The actin regulator coronin 1A is mutant in a thymic egress-deficient mouse strain and in a patient with severe combined immunodeficiency

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Mice carrying the recessive locus for peripheral T cell deficiency (*Ptcd*) have a block in thymic egress, but the mechanism responsible is undefined. Here we found that *Ptcd* T cells had an intrinsic migration defect, impaired lymphoid tissue trafficking and irregularly shaped protrusions. Characterization of the *Ptcd* locus showed a point substitution of lysine for glutamic acid at position 26 in the actin regulator coronin 1A that enhanced its inhibition of the actin regulator Arp2/3 and resulted in its mislocalization from the leading edge of migrating T cells. The discovery of another coronin 1A mutant during an *N*-ethyl-*N*-nitrosourea-mutagenesis screen for T cell-lymphopenic mice prompted us to evaluate a T cell-deficient, B cell-sufficient and natural killer cell-sufficient patient with severe combined immunodeficiency, whom we found had mutations in both *CORO1A* alleles. Our findings establish a function for coronin 1A in T cell egress, identify a surface of coronin involved in Arp2/3 regulation and demonstrate that actin regulation is a biological process defective in human and mouse severe combined immunodeficiency.

After developing in the thymus, mature thymocytes upregulate sphingosine 1-phosphate receptor type 1 (S1P₁) and exit into the circulation to populate the naive T cell compartment. S1P₁ and its ligand, sphingosine 1-phosphate (S1P), are required for the egress of mature thymocytes¹. FTY720, a small-molecule immunosuppressant being tested in clinical trials for the treatment of autoimmune disease, inhibits egress by modulating S1P₁ function¹. Beyond the requirement for S1P₁, little is understood about how T cells migrate from the thymus.

The cataract Shionogi (CTS) strain was initially isolated in the 1960s from a closed colony of ICR mice on the basis of its cataracts and microphthalmia². The CTS strain was later established to have a defect in thymic egress³ after it failed to reject major histocompatibility complex-disparate skin grafts⁴. The nature of this recessive defect, called 'peripheral T cell deficiency' (*Ptcd*), and its function in thymic egress have remained unclear.

Coronins are actin regulators found in all eukaryotes⁵. In addition to binding F-actin, coronin proteins associate with and inhibit the nucleation-promoting Arp2/3 complex. Seven coronin family members exist in mammals, including coronin 1A (Coro1A), which is

expressed mainly in hematopoietic cells. Coro1A-deficient mice have fewer peripheral T cells due to more apoptosis^{6,7}, and one study has attributed this to an excessive accumulation of F-actin⁶. T cell migration is also reported to be defective^{6,7}, but this has been called into question⁸. Those last authors have also questioned whether Coro1A deficiency alters F-actin dynamics and instead link the greater apoptosis to a T cell antigen receptor (TCR) signaling defect⁸. These conflicting reports of Coro1A-deficient mice have complicated the understanding of the function of this actin regulator in T cell biology.

Here we report that *Ptcd* T cells had an intrinsic migration defect that impaired thymic egress and trafficking through lymph nodes. We 'narrowed' the *Ptcd* locus and identified a point mutation in the gene encoding Coro1A (*Coro1a*) that resulted in mislocalization of the protein in T cells and greater inhibition of Arp2/3 by Coro1A in biochemical assays. In a parallel effort to identify additional trafficking mutants by screening mice carrying *N*-ethyl-*N*-nitrosourea (ENU)-induced mutations for altered peripheral T cell numbers, we identified a strain with 90% less Coro1A and a phenotype similar to that of Coro1A-knockout mice. Comparison of *Ptcd* and Coro1A-deficient T cells allowed us to separate the defect in TCR-induced

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Figure 1 *Ptcd* T cells have an intrinsic migration defect. (a,b) Thymocyte and peripheral blood lymphocyte subsets from B6 *Ptcd* and control (heterozygous *Ptcd*/+ (*Het*)) mice (a), or wild-type mice reconstituted with *Ptcd* bone marrow (*Ptcd*→WT) or control bone marrow (*Het*→WT; b). (c) Flow cytometry of S1P₁ on mature CD4 SP thymocytes from *Ptcd* and control mice. Filled histogram (Isotype), isotype-matched control antibody. (d-f) Transwell migration of *Ptcd* and control mature CD4 SP thymocytes to S1P (d) and Transwell migration of thymocytes (e) or naive CD4⁺ splenic T cells or CD19⁺ B cells (f) to the chemokines CXCL12, CCL21 and/or CXCL13 (e,f), presented as percentage of input cells that migrated. In a,b,d-f, bars indicate means; circles indicate individual mice. *, $P < 0.05$. Data are representative of at least two experiments (a,b) or three experiments (c) or are pooled from three or four experiments (d-f).

calcium signaling from the diminished thymocyte survival. In addition to yielding new alleles of *Coro1a*, our mouse forward-genetics approach led us to identify a T cell-deficient, B cell-sufficient and natural killer cell-sufficient (T⁺B⁺NK⁺) patient with SCID who has Coro1A deficiency.

RESULTS

Ptcd is an intrinsic T cell-migration defect

To characterize the cellular basis of the *Ptcd* defect, we first backcrossed the *Ptcd* locus onto the C57BL/6 (B6) strain and confirmed the accumulation of mature single-positive (SP) thymocytes (CD69^{lo}CD62L^{hi}) and associated lower number of peripheral T cells (Fig. 1a and Supplementary Fig. 1 online). Irradiated wild-type mice that had been reconstituted with *Ptcd* bone marrow cells also had an accumulation of mature thymocytes and few circulating T cells (Fig. 1b), whereas reciprocal bone marrow chimeras did not have such defects (Supplementary Fig. 2 online). These results localized the *Ptcd* defect to a hematopoietic lineage-derived cell and indicated that impaired egress of mature thymocytes from the thymus is a pathogenic mechanism.

Although normal amounts of S1P₁ were expressed on *Ptcd* mature SP thymocytes (Fig. 1c), S1P₁ function was impaired. In Transwell

migration assays, *Ptcd* mature CD4 SP thymocytes were less efficient in migrating toward S1P than were cells from control heterozygous littermates (Fig. 1d). The cells also migrated less efficiently toward the chemokines CCL21 and CXCL12. The response of *Ptcd* CD4⁺CD8⁺ double-positive thymocytes to CXCL12 was similarly lower (Fig. 1e), and the migration of naive *Ptcd* splenic T cells was impaired, whereas B cells migrated normally (Fig. 1f). The expression of CCR7 and CXCR4, the respective chemokine receptors that recognize CCL21 and CXCL12, was similar in *Ptcd* and control cells (Supplementary Fig. 3 online). These results indicate that T cells in *Ptcd* mice have a general, cell-intrinsic migration defect that impairs S1P₁ responsiveness and blocks thymic egress.

Ptcd T cells have entry, egress and motility defects

To test for peripheral trafficking defects, we transferred *Ptcd* and control T cells together into wild-type recipients. At 1 h after transfer, the ratio of *Ptcd* T cells to control T cells was lower in peripheral and

Figure 2 *Ptcd* T cells are defective in lymph node trafficking. (a,b) Homing to lymph nodes at 1 h (a) and egress into lymph at 24 h (b) after adoptive cotransfer of *Ptcd* thymocytes (*Ptcd*/*Ptcd*) and heterozygous control thymocytes (*het*; *Ptcd*/+), presented as the ratio of *Ptcd* to control cells, normalized to the injected ratio of *Ptcd* to control cells. To achieve lymph node ratio of about 1:1 at 24 h, initial injections used a ratio of 3:1. MLN, mesenteric lymph node; PLN, peripheral lymph node. (c) Retention of *Ptcd* and control cells in the mesenteric or peripheral lymph nodes 20 h after treatment with integrin-neutralizing antibody. (d-f) Time-lapse two-photon microscopy of the mean velocities (d), turning angles (e) and displacement (f) of *Ptcd* cells and control cells (*Het*; *Ptcd*/+) in explanted lymph nodes. (f) Average of displacement squared (R^2) over time (symbols, means; bars, s.d.). In a-e, bars indicate means; circles indicate individual mice. *, $P < 0.05$. Data are pooled from three experiments (a,b), are from three independent experiments with four mice each (c) or are representative of three independent experiments with at least 40 tracks analyzed in each (d-f).

Figure 3 *Coro1a* is mutated at the *Ptd* locus.

(a) Flow cytometry of thymocytes from affected and unaffected mice generated in the mapping cross between CTS and B6 mice. Mice were considered 'affected' on the basis of a high frequency of SP cells, a high fraction of CD62L^{hi}CD69^{lo} CD4 SP cells and enrichment for CD62L^{hi} staining of CD8 SP cells. Numbers in or adjacent to outlined areas indicate percent cells in each. (b) Critical interval of *Ptd* defined by SNP markers polymorphic between the B6 and CTS strains in various recombinant mice (mouse identifiers, left margin). (c) Dideoxy sequence tracings of DNA from *Ptd* and heterozygous mice identifying a G-to-A mutation at position 76 that resulted in the E26K substitution of CorolA. WT, wild-type DNA from the related nonobese diabetic mouse strain (additional control). (d) CorolA structure, including the *Ptd* point substitution (*). (e) Immunoblot analysis of CorolA and actin in total thymocytes from *Ptd* and control mice, for samples loaded in threefold dilutions (wedges). NC, negative control (*Coro1a*^{-/-}). Data are representative of three experiments.

mesenteric lymph nodes and higher in blood (Fig. 2a), which indicated that *Ptd* cells were less efficient in entering lymph nodes. Next we tested lymph node egress with two approaches. First, we transferred *Ptd* and control T cells together at a ratio of 3:1 to achieve equal proportions in the lymph nodes. At 24 h after transfer, we found a much lower proportion of *Ptd* cells in lymph than in lymph nodes (Fig. 2b), which was consistent with a diminished ability to exit into lymph. As an additional approach, we assessed the retention of *Ptd* T cells in lymph nodes. After transferring *Ptd* and control T cells, we blocked entry into lymph nodes for 20 h with integrin-neutralizing antibodies. Roughly 60% of *Ptd* T cells were retained in the lymph nodes, compared with just 20% of control T cells (Fig. 2c). These results collectively demonstrate that *Ptd* T cells are defective in exiting lymph nodes.

Two-photon microscopy of explanted lymph nodes from mice that had received fluorescence-labeled cells showed that whereas control cells moved at a median velocity of 9.5 μ m/min, *Ptd* cells moved at a lower median velocity of 6.6 μ m/min (Fig. 2d and Supplementary Movie 1 online). In addition, *Ptd* cells had larger turning angles (Fig. 2e), indicative of less-directed paths, and the cells failed to efficiently displace over time (Fig. 2f). These results demonstrate that in addition to blocking thymic egress, the *Ptd* defect impairs trafficking through lymph nodes.

Coro1a is mutated at the *Ptd* locus

The *Ptd* locus has been mapped to a 10.9-megabase region of chromosome 7 containing over 300 open reading frames⁹. To refine the locus, we did a mapping cross between CTS and B6 mice, using thymocyte accumulation to phenotype mice (Fig. 3a). We noted that the cataracts and microphthalmia were present in F₁ hybrids and failed to segregate together with the recessive *Ptd* trait (data not shown), consistent with an early report that these eye traits are autosomal dominant¹⁰. After analyzing over 900 meiosis events, we further mapped the *Ptd* locus to a critical interval of 950 kilobases (Fig. 3b). This gene-rich interval contained 37 open reading frames,

including *Coro1a* (Supplementary Fig. 4 online). *Coro1a* was a likely candidate gene for *Ptd* because of its abundance in T cells¹¹ and the association of coronin family molecules with actin-based motility⁵. Sequencing of *Ptd* DNA showed a G-to-A mutation in exon 2 of *Coro1a* (Fig. 3c), which resulting in a nonconservative glutamic acid-to lysine substitution at residue 26 in the β -propeller domain of the protein (Coro1A^{E26K}; Fig. 3d). This residue was conserved in all annotated CorolA sequences and the charge of the residue was also conserved in a large number of orthologs (Supplementary Fig. 5 online) and was in a surface-exposed loop¹² adjacent to a region that in the close relative CorolB has been indicated to be an actin-binding site¹³. Immunoblot analysis showed that the abundance of CorolA in total thymocytes did not vary between *Ptd* and controls (Fig. 3e).

To exclude the possibility that substitutions other than Coro1A^{E26K} were responsible for the *Ptd* phenotype, we tested for genetic complementation by crossing *Ptd* mice with *Coro1a*^{+/-} mice. As expected, the *Coro1a* wild-type allele complemented *Ptd* defects in T cell lymphopenia and F-actin accumulation, whereas the *Coro1a*-null allele failed to complement (Supplementary Fig. 6a,b online). In addition, mature thymocyte numbers were slightly higher in *Ptd*^{-/-} mice than in *Ptd*^{+/+} mice (Supplementary Fig. 6c). Although these studies do not exclude the possibility that the *Ptd* interval contains additional genetic modifiers, the inability of the *Coro1a*-null allele to 'rescue' several of the *Ptd* defects provides strong evidence that the Coro1A^{E26K} substitution results in the *Ptd* phenotype.

ENU-mutant Koyaanisqatsi is a CorolA hypomorph

In parallel to the studies reported above, we did an ENU-mutagenesis screen to identify regulators of lymphocyte trafficking and egress. We initially identified 'Koyaanisqatsi' (*Koy*) as a recessive mutant strain of mice with a circulating T cell deficiency (Fig. 4a). Further characterization of *Koy* mice showed that they had fewer mature thymocytes and peripheral T cells (Fig. 4b and Supplementary Fig. 7 online). In Transwell migration assays, *Koy* thymocytes and naive T cells migrated

Figure 4 The ENU mutant *Koy* is a Coro1A hypomorph. **(a)** Flow cytometry of B220 and TCR $\alpha\beta$ in T lymphocytes (left) and analysis of lymphocyte subsets (right) in the peripheral blood of *Koy* and littermate control mice. Numbers adjacent to outlined areas (left) indicate percent B220⁺TCR $\alpha\beta$ ⁺ cells (top left) or B220⁺TCR $\alpha\beta$ ⁻ cells (bottom right). **(b)** Flow cytometry of thymocyte subsets in *Koy* and control mice. **(c)** Transwell migration of *Koy* or control double-positive thymocytes, mature CD4 SP thymocytes or splenic naive CD4⁺ T cell toward CXCL12 or CCL21, presented as the percentage of input cells that migrated. Bars indicate means; circles indicate individual mice (**a–c**). **(d)** Flow cytometry of phalloidin staining in mature CD4 SP thymocytes from *Koy* and control mice. Each line represents an individual mouse. **(e)** Dideoxy sequence tracings of genomic DNA from tail tissue of *Koy* and control mice. **(f)** Immunoblot analysis of Coro1A and actin in cells from *Koy* and control mice, as described in **Figure 3f**. Het, *Ptcd*^{+/+} (control); WT, wild-type B6. *, *P* < 0.05. Data are from at least three experiments (**a–c**) or are representative of at least three experiments (**d–f**).

much less efficiently than did cells from littermate control mice (**Fig. 4c**). These findings in *Koy* mice, along with more phalloidin staining of *Koy* mature thymocytes (**Fig. 4d**), resembled those reported for *Coro1a*^{-/-} mice generated by gene targeting⁶. After sequencing *Koy* DNA, we identified an A-to-T substitution in exon 7 of *Coro1a* (**Fig. 4e**) that resulted in an aspartic acid-to-valine

substitution at residue 278. This residue is located at a contact surface predicted to be critical for the proper assembly and stability of Coro1A¹², and immunoblot analysis of total thymocytes showed that *Koy* cells had roughly one tenth as much Coro1A as wild-type cells had (**Fig. 4f**). These data indicate that *Koy* is a hypomorphic mutant of Coro1A.

Figure 5 Allelic comparison of cell survival, F-actin accumulation and Ca²⁺ flux. **(a,b)** Apoptosis of wild-type (WT), Coro1a-knockout (KO) and *Ptcd* mature CD4 SP thymocytes and naive CD4⁺ T cells after 4 h of incubation on ice or at 37 °C, assessed as loss of mitochondrial membrane potential ($\Delta\Psi$; **a**) or positive annexin V staining (AnnV⁺; **b**). *, *P* < 0.05. Data are pooled from three independent experiments. **(c)** Flow cytometry of phalloidin binding in wild-type, *Coro1a*-knockout and *Ptcd* mature CD4 SP thymocytes and naive CD4⁺ T cells. Data are representative of at least three experiments. **(d)** Intracellular Ca²⁺ concentration ([Ca²⁺]_i) in wild-type, *Coro1a*-knockout and *Ptcd* mature CD4 SP thymocytes and naive CD4⁺ T cells. Xlink, crosslinking of prebound anti-CD3 or anti-CD3 plus anti-CD4 by the addition of streptavidin (time, downward arrow). Dead and dying cells were excluded by propidium iodide staining. Data are representative of three experiments.

Figure 6 The E26K substitution alters the cellular distribution of Coro1A and the regulation of Arp2/3 by Coro1A. (a–c) Immunofluorescence microscopy of T cells migrating on intercellular adhesion molecule-coated coverslips in CCL21 (1 µg/ml), stained for F-actin and Coro1A (a) or the Arp2/3 subunit p34 (p34Arc; b), and still images from brightfield time-lapse microscopy (c). Scale bars, 5 µm; 'time stamps' (c), minutes:seconds. (d) Cosedimentation analysis of actin alone or purified coronin proteins and F-actin. S, supernatant; P, pellet. (e) Immunoblot analysis of GFP and p34 in lysates of human embryonic kidney 293FT cells expressing various green fluorescent protein (GFP)-tagged constructs of Coro1A, detected in total cell lysates (left) and after immunoprecipitation with anti-p34 (IP: p34; right). *, GFP degradation bands. (f) Arp2/3-induced actin polymerization assay with various concentrations of purified coronin proteins. AVA, standard mixture of actin, plus the verprolin homology, cofilin homology, acidic domain (VCA) and the Arp2/3 complex; WT, wild-type Coro1A; E26K, Coro1A^{E26K}. (g) Microscopy of *Ptc*^{d/+} and *Ptc*^{d/-} T cells stained as described in a. Original magnification, ×25. Data are representative of three (a–c), or at least three (d–g) experiments.

Survival and Ca²⁺ flux of Coro1A mutants

Direct comparison of *Ptc*^d, *Koy* and *Coro1a*^{-/-} mice confirmed that the E26K substitution was not a phenocopy of the Coro1A-deficient state. Although *Ptc*^d mutants had an accumulation of mature thymocytes (Fig. 1a), Coro1A-deficient mice had fewer mature thymocytes (Fig. 4b) due to reduced cell survival⁶. Analysis of cell viability by measurement of loss of mitochondrial membrane potential (Fig. 5a) and annexin V staining (Fig. 5b) showed that mature thymocytes from *Ptc*^d mice were similar to those from wild-type control mice, whereas mature thymocytes from *Coro1a*^{-/-} mice were less viable. However, *Ptc*^d splenic T cells had a survival defect similar to that of *Coro1a*^{-/-} cells (Fig. 5a,b).

A published study has attributed the survival defect of *Coro1a*^{-/-} T cells to 'downstream' effects of excessive accumulation of F-actin⁶. Consistent with that interpretation, mature *Ptc*^d thymocytes had only slightly more F-actin, as assessed by phalloidin staining (Fig. 5c), whereas mature *Coro1a*^{-/-} thymocytes and the similarly apoptosis-prone *Ptc*^d and *Coro1a*^{-/-} splenic T cells all had approximately twofold more phalloidin staining (Fig. 5c). However, the correlation between F-actin accumulation and less viability did not seem to hold true for *Ptc*^{d/-} T cells, as they had more phalloidin staining similar to that of *Coro1a*^{-/-} T cells and yet were not fewer in number in the thymus (Supplementary Fig. 6). A study has suggested that the lower viability of *Coro1a*^{-/-} cells reflects a requirement for Coro1A in TCR-induced

Ca²⁺ flux rather than in F-actin regulation⁸. However, *Ptc*^d and Coro1A-deficient mature CD4 SP thymocytes showed similar defects in Ca²⁺ flux after CD3 crosslinking (Fig. 5d). Furthermore, both *Ptc*^d and Coro1A-deficient cells showed minimal defects compared with wild-type cells after combined crosslinking of CD3 and CD4 (Fig. 5d), a treatment that may more closely mimic major histocompatibility complex class II-peptide engagement¹⁴. These findings dissociate the function of Coro1A in TCR-induced Ca²⁺ signaling from its function in thymocyte viability but leave unclear whether Coro1A acts through F-actin regulation or by some other mechanism to promote T cell survival.

Coro1A^{E26K} is mislocalized in *Ptc*^d T cells

Coro1B regulates F-actin dynamics at the leading edge of migrating fibroblasts¹⁵, and we found that in migrating wild-type T cells, Coro1A was situated in F-actin-rich and Arp2/3-rich leading-edge protrusions (Fig. 6a,b). The distribution of Coro1A^{E26K} in migrating *Ptc*^d T cells was considerably altered and little of the protein could be detected at the leading edge (Fig. 6a), whereas Arp2/3 continued to concentrate in this region (Fig. 6b). Coro1A^{E26K} also similarly mislocalized when expressed in fibroblasts (Supplementary Fig. 8 online), which indicated an intrinsic failure to distribute at the leading edge.

Real-time live cell imaging showed that protrusions from migrating *Ptc*^d T cells were irregularly shaped and often larger in size than

Figure 7 Deficiency of Coro1A in a T-B⁺NK⁺ patient with SCID. **(a)** Family pedigree (top) and dideoxy sequence tracings (below) of a T-B⁺NK⁺ patient with SCID and family members. Red arrow indicates the location of the deletion of CT between nucleotides 248 and 249 in the coding DNA sequence in the patient that led to a premature truncation after ten missense codons; the father is heterozygous for this (that tracing includes both the mutated (top) and normal (bottom) copies). Red outlines enclose the CT nucleotides missing in the patient and father. **(b)** Immunoblot analysis of Coro1A and actin in EBV-transformed B cell lines derived from various subjects. Data are representative of three experiments. **(c)** Quantitative PCR signals from primer sets of human genes encoding S1P₁ (*S1PR1*), coronin 7 (*CORO7*) and Coro1a (*CORO1A*) from buccal swabs of genomic DNA from the patient with SCID and a normal control. Bars indicate means; circles indicate separate PCR reactions. Data are from three experiments.

protrusions from wild-type cells, consistent with a failure to properly regulate the actin cytoskeleton (**Fig. 6c** and **Supplementary Movies 2–4** online). We noted similarly dysmorphic protrusions from *Ptcd*[−] compound mutant T cells (**Supplementary Fig. 6d**). The short *in vitro* survival of Coro1A-deficient T cells made assessments difficult, but these cells seemed more severely compromised in their migration than *Ptcd*[−] T cells were (**Supplementary Movie 5** online). These results collectively establish that Coro1A^{E26K} fails to distribute correctly in migrating T cells and that the cells have aberrant shape regulation.

Coro1A^{E26K} enhances Arp2/3 inhibition

To understand the biochemical effect of the E26K substitution, we first assessed the ability of Coro1A to bind F-actin. In cosedimentation assays, Coro1A^{E26K} associated normally with F-actin (**Fig. 6d**). In contrast, Arp2/3 coimmunoprecipitated more Coro1A^{E26K} than wild-type Coro1A from cell lysates (**Fig. 6e**). The corresponding substitution in Coro1B also resulted in more association with Arp2/3 (**Supplementary Fig. 9** online). Consistent with more interaction with Arp2/3, Coro1A^{E26K} was more efficient than wild-type Coro1A in inhibiting Arp2/3 activity in actin-polymerization assays (**Fig. 6f**).

To examine how this biochemical gain-of-function substitution manifested as a recessive *Ptcd* allele, we analyzed T cells from *Ptcd*^{+/+} and *Ptcd*[−] mice. As expected, the Coro1A distribution in *Ptcd*[−] T cells (**Fig. 6g**) was similar to that in *Ptcd*^{+/+} T cells (**Fig. 6a**). In *Ptcd*^{+/+} T cells, Coro1A was both present at and mislocalized away from the leading edge (**Fig. 6g**). These results suggest that in *Ptcd*^{+/+} cells, wild-type Coro1A can access the leading edge to regulate actin dynamics. In summary, these biochemical studies indicate that the highly conserved Glu26 residue in Coro1A is important for the regulation of Arp2/3.

Coro1A is deficient in a T-B⁺NK⁺ patient with SCID

After finding that point mutations in *Coro1a* resulted in severe T lymphopenia in both *Ptcd* and *Koy* mice, we sought to determine whether *CORO1A* mutations could also be responsible for human cases of primary T cell immunodeficiency with undefined etiologies. Thus, we sequenced *CORO1A* in DNA from 16 SCID patients lacking mutations in previously recognized genes associated with T-B⁺NK⁺ SCID. Although we failed to detect any *CORO1A* mutations in 15 samples, we did identify a single patient with a two-nucleotide deletion in exon 3 (deletion of CT between nucleotides 248 and 249

in the coding DNA sequence; **Fig. 7a**) that resulted in a frame shift and premature stop codon in the area encoding the Coro1A β-propeller domain (with the first amino acid affected by the frame shift being a substitution of arginine for proline at position 83, and the creation of a new reading frame that ended at the first stop codon at position 10 (with arginine now amino acid 1)). Consistent with a destabilizing truncation, immunoblot analysis of Epstein Barr virus (EBV)-immortalized B lymphoid cell lines derived from the patient's peripheral blood showed an absence of Coro1A protein (**Fig. 7b**). When this patient was 13 months old, she was hospitalized with a severe varicella infection after vaccination; she was later diagnosed as an atypical T-B⁺NK⁺ patient with SCID and was treated by allogeneic bone marrow transplantation. We found that her father was heterozygous for the two-base pair deletion in *CORO1A* but her mother and sister were not (**Fig. 7a**).

To assess the gene-copy number of *CORO1A* in the patient, we did quantitative PCR of genomic DNA with nonoverlapping primers spanning intron 1 through exon 11 of *CORO1A* and noted 50% less product relative to that of a healthy control (**Fig. 7c**). In contrast, signals for the human genes encoding S1P₁, on chromosome 1, and coronin 7, located telomeric to *CORO1A* on chromosome 16p, did not differ from the quantitative PCR signal in control cells. Along with reports of copy-number variation in chromosome 16p11.2, where *CORO1A* is located^{16–18}, these data suggest that our patient, who inherited a two-base pair paternal deletion in one allele of *CORO1A*, also has deletion of the other *CORO1A* allele.

DISCUSSION

Using a forward-genetics approach, we have identified a unique *Coro1a* allele that allowed us to establish a requirement for this actin-regulatory protein in the egress of T cells from the thymus and lymph nodes. Two-photon microscopy showed that Coro1A was needed for normal T cell motility in the lymph node, and *in vitro* motility studies indicated its involvement in regulating cell shape. Moreover, this mutation identified a site on Coro1A critical for Arp2/3 regulation and for controlling Coro1A distribution in the cell. Finally, we demonstrated that Coro1A deficiency in a human was associated with a peripheral T cell deficiency of sufficient severity to cause T-B⁺NK⁺ SCID.

The identification of a critical function for Coro1A in egress from the thymus and lymph nodes provides evidence that migration from these tissues depends on proper regulation of Arp2/3-mediated actin branching, most likely in response to S1P. The protein mDia1, a member of the formin family of actin-nucleating proteins that promote the formation of unbranched actin filaments, is also needed for thymic egress¹⁹. Thus, the formation of both correctly branched as well as unbranched actin networks seems to be required for exit from

this organ. The function of mDia1 in lymph node egress has not been assessed. The small G proteins Rac and Cdc42 induce activation of the Wiskott-Aldrich syndrome protein-Arp2/3 system and, consistent with our results here, the Rac activator Dock2 is needed for lymph node egress²⁰. Although the function of Dock2 in thymic egress has not been determined, given the need for Coro1A, we anticipate that Dock2 is also involved. Lymphocyte protrusions initiate diapedesis²¹, and our findings suggest that Coro1A-dependent control of actin may be critical in the formation of protrusions during T cell egress.

The requirement for Arp2/3-mediated actin branching for T cell-migration responses to chemoattractants has been supported by findings obtained with cells deficient in Dock2, Wiskott-Aldrich syndrome protein and Wiskott-Aldrich syndrome protein-interacting protein^{20,22,23} as well as Coro1A-deficient cells^{6,7}. Our results obtained with *Ptcd* T cells add to those findings and suggest an important function for Coro1A-mediated Arp2/3 regulation in the control of T cell shape. A published study has argued that F-actin dynamics are unaltered in Coro1A-deficient T cells, although cell migration in response to chemoattractants was not examined⁸. The basis for these discrepant findings is unclear, but it seems possible that the very poor survival of Coro1A-deficient naive T cells and their consequent depletion from T cell preparations relative to effector and/or memory T cells confounded some of the comparisons. In our studies here, we were careful to exclude both dead cells and effector or memory cells from our analysis of naive T cells. Although the migration of *Ptcd* T cells was much lower *in vitro* and in the lymph node, it was not absent, and this might explain the continued ability of the *Ptcd* mouse thymus to organize into cortex and medulla³. It seems likely that the lack of notable defects in *Ptcd* and Coro1A-deficient B cells is due to redundancy with other coronin family members, and this adds to other data establishing differences in requirements for B cell and T cell motility^{19,24,25}. Whether substitutions in Coro1A or other actin-regulatory molecules cause defects in common lymphoid progenitors remains to be determined. Although bone marrow chimera-reconstitution efficiencies were similar for *Ptcd* and control donors, our studies did not formally address whether the entry of early thymic precursors is impaired. Future studies should include competition assays with mixed-bone marrow chimeras to evaluate the proportion of *Ptcd*-derived cells in bone marrow and thymus. Leukocyte migration in lymphoid tissue has been described as phases of force-generating actin protrusions followed by myosin-dependent contractions²⁶ that aid the squeezing of the cell nucleus through three-dimensional matrices. In this context, it is notable that *Ptcd* T cells not only had large and dysmorphic protrusions but also failed to elongate their nuclei. Thus, remodeling of the actin cytoskeleton by Coro1A may contribute to both protrusion and contraction during the migration of T cells through lymphoid tissues.

The mechanism by which coronin proteins inhibit Arp2/3-mediated actin branching is complex and incompletely understood. Although several studies have shown that coronin proteins are able to directly bind Arp2/3 and possibly inhibit function before F-actin binds^{27,28}, work with Coro1B in fibroblasts suggests that it acts by displacing Arp2/3 from actin branch points²⁹. We have demonstrated that a Coro1A surface proximal to the predicted F-actin-binding region¹³ was involved in Arp2/3 regulation. Our findings emphasize the importance of characterizing the highly conserved Glu26-containing surface of the type I coronin subfamily in future structure-function studies focusing on how an oligomeric coronin complex dislodges Arp2/3 from F-actin. The failure of Coro1A^{E26K} to localize normally to the leading edge of migrating cells has identified what may be another level of coronin regulation. Further studies are needed to

determine whether interactions with the cofilin phosphatase slingshot⁵ or other still-unknown coronin-binding partners are altered by the substitution.

Coro1A deficiency caused a substantial defect in T cell survival, but the basis for this effect is not yet resolved. Although the *Ptcd* is an important allelic comparison that 'disconnected' the survival and Ca²⁺-flux defects, the greater phalloidin staining of *Ptcd* mature thymocytes also failed to support the idea of a simple correlation between survival and F-actin accumulation. The possibility remains that Coro1A directly affects cell survival independently of effects on F-actin, and analysis of additional Coro1A substitutions may yield new allelic insights. The continued ability of *Ptcd* thymocytes to maintain nearly normal total cellular F-actin despite mislocalization of Coro1A might be explained by an ongoing association of low but sufficient amounts of Coro1A^{E26K} to achieve partial regulation of Arp2/3 at the leading edge, or it might reflect correspondingly greater actin-debranching and depolymerization in the cell body. Alternatively, the *in vitro* assay for examining colocalization of Arp2/3 and Coro1A may not accurately reflect how these proteins distribute *in vivo* in migrating thymocytes. The similar partial deficiencies in TCR-induced Ca²⁺ flux in Coro1A^{E26K} and *Coro1a*^{-/-} T cells are in agreement with emerging evidence that an intact actin cytoskeleton is important for Ca²⁺ flux³⁰. However, our finding that CD3-CD4 co-crosslinking led to equivalent Ca²⁺ flux in wild-type, Coro1A^{E26K} and *Coro1a*^{-/-} thymocytes, along with a published report of normal proliferation of *Coro1a*^{-/-} T cells stimulated by antigen-presenting cells⁶, suggests that Coro1A may have a limited function in TCR signaling during encounters with cognate antigen. We anticipate that single-cell analysis of the release of Ca²⁺ stores and the activity of Ca²⁺ release-activated channels in *Ptcd* and Coro1A-deficient mature thymocytes and T cells, along with similar studies of T cells with other defects in actin-regulatory proteins, will add to the understanding of how the actin cytoskeleton contributes to calcium signaling.

An important challenge of studies of mouse models is 'translation' of the findings to humans. The identification of a patient with a peripheral T cell deficiency who lacks expression of Coro1A suggests that Coro1A has similar functions in humans and mice. Although the human X-linked Wiskott-Aldrich syndrome has long associated actin biology with hematopoietic cell dysfunction, the Coro1A-deficient patient we studied here allowed us to link regulation of the actin cytoskeleton with SCID³¹. T⁺B⁺NK⁺ SCID has been defined before by mutations resulting in impaired TCR or interleukin 7 receptor signaling³². Other forms of SCID involve defects in purine metabolism, DNA recombination or cytokine signaling³³. Mutations in genes involved in these pathways result in apoptosis through purine toxicity, deficient cytokine signaling or failure to rearrange a signaling-competent antigen receptor. Given the results of our study of Coro1A deficiency, we can also include apoptosis via actin dysregulation as a cause of SCID. A report classifying a *Coro1a*-null allele as an auto-immune-suppression locus in mice⁷ is consistent with the idea that Coro1A defects cause immunodeficiency. We predict that substitutions in other hematopoietic cell-specific actin-associated molecules will be found to cause SCID. Because the pericentromeric segment of human chromosome 16 that contains *CORO1A* is prone to copy-number variation¹⁶⁻¹⁸, we also anticipate that additional cases of patients with lower T cell function will involve *CORO1A* mutations.

METHODS

Mice, ENU mutagenesis and bone marrow chimeras. B6 and B6-CD45.1 mice were from the Jackson Laboratories, the National Cancer Institute or a colony maintained at the University of California, San Francisco. UBC-GFP mice were

from Jackson Laboratories. Cataract-Shionogi (CTS/Shi) mice were from TGC. *Ptcd* was backcrossed six to ten generations onto the B6 strain with single-nucleotide polymorphism (SNP) markers as described below. *Coro1a*^{-/-} mice, which were backcrossed ten generations with B6 mice, were generated as described⁶. *Koy* mice were identified by an ENU-mutagenesis screen done as described³⁴ with B6 mice in a colony maintained at the Australian National University. Male B6 mice were treated with three doses, 1 week apart, of 100 mg ENU (Sigma) per kg body weight in 10% (vol/vol) ethanol in citrate buffer, pH 5.0, to produce G₀ mice. After a refractory period of 8 weeks, these G₀ males were mated with B6 females to generate G₁ offspring. Two unrelated G₁ mice were crossed, producing G₂ mice, which were then intercrossed to yield G₃ mice. Screening of these G₃ mice by flow cytometry identified the *Koy* phenotype. All mice were housed in specific pathogen-free conditions, and all protocols were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco, or the Animal Experimentation Ethics Committee of Australian National University. Bone marrow chimeras were generated with 2×10^6 to 3×10^6 bone marrow cells from donor mice transplanted intravenously into lethally irradiated congenic host mice. Chimeras were given prophylactic water containing antibiotics and were analyzed 8–12 weeks after transplantation.

Flow cytometry, intracellular staining and measurement of Ca²⁺ flux. Polyclonal antibody to S1P₁ (anti-S1P₁) has been described³⁵. Phalloidin-fluorescein isothiocyanate was from Sigma. Indo-1 was from Invitrogen. Kits for intracellular staining and annexin V staining were from BD Biosciences. The JC-1 mitochondrial membrane potential assay kit was from Cayman. All other antibodies used for flow cytometry were from BD Biosciences, Invitrogen, eBiosciences or BioLegend. Ca²⁺-flux assays were adapted from published methods for thymocytes¹⁴. Cells were loaded for 30 min at 30 °C with Indo-1 in the presence of biotinylated anti-CD3 (2C11; BD), fluorescein isothiocyanate-anti-CD8 (53-6.7; BD) and Alexa Fluor 647-anti-CD4 (M4-5; BD). Thymocytes were additionally stained with phycoerythrin-anti-CD62L (Mel14; BD) and splenocytes were additionally stained with phycoerythrin-anti-CD44 (IM7; BD). Cells were washed and kept on ice until analysis. For crosslinking of CD3 and CD4, cells were additionally pretreated for 5 min with biotinylated anti-CD4 (RM4-4; BD). Cells were mixed with prewarmed media containing propidium iodide for exclusion of dead cells and were warmed to 37 °C. Baseline Ca²⁺ concentrations were assessed for 30 s before crosslinking with streptavidin (50 µg/ml; Pierce). All flow cytometry was done on a FACSCalibur or an LSRII (BD).

Purification of recombinant protein. Coronin recombinant protein was expressed and purified with a mammalian expression system as described for Coro1B¹⁵. Purified protein was quantified by absorbance at 280 nm and the predicted extinction coefficients of 62,910 M⁻¹cm⁻¹ (Coro1A) and 65,890 M⁻¹cm⁻¹ (Coro1B).

Immunoprecipitation. Cells were washed twice with PBS and were lysed with a KCl buffer (20 mM HEPES, pH 7.0, 100 mM KCl, 0.5% (vol/vol) Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml of 1,10-phenanthroline, 10 µg/ml of aprotinin, 10 µg/ml of leupeptin, 10 mM sodium fluoride and 2 mM sodium orthovanadate). Lysates were cleared for 5 min at 13,000g and were incubated with 0.5 µg primary antibody for 16 h at 4 °C, followed by the addition of 20 µl of a slurry of ImmunoPure-immobilized protein A/G beads (Pierce) and further incubation for an additional hour at 4 °C. Immune complexes were collected, were washed three times with KCl buffer, were separated by SDS-PAGE and were transferred to a polyvinylidene difluoride membranes (Bio-Rad) for immunoblot analysis.

Actin-assembly assay. Arp2/3-nucleation reactions induced by the verprolin homology, cofilin homology, acidic domain were done as described¹⁵: recombinant coronin and Arp2/3 (20 nM) were mixed in MKEI-50 buffer (20 mM imidazole-KOH, pH 7.0, 50 mM KCl, 2 mM MgCl₂ and 1 mM EGTA) and were incubated for 5 min at 25 °C; reactions were initiated by the simultaneous addition of 1.5 µM actin (5% labeled with pyrene, primed for 90 s with 1 mM EGTA and 0.1 mM MgCl₂) and 1 nM glutathione S-transferase-verprolin homology, cofilin homology, acidic domain. The delay between mixing of reactants and recording of fluorescence was 15 s. Fluorescence was converted to

the molar concentration of F-actin according to the fluorescence of completely polymerized actin (24 h after reaction) and unpolymerized actin, with the assumption a critical concentration of 0.1 µM.

Immunoblot analysis. Lysates from mouse thymocytes and human EBV-immortalized cell lines were prepared in radioimmunoprecipitation buffer (1% (vol/vol) Nonidet P-40, 0.5% (wt/vol) sodium deoxycholate, 0.1% (vol/vol) SDS, 50 mM Tris, pH 8.0, 150 mM NaCl and 0.02 NaN₃) and were resolved by PAGE (NuPage; Invitrogen), then were transferred (XCell; Invitrogen) to Immobilon-FL membranes (Millipore), were blocked and probed with LICOR buffer and were analyzed on an Odyssey infrared imager (LICOR). Anti-Coro1A (07-493) was from Upstate Biotechnology, anti-actin (A2066) was from Sigma, and secondary IRDye antibody (611-730-127) was from Rockland.

Transwell migration. Thymocytes and splenocytes collected in migration medium (RPMI medium (Cellgro) supplemented with 10 mM HEPES, pH 7.2 (Cellgro), 100 U/ml of penicillin-streptomycin (Cellgro) and 0.5% (vol/vol) fatty acid-free BSA (Calbiochem)) were used for assays of migration through Transwells (5 µm; Corning). For analysis of migration to S1P, thymocytes were assayed immediately after isolation. For analysis of migration to chemokines, thymocytes or splenocyte samples in which erythrocytes were lysed were resensitized in migration media for 30 min at 37 °C. S1P (Sigma), the human chemokine SDF-1α (Peprotech) and mouse CCL21 and CXCL13 (R&D Systems) were used at various concentrations.

Adoptive cell transfer and treatment. Thymocytes were labeled with the cytosolic dye CFSE (carboxyfluorescein diacetate succinimidyl diester) or CellTracker orange CMTMR ((5-(and-6)-(((4-chloromethyl)benzoyl)amino)-tetramethylrhodamine); Molecular Probes) as described³⁶. For adoptive transfer, mice were injected intravenously with about 1×10^7 to 3×10^7 cells in about 0.3 ml media. For homing assays, mice were analyzed 1 h after transfer. For assays of peripheral egress, mice were analyzed 24 h after transfer or were treated intravenously for 20 h with entry-blocking antibodies and were analyzed after 44 h. Antibodies used for *in vivo* entry blockade have been described³⁷.

Two-photon microscopy. Thymocytes from donor mice were isolated and were labeled with CellTracker orange CMTMR (Invitrogen-Molecular Probes) as described³⁸, then were injected intravenously into B6 recipient mice along with thymocytes from UBC-GFP mice. After 12–24 h, inguinal lymph nodes were isolated for two-photon microscopy, and axillary and brachial lymph nodes were isolated for flow cytometry. Flow cytometry confirmed that over 90% of CMTMR⁺ cells in lymph nodes were mature SP thymocytes (CD4⁺CD8⁻CD69^{lo}CD62L^{hi} or CD4⁺CD8⁺CD69^{lo}CD62L^{hi}). Explants were prepared as described³⁹. Inguinal lymph nodes were perfused with RPMI medium (Cellgro) at 36–37 °C, were aerated with 95% O₂ and 5% CO₂ and were imaged from the cortical side of the lymph node through the capsule. A 'custom' two-photon microscope system was used for imaging, with the 5-W MaiTai Ti:Sapphire laser (Spectra-Physics) tuned to an excitation wavelength of 890 nm. Images were captured with Video Savant software (IO Industries). For time-lapse imagery, 30–36 planes 3 µm apart were collected every 20 s. Each xy plane spanned 240 µm × 288 µm at a resolution 0.6 µm per pixel. Data were further processed with MetaMorph software (Molecular Devices). Imaris software (Bitplane AG) was used for three-dimensional volume rendering and cell tracking. Matlab software was further used to calculate cell velocities, turning angles and displacement. Statistical significance was evaluated with a paired two-tailed Student's *t*-test.

Genetic mapping, SNP analysis, human samples and sequencing. CTS/Shi mice were crossed with B6 mice to generate an F₁ generation. The *Ptcd* locus was mapped with mutant F₂ progeny of F₁ intercrosses, F₁ mice backcrossed to nonparental CTS mice, or mutant F₂ mice crossed with nonparental F₁ mice. Mice were phenotyped by analysis of thymocyte subset frequencies by flow cytometry. Genomic DNA was isolated from tail tissue (Wizard Genomic kit; Promega) and the Amplifluor HT kit (Chemicon, Millipore) and an ABI7300 (Applied Biosystems) were used according to the manufacturers' instructions for SNP reactions. Mapping SNPs were selected on the basis of polymorphisms between B6 mice and BUB/J, a strain closely related to CTS/Shi. SNP primers were designed on the AssayArchitect website. Patients with SCID and their

parents, where available, were enrolled with informed content in studies with Institutional Review Board–approved protocols for determination of genotype and genotype-phenotype correlations. Blood samples were immortalized with EBV to create B cell lines. Human genomic DNA was isolated from buccal swabs or EBV-immortalized lines with a Puregene kit (Gentra Systems). An ABI7300 was used for quantitative PCR of genomic DNA. Human *CORO1A* and mouse *Coro1a* exons and surrounding splice regions were amplified by PCR from genomic DNA, were resequenced and were analyzed on 4Peaks software (mekentosj) relative to the reference human genomic and cDNA sequences NM_007074 and NM_007074.2, respectively (primers, **Supplementary Table 1** online).

Brightfield and immunofluorescence microscopy. For fixed immunofluorescence imaging, 1×10^6 to 2×10^6 lymphocytes from peripheral and mesenteric lymph nodes were seeded onto chambered coverslips (Lab-tek, Nunc) pre-coated with recombinant mouse intercellular adhesion molecule 1–Fc fragment (10 μ g/ml; R&D Systems) and were allowed to settle and migrate for 1 h at 37 °C in the presence of CCL21 (1 μ g/ml; R&D Systems) in RPMI medium (Cellgro) supplemented with 1% (vol/vol) FCS (Invitrogen), 10 mM HEPES, pH 7.2 (Cellgro) and 100 U/ml of penicillin-streptomycin. Cells were then fixed with 4% (wt/vol) paraformaldehyde (Sigma) and were made permeable with 0.5% (vol/vol) Triton-X100 (Sigma) in PBS. Coverslips were then stained with a combination of the following: anti-Coro1A (rabbit polyclonal antibody; 07-493; Millipore, Upstate) or anti-p34Arc (rabbit polyclonal antibody to the Arp2/3 subunit p34; 07-227; Millipore; Upstate), fluorescein isothiocyanate-conjugated phalloidin (Sigma), Alexa Fluor 647-conjugated anti-CD4 (RM4-5; BD), Alexa Fluor 647-conjugated anti-CD8 (53-6.7; BD), biotinylated goat anti-rabbit (BD), streptavidin-indocarbocyanine (Jackson Immuno) or 4,6-diamidino-2-phenylindole (Molecular Probes). The z-stacks were captured on an Axiovert Z1 (Zeiss) and were deconvoluted with Axiovision Software (Zeiss). For real-time imaging, 5×10^5 lymphocytes from peripheral and mesenteric lymph nodes were stained with phycoerythrin-conjugated anti-CD19 (1D3; BD) and Alexa Fluor 647-conjugated anti-CD4 and anti-CD8 and were seeded on intercellular adhesion molecule-coated chambered coverslips with CCL21 as described above with the addition of 0.1% (wt/vol) NuSieve GTG Low Melt agarose (FMC BioProducts) and 4,6-diamidino-2-phenylindole. Cells were maintained at 37 °C and 5% CO₂ during the 3–4 h of imaging in an environmentally controlled stage on an Axiovert Z1. Each 5-minute movie was generated at time-lapse intervals of 10 s, with assembly of a z-stack at each time point from six brightfield xy views with a z-step of 0.5 μ m. After the time lapse, a fluorescence image was captured to identify live T cells. Movies and images were processed with Axiovision (Zeiss) and were exported for annotation and splicing with AfterEffects (Adobe).

Note: Supplementary information is available on the Nature Immunology website.

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