Dab1 tyrosine phosphorylation sites relay positional signals during mouse brain development

Brian W. Howell*†, Tara M. Herrick*, Jeffrey D. Hildebrand*, Yanni Zhang* and Jonathan A. Cooper*

Background: The extracellular protein Reln controls neuronal migrations in parts of the cortex, hippocampus and cerebellum. *In vivo*, absence of Reln correlates with up-regulation of the docking protein Dab1 and decreased Dab1 tyrosine phosphorylation. Loss of the Reln receptor proteins, apolipoprotein receptor 2 and very low density lipoprotein receptor, results in a Reln-like phenotype accompanied by increased Dab1 protein expression. Complete loss of Dab1, however, recapitulates the Reln phenotype.

Results: To determine whether Dab1 tyrosine phosphorylation affects Dab1 protein expression and positioning of embryonic neurons, we have identified Dab1 tyrosine phosphorylation sites. We then generated mice in which the Dab1 protein had all the potential tyrosine phosphorylation sites mutated. This mutant protein is not tyrosine phosphorylated during brain development and is not upregulated to the extent observed in the *Reln* or the *apoER2* and *VLDLR* receptor mutants. Animals expressing the non-phosphorylated Dab1 protein have a phenotype similar to the *dab1*-null mutant.

Conclusions: Dab1 is downregulated by the Reln signal in neurons in the absence of tyrosine phosphorylation. Dab1 tyrosine phosphorylation sites and not downregulation of Dab1 protein are required for Reln signaling.

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Background

Tyrosine kinase signaling is critical at many stages of embryonic development. Receptor tyrosine kinases (TKs) and receptor protein tyrosine phosphatases (PTPs) catalyze the phosphorylation and dephosphorylation of intracellular proteins in response to extracellular stimuli. Non-receptor TKs, such as Src and Abl, are regulated indirectly by transmembrane signals from adhesion receptors as well as extracellular soluble ligands [1,2]. Tyrosine phosphorylation of specific proteins is important for their consequent recruitment to signaling complexes that regulate cytoplasmic and nuclear events. Ultimately, TKs and PTPs regulate cell proliferation, malignant transformation, survival, apoptosis, and differentiation (reviewed in [3–5]). In addition, TKs and PTPs regulate the movement of cells and, in the nervous system, the migration of axonal growth cones [6].

Recent evidence has implicated a cytoplasmic adaptor or docking protein, Dab1, in directing the migrations that occur during mammalian brain development [7,8]. Dab1 is tyrosine phosphorylated when many neuronal migrations are taking place [9]. In mice that lack Dab1, laminar organization of the cortex, hippocampus and cerebellum is abnormal [8,10–17]. Abnormalities include a failure of cortical plate neurons to invade the pre-plate and an inversion of the normal temporal order with which cortical layers are formed [18]. In the hippocampus, migrating pyramidal and granule cells fail to find their correct positions, and in the cerebellum, Purkinje cells do not migrate far enough from their birthplace in the neuroepithelium. These phenotypic characteristics are apparently identical to those seen in mice mutant for the *Reln* gene or doubly mutant for the *apoER2* and *VLDLR* genes [19–24]. The identity of phenotypes has led to the model that the *dab1*, *Reln*, and *apoER2/VLDLR* genes operate on the same pathway, and this pathway is amply supported by biochemical evidence.

The Dab1 p80 protein is expressed in migrating cortical neurons from one of several alternatively spliced mRNAs [9–11]. All Dab1 protein isoforms can bind to phospholipids and to the unphosphorylated sequence F/YXNPXY (in the single-letter amino-acid code, where X is any amino acid), found in the cytoplasmic tails of members of the low density lipoprotein receptor (LDLR) and amyloid precursor protein families [20,25–27], through an amino-terminal phosphotyrosine binding (PTB or PID) domain [28]. The extracellular domains of the LDLR family proteins apolipoprotein receptor 2 (apoER2) and very low density lipoprotein receptor (VLDLR) bind to a variety of extracellular molecules, including apoE-containing lipoproteins and the Reln protein, which they transport into cells via coated pits [29,30]. Reln is an extracellular protein

secreted by specialized early neurons that appear to serve as guideposts for subsequent migrations [19,31,32]. Thus, Reln has the potential to signal by binding to the apoER2 and VLDLR receptors, transmitting a signal to Dab1 physically linked to their cytoplasmic tails.

We have previously shown that Reln signaling correlates with an increase in Dab1 tyrosine phosphorylation [33]. The tyrosine phosphorylation of Dab1 isolated from embryonic brains is reduced in *Reln* mutants. In addition, incubation of embryonic cortical neurons with Reln leads to a rapid induction of Dab1 tyrosine phosphorylation, which is blocked by preventing Reln interaction with the apoER2 and VLDLR receptors [29,30]. Reln signaling therefore correlates with Dab1 tyrosine phosphorylation. The biological role of Dab1 phosphorylation is, however, not known.

Dab1 is related to a *Drosophila* protein, Dab, that is also tyrosine phosphorylated [34] and acts in conjunction with the *Drosophila* Abl TK in central nervous system development [35,36]. *Drosophila* Dab also physically interacts with the Sevenless receptor TK and is involved in ommatidial development [37]. Thus, there is a precedent for the regulation of Dab protein function by tyrosine phosphorylation.

Here we assess the importance of Dab1 tyrosine phosphorylation in the developing mouse. Phosphorylation sites were identified on Dab1. Using gene targeting in embryonic stem cells, we generated mice that expressed either the wild-type Dab1 p80 protein or a mutant protein that lacked tyrosine phosphorylation sites from the endogenous *dab1* promoter. Animals expressing wild-type Dab1 protein from the targeted locus developed normally. However, animals making only the unphosphorylated mutant Dab1 showed aberrant brain development that phenocopied the null mutant.

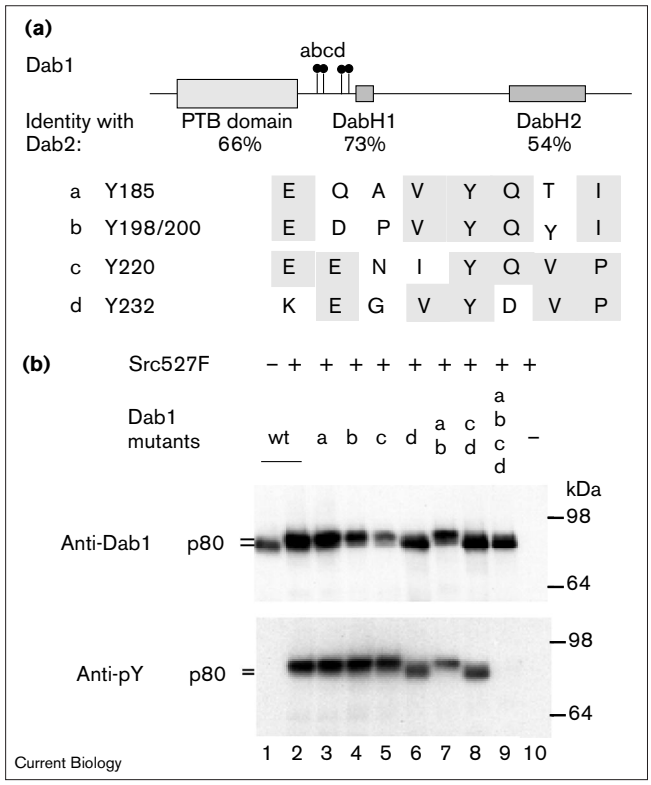
Results

Identification of phosphorylation sites

Inspection of the Dab1 primary sequence reveals four tyrosines, Tyr185, Tyr198, Tyr220, and Tyr232, that are clustered close to the PTB domain (Figure 1a). The sequences surrounding these tyrosines show features characteristic of sites for phosphorylation by many TKs, including an acidic residue at position –3 or –4 relative to the tyrosine [38,39], an aliphatic amino acid at position –1, and a hydrophobic residue or proline at position +3. These are hallmarks of substrates for TKs of the Src and Abl families [38,40–42]. To test whether these tyrosines might be the target for phosphotransfer, several *dab1* mutants were made encoding phenylalanine in place of tyrosine. The potential for phosphorylation was tested for each single mutant and compound mutants in cells that expressed activated Src. Constitutively active Src is known to stimulate kinases in the Abl, Fak and Btk families [43–45] and was therefore used in the hope of identifying the majority of phosphorylation sites on Dab1. Because the sequence around Tyr198 resembles a sequence in the platelet-derived growth factor receptor containing two tyrosines, both of which are thought to be phosphorylated [46], Tyr200 was altered simultaneously with Tyr198.

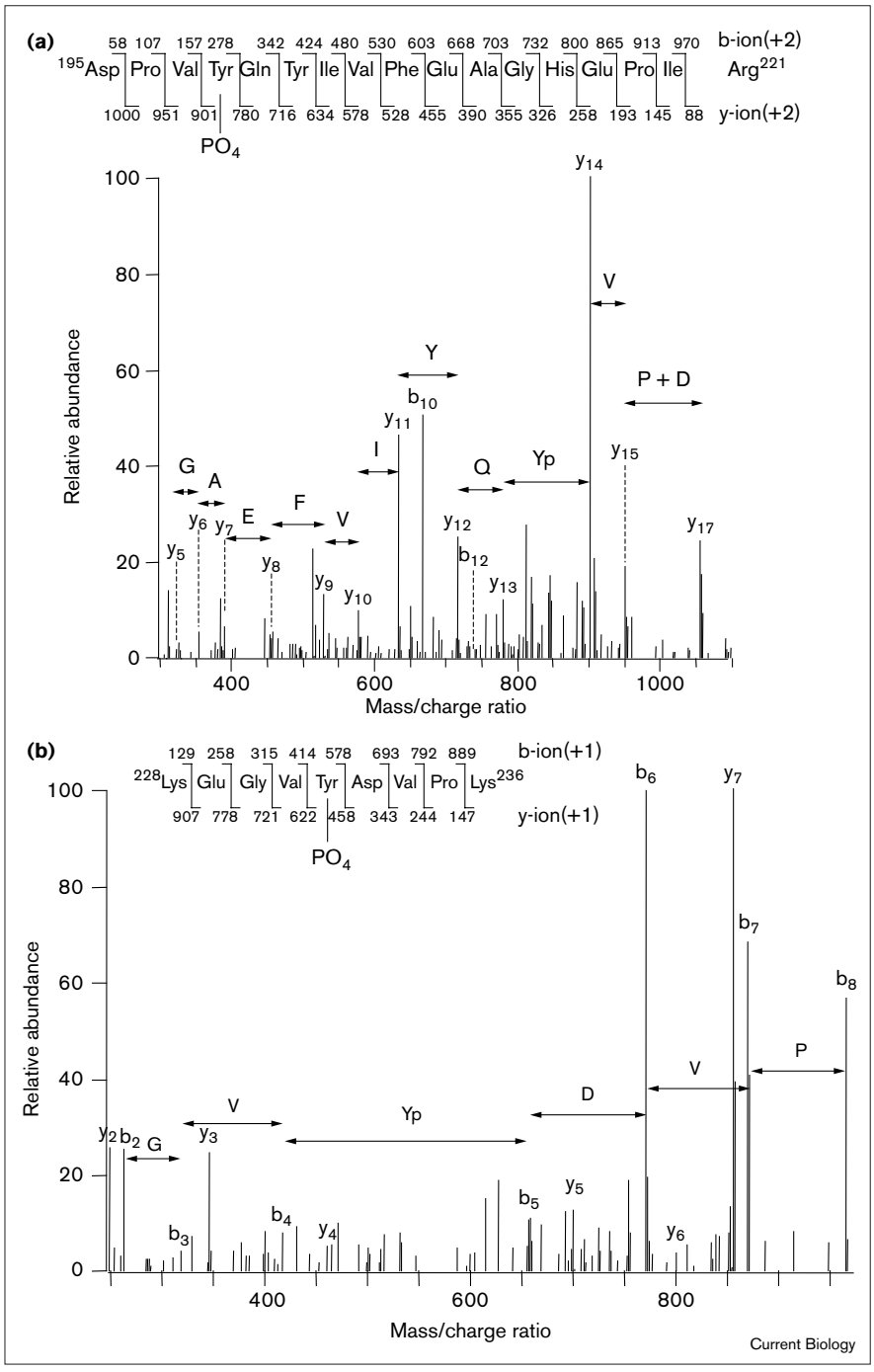
Dab1 expressed in 293T cells is not detectably tyrosine phosphorylated unless co-expressed with the activated Src527F mutant (Figure 1b, lanes 1 and 2). Interestingly, phosphorylation was accompanied by reduced mobility in gel electrophoresis. Mutation of Tyr185, Tyr220 or Tyr232 singly, or Tyr198 and Tyr200 together, did not prevent tyrosine phosphorylation of Dab1. Tyr185 and Tyr198 are in similar sequence motifs, as are Tyr220 and Tyr232

Figure 1



Mutational analysis of tyrosine phosphorylation of Dab1. **(a)** Diagram of Dab1 p80 protein structure, showing three regions of homology to a related mouse gene, Dab2, and the percentage identity. The remainder of the Dab1 protein sequence is not conserved with Dab2. Four potential phosphorylated motifs, including five tyrosines, are shown at positions a, b, c and d. The sequences surrounding the tyrosines are shown below; identical residues are shaded. **(b)** The tyrosines shown in (a) were mutated to phenylalanine singly or in combination. Expression of wild-type Dab1 alone in 293T cells did not lead to tyrosine phosphorylation (lane 1). Coexpression with Src527F led to the phosphorylation of wild-type Dab1 (lane 2), the single and double mutants (lanes 3–8), but not the 5F mutant (lane 9). No equivalent tyrosine phosphorylated proteins were detected in the cells transfected with Src527F alone (lane 10).

Figure 2

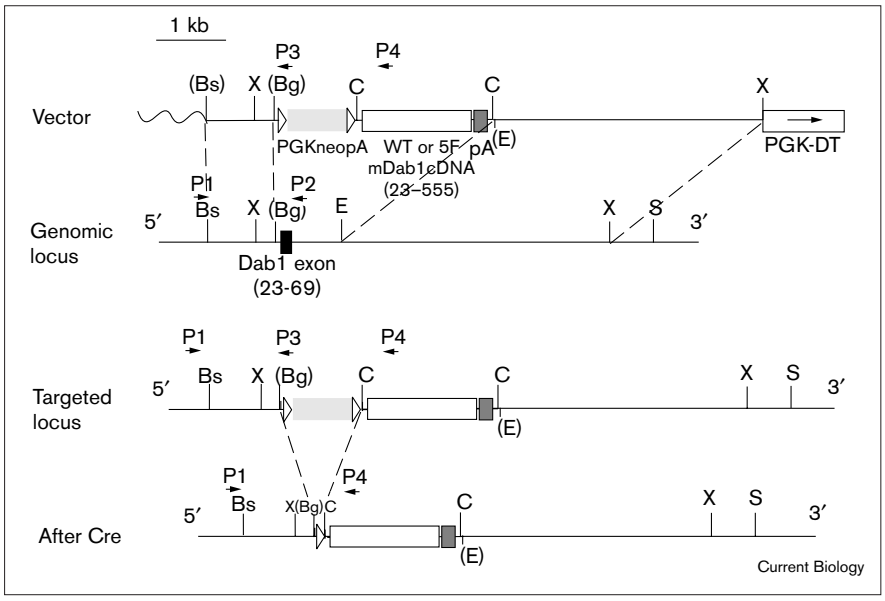


Evidence for phosphorylation of Dab1 at Tyr198 and Tyr232 and binding of Dab1 to SH2 domains *in vitro*. **(a)** Tandem mass spectrometry (MS/MS) analysis of an endoproteinase Asp-N phosphopeptide generated from Dab1 isolated from Src527F-expressing 293T cells. Ions marked are the +2 ions resulting from y- and b-fragmentation of the phosphopeptide shown at the top, which corresponds to the phosphorylation of Tyr198 (y-ions are fragments from the carboxy-terminal side of a peptide bond cleavage; b-ions are from the amino-terminal side). This region of the spectrum also contained several +1 y- and b-ions from the same phosphopeptide, which are not marked for clarity. **(b)** MS/MS spectrum of a trypsin peptide corresponding to Tyr232. Ions shown are +1 y- and b-ions.

(Figure 1a). Paired mutants were analyzed because the similar motifs might be coordinately phosphorylated. These compound mutants were, however, still phosphorylated in the Src527F expressing cells (Figure 1b). Mutation of Tyr232 alone or in conjunction with Tyr220 prevented the phosphorylation-induced mobility shift of Dab1, suggesting that Tyr232 is phosphorylated and responsible for the mobility shift. Mutation of all five tyrosines (5F mutant) decreased tyrosine phosphorylation by Src527F to an undetectable level. This indicates that no other tyrosine residues in Dab1 are targets for Src527F or downstream kinases.

To directly identify phosphorylation sites, we purified Dab1 from Src527F-expressing cells. Peptides were generated with trypsin or endoproteinase Asp-N, purified, and analyzed by electrospray mass spectrometry. Two

Figure 3



Strategy for the replacement of the exon encoding residues 23–69 (black bar) with *dab1* cDNAs. The targeting vector consisted of part of a *dab1* gene intron, a loxP site (triangle), a cassette containing the PGK promoter, the neomycin resistance gene, and a polyadenylation signal (dark grey box), a second loxP site (triangle), a splice acceptor and the sequence encoding residues 23–555 of the Dab1 p80 splice form (white box), some 3′ untranslated region, a triply repeated polyadenylation signal (light gray), and additional genomic sequence. The PGK-driven diphtheria toxin gene (DT) expression was used for selection against nonhomologous recombination. Following homologous recombination, the targeted locus was treated with Cre recombinase to produce the modified allele. Restriction enzyme sites used for mapping: Bs, *Bse*RI; X, *Xba*I; Bg, *Bgl*I; C, *Cla*I; E, *Eco*RI; S, *Sal*I. P1–P4 are oligonucleotides used for PCR analysis (see Materials and methods).

phosphopeptides corresponding to phosphorylation of Tyr198 and Tyr232 were identified (Figure 2a,b). This assay cannot preclude the possibility that Tyr185 and Tyr220 may also be phosphorylated by Src or other kinases, since phosphorylation of a particular peptide can change its physical properties so that it is not recovered after digestion or is not in the appropriate charge-to-mass range for mass spectrometry. Nonetheless, this assay provides evidence that at least two of the sites identified using mutagenesis are phosphorylated when Src is active. In neurons, it is possible that only a subset of the tyrosines identified by this analysis are phosphorylated.

Importance of Dab1 tyrosine phosphorylation for brain development

To determine the role for tyrosine phosphorylation in Dab1 function during development, we expressed a Dab1 protein that lacked tyrosine phosphorylation sites in mutant mice. We used the 5F mutant because it was unlikely to be phosphorylated during embryogenesis. To ensure expression in the appropriate cell types and at normal levels, we expressed the mutant from the endogenous *dab1* locus. A targeting vector was designed to substitute the exon encoding residues 23–69 with a partial cDNA (Figure 3). The introduced DNA consisted of the endogenous splice acceptor and a partial cDNA coding for the p80 splice form of Dab1 from residue 23 to the carboxyl terminus (residue 555). A reiterated polyadenylation cassette followed the stop codon (Figure 3). To control for possible differences in gene expression and for the elimination of *dab1* gene splicing variants, both 5F mutant and wild-type versions of the targeting vector were created. Following homologous recombination in embryonic stem (ES) cells, the locus was modified using Cre recombinase (Figure 3 and see Materials and methods). The final locus contains a single loxP site in the intron separating the exons encoding residues 1–22 from the inserted cDNA.

Table 1

***Dab1-wtki* and *Dab1-5Fki* alleles have no associated embryonic lethality.**

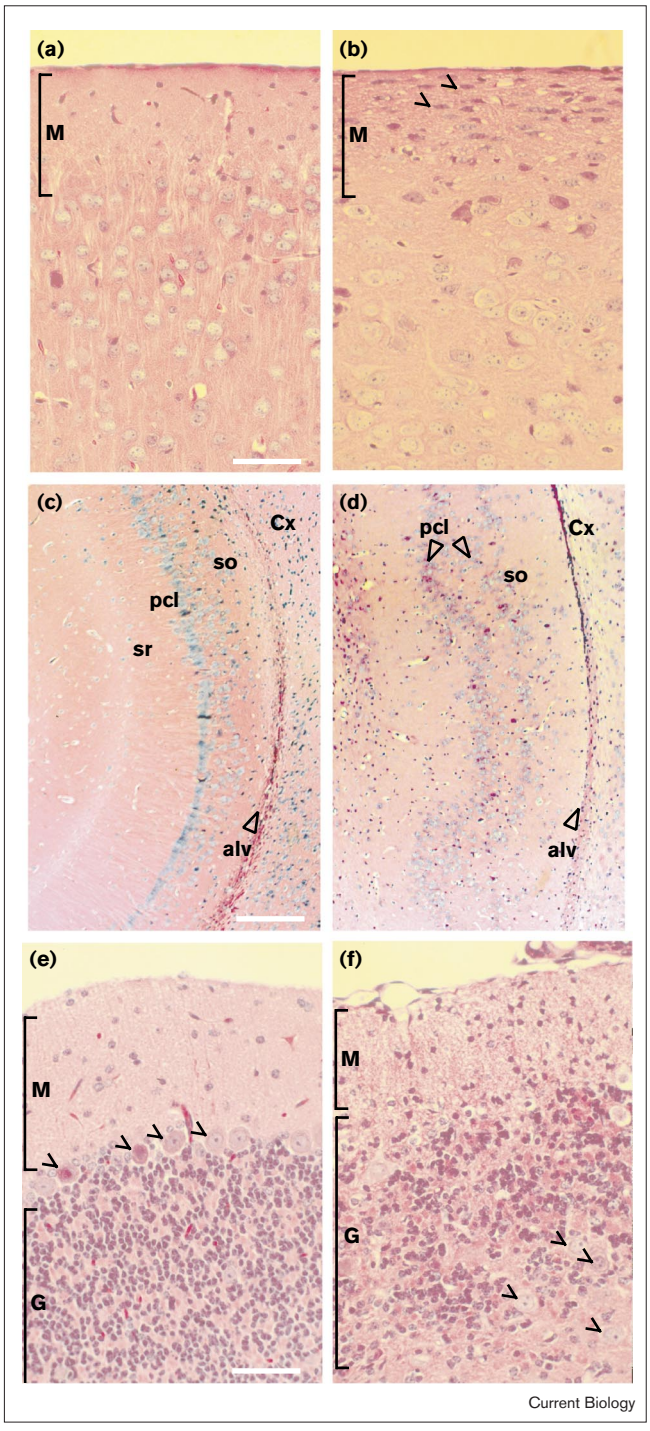
	+/+	*ki*/+	*ki*/*ki*	Total
wtki	15 (14.25)	27 (28.5)	15 (14.25)	57
5Fki	26 (19.75)	33 (39.5)	20*(19.75)	79

Heterozygous parents (*Dab1-ki*/+) were bred, and viable pups were genotyped at 10–14 days after birth. Numbers of pups of each genotype are shown, and numbers predicted from Mendelian inheritance are in parentheses.*All *Dab1-5Fki* homozygotes exhibited behavioral abnormalities and died approximately 20–30 days after birth.

Standard blastocyst injection and transfer techniques were used to generate chimeric mice harboring the wild-type knock-in (*wtki*) or mutant knock-in (*5Fki*) *dab1* alleles. Germline transmission was detected by back-crossing these animals onto the C57BL/6 background; approximately 50% of the agouti animals carried the hybrid alleles (data not shown). Inter-breeding of these progeny produced wild-type, heterozygous and homozygous animals with the expected frequencies (Table 1). These animals were compared with the *dab1-1* null mutant [10], which is phenotypically very similar to alleles in which *dab1* splicing is defective, *dab1-scm* and *dab1-yot* [11,12,15,16].

Figure 4

Histology of *wtki* and *5Fki* mutant brains at postnatal day 20. **(a)** The cerebral cortex of the *wtki* brain appears normal, including a pronounced marginal zone (M). **(b)** The cerebral cortex of the *5Fki* mutant is disordered and the marginal zone contains many cells (arrowheads). **(c)** CA2 region of caudal part of hippocampus of *wtki* brain. Abbreviations: sr, stratum radiatum; pcl, pyramidal cell layer; so, stratum oriens; Cx, cortex; alv, alveolus. **(d)** CA2 region of *5Fki* mutant. The pcl is disorganized. **(e)** Cerebellum of *wtki* brain. Note the characteristic monolayer of Purkinje neurons (arrowheads) beneath a cell-poor molecular layer (M) and above the layer of granule cell neurons (G). **(f)** The *5Fki* mutant develops quite differently, with the Purkinje cells residing in deep clusters surrounded by granule cells. The granule cells have migrated inwards a short distance, leaving a thin molecular layer. Scale bars represent (a,e) 50 μm; (c) 200 μm. Hematoxylin and eosin stain.

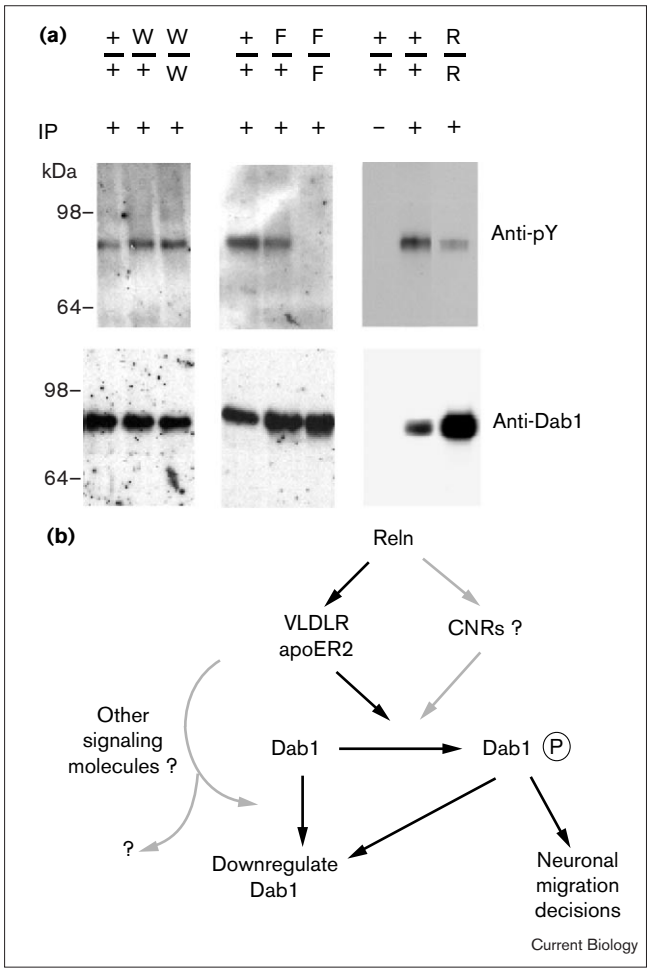


Mice that are homozygous for the *dab1-wtki* allele appeared to be normal in every respect, as did trans-heterozygotes, *dab1-wtki/dab1-1*. Thus, the *dab1-wtki* allele is not haploinsufficient and retains full activity. The brains of *wtki* heterozygous and homozygous animals appeared to be normal in size and overall structure (data not shown). We examined the cerebral cortex, hippocampus and cerebellum of these animals (Figure 4a,c,e). There was no evidence of any developmental abnormality.

In contrast, mice that were homozygous for the *dab1-5Fki* allele mimicked *dab1-1* null animals and displayed a pronounced ataxic behavior characterized by frequent loss of balance, tremulous locomotion, tendency to drag hind limbs and hyperextension of the tail. This phenotype is recessive, but was also seen in *dab1-5Fki/dab1-1* transheterozygotes. Also like the null animals, the *dab1-5Fki* homozygous mice died of unidentified causes at postnatal day 20–30 when they were maintained in a 129Sv/ C57BL hybrid strain background. This phenotype, but not the behavioral or developmental defects, could be rescued by backcrossing the *dab1-1* allele onto the BALB/cByJ background. Homozygous *dab1-1* animals on this background survived in excess of one year and the females are fertile but failed to foster pups (B.W.H. and J.A.C., unpublished observations).

The brains of *5Fki* homozygous mutants were dramatically altered. At the gross structural level the cerebellum is without foliation, and about one-fifth the normal size (data not shown). Histologically, there were obvious defects in organization of the cerebral cortex, hippocampus and cerebellum (Figure 4b,d,f). These defects are very consistent with those observed for animals that completely lack Dab1 expression. In the cerebral cortex, the six characteristic layers were disorganized (Figure 4b). Whereas the cortical marginal zone (the outermost layer) contained few cells in control or *wtki* animals (Figure 4a, layer M), it was packed with cells in the *5Fki* mutant (Figure 4b, arrowheads). The *5Fki* mutant also had ectopic nerve tracts in an intermediate plexiform zone, proximal to the pial surface (data not shown). The CA2 hippocampal pyramidal neurons were organized into a tight layer in normal and *wtki* animals (Figure 4c, pcl), but formed two or three wavy lamina in the *5Fki* mutant (Figure 4d). The granule cells of the dentate gyrus were also disordered in the *5Fki* mutant, similarly to the null mutant (data not shown). The Purkinje cells of the cerebellum were organized in a monolayer in normal and *wtki* animals (Figure 4e, arrowheads) but were found in internal clusters in the *5Fki* mutant (Figure 4f).

Figure 5



Regulation of Dab1 protein expression and phosphorylation. **(a)** Comparison of Dab1 protein and phosphotyrosine levels in wild-type, *wtki* (W), *5Fki* (F), and *Reln* (R) mutant mice brains. Embryos were collected at E16 from crosses between heterozygous animals, the isolated forebrains were lysed in RIPA buffer, and Dab1 protein was purified by immunoprecipitation (IP) from normalized lysates. The immunoprecipitates were resolved by PAGE and immunoblotted with an anti-phosphotyrosine antibody (pY, monoclonal 4G10, upper panel) or an anti-Dab1 antibody (polyclonal B3, lower panel). **(b)** Model for bifurcating pathways at the level of LDL family receptors. The regulation of neuronal migrations depends on Dab1 tyrosine phosphorylation (P), but the down-regulation of Dab1 protein levels only partially depends on Dab1 tyrosine phosphorylation. This suggests that the LDL family receptors transmit a second signal, independent of Dab1 tyrosine phosphorylation but potentially involving Dab1 protein, to a pathway that regulates Dab1 protein levels. This second pathway could also regulate other events during brain development. Black arrows indicate relationships supported by genetic and biochemical evidence; grey arrows indicate proposed pathways.

The cerebellar granule cells (Figure 4f, layer G) were in a less organized layer than normal but were removed from the external limit of the cerebellum by a thin molecular layer (layer M). All of the defects observed were the same as in *dab1-1* null animals.

To test whether the wild-type and 5F mutant p80 proteins were expressed properly, and to determine whether the 5F mutant protein was phosphorylated, samples were prepared from mutant embryos at a time when Reln is acting on nascent neurons. Crosses from heterozygous *dab1-wtki* or *-5Fki* parents were prepared and embryos collected at embryonic day (E)16. Lysates were prepared from brains of embryos that were wild-type, heterozygous, or homozygous for each allele, and Dab1 protein was immunoprecipitated with anti-carboxy-terminal Dab1 antibody. Dab1 protein and phosphotyrosine levels were assayed in these samples (Figure 5a). The *wtki* heterozygous and homozygous animals had Dab1 p80 protein and phosphorylation levels that closely matched the levels from the endogenous gene. Approximately 1.5- to 2-fold increases in Dab1 expression were reproducibly observed in homozygous *5Fki* mutant brains compared with *wtki* brains (Figure 5a, lower panel). However, the increase in Dab1 protein content did not approach the 3- to 6-fold increase observed in Reln or ApoER2/VLDLR mutant embryos [18,29,33] (Figure 5a, right panel). As anticipated, no phosphotyrosine was detected in the Dab1 5F mutant protein (Figure 5a, upper panel). This shows that all the tyrosine residues that are phosphorylated *in vivo* at E16 had been substituted in the 5F allele. Thus, the phenotype of the *dab1-5Fki* animals is not attributable to a failure to express the mutant protein, but correlates with a lack of tyrosine phosphorylation.

Discussion

This work establishes that Dab1 is phosphorylated on one or more tyrosines located in a small region adjacent to the PTB domain. Previously it was shown that the targeted disruption of the *dab1* gene causes a characteristic brain development phenotype [10]. The *dab1-scm* and *dab1-yot* alleles, which display defective splicing of *dab1* mRNA, have very similar phenotypes [11,12]. Here we demonstrate that a wild-type *dab1* hybrid gene expressed at the *dab1* locus rescues this phenotype and restores endogenous levels of Dab1 p80 protein expression and tyrosine phosphorylation at E16. A 5F mutant hybrid gene was not capable of rescuing normal brain development (Figure 4). Since 5F Dab1 p80 protein levels were at least as great as the wild type (Figure 5a), lack of protein expression is not the cause of the observed defects. The 5F mutant molecules were not tyrosine phosphorylated during embryonic brain development (Figure 5a). This suggests that phosphorylation of Dab1 is critical to its role in Reln signaling.

Dab1 tyrosine phosphorylation is reduced, but not eliminated, in *Reln* mutants [33] (Figure 5a). This indicates that Reln-independent as well as Reln-dependent tyrosine phosphorylation of Dab1 occurs. The 5F mutation precludes both basal and Reln-induced tyrosine phosphorylation. The function of Reln-independent tyrosine phosphorylation of Dab1 is unclear. It cannot be sufficient

for neuronal positioning, because *Reln* mutants have basally phosphorylated Dab1 yet exhibit the same phenotype as *dab1* nulls or the *5Fki* mutant. However, our data do not exclude the possibility that Reln-independent basal phosphorylation of Dab1 may be required for subsequent Reln-induced events. Moreover, we cannot rule out the possibility that the 5F mutant protein adopts a different conformation than the wild type and such a change might alter its activity.

The kinase or kinases that phosphorylate Dab1 in response to Reln are not known. Our results indicate that the Reln-dependent phosphorylation sites correspond to one or more of the sites phosphorylated by Src or a Src-activated TK. The Src-related kinase Fyn has been shown to interact with the cytoplasmic domain of the Reln-interacting CNR molecules, which may participate in the Reln response [47]. However, genetic evidence that CNRs or Fyn are required for Dab1 tyrosine phosphorylation or neuronal positioning is currently lacking. In *Drosophila*, Dab interacts genetically with Abl during CNS development [35]. In mice, the loss of Abl does not cause a *dab1*-like phenotype, possibly because of redundancy with its relative Arg. Mutation of Abl and Arg simultaneously leads to embryonic death prior to the neuronal migrations regulated by Dab1 [48]. We have not detected any genetic interactions between mammalian Abl and Dab1. However, Dab1 is tyrosine phosphorylated in cells expressing activated versions of Abl, such as Bcr-Abl (B.W.H. and J.A.C., unpublished observations).

The ability of the wild-type Dab1 p80 isoform to rescue the null phenotype means that other isoforms of Dab1, produced by alternative splicing [9], are not needed for brain development when the p80 form is present. The ability of the hybrid gene to rescue also argues against the possibility that the phenotype of the knock-out is due to the inadvertent inactivation of an uncharacterized gene in the vicinity of *dab1*. The close-to-endogenous levels of Dab1 expression rule out the possibility of gain-of-function or loss-of-function effects. This establishes this technique as a valid approach to investigate the developmental role of various *dab1* mutants.

In addition to regulating Dab1 protein phosphorylation, Reln and VLDLR/apoER2 inversely regulate Dab1 protein levels [18,20,33]. In *Reln* or *VLDLR/apoER2* mutants, Dab1 protein levels are increased 3- to 6-fold, without a detectable increase in *dab1* mRNA [18,20]. Increased levels of Dab1 protein could result from increased stability of Dab1, for example, if tyrosine phosphorylation targets Dab1 for degradation, or from changes in gene expression. However, little or no decrease in Dab1 protein is observed 4 hours after Reln stimulation of cultured neurons [33]. Our new results suggest that downregulation is not wholly dependent upon Dab1 tyrosine phosphorylation, since elimination of Dab1 phosphorylation in the *dab1-5Fki* homozygous mutant animals led to only a slight increase in Dab1 protein levels. As the *dab1-5Fki* mutant has virtually normal levels of Dab1 protein, it is unlikely the Reln signal is relayed by decreased Dab1 protein levels. Instead, the regulation of Dab1 protein levels is probably part of a feedback circuit to regulate signaling through the tyrosine phosphorylation pathway. The constancy of the Dab1 protein level in the 5F mutant implies that Reln and VLDLR/apoER2 regulate Dab1 protein levels independent of Dab1 tyrosine phosphorylation, perhaps through a bifurcation of pathways at the level of the VLDLR/apoER2 (Figure 5b). Such a second signaling pathway could regulate other changes in neurons triggered by Reln, independent of Dab1 (Figure 5b).

Conclusions

We have established that Dab1 tyrosine phosphorylation sites transmit the developmental signal for neuronal positioning. The downregulation of Dab1 protein, which occurs during normal brain development and is preserved in the 5F mutant animals, is not sufficient to regulate neuronal positioning. Reln-dependent phosphorylation of Dab1 probably regulates the interaction with downstream effectors. Since Dab1 has multiple putative SH2 and SH3 domain interaction sites, it may foster the formation of large signaling complexes. Like other docking proteins Dab1 may act to bring kinases in proximity to substrates and/or localize effector molecules to membrane-anchored receptors. The phenotypes of the *Reln*, *dab1*, and the *VLDLR/apoER2* double mutants are complex. It will be interesting to determine whether these phenotypes are manifested by a single biological phenomenon such as the modulation of cellular adhesion or cytoskeletal dynamics, or whether multiple cellular alterations are coordinated by Reln and its signaling partners.

Materials and methods

Plasmid construction

Tyrosine-to-phenyalanine point mutations were generated in the pBSDab555 cDNA [9] by the QuikChange (Stratagene) technique. Briefly, complementary oligonucleotides containing the desired mutations flanked by 12 nucleotides were annealed to the parental plasmids and extended with the Pfu DNA polymerase (Stratagene) for 20 thermocycles. The parental DNA was removed by *Dpn*I digestion and remaining DNA was used to transform *Escherichia coli* by standard techniques. The restriction sites *Xba*I, *Bbs*I, *Xmn*I and *Taq*I were introduced to mark the tyrosine-to-phenylalanine mutations at positions 185, 198/200, 220 and 232, respectively, and did not result in coding sequence changes. Sequence analysis established that no extraneous mutations were introduced. The *dab1* coding sequences were amplified by PCR with the Pfu DNA polymerase and ligated into the PECE vector downstream of the SV40 promoter [49].

The knock-in vectors were generated from the p80KO1 vector previously used to create the *dab1-1* null allele. This vector replaces the splice acceptor and the exon coding for residues 23–69 of the *dab1* gene with a neomycin-resistance cassette (loxP-PGK-neo-poly(A)-loxP) [10]. p80KO1 was altered as follows. The WT or 5F cDNAs in pBS were first modified by cloning a *HindIII–Kpn*I fragment containing a

triple poly(A) signal [50], 3′ to the cDNA. The sequence encoding residues 23–555, some 3′ untranslated region, and the triple poly(A) signal, was then amplified using Pfu polymerase and ligated into the *Cla*I site in p80KO1 between the neomycin resistance cassette and the 3′ homologous genomic fragment, to generate p80WTKI and p805FKI, respectively. The partial *dab1* cDNAs were amplified by the oligonucleotides 5′-GCCATCGATTTCAGCGTGCTTGTTTCTGAACCCATTTCTCTTCTTTTTCAGGTCAGGATCGCAGCGAAGCCACTTTGAT-3′, which restores the splice acceptor and 5′-GGCGATCGATGAGCTCGGTACCCGGGATCCAGACA-3′, which is homologous to the 3′ end of the triple poly(A) signal. The entire sequence of *dab1* was confirmed to be correct in both constructs. The resulting constructs are shown in Figure 3.

Generation of mutant mice

AK7 (129Sv/Sor) embryonic stem (ES) cells (1×10^7) were electroporated with 20 μg of either p80WTKI or p805FKI vector that was linearized at the unique *Xho*I site that lies 3′ to the diphtheria toxin gene. Homologous recombinants were identified among the G418 resistant colonies as described previously for the null allele [10] using one oligonucleotide primer that binds outside of the region of the targeting construct (P1, 5′-GTCAGGCTTCCTAAGTAGAAAGGA-3′, Figure 3) and another that binds the PGK promoter (P3, 5′-GGGAAAAGCGCCTCCCCTACCCCG-3′). The endogenous gene was amplified by P1 and P2 (5′-TTCCAGGAGCGAAATCACTCAACC-3′). Individual clones of ES cells containing homologous recombinants were then transiently transfected with 20 μg PGKcre [51], and 2 μg of pBabePURO, and plated at a density of 2.5×10^4 cells per ml in media containing 1 μg/ml puromycin. Cre mediated recombinants were identified by PCR using an oligonucleotide (P4, Figure 3; 5′-TCATGAACAGCATGGTGATGCT-3′) that binds to the *dab1* cDNA 3′ to the disrupted exon sequence. In the wild-type gene, P4 anneals to an exon that is too great a distance from the P1 primer to support PCR (data not shown). Chimeric animals were generated by standard blastocyst injection and transplantation into foster mothers [52].

Transfections and biochemistry

For high level expression and Dab1 phosphorylation, 293T cells (4×10^6 cells on 100 mm dishes) were transfected with 10 μg of each of pLXSHD Src 527F, and PECE *dab1* (wild-type or mutant) by the calcium phosphate method [9]. After 18–24 h the media were changed to remove the precipitate. Cells were washed with PBS and lysed in 1 ml of RIPA buffer (0.15 M NaCl, 1% Triton X100, 1% sodium deoxycholate, 0.1% SDS, 10 mM sodium phosphate (pH 7.0), 2 mM EDTA, 14 mM 2-mercaptoethanol, 50 mM NaF, 2 mM Na_3VO_4, 1 mM phenylarsine oxide, 20 μg/ml aprotinin, 10 μg/ml pepstatin, and 10 μg/ml leupeptin) after 48 h.

Brain lysis and Dab1 immunoprecipitation was done essentially as previously described, using carboxy-terminal peptide antibody to Dab1 [29,33].

Histology

Animals were fixed by perfusion with 4% paraformaldehyde in phosphate-buffered saline at 4°C. Haemotoxylin and eosin stains were done following standard protocols.

Phosphopeptide analysis

Phosphorylated Dab1 p80 was immunoprecipitated from 293T cells transfected with pLXSHD Src 527F and PECE Dab1 and separated by SDS–PAGE. An estimated 250–500 ng protein were excised from stained gels. In-gel digestion, mass spectrometry analysis and database searches were as described [53]. The digested peptide mixture was extracted and analyzed by microcapillary LC coupled inline with mass spectrometry (Finnigan LCQ ion trap). Peptides were concentrated on the Monitor C18 column (100 μm i.d. and 12 cm in length, in-house made), and salt and hydrophilic molecules were washed away. Separation was accomplished by applying a gradient (4% to 50% acetonitrile over 20 min; Magic 2000, Michrom Bioresources, Inc.). Eluting peptides were electrosprayed to mass spectrometry via a μESI ion source. The instrument was triggered to switch from MS to tandem MS/MS mode for generating collision-induced dissociation (CID) spectra of automatically selected analyte ions. The CID spectra generated during the experiment were searched against protein databases using Sequest program.

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