to investigate whether other mouse homologues of fly modifiers of Abl, such as *prospero*[27,28] and *ena*[29,30], are involved in Reelin function. □

Methods

Mouse crosses and PCR mapping. Genetic mapping was performed in a cross between *scrambler* (C3HeB/FeJLe background) and C57BL/6J mice. DNA was extracted from tissues isolated from F_2 progeny and genotyped by PCR analysis. 500 ng of genomic DNA was amplified by PCR as described[31]. Reaction products were electrophoresed on 8–10% denaturing PAGE, the gels were dried and exposed to Kodak X-OMAT film for 8–16 h at room temperature. The exon of *mdab1* present in YAC403F6 and BAC254G15 was amplified using two primers: exon 1, (GTCAGGATCGCAGCGAAGCCACTTTG); and exon 2, (CCTTGAGCTTCATCATGGAATCTTG), which amplify the region between 331 and 471 (positions relative to the first nucleotide of the mDab1 Genbank file, accession number Y08379).

Northern blot hybridization. Total RNA was isolated from brains of postnatal-day-14 mice and poly(A)$^+$ mRNA was prepared from postnatal-day-7 mice using an Invitrogen kit following the manufacturer's instructions. 10 μg of total or 3 μg poly(A)$^+$ mRNA was loaded per lane on a 1.0% agarose–formaldehyde gel and hybridized with a full-length *mdab1* or *reelin* cDNA probe as described[12]. The same blots were reprobed with riboprobes synthesized from the pTRI-GAPDH template (Ambion) using T7 RNA polymerase (Promega).

RT-PCR analysis. 100 ng poly(A)$^+$ RNA isolated as described was reverse-transcribed and 1 : 20 of each RT reaction was subjected to RT-PCR analysis with the Takara LA-PCR kit using cycle conditions recommended by the manufacturer. Three PCR primer pairs spanning the coding region of *mdab1* were used. The starting and ending bases of each primer pair are as follows, with the forward and reverse primers in each pair, respectively, listed in 5′ to 3′ orientation. Primer pair 1: 19–48, 471–447; primer pair 2: 331–356, 1,123–1,091; primer pair 3: 1,091–1,123, 2,116–2,092.

Brain extract preparation and immunoblot analysis. Protein extracts of mouse brains were prepared by Dounce-homogenizing brains snap-frozen in liquid nitrogen in 500 μl ice-cold lysis buffer (0.1% N-P40, 250 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 2 mM PMSF, 20 μM leupeptin, 50 mM NaF) per 100 mg tissue. Extracts were cleared by microcentrifugation at 14,000 r.p.m. for 30 min. 100 μg protein extract was loaded per lane of a 4–12% polyacrylamide gradient gel (Novagen), electrotransferred to nitrocellulose membranes, incubated with a rabbit polyclonal antibody directed against a the PTB domain of mDab1 (antibody B3; ref. 12), or with an anti-Cdk5 antibody (Santa Cruz), and visualized by enhanced chemiluminescence (ECL, Boehringer Mannheim).

***In situ* hybridization.** Timed pregnant females were killed by cervical dislocation. Embryos were fixed overnight in 4% paraformaldehyde (in 0.1M phosphate buffer; pH 7.2), cryoprotected with 20% sucrose in buffer, embedded, and sectioned (10 μm). Radioactive *mdab1* (nucleotides 1,935–2,116) and *reelin* (nucleotides 5,818–5,973) sense and antisense probes were generated by *in vitro* transcription using ^{33}P-UTP. Hybridization was done overnight at 60 °C in a humid chamber as described[1]. After washing, sections were counterstained with 0.1% toluidine blue and coverslipped.

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Neuronal position in the developing brain is regulated by *mouse disabled-1*

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During mammalian brain development, immature neurons migrate radially from the neuroectoderm to defined locations, giving rise to characteristic cell layers[1,2]. Here we show that targeted disruption of the *mouse disabled1* (*mdab1*) gene[3] disturbs neuronal layering in the cerebral cortex, hippocampus and cerebellum. The gene encodes a cytoplasmic protein, mDab1 p80, which is expressed and tyrosine-phosphorylated in the developing nervous system[3]. It is likely to be an adaptor protein, docking to others through its phosphotyrosine residues and protein-interacting domain[4]. The *mdab1* mutant phenotype is very similar to that of the *reeler* mouse[5–7]. The product of the *reeler* gene, Reelin,

is a secreted protein that has been proposed to act as an extracellular signpost for migrating neurons[8–10]. Because mDab1 is expressed in wild-type cortical neurons, and Reelin expression is normal in *mdab1* mutants, mDab1 may be part of a Reelin-regulated or parallel pathway that controls the final positioning of neurons.

To determine the function of mDab1 p80, we made a targeted disruption of the first 47 codons of the protein-interacting (PI/PTB) domain (Fig. 1a). Mice heterozygous for the altered allele (*mdab1-1*) were generated by standard blastocyst manipulation and mouse breeding (Fig. 1b). In over 200 births, homozygous *mdab1-1* mutants were born with the expected frequency. Western blot analysis of brain lysates showed that the mDab1 p80 protein is absent in the homozygotes (Fig. 1c).

The *mdab1-1* homozygotes seem normal until 10 days postpartum (P10). By P15, it is apparent that *mdab1-1* homozygotes are ataxic: they tremble, walk with a wide gait, drag their hind limbs, and frequently flip onto their backs. The mice generally die between P20 and P30.

The brains of *mdab1-1* mutant mice have multiple defects when studied at P25 (Fig. 2, and data not shown). The cerebral cortex of the *mdab1-1* mutant (Fig. 2b) lacks the distinct cell layers of the wild type (Fig. 2a). The cell-poor layer (marginal zone) under the pial (outer) surface is infiltrated by neurons in the mutant. In addition, fibre bundles are detected running close to the pial surface, suggesting that afferent fibres are running obliquely instead of radially through the cortex[11]. The hippocampus and the dentate gyrus in the *mdab1-1* mice are also indistinct. Normally, large pyramidal neurons form discrete layers marking the dentate gyrus and CA1 and CA3 regions of the hippocampus (Fig. 2c), but in the mutant the large pyramidal neurons are dispersed, although vestiges of the normal structures are visible (Fig. 2d).

Cerebellar development is also severely affected. A normal cerebellum at birth consists of a sheet of Purkinje cells several cells thick covered by a superficial external granular layer. Starting at about P5, the Purkinje cells disperse to form a monolayer and the granule cells of the external granular layer proliferate and migrate inwards to form the inner granule layer. By P25, the normal cerebellum has an outer, cell-poor layer (the molecular layer), a single layer of Purkinje cells with dendritic arbors extending into the molecular layer, a broad inner granule layer, and an underlying layer of white matter (Fig. 2e). The mature cerebellum covers the midbrain. In contrast, the cerebellum of a *mdab1-1* mutant at P25 is small and unfoliated, and the midbrain is exposed. The Purkinje cells are present in a

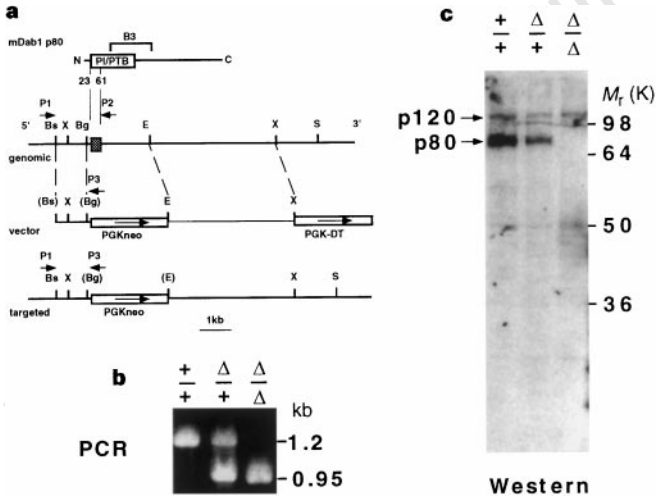

Figure 1 Disruption of the *mdab1* gene. **a**, Structure of the mDab1 p80 protein, with PI/PTB domain, binding site for B3 antibody, and the disrupted exon indicated. The knockout vector was designed with the phosphoglycerate kinase (PGK) promoter driving neomycin phosphotransferase in place of 2 kb of genomic sequences that contained the first exon of the PI/PTB domain. Homologous sequences of 0.9 kb (5′) and 4 kb (3′) flank the PGKneo cassette. Non-homologous integrants were counter-selected with the PGK-diphtheria toxin (DT) cassette. **b**, PCR genotyping of neonates. Oligonucleotides P1 and P2 hybridize to genomic sequences outside the homologous regions, as shown in **a**, and produce a 1.2-kb band by PCR amplification of the wild-type *mdab1* allele. Oligonucleotides P1 and P3 (hybridizing to the 5′ end of the PGK promoter) amplify a 0.95-kb fragment from the *mdab1-1* mutant allele. **c**, Western blot of brain lysate from neonate F_1 littermates probed with anti-mDab1 (B3) polyclonal antibody. mDab1 p80 is underexpressed in heterozygous animals and absent in the *mdab1-1* homozygote. The 120K immunoreactive protein present in wild-type and mutant animals is expressed early in development but not in adults[3], and is either a spliced *mdab1* gene product lacking the PI/PTB domain, or the product of a closely related gene.

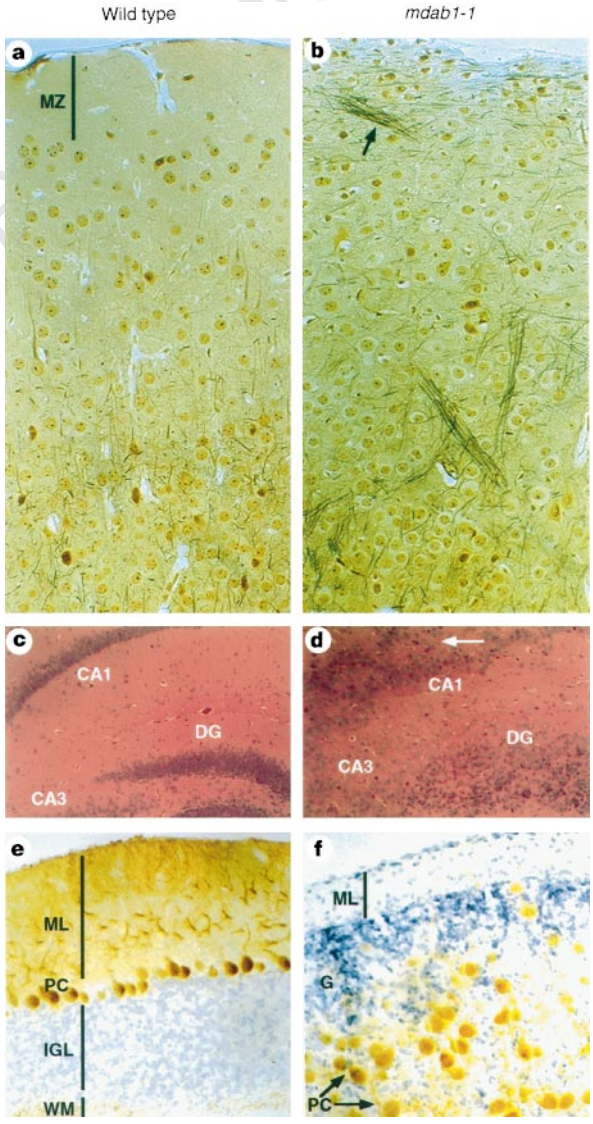

Figure 2 Alterations in the *mdab1-1* mutant brain. Coronal sections of neocortex (Bielschowski stain) (**a**, **b**), hippocampus (haematoxylin and eosin stain) (**c**, **d**) and cerebellum (**e**, **f**) (brown, anti-Calbindin antibody; blue, Nissl stain) of wild-type (**a**, **c**, **e**) and *mdab1-1* (**b**, **d**, **f**) mice. Arrows mark aberrant nerve fascicles (**b**) and split CA1 tract (**d**) in the mutant. In **f**, note that Nissl-stained granule cells are superficial to misclocalized calbindin-containing Purkinje cell bodies and dendritic arbors. Abbreviations: MZ, marginal zone; DG, dentate gyrus; ML, molecular layer; PC, Purkinje cells; G, granule cells; IGL, inner granule layer; WM, white matter.

central mass and their dendrites are oriented randomly (Fig. 2f), and they appear to have matured normally, because they express both calbindin (Fig. 2f) and zebrin II (ref. 12; data not shown). The granule cells are few in number and, although some migrate inwards, most remain superficial to the Purkinje cells (Fig. 2f). The anomalies in granule-cell proliferation and relative position may be secondary to the failure of the Purkinje cells to disperse into a monolayer[11].

In the neocortex, the position and fate of a neuron is strongly correlated with its birthdate[2]. Birthdate studies have been done by labelling mice *in utero* with DNA precursors, and identifying labelled neurons in the cortex after birth[13]. Cells undergoing their last S phase during the labelling period retain the label in their DNA, whereas cells that continue to cycle dilute the label over time. To test whether cortical neurons migrate correctly in *mdab1-1* mutant mice, we treated mice *in utero* with 5-bromodeoxyuridine (BrdU) and analysed their brains at P25. Nuclei that were labelled with BrdU at embryonic day 11 (E11) were found deep in the cortex of wild-type animals (layer VI)[13], but were superficial in the *mdab1-1* mutants (Fig. 3a, b). Conversely, neurons labelled at E16 were predominantly in the superficial cortex (layers II–III) of wild-type mice[13], but remain deep in the cortex of *mdab1-1* mutant littermates (Fig. 3c, d). This shows that the final positions of cortical neurons are abnormal in *mdab1-1* mutant mice. Because large pyramidal cells, normally located deep in the cortex, are found near the surface of the mutant cortex (Fig. 2b, and data not shown), the abnormal layering of the mutant cortex may result from altered migration of neurons without an alteration in fate. Experiments with molecular markers will be needed to test whether cell fate is also altered.

These observations indicate that *mdab1-1* mutant mice resemble *reeler* mutants in neuroanatomy and neuron birthdates[14–16]. Indeed, detailed histology of mature *mdab1-1* and *reeler* cerebella shows identical morphologies and the same abnormal compartmentation of Purkinje cells (data not shown). In both cases, the defects may be a consequence of altered neuronal migration. This suggests that the proteins mDab1 and Reelin could be involved in the same or parallel pathways that regulate neuronal migration. Reelin has been proposed to serve as a marker for the locality at which radially migrating cortical neurons come to rest[8,9]. Reelin is expressed by pioneer Cajal–Retzius neurons that occupy the cell-poor marginal zone of the neocortex. The *mdab1-1* phenotype raises the possibility that mDab1 might be needed either to make or to respond to the Reelin signal, or to convey another signal that acts in concert with the Reelin pathway.

mDab1 does not seem to be required for Cajal–Retzius cell migration or Reelin production, because indirect immunofluorescence shows that the Cajal–Retzius cells are present in the marginal zone and express Reelin in the *mdab1-1* neocortex at E16 (Fig. 4a, b). To test whether mDab1 might be in the cells responding to the Reelin signal, we localized mDab1 in the E16 cortex by immunofluorescence (Fig. 4c–e), and detected it in the cytoplasm of nearly all neurons in the developing cortical plate (Fig. 4c). mDab1 was found throughout the cortical plate, intermediate zone, and ventricular zone of the wild-type embryos (Fig. 4d). The reduced staining of mutant embryos (Fig. 4e) shows that staining is specific. Thus mDab1 is expressed by cortical neurons, consistent with it acting in response to external signals such as Reelin.

In the E16 wild-type but not mutant cerebellum, mDab1 was expressed in the cerebellar anlage, where the Purkinje cells are coalescing (Fig. 4f, g). Expression of mDab1 was detected in the external granular layer, and it is unlikely that Bergmann glia express mDab1 because they have cytoplasmic projections that would have been detected in the external granular layer. Because Purkinje cells in *mdab1-1* mutants are malpositioned at P0 before the start of granule cell ingress (data not shown), the primary defect in the mutant cerebellum may be the result of defects in the Purkinje cells. It has been shown previously that granule cells depend on adjacent Purkinje cells for trophic support[17], and that granule cells make Reelin[18]. Because Reelin expression is not altered in the *mdab1-1* mutants, and because mDab1 is expressed by the affected cell types in the neocortex and cerebellum, it seems likely that mDab1 acts cell-autonomously.

mDab1 has no known catalytic activity, so it may function as a docking protein to link proteins together through its amino-terminal PI/PTB domain and tyrosine-phosphorylated motifs[3].

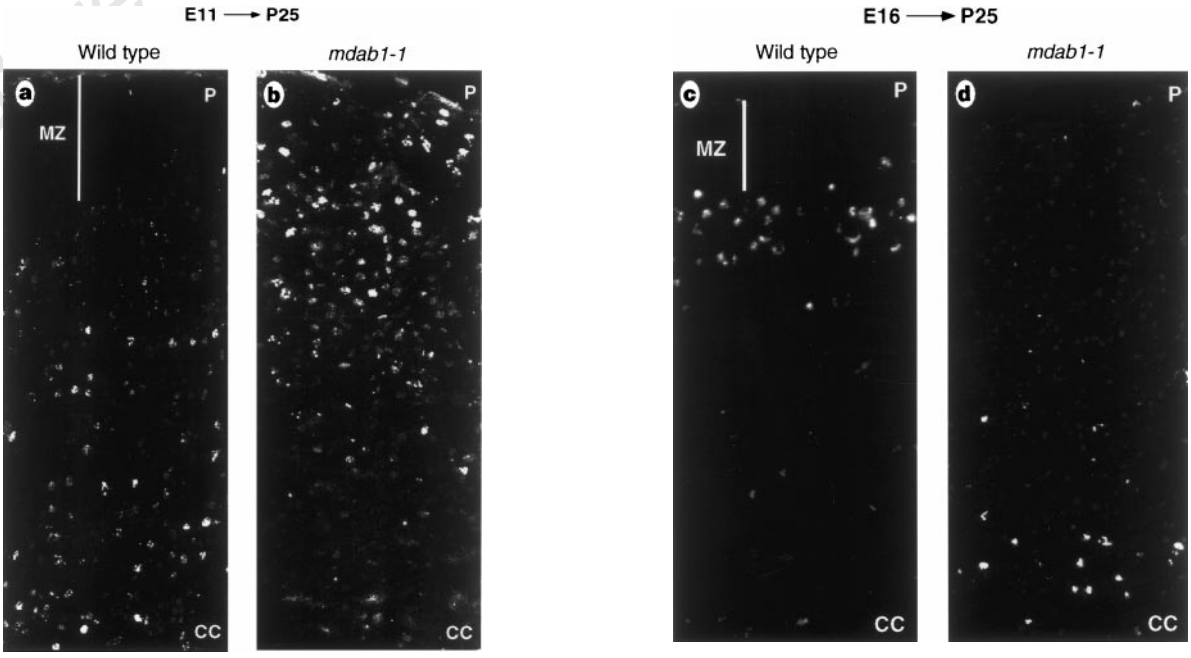

Figure 3 Altered positions of *mdab1-1* neurons in cerebral cortex. Animals were labelled with BrdU *in utero* at E11 (**a**, **b**) or E16 (**c**, **d**) and killed at P25. Coronal sections of cortices at the level of the hippocampus were prepared from wild-type (**a**, **c**) and mutant (**b**, **d**) littermates, and immunostained with anti-BrdU antibodies (white). P, pia; CC, corpus callosum.

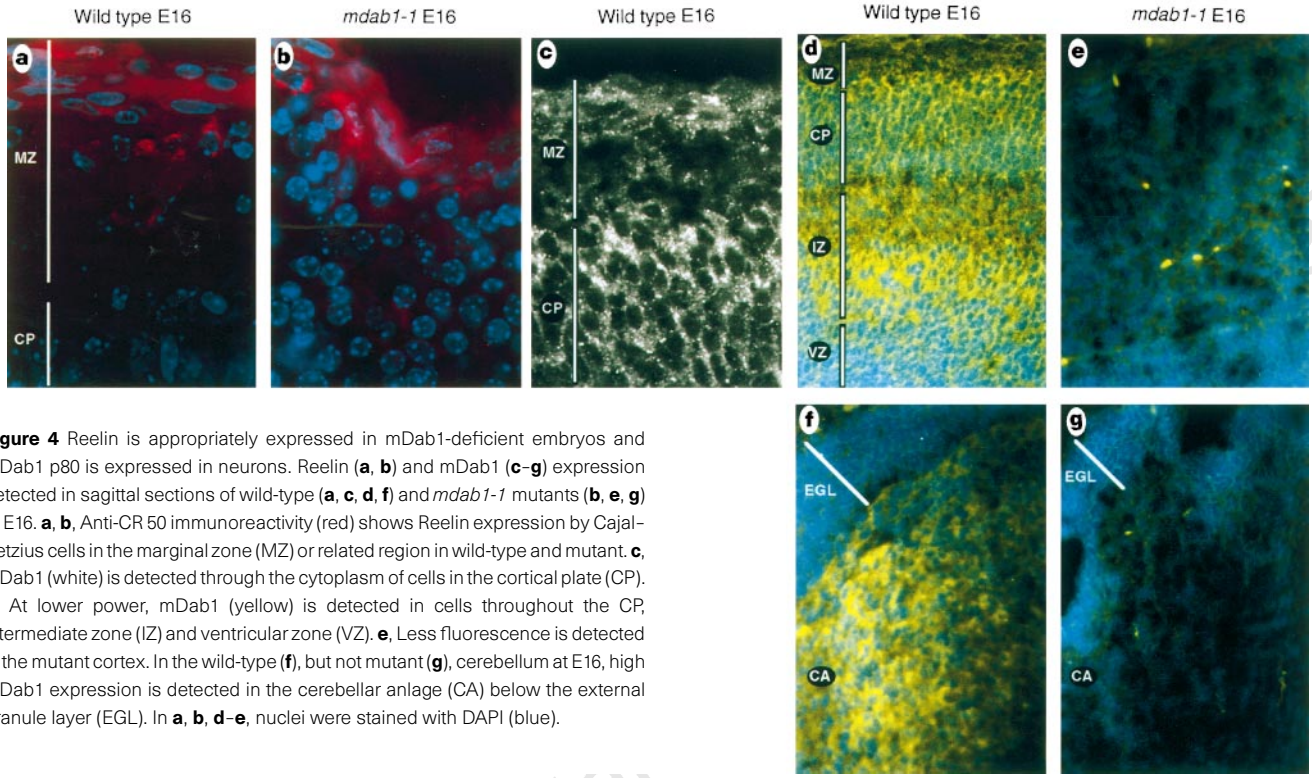

Figure 4 Reelin is appropriately expressed in mDab1-deficient embryos and mDab1 p80 is expressed in neurons. Reelin (**a**, **b**) and mDab1 (**c**–**g**) expression detected in sagittal sections of wild-type (**a**, **c**, **d**, **f**) and *mdab1-1* mutants (**b**, **e**, **g**) at E16. **a**, **b**, Anti-CR 50 immunoreactivity (red) shows Reelin expression by Cajal–Retzius cells in the marginal zone (MZ) or related region in wild-type and mutant. **c**, mDab1 (white) is detected through the cytoplasm of cells in the cortical plate (CP). **d**, At lower power, mDab1 (yellow) is detected in cells throughout the CP, intermediate zone (IZ) and ventricular zone (VZ). **e**, Less fluorescence is detected in the mutant cortex. In the wild-type (**f**), but not mutant (**g**), cerebellum at E16, high mDab1 expression is detected in the cerebellar anlage (CA) below the external granule layer (EGL). In **a**, **b**, **d**–**e**, nuclei were stained with DAPI (blue).

This function may be regulated by extracellular signals. Thus proteins that modify and bind to mDab1, including non-receptor tyrosine kinases such as Src and Abl[3], may regulate neuronal migration. Mutations in other components of the signalling pathway might be expected to cause a phenotype like that of *mdab1*. Mutations in *src* and *abl* do not affect brain development[19–21], but these tyrosine kinases may be redundant. Phenotypes resembling that of *mdab1-1* mutants are seen with mutations in *scrambler* (ref. 22), *yotari* (ref. 23), Cdk5 (ref. 24) and *p35* (ref. 25); *scrambler* and *yotari* are alleles of *mdab1* (ref. 26). Cdk5 and p35 are the catalytic and regulatory subunits of a serine/threonine kinase that could potentially operate in a common signalling pathway with mDab1 and Reelin. It remains possible, however, that these molecules operate on mechanistically distinct processes that control the laminar organization of neurons.

Disabled, the *Drosophila* homologue of *mdab1*, is required for axonal pathfinding or fasciculation, and acts together with *abl*[27,28]. Redundant pathways regulate pathfinding[29], but it is not clear from this whether Abl and Disabled are on the same or parallel pathways. However, there are a number of parallels between axonal pathfinding and cell migration, and Disabled-like molecules may be involved in both processes. Pathfinding defects have not yet been observed in the *mdab1-1* mice. The *reeler* mutation affects some neuronal connections[30] but not others[11]. Identification of mDab1 binding partners and a receptor for Reelin will help us to understand how the activities of these proteins coordinate neuronal migration and axonal guidance. □

Methods

Production of mutant mice. The *mdab1-1* targeting vector was constructed in the PGKneolox2DTA vector. A 0.9-kb *Bse*RI–*Bgl*I fragment, which corresponds to intronic sequences 5′ to the exon encoding residues 23–69 of the *mdab1* gene, was blunted and ligated into *Not*I-digested vector. A 4-kilobase *Eco*RI–*Xba*I fragment from the *mdab1* gene 3′ to the same exon was linkered and ligated 3′ into the *Sal*I site between the PGKneo and PGK-DT sequences producing p80KO1. AK7 embryonic stem (ES) cells (1×10^7) were electroporated with linearized p80KO1 (20 μg). Cell culture and blastocyst injections were done as previously described[19]. Oligonucleotides used for PCR genotyping (Fig. 1) were P1 (5′-GTCAGGCTTCCTAAGTAGAAAGGA-3′), P2 (5′-TTCC AGGAGCGAAATCACTCAACC-3′) and P3 (5′-GGGAAAAGCGCCTCCCC TACCCGGT-3′). Similar phenotypes were observed with two independently-derived ES clones, and in 129Sv congenic or C57Bl6/129Sv hybrid genetic backgrounds.

Histology and immunohistochemistry. Animals were fixed by perfusion with 4% paraformaldehyde in phosphate-buffered saline at 4 °C, except for anti-mDab1 immunofluorescence studies in which a solution of dimethyl sulphoxide and methanol (1 : 4) was used. Haematoxylin and eosin, Nissl and Bielschowski stains were done following standard protocols. Anti-CR 50, anti-BrdU (Becton Dickinson) and anti-calbindin immunohistochemistry have been described previously[9,25]. An antibody specific to mDab1 p80 was generated by depleting anti-mDab1 (B3) antibodies of reactivity to p120 by adsorption with lysates from *mdab1-1* mutant brains, and used essentially as described[3]. For birthdate analysis, BrdU was injected into pregnant mice (0.15 mg per g body weight) at the stage indicated[16]. Immunofluorescence images were collected using either Delta Vision (Applied Precision) or confocal microscopes (Fig. 4c; BioRad).

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A CD4$^+$ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis

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Induction and maintenance of peripheral tolerance are important mechanisms to maintain the balance of the immune system. In addition to the deletion of T cells and their failure to respond in certain circumstances, active suppression mediated by T cells or T-cell factors has been proposed as a mechanism for maintaining peripheral tolerance[1]. However, the inability to isolate and clone regulatory T cells involved in antigen-specific inhibition of immune responses has made it difficult to understand the mechanisms underlying such active suppression. Here we show that chronic activation of both human and murine CD4$^+$ T cells in the presence of interleukin (IL)-10 gives rise to CD4$^+$ T-cell clones with low proliferative capacity, producing high levels of IL-10, low levels of IL-2 and no IL-4. These antigen-specific T-cell clones suppress the proliferation of CD4$^+$ T cells in response to antigen, and prevent colitis induced in SCID mice by pathogenic CD4$^+$CD45RB^{high} splenic T cells. Thus IL-10 drives the generation of a CD4$^+$ T-cell subset, designated T regulatory cells 1 (Tr1), which suppresses antigen-specific immune responses and actively downregulates a pathological immune response *in vivo*.

Immune tolerance towards self antigens is dependent on the ability of the immune system to discriminate between self and non-self[2,3]. This occurs mainly through clonal deletion in the thymus of self-reactive T lymphocytes at an early stage of development. However, the immune system is also exposed to extrathymic self antigens and to repetitive stimulation by non-pathogenic antigens through inhalation and ingestion of foreign substances. To avoid chronic cell activation and inflammation, the immune system must develop unresponsiveness to such stimuli. Several mechanisms of peripheral tolerance have been proposed, including T-cell anergy[4,5], T-cell deletion[2], and active immune suppression[1]. Understanding these mechanisms of tolerance induction is clinically relevant for the treatment of autoimmune diseases and in transplantation, where the graft must ultimately be recognized as self.

The induction of anergy[5] and cell deletion by apoptosis[2] have been documented both *in vitro* and *in vivo*, but the analysis of active immune suppression mediated by T cells has been hampered by the inability to generate and clone these cells *in vitro*. The importance of cytokines in the development of specialized Th cells is now clear. Th1 cells are induced by activation in the presence of IL-12, whereas IL-4 drives the differentiation of Th2 cells[6]. Here we show that repetitive stimulation of CD4$^+$ T cells in the presence of IL-10 induces the differentiation of a unique subset of T cells with immunoregulatory properties.

Ovalbumin (OVA)-specific naive CD4$^+$ T cells obtained from the $\alpha\beta$ T-cell antigen receptor (TCR) DO11-10 transgenic mice[7] repeatedly stimulated with splenic antigen-presenting cells (APCs) and OVA peptide in the presence of IL-10, or IL-4 and IL-10, displayed a cytokine profile distinct from that of the classical Th0, Th1 or Th2 phenotype[8]. These T cells produce high levels of IL-10 and IL-5 and low levels of IL-2 and IL-4 (Fig. 1). Another characteristic of these T-cell populations is that they proliferated poorly in response to antigenic stimulation (Fig. 2a). In contrast, CD4$^+$ T cells isolated from OVA-specific TCR transgenic mice repeatedly stimulated with OVA peptide in the presence of IL-4 alone displayed the typical Th2-type profile, secreting IL-4, IL-5 and IL-10 (Fig. 1)[9].

Analysis of T-cell clones isolated from the mouse CD4$^+$ T-cell populations that were repeatedly stimulated with OVA peptide and splenic APCs in the presence of IL-10 showed that half of the CD4$^+$ T-cell clones obtained displayed this cytokine profile. They produced high levels of IL-10 and undetectable levels of IL-2 and IL-4, whereas the levels of production of interferon (IFN)-γ and transforming growth factor (TGF)-β were comparable with those of Th0 and Th1 clones, respectively (Table 1). These T-cell clones also proliferated poorly in response to antigen-specific stimulation (Fig. 2a), which may explain the previous failure to isolate these cells. Only Th1, Th2 and Th0 clones were isolated from T-cell populations cultured in the absence of IL-10 (Table 1). Thus chronic activation of mouse CD4$^+$ T cells in the presence of IL-10 results in the generation of a T-cell subset with a unique cytokine profile and low proliferative capacity. These cells are designated Tr1 cells on the basis of the their function.

Tr1 cells can also be generated from human peripheral blood. Both alloantigen-specific CD4$^+$ T-cell clones (JDV24) and non-alloantigen-specific T-cell clones (JDV15, JDV308 and JDV94)

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