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Serine phosphorylation regulates disabled-1 early isoform turnover independently of Reelin

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ABSTRACT

The Reelin-Disabled 1 (Dab1) signaling pathway plays an important role in neuronal cell migration during brain development. Dab1, an intracellular adapter protein which is tyrosine phosphorylated upon Reelin stimulation, has been directly implicated in the transmission and termination of Reelin-mediated signaling. Two main forms of Dab1 have been identified in the developing chick retina, an early isoform (Dab1-E) expressed in progenitor cells and a late isoform (Dab1-L, a.k.a. Dab1) expressed in differentiated cells. Dab1-E is missing two Src family kinase (SFK) phosphorylation sites that are critical for Reelin-Dab1 signaling and is not tyrosine phosphorylated. We have recently demonstrated a role for Dab1-E in the maintenance of retinal progenitor cells. Here, we report that Dab1-E is phosphorylated at serine/threonine residues independent of Reelin. Cdk2, highly expressed in retinal progenitor cells, mediates Dab1-E phosphorylation at serine 475 which in turn promotes ubiquitination-triggered proteasome degradation of Dab1-E. Inhibition of protein phosphatase 1 and/or protein phosphatase 2A leads to increased Dab1-E instability. We propose that Dab1 turnover is regulated by both Reelin-independent serine/threonine phosphorylation and Reelin-dependent tyrosine phosphorylation.

1. Introduction

The cytoplasmic adaptor protein, Disabled-1 (Dab1), regulates the proper positioning of migrating neurons in response to Reelin signaling [1,2]. Dab1 contains an N-terminal protein interaction/phosphotyrosine binding (PI/PTB) domain, which associates with the NPxY motifs of the two Reelin receptors, very low density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2) [3,4]. The N-terminal domain is followed by a tyrosine-rich region, which consists of five highly-conserved tyrosine (Y) residues (Y185, Y198, Y200, Y220 and Y232) corresponding to two consensus Src family kinase (SFK) recognition sites (Y185 and Y198/Y200) and two consensus Abl recognition sites (Y220 and Y232) [5]. At least three of the four tyrosine phosphorylation sites (Y198, Y220 and Y232) are phosphorylated by SFKs and/or involved in the activation of SFKs in cultured neurons upon Reelin stimulation [6–8]. Tyrosine phosphorylation of Dab1 is essential for Reelin signaling, as mice expressing Dab1 with substitutions at these five tyrosine residues have neuronal cell positioning defects similar to those observed in Reelin-deficient (*reeler*) and *Dab1* −/− mice [9].

Tyrosine-phosphorylated Dab1 transmits the Reelin signal by activating a host of downstream effectors, including SFK, phosphatidylinositol 3 kinase (PI-3 K)/Akt, mTOR, CrkL/C3G/Rap and LIMK1 (LIM kinase 1) [7,10–14]. These events ultimately lead to cytoskeleton remodeling and correct neuronal positioning during development. Importantly, tyrosine-phosphorylated Dab1 also down-regulates Reelin signaling by recruiting SOCS (suppressors of cytokine signaling) proteins, adaptors for cullin-based E3 ligase complexes, thus targeting itself for ubiquitination and degradation [15–17]. This negative feedback mechanism prevents "overmigration" of neurons and ensures precise positioning of migrating neurons during development [17,18].

In addition to tyrosine phosphorylation, Dab1 is phosphorylated by the serine/threonine (S/T) kinase cyclin dependent kinase 5 (Cdk5) [19,20]. In particular, S491 in the C-terminus of Dab1 has been shown to be an important Cdk5 phosphorylation target both *in vitro* and *in vivo* [19]. Like Reelin and Dab1, Cdk5 plays an important role in neuronal cell positioning by phosphorylating substrates involved in cytoskeleton reorganization and cell migration. However, whether Dab1 serves as a convergence point for Reelin and Cdk5 signaling to fine tune neuronal cell migration is not clear at the present time. There is evidence implicating S/T phosphorylation in the modulation of Dab1 tyrosine phosphorylation [16,20]. Moreover, Dab1 levels have been shown to be either elevated or unaltered in *Cdk5* −/− mice

Abbreviations: ApoER2, apolipoprotein E receptor 2; CsA, cyclosporine A; Cdk, cyclin dependent kinase; Dab1, Disabled-1; CHX, cycloheximide; CPTS, 3,4',4″,4-copper phthalocyanine tetrasulfonic acid, tetrasodium salt; CsA, cyclosporine A; ED, embryonic day; GSK3, glycogen synthase kinase 3; LIMK1, LIM kinase 1; NEM, *N*-ethylmaleimide; OA, okadaic acid; PBD, polo-box domain; P1, post-hatching day 1; PBS, phosphate buffered saline; PI/PTB, protein interaction/phosphotyrosine binding; PI-3 K, phosphatidylinositol 3 kinase; PKA, protein kinase A; PKC, protein kinase C; Plk, polo-like kinase; PPase, phosphatase; RAP, receptor-associated protein; S/T, serine/threonine; SOCS, suppressors of cytokine signaling; SFK, src family kinase; VLDLR, very low density lipoprotein receptor.

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depending on the study, further confounding the importance of Dab1 S/T phosphorylation in Dab1 function [19–22].

We have identified two alternatively-spliced Dab1 isoforms in the developing chick retina: Dab1-E expressed in undifferentiated retinal progenitor cells, and Dab1-L expressed in differentiated ganglion, horizontal and amacrine cells [23,24]. Dab1-L, commonly referred to as Dab1, contains the five tyrosine residues described above, whereas Dab1-E is missing two SFK tyrosine phosphorylation sites, but retains two Abl tyrosine phosphorylation sites. In addition, Dab1-E has a 19 aa region encoded by alternatively-spliced exon 9a. Dab1-L is tyrosine phosphorylated upon Reelin stimulation and recruits Crk adaptor proteins, whereas Dab1-E is not tyrosine phosphorylated, nor does it associate with Crk proteins. Knockdown of Dab1-E in the developing chick retina results in a decrease in the number of retinal progenitor cells [24]. Here, we demonstrate that there are multiple phosphorylated forms of Dab1-E in the developing chick retina. In contrast to Dab1-L, Dab1-E phosphorylation exclusively involves S/T residues and is independent of Reelin. Unlike Dab1-L which is phosphorylated at S/T residues by Cdk5, Dab1-E appears to be primarily phosphorylated by Cdk2. Dab1-E S475, the counterpart of Dab1-L S491, is a major site of phosphorylation, which in turn destabilizes Dab1-E protein. We also demonstrate that Dab1-E stability is controlled by ubiquitination-mediated proteasome degradation and protein phosphatases 1 and/or 2A.

2. Materials and methods

2.1. DNA constructs

pEGFP-C1-Dab1-E and pEGFP-C1-Dab1-L have been previously described [22]. DNA constructs expressing GST-Dab1-E middle (residues 140–263) and GST-Dab1-E-C-terminus (residues 441–535) were generated by cloning the corresponding PCR fragments from pEGFP-C1-Dab1-E into pGEX-4 T2 at the BamHI and EcoRI sites. Full-length Dab1-E cDNA derived from pEGFPC1-Dab1-E was subcloned into pCMV-Tag4A at the BamHI and XhoI sites to produce the FLAG-tagged Dab1-E. pGEX-4 T2-Dab1-E-S475A, pEGFP-C1-Dab1-E-S475A and other pCMV-Tag4A-Dab1-E mutants were made by quick-change site-directed mutagenesis (Stratagene). Cdk2-HA (plasmid 1884), Cdk2-HA dominant negative (plasmid 1885) and HA-Ubiquitin (plasmid 18712) constructs were obtained from Addgene [25,26]. The construct expressing GST-RAP (receptor-associated protein) was a gift from Dr. Joachim Herz (University of Texas, Southwestern Medical Center).

2.2. Antibodies and pharmacological reagents

The rabbit anti-Dab1-E and rabbit anti-DDX1 antibodies have been described previously [24,27]. The following antibodies were used for western blot analysis: rabbit anti-Dab1-E (1:400), rabbit anti-Dab1 (100-4101-225, Rockland, 1:5000), mouse anti-actin (A5441, Sigma, 1:200,000), rabbit anti-Cdk2 (sc-163, Santa Cruz, 1:400), mouse anti-Cdc2/Cdk1 (CC16, Calbiochem, 1:100), mouse anti-Cdc2 p34 (sc-54, Santa Cruz, 1:200), rabbit anti-Cdk4 (sc-260, Santa Cruz, 1:200), rabbit anti-Cdk4 (06-139, Millipore), rabbit anti-Cdk5 (sc-173, Santa Cruz, 1:400), rabbit anti-p35 (sc-820, Santa Cruz, 1:200), mouse anti-Cyclin A (ab39, Abcam, 1:500), rabbit anti-Cyclin B1(sc-752, Santa Cruz, 1:200), mouse anti-Cyclin D1/2 (05-362, Upstate, 1:1000), mouse anti-Cyclin E (554182, BD Bioscience, 1:500), rabbit anti-pS491 Dab1 (ab5776, Abcam, 1:1000), mouse anti-phosphotyrosine antibody (pY-100 9411, Cell Signaling Technology, 1:1000) and rabbit anti-phosphoserine CDK substrate antibody (2324, Cell Signaling Technology, 1:1000). The following antibodies were used for immunohistochemical analysis: rabbit anti-pS491 Dab1 (ab5776, Abcam, 1: 200), rabbit anti-Cdk2 (sc-163, Santa Cruz, 1:400) and rabbit anti-Dab1-E (1:400).

MG132, okadaic acid (OA), roscovitine [2-(R)-(1-Ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine], purvalanol A [2-(1R-Isopropyl-2-hydroxyethylamino)-6-(3-chloroanilino)-9-isopropylpurine], glycogen synthase kinase 3 (GSK3) inhibitor and the serine/threonine kinase inhibitor set were obtained from Calbiochem. Cycloheximide (CHX), cyclosporine A (CsA) and *N*-ethylmaleimide (NEM) were obtained from Sigma.

2.3. Cell culture, drug treatment and DNA transfection

Hela and HEK293T cells were maintained in DMEM supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). Primary retinal cultures were prepared from embryonic day (ED) 5 or ED10 chick retinas dissociated with trypsin as previously described [8]. The treatments and transfections were carried out 24 h after plating. For drug treatment, cells were treated with different kinase inhibitors, CHX, MG132, Reelin, or RAP, as indicated. For transfection, the DNA was introduced into cells by calcium phosphate-mediated DNA precipitation and removed after 16–18 h. Cells were lysed in RIPA buffer [50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 1 mM PMSF, 1 mM Na_3VO_4 and 1 × Complete protease inhibitor cocktail (Roche)] followed by immunoprecipitation or western blot analysis.

2.4. Purification of GST fusion protein

pGEX constructs encoding the GST-fused middle (140–263 aa) or C-terminus (441–535 aa) of chicken Dab1-E, and RAP, were transformed into the *E. coli* strain BL21. Expression of the fusion protein was induced with 1 mM IPTG for 4 h at 30 °C. Cells were resuspended in phosphate buffered saline (PBS) containing 1 mM phenylmethylsulfonyl fluoride and 2 mM DTT and lysed by sonication (40% output for 10 bursts). Triton X-100 was added to a final concentration of 1% to increase protein solubility. Cleared lysates were incubated with glutathione-Sepharose beads (GE Healthcare) and bound proteins were eluted in 10 mM reduced glutathione (Sigma). The eluants were concentrated using Centricon-30 (Millipore) with three buffer exchanges in PBS.

2.5. Western blot analysis, immunoprecipitation and phosphatase treatment

Chick retinal tissue and cultures were lysed in RIPA buffer. For western blotting, lysates were either used fresh, or stored at −80 °C before use. For immunoprecipitation, cell lysates were precleared with protein A (for primary antibodies raised in rabbit) or protein G (for primary antibodies raised in mouse) Sepharose beads (GE Healthcare) for 1 h at 4 °C, incubated with primary antibodies or IgG control overnight at 4 °C. The immunocomplexes were then collected with protein A or protein G Sepharose beads. Immunoprecipitates or cell lysates were separated by SDS-PAGE, blotted onto nitrocellulose or PVDF membranes and immunostained with antibodies as indicated. For phosphatase treatment, Dab1 immunoprecipitates bound to protein A Sepharose beads were washed in lysis buffer three times and incubated in phosphatase buffer [50 mM Tris–HCl pH 7.5, 100 mM NaCl, 2 mM dithiothreitol (DTT), 0.1 mM EGTA, 0.01% Brij-35 and 20 μM $MnCl_2$] containing 400 U protein phosphatase (λ PPase, New England Biolabs) at 30 °C for 1 h.

2.6. In vitro kinase assay

ED5 chick retinas were lysed in ELB buffer (50 mM HEPES pH 7.2, 250 mM NaCl, 0.5% NP-40, 5 mM NaF, 0.5 mM DTT, 1 mM PMSF, 1 mM Na_3VO_4 and 1 × Complete protease inhibitor cocktail). Endogenous Cdk1, Cdk2, Cdk4 and Cdk5 proteins were immunoprecipitated from precleared retinal lysates as described above. The immunocomplexes were washed three times in lysis buffer and twice in kinase buffer (50 mM HEPES pH 7.2, 10 mM $MgCl_2$, 1 mM DTT). The immunoprecipitates were incubated with 2 μg of GST-fused Dab1-E fragments in 30 μl kinase buffer supplemented with 10 μM cold ATP and 5 μCi [γ-^{32}P]-ATP at 30 °C for

30 min. Two μg histone H1 (New England Biolabs) and GST were used as positive and negative controls, respectively. To examine the effect of Cdk inhibition on Dab1-E phosphorylation, 20 μM roscovitine was added to the kinase buffer. The reaction was terminated by the addition of 30 μl 2×SDS sample buffer. Samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Proteins were visualized by 3,4',4″,4-copper phthalocyanine tetrasulfonic acid, tetrasodium salt (CPTS) staining and [γ-^{32}P]-ATP incorporation was analysed by autoradiography.

2.7. Inorganic ^{32}P ($^{32}P_i$) labeling of retinal cultures

ED5 retinal cells were cultured for 24 h and labeled with 2 mCi $^{32}P_i$ (PBS13, GE healthcare) in phosphate-free medium supplemented with 10% dialyzed fetal calf serum (Invitrogen) for 1 h at 37 °C. Cells were washed in ice-cold Tris-buffered saline (TBS, pH 7.5) and lysed in RIPA buffer. Dab1 or IgG immunoprecipitates were resolved by SDS-PAGE and transferred to a PVDF membrane followed by immunostaining with anti-Dab1 antibody. ^{32}P-labeled proteins were visualized by autoradiography.

2.8. Ubiquitination assays

HEK293T cells were transfected with pcDNA3-Dab1-E or HA-Ubiquitin alone, or co-transfected with pcDNA3-Dab1-E or pcDNA3-Dab1-L and HA-Ubiquitin constructs using calcium phosphate-mediated DNA precipitation. Forty-eight h after transfection, cells were treated with 10 μM MG132 for 4 h followed by cell lysis in RIPA buffer containing protease inhibitors and 10 mM NEM to inhibit deubiquitinases. To immunoprecipitate the Dab1 protein, 200 μg of precleared cell lysates were incubated with 2 μl rabbit anti-Dab1 at 4 °C for 2 h, and the immunocomplexes were collected using 50 μl of Protein A Sepharose beads, followed by western blot analysis.

2.9. Immunohistochemical analysis

Chick embryos or eyes were collected at ED5 or ED7, fixed in 10% formalin and embedded in paraffin. Tissue sections (5 μm) were deparaffinized in xylene. Antigen retrieval was by microwaving in a pressure cooker in 0.01 M citrate pH 6.0 for 20 min, followed by blocking in 500 mM glycine. Sections were immunostained with primary antibodies as indicated. The signal was detected using the Dakocytomation Envision + anti-rabbit secondary system. Tissue sections were counterstained with hematoxylin to label the nuclei.

2.10. Quantitative and statistical analysis

The density of protein bands from at least three independent experiments was quantified using Image J software. ANOVA (analysis of variance) and *t* test were used to compare Dab1-E protein levels.

3. Results

3.1. Phosphorylation of Dab1-E serine/threonine, but not tyrosine, in the developing chick retina

Our previous studies have shown that Dab1-E, unlike Dab1-L, is not tyrosine phosphorylated in the developing chick retina [24]. Western blot analysis of Dab1 immunoprecipitates from ED10 retina using anti-Dab1-E antibody reveals up to four Dab1-E bands (depending on the SDS-PAGE conditions) suggesting posttranslational modification and/or multiple isoforms of Dab1-E (Fig. 1A, left panel). Immunostaining the same blot with anti-phosphotyrosine antibody confirmed that only Dab1-L (top band detected with anti-Dab1 antibody, as shown in the middle panel of Fig. 1A) is tyrosine phosphorylated (Fig. 1A, right panel).

We then carried out $^{32}P_i$ metabolic labeling and dephosphorylation assays to determine whether Dab1-E was phosphorylated on S/T residues. Since Dab1-E is predominantly expressed at early stages of retinal development, we prepared primary retinal cells from ED5 chick embryos and cultured them in $^{32}P_i$ for 1 h. Immunoprecipitation of endogenous Dab1 proteins was carried out, followed by autoradiography and western blot analysis. Autoradiography revealed a strong phosphorylated band corresponding to the slowest migrating form of Dab1-E in the Dab1 immunoprecipitates (Fig. 1B). Additional weaker bands were also observed in the Dab1 immunoprecipitates, indicating that these bands also represent phosphorylated forms of Dab1-E.

To further assess Dab1-E phosphorylation status in retina, Dab1 immunoprecipitates from ED5, ED10 and ED15 chick retina were treated with λ PPase. As shown in Fig. 1C, four Dab1-E bands are present at both ED5 and ED10. However, at ED15, the Dab1-E signal is barely detectable. In contrast, Dab1-L is not detected at ED5, but is present at ED10 and ED15 (see thick arrowheads in the bottom panel). PPase treatment results in the collapse of the Dab1-E bands into a single band,

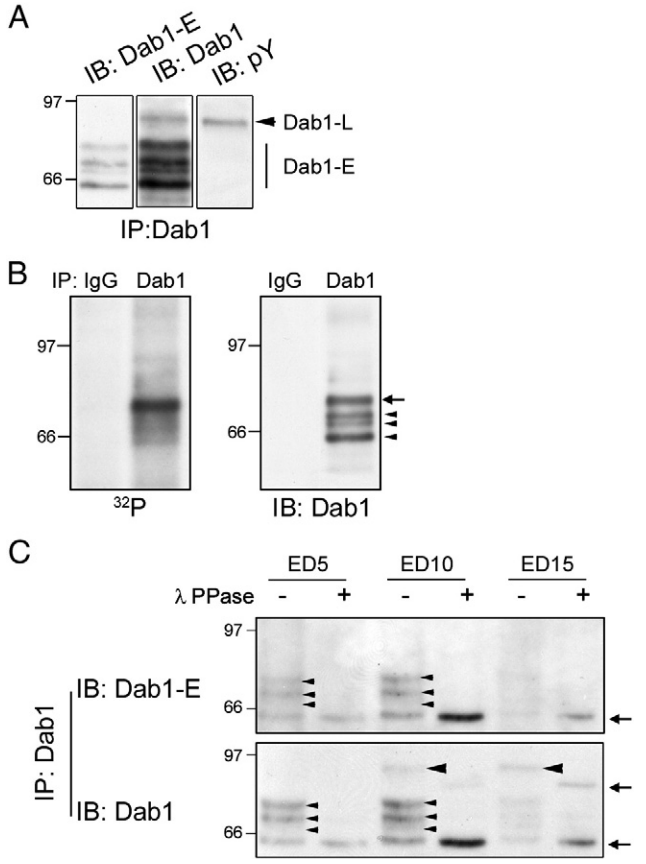

Fig. 1. Dab1-E phosphorylation in the developing retina. Dab1 proteins were immunoprecipitated from ED10 retinal lysates using anti-Dab1 antibody and analyzed by western blotting. The blot was immunostained with anti-Dab1-E antibody (left panel), anti-Dab1 antibody (middle panel) and anti-phosphotyrosine antibody (right panel). The arrowhead indicates Dab1-L, whereas the vertical bar indicates the different forms of Dab1-E. (B) ED5 chick retinal cells were metabolically labeled with ^{32}Pi. Endogenous Dab1 proteins were immunoprecipitated with anti-Dab1 antibody. The immunoprecipitates were resolved by SDS-PAGE and transferred to a PVDF membrane. Phosphorylated Dab1 proteins were visualized by autoradiography. The membrane was immunostained with anti-Dab1 antibody. The arrow indicates the major Dab1-E band labeled with ^{32}Pi, whereas arrowheads indicate weaker bands labeled with ^{32}Pi. (C) Endogeneous Dab1 was immunoprecipitated from lysates prepared from ED5, ED10 and ED15 chick retinas. The immunoprecipitates were treated with lambda phosphatase (λ PPase) (+) or left untreated (−). Blots were immunostained with anti-Dab1-E antibody (top panel) and anti-Dab1 antibody (bottom panel). Thin arrowheads indicate phosphorylated Dab1-E and thick arrowheads indicate phosphorylated Dab1-L, whereas arrows point to dephosphorylated Dab1-E and -L.

indicating multiple phosphorylated forms of Dab1-E, in agreement with the $^{32}P_i$ metabolic labeling experiments. PPase treatment also significantly alters the migration of Dab1-L (Fig. 1C, bottom panel), consistent with previous reports indicating that Dab1 (-L) is phosphorylated [16,24]. These results show that Dab1-E is phosphorylated on multiple S/T residues.

3.2. Inhibition of Cdk, but not Reelin signaling, reduces Dab1-E serine/threonine phosphorylation

Reelin induces Dab1 (-L) tyrosine phosphorylation but has no effect on its serine phosphorylation [3,6]. To examine whether Reelin regulates Dab1-E S/T phosphorylation, we treated primary retinal cells with recombinant RAP at ED10, a stage when Dab1-E, Dab1-L and Reelin signaling components are all expressed. RAP is a receptor chaperone that blocks Reelin signaling by interfering with Reelin binding to its receptors VLDLR and ApoER2 [28]. As shown in Fig. 2A (top panel), levels of tyrosine-phosphorylated Dab1-L were reduced in RAP-treated retinal cells; however, neither Dab1-E levels nor its phosphorylation was affected by RAP treatment even when cells were treated with higher doses of RAP (Fig. 2A, bottom panel). Similar results were observed in retinal cultures treated with the SFK inhibitor PP2 (data not shown). As well, Reelin treatment of ED10 primary retinal cells had no effect on Dab1-E phosphorylation [24], in support of Dab1-E S/T phosphorylation being independent of Reelin.

To identify potential kinases responsible for Dab1-E phosphorylation, we carried out bioinformatic analysis using the NetPhos, Motif Scan and Scansite software programs to predict putative kinase phosphorylation motifs in Dab1-E. Protein kinase A (PKA), protein kinase C (PKC), glycogen synthase kinase 3 (GSK3) and Cdk were all found to be strong candidates for Dab1-E phosphorylation at multiple sites. We then examined whether Dab1-E phosphorylation was affected in ED5 primary retinal cultures upon inhibition of these different kinases. Treatment of cells with the two Cdk inhibitors, roscovitine (Rosc) and purvalanol A (Purv), significantly reduced the levels of the slowest migrating Dab1-E band, presumably the most highly-phosphoryated form of Dab1-E. In contrast, solvent control (DMSO), PKA, PKC and GSK3 inhibitors had no effect on the Dab1-E banding pattern (Fig. 2B). These results implicate Cdk in Dab1-E phosphorylation.

3.3. Cdk2 is involved in Dab1-E phosphorylation in the developing retina

Previous studies have shown that Cdk5 phosphorylates Dab1 (i.e. Dab1-L) in a Reelin-independent manner [19]. Hence, Cdk5 is a strong candidate for Dab1-E phosphorylation. However, Cdk5 is mainly active in postmitotic neurons where its activators p35 and p39 are expressed whereas Dab1-E is preferentially found in proliferating retinal progenitor cells. To determine whether Cdk5 is involved in Dab1-E phosphorylation, we carried out western blot analysis to examine the levels of Cdk5 and its activator p35 in the developing chick retina. As shown in Fig. 3A, Cdk5 is expressed throughout retinal development from ED5 to post-hatching day 1 (P1). Importantly, p35 is primarily expressed at later stages of development (ED10-P1), thus correlating with the Dab1-L, but not Dab1-E, expression pattern. These data support a role for Cdk5 in phosphorylating Dab1-L in the retina.

We then examined the expression of other Cdks including Cdk1, Cdk2, Cdk4 and their regulatory subunits in the developing chick retina. Although cyclin D, the Cdk4 regulatory subunit, was highly expressed at early stages of retinal development, we were not able to detect Cdk4 using two anti-Cdk4 antibodies. As Cdk4 has previously been shown to be expressed in newborn rodent retina [29], it is likely that our two antibodies do not recognize chicken Cdk4. Neither Cdk1 nor its regulatory subunit cyclin B was detected in the chick retina (data not shown). Cdk2 and its regulatory subunit cyclin E were both

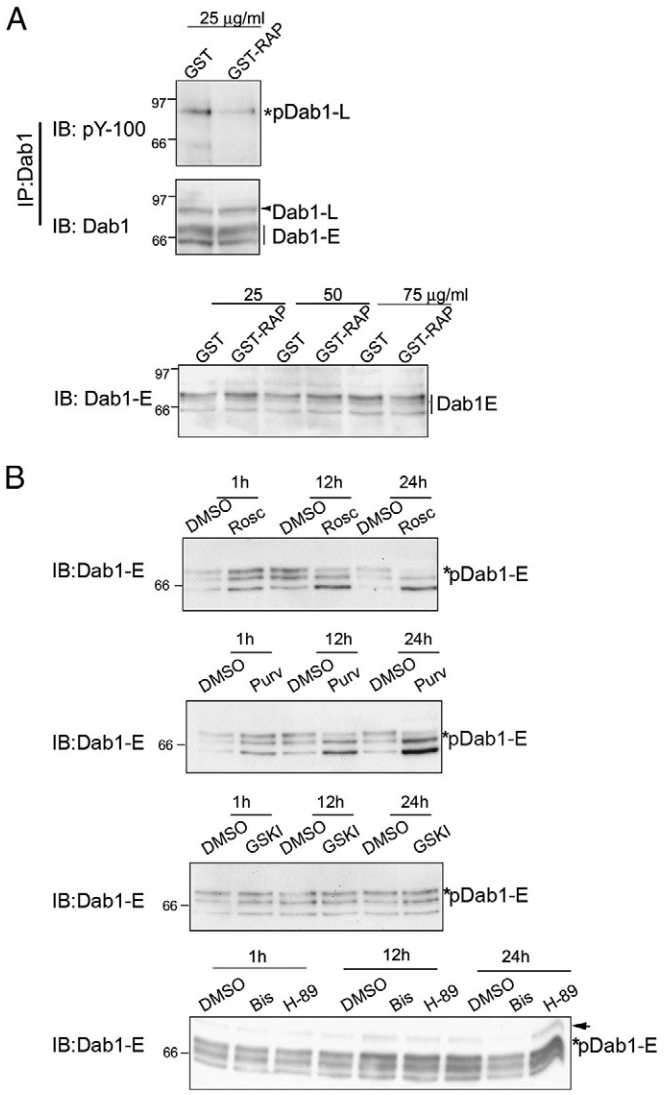

Fig. 2. Cdk inhibition reduces Dab1-E phosphorylation. (A) Lysates were prepared from ED10 retinal cultures treated with 25 µg/ml of GST or GST-RAP for 24 h and subjected to immunoprecipitation with anti-Dab1 antibody. The immunoprecipitates were resolved by 8% SDS-PAGE and blots immunostained with anti-phosphotyrosine and anti-Dab1 antibodies (top panel). ED10 retinal cultures were treated with GST or GST-RAP for 24 h at the indicated concentrations. Western blot analysis was carried out using anti-Dab1-E antibody. (B) ED5 retinal cultures were treated with DMSO, 20 µM roscovitine (Rosc, Cdk inhibitor), 20 µM purvananol A (Purv, Cdk inhibitor), 20 µM GSKI (Gsk 3 inhibitor), 1 µM bisindolylmaleimide I (Bis, PKC inhibitor) or 1 µM H-89 (PKA inhibitor) for 1, 12 and 24 h. Cell lysates were resolved by 8% SDS-PAGE and immunoblotted using an anti-Dab1-E antibody. The arrow indicates a non-specific band labeled by the antibody.

detected at early stages of retinal development (Fig. 3A). Immunohistochemical analysis of ED7 retinal tissue sections with anti-Dab1-E and anti-Cdk2 antibodies revealed that both Cdk2 and Dab1-E are expressed in proliferating retinal progenitor cells, but not in differentiated ganglion cells (Fig. 3B). Together, these data suggest a connection between Cdk2 and Dab1-E phosphorylation.

Next, we assessed Cdk activity at early stages of chick retinal development. Using the well-known Cdk substrate, histone H1, we assayed the activity of Cdk2 and Cdk5 complexes immunoprecipitated from ED5 chick retina. The Cdk2 immunocomplex was much more efficient in phosphorylating histone H1 than the Cdk5 immunocomplex at this developmental stage (Fig. 3C).

Cdks phosphorylate serines and threonines located upstream of a proline residue (S/TP) [30]. Both Dab1-E and Dab1-L contain eleven such serine and threonine residues. These serine and threonine sites are clustered in two regions, with 4 sites located in the middle of Dab1, and 6

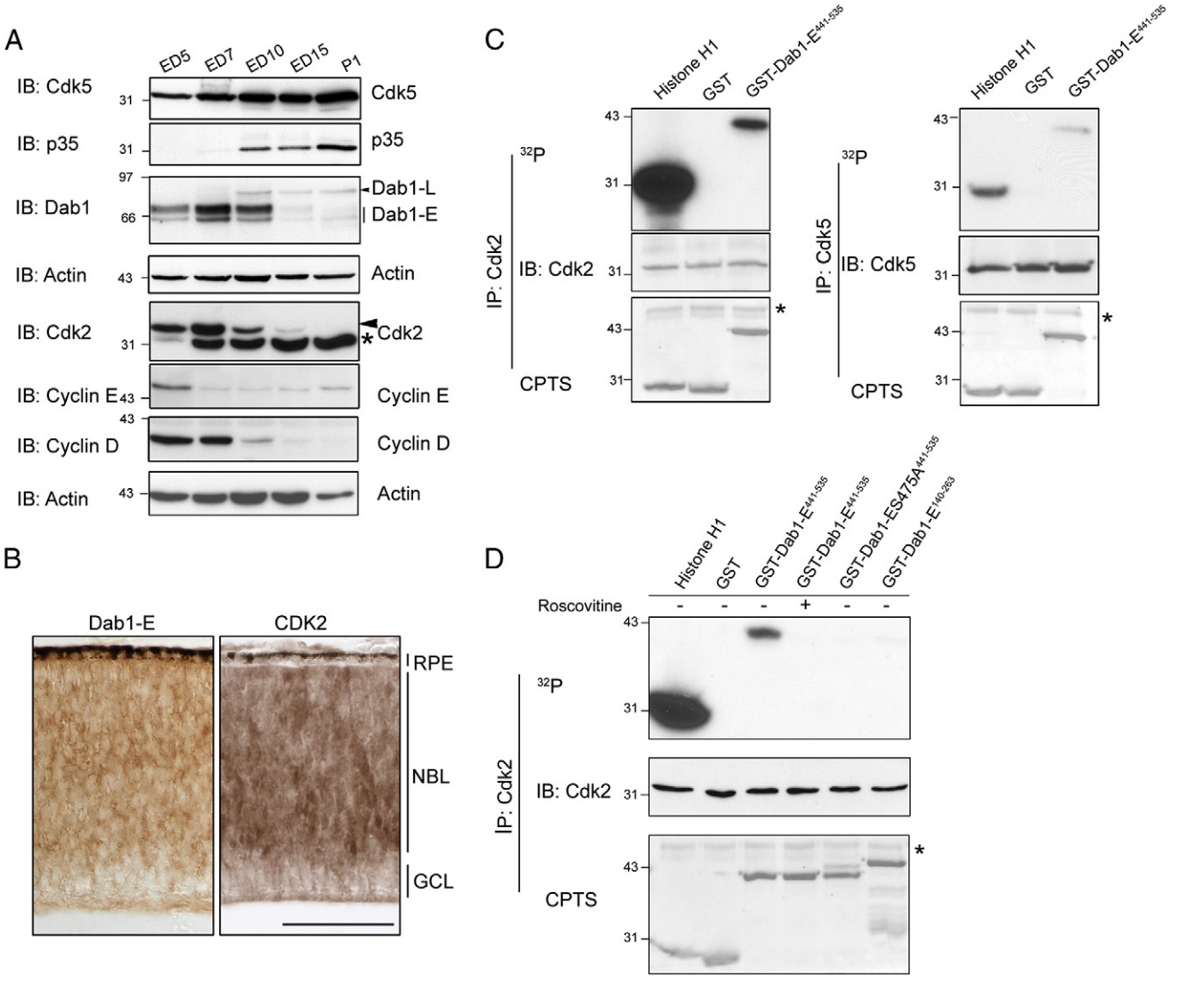

Fig. 3. Cdk phosphorylates Dab1-E *in vitro*. (A) Expression of Cdk and cyclins in the developing retina. Retinal lysates prepared from ED5, ED7, ED10, ED15 and P1 chick embryos were resolved by SDS-PAGE and blots immunostained with anti-Cdk5, anti-p35, anti-Dab1, anti-Cyclin D1, anti-Cyclin E, anti-Cdk2 and anti-actin antibodies. The two Cdk2 bands (indicated by arrowhead and asterisk) may represent posttranslationally-modified or alternatively-spliced Cdk2 isoforms. (B) Immunohistochemical analysis of Dab1-E and Cdk2 in ED7 chick retina. Tissue sections from formalin-fixed paraffin-embedded ED7 chick retina were immunostained with anti-Dab1-E or anti-Cdk2 antibody. Abbreviations: RPE, retinal pigmented epithelium; NBL, neuroblastic layer; GCL, ganglion cell layer. Scale bar, 50 μm. (C) Endogenous Cdk2 and Cdk5 proteins were immunoprecipitated from ED5 retinal tissue lysates and incubated with histone H1, GST and GST-Dab1-$E^{441-535}$, in the presence of [γ-^{32}P] ATP. The complexes were separated in a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Protein phosphorylation was visualized by autoradiography. Histone H1, GST and GST-Dab1-E fragments were visualized by CPTS staining. Cdk2 and Cdk5 identity was verified by immunostaining the membranes with anti-Cdk2 and anti-Cdk5 antibodies. (D) The Cdk2 complex was immunoprecipitated and incubated with putative substrates as indicated in (B) in the absence/presence of 20 μM roscovitine. Autoradiography, CPTS and western blot analysis are described in (B).

sites in the C-terminus (Fig. 4B). We immunoprecipitated the endogenous Cdk2 and Cdk5 complexes from ED5 chick retina and tested their ability to phosphorylate Dab1-E protein *in vitro*. The immunocomplexes were incubated with recombinant GST-fused Dab1-E middle (residues 140-263, containing 4 Cdk sites) and Dab1-E C-terminal (residues 441-535, containing 6 Cdk sites) fragments. The GST-Dab1-E C-terminal fragment, but not the Dab1-E middle fragment, was phosphorylated by both the Cdk5 and Cdk2 immunocomplexes, with the Cdk2 immunocomplex demonstrating considerably higher activity (Fig. 3C and D, data not shown). In the presence of 20 μM roscovitine, the phosphorylation of Dab1-E C-terminus by Cdk2 was abolished, confirming that Cdk activity is required for Dab1-E phoshorylation (Fig. 3D). Combined with the spatial expression pattern of Cdk2, these phosphorylation data indicate that Cdk2 is a good candidate for Dab1-E phosphorylation at S/T residues at early developmental stages of chick retina.

3.4. Serine 475 is a critical residue for Dab1-E phosphorylation both in vitro and in vivo

To identify the Cdk S/T phosphorylation sites, we substituted the 11 S/T residues with alanines in FLAG-tagged Dab1-E expression constructs. Analysis of exogenously expressed wild-type Dab1-E (with or without FLAG tag) in either retinal cultures or HeLa cells by western blotting revealed two forms of Dab1-E (Fig. 4A). λPPase treatment resulted in the collapse of the upper form into the lower form, indicating that exogenous Dab1-E can be phosphorylated (Fig. 4A). Western blot analysis of FLAG-tagged Dab1-E wild-type and S/T mutants in transfected HEK293T cells indicate a similar migration pattern to that of wild-type Dab1-E for the majority of Dab1-E mutants (Fig. 4B, lanes 1–8, 10). However, substitution of S475 with alanine (S475A mutant, lane 9) significantly reduced the levels of the slower migrating band, indicating that S475 is a critical

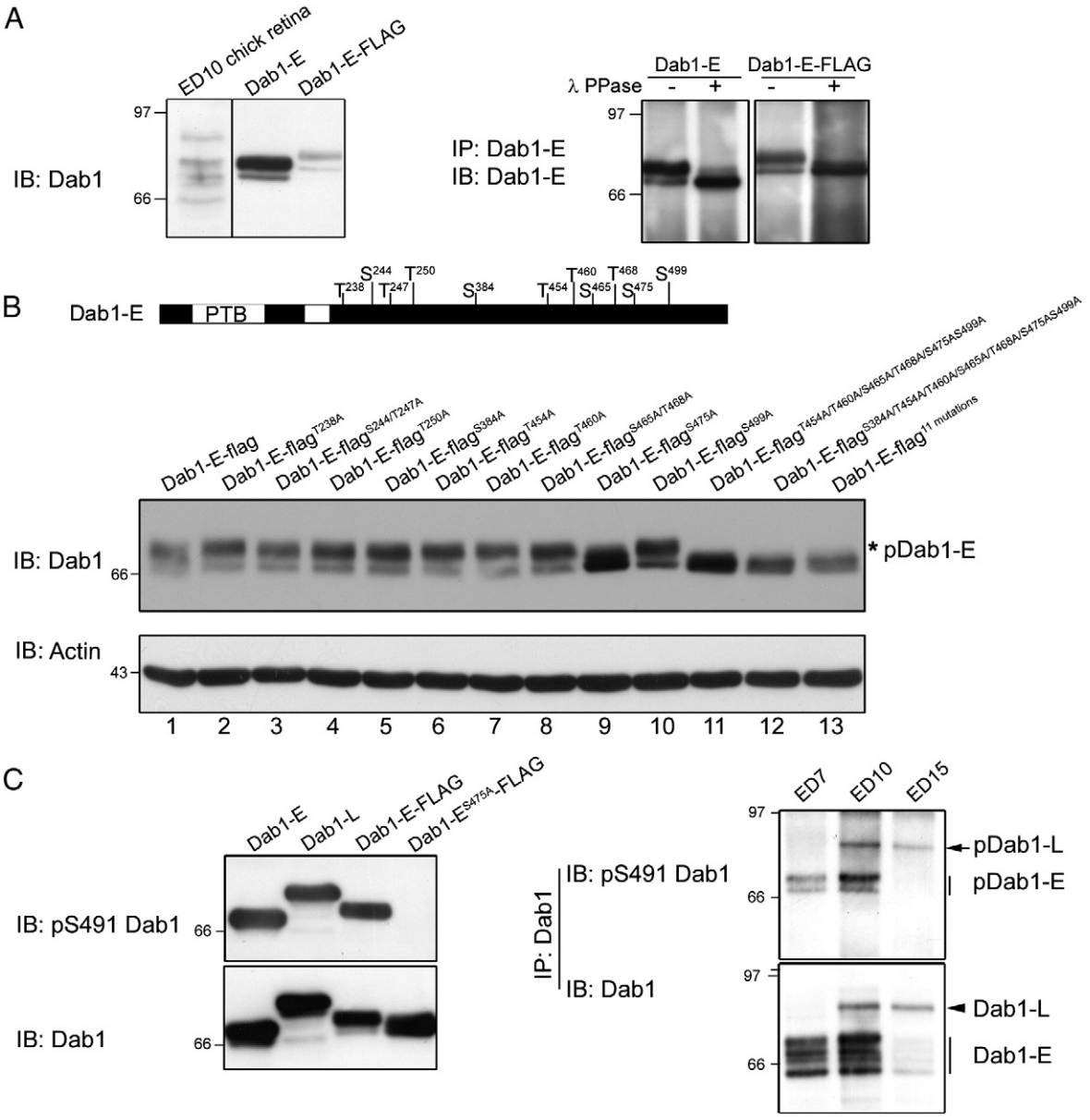

Fig. 4. Dab1-E serine 475 phosphorylation in transfected cells and in the retina. (A) ED10 chick retinal lysates and lysates prepared from HeLa cells transfected with pcDNA3-Dab1-E and pCMV-Tag4A-Dab1-E (Dab1-E-FLAG) expression constructs were subjected to western blot analysis using anti-Dab1 antibody (left panel). Dab1-E immunoprecipitations were carried out using HeLa cells transfected with pcDNA3-Dab1-E or pCMV-Tag4A-Dab1-E expression constructs. The immunoprecipitates were treated with λ protein phosphatase (λ PPase +) or left untreated (−) before separating the proteins in an 8% SDS-polyacrylamide gel and immunoblotting with anti-Dab1-E antibody. (B) Schematic representation of putative Cdk phosphorylation sites in Dab1-E protein (top panel). Lysates prepared from HeLa cells transfected with pCMV-Tag4A-Dab1-E wild-type and mutants were subjected to western blot analysis using anti-Dab1 and anti-actin antibodies (bottom panel). The asterisk indicates phosphorylated Dab1-E. (C). Protein extracts prepared from HEK293T cells transfected with pcDNA3-Dab1-E, pcDNA3-Dab1-L, pCMV-Tag4A-Dab1-E wild-type and pCMV-Tag4A-Dab1-E^{S475A} were analysed by western blotting using the anti-S491 Dab1 phosphorylation specific antibody (pS491Dab1, left panel). Dab1 immunoprecipitates prepared from ED7, ED10 and ED15 chick retina lysates were resolved by 8% SDS-PAGE (20×20 cm), transferred to a PVDF membrane and immunostained with anti-pS491 Dab1 antibody and Dab1 antibody.

residue for Dab1-E phosphorylation. It is noteworthy that there is still a residual upper band in the S475 mutant. This upper band disappeared completely only when all six Cdk sites in the C-terminus of Dab1-E were substituted with alanines (Fig. 4B, lane 11), suggesting multiple sites of Dab1-E phosphorylation.

The counterpart of S475 (Dab1-E) is S491 in Dab1-L. We used anti-Dab1 pS491 antibody, which specifically recognizes phosphorylated S491, to examine Dab1-E S475 phosphorylation *in vivo*. As shown in Fig. 4C (left panel), anti-Dab1 pS491 antibody recognizes both exogenous wild-type Dab1-E and Dab1-L, but not the Dab1-E-S475A mutant, in HEK293T transfected cells. As expected, immunoblotting analysis of Dab1 immunoprecipitates from ED7, ED10 and ED15 chick retinal lysates using this phospho-antibody reveals S491-phosphorylation of Dab1-L at ED10 and ED15. Importantly, the two slower migrating forms of Dab1-E in ED7 and ED10 retina were both labeled by the anti-Dab1 pS491 antibody, providing direct evidence that Dab1-E is phosphorylated at S475 in this tissue (Fig. 4C, right panel). The fact that two bands with different migration rates are recognized by the pS491 antibody suggests phosphorylation of residues other than S475 in Dab1-E.

3.5. Cdk2 phosphorylates Dab1-E at serine 475 in the developing retina

Our kinase assays show that Cdk2 is the most active Cdk in ED5 chick retina and that Cdk2 phosphorylates the C-terminus of Dab1-E *in vitro* (Fig. 3C and D). To determine whether Cdk2 phosphorylates Dab1-E S475 *in vivo*, we transfected primary ED5 retinal cultures with

a Cdk2 dominant negative mutant and examined the levels of Dab1-E S475 phosphorylation in these transfectants. A significant reduction in the levels of Dab1-E phosphorylated at S475 was observed in Dab1-E immunoprecipitates (Fig. 5A), indicating that Cdk2 mediates Dab1-E phosphorylation at S475 in retinal cultures.

Next, we immunostained consecutive ED5 retinal tissue sections with anti-pS491 Dab1 and anti-Cdk2 antibodies. As shown in Fig. 5B, phospho-S491 Dab1 antibody labeled both proliferating retinal progenitor cells and differentiated ganglion cells. In contrast, Cdk2 was detected in proliferating retinal progenitor cells known to be positive for Dab1-E, but not in the differentiated ganglion cells where Dab1-L is expressed. Thus, the cellular distribution of Cdk2 is in agreement with its proposed role in phosphorylating Dab1-E at S475 in retinal progenitor cells. Our data also indicate that a kinase other than Cdk2 (likely Cdk5) is involved in phosphorylating Dab1-L at S491 in postmitotic neurons, such as ganglion cells, as previously reported [19].

3.6. S475 phosphorylation destabilizes Dab1-E protein and promotes ubiquitination-mediated proteasome degradation

Previous studies have suggested a potential but controversial role for S491 Dab1 phosphorylation in regulating Dab1 levels, with two reports indicating increased levels of Dab1 in *Cdk5* −/− mouse brain [19,20], and two reports indicating no change in Dab1 levels in these mice [21,22]. In support of a role for Cdk in regulating Dab1 levels, we have observed that inhibition of Cdk in primary retinal cultures results in a significant increase in Dab1-E (Fig. 2B). Furthermore, we have observed increased levels of Dab1-E S475A mutant protein compared to wild-type protein in transfected HeLa and HEK293T cells (compare lanes 1 and 9 in Fig. 4B and lanes 3 and 4 in Fig. 4C), suggesting that S475 phosphorylation may destabilize Dab1-E protein and/or enhance its turnover.

To test the effect of serine/threonine phosphorylation on Dab1-E protein stability, we treated primary ED5 retinal cultures with cycloheximide (CHX), an inhibitor of *de novo* protein synthesis. As shown in Fig. 6A, a preferential decrease in the levels of the slower migrating form of Dab1-E relative to the two faster migrating bands was noted in the presence of CHX. These results indicate that the slower migrating form of Dab1-E, which is phosphorylated at S475 and likely at other S/T residues, is less stable than the other phosphorylated and/or nonphosphorylated forms of Dab1-E.

To further address the role of S/T phosphorylation in regulating Dab1-E protein turnover, ED5 primary retinal cultures were treated with S/T protein phosphatase inhibitors okadaic acid (OA), a protein phosphatase 2A (PP2A) and protein phosphatase 1 (PP1) inhibitor, or cyclosporine A (CsA), an inhibitor of calcineurin/protein phosphatase 2B (PP2B). If S/T phosphorylation destabilizes Dab1-E protein, one would expect that inhibition of PPase would lead to increased Dab1-E phosphorylation and decreased protein levels. As shown in Fig. 6B, OA treatment results in loss of the faster migrating Dab1-E bands which represent the less-phosphorylated and non-phosphorylated forms of Dab1-E. Importantly, this upward migration shift was accompanied by an overall reduction in Dab1-E protein levels, in agreement with the idea that S/T phosphorylation destabilizes Dab1-E protein. In contrast, CsA treatment had no effect on the Dab1-E banding pattern and levels (Fig. 6B), indicating that Dab1-E protein levels are highly sensitive to PP2A/PP1, but not PP2B, phosphatase activity. We also treated retinal cultures with DMSO, OA and OA in the presence of CHX to inhibit protein synthesis. As shown in Fig. 6C, similar decreases in Dab1-E levels were observed whether cells were treated with OA alone or OA plus CHX.

Next, we examined whether the single band observed upon OA treatment contained S475-phosphorylated Dab1-E. As shown in Fig. 6C, the single Dab1-E band observed in OA-treated cells was recognized by the pS491 antibody. Furthermore, addition of the proteasome inhibitor MG132 prevented OA-induced decrease in S475 phosphorylated-Dab1-E levels (Fig. 6D, $P<0.01$, ANOVA and t test). Together, these data demonstrate that S/T phosphorylation destabilizes Dab1-E and may target it for proteasome-mediated degradation.

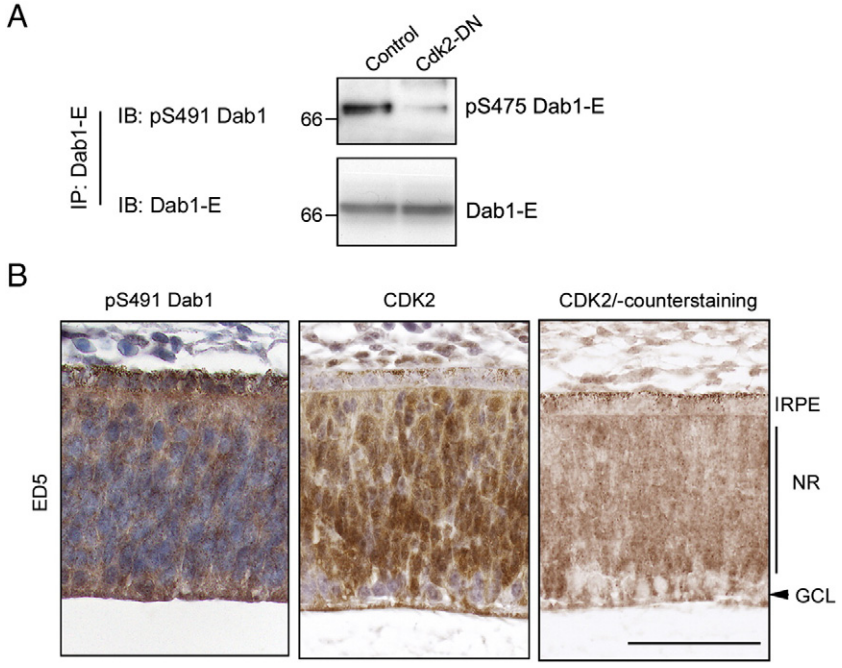

Fig. 5. Cdk2 phosphorylates Dab1-E in the retina. (A) Dab1-E was immunoprecipitated from ED5 retinal cultures transfected with pcDNA3 and pcDNA3-HA-Cdk2 dominant negative (Cdk2-DN) expression constructs. The immunoprecipitates were analysed by western blotting using anti-pS491 Dab1 (pS475 Dab1-E) and anti-Dab1-E antibodies. (B) Immunohistochemical analysis of pS491 Dab1 and Cdk2 in ED5 chick retina. Consecutive tissue sections from formalin-fixed paraffin-embedded ED5 chick retina were immunostained with anti-pS491 Dab1 or anti-Cdk2 antibody. Sections were either counterstained with hematoxylin (left and middle panels) or not counterstained (right panel) in order to better visualize the cellular distribution of Cdk2 protein in the retina. Abbreviations: RPE, retinal pigmented epithelium; NR, neural retina; GCL, ganglion cell layer. Scale bar, 50 μm.

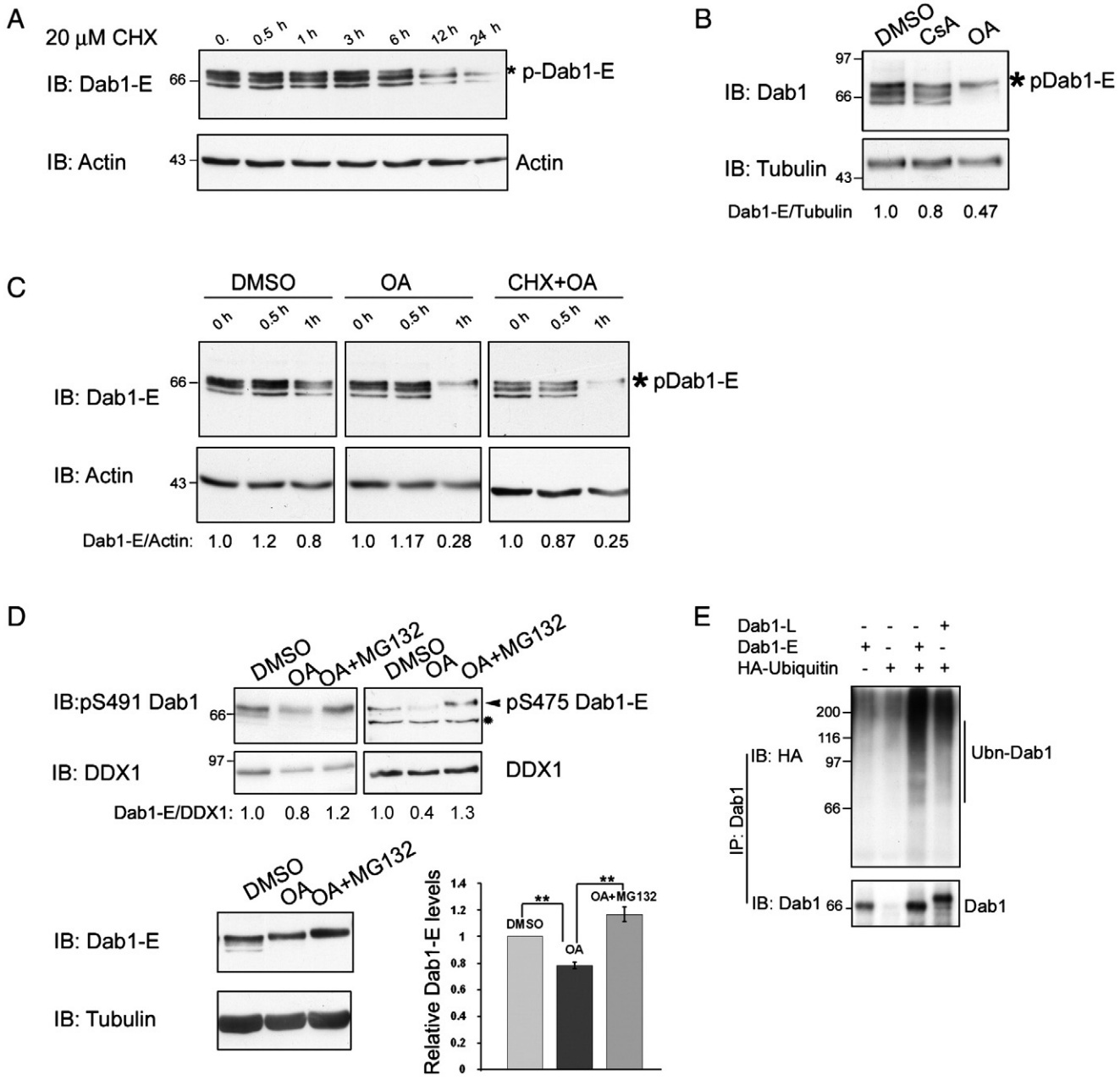

Fig. 6. Serine 475 phosphorylation destabilizes Dab1-E and promotes ubiquitination. (A) Lysates were prepared from ED5 retinal cultures treated with 20 μM cycloheximide (CHX) for 0 to 24 h (as indicated) and subjected to western blot analysis using anti-Dab1-E and anti-actin antibodies. (B) ED5 retinal cultures were treated with DMSO, 1 μM cyclosporin A (CsA) or 250 nM okadaic acid (OA) for 1 h. Cell lysates were resolved by 8% SDS-PAGE, transferred and immunostained with anti-Dab1-E and anti-tubulin antibodies. Protein band density was quantified by Image J. Values indicate the ratio between Dab1-E and tubulin levels, with the ratio arbitrarily set at 1 for DMSO-treated samples. (C) ED5 retinal cultures were treated with DMSO, 250 nM okadaic acid (OA) or 250 nM OA in the presence of 20 μM CHX for the indicated times. Western blot analysis and quantitation were carried out as in (B), with actin used as the loading control. (D) ED5 retinal cultures were treated with DMSO, 100 nM okadaic acid (OA) or 100 nM OA in the presence of 10 μM MG132 for 4 h. Western blot analysis and quantitation were carried out as in (B), with DDX1 and tubulin serving as loading controls, respectively. The histogram shows relative Dab1-E protein levels from three independent experiments. Significant differences between DMSO, OA and OA + MG132 treated cultures are indicated by double asterisks ($P<0.01$, ANOVA and t test). (E) HEK293T cells were transfected with pcDNA3-Dab1-E, pcDNA3-HA-Ubiquitin (HA-Ubiquitin), pcDNA3-HA-Ubiquitin and pcDNA3-Dab1-E, or pcDNA3-HA-Ubiquitin and pcDNA3-Dab1-L. Dab1 proteins were immunoprecipitated from cell lysates using anti-Dab1 antibody and immunoprecipitates analysed by western blotting using anti-HA and anti-Dab1 antibodies.

Proteasome-mediated protein degradation usually involves ubiquitin conjugation to the target protein. We next addressed whether Dab1-E can be ubiquitinated by co-transfecting HEK293T cells with a pcDNA3-Dab1-E expression construct and a HA-tagged ubiquitin construct. Cells co-transfected with the pcDNA3-Dab1-L and ubiquitin constructs were included as a positive control. Transfected cells were incubated with MG132 for 4 h and cell lysates immunoprecipitated with anti-Dab1 antibody and subjected to western blot analysis using an anti-HA antibody (Fig. 6E, top panel) or anti-Dab1 antibody (Fig. 6E, bottom panel). A ladder of polyubiquitinated Dab1 was detected in the immunoprecipitates prepared from Dab1-E or Dab1-L and ubiquitin double-transfected cells, indicating that both Dab1-E and Dab1-L serve as a substrate for the ubiquitination machinery.

4. Discussion

Fine tuning of Reelin signaling and the correct positioning of neurons during development depend on precise regulation of Dab1 levels [15–17]. Reelin-induced Dab1 tyrosine phosphorylation is a key factor in regulating its levels, as tyrosine-phosphorylated Dab1 recruits SH2

domain-containing E3 ligase complexes and targets itself for rapid proteasome degradation. In contrast to the commonly studied Dab1 (Dab1-L), Dab1-E is not tyrosine phosphorylated in response to Reelin and appears to play a role in the maintenance of retinal progenitor cells rather than neuronal cell migration and positioning [24]. We show that, like Dab1-L, Dab1-E activity is modulated by phosphorylation and degradation via the proteasome; however, regulation of Dab1-E activity is driven by S/T phosphorylation rather than tyrosine phosphorylation. Although S/T phosphorylation of Dab1 has previously been documented in the literature [16,19,20,31], this is the first study demonstrating a direct role for S/T phosphorylation in the regulation of Dab1 levels.

Previous reports have alluded to serine phosphorylation playing a role in the regulation of Dab1 levels. For example, reduced S491 phosphorylation and elevated Dab1-L protein levels are observed in ectopically positioned neurons in *Cdk5*−/− mice [19]. Furthermore, p45, a truncated form of Dab1 missing the C-terminal S/T residues (including S491), is expressed at higher levels than full-length Dab1, suggesting that the C-terminus reduces Dab1 protein stability [32]. However, as most of the work carried out to date on Dab1 has focused on Reelin-induced Dab1 tyrosine phosphorylation-mediated degradation, we still have little mechanistic insight into the role of S/T phosphorylation in regulating Dab1 levels. Since Dab1-E is not tyrosine-phosphorylated, our data clearly demonstrate a role for S/T phosphorylation, particularly S475, in the regulation of Dab1-E turnover. We postulate that the same proteolysis mechanism may apply to Dab1-L as this isoform is also phosphorylated at S491, the counterpart of S475. Thus, Dab1-L levels may be regulated by both Cdk5-mediated S491 phosphorylation and Reelin-induced tyrosine phosphorylation. Regulation of Dab1-L levels by both Cdk5 and Reelin would explain why increased Dab1 levels are observed in *Cdk5*−/− mice [19] even though Cdk inhibition has no effect on Reelin-induced Dab1 degradation [15].

A computer-based motif search has revealed one PEST motif in the central region and three PEST motifs in the C-terminal region of Dab1 (Fig. 7). PEST motifs are hydrophilic stretches rich in proline (P), glutamate (E), aspartate (D), serine (S) and threonine (T) residues, frequently targeted by the ubiquitination-proteasome degradation pathway [33]. These motifs are usually dormant, but phosphorylation can unmask dormant PEST signals, likely through direct recruitment of E3 ubiquitin ligases or phosphorylation-induced conformational changes, thus priming the protein for proteolysis [33]. As S475/S491 is located in the 10-amino acid region separating PEST motifs 2 and 3 (Fig. 7), phosphorylation of this site may activate the latent PEST signals, thus triggering ubiquitination and targeting Dab1 for degradation. Our *in vitro* data demonstrate that Dab1-E, like Dab1-L, can be polyubiquitinated, in support of both these proteins being targets for proteasome degradation. Interestingly, the Reelin-responsive site Y220 is located within PEST motif 1 and Y220 phosphorylation has been proposed to generate a recognition signal for the ubiquitin-proteasome machinery, leading to Reelin-triggered Dab1 degradation [15]. Loss of Y220 and PEST motif 1 in Dab1-E suggests that Dab1-E turnover is primarily regulated through S/T phosphorylation.

Cdk2 is central to G1/S transition and S-phase progression through the cell cycle. The Cdk2-cyclin E complex has recently been shown to play an important role in regulating the cell cycle of chondroitin sulfate proteoglycan NG2+ progenitor cells, a major population of proliferating cells located in the anterior subventricular zone of the olfactory bulb [34]. Loss of Cdk2 reduces self-renewal potential and promotes differentiation of NG2+ progenitor cells in adult brain [35]. Like Cdk2-cyclin E, Dab1-E appears to play a role in the maintenance of the retinal progenitor pool since Dab1-E knock-down reduces cell proliferation and promotes ganglion cell differentiation in the retina [24]. Thus, we propose that Cdk2-mediated Dab1-E phosphorylation and degradation is involved in regulating cell cycle progression. It has recently been shown that overexpression of a Dab1-E-like isoform, Dab1.7bc, causes aberrant neuronal positioning in the mouse cortex [36], in support of the idea that Dab1-E and Dab1-L levels must be tightly regulated for normal neuronal migration.

Our results demonstrate that Cdk2-mediated Dab1-E phosphorylation at S475 is critical for the regulation of Dab1-E turnover. Other kinases may also be involved in Dab1-E phosphorylation, as Dab1-E is phosphorylated at multiple sites in the developing retina. It has previously been shown that Cdks can function as priming kinases for polo-like kinases (Plks), a family of S/T kinases that play critical roles in the regulation of the cell

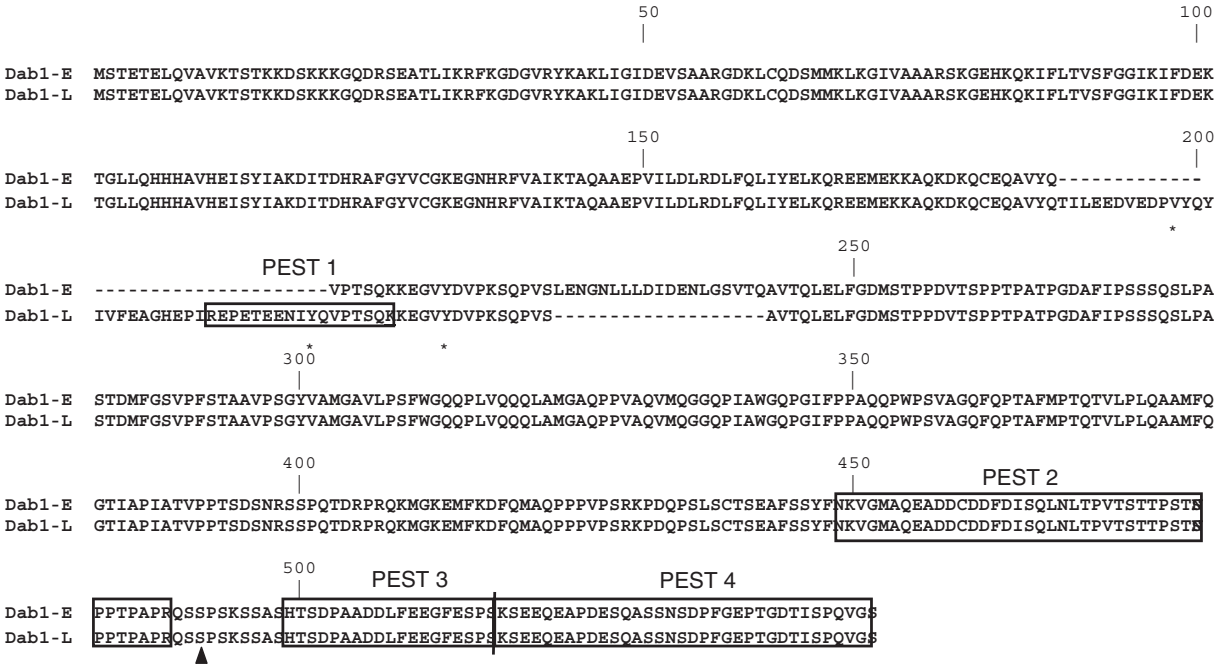

Fig. 7. The protein sequences of both Dab1-E (AY242122) and Dab1-L (AY242123) were scanned for potential PEST motifs using a web-based algorithm, ePESTfind (http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind). Four potential PEST motifs with a PESTfind Score >5 are highlighted in black boxes. Tyrosines that are phosphorylated upon Reelin stimulation in Dab1-L are marked with asterisks, whereas the S475/S491 residue phosphorylated by Cdk in Dab1-E/Dab1-L is indicated with a triangle. Amino acid numbering is based on the Dab1-L sequence.

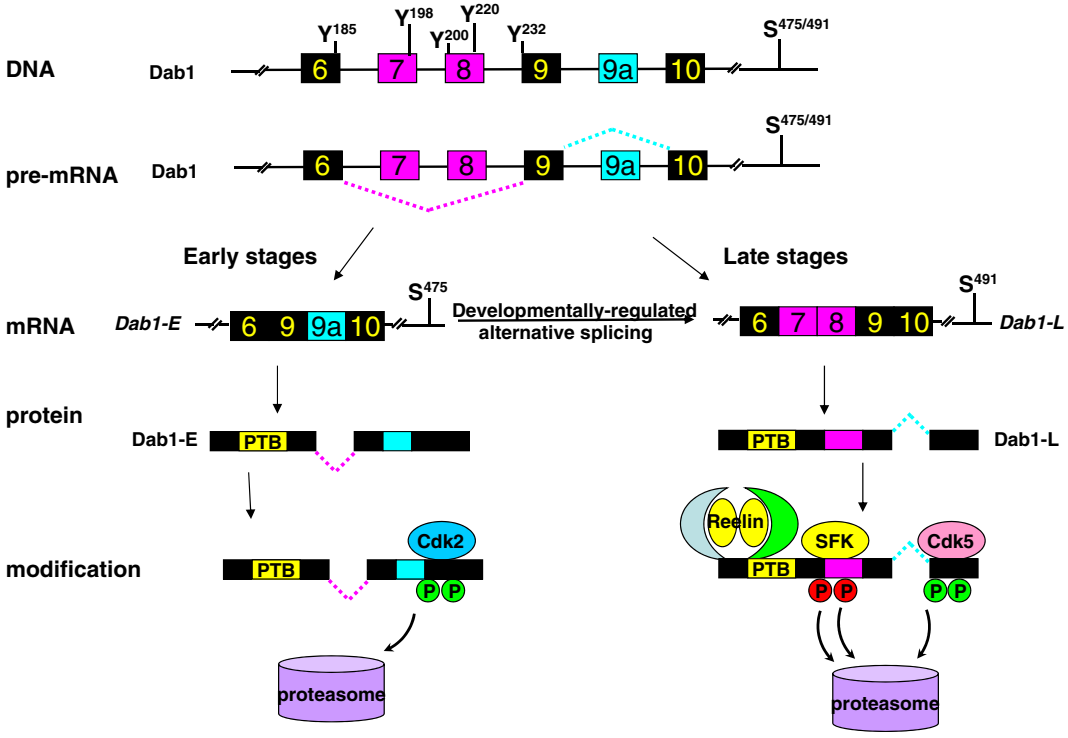

Fig. 8. Model depicting the dual regulatory mechanism controlling Dab1-E and Dab1-L levels during development. The Dab1 gene structure (exons 6, 7, 8, 9, 9a and 10), pre-mRNA (dotted lines indicate alternative splicing), mRNA and protein are diagrammatically represented. At early developmental stages, Dab1-E is the main isoform expressed in the retina. As development progresses, alternative splicing results in a switch from Dab1-E to Dab1-L isoform. S/T phosphorylation of Dab1-E occurs independently of Reelin, whereas tyrosine phosphorylation of Dab1-L is dependent on Reelin stimulation. Dab1 levels are tightly regulated by Reelin-induced Dab1-L tyrosine phosphorylation and Cdk-mediated S/T serine phosphorylation through proteasome degradation.

cycle [37–39]. Plks have a C-terminal polo-box domain (PBD) which binds preferentially to peptides containing the consensus sequence S(pS/pT)P [37,38]. Importantly, Cdk-mediated Dab1-E/L phosphorylation at S475/S491 (SSP) generates a PBD-binding site (SpSP). It is therefore possible that phosphorylated Dab1-E/L can recruit Plk for additional phosphorylation at S/T sites. In keeping with this idea, Plks are differentially expressed in the developing brain, with Plk1 preferentially associated with proliferating cells and Plk2 primarily found in differentiated neurons [40]. Cdk2 and Cdk5 may thus work in conjunction with Plk1 and Plk2 to phosphorylate Dab1-E and Dab1-L, respectively.

In summary, we have found that Cdk2-mediated S/T Dab1-E phosphorylation regulates its turnover independently of Reelin signaling. A main site of S/T phosphorylation in Dab1-E is S475, the counterpart of S491 previously shown to be phosphorylated in Dab1-L. Based on the results reported here combined with data from previous studies, we propose that Dab1 protein levels are under dual regulation by Reelin-independent S/T phosphorylation and Reelin-dependent tyrosine phosphorylation (Fig. 8). At early developmental stages, Dab1-E levels are regulated by Cdk2-mediated S/T phosphorylation. As development proceeds, alternative splicing is activated resulting in the production of Reelin-responsive Dab1-L, whose levels are regulated by both Cdk5-mediated serine phosphorylation and Reelin-induced tyrosine phosphorylation. This dual mechanism may be critical to the control of developmental stage-specific events that are dependent on Dab1-E and Dab1-L functions.

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