$3 recombinations, genomic DNA from FACS-sorted $CD19^+$/$c\text{-}kit^+$ pro-B cells was analyzed using the primers $V_H$1-FR3 fwd (GAG GAC TCT GCR GTC TAT TWC TGT GC (5)) and $J_H$3 rev (CCC TGA CCC AGA CCC ATG T) and the fluorescently labeled $J_H$3 probe (6FAM-TTC AAC CCC TTT GTC CCA AAG TT-TAM). The amount of germline or $DQ_{52}J_H$ rearranged IgH loci was measured on genomic DNA isolated from sorted $CD19^+$/$c\text{-}kit^+$/surface $IgM^-$ pro-B cells using the primers $DQ_{52}$ fwd (CAA GAG ATG ACT GGC AGA TTG G) and $DQ_{52}$ rev (TCA AAA CCT TGC ACC AGT CAG A) and the fluorescently labeled $DQ_{52}$ probe (6FAM-ATA CCC ATA CTC TGT GGC TAG TGT GAG GTT TAA GCC-TAM). These primers amplify a sequence 5′ of the $D_{Q52}$ gene segment, including its 5′ RSS, which

is only present on germline or $D_{Q52}J_H$ rearranged IgH loci, because it is deleted in all other D-to-$J_H$ rearrangements or a $V_H$-to-$D_{Q52}$ rearrangement.

### Quantification of Ter3 H chain-mRNA amounts

To compare the amounts of Ter3 transcripts to that of endogenously encoded sense H chain-transcripts, Ter3 mice were bred to quasi monoclonal (QM) mice carrying a copy of the productive wildtype $V_H$17.2.25 VDJ exon (i.e., without a nonsense codon) homologously targeted into the IgH locus (2). Thereby, abundances of nonsense $V_H$17.2.25-H chain (Ter3) mRNA expressed from the Ter3 transgene and sense $V_H$17.2.25-H chain mRNA from the endogenous IgH locus can be compared. Total RNA was isolated from sorted c-kit$^+$/CD19$^+$ bone marrow and splenic CD19$^+$/$V_H$17.2.25 idiotype$^+$ B cells from IgH$^{QM/wt}$, Ter3$^{Tg}$ mice. Sense and nonsense transcripts were amplified together by RT-PCR with primers binding to the 5' UTR of both sense $V_H$17.2.25-H chain and Ter3 mRNA (5'UTR of 17.2.25 fwd, CTA CAG ACA CTG AAT CTC AAG GTC C) and the Cμ1 region (mCμ1 rev, GAA GGA AAT GGT GCT GGG CAG G). The 567bp PCR product was gel purified, digested with *Fsp*I, which due to the presence of a mutated Ala codon cleaves only the nonsense Ter3 but not sense $V_H$17.2.25-H chain PCR product into a 188bp and a 379bp fragment (Fig. S1), and re-analyzed on an EtBr agarose gel. The relative amount of Ter3 nonsense mRNA was determined by dividing the band intensity of the 567bp product in digested samples (containing only sense $V_H$17.2.25-H chain mRNAs) by that in undigested samples (containing both sense and nonsense $V_H$17.2.25 transcripts). The specificity of the digest was controlled with cDNA from Ag8 hybridoma cells transfected with expression plasmids encoding either the sense (pμGPT) or nonsense form (pμTer3) of $V_H$17.2.25 transcripts.

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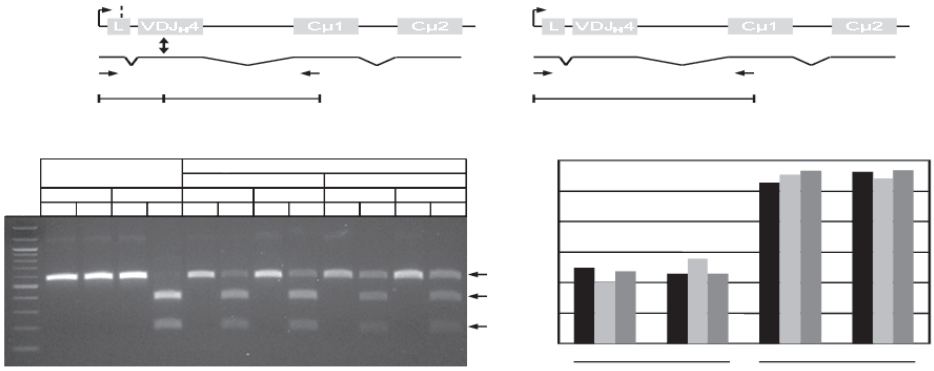
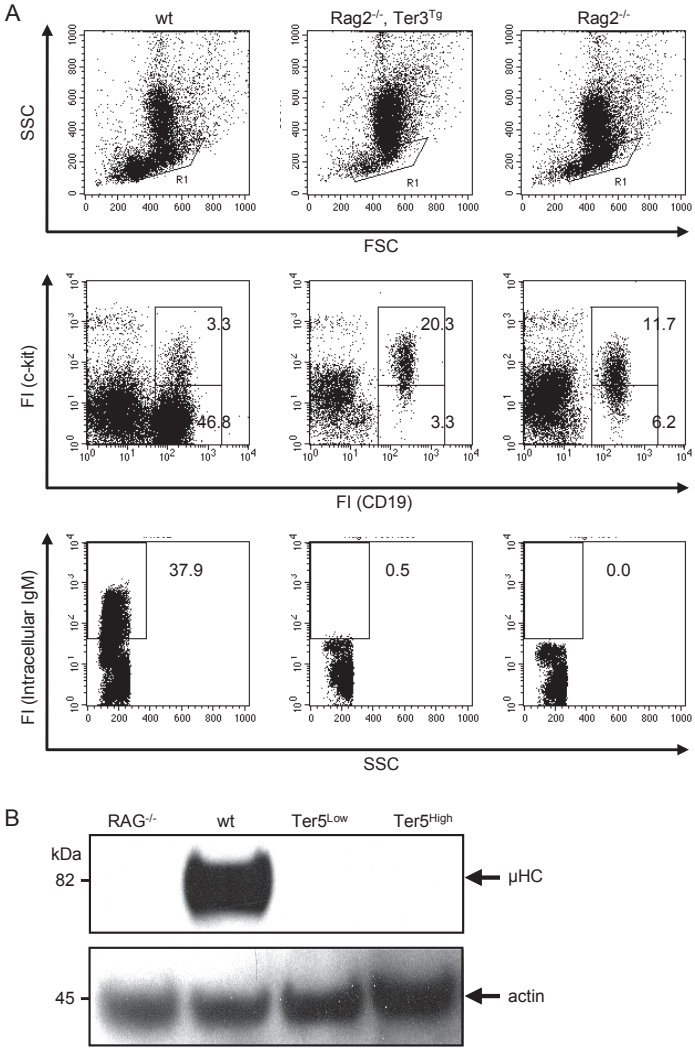
# SUPPLEMENTAL TABLES AND FIGURES

**Supplementary Table 1.** Average cell numbers of B cell populations in the bone marrow of 6-week-old Ter3 mice and littermate controls. Data are from a representative litter (mean ± SD).

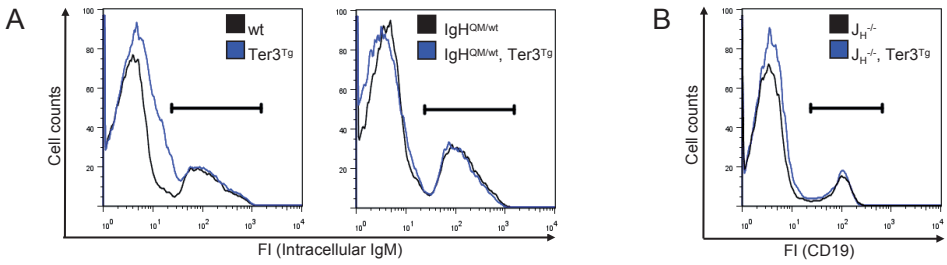
| Cells x$10^5$ | Wildtype | $Ter3^{Tg}$ | $Ter3^{Tg/Tg}$ |
|---|---|---|---|
| c-$kit^+$ | 5.25 (±3.0) | 6.84 (-) | 12.66 (±2.36) |
| $CD25^+$ | 43.85 (±28.27) | 36.32 (-) | 17.27 (±4.66) |
| $\mu HC^+$ | 18.73 (±12.64) | 18.98 (-) | 8.33 (±1.65) |



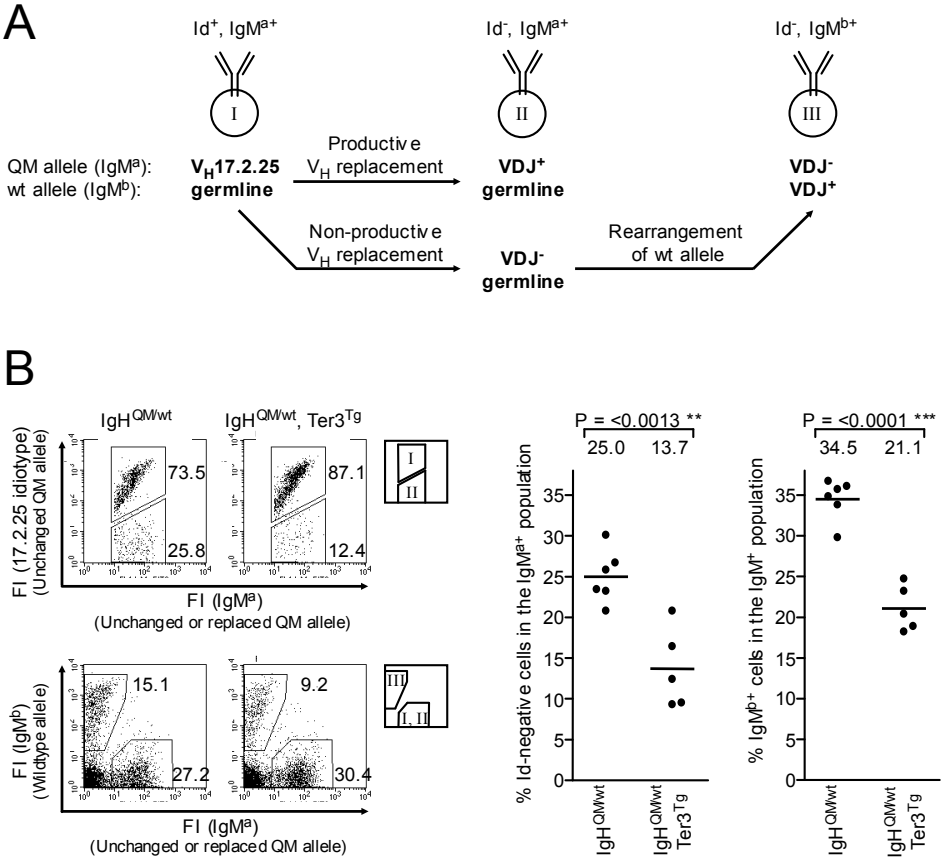

**Supplementary Figure 1**. Comparison of the abundance of nonsense H chain transcripts from the Ter3 transgene ($V_H$17.2.25 VDJ exon with stop = Ter3) with that of sense H chain transcripts from the QM allele (productive $V_H$17.2.25 VDJ targeted into the IgH locus). (**a**) Genomic organization of the wild-type sense and Ter3 nonsense $V_H$17.2.25 VDJ exon in the respective transgenes and pμ expression vectors. The conversion of a downstream ATG into an Ala codon created a FspI restriction site in the nonsense allele, which distinguishes the two $V_H$17.2.25 exons. PCR primer pairs are indicated by arrows. (**b**) RT-PCR analysis of $V_H$17.2.25 mRNA in Ag8 plasmacytoma cells transfected with the sense pμ and nonsense pμTer3 plasmid and in B lymphoid populations isolated from Ter3 transgenic mice heterozygous for the knock-in wild-type $V_H$17.2.25 VDJ exon ($IgH^{QM/wt}$, $Ter3^{Tg}$ mice). RNA was isolated from transfected Ag8 cells as well as sorted c-$kit^+$ pro-B cells and $V_H$17.2.25 $idiotype^+$ splenic B cells. $V_H$17.2.25 H chain mRNA was amplified by RT-PCR with the primers indicated in panel A and purified and electrophoretically separated before (-) or after (+) FspI digest. The ethidium bromide-stained gel was scanned, and ratios of sense $V_H$17.2.25-H chain to total $V_H$17.2.25-H chain mRNA were calculated by dividing the intensities of the bands for the full-length 567-bp product in digested samples (containing only indigestible sense $V_H$17.2.25 H chain mRNA) by that from undigested samples (containing both wild-type and Ter3 H chain mRNA). Results are from three assays, with RNA from two mice (#1 and #2). The analysis revealed a threefold increase in the abundance of Ter3 mRNA in c-$kit^+$ pro-B cells and about equal amounts of both Ter3 and sense $V_H$17.2.25-H chain transcripts in sorted $CD19^+/V_H$17.2.25 $idiotype^+$ splenic B cells.




**Supplementary Figure 2**. Ter3 and Ter5 μ mRNAs are not translated into H chain protein. (**a**) Bone marrow cells of 6-week-old mice were either membrane stained for c-kit and CD19 or fixed and stained for intracellular H chain using a polyclonal goat anti-IgM antibody. Fluorescence intensities (Fl) of cells in the lymphocyte gate were determined. The percentages of cells in the individual gates are indicated. FSC, forward scatter; SSC, side scatter. (**b**) Lysates prepared from bone marrow cells of RAG-deficient ($Rag^{-/-}$), wild-type (wt) and homozygous $Ter5^{Low}$ and $Ter5^{High}$ mice were subjected to Western blot analysis using a polyclonal goat anti-μH chain antibody. Actin signals served as control for the integrity and quantity of loaded protein.




**Supplementary Figure 3**. Ter3 mRNA does not affect expression of IgM and CD19 in Ter3 transgenic mice. (**A**) Bone marrow cells from 6-week-old mice of the indicated genotypes stained for intracellular H chain. (**B**) Bone marrow cells from $J_H$-deficient mice membrane stained for CD19.




**Supplementary Figure 4.** Ter3 transcripts interfere with $V_H$ replacement. (A) The $V_H$ exon of the productive knock-in $V_H$17.2.25-H chain gene (QM allele) can be modified by $V_H$ replacement. As a result, B cells in heterozygous $IgH^{QM/wt}$ mice can express any of three types of H chains: (I) the unchanged $V_H$17.2.25-H chain with $V_H$17.2.25 idiotype ($Id^+$) and $IgM^a$ allotype, (II) an H chain with a productively replaced $V_H$ domain ($Id^-$) and $IgM^a$ allotype, or (III) an H chain with $IgM^b$ allotype encoded by a productively rearranged wild-type (wt) allele in cells with a nonproductive replacement ($VDJ^-$). (B) The effect of the Ter3 transgene on the frequency of $V_H$ replacement was determined in the bone marrow of 6-week-old mice by flow cytometry in two ways. In the upper panel, the frequency of $V_H$17.2.25-$Id^+$ cells expressing the unchanged QM allele was determined within the $IgM^a$-positive population. The lower panel shows the analysis of $IgM^a$-positive cells expressing the QM allele and $IgM^b$-positive cells expressing a productively rearranged wild-type allele. Expression of the Ter3 transgene reduced the frequencies of both idiotype-negative cells in the $IgM^a$-positive B cell population and $IgM^b$-positive cells in the total B cell population. These data suggest a suppressive effect of Ter3 mRNA on VDJ recombination. Results for the entire litter are presented in the diagrams to the right; one dot represents one mouse.