

Tissue Hyperplasia and Enhanced T-Cell Signalling via ZAP-70 in c-Cbl-Deficient Mice

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The c-Cbl protein is tyrosine phosphorylated and forms complexes with a wide range of signalling partners in response to various growth factors. How c-Cbl interacts with proteins, such as Grb2, phosphatidylinositol 3-kinase, and phosphorylated receptors, is well understood, but its role in these complexes is unclear. Recently, the *Caenorhabditis elegans* Cbl homolog, Sli-1, was shown to act as a negative regulator of epidermal growth factor receptor signalling. This finding forced a reassessment of the role of Cbl proteins and highlighted the desirability of testing genetically whether c-Cbl acts as a negative regulator of mammalian signalling. Here we investigate the role of c-Cbl in development and homeostasis in mice by targeted disruption of the c-Cbl locus. c-Cbl-deficient mice were viable, fertile, and outwardly normal in appearance. Bone development and remodeling also appeared normal in c-Cbl mutants, despite a previously reported requirement for c-Cbl in osteoclast function. However, consistent with a high level of expression of c-Cbl in the hemopoietic compartment, c-Cbl-deficient mice displayed marked changes in their hemopoietic profiles, including altered T-cell receptor expression, lymphoid hyperplasia, and primary splenic extramedullary hemopoiesis. The mammary fat pads of mutant female mice also showed increased ductal density and branching compared to those of their wild-type littermates, indicating an unanticipated role for c-Cbl in regulating mammary growth. Collectively, the hyperplastic histological changes seen in c-Cbl mutant mice are indicative of a normal role for c-Cbl in negatively regulating signalling events that control cell growth. Consistent with this view, we observed greatly increased intracellular protein tyrosine phosphorylation in thymocytes following CD3 ϵ cross-linking. In particular, phosphorylation of ZAP-70 kinase in thymocytes was uncoupled from a requirement for CD4-mediated Lck activation. This study provides the first biochemical characterization of any organism that is deficient in a member of this unique protein family. Our findings demonstrate critical roles for c-Cbl in hemopoiesis and in controlling cellular proliferation and signalling by the Syk/ZAP-70 family of protein kinases.

The c-Cbl protooncogene is the cellular homolog of the acutely transforming v-Cbl oncogene, which was originally identified in the murine Cas NS-1 retrovirus (27). v-Cbl induces pre-B-cell and myeloid tumors in mice, transforms rodent fibroblast cell lines, and encodes a protein of 357 amino acids which encompasses the amino-terminal region of c-Cbl (2). Recently the 913-amino-acid product of c-Cbl has been identified as a ubiquitous substrate of protein tyrosine kinases that is rapidly phosphorylated following stimulation of growth factor, antigen, and integrin receptors (4, 11, 13, 17, 25, 33, 36–38, 40, 52, 60). c-Cbl protein has no known catalytic function but contains a novel phosphotyrosine binding (PTB) domain, a RING finger domain, an extensive proline-rich region, and a carboxy-terminal leucine zipper. To date four Cbl proteins have been described: c-Cbl and Cbl-b in mammals, *Drosophila* Cbl, and *Caenorhabditis elegans* Cbl (known as Sli-1) (2, 19, 21, 34, 64). The partial sequence of a third human Cbl homolog has recently been lodged in the National Institutes of Health expressed sequence tag (EST) database (AA112513). All Cbl proteins are highly conserved in their amino-terminal

PTB and RING finger domains but are divergent at their carboxy termini. The carboxy-terminal half of c-Cbl contains proline-rich SH3-binding domains, three major sites of tyrosine phosphorylation for SH2 domain interactions, and the binding site for interactions with 14-3-3 proteins (30).

While the multiple complexes that c-Cbl is found in suggest diverse roles in signal transduction, a definitive function for c-Cbl has not emerged. The most revealing clue about the function of Cbl proteins came originally from genetic studies in *C. elegans*, where Sli-1 was identified as a negative regulator of the epidermal growth factor (EGF) receptor tyrosine kinase (Let-23) (20). These experiments demonstrated that Sli-1 acts at the level of Let-23 and the *C. elegans* Grb2 homolog (Sem5), consistent with mammalian studies that place Cbl at an early point in tyrosine kinase-mediated signal transduction (64). Recent studies have provided initial evidence that mammalian c-Cbl may also negatively regulate the activity of cytoplasmic protein tyrosine kinases. Overexpression of c-Cbl in mast cells suppresses Syk kinase activity (39) and reduces Ras-dependent AP1 activation following T-cell ligation in Jurkat cells (43). In addition, treatment of cells with antisense c-Cbl can enhance the activation of the JAK-STAT pathway (56). It is unclear whether these effects on kinase activity are direct. Interestingly, c-Cbl has been shown to be transiently ubiquitinated in colony-stimulating factor 1 (CSF-1) stimulated macrophages, leading to the suggestion that c-Cbl may negatively regulate

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signalling in these cells by participating in ubiquitin-mediated degradation of the CSF-1 receptor (60).

Studies of oncogenic forms of c-Cbl have also provided clues to Cbl function. c-Cbl can be rendered oncogenic either by a large carboxy-terminal truncation (v-Cbl) or by a small internal deletion immediately amino terminal to the RING finger (70Z-Cbl) (1, 2, 27). Studies of v-Cbl demonstrated that the PTB domain forms a direct association with the EGF receptor and the ZAP-70 tyrosine kinase (4, 17, 31, 53). These findings indicate that v-Cbl may compete with c-Cbl for binding sites on activated receptor complexes, thereby blocking regulation by c-Cbl and resulting in transformation. In contrast, transformation by 70Z-Cbl appears to involve a positive signalling mechanism. Expression of 70Z-Cbl causes hyperphosphorylation of a range of substrates by enhancing the kinase activity of the platelet-derived growth factor and EGF receptors (3, 54). These studies indicate that 70Z-Cbl has lost its ability to function as a negative regulator but still provides docking sites for complexes that mediate growth-stimulatory signals. This notion is supported by the finding that 70Z-Cbl, but not c-Cbl or v-Cbl, induces transcriptional activation of the nuclear factor of activated T cells (NFAT) (29). 70Z-Cbl-mediated NFAT activation was markedly enhanced by stimulation with calcium ionophore and abrogated by the expression of a dominant-negative Ras, implicating 70Z-Cbl in the Ras signalling pathway. Reduced activation of Erk2 and decreased stimulation of a Ras-sensitive AP1 reporter in c-Cbl-overexpressing Jurkat cells (43) is also consistent with a role for c-Cbl in T-cell receptor (TCR)-mediated Ras activation.

These studies have led to the realization that c-Cbl may function as a negative regulator of protein tyrosine kinases and also to provide docking sites for a multitude of signalling proteins. To study these proposed roles in detail, we have generated mice that are deficient in c-Cbl. The results obtained with these animals provide compelling evidence that mammalian c-Cbl is involved in the control of tissue cellularity and that it functions as a negative regulator of protein tyrosine kinases.

MATERIALS AND METHODS

c-Cbl targeting vector and isolation of targeted ES cells and mice. Murine c-Cbl genomic clones were isolated from a λ UNI-ZAP 129Sv library (Stratagene) by hybridization with a 32 P-labelled murine c-Cbl cDNA, and the positions of the coding exons were determined by standard procedures (44). A 7-kb *Xba*I fragment was subcloned into the *Xba*I site of pBSII (Stratagene), and from this an *Eco*RI-*Hind*III fragment that contained a c-Cbl coding exon, beginning at amino acid codon 146, was subcloned into pALTER (Promega). A *Cl*aI site was introduced at amino acid codon 153 by site-directed mutagenesis, converting the sequence CAAATTATCCCTGATCTT to CAAATTATCGAGGATCTT. This fragment was returned to the 7-kb *Xba*I fragment, and a 1.8-kb pGKNeo-selectable marker was inserted between the *Cl*aI and *Hind*III sites, removing amino acid codons 153 to 190 and creating an in-frame stop codon within the pGK promoter. The construct was linearized with *Xho*I and electroporated into W9.5 embryonic stem (ES) cells (26). Targeted ES cells were selected in G418 at 200 μ g/ml. DNA was obtained for Southern analysis of these clones by using proteinase K and sodium dodecyl sulfate (SDS) and was restricted with *Bam*HI. To identify targeted clones, filters were probed with a 32 P-labelled genomic fragment outside the targeting construct (Fig. 1A). As predicted, targeted clones gave a new band of 4.2 kb. Fifteen clones were obtained at a frequency of 1 clone per 27 Neo^r ES cell clones.

Four G418-resistant clones that had only single integration events, as evidenced by probing a Southern blot with neomycin-specific probe, were used to generate chimeric mice by microinjection of embryonic day 3.5 C57BL/6J blastocysts. Male chimeras were mated to C57BL/6J females, and germ line transmission of the ES cell genome was detected by coat color. Southern blot analysis of the tail DNA of pups was performed by standard procedures (44), and approximately half of the agouti pups were heterozygous for the c-Cbl mutation. C57BL/6J and 129Sv mice were obtained from the Animal Resource Center, Murdoch, Western Australia. Western blot analysis of tissues from homozygous mutant mice was performed essentially as described previously (59) but with an antiserum directed to a C-terminal c-Cbl peptide, C15 (Santa Cruz).

Thymocyte stimulation by antibody cross-linking. Single-cell suspensions of thymocytes were prepared at 5×10^7 /ml in RPMI 1640 supplemented with 5%

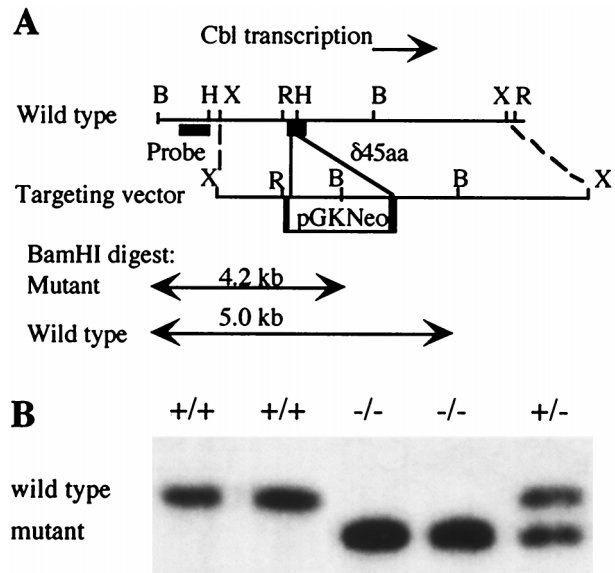


FIG. 1. Targeted disruption of the c-Cbl locus. (A) Mutation strategy in which c-Cbl coding sequences were replaced by a neomycin resistance cassette (pGKneo). Predicted sizes are shown for the wild-type and mutant alleles following *Bam*HI digestion of genomic DNA and probing with the indicated fragment outside the targeting construct. B, *Bam*HI; H, *Hind*III; X, *Xba*I; R, *Eco*RI. (B) Southern blot analysis of tail DNA from progeny of an intercross of heterozygous mice, restricted with *Bam*HI and probed with the fragment indicated in A. Mice bearing the mutant allele showed the predicted restriction fragment.

fetal calf serum (FCS) (RPMI-5% FCS). Hamster anti-CD3 ϵ (500A2) or biotinylated anti-CD3 ϵ (500A2) and anti-CD4 (L3T4) antibodies were added to the cells at 10 μ g/ml, and the cells were incubated on ice for 10 min and washed once in RPMI-5% FCS. Cross-linking was carried out by addition of 12.5 μ g of anti-hamster immunoglobulin G (IgG) antibodies/ml in RPMI-5% FCS or 40 μ g of streptavidin/ml in RPMI. The cells were incubated on ice for 10 min before stimulation at 37°C for 5 min or various times as indicated. All antibodies were obtained from PharMingen.

Immunoprecipitations and immunoblotting. Following stimulation, the thymocytes were washed once in ice-cold phosphate-buffered saline and then lysed at 3×10^7 to 5×10^7 cells/ml in ice-cold Nonidet P-40 (NP-40) lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 2 mM EDTA, 1 mM sodium orthovanadate, 1% NP-40) supplemented with 10 μ g of aprotinin/ml, 10 mM NaF, and 1 μ g (each) of chymostatin, leupeptin, and pepstatin/ml. After being incubated for 10 min on ice, the lysates were cleared by centrifugation at $14,000 \times g$ for 8 min. The cleared lysates (0.5-ml aliquots) were then analyzed by immunoprecipitation and immunoblotting as previously described (4). Anti-Lck antibodies were purchased from Zymed, anti-SLP-76 and anti-Fyn antibodies were purchased from Santa Cruz, anti-ZAP-70 antibodies were purchased from Transduction Laboratories and L. Samelson, and anti-phosphotyrosine (4G10) antibodies were purchased from B. Druker.

Immune complex kinase assays. Anti-Lck immunoprecipitates from unstimulated or anti-CD3- and anti-CD3-CD4-stimulated thymocytes were washed three times in NP-40 lysis buffer and then washed once in kinase buffer (20 mM MOPS [morpholinepropanesulfonic acid] buffer [pH 7.0], 5 mM MgCl₂, 5 mM MnCl₂). Immunoprecipitates were then incubated with 25 μ l of kinase buffer containing 12.5 μ Ci of [γ - 32 P]ATP (4,000 Ci/mmol; Bresatec) for 10 min at room temperature with occasional mixing. The kinase reaction was stopped by addition of 1 ml of ice-cold modified RIPA buffer (20 mM MOPS [pH 7.0], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS), the mixture was centrifuged briefly, and the supernatant containing unincorporated radioisotope was discarded. Immunoprecipitates were washed a further three times in RIPA buffer, by which time minimal radioactivity was detected in the discarded supernatant. Samples were resuspended in 30 μ l of 1 \times Laemmli sample buffer, incubated at room temperature for 10 min, and then boiled for 3 min, and the supernatant was transferred to a fresh tube. The samples were separated by electrophoresis through a SDS-10% polyacrylamide gel, dried at 80°C under vacuum for 30 min, and analyzed by autoradiography after a 30-min exposure.

Thymocyte proliferation assay. Thymocytes (4×10^5) from normal or c-Cbl-deficient mice were cultured in 200 μ l of RPMI-10% FCS with plate-bound anti-TCR $\alpha\beta$ (H57-597) antibodies for up to 3 days in round-bottom 96-well culture dishes (Nunc). Eight hours before being harvested, the cells were pulsed with 0.6 μ Ci of [3 H]thymidine and the radioactivity of individual filters was measured by liquid scintillation counting. All cultures were in triplicate, and the

TABLE 1. Numbers of offspring of *c-Cbl*^{-/-} mice and control animals^a

Genotype	Total no.	Ratio	
		Expected	Actual
+/+	63	1	1.05
+/-	121	2	2.01
-/-	56	1	0.94

^a From heterozygous intercross.

results are presented as mean counts per minute \pm standard error of the mean from thymuses of 10 wild-type and 10 *c-Cbl*^{-/-} mice.

Assay of myeloid progenitors. Bone marrow and spleen cell suspensions (2,500 and 10,000 cells per dish, respectively) were assayed for the presence of macrophage lineage progenitor cells in a double-layer nutrient agar culture system exactly as previously described (5). A predetermined optimal concentration of partially purified pregnant mouse uterus extract was used as a source of macrophage CSF (6). Colonies of at least 50 cells were scored after 14 days of incubation. The values are means \pm standard errors of the mean for mice 7 to 9 weeks of age except where indicated. The statistical significance was determined by the Student *t* test, using the SigmaStat statistical package (version 2.0; Jandel Corp., St. Rafael, Calif.).

Histological preparations. Standard histology was performed as described previously (59). Tartrate-resistant acid phosphatase stains for osteoclasts were performed with reagents from Sigma, according to the manufacturer's protocols. Whole-mount stains of mammary fat pads were performed as described previously (58).

Flow cytometric analysis. Immunophenotyping was performed with a FAC-StarPlus flow cytometer. List files of at least 10,000 events were collected for each cell population and were analyzed with Lysis II software. CD4 and CD8 cell subsets and B220-positive cells expressing IgM or IgD were analyzed by two-color fluorescence (fluorescein isothiocyanate or phycoerythrin) following fluorescence compensation. Monoclonal antibodies directed against murine CD3 ϵ (145-2C11), CD4 (GK1.5), CD8 (53-6.7), TCR β (H57), IgM (R6-60.2), and IgD were purchased from PharMingen. The monoclonal antibody directed against B220 (RA36B2) (9) was purified from rat hybridoma conditioned medium. Streptavidin-phycoerythrin was purchased from Caltag Laboratories.

RESULTS

Targeting of the *c-Cbl* locus. To generate *c-Cbl*-deficient mice, we first sought to disrupt one allele of the gene in ES cells by homologous recombination of a targeting construct into the *c-Cbl* locus (Fig. 1A). Following electroporation of the targeting construct, multiple ES cell clones were obtained in which the *c-Cbl* gene was precisely disrupted. Four such clones were used to generate mice with a germ line mutation. Homozygous *c-Cbl*^{-/-} mice, detected by Southern blot analysis (Fig. 1B), were produced in expected numbers from an intercross of heterozygotes (Table 1). *c-Cbl* is widely expressed in adult mouse tissues but is most abundant in the testes and thymus (28). Despite this, homozygous *c-Cbl*^{-/-} male and female animals were fertile, and the litter sizes of *c-Cbl*^{-/-} intercrosses were comparable to those of wild-type controls (Table 2). Homozygous mutant animals had normal growth rates and longevity (data not shown) and were externally indistinguishable from their wild-type littermates.

To further confirm that the gene had been functionally disrupted, Western blot analysis was performed on tissue lysates

TABLE 2. Litter numbers and sizes of *c-Cbl*^{-/-} mice and control animals

Genotype of parents	Avg litter size	No. of litters
+/+	7	11
+/-	9	13
-/-	6	11

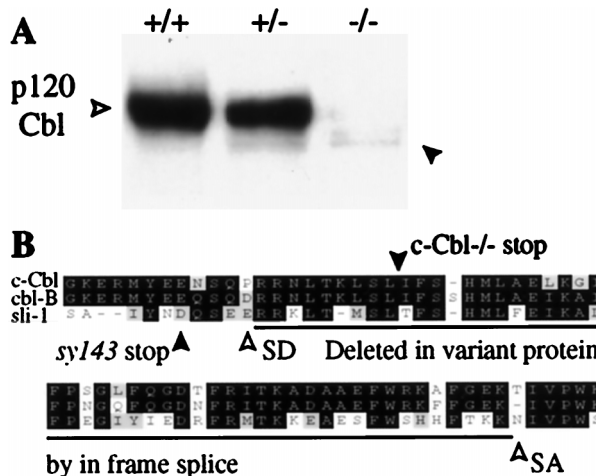


FIG. 2. Western blot analysis of *c-Cbl* mutant mice. (A) Protein levels of *c-Cbl* were greatly reduced in homozygote mutant thymocytes (open arrowhead), although a trace amount of a slightly smaller protein remained (solid arrowhead). Note that the smaller protein is only apparent in homozygous and heterozygous mutant mice. (B) Alignment showing the positions of splice donor (SD) and splice acceptor (SA) sites adjacent to the targeting site (*c-Cbl*^{-/-} stop) and the sequences deleted in the smaller protein (panel A, solid arrowhead). The majority of the *c-Cbl* protein was truncated by insertion of a Neo^r cassette several residues downstream of a site corresponding to a null allele in *Sli-1* (*sy1143* stop). Cloning of the mRNA for the residual mutant protein demonstrated that it was produced by an atypical, in-frame splicing event that was generated only from the mutant allele. Black boxes, identical amino acids; grey boxes, conservative amino acid differences; white boxes, nonconservative amino acid differences.

with an antiserum directed to the C terminus of *c-Cbl* which does not cross-react with the related *Cbl-b* protein. There was a complete absence of full-length protein in all tissues tested in homozygous mutant mice. Trace amounts of a smaller protein were apparent, however, in the thymus, where *c-Cbl* is especially abundant and easy to detect (Fig. 2A). A survey of other tissues, including those of the spleen, lymph node, breast, and brain, also showed very weak expression of the smaller protein in proportion to the normal levels of the protein in wild-type tissue (data not shown). The residual protein was seen only in *c-Cbl*^{+/-} and *c-Cbl*^{-/-} mice and therefore appeared to be a product of the mutant allele rather than a normal splice variant. Consistent with this notion, the transcript corresponding to this protein was cloned only from mutant mice when reverse transcription and PCR amplification were performed. DNA sequence analysis demonstrated that the smaller protein was encoded by an in-frame splicing event that deleted the targeted exon (Fig. 2B). The predicted protein lacked highly conserved residues in the region of the PTB domain of *c-Cbl* (Fig. 2B) (31, 53). Thus, the *c-Cbl* mice lack any wild-type *c-Cbl* protein and produce trace amounts of an abnormal protein that lacks highly conserved residues within the PTB domain, indicating that these mice are likely to be null for *c-Cbl* function.

Hemopoietic and mammary hyperplasia. We performed a histological survey of neonatal and adult mice to identify tissue abnormalities in *c-Cbl*-deficient mice. A prominent feature of *c-Cbl*^{-/-} mice was a severe splenic disorder characterized by splenomegaly (Table 3 and Fig. 3), fibrosis, and extensive extramedullary hemopoiesis (EMH), in which large numbers of megakaryocytes and normoblasts were apparent in the spleen parenchyma. Increased EMH in the spleens and livers of mutants was apparent from birth (data not shown). EMH can occur as a secondary response to failure of bone marrow hemopoiesis or due to anemia, for example, following hemorrhage. However, erythrocyte numbers and other blood param-

TABLE 3. Hemopoietic parameters of wild-type and *Cbl*^{-/-} mice

Parameter	Value (\pm SD) for genotype:		P value
	+/+	-/-	
Blood			
Leukocytes (10^6 /ml)	8.5 \pm 1.2	10.9 \pm 1.3	NS ^b
Erythrocytes (10^9 /ml)	9.3 \pm 0.5	9.3 \pm 0.6	NS
Platelets (10^9 /ml)	1,091 \pm 39	1,755 \pm 87	<0.001
Reticulocytes (10^9 /ml)	0.39 \pm 0.03	0.40 \pm 0.06	NS
Packed cell vol (%)	46.9 \pm 1.2	46.3 \pm 0.9	NS
Spleen			
Wt (mg)	79.6 \pm 5.8	136.6 \pm 9.7	<0.001
CFC/ 10^4 cells	30.5 \pm 8.6	65.8 \pm 17.8	NS ^c
Bone marrow			
Cells/femur (10^7)	2.3 \pm 0.3	2.0 \pm 0.2	NS
CFC/femur (10^6) ^a	5.55 \pm 1.54	6.02 \pm 1.38	NS
Thymus cellularity (10^7)			
5-5.5 wk old	16.3 \pm 2.0	24.8 \pm 1.3	<0.005
7-9 wk old	16.1 \pm 1.6	14.9 \pm 1.5	NS
Lymph node cellularity (10^6)	16.3 \pm 2.2	31.7 \pm 3.7	0.004

^a CFC responsive to CSF-1 alone were assayed as an index of the committed progenitor cell compartment (5).

^b NS, not significant.

^c The incidence of spleen CFC appeared elevated in *Cbl*^{-/-} mice but did not reach statistical significance ($P = 0.1$).

eters examined were comparable in mutant and wild-type animals, except for an increase in circulating platelets in *c-Cbl*^{-/-} mice (Table 3). The thrombocytosis seen in *c-Cbl*^{-/-} mice may reflect the large numbers of splenic megakaryocytes seen in these animals (Fig. 3D). Bone marrow cellularity and the incidence of bone marrow progenitor colony-forming cells (CFC) were not significantly different in wild-type and *c-Cbl*^{-/-} mice (Table 3). There was an increase in the number of CFC in some *c-Cbl*^{-/-} mice, but due to a large variability between animals, the changes noted were not significantly different from those of the controls. Lymph nodes were significantly enlarged in *c-Cbl*^{-/-} mice and were comprised of increased numbers of both T and B cells (Table 3 and data not shown). Interestingly, mice aged between 5 and 5.5 weeks had significantly increased numbers of thymocytes, but by 7 to 9 weeks of age, thymic cellularity in *c-Cbl*^{-/-} mice was comparable to that in their wild-type littermates (Table 3).

We observed that the mammary fat pads of virgin adult female mice were slightly thickened compared with those of controls. When stained as whole-mount preparations to reveal the ductal development, a striking increase in duct density and branching was observed in mature virgin *c-Cbl*^{-/-} mice compared with that in controls (Fig. 3E to H). Examination of other branching and secretory tissues, including the glands associated with the male reproductive system and the salivary glands, did not reveal any morphological difference between wild-type and *c-Cbl*^{-/-} mice (data not shown). *c-Cbl* expression was readily detectable in wild-type animals by Western blot analysis in all tissues affected by the *c-Cbl* mutation (data not shown).

The splenic, lymphoid, and mammary changes we observed appeared to uniformly affect each type of tissue and were not suggestive of focal clonal expansion(s). Whether these abnormalities represent low-grade neoplastic changes is unclear; however, we have not observed the development of progres-

sive, malignant tumors in *c-Cbl*^{-/-} mice maintained up to 12 months of age.

Bone development and remodelling. *c-Cbl* has been reported to be required for osteoclast activity in an in vitro bone resorption assay (51). To investigate whether there were any skeletal abnormalities in mutant animals, mice were examined by high-resolution X ray and histologically to reveal general morphology and osteoclasts. X-ray analysis showed distinct marrow cavities in the long bones of mutant animals and demonstrated that their general skeletal architecture was normal (Fig. 4A and B). This was confirmed by hematoxylin and eosin staining of decalcified femurs and tibias from juvenile and adult mice and serial sections through neonatal mice (Fig. 4C and D and data not shown). Finally, bone sections stained for tartrate-resistant acid phosphatase as a marker of osteoclasts revealed normal numbers and morphology of osteoclasts in mutant animals compared with those in their littermates (Fig. 4E and F). A more detailed in vitro analysis of osteoclast activity and a histomorphometric analysis of bones from mutant mice may be used to reveal a subtle requirement for *c-Cbl* in bone remodelling.

Abnormalities in T-cell profiles. *c-Cbl* is highly expressed in the thymus and has been implicated in TCR and B-cell receptor signalling (11, 13, 22). To investigate its role in T- and B-cell development, and to further characterize the hemopoietic abnormalities in the *c-Cbl*^{-/-} mouse, we performed flow cytometric analyses on 7- to 9-week-old mice. Although *c-Cbl*^{-/-} mice had normal numbers of thymic cells (Table 3), there was a pronounced alteration in their phenotype. There was a large increase in the number of TCR β - and CD3 ϵ -expressing thymocytes in the *c-Cbl*^{-/-} mice compared with those in their wild-type littermates (Fig. 5). The percentage of CD4-CD8 double-positive and CD4 and CD8 single-positive thymic cells was largely normal in *c-Cbl*^{-/-} mice, except for a tendency for fewer CD8 single-positive cells. The slight reduction in CD8 single-positive cells was not statistically significant when data from a large number of animals was compared and may therefore reflect a chance occurrence or a phenotype that occurs at low penetrance in the *c-Cbl*^{-/-} mice (data not shown). Although CD4/CD8 ratios were largely unaffected in *c-Cbl*^{-/-} mice, there was a consistent increase in the intensity of CD4 and CD8 staining in the double-positive thymocytes, but not in single-positive cells (Fig. 5). Similar changes in the TCR β -CD ϵ and CD4-CD8 profiles were observed in the spleens of *c-Cbl*^{-/-} mice, but they were less pronounced and a proportion of animals appeared largely normal (data not shown).

Whereas the thymic-T-cell changes seen in *c-Cbl*^{-/-} mice were highly reproducible, more variable changes were observed in B cells. The mesenteric lymph nodes of *c-Cbl*^{-/-} mice demonstrated absolute increases in both B and T cells (Table 3). The distribution of immature and mature B cells in the spleen, lymph nodes, and bone marrow, assessed by IgM, IgD, and B220 staining, was generally normal (data not shown). However, some *c-Cbl*^{-/-} mice had a larger proportion of IgM-positive, IgD-negative cells in the bone marrow and spleen. Antibody stains for cells in the granulocyte and macrophage/monocyte lineages did not reveal any abnormalities in mutant hemopoietic tissues (data not shown). Depletion of *c-Cbl* therefore had its most apparent impact on cells in the lymphoid lineage, most notably in the T-cell compartment.

Given the changes observed by flow cytometric analysis, we investigated the functional integrity of the immune system by measuring antibody responses to antigenic challenge. Mice were immunized with the hapten nitrophenol coupled either to

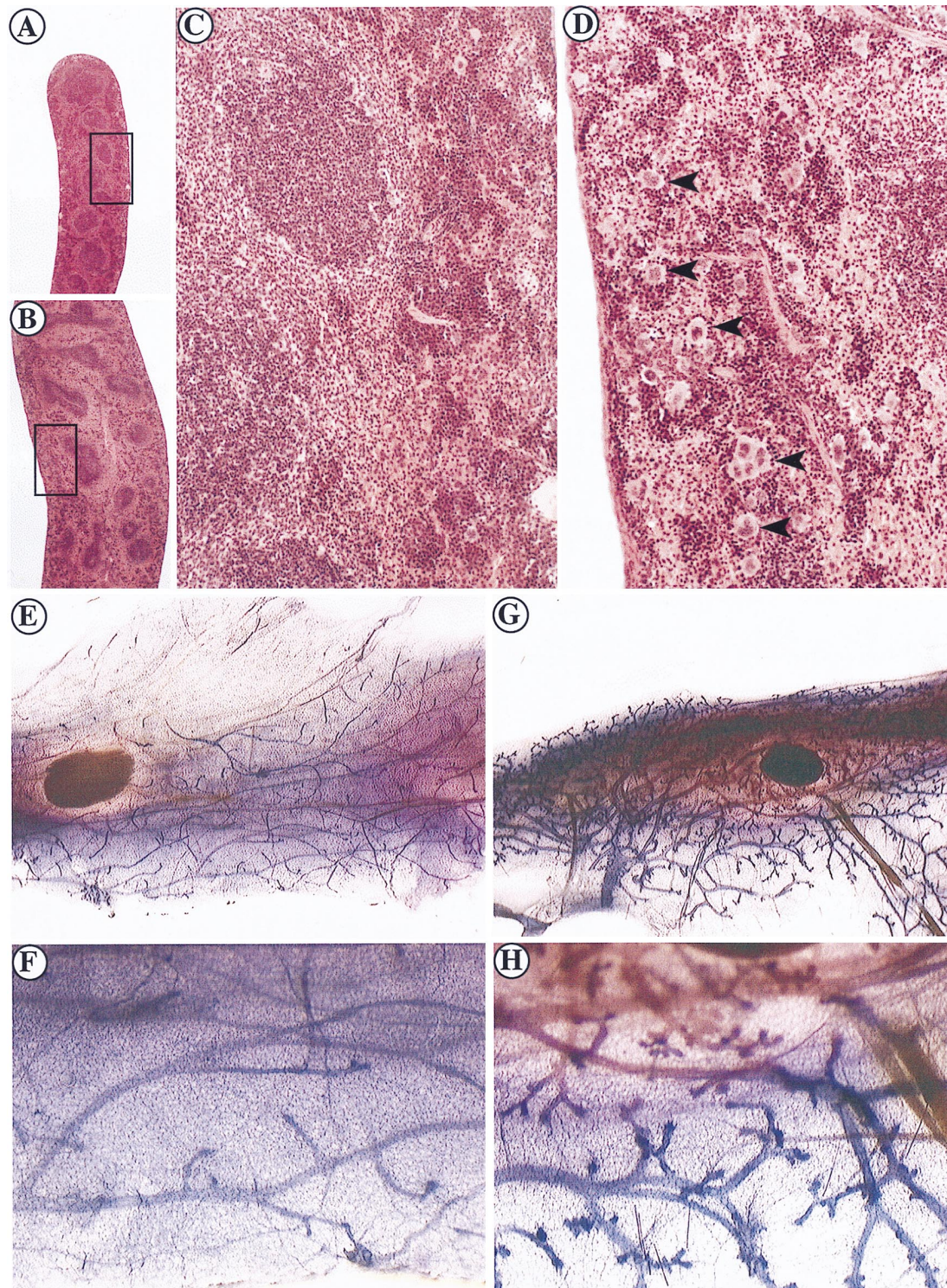


FIG. 3. Primary splenomegaly, EMH, and mammary duct hyperplasia in *c-Cbl*^{-/-} mice. (A to D) Hematoxylin and eosin staining of splenic sections from wild-type (A and C) and *c-Cbl*^{-/-} (B and D) mice. (A and B) Low-power magnification demonstrating splenomegaly and disruption of the architecture of the spleen of a *c-Cbl*^{-/-} mutant. (C and D) High-power magnification of the boxed regions in panels A and B showing EMH with large numbers of megakaryocytes (arrowheads) in the spleen of a *c-Cbl*^{-/-} mutant (D) compared with that of its normal littermate (C). (E to H) Whole-mount stains of the abdominal mammary fat pads of virgin 13-week-old female mice. Excessive ductal branching was seen in *c-Cbl*^{-/-} mice (G and H) compared to that in age-matched wild-type mice (E and F).

lipopolysaccharide or keyhole limpet hemocyanin, in order to measure T-cell-independent and T-cell-dependent antigens, respectively. The immunized mice were then assayed for their isotopic responses over a 3-week period. The magnitude and

timing of the responses to these antigens were comparable in mutant and wild-type animals (data not shown). In vitro proliferation of thymocytes and peripheral T cells in response to TCR cross-linking was also investigated. While there was a

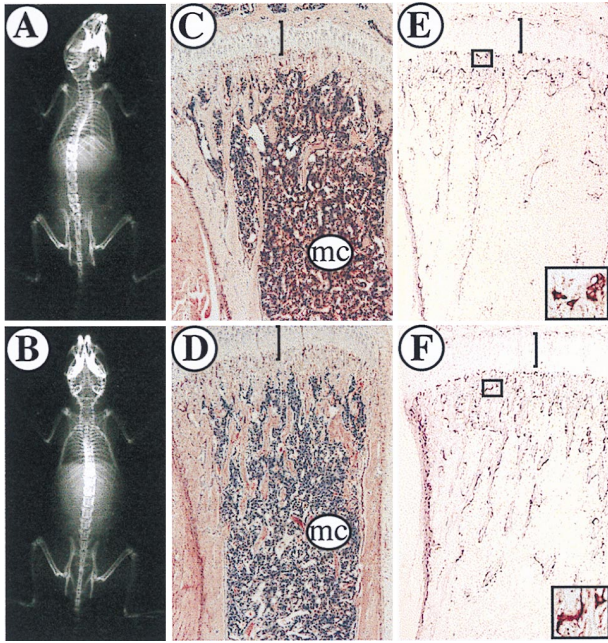


FIG. 4. Bone morphology in *c-Cbl*^{-/-} and wild-type control mice. (A and B) X-ray analysis of 7-week-old wild-type (A) and *c-Cbl*^{-/-} (B) mice showing comparable skeletal morphology. (C and D) Hematoxylin and eosin staining of the femoral heads of a 6-week-old wild-type mouse (C) and its *c-Cbl*^{-/-} littermate (D). The morphologies of the growth plate (bar) and marrow cavity (mc) were comparable. (E and F) Sections comparable to those in panels C and D stained for tartrate-resistant acid phosphatase to reveal osteoclasts. The numbers and morphologies (high-power insets of boxed regions) of osteoclasts were comparable in wild-type (E) and *c-Cbl*^{-/-} (F) mice.

trend towards a higher proliferative response in *c-Cbl*^{-/-} thymocytes, this was not statistically significant (Fig. 6). Further evaluation of response to limiting amounts of cross-linking antibodies or to ovalbumin with T cells from antigen-primed animals failed to detect significant proliferative differences between wild-type and *c-Cbl*^{-/-} cells (data not shown).

CD3-mediated activation of ZAP-70 in *c-Cbl*-deficient thymocytes. *c-Cbl* is a prominent target of the TCR-regulated Fyn kinase (55). Overexpression of *c-Cbl* reduces Fcε Receptor I-mediated phosphorylation of the Syk tyrosine kinase in mast cells (39) and decreases TCR-mediated, Ras-dependent activation of AP1 (43). To investigate the intracellular consequences of loss of *c-Cbl*, we examined signalling events in thymocytes before and after TCR activation by cross-linking with anti-CD3 antibodies. Immunoblotting of lysates with anti-phosphotyrosine antibodies revealed that unstimulated *c-Cbl*^{-/-} thymocytes had higher basal levels of tyrosine-phosphorylated proteins in the 50- to 60-kDa molecular mass range compared with normal cells (Fig. 7A). Anti-CD3 cross-linking of thymocytes from normal mice resulted in a large induction of *c-Cbl* tyrosine phosphorylation, consistent with Fyn kinase activation, and an increase in the tyrosine phosphorylation of proteins in the 50- to 60-kDa molecular mass range. In contrast, stimulation of *c-Cbl*^{-/-} thymocytes through the TCR-CD3 complex revealed the tyrosine phosphorylation of additional substrates not evident in normal cells. Most striking was the massive induction in tyrosine phosphorylation of three polypeptides with molecular masses of 70, 75, and 80 kDa and the hyperphosphorylation of a protein of a mobility equivalent to that of pp36-38 (48) (Fig. 7A).

The electrophoretic mobility of these proteins raised the possibility that anti-CD3 stimulation of *Cbl*-deficient thymo-

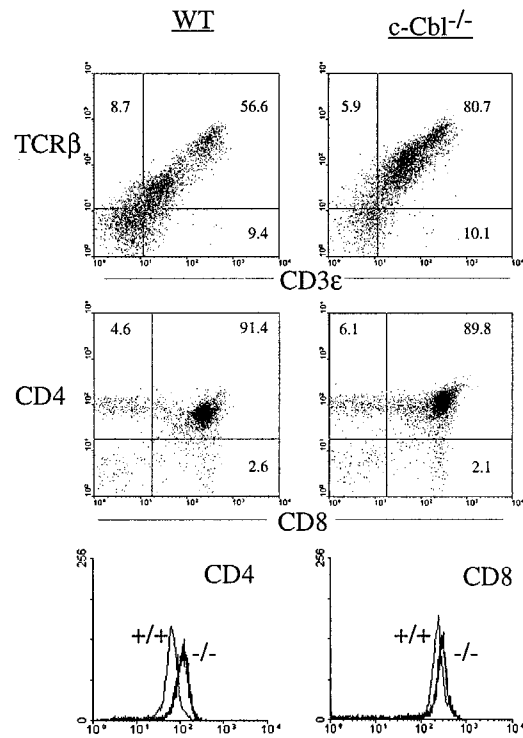


FIG. 5. Altered T-cell parameters in *c-Cbl*^{-/-} mice. Flow cytometry profiles from wild-type (WT) and *c-Cbl*^{-/-} cells as indicated. The flow cytometry profiles were consistent in 12 or more mice of each genotype.

cytes had inappropriately phosphorylated and activated the ZAP-70 protein tyrosine kinase, which is known to phosphorylate the substrates SLP-76 and pp36-38 (44a, 62). ZAP-70 in normal thymocytes is activated via the CD4-coupled tyrosine

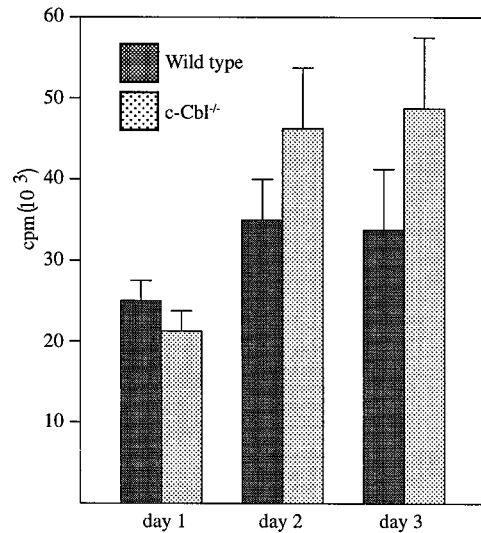


FIG. 6. Analysis of thymocyte proliferation in response to anti-TCR stimulation. Thymocytes from wild-type and *c-Cbl*^{-/-} mice were cultured with plate-bound anti-TCRαβ (H57-597) antibodies for the indicated times and pulsed with 0.6 mCi of [³H]thymidine 8 h before being harvested. All cultures were in triplicate, and the results represent mean radioactive values ± standard errors of the mean from cells harvested from the thymuses of 10 wild-type and 10 *c-Cbl*^{-/-} mice. No response was observed in the absence of antibody stimulation.

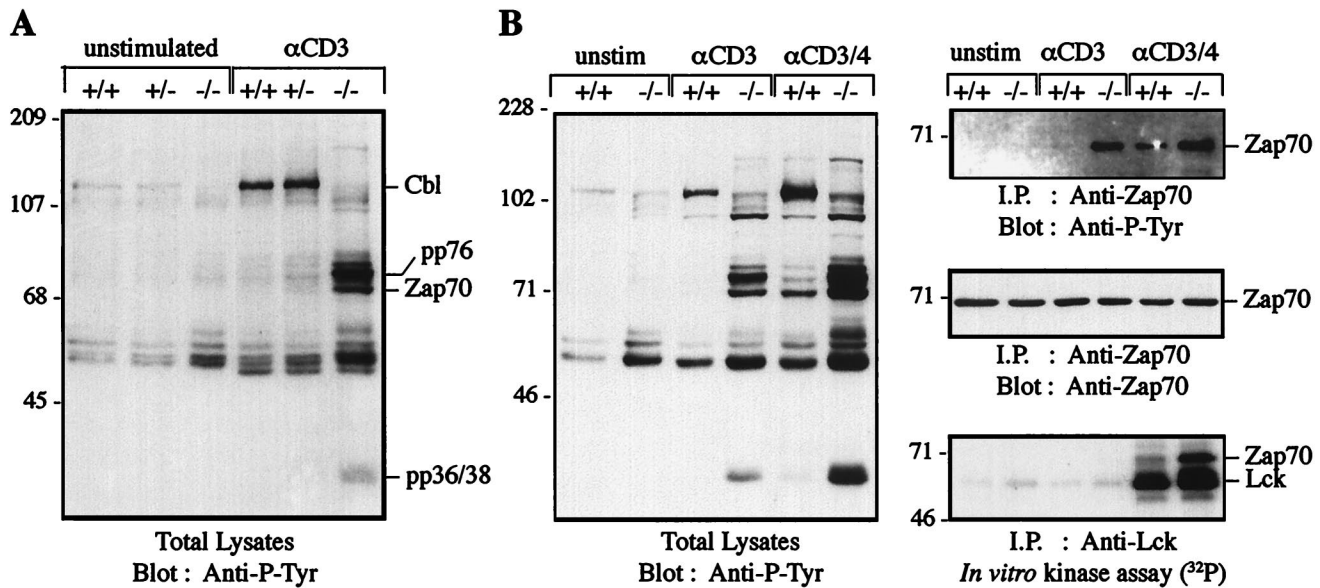


FIG. 7. (A) Anti-CD3 stimulation of thymocytes from *c-Cbl*-deficient mice induces the tyrosine phosphorylation of proteins that are not detectably phosphorylated in normal thymocytes. Thymocytes from *c-Cbl*^{+/+}, *c-Cbl*^{+/-}, and *c-Cbl*^{-/-} mice were left unstimulated or stimulated with anti-CD3 (α CD3) antibodies for 5 min at 37°C before lysis and immunoblotting with anti-phosphotyrosine (Anti-P-Tyr) antibodies. The 76-kDa phosphoprotein from the stimulated *c-Cbl*^{-/-} thymocytes has a mobility equivalent to that of SLP-76. (B) Anti-CD3-induced phosphorylation of ZAP-70 in *c-Cbl*-deficient thymocytes is not dependent on Lck activation. Thymocytes from *c-Cbl*^{+/+} or *c-Cbl*^{-/-} mice were left unstimulated (unstim) or stimulated with anti-CD3 or anti-CD3-CD4 antibodies for 5 min at 37°C. Cell lysates were either analyzed by immunoblotting with anti-phosphotyrosine antibodies or immunoprecipitated (i.p.) with anti-Lck or anti-ZAP-70 antibodies. The anti-Lck immunoprecipitates were analyzed in an immune complex kinase assay for Lck kinase activity, and the anti-ZAP-70 immunoprecipitates were analyzed by anti-phosphotyrosine immunoblotting.

kinase Lck (63). We compared the effects of anti-CD3 and anti-CD3-CD4 stimulation of thymocytes from normal and *c-Cbl*-deficient mice (Fig. 7B). Again anti-CD3 stimulation of normal thymocytes resulted in the phosphorylation of a limited number of substrates, with *c-Cbl* being most prominent, whereas in *c-Cbl*-deficient thymocytes tyrosine phosphorylation of the 70- to 80-kDa substrates predominated. Immunoprecipitation of ZAP-70 and immunoblotting with anti-phosphotyrosine antibodies confirmed the lack of activated ZAP-70 in anti-CD3-stimulated normal thymocytes, whereas in *c-Cbl*-deficient thymocytes ZAP-70 is inappropriately activated (Fig. 7B). Following costimulation of normal thymocytes with anti-CD3-CD4, there was an induction of a hyperphosphorylated 70-kDa protein and, to a lesser extent, proteins of approximately 75 and 80 kDa. Anti-ZAP-70 immunoprecipitation of costimulated normal thymocytes confirmed the identity of this protein as tyrosine-phosphorylated ZAP-70. The immunoprecipitation also showed that ZAP-70 tyrosine phosphorylation was slightly enhanced in the *c-Cbl*-deficient thymocytes, but there was a disproportionately large increase in the phosphorylation of the 75-, 80-, and 36- to 38-kDa proteins compared to that in normal thymocytes (Fig. 7B).

These findings suggested that *c-Cbl* was functioning as a negative regulator of ZAP-70. To test whether this regulation is a direct effect or is mediated indirectly by *c-Cbl*'s effect on Lck, we analyzed Lck in an immune complex kinase assay. Lck is an upstream activator of ZAP-70 that phosphorylates tyrosine 493 in the kinase domain of ZAP-70, resulting in catalytic activation of ZAP-70 (8, 61). The results of the immune complex kinase assay shown in Fig. 7B demonstrated a lack of activated Lck in both normal and *c-Cbl*-deficient thymocytes stimulated by anti-CD3 antibodies, consistent with the requirement for CD4 cross-linking for Lck activation (63). Thus, it appears that the inappropriate activation of ZAP-70 via CD3

in the *c-Cbl*-deficient thymocytes was occurring without the overt activation of Lck. In contrast costimulation by anti-CD3-CD4 antibodies results in a large induction of Lck kinase activity in both normal and *c-Cbl*-deficient thymocytes. Lck induction and ZAP-70 phosphorylation were slightly higher in the *c-Cbl*-deficient thymocytes, but there was a disproportionately increased association of Lck and ZAP-70. Furthermore, there is a very large increase in the level of phosphorylation of the 75- to 80-kDa proteins in *c-Cbl*^{-/-} cells relative to that in wild-type control cells, which was disproportionate to the level of ZAP-70 phosphorylation. Taken together these findings suggest that the depletion of *c-Cbl* greatly enhances ZAP-70 activity via a mechanism that independent of its immediate upstream activator.

To test the possibility that this enhanced phosphorylation could be a consequence of decreased tyrosine phosphatase activity rather than a deregulation of ZAP-70 activity, we compared a time course of tyrosine phosphorylation of proteins in anti-CD3e-stimulated thymocytes. Dephosphorylation of prominent substrates occurred at a comparable rate in both wild-type and *c-Cbl*^{-/-} thymocytes (Fig. 8A), indicating that increased kinase activity is responsible for the increased level of phosphoproteins seen in *c-Cbl*^{-/-} thymocytes. Furthermore, there appears to be some degree of specificity, as the enhancement in tyrosine phosphorylation due to *c-Cbl* depletion appears to be restricted to about four substrates, which is in contrast to the array of hyperphosphorylated proteins in pervanadate-treated thymocytes (data not shown).

Consistent with the increased phosphorylation seen in *c-Cbl*^{-/-} thymocytes we also found an increased level of activated Erk2 following anti-CD3 stimulation (Fig. 8B). This finding is consistent with the suppression of Erk2 activity by *c-Cbl* overexpression in Jurkat T cells (43) and the ability of 70Z-Cbl to induce NFAT activation via the Ras pathway (29). Collec-

expression, and c-Cbl has been strongly implicated in TCR signalling. It was therefore of considerable interest to investigate the consequences of c-Cbl depletion for T-cell development. Development of a functional T-cell repertoire occurred normally in *c-Cbl*^{-/-} mice as evidenced by a correct ratio of CD4 and CD8 single- and double-positive cells and normal in vivo responses to antigen. That T-cell development was not severely affected in *c-Cbl*^{-/-} mice is surprising for several reasons: the surface expression of CD4, CD8, and TCR was increased in *c-Cbl*^{-/-} thymocytes; there was hyperphosphorylation of intracellular proteins in response to receptor cross-linking; and there was a marked qualitative difference between wild-type and *c-Cbl*^{-/-} thymocytes in that the latter responded acutely to a TCR-mediated signal in the absence of the normal requirement for coreceptor aggregation (63). Perhaps c-Cbl occupies an unusual niche as a negative regulator that finely tunes T-cell development rather than being one of the critical positive components of T-cell signalling for which a severe loss-of-function phenotype is normally seen (reviewed in reference 41). Alternatively, redundancy with Cbl-b may allow cells to accommodate the loss of c-Cbl, as is seen with Lck and Fyn in mice that are mutant for these genes (18).

Increased expression of the TCR and its coreceptors in c-Cbl mutant mice is of interest given the proposed role of c-Cbl in mediating degradation of the CSF-1 receptor (60). The increased expression of CD4 and CD8 in the double-positive thymocytes closely resembles that seen in mice that are deficient in a specific splice variant of CD45 (23). The altered CD4-CD8 expression seen in CD45 mutant mice may be a consequence of the profound block in thymocyte development that occurs at the transition from immature CD4-CD8 double-positive cells to mature, single-positive cells. However, it is notable that *c-Cbl*^{-/-} mice do not display a similar block in their development. Whether the cell surface changes observed in the *c-Cbl*^{-/-} mice reflects a direct effect of loss of c-Cbl, such as increased stability of these proteins, or an altered T-cell developmental sequence requires further investigation.

c-Cbl phosphorylation is reduced in osteoclast-like cells from c-Src mutant mice, indicating that c-Cbl may be involved in signalling by Src (51), which in turn is required for osteoclast activity and bone remodelling (50). Consistent with this notion, in vitro bone resorption by osteoclasts was inhibited with antisense oligonucleotides to *c-Cbl*. Despite these findings we did not observe any bone defects in *c-Cbl* mutant mice. Although Cbl-b expression was not assessed in the antisense experiments, the oligonucleotides used were directed to sequences that were nonhomologous between the Cbl-b and c-Cbl genes. In addition, c-Cbl expression was more profoundly reduced in this study than in the experiments by Tanaka et al. (51). Thus, a difference in the degree or scope of attenuation of Cbl protein expression does not readily explain the discrepancy between the two studies. Rather this difference more likely reflects the assays used, one in vitro and the other measuring steady-state bone morphology in vivo. Detailed bone morphometry and in vitro analysis of *c-Cbl*^{-/-} osteoclast activity is currently in progress to further characterize the role of c-Cbl in these cells.

c-Cbl as a negative regulator of ZAP-70. Our analysis of signalling via the TCR-CD3 complex and the CD4 receptor on thymocytes has provided compelling evidence that c-Cbl acts as a negative regulator of the ZAP-70 protein tyrosine kinase. The initial analysis of CD3-mediated signalling revealed a marked induction in ZAP-70 tyrosine phosphorylation (Fig. 7). Three other proteins in c-Cbl-deficient thymocytes also revealed a massive induction in tyrosine phosphorylation following CD3 cross-linking. Based on their relative mobilities, two

of these proteins may represent the ZAP-70 substrates SLP-76 and pp36-38 (or LAT) (44a, 62). Importantly, costimulation of normal thymocytes with anti-CD3-CD4 antibodies induced a level of phosphorylation of ZAP-70 that was only slightly lower than that seen in c-Cbl-deficient thymocytes (Fig. 7B). Despite this, phosphoproteins other than ZAP-70 showed a massive increase in c-Cbl-deficient thymocytes costimulated with anti-CD3-CD4. Thus, the activating signal appears to be enhanced by c-Cbl depletion beyond that accounted for by an activation of ZAP-70 alone.

A striking aspect of the response of *c-Cbl*^{-/-} thymocytes to receptor cross-linking was that loss of c-Cbl abrogated the normal requirement for CD4-TCR costimulation for ZAP-70 activation in thymocytes (63). TCR signalling in these cells was not completely deregulated, however, as a signal through CD3 was still necessary for ZAP-70 activation. In normal CD4⁺ CD8⁺ thymocytes ZAP-70 forms an inactive complex with the TCR ζ chain and cannot be activated by CD3 cross-linking alone because CD4-Ia interactions sequester available Lck (63). We considered it possible that loss of c-Cbl increased the availability of Lck or that Lck was being activated by CD3 alone in *c-Cbl*^{-/-} thymocytes. We were also concerned that a trivial explanation for these findings was that they simply reflected the increased number of TCR-expressing thymocytes in *c-Cbl*^{-/-} mice. This does not appear to be the case, however, as Lck immune complex kinase assays revealed no obvious difference in Lck activity between normal and c-Cbl-deficient thymocytes following CD3 stimulation (Fig. 7B). We would expect that if the altered signalling observed in *c-Cbl*^{-/-} thymocytes reflected an expanded TCR-expressing pool, then Lck activation would parallel the other intracellular changes we observed. The profound CD3-mediated intracellular phosphorylation that occurred in *c-Cbl*^{-/-} thymocytes in the absence Lck activation indicated that signalling through ZAP-70 had become uncoupled from Lck activation in these cells. This implied that a kinase other than Lck may be responsible for inappropriate activation of ZAP-70 in CD3-cross-linked *c-Cbl*^{-/-} thymocytes. Several reports have described an interaction between c-Cbl and the CD3 ϵ chain-associated Src family kinase, Fyn (14, 16, 55). Although it is possible that the loss of c-Cbl increases the availability of Fyn to phosphorylate ZAP-70 following CD3 ϵ cross-linking, we have found no evidence of this (data not shown). Alternatively there may be a direct loss of ZAP-70 regulation so that a normally weak signal via CD3 is sufficient to trigger its activation. Consistent with the notion that loss of c-Cbl may impact directly on ZAP-70 regulation is the recent finding that the c-Cbl PTB domain binds selectively to Tyr-292 of ZAP-70 (32). Phosphorylated Tyr-292 is thought to negatively regulate ZAP-70 through the binding of regulatory molecules (24). We aim to further test this hypothesis by generating thymocyte cell lines from *c-Cbl*^{-/-} mice into which wild-type and mutant alleles of the gene can be introduced.

Experiments first performed in *C. elegans*, where the weak signal from a reduction-of-function Let-23 receptor mutant is rescued by the depletion of Sli-1, provided the first indication that Cbl proteins may negatively regulate tyrosine kinases (64). Our findings, together with studies revealing the suppressive effect of c-Cbl overexpression on Syk kinase activity, provides compelling evidence for c-Cbl to be classified as a negative regulator of the Syk/ZAP-70 family of tyrosine kinases. Peptides closely matching the Tyr-292 negative regulatory sequence are represented in several other proteins to which c-Cbl binds, including the EGF receptor, Syk, and platelet-derived growth factor alpha (32), suggesting a potential general mechanism for c-Cbl regulation of protein tyrosine ki-

nases. While our observations support this general mechanism for c-Cbl action, the moderate phenotype of the *c-Cbl*^{-/-} mice suggests that substantial functional redundancy may exist with other Cbl family members. Further elucidation of the mechanism by which this unique family of proteins regulates intracellular signalling is likely to be of considerable importance, given the central role that tyrosine kinases play in controlling cell survival, growth, and differentiation.

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