The Dependence Receptor UNC5H2/B Triggers Apoptosis via PP2A-Mediated Dephosphorylation of DAPK Kinase

Céline Guennebeaud, 1,5 David Goldschneider, 1,5 Marie Castets, 1,5 Catherine Guix, 1 Guillaume Chazot, 1 Céline Delloye-Bourgeois, 1 Avital Eisenberg-Lerner, 2 Galit Shohat, 2 Mingjie Zhang, 3 Vincent Laudet, 4 Adi Kimchi, 2,5 Agnès Bernet, 1,6 and Patrick Mehlen 1,*

1Apoptosis, Cancer and Development Laboratory-Equipe Labellisée “La Ligue,” CNRS UMR5238, Université de Lyon, Centre Léon Bérard, 69008 Lyon, France
2Department of Molecular Genetics, Weizmann Institute of Science, Rehovot 76100, Israel
3Division of Life Science, Molecular Neuroscience Center, Hong Kong University of Science and Technology, Hong Kong
4Molecular Zoology, Institut de Géonomique Fonctionnelle de Lyon-CNRS UMR5242, INRA IFR128 BioSciences Lyon-Gerland, Université de Lyon-Ecole Normale Supérieure de Lyon, 46 Allié d’Italie, 69364 Lyon Cedex 07, France
5These authors contributed equally to this work
*Correspondence: mehlen@lyon.fnclcc.fr
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SUMMARY

The UNC5H dependence receptors promote apoptosis in the absence of their ligand, netrin-1, and this is important for neuronal and vascular development and for limitation of cancer progression. UNC5H2 (also called UNC5B) triggers cell death through the activation of the serine-threonine protein kinase DAPk. While performing a siRNA screen to identify genes implicated in UNC5H-induced apoptosis, we identified the structural subunit PR65b of the holoenzyme protein phosphatase 2A (PP2A). We show that UNC5H2/B recruits a protein complex that includes PR65b and DAPk and retains PP2A activity. PP2A activity is required for UNC5H2/B-induced apoptosis, since it activates DAPk by triggering its dephosphorylation. Moreover, netrin-1 binding to UNC5H2/B prevents this effect through interaction of the PP2A inhibitor CIP2A to UNC5H2/B. Thus we show here that, in the absence of netrin-1, recruitment of PP2A to UNC5H2/B allows the activation of DAPk via a PP2A-mediated dephosphorylation and that this mechanism is involved in angiogenesis regulation.

INRODUCTION

The Death-Associated-Protein kinase (DAPk) (Deiss et al., 1995; Feinstein et al., 1995) is a crucial intracellular protein that mediates cell death induction through a wide spectrum of apoptotic and nonapoptotic signals via its serine-threonine kinase activity (Cohen et al., 1999; Deiss et al., 1995; Eisenberg-Lerner and Kimchi, 2007; Gozuaçik et al., 2008). As such, DAPk is implicated in the suppression of both early-stage oncogenesis (Raveh and Kimchi, 2001) and metastasis (Inbal et al., 1997).

In accordance with these proposed roles in oncogenesis, DAPk is silenced—mainly by promoter methylation—in a broad spectrum of human tumors (Gozuaçik and Kimchi, 2006; Kawaguchi et al., 2004). However, while DAPk is a key player in cell death regulation, very few direct activators of its catalytic activity have been identified: these include calmodulin (CaM), which binds to DAPk’s CaM regulatory region and is activated by a calcium spike (Cohen et al., 1997); ERK kinase, which binds to the DAPk death domain and activates its catalytic activity through phosphorylation (Cohen et al., 1997); ERK kinase, which binds to the DAPk death domain and activates its catalytic activity through phosphorylation (Chen et al., 2005); and the dependence receptor UNC5H2, which also binds DAPk via its death domain (Llambi et al., 2005).

Dependence receptors such as UNC5H2 share the property of apoptosis induction in the absence of ligand, with a block of apoptosis induction by ligand binding. Examples of dependence receptors include p75ntr, Patched (Ptc), Neogenin, RET, TrkC, EPHA4, and Aik tyrosine kinase receptors, as well as the netrin-1 receptors Deleted in Colorectal Cancer (DCC) and UNC5H (i.e., UNC5H1, UNC5H2, UNC5H3, and UNC5H4, also called UNC5A, UNC5B, UNC5C, and UNC5D) (Bordeaux et al., 2000; Furne et al., 2009; Goldschneider and Mehlen, 2010; Mehlen et al., 1998).

The DCC and UNC5H receptors are the prototype dependence receptors. They share the same ligand, netrin-1, which is a diffusible laminin-related protein. Netrin-1 has been shown to play a major role in the control of neuronal navigation during nervous system development, by interacting with DCC (Keino-Masu et al., 1996; Serafini et al., 1996; Forcet et al., 2002) and UNC5H (Ackerman et al., 1997; Hong et al., 1999). Recently, netrin-1 has also been shown to contribute to the patterning of developing nonneuronal epithelial tissues such as mammary gland, pancreas, and lung, by regulating diverse processes such as cell adhesion, motility, proliferation, and differentiation (for reviews, see Cirulli and Yebra [2007] and Mehlen and Furne [2005]). However, netrin-1 has also emerged as a cell survival cue, via a mechanism that features a block of the proapoptotic activity of its receptors DCC and UNC5H (Llambi et al., 2001; Mehlen et al., 1998; Tang et al., 2008; Tanikawa et al., 2003; Wang et al., 2008).
The proapoptotic activity of these unbound netrin-1 receptors has been shown to act as a mechanism for eliminating tumor cells that would otherwise invade or metastasize to regions of reduced netrin-1—thus these receptors tie the cells to regions of ligand availability (Bernet et al., 2007; Delloye-Bourgeois et al., 2009a; Fitamant et al., 2008; Mazelin et al., 2004). However, the proapoptotic function of DCC and UNC5H is not limited to cancer regulation but is also implicated in developmental programmed cell death. The proapoptotic activity of netrin-1 dependence receptors is believed to be important for adequate neuronal migration and localization during nervous system development (Furne et al., 2008; Mehlen and Brederesen, 2004; Tang et al., 2008), as well as for developmental angiogenesis regulation (Castets et al., 2009). In particular, it has been shown that netrin-1 acts as a survival cue for endothelial cells by preventing apoptosis mediated by UNC5H2. More specifically, netrin-1 inactivation in zebrafish is associated with vessel defects that can be rescued by inhibiting apoptosis, using chemical reagents or inactivation of UNC5H2 (Castets et al., 2009). Interestingly, DAPk inactivation also corrects the defects observed in netrin-1 knockdown zebrafish embryos, demonstrating the biological relevance of the UNC5H2.DAPk pathway during developmental angiogenesis (Castets et al., 2009).

Understanding the mechanisms by which UNC5H2 mediates apoptosis via DAPk is therefore of interest, since these mechanisms are likely to be involved in several developmental and pathological processes. UNC5H2 has been shown to recruit DAPk via their respective death domains and to activate this kinase by inhibiting its autophosphorylation. Indeed, DAPk displays an inhibitory autophosphorylation on serine 308 within the CaM regulatory region, previously shown by mutational analysis to mask its catalytic domain (Shohat et al., 2001). However, the mechanism(s) by which UNC5H2 mediates DAPk serine 308 dephosphorylation have been unknown. Here we show that UNC5H2 interacts with the structural subunit PR65b (PPP2R1B) of the holoenzyme protein phosphatase PP2A.

PP2A is a ubiquitously expressed serine-threonine phosphatase that accounts for a large fraction of phosphatase activity in eukaryotic cells. However, PP2A is not a single molecular species but rather a collection of oligomeric enzymes, each of which contains a common catalytic subunit, PP2Ac. This catalytic subunit interacts with the PR65 scaffold protein and with a wide family of regulatory B subunits (Janssens and Goris, 2001). Two alternative genes, PR65a and PR65b, encode two forms of the PR65 scaffold protein. While most PP2A holoenzymes contain the PR65a isoform, PR65b appears to play a key regulatory role in cancer. Indeed, PR65b is decreased or mutated in a large fraction of human cancers and has recently been causally linked to cancer development (Eichhorn et al., 2009). Furthermore, PP2A inactivation in cancer also occurs frequently through upregulation of CIP2A, a PR65 interactor and PP2A inhibitor (Juntilla et al., 2007). Here we show that UNC5H2, DAPk, PR65b, PP2Ac, and CIP2A form a dynamic protein complex that regulates UNC5H2-mediated apoptosis. Moreover, we present evidence to support the view that PP2A recruitment to UNC5H2 via PR65b leads to DAPk dephosphorylation and to its subsequent activation.

RESULTS

PR65b Is Required for UNC5H2-Induced Apoptosis

In an attempt to understand the mechanisms allowing UNC5H receptors to trigger apoptosis, we performed a siRNA screen. We took advantage of the fact that a large fraction of tumors and cancer cell lines display autocrine production of netrin-1, thus blocking UNC5H-induced apoptosis (Fitamant et al., 2008; Delloye-Bourgeois et al., 2009a, 2009b). Treatment with a decoy protein, DCC-5Fbn, which interferes with netrin-1, was shown to trigger apoptosis of different netrin-1-expressing cancer cell lines, by restoring UNC5H-induced cell death (Figure 1A and Delloye-Bourgeois et al., 2009a, 2009b; Fitamant et al., 2008). The DCC-5Fbn-sensitive Cal51 breast cancer cells, which express netrin-1 and UNC5H1, UNC5H2, and UNC5H3, but not DCC (data not shown), were then infected with a shRNA-GFP lentivirus library with 25,500 unique clones and further treated with DCC-5Fbn (Figure 1B). Surviving cells corresponded to those that had escaped UNC5H-induced cell death, and these were individually cloned. Corresponding shRNAs were sequenced from resistant clones (Figure 1B, with one example in Figure 1C). Table S1 (available online) provides a list of 48 different genes identified by this screen, putatively targeted by the shRNAs expressed in DCC-5Fbn-resistant clones. Three genes were identified twice in this screen. PR65b, the scaffold protein for PP2A, was chosen for further evaluation, since it was targeted twice in the screen with two different shRNA and because UNC5H2-induced apoptosis is known to be dependent on DAPk dephosphorylation (Liambi et al., 2005).

We first determined whether the two siRNAs encoded by the shRNAs sequenced in the DCC-5Fbn-resistant clones (Table S2) were indeed efficient in inhibiting PR65b expression and UNC5H-dependent apoptosis. Cal51 cells were transfected with a netrin-1 siRNA, alone, or in combination with PR65b siRNAs. Both PR65b siRNAs markedly inhibited PR65b expression and were associated with a decrease in PP2A activity (Figures 1D and 1E). Cell death was then analyzed via a caspase activity assay. While netrin-1 siRNA increased caspase activity, cotransfection of each PR65b siRNA completely blocked this effect (Figure 1F). Similar results were obtained with two other siRNAs specifically designed to inhibit PR65b expression (Figures S1A and S1B). A similar experiment was performed on another tumor cell line that also displayed autocrine production of netrin-1: H358 lung cancer cells are dependent on their autocrine secretion of netrin-1 for survival, and netrin-1 inhibition triggers UNC5H1- and UNC5H2-induced apoptosis in these cells (Delloye-Bourgeois et al., 2009a, 2009b). Similarly to Cal51 cells, netrin-1 silencing in H358 cells was associated with caspase-3 activation, while PR65b silencing prevented this effect (Figures 1C and 1D). Similarly, treatment of H358 or Cal51 cells with 5nM okadaic acid, a PP2A inhibitor, prevented apoptosis triggered by netrin-1 siRNA (Figures S1C and S1D and data not shown). Thus, PR65b and PP2A activity are probably required for netrin-1 withdrawal-induced apoptosis.

To further define PR65b and PP2A involvement in UNC5H-induced apoptosis, we took advantage of the HEK293T experimental cell system, in which UNC5H2 transfection was shown to trigger apoptosis (Liambi et al., 2001, 2005; Tanikawa
Figure 1. Identification of PR65β as a Mediator of UNC5H-Induced Apoptosis Using a shRNA Screen

(A) Cell system used for UNC5H apoptotic signaling effector screening. The breast cancer cell line Cal51, which expresses UNC5H and netrin-1, was incubated with the DCC-5Fbn decoy protein interfering with netrin-1 to induce UNC5H-mediated apoptosis.

(B) Lentiviral infection of Cal51 cells using a shRNA library and selection of clones resistant to UNC5H-induced apoptosis.

(C) Sensitivity of Cal51 parental (WT) cell line and of a DCC-5Fbn-resistant infected clone to UNC5H-induced apoptosis after DCC-5Fbn treatment. Cell survival was quantified with MTT assay. Results are means ± standard error of the mean (SEM) (n = 3).

(D) Efficiency of netrin-1 and PR65β siRNAs in the Cal51 cell line. Quantification of mRNA levels was performed by Q-RT-PCR and compared to HPRT as a control, housekeeping gene.

(E) PR65β siRNAs reduce PP2A phosphatase activity in Cal51 cells. PP2A activity was measured using an ELISA-based PP2A activity measurement (see Experimental Procedures). Results are means ± SEM (n = 3).

(F) Caspase-3 activation induced by netrin-1 silencing is significantly reduced by silencing of PR65β in Cal51 cells. Two different siRNAs are used. Results are displayed as means ± SEM (n = 3). All p values were obtained by Mann Whitney U test.
et al., 2003; Wang et al., 2009) and in which the role of DAPk in UNC5H2-induced cell death was reported (Llambi et al., 2005).

PR65β siRNAs transfection into HEK293T cells significantly reduced PR65β, at both the mRNA (Figure S2A) and protein (Figure 2A) levels. As noted above, two isoforms of PR65 exist, PR65α and PR65β, and, while one antibody specifically detected the α isoform specifically, the PR65 antibodies used in this study did not discriminate the β from the α isoform. As shown in Figure 2A and Figure S2B, PR65β siRNA showed no effect on PR65α mRNA or protein level (Figure 2A), demonstrating its specificity for the PR65β subunit. Furthermore, this decrease in PR65β mRNA and protein was associated with a decrease in PP2A phosphatase activity (Figure 2B and data not shown).

We next confirmed PR65β involvement in cell death induced by UNC5H in HEK293T cells. Forced expression of UNC5H2 triggered HEK293T cells apoptosis, as determined by TUNEL staining (Figure 2C) and caspase-3 activity (Figure 2D). Silencing of PR65β by siRNA was sufficient to inhibit UNC5H2-mediated cell death (Figures 2C and 2D). Similar data were obtained with UNC5H1 (Figure 2C and data not shown) and with three other PR65β siRNAs (Figure S2C). As a negative control, apoptosis induced by forced expression of Patched (Ptc), another dependence receptor (Mille et al., 2009b; Thibert et al., 2003), was not significantly inhibited by silencing of the PR65β subunit (Figure 2C and Figure S2D). Of interest, forced expression of UNC5H2 was associated with an increase in PP2A phosphatase activity (Figure 2E). Because this increased PP2A activation could reflect

Figure 2. Involvement of PR65β in UNC5H2-Mediated Apoptosis

(A) Efficiency of PR65β siRNA on PR65β expression at protein level in HEK293T cells. Immunoblot analysis reveals specific decrease of PR65β expression after PR65β siRNA transfection, whereas PR65α expression is not modified. β-Actin detection is used as a control.

(B) ELISA-based PP2A activity assay showing that PR65β siRNA is sufficient to decrease global PP2A phosphatase activity in HEK293T cells.

(C) Silencing of PR65β prevents UNC5H2-induced apoptosis in HEK293T cells. TUNEL assay was used to monitor the effect of PR65β siRNA on apoptosis induced by transfection of the dependence receptors UNC5H1, UNC5H2, or Ptc. Results are means ± SEM of the percentage of TUNEL-positive cells in each condition (n = 3). Representative fields are presented on the right panel.

(D) Silencing of PR65β prevents caspase-3 activation induced by forced expression of UNC5H2 in HEK293T cells. Results are means ± SEM of caspase-3 activity relative to control (n = 5). Transfection efficiency was assessed by western blot and presented in the lower panel.

(E) UNC5H2 expression triggers activation of PP2A in HEK293T cells. This PP2A phosphatase activity is not blocked by the general caspase inhibitor z-VAD-fmk. PP2A phosphatase activity was measured using an ELISA-based PP2A activity measurement (see Experimental Procedures). Results are means ± SEM (n = 5). All p values were calculated using Mann-Whitney U test.
an indirect effect due to apoptosis amplification rather than a direct activation of PP2A by UNC5H2, a similar experiment was performed in the presence of the general caspase inhibitor z-VAD-fmk. As shown in Figure 2E, z-VAD-fmk had no effect on PP2A phosphatase activation by UNC5H2, thus supporting the view that UNC5H2 directly triggers PP2A activation. Thus, taken together, these data show that PR65β and PP2A activity are required for UNC5H1/2-mediated apoptosis.

UNC5H2, PR65β, PP2Ac, and DAPk Form a Protein Complex in the Absence of Netrin-1

We next investigated whether PR65β interacts with UNC5H2. As shown in Figure 3A, transfected UNC5H2 efficiently immunoprecipitated endogenous PR65β in HEK293T cells. Even though PR65α is the most abundant isoform reported in most cell lines, we failed to detect an UNC5H2/PR65α interaction, supporting the view that UNC5H2 specifically interacts with PR65β. However, the presence of netrin-1 inhibited the pull-down of UNC5H2 with PR65β. Interaction between UNC5H2 and PR65β was further analyzed by confocal analysis (Figure 3B). In agreement with the immunoprecipitation data, the addition of netrin-1 disrupted the UNC5H2/PR65β colocalization, supporting the view that netrin-1 prevents interaction between UNC5H2 and PR65β (Figures 3A and 3B). To determine whether the interaction between UNC5H2 and PR65β is direct or not, we performed a targeted yeast two-hybrid assay using the
intracellular domain of UNC5H2 as a bait and PR65β as a prey. As shown in Figure 3C, expression of the UNC5H2 intracellular domain together with PR65β promoted Ga4-dependent transcription in yeast, even though this transcription was of reduced intensity compared to the one detected for UNC5H2 and DAPk coexpression used here as a positive control (Figure 3C). Thus the interaction between UNC5H2 and PR65β is likely to be direct. Interaction between UNC5H2 and PR65β was not only detected in cell culture but also in vivo: in adult mouse brain extract, pull-down of UNC5H2 allowed the immunodetection of PR65β, while immunoprecipitation of PR65β pulled down UNC5H2 (Figure 3D).

Because UNC5H2 was shown to interact with and activate DAPk, we next determined whether UNC5H2, PR65β, and DAPk form a ternary complex acting on DAPk activation. As a first approach, we analyzed whether DAPk and PR65β colocalize when netrin-1 is absent. As shown in Figure 4A, DAPk colocalized with PR65β in the absence of netrin-1 treatment, whereas addition of netrin-1 disrupted DAPk/PR65β colocalization in HEK293T cells. We have previously shown that UNC5H receptors are localized to lipid rafts and that the interaction between UNC5H2 and DAPk is dependent on lipid raft integrity (Maise et al., 2008). Here, we found that DAPk, PR65β, and the catalytic subunit of PP2A (PP2Ac) were all partially localized to detergent-resistant membranes that contained lipid rafts and were enriched in the fractions that contained the lipid raft marker caveolin-1 (Figure 4B). When lipid rafts were disrupted by incubating cells with methyl-β-cyclodextrin (MβCD), which removes cholesterol from the plasma membrane, the presence of DAPk, PR65β, and PP2Ac in the lipid raft fractions was abolished (Figure 4B).

We then assessed whether PP2A is part of the UNC5H2-DAPk complex. As shown on Figure 4C, immunoprecipitation of PP2Ac efficiently pulled down UNC5H2 and DAPk in the absence of netrin-1 in HEK293T cells. In agreement with the UNC5H2/PR65β interaction data, the presence of netrin-1 disrupts PP2Ac from UNC5H2 and DAPk. Moreover, PP2Ac/UNC5H2 interaction was also detected in mouse brain extract (Figure 4D). The overall model thus suggests that in the absence of netrin-1, UNC5H2-DAPk recruits both PR65β and PP2Ac. We thus assessed whether a PP2A activity was observed in the UNC5H2 complex. To do so, we performed UNC5H2 immunoprecipitation on UNC5H2 expressing HEK293T cells and measured PP2A activity in the absence of netrin-1 in HEK293T cells transfectected with UNC5H2-V619Q, PR65β was not confined to the original low molecular weight fraction. Moreover, PR65β immunoprecipitation performed on these large size fractions pulled down both DAPk and UNC5H2 (Figure 4G). Moreover, while measuring PP2A activity in the different fractions, we detected higher PP2A activity in the high molecular weight UNC5H2/DAPk fractions, as compared to low molecular weight fractions (Figure 4H). Thus, UNC5H2, PP2A, and DAPk are included in a large size protein complex in the absence of netrin-1, but not in the presence of netrin-1.

It was recently shown that the UNC5H2 intracellular domain can be folded into a closed conformation that prevents apoptosis—i.e., the folding of the ZU5 domain masks the proapoptotic death domain (Wang et al., 2009). In the absence of netrin-1, the UNC5H2 receptor is thought to adopt an open conformation, allowing the exposure of the death domain and resultant apoptosis induction. We therefore took advantage of an UNC5H2 mutant that is in a forced open conformation (UNC5H2-V619Q) (Wang et al., 2009). Size exclusion assays on the DAPk/UNC5H2/PR65β complex were once again performed, using this UNC5H2-V619Q mutant instead of the wild-type UNC5H2. As expected, while PR65β was once again detected in the two peaks of exclusion size (<400 kDa and the one corresponding to the UNC5H2/DAPk fractions [400–1000 kDa]) in the absence of netrin-1 in HEK293T cells transfected with UNC5H2-V619Q, PR65β was not confined to the low molecular weight fraction (<400 kDa) when netrin-1 was added, as it had been with wild-type UNC5H2 (Figure 4F). Together, these data support the following model: in the presence of netrin-1, UNC5H2 is in a closed conformation that prevents the recruitment of the PP2A complex, while in the absence of netrin-1, the UNC5H2 intracellular domain opens, allowing PP2A recruitment to the UNC5H2/DAPk complex.

PP2A Mediates UNC5H2-Induced DAPk Dephosphorylation

Because DAPk dephosphorylation is a prerequisite for its activation (Bialik and Kimchi, 2006) and because this dephosphorylation occurs upon netrin-1 withdrawal in UNC5H2-expressing cells (Llambi et al., 2005), we investigated the possibility that PP2A may be required for DAPk dephosphorylation. As a first approach, PP2A activity was increased by overexpression of its catalytic subunit. As shown in Figure 5A, enforced PP2A expression was associated with a reduction of DAPk serine phosphorylation (p-S308). Conversely, silencing of PR65β by siRNA and treatment with okadaic acid each increased DAPk serine 308 phosphorylation in HEK293T cells (Figure 5B). We then expressed UNC5H2 and DAPk in HEK293T cells and analyzed the DAPk p-S308 level after PR65β silencing. As shown in Figure 5C and Figure S3A, while transfection of UNC5H2 induced a decrease in the number of p-S308 HEK293T-positive cells, PR65β silencing by siRNA prevented this reduction. Similarly, fewer cells were stained with p-S308-specific antibody after netrin-1 inhibition by a siRNA approach in the netrin-1-expressing H358 cells, while this effect was not observed upon cotransfection with PR65β siRNA (Figures S3B and S3C). Altogether, these data support the view that in the absence of netrin-1, PP2A is recruited to the UNC5H2/DAPk complex and mediates DAPk dephosphorylation.
Figure 4. UNC5H2, PR65β, PP2Ac, and DAPk Form a Protein Complex Localized in Lipid Rafts in the Absence of Netrin-1

(A) Colocalization assay between PR65β and DAPk in HEK293T cells in the presence or absence of netrin-1. Cells were transfected with Flag-DAPk and with or without netrin-1. Flag-DAPk (red) and PR65β (green) were detected by immunostaining using specific antibodies. Representative images obtained by confocal microscopy are shown. Insets in the merge: magnification of the dotted square.

(B) DAPk, PR65β, and PP2Ac are partially localized in lipid rafts. HEK293T cells were treated with Methyl-β-Cyclodextrin (MβCD) or Xylazine (Xyl) as a control for 1 hr. Low-density detergent-insoluble membranes were fractionated by a discontinuous sucrose density gradient centrifugation as detailed in Experimental Procedures. Fractions were collected and separated by SDS-PAGE, and endogenous DAP-kinase (DAPk), PP2Ac, PR65β, and caveolin-1 (CAV-1) were detected by western blot. HF (heavy nonraft fraction) and DRM (detergent-resistant membranes) are shown.

(C) Immunoprecipitation of UNC5H2, DAPk, and PP2Ac in HEK293T transfected cells. PP2Ac was immunoprecipitated using specific antibody, and it efficiently pulled down HA-UNC5H2 and endogenous DAPk, in the absence of netrin-1 (n = 3).

(D) Immunoprecipitation of UNC5H2 with PP2Ac in adult mouse brain lysates. A pull-down of PP2Ac coimmunoprecipitated UNC5H2 as revealed by western blot using a specific UNC5H2 antibody.

(E) PP2A activity is detected in the UNC5H2 pull-down. UNC5H2 pull-down was performed from HEK293T transfected with UNC5H2 (or mock vector), PP2Ac, and PR65β and PP2A phosphatase activity was measured using an ELISA-based PP2A activity assay (see Experimental Procedures) on the resulting pull-down. PP2A phosphatase pulled down activity is decreased by netrin-1. Results are means ± SEM (n = 4).

(F) Size-exclusion assay supporting the PR65β recruitment in an UNC5H2/DAPk complex in the absence of netrin-1 in HEK293T cells. Cells were transfected with UNC5H2-HA and DAPk-FLAG constructs with or without netrin-1. Lysates were subjected to size-exclusion chromatography. UNC5H2, DAPk, and PR65β were detected by immunoblots on the different collected fractions (n = 3). UNC5H2, DAPk, and PR65β are detected in the high molecular weight fractions in the absence of netrin-1, whereas PR65β moves into low molecular weight fractions in the presence of netrin-1. The lower panel represents fractions from size exclusion chromatography performed from HEK293T transfected with a constitutively open UNC5H2 mutant, HA-UNC5H2-V619Q. PR65 immunoblot is shown, indicating that this mutant recruits PR65 into the high molecular weight fractions regardless of the presence or absence of netrin-1 (n = 3).

(G) IP: Anti-UNC5H2

(H) PP2A activity is higher in UNC5H2 containing fractions in size exclusion assay. PP2Ac was immunoprecipitated in high molecular weight fractions (>440 kDa) containing UNC5H2 and in low molecular weight fractions (~66 kDa) and PP2A activity was measured as described in the Experimental Procedures. Results are means ± SEM (n = 4, p < 0.05 U test).
CIP2A Was Recently Identified as an Interactor of PR65 that Represses PP2A Activity in Various Cancer Cells (Junttila et al., 2007). We therefore investigated whether CIP2A may be involved in UNC5H2-induced death signaling regulation. Coimmunoprecipitation in CIP2A/UNC5H2 HEK293T-transfected cells showed that CIP2A was weakly pulled down with UNC5H2 in the absence of netrin-1 (Figure 6A). However, the addition of netrin-1 was associated with a clearly increased interaction between UNC5H2 and CIP2A (Figure 6A), suggesting that netrin-1 favors the recruitment of this PP2A inhibitor. We then analyzed whether CIP2A antagonizes PP2A-mediated dephosphorylation of DAPk and UNC5H2-induced apoptosis. As shown in Figures 6B and 6C, enforced expression of CIP2A in HEK293T cells significantly prevented UNC5H2-induced apoptosis, while it had no effect on Ptc-induced apoptosis. Conversely, silencing of CIP2A by siRNA (Figure 6D and Figure S4A) was associated with an increase in UNC5H2-induced apoptosis (Figure S4B). We therefore analyzed the DAPk p-S308 level in UNC5H2/DAPk HEK293T-transfected cells after CIP2A silencing. As shown in Figure 6E, CIP2A siRNA transfection was associated with an increased inhibition of DAPk autophosphorylation. These data support the view that CIP2A is a negative regulator of UNC5H2-induced apoptosis because it is recruited to UNC5H2 in the presence of netrin-1 and inhibits PP2A-mediated dephosphorylation of DAPk.

PP2A Activity Is Required for the Dependence Receptor Activity of UNC5H2 during Developmental Angiogenesis

To evaluate the relevance of PP2A/PR65β involvement in UNC5H2 proapoptotic activity in vivo, we took advantage of the recent observation that netrin-1 acts as a survival factor for endothelial cells during developmental angiogenesis by inhibiting UNC5H2-induced apoptosis (Castets et al., 2009). We first observed that PR65β silencing by siRNA promotes survival of
HUAEc endothelial cells in vitro (Figure 7A), similarly to what had been observed in response to treatment with netrin-1 or UNC5H2 silencing by siRNA (Figure 7B and Castets et al., 2009). We then investigated whether the angiogenic defects observed in vivo upon netrin-1 disruption, which have been shown to result at least partially from UNC5H2-induced endothelial cell apoptosis, could be rescued by inhibition of PR65β.

Silencing of netrin-1α, using different netrin-1α morpholinos, is associated with vascular developmental defects during zebrafish development (Lu et al., 2004; Wilson et al., 2006). We have shown that these vessel defects observed after netrin-1 reduction can be blocked by treatment with a caspase inhibitor or the simultaneous disruption of UNC5H2 expression (Castets et al., 2009).

Using a fli:egfp transgenic zebrafish targeting the expression of GFP specifically in blood vessels (Lawson and Weinstein, 2002), we confirmed at 30–60 hr postfertilization (hpf) that netrin-1α silencing using a translation-blocking morpholino (tsl-netrin-1α) led to the disorganization of intersegmental vessels (ISVs) in 57% of the embryos injected and to the absence of the dorsal anastomotic vessel (DLAV) (Figure 7B).
As described previously (Castets et al., 2009), we also observed that simultaneous injection of UNC5H2 morpholinos was able to correct the defects induced by tsl-netrin-1a morpholino injection (data not shown). We then analyzed the effect of PR65β morpholinos on the tsl-netrin-1a blocking morpholino-associated vessel defects. As shown in Figure 7B, the PR65β morpholino reversed the ISV defects associated with netrin-1α silencing (p < 0.001), whereas this effect was blocked by the injection of human PR65β mRNA. This confirms that, in vivo, UNC5H2 proapoptotic activity is dependent on PP2A activation. Moreover, while netrin-1α silencing was associated with increased caspase activity (Castets et al., 2009), the PR65β morpholino blocked this effect (Figure 7C), similarly to what had been observed with UNC5H2 or DAPk morpholinos (Castets et al., 2009). Furthermore, the injection of human PR65β mRNA in this setting restored the caspase activity level observed upon netrin-1 silencing. Thus PP2A, as a regulator of UNC5H2-mediated cell death, is implicated in angiogenesis regulation.

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DISCUSSION

By means of an unbiased shRNA screen, we have identified PP2A as a mediator of the death signaling induced by the dependence receptor UNC5H2. A model predicted from the data presented above is depicted in Figure 7D: an interaction between UNC5H2 and DAPk, which requires their respective death domains, occurs independently of netrin-1 (Liambi et al., 2005). However, the association of the UNC5H2/DAPk complex with the PP2A complex, the latter of which includes PR65α and PP2Ac, is negatively regulated by netrin-1. In the presence of netrin-1, the PP2A complex is repressed by the recruitment of CIP2A into the UNC5H2/DAPk complex and, in this setting, DAPk is autophosphorylated on serine 308, thus remaining inactive. Conversely, netrin-1 withdrawal is associated with a conformational change in UNC5H2, exposure of the death domain, CIP2A release, and PP2A recruitment, leading to DAPk dephosphorylation at Ser308 and resultant activation of DAPk. The activation process appears to take place in lipid rafts, probably because other proteins localized to lipid rafts are important to support this dephosphorylation. One may then ask how the loss of interaction between UNC5H2 and its ligand is associated with the recruitment of PP2A to the UNC5H2-DAPk complex. One possibility is that, in the presence of netrin-1, UNC5H2 has been shown to multimerize (Mille et al., 2009a), and this multimerization of UNC5H2 might conceivably prevent PP2A recruitment due to steric hindrance. Alternatively, it has been demonstrated that in non-proapoptotic conditions, the intracellular domain of UNC5H2 forms a structural supramodule that masks and inhibits its own death domain (Wang et al., 2009). It is therefore tempting to speculate that the presence of netrin-1 prevents PP2A recruitment because of this inhibitory structural supramodule, while netrin-1 withdrawal is associated with the opening of this structural supramodule and the unmasking of the proapoptotic death domain. In support of this view, we have shown here in the size exclusion assays that, unlike wild-type UNC5H2, a mutant of UNC5H2 that maintains an open conformation fails to lose its association with PR65α after netrin-1 binding.

Our data thus support PP2A as a mediator of UNC5H2-induced apoptosis. PP2A activity has been suggested to be an important mediator of cell death both in vitro and in vivo (Garcia et al., 2003; Kong et al., 2004; Li et al., 2002; Silverstein et al., 2002; Strack et al., 2004). Of interest, PP2A-like activity has been implicated in the two classic pathways for caspase-dependent apoptosis—i.e., the extrinsic pathway, with the effect of preventing apoptosis induced by the extrinsic pathway, the effect of preventing apoptosis, and the intrinsic pathway, with the demonstrated effect of PP2A on Bcl-2 or Bax (Chatfield and Eastman, 2004; Deng et al., 2009). PP2A-like activity has also been implicated in the activation of DAPk in ER stress-induced caspase-independent cell death (Gozuacik et al., 2008). Here, we show that PP2A is directly implicated in the dependence receptor pathway.

Two questions arise from this work. First, is PP2A involvement a general phenomenon for all dependence receptors, or is its involvement restricted to the ones using DAPk to trigger apoptosis? Notably, the repulsive guidance molecule (RGM) dependence receptor neogenin has also been shown to interact with DAPk and to require DAPk to trigger apoptosis (Fujita et al., 2008). However, this is probably not a general requirement since, as shown above, the dependence receptor Patched triggers apoptosis independently of PP2A activity (Figure 2). Second, what molecules constitute the PP2A complex recruited to UNC5H2 to dephosphorylate DAPk? It is widely accepted that PP2A is not a single protein complex, but actually regroups many different protein complexes that depend mainly on the type of PR65 isoform. Regarding the PR65 isoforms, although most reports to date describe PR65α as the more abundant isoform, we failed to implicate PR65α in UNC5H2-induced apoptosis. Perhaps this is related to the finding that, similarly to UNC5H and DAPk, PR65α appears to play a central regulatory role in cancer (Eichhorn et al., 2009). Regarding the regulatory B subunits, 15 genes have been identified to date in the human genome that encode at least 26 different alternative transcripts and splice forms representing the B subunits of the PP2A holoenzyme (Eichhorn et al., 2009). Future work will be needed to define which is (or are) the preferential subunit(s) required for UNC5H2-dependent PP2A-mediated activation of DAPk. The work presented here has implicated one important negative regulator of PP2A, CIP2A. We have shown that CIP2A antagonizes UNC5H2-induced apoptosis, probably because it inhibits UNC5H2-dependent PP2A activation. We have also observed that, in the absence of netrin-1, UNC5H2 interacts with PR65α but little if any with CIP2A. Conversely, the presence of netrin-1 induces CIP2A recruitment to UNC5H2, while PR65α dissociates from the receptor complex. CIP2A is known to block PP2A activity by interacting with PR65 (Junttila et al., 2007). Our results suggest the possibility of at least two pools of CIP2A: one pool that disrupts PP2A interaction with UNC5H2 when netrin-1 is bound to its receptor, and one pool bound to PP2A (but unbound to UNC5H2) inhibiting PP2A activity in the cytosol. Although CIP2A is unlikely to account for all of the inhibitory effects of netrin-1 on PP2A activation and PP2A-mediated dephosphorylation of DAPk (see Figures 6B and 6E), it is of interest to note that both netrin-1 and CIP2A display putative pro-oncogenic activities: CIP2A appears to be upregulated in a wide fraction of cancer cell lines, and CIP2A was thus proposed to play a pro-oncogenic role by stabilizing c-Myc via the inhibition of PP2A (Junttila et al., 2007). Whether CIP2A acts as an oncogene by preventing UNC5H2-mediated apoptosis, which has been proposed to act as a safeguard mechanism against tumor progression (Bernet et al., 2007; Mazelin et al., 2004; Mehlen and Puisieux, 2006), remains to be investigated.

It is interesting to note that a series of reports has shown a causal role for PP2A inhibition in cellular transformation and cancer progression (Arroyo and Hahn, 2005; Junttila et al., 2007; Sablina et al., 2007). Thus, determination of the PP2A protein complex regulating UNC5H2-dependent DAPk activation may represent a therapeutic target. The pair netrin-1/UNC5H has indeed recently emerged as a key player in cancer progression (Bernet et al., 2007; Mazelin et al., 2004), with the finding that numerous tumor types produce netrin-1 in an autocrine fashion as a selective advantage for growth (Delloye-Bourgeois et al., 2009a, 2009b; Fitamant et al., 2008). The working hypothesis is that this netrin-1 expression maintains survival of
tumor epithelial cells by blocking UNC5H-mediated cell death. In this respect, developing an agonist of the UNC5H/PP2A interaction, an antagonist of UNC5H/CIP2A or more generally an activator of the functional PP2A complex involved in mediating DAPK dephosphorylation, may represent a powerful antitumor approach.

Interestingly, the netrin-1/UNC5H2 pair not only controls survival of epithelial tumor cells but also that of endothelial cells and thus, potentially, of angiogenic support of tumor cell survival. Indeed, netrin-1 was recently proposed to play an important role in embryonic and pathological angiogenesis (Cirulli and Yebra, 2007; Lu et al., 2004; Wilson et al., 2006). However, reported data led to the apparently contradictory conclusions that netrin-1 is either a pro- or an antiangiogenic factor. We recently proposed to reconcile these apparent opposing observations by demonstrating that netrin-1 acts as a survival factor for endothelial cells, blocking the proapoptotic effect of the dependence receptor UNC5H2 (Castets et al., 2009). Similarly, we show here that in the setting of endothelial cell apoptosis during angiogenesis, PP2A is required for UNC5H2-induced apoptosis. This has dual implications: first, this is the initial description of PP2A involvement in the regulation of angiogenesis. Second, although the role of PP2A has been demonstrated here only during developmental angiogenesis, it is tempting to speculate that it may have a similar role during pathological angiogenesis. As a consequence, targeting the PP2A/UNC5H interaction or PP2A activity in tumors with autocrine netrin-1 expression might have an impact on the survival not only of tumor epithelial cells but also of endothelial cells constituting the vascular support for their respective neoplasms. PP2A and the PP2A/UNC5H interaction may thus represent appealing targets for anticancer therapy.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**

HEK293T and Cal51 cells were grown in DMEM (Invitrogen), supplemented with 10% FBS (Cambrex). H358 cells were grown in RPMI 1640 glutamax (Invitrogen) supplemented with 10% FBS. SiRNA and plasmid constructs (UNC5H2-HA, DAPk-FLAG, Netrin-1, and HA-UNC5H2-V619Q mutant) were transfected using, respectively, Lipofectamine 2000 and Lipofectamine Plus Reagent according to manufacturer’s instructions (Invitrogen). Sequences of the four siRNAs directed against PR65/si were provided in Table S2.

**Lentivirus Library and Cal51 Infection**

The 8.5K Lentivirus Library was obtained from System Bioscience. This library encodes for shRNAfused to GFP and encapsulated in FIV (Feline Immunodeficiency virus) pseudotyped targeting 8500 human genes. Multiplicity of infection (MOI) of Cal51 cell line was determined using the control pFIV-cop-GFP vector provided. Cal51 cells were then infected (MOI = 2) with the lentivirus library. Cells were treated with DCC-5Fbn at 0.8 μg/ml for 72 hr and GFP expression was checked. Surviving clones were isolated and amplified and their resistance to DCC-5Fbn-induced cell death was checked by MTT assay.

**Cell Death Assays**

DCC-5Fbn decoy peptide was produced as described before (Delloye-Bourgeois et al., 2009a; Fitamant et al., 2008). MTT assays were performed on Cal51 clones cultured in serum-free media in 96-well plates using CellTiter96 Non-Radioactive Cell Proliferation Assay (Promega). Caspase-3 activity was performed as described before (Llambi et al., 2005; Tauszig-Delamasure et al., 2007) using the Ac-DEVD-AMC substrate assay (Gentaur, Belgium).

**TUNEL immunostaining**

TUNEL immunostaining was performed using TUNEL assay kit (Roche) on fixed and permeabilized cells (4% paraformaldehyde, PBS1x/Triton0.2%) as described previously (Llambi et al., 2005; Tauszig-Delamasure et al., 2007). Biotinylated DNA’s extremites were revealed using Cy-3 coupled Streptavidine (Jackson ImmunoResearch) at a dilution range of 1:1000.

**Immunostaining**

Before immunostaining, cells were cytopsined, fixed 20 min in 4% paraformaldehyde, and permeabilized in PBS0.2%Triton. PR65/si (Santa Cruz, 1:250), DAPk (Sigma, 1:200), and phosphoserine-308 DAPk (Sigma, 1:200) staining were realized using specific antibodies. Fluorescence labelings were obtained using corresponding secondary antibodies coupled to alexa 488 at a dilution of 1:400 (Jackson Immunoresearch).

**Colocalization Assays**

HEK293T cells were cultured on coverslips and transfected with DAPk-Flag, UNC5H2-HA (Llambi et al., 2005), and netrin-1 or the corresponding pcDNA-3 empty vector. Cells were fixed on 4% paraformaldehyde 48 hr posttransfection and permeabilized using PBS1x/Triton0.2%. Cells were then labeled using anti-HA (Sigma), anti-Flag (Sigma), or PR65/si (Santa Cruz) antibodies and nuclei were labeled with Dapi. Images were obtained by confocal microscopy and analyzing using ImageJ software.

**Immunoprecipitation**

Immunoprecipitations were carried out on HEK293T cells transfected with UNC5H2-HA-and DAPk-FLAG constructs, as described previously (Llambi et al., 2005; please also see Supplemental Experimental Procedures). For in vivo immunoprecipitation, mice brains from OF1 adult mice (Charles River Laboratories) were dissected, cut in small pieces, and lysed in buffer described above before sonication. Immunoprecipitations were performed using anti-UNC5H2 (R&D system), anti-PR65/si (Santa Cruz), anti-PP2Ac (R&D), or control antibodies using μMACS Protein G MicroBeads system (Miltenyi Biotec).

**Two-Hybrid Screen**

The two-hybrid system matchmaker II (Clontech) was used according to the manufacturer’s instructions and as described in Supplemental Experimental Procedures.

**Gel Filtration**

HEK293T cells were transfected with DAPk-FLAG and UNC5H2-HA or UNC5H2-V619Q-HA expressing constructs, and with pcDNA3 or netrin-1 expressing vectors. Cells were collected 48 hr posttransfection, washed with PBS1x, lysed as described for immunoprecipitation 1 hr at +4°C, and sonicated. Supernatants of each lysate were collected and used to perform size-exclusion chromatography on High PrepTM SephacrylTM S300 (HR 16/60, Amersham) using an FPLC protein purification system (Biologic HR, Biorad). Column was calibrated with protein standards (29–2000 kDa) according to manufacturer’s instructions (Sigma). Fractions were analyzed on Criterion XT Precast gels (Bis-Tris 4%–12%; Biorad) by western blotting. Aliquots from the 7–12 fractions were also pooled to perform coimmunoprecipitation experiments as described above.

**PP2A Activity Assay**

PP2A activity was measured using Active PP2A DuoSet IC kit according to manufacturer’s instructions (R&D system) and as described in Supplemental Experimental Procedures.

**Quantitative RT-PCR**

Total mRNAs were extracted from cells or zebrafish using Nucleospin RNAII kit (Macherey-Nagel) and 1 μg was reverse-transcribed using the iScript cDNA Synthesis kit (Bio-Rad). Real-time quantitative RT-PCR was performed on a LightCycler 2.0 apparatus (Roche) using the Light Cycler FastStart DNA Master SYBERGreen I kit (Roche). Oligonucleotides sequences are available on request.
Lipid Raft Disruption and Isolation of Low-Density Triton X-100-Insoluble Caveolin-Rich Lipid Rafts

HEK293T cells were treated with methyl-β-cyclodextrin (8 mM) or xylazine (5 μg/μl) as a control for 1 hr. For isolation of the lipid-raft associated proteins, cells were scraped on ice in 1 ml of ice-cold lysis buffer (25 mM MES [pH 6.5], 150 mM NaCl, 1% Triton X-100, 1 mM Na3VO4, 1 mM Na3P2O7) supplemented with protease inhibitors. Following Dounce homogenization, extracts were adjusted to 40% (w/v) sucrose and loaded on the bottom of a discontinuous 30% (w/v) to 5% (w/v) sucrose gradient. The tubes were then ultracentrifuged in a SW-41 rotor at 39,000 × g for 20 hr at 4 °C. Ten fractions were collected from the top of the tube (fractions 1–3 were combined). Fractions 1–5 correspond to detergent-resistant membrane (DRM) while fractions 8–10 correspond to heavy nonraft fraction (HF). Samples from each of the fractions were subjected to SDS-PAGE and western blot analysis.

Morpholino Knockdown of Zebrafish Embryos

fli1:eGFP transgenic zebrafish embryos were collected and injected (between the 1- and 4-cell stage) with netrin-1a translation blocking morpholino (Castets et al., 2009; Lu et al., 2004) and/or PR65/66 morpholinos (GeneTools design), at 6 and 3 ng respectively. For PR65/66 morpholino rescue experiments, cDNA encoding the human PR65/66 (Addgene) was subcloned into a PCS2+ vector. For synthesizing capped mRNAs, the PCS2+ plasmid was linearized with NotI and transcribed with SP6 RNA polymerase (SP6 in vitro transcription kit; Ambion). RNAs were injected at 0.1 μg/μl. Morphology and vessel abnormalities were then assessed 36 hr postfecundation by fluorescent microscopy. Caspase-3 activity measurement was performed as described previously (Castets et al., 2009).

SUPPLEMENTAL INFORMATION

Supplemental Information includes two tables, four figures, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.molcel.2010.11.021.

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