Attenuation of Notch signalling by the Down-syndrome-associated kinase DYRK1A

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Summary

Notch signalling is used throughout the animal kingdom to spatially and temporally regulate cell fate, proliferation and differentiation. Its importance is reflected in the dramatic effects produced on both development and health by small variations in the strength of the Notch signal. The Down-syndrome-associated kinase DYRK1A is coexpressed with Notch in various tissues during embryonic development. Here we show that DYRK1A moves to the nuclear transcription compartment where it interacts with the intracellular domain of Notch promoting its phosphorylation in the ankyrin domain and reducing its capacity to sustain transcription. DYRK1A attenuates Notch signalling in neural cells both in culture and in vivo, constituting a novel mechanism capable of modulating different developmental processes that can also contribute to the alterations observed during brain development in animal models of Down syndrome.

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Introduction

The myriad of stimuli received by cells shape their response to the environment in a manner that depends both on the strength of these signals and on their particular history. These incoming signals must be integrated so that a given cell can coordinate with its neighbours to shape the organism and react correctly to these stimuli.

Although many signalling components have been defined, we still know little about how this integration may occur. The Notch signalling pathway may be particularly important as all metazoans use it to mediate local cell-cell interactions in many cell lineages. Notch signalling is often used iteratively, and the same pathway that controls the proliferation of progenitor cells can also regulate binary cell fate decisions and subsequent differentiation steps (reviewed by Bray, 2006; Louvi and Artavanis-Tsakonas, 2006).

Notch is a single-pass transmembrane receptor activated extracellularly by the Delta and Serrate ligand families. This activation leads to the proteolytic cleavage and release of the Notch intracellular domain (NICD) (Schroeter et al., 1998; Struhl and Adachi, 1998), which in turn acts as a transcriptional regulator in the nucleus (reviewed by Bray, 2006; Louvi and Artavanis-Tsakonas, 2006). The NICD can associate with CBF1 (also called CSL after CBF1, Su(H) and Lag-1) and when coupled with MAML, it acts as a transcriptional activator (Smoller et al., 1990; Fortini and Artavanis-Tsakonas, 1994; Nam et al., 2006; Wilson and Kovall, 2006), for a review see (Bray, 2006; Louvi and Artavanis-Tsakonas, 2006). Transcription terminates when the NICD is phosphorylated and degraded in a proteasome-dependent manner (Fryer et al., 2004).

DYRK1A is a nuclear serine/threonine protein kinase of the evolutionarily conserved DYRK kinase family. The *Drosophila* homologue *minibrain* (*mnb*) was originally identified as a mutant affecting postembryonic neurogenesis, with adult mutant flies developing small optic lobes and brain hemispheres (Tejedor et al., 1995). The vertebrate homologue is specifically expressed in the central nervous system and it is widely expressed during neurogenesis (Hammerle et al., 2002; Hammerle et al., 2003b; Marti et al., 2003; Hammerle et al., 2008). Heterozygous mouse mutants also show specific alterations in the brain and overexpression of *Dyrk1a* causes developmental defects (Fotaki et al., 2002; Hammerle et al., 2003a), strikingly at sites where Notch signalling influences development. Like other members of the family, DYRK1A can modify transcription factors, chromatin remodelling proteins, or components of signalling cascades such as Ras-BRaf-MEK1 (Galceran et al., 2003; von Groote-Bidlingmaier et al., 2003; Sitz et al., 2004; Kelly and Rahmani, 2005; Arron et al., 2006; Gwack et al., 2006).

Notch signalling is controlled at several regulatory checkpoints that include ligand and receptor expression, localization, posttranscriptional modification, trafficking or nuclear association, and stability. The NICD is normally hyperphosphorylated in the nucleus (Kidd et al., 1998; Redmond et al., 2000) and several kinases can induce phosphorylation of the NICD, thereby influencing the output of Notch signalling (Foltz and Nye, 2001; Ingles-Esteve et al., 2001; Foltz et al., 2002; Espinosa et al., 2003). Phosphorylation of the NICD PEST (proliner, glutamic acid, serine- and threonine-rich) domain induces proteasome-mediated degradation (Fryer et al., 2004). Here, we show that DYRK1A can also regulate Notch signalling in a kinase-dependent manner. Indeed, DYRK1A physically interacts with the NICD inducing its phosphorylation in the Ankyrin domain, thereby attenuating Notch signalling.

Results

Colocalization of DYRK1A and components of the Notch signalling pathway

Notch signalling and DYRK1A activity are both required for the correct development of the nervous system. In the developing mouse neocortex at embryonic day 14, when both neurogenesis and

neuronal differentiation are active, *Notch1* transcripts were detected in the ventricular and subventricular areas (Fig. 1A,B). Similarly, the *Dll1* ligand and *Hes1*, a target of Notch signalling, were expressed in a punctate pattern in the same area (Fig. 1C-F) (Henrique et al., 1995). *Dyrk1a* was detected in the same regions as these components of Notch signalling and in the cortical plate (Fig. 1G,H) (Hammerle et al., 2008), and similar *Dyrk1a* expression was observed in homologous regions in the chick (Hammerle et al., 2003b). Thus, *Dyrk1a* is expressed in regions where Notch signalling occurs and coincides with *Hes1* and *Notch1* in the ventricular zone. Hence, cells that receive a Notch signal could simultaneously be expressing *Dyrk1a*.

DYRK1A modulates endogenous Notch signalling

We investigated the influence of DYRK1A on Notch signalling in a coculture system where the Notch-expressing human neuroblastoma cell line SH-SY5Y comes into contact with control L cells (L-tk$^-$) or those expressing the ligand Jagged 1 [SN3T9 (Lindsell et al., 1995)]. The endogenous presence of *Notch* and *Dyrk1a* expression is shown in supplementary material Fig. S1. We detected significant activation of a Notch reporter driven by multimerized CSL binding sites when SH-SY5Y cells were cocultured with Jagged-1-expressing cells. However, this activation diminished in a dose-dependent manner when cells were co-transfected with a DYRK1A expression plasmid (Fig. 2A). Similar results were obtained when different ligands (Dll1) and different Notch-expressing cell lines (C2C12) were tested (data not shown). DYRK1A also acted on a natural promoter such as a fragment of the *Hes1* gene promoter: its activity in the presence of DYRK1A and Jagged 1 was similar to that of uninduced cells (Fig. 2A).

To analyze the role of DYRK1A in Notch signalling we performed the same experiment in the presence of an siRNA directed against the endogenous *Dyrk1a* gene (siDYRK1A). siDYRK1A caused a reduction of the expression of the endogenous gene in C2C12 cells as measured by RT-PCR (supplementary material Fig. S1) and an increase in their response to Jagged 1 with respect to a control siRNA (Fig. 2B). A similar effect could be observed in SH-SY5Y cells when exposed to Jagged 1 (Fig. 2B). Thus, the attenuation of Notch signalling caused by the presence of DYRK1A and the enhancement of its response when the endogenous DYRK1A protein is removed point to a direct role of DYRK1A in the modulation of the output of the Notch signalling pathway.

DYRK1A interacts with the NICD of Notch1

We analyzed different truncated versions of Notch1 to determine where DYRK1A acts in the Notch signalling cascade. DYRK1A attenuated the activity of a membrane-tethered form lacking most of the extracellular domain of Notch [NΔE (Kopan et al., 1996)]. This form still needs the activity of γ-secretase to be released from the membrane but it is cleaved independently of the presence of a ligand and mimics the activated receptor (Schroeter et al., 1998) (Fig. 2C,D). Similarly, a form of the NICD that lacks all of the extracellular and transmembrane domains can be attenuated by DYRK1A expression (Fig. 2C,D). Hence, DYRK1A does not appear to act through Notch processing at the membrane. Moreover, the removal of the PEST and OPA (glutamine-rich) regions did not alter the ability of DYK1A to downregulate Notch signalling (NICD-S; Fig. 2C,D). A further isoform that contains the cytoplasmic region between the γ-secretase processing site and most of the seventh ankyrin repeat was unable to drive transcription from the reporters used (data not shown) and therefore, could not be tested for downregulation by DYRK1A (NICD-ANK; Fig. 2C and data not shown) (Kurooka et al., 1998).

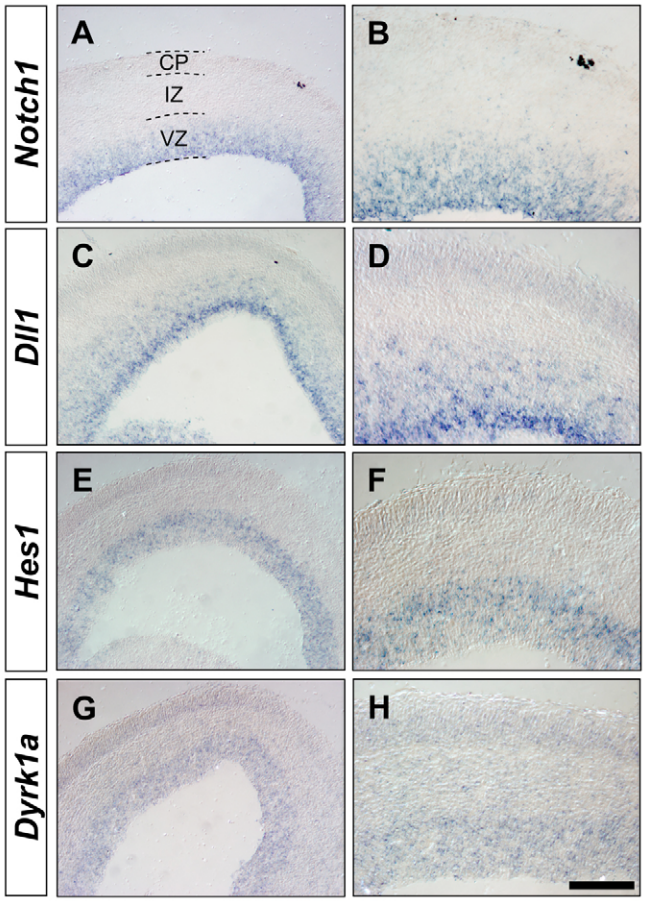

Fig. 1. *Dyrk1a* is coexpressed with components of the Notch signalling pathway in the ventricular zone of the developing cortex. In situ hybridization for elements of the Notch signalling pathway and *Dyrk1a* in the developing neocortex of wild-type E14 mouse embryos. The specific RNA probes used are indicated on the left, and the ventricular and subventricular zone (VZ) of the cortex, the intermediate zone (IZ) and the cortical plate (CP) are defined by the dotted lines in A. (A,B) *Notch1* is expressed in the ventricular and subventricular zone (VZ) of the cortex whereas its expression in the intermediate zone (IZ) and cortical plate (CP) is negligible. (C,D) Notch ligand *Dll1* is mainly restricted to the most ventricular region with some scattered positive cells in the subventricular zone. (E,F) *Hes1*, a target of Notch signalling is expressed in the ventricular and subventricular region. (G,H) *Dyrk1a* is expressed in both the cortical plate and the ventricular and subventricular zones. Expression in the ventricular zone shows also a 'speckled' pattern and extends over the whole area. Right panels (B,D,F,H) are higher magnifications of the left panels (A,C,E,G). Scale bar: 100 μm (B,D,F,H) and 200 μm (A,C,E,G).

Accordingly, the repression of Notch signalling in the presence of DYRK1A does not involve membrane processing or the regions that control the half-life and stability of the NICD (PEST and OPA). Hence, the region including the CSL binding domain and the ankyrin repeats may be the minimal regions associated with this modulation.

Kinase activity of DYRK1A is necessary to modulate Notch signalling and phosphorylate the intracellular domain of Notch

DYRK1A is a dual specificity kinase capable of phosphorylating its own tyrosine residues and the serine/threonine residues of exogenous substrates. Furthermore, DYRK1A can activate some

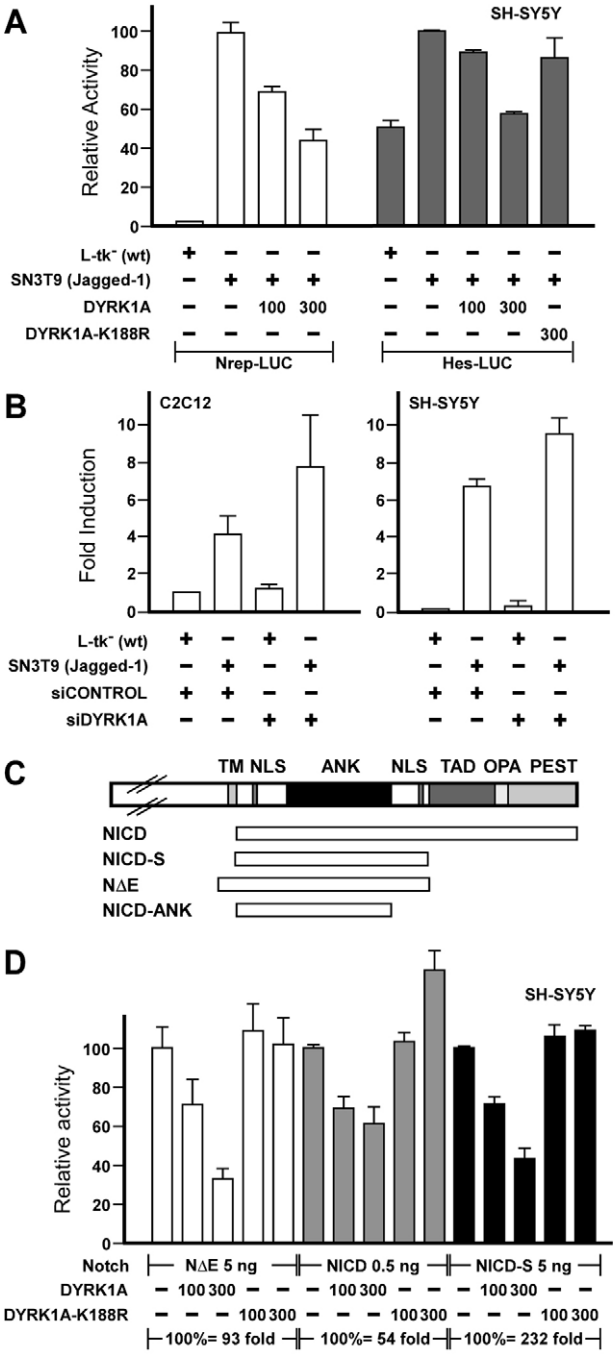

Fig. 2. (A) DYRK1A attenuates Notch signalling in cultured cells. SH-SY5Y cells were transfected with the Nrep (pGa981-6, open bars) or Hes-LUC (filled bars) luciferase reporter genes along with expression plasmids encoding DYRK1A or its K188R substitution (amounts indicated in ng). The cells were then cocultured with Jagged-1-expressing SN3T9 or parental L-tk⁻ cells (Lindsell et al., 1995) and the relative luciferase activity was quantified. The levels of luciferase were normalized for the expression of a co-transfected CMV-driven Renilla luciferase. Relative activity is relative to the level of luciferase from cells transfected with each reporter and cultured in the presence of Jagged-1-expressing cells (100%). Values are means and errors of 2-3 experiments. (B) Removal of endogenous DYRK1A causes an increase in the strength of Notch signalling. C2C12 and SH-SY5Y cells were transfected with Nrep and the indicated siRNA with Lipofectamine 2000, and after incubation for 8 hours complexes were removed and cells were and cocultured with Jagged-1-expressing or parental L-tk⁻ cells. Luciferase was measured as in A. Fold induction is relative to cells transfected with Nrep and incubated with control L-tk⁻ cells. (C) Scheme of the relevant domains in Notch1 protein and the fragments used: TM, transmembrane domain; NLS, nuclear localization signal; ANK, ankyrin domain; TAD, transactivation domain. The OPA and PEST regions are represented by shaded rectangles and based on the previous description by Kurooka et al. (Kurooka et al., 1998). The extracellular domain of Notch is not represented to scale (indicated by the diagonal lines). The fragments of the NICD are represented by open rectangles; the NICD contains amino acids 1751-2531 from the mouse gene; NICD-S, 1751-2184; NDE, 1704-2184 and NICD-ANK, 1751-2098. (D) DYRK1A interacts with the RAM and ANK domains of the NICD. SH-SY5Y cells were transfected with Nrep, CMV-driven Renilla luciferase and the indicated DYRK1A and Notch expression vectors (amounts indicated in ng), and luciferase activity was quantified. Relative activity is relative to the normalized level of luciferase from cells transfected with the Nrep reporter and the corresponding NICD fragment. The fold induction for each NICD fragment over the control situation (no NICD; 100%) is indicated. Values are means, and error bars indicate the standard error from 2-3 experiments.

substrates in the absence of detectable kinase activity (Sitz et al., 2004; Kelly and Rahmani, 2005), and the K188R substitution in its ATP binding motif abolishes most of its kinase activity while retaining these other kinase-independent functions (Wiechmann et al., 2003). This K188R substitution did not significantly repress the output of Notch signalling in SH-SY5Y cells following Jagged 1 activation (Fig. 2A). Similarly, its effect on the active forms of Notch tested indicates that the kinase-dead form of DYRK1A is not causing a major effect on their activity (Fig. 2D). Thus, the activity of DYRK1A on Notch signalling depends mostly on its kinase activity.

In the presence of DYRK1A, we detected a small shift in the electrophoretic mobility of the NICD, which was reversed by alkaline phosphatase (AP) but not in the presence of phosphatase inhibitors (Fig. 3A). The NICD-S truncation behaved similarly and the shortest fragment to show a change in mobility was NICD-ANK (Fig. 3B), a transcriptionally inactive form of the NICD. Likewise, other members of the DYRK family also modified the migration of NICD-S in the same manner (supplementary material Fig. S2). Thus, this change in mobility and the attenuation of Notch signalling appear to be related, suggesting that phosphorylation of a region outside the PEST domain by DYRK kinases impairs the capacity of the NICD fragment to activate transcription.

DYRK1A can directly phosphorylate NICD

In kinase assays, there were two major phosphorylation sites of the NICD, the OPA/PEST region and the RAM-ANK fragment (Fig. 4A). Although the former was mildly phosphorylated in the absence of DYRK1A and it is probably the substrate of another kinase, the phosphorylation of the latter appeared to be fully dependent on DYRK1A. Indeed, the RAM-ANK fragment was not phosphorylated by the kinase-deficient DYRK1A-K188R. Thus, DYRK1A appears to directly phosphorylate the NICD outside of the PEST region, in a domain that contains the CSL interaction domain and the ankyrin repeats.

To characterize the phosphorylation sites in the NICD we performed 2-D electrophoresis to analyze its degree of phosphorylation. In the absence of exogenous DYRK1A, the NICD-S shows several spots indicative of different phosphorylation states (Fig. 4B). The presence of exogenous DYRK1A induced a mobility change consistent with increased phosphorylation and the presence of multiple DYRK1A-dependent phosphorylation sites. When DYRK1A-expressing C2C12 cells were used, most of the NICD-S protein migrated as the hyperphosphorylated form.

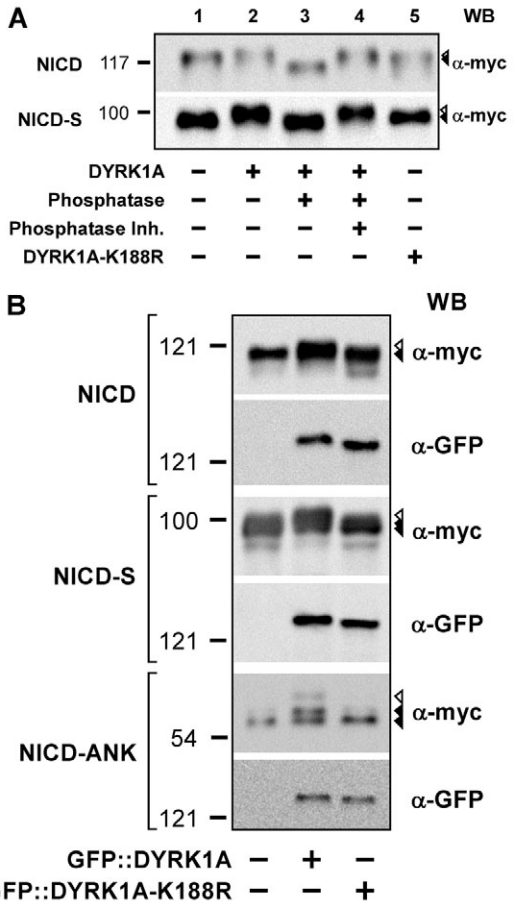

Fig. 3. (A) DYRK1A phosphorylation of the NICD shifts its mobility. Whole extracts of transfected HEK293 cells were treated with alkaline phosphatase in the presence or absence of inhibitors. (Upper panel) Cells transfected with the Myc-tagged NICD isoform; (lower panel) with the Myc-tagged NICD-S isoform. Open arrowheads indicate the migration of the NICD in the presence of DYRK1A, the filled arrowheads indicate the normal migration in its absence. Molecular mass marker positions are indicated on the left, in kDa. (B) Retardation of the NICD depends on the presence of the ANK and RAM domains. Whole extracts of HEK293 cells transfected with the Myc-tagged Notch isoforms, indicated at left, and the GFP linked kinases, indicated at bottom, were probed for the NICD constructs (anti-Myc upper panel in each section) and DYRK1A (anti-GFP lower panels). Retardation only occurs in the presence of DYRK1A (arrowheads on the right). Note that both NICD-S and NICD-ANK migrate as doublets (filled arrowheads). The K188R substitution shows a faster migration most probably because of its inability to autophosphorylate.

However, treatment of C2C12 cells with siRNA directed against DYRK1A caused the appearance of underphosphorylated forms (Fig. 4B).

We analyzed the potential consensus DYRK1A phosphorylation sites in the NICD and we found at least five evolutionarily conserved threonine sites that could match the consensus RPX**S**/**T**P. Mutational analysis of these sites indicated that individual or paired alanine substitutions (one substitution in each of two ankyrin domains) did not affect their attenuation by DYRK1A (not shown). This suggests that DYRK1A phosphorylates multiple sites in the NICD. In agreement with this, individual mutations in a total of 18 different serines or threonines that conform to the consensus phosphorylation site did not reduce the degree of DYRK1A-mediated attenuation of Notch signalling.

Not only did the NICD appear to be directly phosphorylated by DYRK1A but in cells co-transfected with both proteins, a small amount of the NICD specifically associated with DYRK1A (Fig. 5A). However, a stable interaction was clearly observed between the NICD and the DYRK1A-K188R substitution (Fig. 5A), suggesting that complexes with phosphorylation intermediates may be stabilized when the enzyme lacks its kinase activity. A similar interaction with DYRK1A was also observed with the NICD-S fragment (Fig. 5A).

Since phosphorylation can regulate stability of the NICD, we determined the effect of DYRK1A on the half-life of the NICD in the absence of protein biosynthesis. In the presence of DYRK1A the half-life of the NICD was not reduced (Fig. 5B). Interestingly, the slow migrating form of the NICD seems to be slightly protected from degradation excluding the possibility that the attenuation of Notch signalling was due to a decrease in NICD stability.

DYRK1A and NICD colocalize in the cell

We also investigated whether the NICD and DYRK1A colocalize in the nucleus. DYRK1A nuclear localization is similar to that of the Sc35 splicing factor (Alvarez et al., 2003; de Graaf et al., 2004). The punctate distribution of the NICD in the nucleus of transfected cells did not colocalize with the Sc35 splicing factor consistent with its proposed association with the transcription machinery (Fig. 6Ab-d) (Hall et al., 2006; Hooper et al., 2007). Endogenous Notch showed a similar distribution in C2C12 cells with small puncta located close to Sc35 speckles (Fig. 6Ba-c). As expected, GFP::DYRK1A colocalized with nuclear Sc35 (Fig. 6Ae-h) (Alvarez et al., 2003; de Graaf et al., 2004) similarly to endogenous DYRK1A (Fig. 6Bd-f). Interestingly, when DYRK1A and the NICD were coexpressed, a small fraction of DYRK1A appeared to be associated with the NICD and not with Sc35 (Fig. 6Ai-l and insets therein). Splicing and transcription are normally physically associated and cooperate in controlling gene expression (Hall et al., 2006; Misteli, 2007). This data is in agreement with our data showing an interaction between the NCID and DYRK1A (Fig. 5A). Given the increased association of the NICD with the kinase-deficient DYRK1A (Fig. 5A), we predicted an increased colocalization of the NICD with DYRK1A-K188R as shown in Fig. 6Am-p. Both data are consistent with the presence of an enzymatically frozen intermediate (Fig. 5A) and strongly suggest that DYRK1A transitorily leaves the splicing compartment to contact the transcription machinery where it can transiently associate with the NICD.

DYRK1A can attenuate Notch signalling in cell culture and in vivo

We next investigated whether DYRK1A could attenuate Notch signalling in cultured cells. To address this point we took advantage of the described impairment in neuritogenesis mediated by Notch (Franklin et al., 1999). Indeed, as shown in Fig. 7, whereas Notch signalling impaired neurite formation in N2a neuroblastoma cells cultured in reduced serum (16.2% versus 25.4% for controls, $P \leq 0.05$; $n=3$), the presence of DYRK1A reversed this deficit (30.8% neuritogenesis, $P \leq 0.02$; $n=3$). As expected, the proportion of cells with neurites was similar to that of control N2a cells ($P \leq 0.05$; $n=3$; Fig. 7A).

Once shown that DYRK1A could release the Notch-mediated impairment in neuritogenesis, we examined whether it was sufficient to attenuate endogenous Notch signalling in vivo. Notch signalling is active in the developing neural tube of the

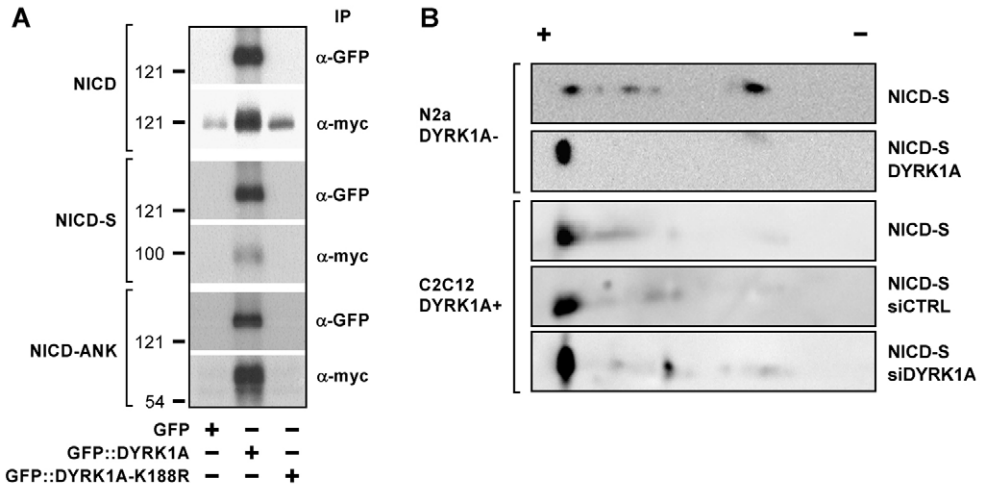

Fig. 4. (A) In vitro phosphorylation of the NICD by DYRK1A. Immunopurified GFP-fused kinases (indicated at the bottom) and Myc-tagged substrates (plasmids indicated on the left) were isolated from whole cell extracts of transfected HEK293 cells. Beads loaded with the immunopurified proteins were mixed and subjected to a kinase reaction in presence of radioactive [γ-^{32}P]ATP. Enzymes and substrates were separated by immunoprecipitation (indicated on the right) and were assessed separately. Autoradiograms of the resulting immunoprecipitations (IP) indicate the phosphorylation levels of the Notch (α-Myc IP) and DYRK1A isoforms (α-GFP IP). Note the lack of phosphorylation of NICD-S and NICD-ANK incubated with DYRK1A-K188R or GFP immunocomplexes. Only DYRK1A shows autophosphorylation. (B) 2-D electrophoresis analysis of migration of the NICD-S in the presence of DYRK1A. Plasmids and siRNAs, indicated on the right, were transfected in the cell lines indicated on the left; endogenous expression of DYRK1A is given below. The presence of exogenous DYRK1A induces a shift towards the acidic region of the gradient labelled with a + over the panels. siRNA against DYRK1A prevents the conversion of the NICD into more acidic protein spots.

chick embryo, as evidenced by the expression of some of its targets (Fior and Henrique, 2005; Hammerle and Tejedor, 2007). Thus, we over-expressed DYRK1A in one side of the chicken neural tube through in ovo electroporation to assess whether it had an effect on the expression of the Notch signalling target *Hes5-1*. As shown in Fig. 7B, *Hes5-1* expression was dramatically reduced in the electroporated side indicating that DYRK1A downregulates Notch signalling in vivo (Fig. 7B). Interestingly, the electroporation of the inactive DYRK1A-K188R mutant form did not modify *Hes5-1* expression, indicating its failure in attenuating Notch signalling in vivo.

Discussion

The endogenous expression patterns of DYRK1A and components of the Notch signalling pathway in the mouse neocortex indicate that they can be simultaneously expressed in the same cells. This work also shows that DYRK1A and Notch can physically interact and reveals a novel role for DYRK1A in attenuating Notch signalling both in vitro and in vivo.

DYRK1A attenuates Notch signalling in vitro

Our data show that DYRK1A attenuates Notch signalling both in a neuroblastoma cell line containing the endogenous components

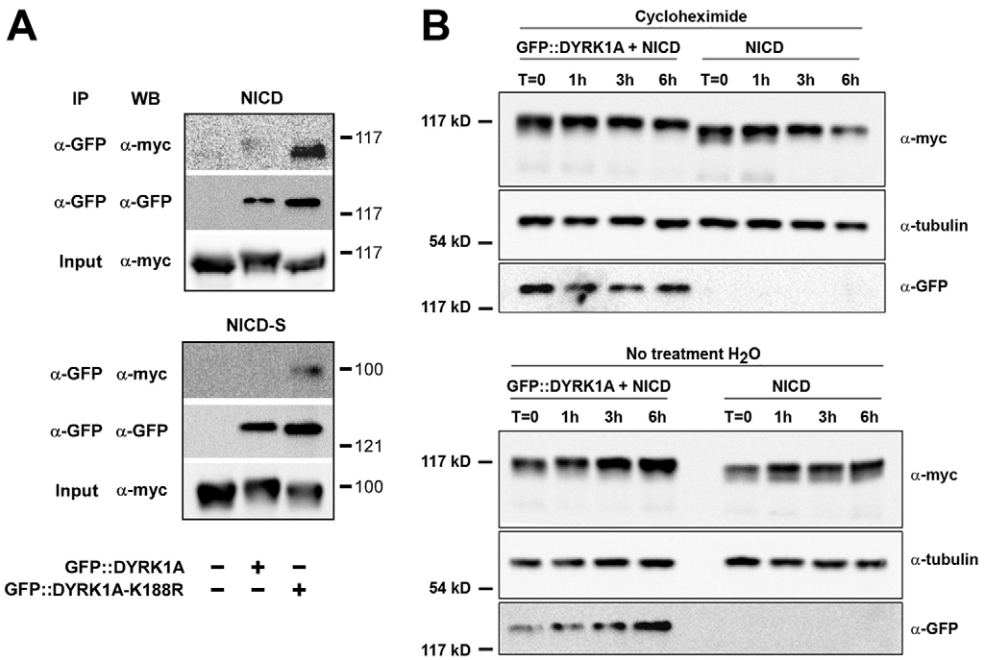

Fig. 5. (A) DYRK1A and the NICD interact in vitro. The two upper samples of each panel are the same western blots probed for the NICD (anti-Myc) and DYRK1A (anti-GFP). The lower part of each panel had one tenth of the input used for immunoprecipitation. Whole cell extract from HEK293 cells transfected with Myc-tagged NICD (upper panel) or Myc-tagged NICD-S (lower panel) and the indicated form of DYRK1A, as shown at the bottom, were immunoprecipitated with α-GFP antibodies and immunoblots were probed with the indicated antibodies. Molecular mass standards are indicated in kDa on the right. (B) Effect of DYRK1A expression on Notch stability. HEK293 cells were transfected with the indicated plasmids and 16 hours after transfection were incubated with cycloheximide (100 μg/ml) or the solvent (water) for the indicated time. Note the change in mobility of the NICD in the presence of DYRK1A. No significant changes were observed between the stability of NICD in the presence or absence of DYRK1A.

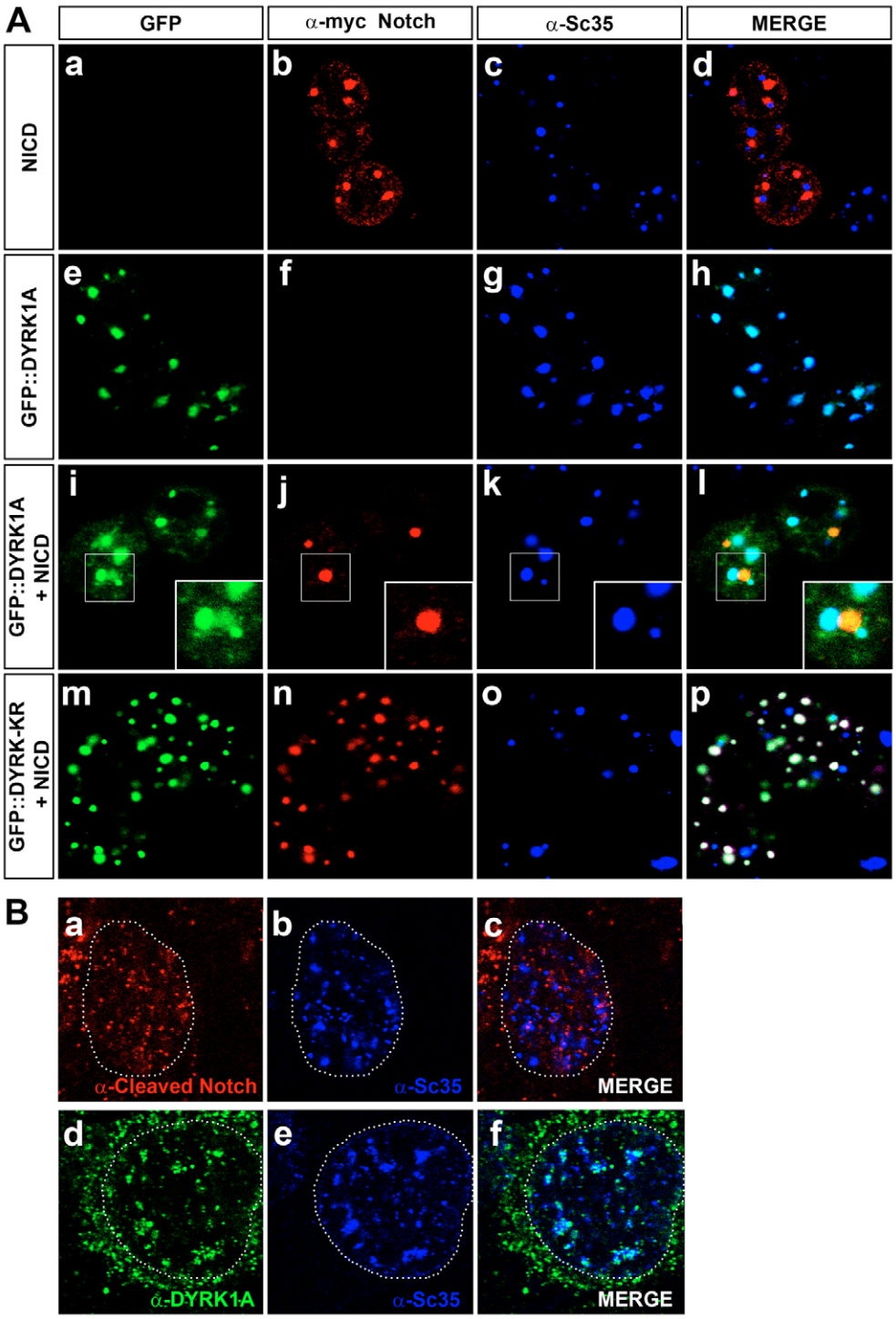

Fig. 6. (A) DYRK1A and the NICD colocalize in the cell nucleus. HEK293 cells were transfected with the expression plasmids encoding the proteins indicated. Single confocal image sections are shown for each channel. GFP detection is of the GFP moiety of the DYRK1A fusions (a,e,i,m), α-Myc antibodies detected the tagged NICD protein (b,f,j,n), α-Sc35 antibodies detect the endogenous Sc35 splicing factor (c,g,k,o); the Merge images (d,h,l,p) are of the whole row. Insets in i-l are magnifications of the regions indicated to illustrate the colocalization of the NICD and DYRK1A in puncta close to the Sc35 speckles. (B) Endogenous NICD and DYRK1A are localized in the nucleus. Endogenously cleaved Notch1 was detected in C2C12 cells in nuclear puncta, in close vicinity to Sc35 speckles (a-c), and some cytoplasmic puncta are also evident. Endogenous DYRK1A distribution is both cytoplasmic and nuclear. Most of the nuclear puncta are located in close association with Sc35 speckles (d-f). Dotted lines delimit the nuclei.

of the signalling pathway and in cells engineered to express them. DYRK1A gain- and loss-of-function experiments show that it attenuates the corresponding Notch output.

The modulation of other signalling events by DYRK1A has been described previously (Galceran et al., 2003; von Groote-Bidlingmaier et al., 2003; Sitz et al., 2004; Kelly and Rahmani, 2005; Arron et al., 2006; Gwack et al., 2006), with the kinase activity of DYRK1A often being found to be dispensable for the regulation (von Groote-Bidlingmaier et al., 2003; Kelly and Rahmani, 2005). By contrast, we show here that the attenuation of Notch signalling by DYRK1A is dependent on its kinase activity, compatible with previous findings indicating that the intracellular domain of Notch is a substrate of several kinases that can modulate its activity (Kidd et al., 1998; Redmond et al., 2000; Foltz and Nye, 2001; Ingles-Esteve et al., 2001; Foltz et al., 2002; Espinosa et al., 2003; Fryer et al., 2004). Interestingly, the domains previously described as targets for phosphorylation [TAD and OPA/PEST (Franklin et al., 1999)] are dispensable for the regulation of Notch signalling by DYRK1A. Indeed, our mapping analysis shows that DYRK1A phosphorylates the RAM-ANK domain.

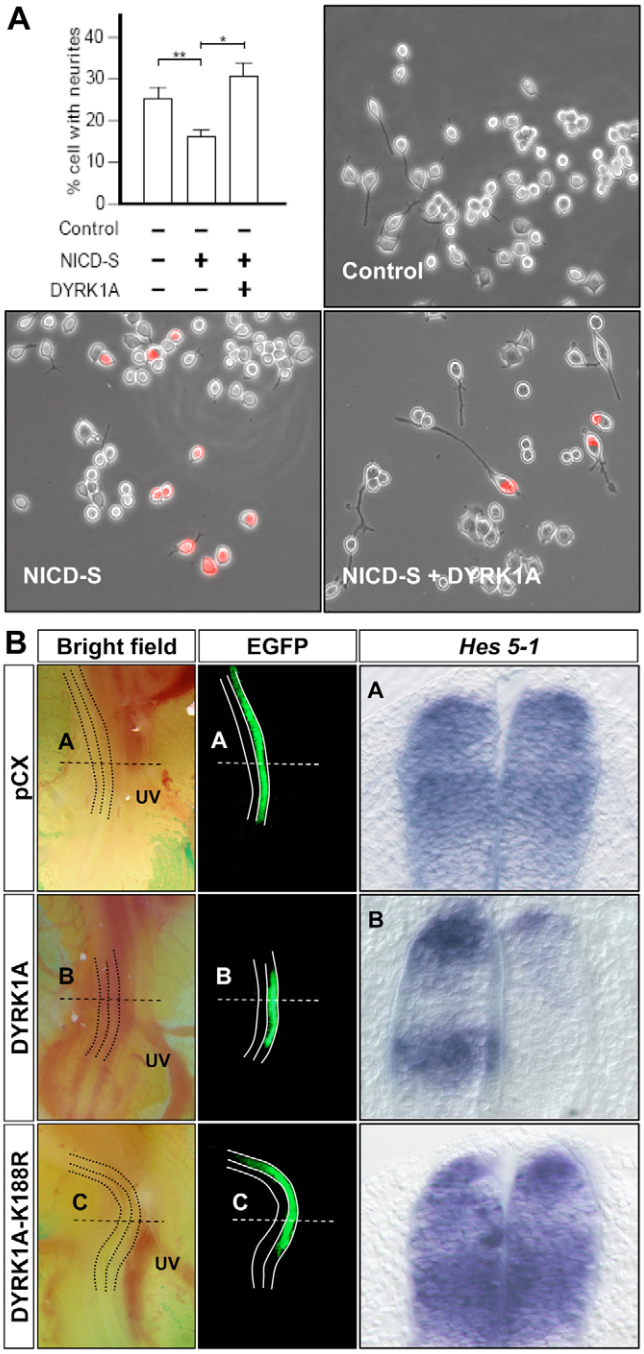

Fig. 7. DYRK1A interferes with Notch signalling in vivo. (A) DYRK1A attenuates the NICD repression on neuritogenesis. Neuro2a cells were transfected with Nrep-RFP, a reporter of Notch activity driving mRFP, plus the plasmids indicated in each panel. Red cells are to those that have received Notch signal. Differentiating Neuro2a cells produce neurites and the NICD represses this process (lower left panel). Co-transfection of DYRK1A relieves this repression (lower right panel) as quantified (mean and s.e.m. of three experiments, Student's *t*-test: $*P \leq 0.02$, $**P \leq 0.05$). (B) DYRK1A reduces Hes 5-1 expression in the developing neural tube. The right hand side of the neural tube of stage HH12 chick embryos was electroporated with a vector encoding GFP plus control empty vectors (pCX) or those encoding wild-type or inactive forms of DYRK1A. The embryos were subsequently allowed to develop until HH17 before assessing the expression of the Notch signalling readout *Hes5-1*. The middle panel shows the expression of the co-electroporated GFP-expressing vector on the right half of the neural tube. Dotted lines in left and middle panels indicate the level of the sections shown in the right panels, and white lines in the middle panels outline the neural tube. When the vector encoding DYRK1A was electroporated, *Hes 5-1* expression was highly reduced in the electroporated side. Electroporation of both the control empty vector (pCX) or of the inactive DYRK1A-K188R mutant form failed to downregulate *Hes 5-1* expression.

To identify the phosphorylation sites in Notch we examined the potential sites based on the described consensus sequence (RPX**S**/**T**P), Notch crystal structure and their conservation in different species (Nam et al., 2006; Wilson and Kovall, 2006). Our data show that single substitutions in any of the putative 18 conserved serines and threonines that could be targets of DYRK1A could not reduce the attenuation caused by the kinase. These substitutions included the best conserved sites corresponding to TP motifs within the TPLH sequence at positions 4-7 of the ANK repeats 1, 3, 4 and 6 of Notch1 (Sedgwick and Smerdon, 1999; Mosavi et al., 2002; Lubman et al., 2005). These results indicate that DYRK1A phosphorylates Notch at multiple sites. Double substitutions in different combinations of ANK repeats dramatically decreased Notch activity, but DYRK1A could still attenuate the diminished response. These data highlight the importance of the threonines in the maintenance of the ANK structure and preclude further analysis of multiple mutations in the context of DYRK1A activity. To overcome this problem, we carried out 2-D electrophoresis analysis and confirmed that DYRK1A induces multiple phosphorylation events in the NICD as shown by the large shift observed when both proteins were coexpressed. Interestingly, whereas the phosphorylation of the PEST region by CDK8 targets the NICD to a degradation pathway (Fryer et al., 2004), phosphorylation events mediated by DYRK1A do not affect the stability of the Notch protein. Thus, DYRK1A attenuates Notch activity by phosphorylation in multiple residues without affecting its stability.

DYRK1A and NICD transiently interact in the nucleus

Although DYRK1A performs some of its activities in a kinase-independent manner (von Groote-Bidlingmaier et al., 2003; Sitz et al., 2004; Kelly and Rahmani, 2005), our data indicate that the relationship between DYRK1A and the NICD needs the kinase activity, suggesting an enzyme-substrate type interaction. Indeed, our data indicate that the interaction is very transient and thus, difficult to detect. The use of the kinase-inactive form of DYRK1A greatly favoured the detection of the complex, consistent with the formation of a stable, albeit unproductive, complex with Notch. Hence, we conclude that DYRK1A and the NICD transiently associate in the nucleus, an association that is stabilized when the kinase is inactive.

DYRK1A attenuates Notch signalling in vivo

Besides the effect that DYRK1A exerts on the transcriptional activity of Notch signalling reporters we have investigated whether the expression of DYRK1A could affect some of the activities that Notch signalling performs in vivo. Notch signalling can prevent the maturation of neurons (Franklin et al., 1999) and we checked whether the reduction in neurite development in cells with an activated form of the NICD could be reversed by the presence of DYRK1A. Indeed, we found that the repression in neuritogenesis was released in the presence of DYRK1A, indicating that the response of the cell to Notch signalling was attenuated.

As Notch signalling is used iteratively to control cell proliferation, determination or differentiation in the developing neural tube, we used it as an in vivo model to study the effect of DYRK1A on Notch signalling. The overexpression of DYRK1A in the developing neural tube repressed the expression of *Hes5-1*, a well known indicator of Notch signalling (Fior and Henrique, 2005; Hammerle and Tejedor, 2007), confirming that DYRK1A is sufficient to attenuate endogenous Notch signalling in vivo.

In *Drosophila* the lack of function mutant *minibrain* (*mnb*), the fly DYRK1A homologue, results in a reduced brain size because of a decrease of the generation of cells during postembryonic development (Tejedor et al., 1995) and similarly, *Dyrk1a*$^{+/-}$ mice also have smaller brains (Tejedor et al., 1995; Fotaki et al., 2002; Hammerle et al., 2003a). Although an abnormal increase in Notch signalling inhibits neuronal differentiation in mice, deficient Notch signalling also leads to a reduction in the number of neurons in the adult cause by the induction of precocious neuronal differentiation (for a review, see Yoon and Gaiano, 2005; Louvi and Artavanis-Tsakonas, 2006). Thus, these data are compatible with our finding that DIRK1A attenuates Notch signalling both in vitro and in vivo. Interestingly, our data may be also relevant for studies of Down syndrome (DS). Indeed, *DYRK1A* is one of the genes located in the Down syndrome critical region (Delabar et al., 1993) and its expression is upregulated in DS individuals (Dowjat et al., 2007; Lockstone et al., 2007). Notch signalling is also altered in the DS condition, although contrasting data have been obtained in studies in humans and mouse models. Although Notch signalling seems to be upregulated in the cortex of DS individuals, it is repressed in the cerebellum of Ts1Cje mice, a model for DS (Dauphinot et al., 2005). Several factors could account for these apparently contradictory results. First, the human DS condition also produces an increase in the expression of the Notch receptor and changes in the expression of other Notch modifiers such as *Dlx* (Dauphinot et al., 2005; Lockstone et al., 2007). The presence of DYRK1A in these cells, with a clear misbalance in gene expression, might result in an attenuation of an otherwise augmented Notch signalling and be coherent with the above mentioned results in which Notch output is upregulated (Fischer et al., 2005; Lockstone et al., 2007) or downregulated (Dauphinot et al., 2005). Nevertheless, we believe that the clear effects of DYRK1A in the attenuation of Notch signalling that we describe here can have a higher impact on DS during embryonic development, when DYRK1A is prominently expressed in Notch expressing areas.

In summary, we show here that DYRK1A is able to attenuate Notch signalling both in neuroblastoma cells and in vivo, providing further insight into the mechanisms by which neurogenesis and other cell decisions mediated by Notch signalling can be modulated both in physiological and pathological conditions. Indeed, its ability to downregulate Notch signalling could contribute to the severe alterations in the formation of certain brain regions observed in animal models and associated with the development of Down syndrome in humans. Finally, the widespread expression of DYRK1A opens up the possibility that it can also modulate Notch signalling in other tissues.

Materials and Methods

In situ hybridization

In situ hybridization in E14 (embryonic day 14) mouse embryos was performed on 20 μm cryosections obtained from embryo brains fixed in 4% formaldehyde. The entire mesenchyme had been removed from the nervous tissue prior to cryoprotection and embedding. Sections were processed as described previously (Galceran et al., 2000). In situ hybridization was carried out using in vitro transcribed and DIG-labelled antisense RNA probes that corresponded to: mouse Notch1, (5020-6955 from NM008714.2), Hes1 (1-702 from NM008235.2), Dll1 (1110-2355 from NM007865), and DYRK1A (1096-1988 from NM007890.1).

Plasmids

Expression plasmids for rat DYRK1A, the K188R substitution of rat DYRK1A, human DYRK1B-p69, human DYRK2 and rat CLK3 fused to pEGFP-C1 (Clontech) were as described previously (Becker et al., 1998; Leder et al., 1999; Wiechmann et al., 2003; de Graaf et al., 2004). The mouse Notch1 expression vectors contained the following isoforms. NΔE: the signal peptide followed by the fragment 1704-2184 (Kopan et al., 1996); NICD: amino acids 1751-2531 in pCMV-Myc (Clontech); NICD-S: amino acids 1751-2184 followed by six Myc tags in pEVRF; and NICD-ANK amino acids 1751-2098 in pCMV-Myc. The Notch reporter construct pGa981-6 [Nrep, described by Minoguchi et al. (Minoguchi et al., 1997)], which contains 12 copies of the CBF binding site. Hes-LUC was constructed by cloning the fragment –200 to +134 of the mouse Hes1 gene into pGL3Basic (Promega). Nrep-RFP is a derivative of pGA981-6 in which the luciferase gene was replaced with mRFP.

Cell culture

Cells were cultured in DMEM or MEM growth medium (Sigma), with 10% FBS (Sigma) at 37°C and in 5% CO_2. SH-SY5H cells were grown in MEM, whereas the C2C12, HEK293, L cell-derived lines and Neuro2A were grown in high glucose DMEM. To differentiate Neuro2A cells the FBS was reduced to 0.2%.

For the coculture assays, 0.8×10^5 SHSY5H cells were transiently transfected with Lipofectamine 2000 (Invitrogen). Parental L cells or JT cells [SN3T9 (Lindsell et al., 1995)] were added after removing complexes. Gene reporters were transfected alone or in combination with the expression plasmids for DYRK1A, DYRK1B, DYRK2 or CLK3 fused to GFP (Becker et al., 1998), or plasmids containing the Notch deletions.

Plasmids and siRNA transfection

Expression plasmids for the different DYRK and Notch isoforms were transiently transfected with Lipofectamine 2000 for reporter assays or with jetPEI (PolyPlus-Transfection) for protein isolation. A CMV-RL control plasmid was used in each transfection experiment to control for the transfection efficiency. Firefly and Renilla luciferase activities were measured with the Dual-Luciferase Reporter assay system (Promega) according to the manufacturer's recommendations.

ON-TARGETplus SMARTpool specific for mouse DYRK1A, and ON-TARGETplus siCONTROL were obtained from Dharmacon and transfected using Lipofectamine 2000. Transfected cells were incubated overnight with the siRNA before being replated in the presence of Jagged-1-expressing cells or control.

Protein extracts, immunoprecipitation and phosphatase assay

HEK293 cells were transfected with expression plasmids using jetPEI (PolyPlus-Transfection). The cells were harvested and extracted in Co-IP150 buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 2 mM sodium pyrophosphate, 20 mM NaF, 1% NP-40, 1 mM sodium orthovanadate, 1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin). The proteins were resolved by SDS-PAGE and transferred to Hybond ECL nitrocellulose membranes (Amersham Biosciences), which were then probed with mouse anti-Myc 9E10 (1:2000; Roche Applied Science) or rabbit anti-GFP (1:1000; A11122 Molecular Probes, Invitrogen). The antibodies were detected with HRP-conjugated secondary antibodies (sheep anti-mouse or anti-rabbit HRP conjugates, 1:5000; Santa Cruz), and visualized by enhanced chemiluminescence (ECL; Amersham Biosciences).

Immunoprecipitation was performed on 200 μg of total protein in Co-IP20 buffer (Co-IP150 with 20 mM NaCl), adding 2 μg of anti-Myc antibodies or 3 μg of anti-GFP antibodies. Complexes were isolated with protein-G-Sepharose (Roche Applied Science) and washed in Co-IP20. Immunoprecipitates were resolved by SDS-PAGE and then examined in western blots with the appropriate antibodies. For alkaline phosphatase treatment, 15 μg of whole cell extracts were incubated with 20 IU of calf intestinal alkaline phosphatase (Roche Applied Science; 1 hour at 37°C) in the presence or absence of 20 mM NaF and 1 mM sodium orthovanadate as phosphatase inhibitors. Proteins were precipitated with TCA and examined in immunoblots.

Two-dimensional electrophoresis

Neuro2a or C2C12 cell extracts for 2-D electrophoresis were obtained from Lipofectamine2000-transfected cells using Sample preparation solution as recommended in the GE Healthcare 2D System (7 M urea, 2M thiourea, 4% CHAPS, 2% IPG buffer, 40 mM DTT). Samples were cleaned and concentrated with the 2D Clean-Up Kit (GE Healthcare) and separated in Immobiline Drystreaps pH 4-7 in the IPGphor III system according to manufacturer's recommendations (GE Healthcare). The second dimension was run in standard PAGE, immunoblotted and probed with 9E10 anti-Myc antibodies.

In-vitro phosphorylation assay

GFP, GFP::DYRK1A (wt) and GFP::DYRK1A-K188R were immunoprecipitated from transiently transfected HEK293 cells with anti-GFP antibodies (Molecular Probes, Invitrogen) and protein G agarose (Roche Applied Science). Myc-tagged

Notch proteins were immunoprecipitated with anti-Myc antibodies (Roche Applied Science) and Prot G-Dynabeads (Dynal, Invitrogen). Matrices were washed three times with high salt buffer (Co-IP 600: Co-IP buffer containing 600 mM NaCl) and equilibrated in phosphorylation buffer (50 mM Tris-HCl, 20 mM NaCl, 10 mM $MgCl_2$, 2 mM sodium pyrophosphate, 20 mM NaF, 1% NP-40, 1 mM sodium orthovanadate, 1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin). Phosphorylation reactions were performed by mixing the Prot-G-Dynabead purified substrates with Prot-G-Sepharose-purified kinases in phosphorylation buffer with 200 μM ATP, 2500 c.p.m./pmol [γ-^{32}P]ATP (Amersham) and 2 μg of rabbit IgG with gentle agitation (1 hour, 30°C). Both matrices were isolated, washed with phosphorylation buffer, and analyzed separately by SDS-PAGE and autoradiography.

In ovo electroporation of chicken embryo

Stage Hamburger and Hamilton (HH) 11-12 embryos were electroporated as described previously (del Barrio and Nieto, 2002). After incubation for 24 hours EGFP-expressing embryos were fixed and processed for in situ hybridization.

Staining and imaging procedures

Immunofluorescence of transiently transfected HEK293 cells was carried out using standard procedures and the following primary antibodies: rabbit anti-Myc (1:400 ab910; Abcam), rabbit anti-DYRK1A (1:50; Cell Signaling), rabbit anti-cleaved Notch1 (1:50; Cell Signaling), and mouse anti-splicing factor Sc35 (1:1000 S4045; Sigma). The secondary antibodies used were Alexa Fluor 568 goat anti-rabbit and Alexa Fluor 647 goat anti-mouse (1:500; Molecular Probes, Invitrogen) and after washing, the coverslips were mounted with Vectashield (Vector). GFP was excited with the 488-nm argon line, and all images were captured using a confocal microscope (Leica Microsystems TCS SL) and acquired with a HCX PL-APO 63×/1.32-0.6 oil immersion lens and Leica LCS software. Images were resized, cropped, labelled and arranged with Photoshop CS2 (Adobe).

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