A mutation in a chromosome condensin II subunit, kleisin β, specifically disrupts T cell development

Katharine M. Gosling*, Lydia E. Makaroff*†, Angelo Theodoratos*, Yong-Hee Kim*, Belinda Whittle‡§, Lixin Rui‡¶, Hua Wu‖**, Nancy A. Hong‖, Gavin C. Kennedy††, Julie-Anne Fritz*, Adele L. Yates‡, Christopher C. Goodnow‡§, and Aude M. Fahrer*‡‡

*School of Biochemistry and Molecular Biology, ‡John Curtin School of Medical Research, and §Australian Phenomics Facility, Australian National University, Canberra ACT 0200 Australia; ‖Phenomix Corporation, San Diego, CA 92121; and ††Plant Industries, Commonwealth Scientific and Industrial Research Organisation, Canberra ACT 2601 Australia

Communicated by Gerald R. Crabtree, Stanford University School of Medicine, Stanford, CA, May 31, 2007 (received for review February 20, 2006)

Condensins are ubiquitously expressed multiprotein complexes that are important for chromosome condensation and epigenetic regulation of gene transcription, but whose specific roles in vertebrates are poorly understood. We describe a mouse strain, nessy, isolated during an ethylnitrosourea screen for recessive immunological mutations. The nessy mouse has a defect in T lymphocyte development that decreases circulating T cell numbers, increases their expression of the activation/memory marker CD44, and dramatically decreases the numbers of $CD4^+CD8^+$ thymocytes and their immediate DN4 precursors. A missense mutation in an unusual alternatively spliced first exon of the kleisin β gene, a member of the condensin II complex, was shown to be responsible and act in a T cell-autonomous manner. Despite the ubiquitous expression and role of condensins, kleisin $\beta^{nes/nes}$ mice were viable, fertile, and showed no defects even in the parallel pathway of B cell lymphocyte differentiation. These data define a unique lineage-specific requirement for kleisin β in mammalian T cell differentiation.

Ncaph2 | splice variation

The development of $\alpha\beta$ T cells in the thymus is one of the best studied models of cell development. Cells entering the thymus begin as $CD4^-CD8^-$ double negative (DN) cells, acquire expression of both markers to become double positive (DP) cells, and then lose expression of either CD4 or CD8 to become cytotoxic or helper T cells, respectively (1). Immature DN cells can be further subdivided into DN1–DN4 based on the expression of CD44 and CD25 surface markers (1). Rearrangement of the T cell antigen receptor (TCR) β-chain occurs at the DN3 stage. Successful TCR_β rearrangement allows progression to the DP stage, where α-chain rearrangement occurs, followed by positive and negative selection of the T cells to ensure correct recognition of MHC molecules while preventing inappropriate reactions to self-peptides (2). It is estimated that only 1% of T cells survive TCR rearrangement and selection (3). Defects in this process can lead to immunodeficiency or autoimmunity (4).

Development of T cells is thus an exquisitely controlled process and has been studied extensively to identify the critical molecules involved. Of particular interest is the growing appreciation of the role of epigenetics in T cell development (5). A single transcription factor (such as GATA-3) is often used at different points in the T cell developmental pathway, and in the developmental pathway of other lineages, in each case promoting different cell fates (6). Furthermore, cells need to ''remember'' cell fate decisions (e.g., differentiation to the CD4 lineage) after many rounds of division, which they accomplish by the remodeling of DNA in a form inherited by daughter cells, into regions accessible, or no longer accessible for gene transcription (5). Genes with epigenetic roles and known functions in T cell development include Brg1 and Mi2β, the ATPase subunits of the BAF and NURD chromatin remodeling complexes (7, 8); SATB-1 (9), a protein found to form a cage-like structure within the nucleus, which selectively tethers DNA, affecting transcription; and Dnmt-1, a protein responsible for the propagation of DNA methylation patterns during cell division (10). In addition, the polycomb group proteins Bmi1, M33, and mel18, which maintain silencing of genes by combining with histone deacetylases or blocking nucleosome remodeling, have also been shown to cause defects in thymocyte differentiation (11). In most of these examples, knockout of the protein is embryonic lethal or causes poor viability in mice, and conditional knockouts are used to study the T cell-specific effects of the gene.

Knockout mice have been invaluable in understanding T cell differentiation, with >100 genes shown to be important in this process. However, this technology has the disadvantage of only being applied to genes already suspected to play a role in T cell development. Knocking out a gene of interest to see whether a phenotype is observed is an example of a reverse-genetics approach. Forward-genetics approaches, which start with a phenotype, have the advantage of not presupposing which genes are involved and can therefore identify completely unexpected components in a process.

This study describes the nessy mutant mouse strain, developed as part of a forward-genetics screen for recessive genes involved in T cell production or homeostasis. We identify the causative defect as being in kleisin-β, a member of the condensin II complex. Condensins are involved in the condensation of chromosomes during mitosis, as well as in epigenetic regulation of gene transcription (12). This paper provides a lineage-specific role for a condensin subunit and identifies a previously unsuspected role for kleisin β in the extensively studied process of T cell development.

Results

Generation and Characterization of the Nessy Mutant Strain. Single-nucleotide substitutions were induced in the uniform sequence of C57BL/6 inbred mice by treating males with ethylnitrosourea and breeding them with C57BL/6 females. Male offspring (G1

Author contributions: K.M.G. and L.E.M. contributed equally to this work; K.M.G., L.E.M., L.R., N.A.H., C.C.G., and A.M.F. designed research; K.M.G., L.E.M., A.T., Y.-H.K., B.W., H.W., G.C.K., A.L.Y., and A.M.F. performed research; K.M.G., L.E.M., A.T., Y.-H.K., B.W., H.W., N.A.H., J.-A.F., A.L.Y., C.C.G., and A.M.F. analyzed data; and C.C.G. and A.M.F. wrote the paper.

The authors declare no conflict of interest.

Abbreviations: DN, double negative; DP, double positive; SMC, structural maintenance of chromosome; TCR, T cell antigen receptor.

†Present address: Department of Immunology, University of Washington, Seattle, WA 98195.

¶Present address: Metabolism Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892.

**Present address: Novartis Institutes for Biomedical Research, Cambridge, MA 02139.

‡‡To whom correspondence should be addressed. E-mail: aude.fahrer@anu.edu.au.

This article contains supporting information online at www.pnas.org/cgi/content/full/0704870104/DC1.

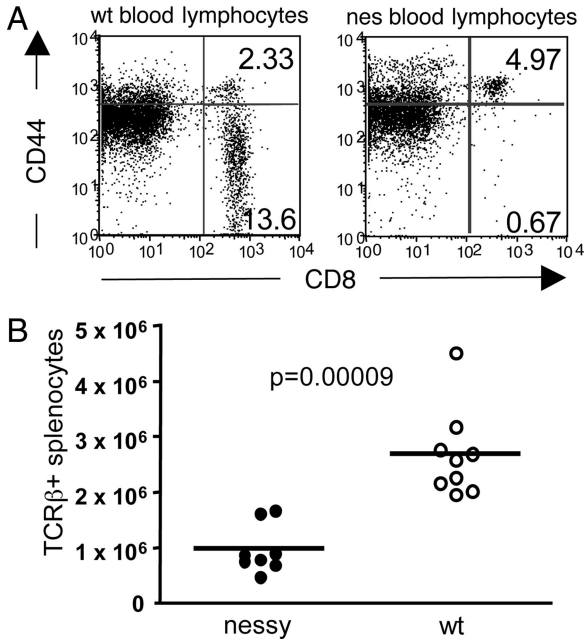

Fig. 1. Blood and spleen phenotype of the nessy mouse. (*A*) The CD44^{high} peripheral blood phenotype, which led to the identification of the nessy strain. Shown is a flow-cytometric analysis of wild-type (wt) and nessy homozygous C57BL/6 mice at 6–8 weeks of age. Dot plots are gated on lymphocytes and are representative of >50 mice. CD44^{low} naive CD8 cells (lower right quadrants) and CD44^{high} activated/memory CD8 T cells (upper right quadrants) are used to identify nessy mice. (*B*) Reduced splenic T cells in nessy mice. Total numbers of T cells from nessy (filled circles) and C57BL/6 (open circles) mice ages 6–12 weeks, determined by α-TCRβ staining. The *P* value was calculated by using a two-tailed Student's *t* test.

mice) were used to found separate inbreeding pedigrees by breeding first with C57BL/6 females and then with their daughters (G2). To identify recessive phenotypes, the third-generation (G3) progeny (homozygous for an estimated 375 sequence variants) were screened by three-color flow cytometry of blood to evaluate expression levels of CD4, CD8, and CD44.

Nessy was initially identified by loss of the main subset of naive CD8 T cells bearing low levels of the T cell activation marker, CD44 (Fig. 1*A*). Heightened mean expression of CD44 on blood CD8 cells was inherited as a recessive Mendelian trait and occurred on CD8 and CD4 T cell subsets in the blood and spleen. Spleen T cell numbers were also decreased in nessy mice by an average of 2.7-fold (Fig. 1*B*).

Further characterization revealed a profound defect in T cell differentiation within the thymus. Nessy thymuses were smaller, with corticomedullary junctions less well defined, and cortical cells sparser than in wild-type mice (Fig. 2*A*). The thymocyte defect was typified by an increased proportion of CD4$^-$CD8$^-$ DN T cell progenitors, with the CD44^{high} phenotype already evident in single-positive thymocytes (Fig. 2*B*). Quantitation of absolute cell numbers revealed a 10-fold reduction in thymocyte numbers, a 5-fold reduction in DN4 cells (CD4$^-$CD8$^-$CD44^{low}CD25^{low}), and a 100-fold reduction in CD4$^+$CD8$^+$ DP cells (Fig. 2*C*). Single-positive cell numbers were also decreased by 50- to 100-fold, reflecting the DP decrease, and suggesting that a substantial recovery in T cell numbers occurs in the periphery (Fig. 1*B*).

Further characterization of thymocytes (Fig. 2*D*) showed increased TCR expression on nessy DP cells (as seen by TCRβ and CD3 staining); increased expression of CD5, an inhibitory receptor whose expression increases in proportion to TCR signal transduction (13); and an increased proportion of DP cells staining with annexin V, consistent with the loss of DP cells through exaggerated apoptosis. IL-7Rα, which is required for thymocyte survival (14), was expressed on nessy thymocytes (Fig. 2*D*), eliminating IL7Rα deficiency as a reason for decreased thymus cellularity.

To see whether a prerearranged TCR could correct the nessy thymic phenotype, nessy mice were crossed to 3A9 TCR transgenic mice (15). As shown in Fig. 2*E*, nessy mice expressing the transgenic TCR (and homozygous for the appropriate H2^k MHC) still show the greatly increased percentage of DN cells and decreased percentage of DP cells typical of nessy (compare with Fig. 2*B*). This finding rules out inefficient TCR rearrangement as the cause of the phenotype.

Culture of bone marrow cells from nessy and wild-type mice on the OP9 delta like 1 cell line allowed differentiation of T cells to the DN4 stage [supporting information (SI) Fig. 6]. Nessy-derived cultures showed the typical percentage increase in DN3 cells. Importantly, no obvious difference in proliferation was seen between nessy and wild-type-derived cultures. To further investigate whether a decrease in thymocyte proliferation was the cause of the nessy phenotype, BrdU incorporation was measured. As shown in Fig. 2*F*, no significant differences were detected in total thymocyte proliferation or in DN3 proliferation. DN4 proliferation was significantly lower in nessy mice (despite a wide spread of values measured in wild-type mice), whereas DP proliferation was significantly increased in nessy mice. Thus, reduced proliferation could potentially account for the decrease in thymocyte numbers at DN4, but not for the most profound decrease in numbers at the DP stage (Fig. 2*C*).

Given the pronounced thymocyte differentiation defect in nessy mice, B cell differentiation was also comprehensively analyzed. As shown in Fig. 3 and SI Fig. 7, no differences were seen between nessy and wild-type B cell subsets in peritoneal exudate cells, spleen cells, or developing B cell populations in the bone marrow. Thus, despite the close parallels between the differentiation of B and T cells, only T cell development is affected in nessy mice. Nessy mice appeared otherwise healthy, and no other defects were observed.

Identification and Verification of the Mutant Gene. The *nessy* mutation was mapped to a 1.2-Mb interval on chromosome 15, which contained no previously known T cell differentiation genes (bases 89241759–90449781, ENSEMBL release 24.33.1). Sequencing of predicted exons within the interval identified a T to A substitution within the first exon of a hypothetical protein-coding gene ENSMUSG00000008690. Three potential mRNA splice variants differing only in exon 1 were identified by aligning sequences from Unigene cluster Mm.143167 (Fig. 4*A*). The *nessy* mutation results in a predicted Ile to Asn substitution at position 15 in the protein encoded by the long mRNA form and no coding change in the short splice product. The intermediate isoform would potentially have a Ser to Thr change at codon 13, assuming translation from a second ATG 8 bases downstream from the first (Fig. 4*A*). Alignment of homologues from other vertebrate species showed conservation of the Ile residue at position 15 in the long form of the gene (Fig. 4*B*).

The nessy Mutation Causes a Cell-Autonomous Disruption of T Cell Differentiation. Bone marrow from B6-*nes*/*nes* mice reproduced the CD44^{high}CD8 T cell phenotype when used to reconstitute the hemopoietic systems of irradiated wild-type B6.SJL-*Ly5*^{a} recipient mice (Fig. 5*A*). When irradiated mice were reconstituted with a mixture of 50% B6-*nes*/*nes* and 50% wild-type B6-*Ly5*^{a} bone marrow, *nes*/*nes*-derived T cells were at a selective disadvantage, accounting for <2.5% of thymocytes and <3.3% of splenic T cells. Nessy-derived T cells from the mixed chimaeras still displayed the characteristic nessy phenotype of high CD44 expression, despite making up such a small percentage of total spleen cells (Fig. 5*A*, two right dot plots). This result indicates that the nessy phenotype is T

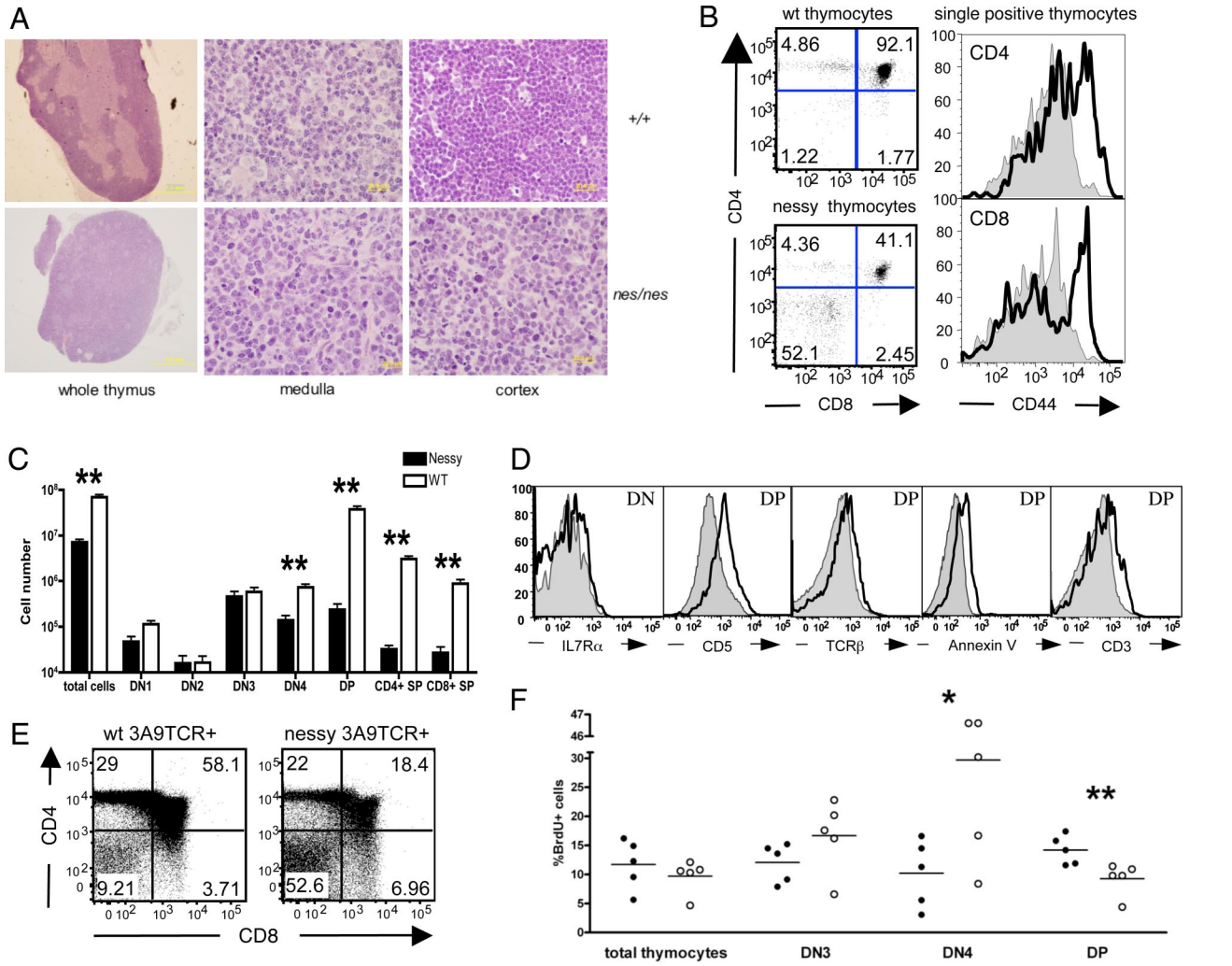

Fig. 2. Defective T cell differentiation in nessy. Histology and flow-cytometric analysis of wild-type (wt) and nessy homozygous C57BL/6 mice at 6–8 weeks of age. Flow-cytometry plots are gated on lymphocytes. (*A*) H&E staining of formalin-fixed thymic sections from wild-type (*Upper*) and nessy (*Lower*) mice. Representative of three mice from each group. (*Left*) Thymus at ×4 magnification. (*Center*) Medulla at ×100 magnification. (*Right*) Cortex at ×100 magnification. (*B*) (*Left*) Major subsets of thymocytes defined by CD4 and CD8 expression. (*Right*) Increased CD44 expression on single-positive thymocytes from nessy mice (black lines) compared with wt mice (filled histograms). (*C*) Cell number (+SEM) in thymocyte subsets, proceeding from the least mature progenitors on the left to the most mature subsets on the right. **, $P < 0.01$ by two-tailed Student's *t* test. DN4, $P = 0.0009$; DP, $P = 0.0001$; CD4 SP, $P = 0.00009$; CD8 SP, $P = 0.001$. Cell subset distribution was determined by flow cytometry with αCD4-FITC, αCD44-TC, αCD25-PE, and αCD8-FITC (for DN subpopulations) or αCD8-PE (for other populations). (*D*) Representative histograms showing binding of αCD5-FITC, Annexin V-FITC, αTCRβ-FITC, αCD3-FITC on $CD4^+CD8+$ DP thymocytes, and αIL-7Rα-FITC on DN thymocytes (because IL-7Rα is not expressed on DP cells). (*E*) Thymocyte profile in 3A9 TCR^+ nes/nes and +/+ (C57BL/6 × B10.Br) mice. Major subsets of thymocytes defined by CD4 and CD8 expression. Cell subset distribution was determined by flow cytometry with αCD4-PECy7, αCD44-TC, αCD25-APC, and αCD8-APCCy7. (*F*) Percentage of cells incorporating BrdU in total thymocytes and in thymocyte subsets from nessy (filled circles) and C57BL/6 (open circles) mice. *, $P < 0.05$; **, $P < 0.01$ by paired two-tailed Student's *t* test. Actual *P* values: total thymocytes $P = 0.12$; DN, $P = 0.14$; DN4, $P = 0.0266$; DP, $P = 0.0075$. Cell subset distribution was determined by flow cytometry with αBrdU-FITC, αCD4-PE-Cy7, αCD8-APC-Cy7, αCD25-APC, and αCD44-PE.

cell-intrinsic. It also demonstrates that the $CD44^{high}$ phenotype is independent of homeostatic proliferation.

Retroviral Rescue of the Nessy Phenotype. To prove that the point mutation identified is the cause of the nessy phenotype, *nes/nes* (*Ly5*^{b}) bone marrow stem cells were transduced with a bicistronic retroviral vector expressing the wild-type long cDNA isoform of ENSMUSG00000008690 and GFP. These cells (comprising both transduced and untransduced populations) were used to reconstitute irradiated B6-*Ly5*^{a} mice. As shown in Fig. 5*B*, retrovirus-transduced splenic *Ly5*$^{b+}$ GFP^+CD8 T cells show a wild-type pattern of CD44 expression, whereas untransduced *Ly5*$^{b+}$$GFP^-$ T cells from the same mice retain the $CD44^{high}$ profile typical of nessy. These experiments prove that the single T to A mutation, resulting in the Ile15Asn substitution, is the cause of the T cell-specific differentiation defect in *nessy* mice.

Identification of the Mutated Gene as Kleisin β, a Component of Condensin II. ENSMUSG00000008690 was annotated as encoding an unknown hypothetical protein in all databases searched, but alignments based on the conserved N- and C-terminal regions led to its identification as encoding kleisin β (16), a subunit of the recently identified condensin II complex (17, 18). Condensins and the closely related cohesins are ubiquitously expressed multiprotein complexes that package chromosomes and hold replicated sister chromatids together between the S and M phases of the cell cycle in eukaryotes (19).

Discussion

The data presented in this study show a completely unexpected, T cell-specific role for kleisin β in T cell development. Kleisin β is a ubiquitous protein known to form part of the condensin II

IMMUNOLOGY

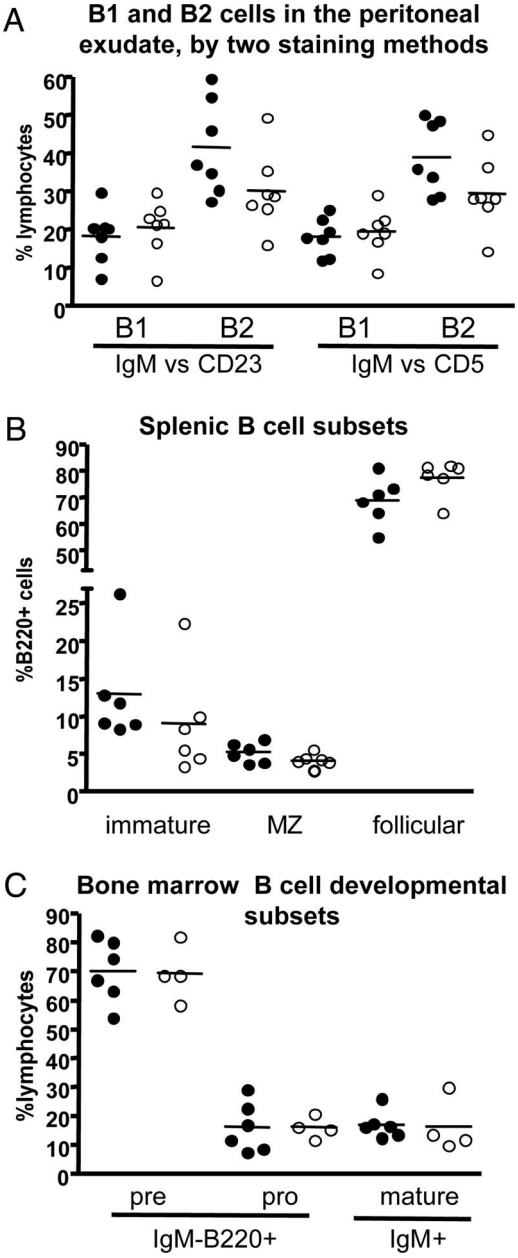

Fig. 3. B cell subsets in the nessy mouse. No significant differences were found between nessy and C57BL/6 mice in any of the subsets examined (*P* values were calculated by using a two-tailed Student's *t* test). Mice were aged between 7 and 14 weeks at the time of death. (*A*) B1 and B2 cells in the peritoneal exudate of nessy (filled circles) and C57BL/6 (open circles) mice. Percentages of B1 and B2 cells were determined by staining with either anti-IgM and anti-CD23 or anti-IgM and anti-CD5 (IgM vs. CD3 B1, $P = 0.58$; B2, $P = 0.09$; IgM vs. CD5 B1, $P = 0.66$; B2, $P = 0.09$). (*B*) Percentages of splenic B cell subsets from nessy (filled circles) and C57BL/6 (open circles) mice determined by staining with anti-CD23 and anti-CD21 to show marginal zone (MZ), follicular, and immature B cells (MZ, $P = 0.14$; follicular, $P = 0.09$; immature, $P = 0.35$). (*C*) Percentages of B cells, pre-B cells, and pro-B cells in the bone marrow of nessy (filled circles) and C57BL/6 (open circles) mice. Percentages were determined by staining with anti-B220, anti-IgM, anti-CD43 (S7), and anti-HSA/CD24 (B cells, $P = 0.88$; pre-B cells, $P = 0.89$; pro-B cells, $P = 0.99$). Representative nessy and C57BL/6 plots for each panel are shown SI Fig. 7.

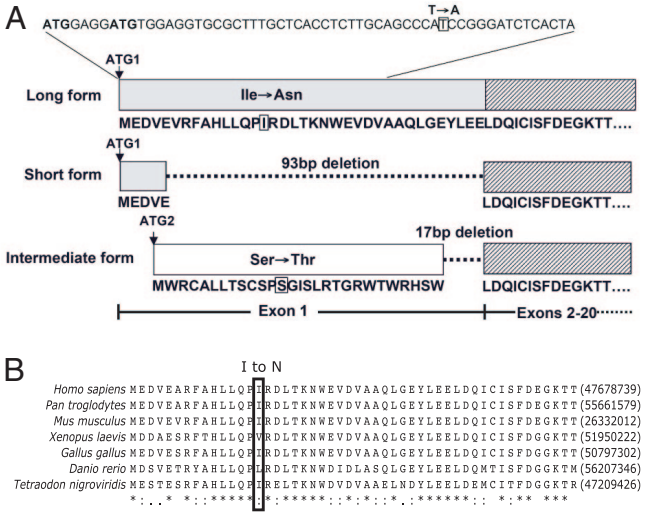

Fig. 4. The nessy point mutation, alternative splicing, and sequence conservation in kleisin β. (*A*) Predicted wild-type and nessy N-terminal sequence of the three hypothetical proteins encoded by alternative exon 1 splicing. The causative mutation and altered amino acids are boxed. (*B*) Clustal W alignment of kleisin β sequences from different organisms, showing sequence conservation of the Ile residue mutated in nessy. Numbers in brackets refer to National Center for Biotechnology Information accession numbers. Asterisks, colons, and periods indicate identity, conservation, or semiconservation of amino acids at each position.

complex. Condensins and cohesins consist of a V-shaped heterodimer of structural maintenance of chromosome (SMC) proteins closed into a ring by a kleisin subunit. The ring is thought to encircle individual or sister chromatids. SMC proteins, which contain ATPase domains, are thought to form molecular motors with the ability to create and change DNA structures (19). Condensins consist of SMC2 and SMC4, closed by kleisin γ (CAP-H) in condensin I and by kleisin β (CAP-H2) in condensin II. The other subunits of these complexes are CAP-D2 and CAP-G in condensin I or CAP-D3 (HCP-6) and CAP-G2 [also called FLJ20311 or more than blood (MTB)] in condensin II (17, 18, 20).

Condensins have been shown to have important roles in chromosome condensation during mitosis (12). Down-regulation of condensin subunits by siRNA in HeLa cells causes defects in chromosome segregation (17, 21–23), with kleisin β down-regulation resulting in >40% of cells failing to align their chromosomes correctly during metaphase (22). Knockouts of SMC2, SMC4, CAP-D2, CAP-G, and kleisin γ have been shown to be lethal in several organisms (19), as has deletion of the condensin II subunit MTB in mice (20).

In addition to roles in chromosome segregation, it has become apparent that condensins also play important roles in the regulation of gene transcription (12). Transcription regulation has been shown by condensin I in yeast, *Caenorhabditis elegans*, and *Drosophila* (12). Transcriptional regulation was found to be mediated through interaction of condensin with a histone deacetylase in yeast (*24*) and with a polycomb group protein in *Drosophila* (25), indicating an epigenetic mechanism. In human cell lines, CAP-G (condensin I) was found to coimmunoprecipitate with the DNA methylase DNMT3B, which also immunoprecipitates SNF2H, a chromatin-remodeling enzyme (*26*).

Evidence is also now accumulating for a role for condensin II in epigenetic regulation. The condensin II subunit CAP-G2 has been shown to bind the chromokinesin KIF4A (27), which, in a separate study, has been shown to coimmunoprecipitate DNMT3B, which in turn coimmunoprecitates the chromatin remodeler SNF2H and the histone deacetylase HDAC-1, as well as SMC2 and SMC4 (26). Most recently, in an important study, the mouse CAP-G2 subunit has been directly shown to mediate transcriptional repression, with histone deacetylase recruitment implicated as the likely mechanism (28). Thus, the condensins,

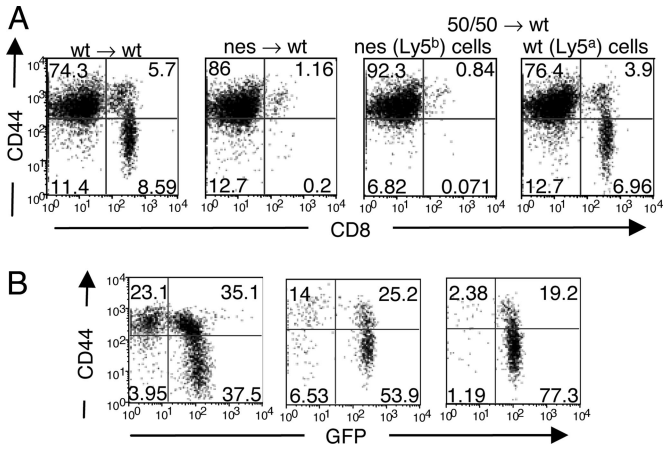

Fig. 5. The nessy phenotype is due to a T cell-intrinsic defect and can be rescued by expression of the wild-type kleisin β sequence. (*A*) The hemopoietic systems of lethally irradiated B6.SJL.*Ly5*^{a} mice were reconstituted with bone marrow cells from a wild-type or *nes*/*nes* C57BL/6 (*Ly5*^{b}) mouse or a 50/50 mixture of bone marrow from *nes*/*nes* C57BL/6 (*Ly5*^{b}) and wild-type B6.SJL.*Ly5*^{a} mice. The left three dot plots are gated on *Ly5*^{b} spleen cells, showing the subsets of CD44^{high} and CD44^{low} CD8 cells. The rightmost dot plot is gated on *Ly5*^{a} spleen cells in the 50/50 chimeras, showing normal CD8 subsets derived from wt precursors, which contrast with the absence of CD44^{low} CD8 cells from *nes*/*nes* precursors. (*B*) Hemopoietic chimeras reconstituted with B6 *nes*/*nes* marrow as in *A*, except that the hemopoietic stem cells were first transduced *in vitro* with a bicistronic retrovirus expressing the wild-type long cDNA from ENSMUSG00000008690 and GFP. Profiles are gated on *Ly5*$^{b+}$ CD8$^+$ *nes*/*nes* splenic lymphocytes and display CD44^{low} and CD44^{high} subsets of CD8 cells differentiated from transduced (GFP$^+$) and nontransduced (GFP$^-$) *nes*/*nes* stem cells. The dot plots show data from three independently reconstituted mice in two separate transduction experiments. Note that the GFP$^-$ cells in the left-hand quadrants of each plot show the typical CD44^{high} nessy profile and serve as internal controls in each case.

which are heteropentameric complexes, appear to form part of even larger complexes, including DNA methylases, histone deacetylases, and polycomb group proteins.

The most likely explanations for the role of kleisin β in the nessy phenotype are therefore a defect in either cell proliferation or epigenetic regulation of gene transcription. Kleisin β is present in all cell types and is required for cell division. One possibility is that the kleisin β mutation is a subtle hypomorphic allele affecting chromosome condensation and mitosis and only becomes rate limiting during the rapid burst of proliferation occurring during T cell development. Although it is true that T cell development entails a substantial amount of cell division, equivalent or higher rates would be required for B cell and embryonic development. The fact that the nessy mouse is viable, fertile, and of normal appearance, and that B cell development is normal (Fig. 3), therefore precludes a generalized defect of the nessy mutation in cell division. These data also argue against a related possibility: that DNA damage, acting through p53, could be responsible for the T cell developmental defect. p53 is known to be important in thymocyte development, partially rescuing development of RAG$^{-/-}$ thymocytes by allowing their progression to the DP stage (29). p53 also has a similar rescuing effect in B cell differentiation in SCID mice (30). Therefore, the normal nessy B cell development (Fig. 3), as well as the failure of a TCR transgene (which represses endogenous TCRβ rearrangement) to rescue the nessy phenotype (Fig. 2*E*), both argue against DNA damage as the cause of the phenotype.

It remains possible, however, that kleisin β has an additional, thymocyte-specific role in cell division. Thymocyte BrdU incorporation was measured to investigate this possibility (Fig. 2*F*). DNA replication in nessy mice was unchanged at DN3, was decreased at DN4, but increased at DP, whereas cell numbers drop at DN4 (to 1/5 of wild type) and drop again substantially at DP (to 1/100 of wt; Fig. 2*C*). Thus, decreased proliferation may contribute to, but is not sufficient to explain, the phenotype (in particular, the sharp reduction in DP cell numbers). The differences in proliferation that are seen in nessy thymocytes could be due to a direct effect of the kleisin β mutation on proliferation, but could also be due to an indirect effect, secondary, for example, to an epigenetic effect or an effect on TCR signaling.

Characterization of the thymic phenotype in the nessy mouse (Fig. 2*D*) suggests that increased TCR signaling may well contribute to the decreased thymic cellularity. Higher expression of TCRβ, CD3, and CD5 on DP cells suggests increased TCR signaling. The increase in annexin V binding (Fig. 2*D*), together with the reduced DP cell numbers (Fig. 2*C*), is also consistent with more signaling through the TCR, leading to increased negative selection. Furthermore, the high expression of CD5 and CD44 on *nes*/*nes* DP and mature T cells, respectively, resembles mouse mutants with exaggerated TCR signaling due to defects in casitas B-lineage lymphoma (cbl) (31) and src-like adaptor protein (32), and double defects in Cbl and Cblb (33). Given the known roles of condensins in epigenetic regulation, altered transcriptional regulation is an attractive hypothesis to explain the apparent increase in TCR signaling. This result would fit with the known roles for epigenetic regulation in thymocyte differentiation detailed earlier. It is true that the nessy phenotype does not resemble either the thymic developmental defects in the SATB1$^{-/-}$ mice (reduced DN cells and a partial block at the DP stage) (34) or the Brg1 conditional knockout mice (an essentially complete block at the DN stage) (35, 36). However, it is unlikely that all epigenetic modifiers will target the same subset of genes or that all mutations in epigenetic modifiers will be phenotypically identical. One hypothesis, then, is that the nessy phenotype is caused by a separation of function allele of kleisin β, which affects its epigenetic function, but not its role in mitosis, reminiscent of the separation of function mutations reported in the *Bacillus subtilis* cohesin/condensin kleisin, ScpA, which affect gene regulation, but not chromosome condensation or segregation (37). The T cell-specific effect of the phenotype in this scheme could be explained by the necessary association of chromatin remodeling complexes with cell type-specific subunits, as has been seen for BAF (7) and polycomb group-silencing proteins (11).

The point mutation we have identified in the nessy mouse causes a separation of function mutation in kleisin β, which leaves its general role in cell division intact, but causes a specific defect in T cell development. The two known roles for condensin II are in cell division and epigenetic regulation. Our data suggest that differences in cell division are not sufficient to explain the phenotype of the nessy mouse. Our study demonstrates the value of ethylnitrosourea mutagenesis in identifying novel proteins involved in complex traits. In addition to not presupposing which genes are important, the genetic variation produced recapitulates natural variation. These point mutations can yield separation of function alleles, illuminating cell type-specific functions while preserving ubiquitous functions. Similar findings are unlikely to be achieved by reverse-genetic studies. In this paper, we demonstrate a lineage-specific role for kleisin β in T cell development. This finding provides a link between the rapidly developing fields of T cell development and chromosome structure.

Materials and Methods

Development of Mouse Strains. Male C57BL/6 mice were treated with ethylnitrosourea as previously described (38). Mice were screened by flow cytometry of blood for CD4, CD8, and CD44. Outlying mice or their littermates were bred to confirm heritability of the trait and to establish each strain. All work was

approved by the Australian National University Animal Ethics committee and carried out in accordance with institutional guidelines.

Mapping. Mutations were mapped by using NOD.H2k as the outcross strain as previously described (38) and identified by sequencing transcripts or predicted ORFs.

Flow Cytometry. Single-cell suspensions were stained with fluorochrome-conjugated antibodies (obtained from BD PharMingen, San Diego, CA). Samples were collected on FACSort or LSRII flow cytometers (Becton Dickinson, San Jose, CA). Data were analyzed by using FloJo software (Treestar, Ashland, OR).

Histology. Thymuses were fixed in neutral buffered formalin, embedded in paraffin, sectioned, and stained with H&E for histological examination.

***In Vitro* T Cell Differentiation.** OP9 *in vitro* T cell differentiation was performed essentially as described (39). Bone marrow cells were isolated from nessy and C57BL/6 mice, and 1×10^5 cells per well of a six-well plate were seeded onto semiconfluent OP9-δ stromal cells. The cells were passaged at least every 8 days, and 1×10^5 or 2×10^5 cells were placed onto new OP9-δ stromal cells after each passage. The remaining cells were analyzed by flow cytometry.

BrdU Incorporation. Mice were injected i.p. twice with 1 mg of BrdU (Roche Diagnostics, Indianapolis, IN) at 30-min intervals and killed 6 h after the second injection. Surface-stained thymocytes were fixed and permeabilized in PBS containing 2% paraformaldehyde plus 0.1% saponin for 30 min on ice, washed, and fixed for a further 5min. Fixed cells were incubated with 50 units of DNase I (Ambion, Austin, TX) for 1 h at 37°C, and BrdU incorporation was detected by using FITC-conjugated anti-BrdU antibody (clone 3D4; BD PharMingen).

Bone Marrow Chimaeras and Retroviral Rescue. Bone marrow chimaeras were performed as previously described (38). Mice were injected with 1.5×10^6 to 2×10^6 bone marrow cells i.v. and analyzed 3–5 months after reconstitution. The MSCV-LTR transfection method has previously been described (40). Kleisin β was amplified from wild-type cDNA using forward primer T (CGA *GTC GAC* GTT CTA GAC ATG GAG GAT GTG GAG) and reverse primer C (ATC *GTC GAC* CTC CAG GTA TAT ACT ACT TTC CA). Engineered SalI restriction sites are italicized. Mice were analyzed 6 weeks after reconstitution.

We thank the staff of the Medical Genome Centre for expert care of mice and Drs. Adrian Liston and Stefan Bröer for critical reading of the manuscript. This work was supported by grants from the Australian National Health and Medical Research Council and the Cancer Council ACT (to A.M.F.) and by grants from the National Institutes of Health and Welcome Trust (to C.C.G.).

1. Rothenberg EV, Dionne CJ (2002) *Immunol Rev* 187:96–115.
2. von Boehmer H, Aifantis I, Gounari F, Azogui O, Haughn L, Apostolou I, Jaeckel E, Grassi F, Klein L (2003) *Immunol Rev* 191:62–78.
3. Egerton M, Scollay R, Shortman K (1990) *Proc Natl Acad Sci USA* 87:2579–2582.
4. Cunningham-Rundles C, Ponda PP (2005) *Nat Rev Immunol* 5:880–892.
5. Su RC, Sridharan R, Smale ST (2005) *Semin Immunol* 17:129–140.
6. Rothenberg EV, Taghon T (2005) *Annu Rev Immunol* 23:601–649.
7. Chi T (2004) *Nat Rev Immunol* 4:965–977.
8. Williams CJ, Naito T, Arco PG, Seavitt JR, Cashman SM, De Souza B, Qi X, Keables P, Von Andrian UH, Georgopoulos K (2004) *Immunity* 20:719–733.
9. Cai S, Han HJ, Kohwi-Shigematsu T (2003) *Nat Genet* 34:42–51.
10. Lee PP, Fitzpatrick DR, Beard C, Jessup HK, Lehar S, Makar KW, Perez-Melgosa M, Sweetser MT, Schlissel MS, Nguyen S, *et al*. (2001) *Immunity* 15:763–774.
11. Raaphorst FM, Otte AP, Meijer CJ (2001) *Trends Immunol* 22:682–690.
12. Hirano T (2005) *Curr Biol* 15:R265–R275.
13. Azzam HS, Grinberg A, Lui K, Shen H, Shores EW, Love PE (1998) *J Exp Med* 188:2301–2311.
14. Fry TJ, Mackall CL (2005) *J Immunol* 174:6571–6576.
15. Ho WY, Cooke MP, Goodnow CC, Davis MM (1994) *J Exp Med* 179:1539–1549.
16. Schleiffer A, Kaitna S, Maurer-Stroh S, Glotzer M, Nasmyth K, Eisenhaber F (2003) *Mol Cell* 11:571–575.
17. Ono T, Losada A, Hirano M, Myers MP, Neuwald AF, Hirano T (2003) *Cell* 115:109–121.
18. Yeong FM, Hombauer H, Wendt KS, Hirota T, Mudrak I, Mechtler K, Loregger T, Marchler-Bauer A, Tanaka K, Peters JM, Ogris E (2003) *Curr Biol* 13:2058–2064.
19. Jessberger R (2002) *Nat Rev Mol Cell Biol* 3:767–778.
20. Smith ED, Xu Y, Tomson BN, Leung CG, Fujiwara Y, Orkin SH, Crispino JD (2004) *Mol Cell Biol* 24:1168–1173.
21. Hirota T, Gerlich D, Koch B, Ellenberg J, Peters JM (2004) *J Cell Sci* 117:6435–6445.
22. Ono T, Fang Y, Spector DL, Hirano T (2004) *Mol Biol Cell* 15:3296–3308.
23. Watrin E, Legagneux V (2005) *Mol Cell Biol* 25:740–750.
24. Machin F, Paschos K, Jarmuz A, Torres-Rosell J, Pade C, Aragon L (2004) *Curr Biol* 14:125–130.
25. Lupo R, Breiling A, Bianchi ME, Orlando V (2001) *Mol Cell* 7:127–136.
26. Geiman TM, Sankpal UT, Robertson AK, Chen Y, Mazumdar M, Heale JT, Schmiesing JA, Kim W, Yokomori K, Zhao Y, Robertson KD (2004) *Nucleic Acids Res* 32:2716–2729.
27. Mazumdar M, Sundareshan S, Misteli T (2004) *J Cell Biol* 166:613–620.
28. Xu Y, Leung CG, Lee DC, Kennedy BK, Crispino JD (2006) *Leukemia* 20:1261–1269.
29. Jiang D, Lenardo MJ, Zuniga-Pflucker JC (1996) *J Exp Med* 183:1923–1928.
30. Guidos CJ, Williams CJ, Grandal I, Knowles G, Huang MT, Danska JS (1996) *Genes Dev* 10:2038–2054.
31. Naramura M, Kole HK, Hu RJ, Gu H (1998) *Proc Natl Acad Sci USA* 95:15547–15552.
32. Sosinowski T, Killeen N, Weiss A (2001) *Immunity* 15:457–466.
33. Naramura M, Jang IK, Kole H, Huang F, Haines D, Gu H (2002) *Nat Immunol* 3:1192–1199.
34. Alvarez JD, Yasui DH, Niida H, Joh T, Loh DY, Kohwi-Shigematsu T (2000) *Genes Dev* 14:521–535.
35. Chi TH, Wan M, Lee PP, Akashi K, Metzger D, Chambon P, Wilson CB, Crabtree GR (2003) *Immunity* 19:169–182.
36. Gebuhr TC, Kovalev GI, Bultman S, Godfrey V, Su L, Magnuson T (2003) *J Exp Med* 198:1937–1949.
37. Dervyn E, Noirot-Gros MF, Mervelet P, McGovern S, Ehrlich SD, Polard P, Noirot P (2004) *Mol Microbiol* 51:1629–1640.
38. Miosge LA, Blasioli J, Blery M, Goodnow CC (2002) *J Exp Med* 196:1113–1119.
39. Cho SK, Zuniga-Pflucker JC (2003) *Meth Enzymol* 365:158–169.
40. Vinuesa CG, Cook MC, Angelucci C, Athanasopoulos V, Rui L, Hill KM, Yu D, Domaschenz H, Whittle B, Lambe T, *et al*. (2005) *Nature* 435:452–458.