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RESEARCH ARTICLE

PTPα is required for laminin-2-induced Fyn–Akt signaling to drive oligodendrocyte differentiation

Philip T. T. Ly, Craig Stewart, Catherine J. Pallen

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ABSTRACT

Extrinsic signals that regulate oligodendrocyte maturation and subsequent myelination are essential for central nervous system development and regeneration. Deficiency in the extracellular factor laminin-2 (Lm2, comprising the α2β1γ1 chains), as occurs in congenital muscular dystrophy, can lead to impaired oligodendroglial development and aberrant myelination, but many aspects of Lm2-regulated oligodendroglial signaling and differentiation remain undefined. We show that receptor-like protein tyrosine phosphatase α (PTPα, also known as PTPRA) is essential for myelin basic protein expression and cell spreading during Lm2-induced oligodendrocyte differentiation. PTPα c with the Lm2 receptors α6β1 integrin and dystroglycan to transduce Fyn activation upon Lm2 engagement. In t PTPα mediates a subset of Lm2-induced signals required for differentiation, includeing mTOR-dependent Akt activation but not Erk1/2 activation. We identify N-myc downstream regulated gene-1 (NDRG1) as a PTPα-regulated molecule during oligodendrocyte differentiation, and distinguish Lm2 receptor-specific modes of Fyn–Akt-dependent and -independent NDRG1 phosphorylation. Altogether, this reveals an Lm2-regulated PTPα–Fyn–Akt signaling axis that is critical for key aspects of the gene expression and morphological changes that mark oligodendrocyte maturation.

INTRODUCTION

Oligodendrocytes (OLs) are a specialized cell type in the central nervous system that ensheathe axons with tightly

myelination and ensures rapid transmission of neural impulses and maintains neuronal integrity by providing metabolic and trophic support to the axons (Nave and Trapp, 2008; Sherman and Brophy, 2005). Myelin deficiency resulting from genetic anomalies as in certain pediatric leukodystrophies or after injury, as in multiple sclerosis, can severely compromise neurological functions. The development of OLs occurs in at least two distinct stages: (1) terminal differentiation of the proliferative oligodendrocyte precursor cells (OPCs), and (2) dramatic morphological changes resulting in extension of elaborate membraneous myelin sheets (Buttery and ffrench-Constant, 1999; Nave and Trapp, 2008).

Receptor-like protein tyrosine phosphatase α (PTPα, PTPRA) is a brain-enriched transmembrane protein and a known activator of Src family kinases (SFKs) in various cell types including OPCs (Pallen, 2003; Wang et al., 2009a). The SFK Fyn is required for brain myelination (Sperber et al., 2001; Umemori et al., 1994), and mice lacking PTPα exhibit reduced brain Fyn activity (Ponniah et al., 1999; Su et al., 1999) as well as impaired OL development and hypomyelination in the forebrain (Wang et al., 2009a). Furthermore, PTPα functions to activate Fyn and downstream signaling to FAK and Rho GTPases to promote *in vitro* OL differentiation and morphological maturation. While many extracellular molecules exert differentiation-promoting or -inhibitory cues that control OL development during CNS myelination (Wheeler and Fuss, 2016; Zuchero and Barres, 2013), the role(s) of PTPα in relaying or responding to these extrinsic signals has not been characterized.

Regulation of CNS myelination by the extracellular matrix (ECM) is incompletely understood, but *in vitro* and *in vivo* studies indicate an important role of the ECM component laminin-2 (Lm2, comprising the α2β1γ1 chains), also known as merosin. In humans, Lm2 deficiency caused by mutations in LAMA2, the gene encoding the α2 chain of Lm2, causes a form of congenital muscular dystrophy that manifests with developmental abnormalities in brain myelination thought to arise from OL dysfunction (Gilhuis et al., 2002; Philpot et al., 1995). Dystrophic mice with Lm2 deficiency (*dy/dy*) also have defects in CNS myelination associated with reduced numbers of mature OLs (Chun et al., 2003; Relucio et al., 2009). Lm2 promotes OPC differentiation, with α6β1 integrin and dystroglycan in OPCs identified as Lm2 receptors with integral roles in relaying Lm2-induced differentiation signals, including activation of Fyn (Buttery and ffrench-Constant, 1999; Colognato et al., 2007; Laursen et al., 2009). Additionally, signaling molecules, such as the Akt family of proteins (hereafter Akt) and Erk1 and Erk2 (Erk1/2, also known as MAPK3 and MAPK1, respectively), are important regula differentiation, including morphological maturation, and respond to Lm2 stimulation (Barros et al., 2009; Colognato and Tzvetanova, 2011; Dai et al., 2014; Gaesser and Fyffe-Maricich, 2016; Guardiola-Diaz et al., 2012). However, the precise molecular mechanisms leading to Lm2-mediated Fyn activation and other key signaling events remain unclear.

PTPα interacts with several types of non-catalytic receptors to mediate ligand-dependent SFK activation (Bodrikov et al., 2005; Ye et al., 2008; Zeng et al., 1999), including with $\alpha_v\beta3$ integrins in fibronectin-stimulated mouse embryonic fibroblasts (von Wichert et al., 2003). Whether PTPα interacts with Lm2 receptors in OPCs and/or has any role in Lm2-induced OPC differentiation has not been examined. To investigate this, we isolated neural stem cells from wild-type (WT) and PTPα$^{-/-}$ (knockout, KO) mice to derive OPCs, and induced OPC maturation in the presence or absence of Lm2 substrate. We find that PTPα is required for Lm2-stimulated differentiation signaling, acting as a previously unidentified

required for Lm2-dependent Akt, but not Erk1/2 activation, and this leads to the phosphorylation of a tumor suppressor protein that may have an important function in OL development. Overall, PTPα functions as a co-receptor that transduces a critical and specific subset of intracellular signals in the extrinsically initiated Lm2-mediated differentiation program.

RESULTS

PTPα is required for Lm2-induced OPC differentiation

To investigate whether PTPα is required for Lm2-induced OPC differentiation, WT and PTPα-null (KO) OPCs were seeded onto poly-D-lysine (PDL)-, or PDL and Lm2 (PDL/Lm2)-coated substrate and differentiated for 5 days, followed by immunostaining for Olig2 (an OL lineage marker), PDGFRα (OPCs) and MBP (mature OLs) (Fig. 1A). Comparable numbers of Olig2+ WT and KO OPCs were present in cultures on PDL or PDL/Lm2, and these contained similar populations of PDGFRα and Olig2 double-positive (PDGFRα/Olig2+) cells (Fig. 1B,C) (P>0.05). PTPα was critical for differentiation, as KO OPCs cultured on PDL or on PDL/Lm2 had severely reduced populations of mature MBP and Olig2 double-positive (MBP/Olig2+) cells (Fig. 1D). Moreover, while KO OPCs exhibited a minimal response to Lm2, Lm2 clearly stimulated the differentiation of WT OPCs; nearly double the population of MBP/Olig2+ OLs were detected in cultures maintained on PDL/Lm2 compared to those on PDL alone (Fig. 1D).

Fig. 1.

PTPα is required for Lm2-induced OPC differentiation. WT and KO OPCs were seeded on PDL- or PDL/Lm2 (Lm2)-coated surfaces, maintained in proliferation medium for 5 days, and then subjected to differentiation for 0 or 5 days (0 day, 5 days). (A) 5 day differentiated WT and KO cultures were stained for PDGFRα, MBP and Olig2. Scale bar: 75 µm. (B) The number of Olig2+ cells, and (C) the percentage of PDGFRα/Olig2+ and (D) MBP/Olig2+ cells in the Olig2+ cell population, in 5 days differentiated WT and KO cultures were quantified. Data represent mean±s.d. for four independent experiments each performed in duplicate with six to eight areas per well per experiment analyzed. ***P*<0.001 (one-way ANOVA followed by Tukey's post hoc test). (E) Western blot analysis of lysates from WT and KO OPCs differentiated on PDL or Lm2 for expression levels of OPC and OL markers. MBP bands are indicated by the arrowheads. The prominent ∼17–18 kDa band in the MBP blot appears to be a non-specific signal that appears when new dilutions of the antibody are used, as it was usually not present in blots for MBP carried out re-using diluted antibody (see other figures). (F) Quantification of MBP+ area per cell after 5 days differentiation on PDL or Lm2. The upper panels depict the boundary of the MBP+ area of representative WT and KO OLs differentiated in the presence of Lm2. Each dot on the lower graph represents a quantified cell and the lines indicate mean±s.d.; 50–150 cells were analyzed from four independent experiments. **P*<0.05; ***P*<0.005; ****P*<0.0005; n.s., not significantly different (one-way ANOVA followed by Tukey's post hoc test).

These findings were supported by western blot analysis of lysates derived from pre- (0 day) and post- (5 days) differentiation cultures of WT and KO OPCs maintained on PDL or PDL/Lm2 (Fig. 1E). MBP and CNP (2′,3′-cyclic-nucleotide 3′-phosphodiesterase, also known as CNPase; another marker of maturing OLs) expression were dramatically increased in WT OLs differentiated on PDL/Lm2 compared to what was seen with PDL alone, but were not detectably upregulated in KO cells. In conjunction, PDGFRα and Olig2 expression declined in differentiated WT cultures, consistent with reports of the reduced expression of these proteins in mature OLs versus immature progenitor cells (Kuhlmann et al., 2008; Zhu et al., 2014), while this was not apparent in KO cultures.

Lm2 enhances morphological elaboration of OPCs during differentiation (Buttery and ffrench-Constant, 1999), marked by a lattice of MBP+ extensions and an overall larger cell area. This aspect of Lm2-stimulated maturation was also regulated by PTPα, as the MBP+ area of WT OLs was significantly larger than that of KO OLs differentiated on PDL/Lm2 (Fig. 1F). A lesser but still significant increase in MBP+ cell area was also found for WT compared to KO cells differentiated on PDL (Fig. 1F).

Altogether, the above results indicate that PTPα is necessary for Lm2-induced extrinsic, as well as intrinsic (on PDL alone), regulation of OL differentiation.

Lm2 receptor profiles in differentiating WT and KO OPCs

OPCs express two types of laminin receptor, dystroglycan [DG, which has α-DG and β-DG subunits, both encoded by *DAG1*] and α6β1 integrin (Colognato et al., 2007; Milner and Ffrench-Constant, 1994). We assessed the expression and cell surface localization of these receptors to determine whether impaired Lm2-induced differentiation of KO OPCs could be due to reduced receptor availability. Immunoblot analysis showed that expression of integrin α6 and β1 subunits is similar between WT and KO OLs after differentiation on PDL/Lm2 for 5 days (Fig. 2A). Likewise, there are no differences in

(Colognato et al., 2007; Laursen et al., 2009), the expression of α6, β1 and α-DG, is upregulated with differentiation on Lm2 in both WT and KO cultures. FACS analysis revealed that cell surface levels of the α6β1 integrin complex and α-DG are similar between Lm2-differentiated WT and KO OLs (Fig. 2B). As Lm2 receptor expression is PTPα independent, defects in receptor downstream signaling appear to account for the abrogated Lm2-induced differentiation of KO OPCs.

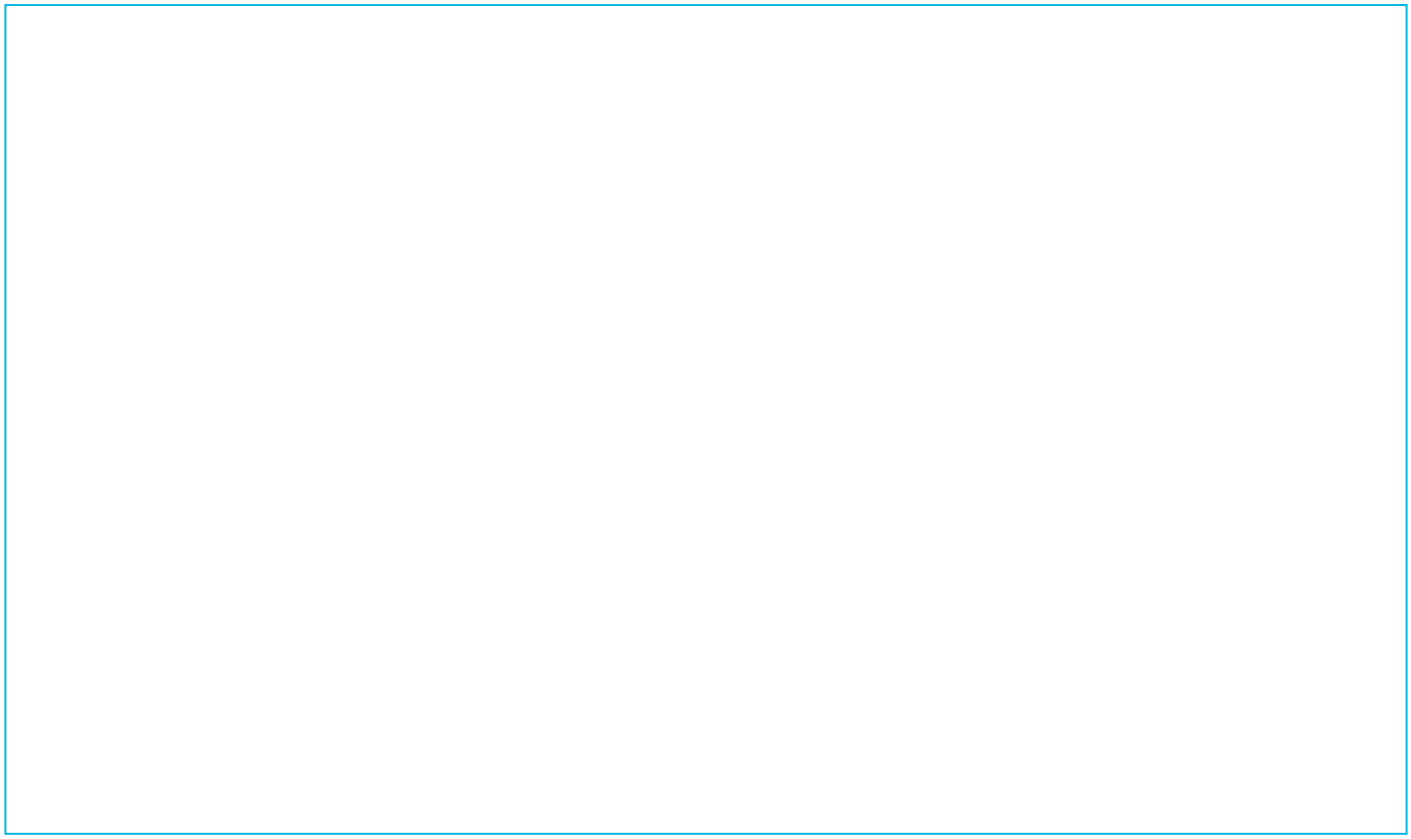

Fig. 2.

PTPα-independent expression of α6β1 integrin and DG is required for Lm2-induced differentiation. WT and KO OPCs were seeded onto PDL/Lm2 substrate, allowed to proliferate and then differentiated for 5 days. (A) Immunoblot analysis of lysates from differentiated WT and KO cultures for α6 and β1 integrin, and α-DG and β-DG expression. (B) FACS analysis of the cell surface expression levels of α-DG and α6β1 integrin in 5 day differentiated WT and KO cells. (C) WT OPCs were differentiated for 5 days on Lm2 in the presence of neutralizing antibodies to β1 integrin and α-DG or with a non-specific IgM antibody, followed by staining for MBP (an OL marker) and Olig2 (an OL lineage marker) and (D) cell counting to determine the percentage of MBP/Olig2+ cells. (E) The MBP+ area per cell in the antibody- or control-treated WT OLs was quantified for at least 150 cells from three independent experiments. Each dot represents a cell and the lines indicate mean±s.d. ***P*<0.001; ****P*<0.0001 (one-way ANOVA followed by Tukey's post hoc test). (F) Immunoblot of MBP expression in WT OLs treated with control or neutralizing antibodies.

To validate the roles of α6β1 integrin and DG in Lm2-dependent neural stem cell-derived OPC differentiation, WT OPCs were differentiated on PDL/Lm2-coated vessels while being treated with function-blocking IgM antibodies to β1 integrin, α-DG or both. An IgM antibody served as a treatment control. After 5 days differentiation and antibody treatment, the cells were immunostained for MBP and Olig2, and the percentage of Olig2+ cells that were MBP+ were quantified (**Fig. 2**C,D). Function-blocking antibodies to β1 integrin, α-DG, or both did not significantly affect the numbers of Olig2+ cells (mean±s.d. cells/field: control 186±64, treatments with β1 antibody 172±35, α-DG antibody 196±15, both antibodies 198±8; *n*=3) or, as previously reported (Colognato et al., 2007; Milner and Ffrench-Constant, 1994), the population of mature cells (MBP/Olig2+) in the cultures (**Fig. 2**D). However, the cell size, as defined by the MBP-positive area, was significantly reduced by treatment with antibody to β1 integrin and/or α-DG as compared to treatment with the non-specific antibody control (**Fig. 2**E). This is in accordance with other reports showing β1-integrin- and α-DG-blocking antibodies disrupt oligodendroglial process formation and MBP+ myelin sheet extension (Colognato et al., 2007; Milner and Ffrench-Constant, 1994). Interestingly, MBP expression was significantly reduced by α-DG-blocking antibody, but not by β1-integrin-blocking antibody (**Fig. 2**F). Together, this indicates that the integrin and DG receptors have overlapping but different roles in regulating OL differentiation, with β1 integrins and DG regulating process elaboration and morphological maturation, and with DG playing an additional important role in gene expression changes, or at least in *Mbp* expression.

PTPα interacts with integrin and dystroglycan in OLs

We investigated whether PTPα physically interacts with either or both types of Lm2 receptor. WT and KO OPCs were differentiated in the presence of Lm2 for 5 days, and treated with cross-linker prior to lysis and immunoprecipitation with control IgG antibodies or antibodies specific to PTPα. Immunoblot analysis revealed that PTPα forms a complex with β-DG and Fyn, as both proteins were detected in PTPα immunoprecipitates (**Fig. 3**A). PTPα is a known activator of Fyn, and Fyn has previously been shown to physically interact with PTPα in developing mouse brains (Bhandari et al., 1998). Here, we demonstrated that PTPα–Fyn interaction also occurs in differentiating OPCs on a Lm2 substrate. Probing the immunoprecipitates for α6 integrin was uninformative, as any potential α6 integrin signal at the expected size of 120 kDa was obscured by a strong uncharacterized band of about the same size that was present in both control IgG and anti-PTPα immunoprecipitates (data not shown).

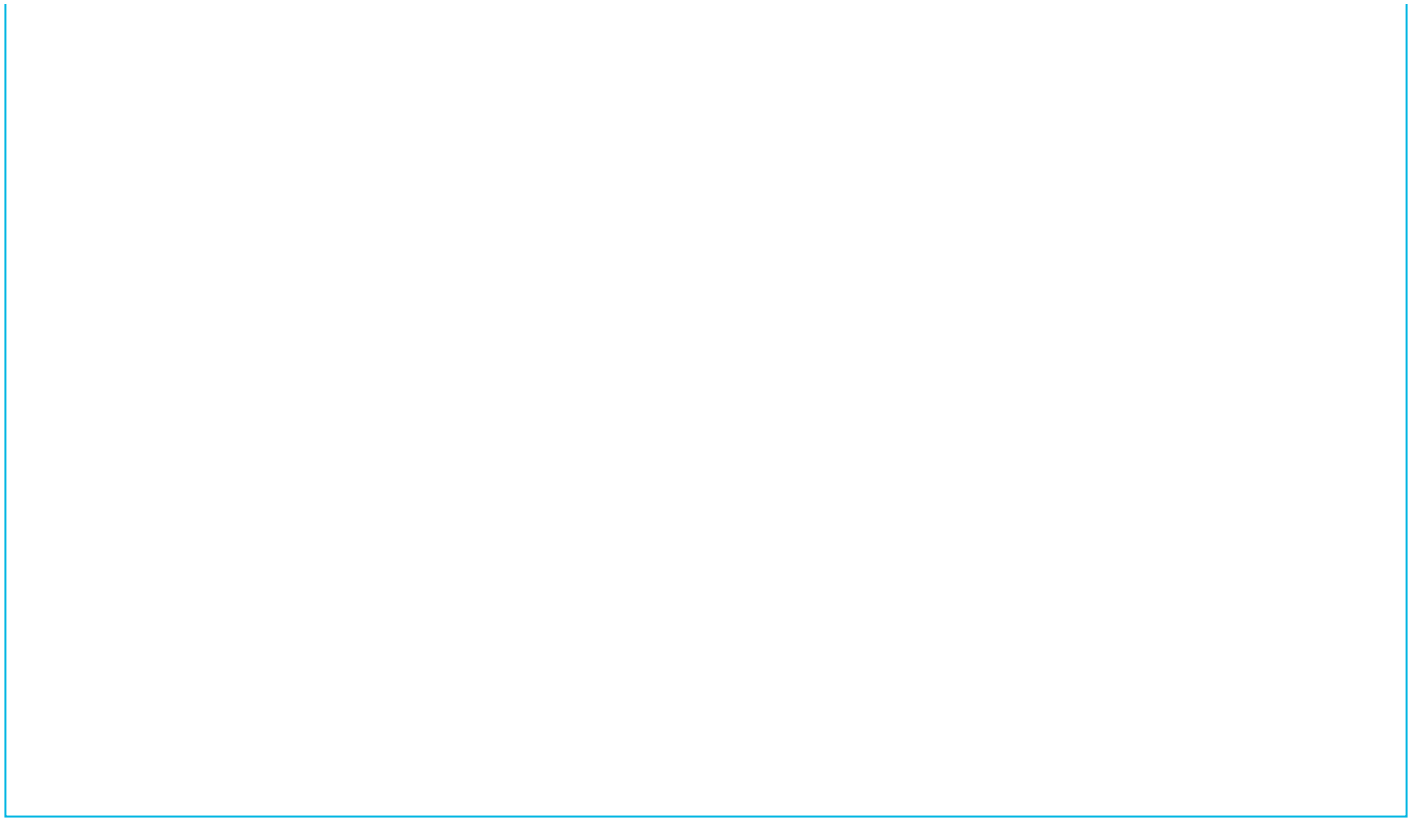

Fig. 3.

PTPα interacts with integrin and dystroglycan during Lm2-induced OPC differentiation. WT and KO OPCs were differentiated on PDL/Lm2 substrates for 5 days. The cells were subsequently cross-linked and lysed. Cell lysates were subjected to immunoprecipitation (IP) with (A) pre-immune rabbit IgG or anti-PTPα antibody or (B) normal mouse IgG or α6 integrin antibody (GoH3 clone). Western blotting was performed with antibodies to PTPα, Fyn, α6 integrin or β-DG. Bands present in the control IgG and IP lanes that are absent in the lysate lanes in the blots for Fyn and β-DG (open arrowheads) likely represent immunoreactivity to antibody heavy chain. Blots shown are representative of at least two independent experiments.

To verify whether PTPα indeed interacts with α6 integrin, lysates from WT and KO OLs were immunoprecipitated with anti-α6 integrin antibody. PTPα co-immunoprecipitated with α6 integrin, indicating that both proteins are part of a complex (Fig. 3B). Fyn co-immunoprecipitated with α6 integrin in WT OLs as previously reported (Laursen et al., 2009) and in KO OLs (Fig. 3B). Interestingly, β-DG also co-immunoprecipitated with α6 integrin from differentiated WT and KO OLs, indicating that these two receptors may relay Lm2 signals as a complex (Fig. 3B). Moreover, since the α6 integrin and β-DG interaction is detected in KO OPCs, this occurs independently of PTPα.

PTPα–Fyn signaling during Lm2-induced OL differentiation

The SFK Fyn is a critical regulator of OL development and myelination (Sperber et al., 2001; Umemori et al., 1994). In contrast to other SFKs, Fyn activity increases with OL differentiation (Peckham et al., 2016; Umemori et al., 1994; Wang et al., 2009a). PTPα is a known activator of Fyn, directly dephosphorylating the inhibitory tyrosine residue in Fyn to enhance kinase activity (Bhandari et al., 1998; Ponniah et al., 1999). We validated this in WT and KO mouse brains using an antibody recognizing the phosphorylated inhibitory tyrosine residue located in the C-terminal region of all SFKs (pSFK^{Y527}). The pSFK^{Y527} levels were similar in post-natal day (P)7 WT and KO brains and MBP expression was low at this age. At P14, pSFK^{Y527} levels were significantly decreased in WT brains relative to KO brains (Fig. 4A,B), indicating PTPα-dependent SFK activation. While various types of brain cells may contribute to this SFK activation, this coincided with a period of active OL development as shown by readily detectable MBP expression in WT brains that was much less evident in KO brains (Fig. 4A). To confirm that this SFK dephosphorylation involved Fyn, brain lysates were subjected to Fyn immunoprecipitation and the level of pSFK^{Y527} (reflecting pFyn^{Y531}) was quantified. The pFyn^{Y531} levels were significantly higher in KO brains, indicating reduced Fyn dephosphorylation and activity (Fig. 4C,D).

PTPα-dependent Fyn activation is concomitant with OPC differentiation *in vivo* and *in vitro*. (A) Lysates from P7 and P14 WT and KO mouse cortices were probed with antibodies recognizing phospho-Y527 SFKs (pSFK^{Y527}), Fyn, CNP and MBP. SFK activation is reflected by dephosphorylation of Y527 and coincides with the MBP expression observed in P14 WT mice. (B) Quantification of pSFK^{Y527} per unit Fyn expression. Bars represent mean±s.d., n=3 animals per genotype. *P<0.05 (unpaired Student's t-test). (C) Fyn was immunoprecipitated from cortical lysates prepared from P14 WT and KO mice and immunoblotted for pSFK^{Y527}. (D) Quantification of Fyn phospho-Y531 (pFyn^{Y531}; recognized by the pSFK^{Y527} antibody) per unit Fyn in immunoprecipitates, as in C, from WT and KO mice. Bars represent mean±s.d., n=3 animals per genotype. *P<0.05 (unpaired Student's t-test). (E–G) WT and KO OPCs were cultured on plates coated with PDL and with/without Lm2 under proliferating (0 day) or differentiating conditions for 5 days. (E) pSFK^{Y527} and Fyn levels were determined by immunoblotting and (F) quantified. (G) Enzymatic assay of Fyn kinase immunoprecipitated from the WT and KO cells. Bars in the graph represent mean±s.d. from three independent experiments, and the upper panel is a representative immunoblot from one such experiment confirming that equivalent levels of Fyn were present in the Fyn immunoprecipitates. (*P<0.05; **P<0.01; ***P<0.001 (ne-way ANOVA followed by Tukey's post hoc test).

To examine PTPα-dependent Fyn activation during Lm2-induced OPC differentiation, WT and KO OPCs were seeded onto PDL or PDL/Lm2-coated dishes, and Fyn activation, as indicated by the level of pSFK^{Y527}, was determined pre- and post-differentiation. Significantly reduced pSFK^{Y527} levels were evident only when WT OPCs were induced to differentiate on PDL (0 days versus 5 days), and in PDL/Lm2-exposed WT OPCs compared to KO OPCs at 5 days (Fig. 4E,F). To more precisely and directly measure Fyn activity, we conducted *in vitro* kinase assays of Fyn immunoprecipitates. Differentiation on PDL increased Fyn activity 3-fold between WT OPCs (0 days) and OLs after 5 days differentiation (Fig. 4G). Even under pre-differentiating (proliferating) culture conditions (0 days), the presence of Lm2 resulted in a 2-fold elevation (P<0.05) of Fyn activity in WT OPCs, and a subsequent 5 days of differentiating culture on PDL/Lm2 modestly increased Fyn activity by a further ∼10% (although this is not a significant change) (Fig. 4G). However, Fyn activity was significantly higher in these WT OLs than in KO OPCs or OLs, and the presence of Lm2 did not increase Fyn activity in KO OPCs or OLs (Fig. 4G). These results confirmed that PTPα-regulated Fyn activation underlies cell-mediated and Lm2-directed differentiation programs during oligodendrogenesis.

PTPα regulates Akt, but not Erk1/2, activation during Lm2-induced OPC differentiation and in the developing brain

Lm2 stimulation activates Akt and Erk1/2 signaling, and these two pathways play key roles in regulating OL development and myelination. Since Lm2-induced OL differentiation requires PTPα, we examined whether Akt and Erk1/2 activities are PTPα-dependent during OPC differentiation. Phosphorylation of Akt at S473 and T308 is required for full kinase activity (Alessi et al., 1996). Under proliferating conditions (0 day) on PDL or PDL/Lm2, WT and KO OPCs displayed simil of Akt^{S473} and Akt^{T308} phosphorylation (Fig. 5A–C). Upon differentiation in the presence of Lm2, Akt^{S473} phosph was significantly increased only in WT OPCs (91.9±14.1% to 167.7±21.9% in 5 days differentiated OLs, mean±s.d., P<0.05) but remained unchanged in the KO cells (99.0±29.0% at 0 day versus 93.1±20.1% at 5 days) (Fig. 5A,B). Interestingly, under differentiating conditions Lm2 increased the phosphorylation of Akt^{T308} by ∼2–2.5-fold in both WT and KO OLs (Fig. 5A,C). We also examined Erk1/2 phosphorylation, an indicator of Erk1/2 activity, and found that Lm2-induced differentiation increased this ∼2-fold by 5 days in WT and KO OLs compared to the respective pre-differentiated 0 day OPCs (P<0.05) (Fig. 5A,D). WT and KO OLs differentiated on PDL alone did not show any significant changes in Erk1/2 activity.

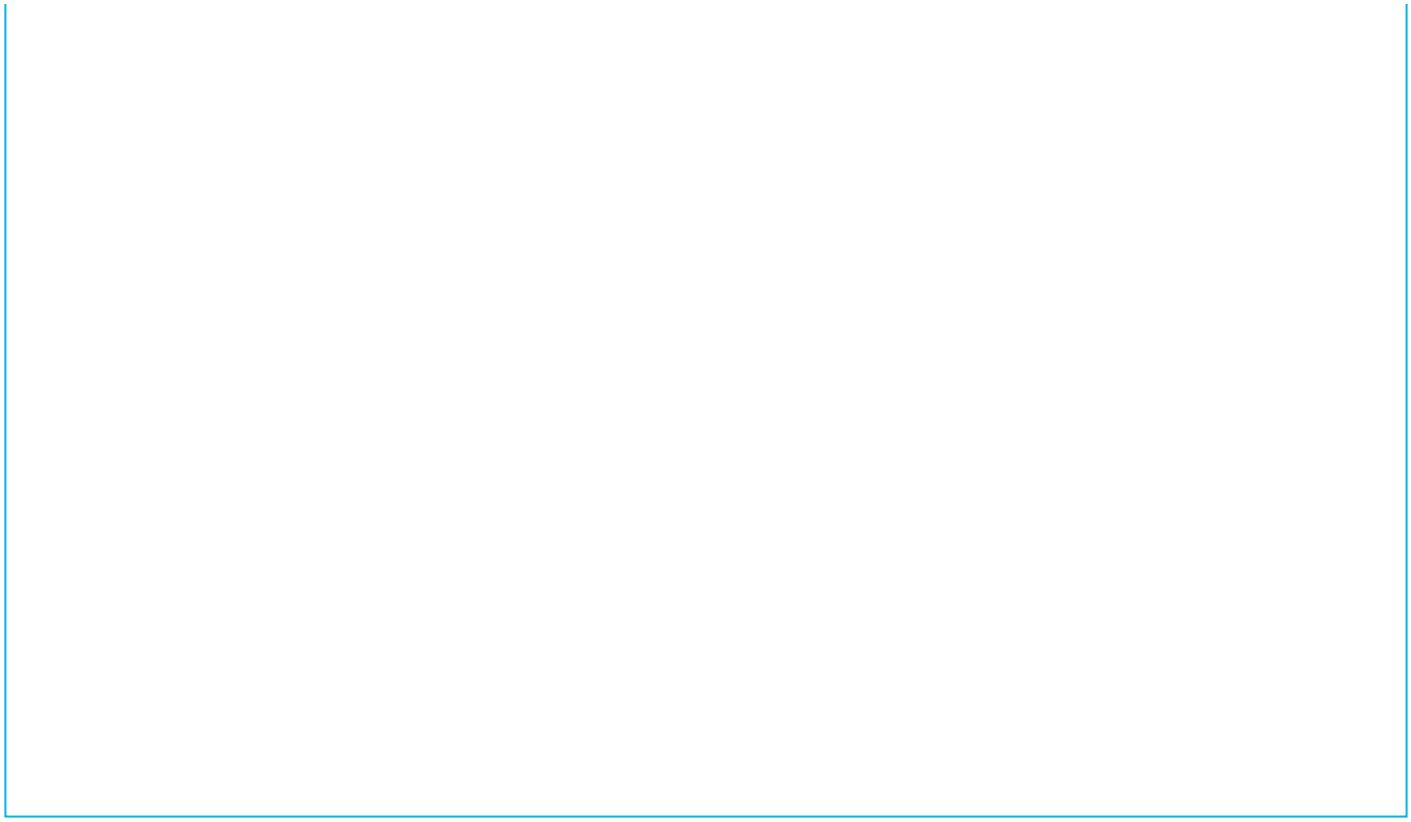

Fig. 5.

Akt but not Erk1/2 activation is dependent on PTPα during OPC differentiation. WT and KO OPCs were seeded onto plates coated with PDL and with/without Lm2, allowed to proliferate and differentiated for 0 day or 5 days. (A) Immunoblot analysis of Akt and Erk1/2 phosphorylation. The levels of (B) Akt^{S473} and (C) Akt^{T308} phosphorylation (pAkt^{S473} and pAkt^{T403}, respectively) were normalized to total Akt expression, and (D) the levels of pErk1/2 were normalized to total Erk1/2 expression using data from the independent experiments. The results from each experiment were normalized to WT cells at 0 day and are represented as a percentage. Bars indicate mean±s.d. *P<0.05 (one-way ANOVA followed by Tukey's post hoc test). (E) Lysates from P7 and P14 WT and KO mouse cortices were probed with antibodies recognizing pAkt^{S473} and pAkt^{T403} and phosphorylated Erk1/2 (pErk1/2$^{T203/Y204}$). Phosphorylation of (F) Akt^{S473}, (G) Akt^{T308}, and (H) Erk1/2 was normalized to the level of the respective total protein. Bars represent mean±s.d., n=8–9 mice per genotype. *P<0.05 (unpaired Student's t-test).

We determined whether PTPα-dependent Akt activation occurred *in vivo* by immunoblotting cortical lysates from P7 and P14 WT and KO mice for Akt phosphorylation. While Akt^{T308} phosphorylation was not affected by age or genotype, Akt^{S473} phosphorylation was significantly reduced in P7 and P14 KO brains compared to WT brains (P<0.05) (Fig. 5E–G). In line with the *in vitro* OPC differentiation findings, Erk1/2 was activated in the brains of WT and KO mice from P7 to P14 (P<0.05), and this occurred independently of PTPα expression (Fig. 5E,H).

Taken together, these results indicate that Lm2 stimulates the activation of Akt and Erk1/2 during OPC differentiation. However, the Lm2 stimulation of Akt^{S473} phosphorylation is uniquely PTPα dependent, as is the phosphorylation of this site in developing brain.

PTPα-mediated Akt^{S473} phosphorylation promotes OL differentiation

To examine the involvement of active Akt in OPC differentiation, we immunostained 5 day differentiated WT and KO OPC cultures for pAkt^{S473}, MBP and the OL lineage marker Sox10. The absence of PTPα limited the differentiation/maturation of OLs as indicated by a significantly reduced proportion of cells that were double-positive for MBP and Sox10 (MBP/Sox10+) (Fig. 6A,B). The population of Sox10+ cells that was positive for pAkt^{S473} was likewise reduced in the KO cultures (WT 24.7±1.5% vs KO 14.7±2.3%), indicating PTPα-dependent S473 phosphorylation (Fig. 6A,C). Furthermore, co-labeling of pAkt^{S473} with MBP/Sox10+ cells (differentiated/mature cells) was significantly lowered in the differentiated KO OLs as compared to WT OLs (Fig. 6D). On the other hand, no significant difference was detected in the populations of WT and KO MBP-negative but Sox10+ cells that expressed pAkt^{S473} (interpreted as undifferentiated OPCs) (Fig. 6D). These findings indicate that a PTPα-regulated increase in Akt^{S473} phosphorylation is specifically associated with maturing or mature OLs.

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Fig. 6.

PTPα-dependent Akt^{S473} phosphorylation is important for OPC differentiation. (A) WT and KO OPCs were differentiated on Lm2 substrate for 5 days and immunostained for MBP, Sox10 and pAkt^{S473}. The insets in the merged channels show the pAkt^{S473} signal in an MBP/Sox10+ OL. (B) The percentage of differentiated cells, as indicated by MBP/Sox10 positivity, was quantified. (C) The levels of pAkt^{S473} in OPCs/OLs (pAkt^{S473} in Sox10+ cells) in WT and KO cultures were quantified. (D) Akt^{S473} phosphorylation in OLs (MBP+/Sox10+) and OPCs (MBP−/Sox10+) in WT and KO cultures was quantified in three independent experiments with cells counted in 6–8 random areas per well per experiment. Bars represent mean±s.d. *P<0.05, **P<0.01 (unpaired Student's t-test). (E) Differentiating WT OPCs were treated with Torin (10 nM) for 5 days. Cell lysates were immunoblotted to detect the phosphorylation of Akt^{S473}, Akt^{T308} and the downstream mTOR target p70S6K^{T389}, and the expression of Akt, p70S6K, and the differentiation markers MBP and CNP.

Akt^{S473} phosphorylation is regulated by a feed-forward mechanism involving mammalian target of rapamycin (mTOR), which is also believed to be the PDK2 enzyme that phosphorylates Akt at this site for full kinase activation (Nave et al., 1999). Inhibition of mTOR with Torin abolished Lm2-upregulated phosphorylation of Akt^{S473}, but not that of Akt^{T308} in WT OLs (Fig. 6E). Torin treatment also abolished phosphorylation of p70S6 kinase (also known as RPS6KB1), a well-characterized immediate downstream effector of mTOR, indicating that mTOR activity is inhibited by drug treatment. Furthermore, Torin treatment prevented OPC maturation, as indicated by the lower MBP and CNP expression levels (Fig. 6E). These results confirmed that Lm2-induced Akt^{S473} phosphorylation is mediated by mTOR signaling and important for differentiation.

NDRG1 is a downstream target of PTPα–Akt signaling

To investigate whether impaired Akt^{S473} phosphorylation results in suboptimal Akt activation during OL differentiation, WT and KO OPC/OL lysates were probed with a phospho-substrate antibody that recognizes conserved motifs phosphorylated by the family of AGC kinases, including Akt. The phosphorylation of several protein bands appeared to be upregulated during OL differentiation (arrowheads, Fig. 7A). Densitometric quantification of the indicated bands (Table S1) revealed PTPα-dependent changes in a prominent ~45 kDa phosphoprotein at all time points (KO 37%, 60% and 31% lower than WT at 0, 3 and 5 days respectively, n=2) (open arrowhead, Fig. 7A). To find out whether these bands represented proteins phosphorylated by Akt, differentiating WT OPC cultures were treated with an Akt1/2 inhibitor. This reduced phosphorylation of the 100, 75, 60 and 45 kDa bands, indicating that these are likely bona fide Akt subs (Fig. 7B).

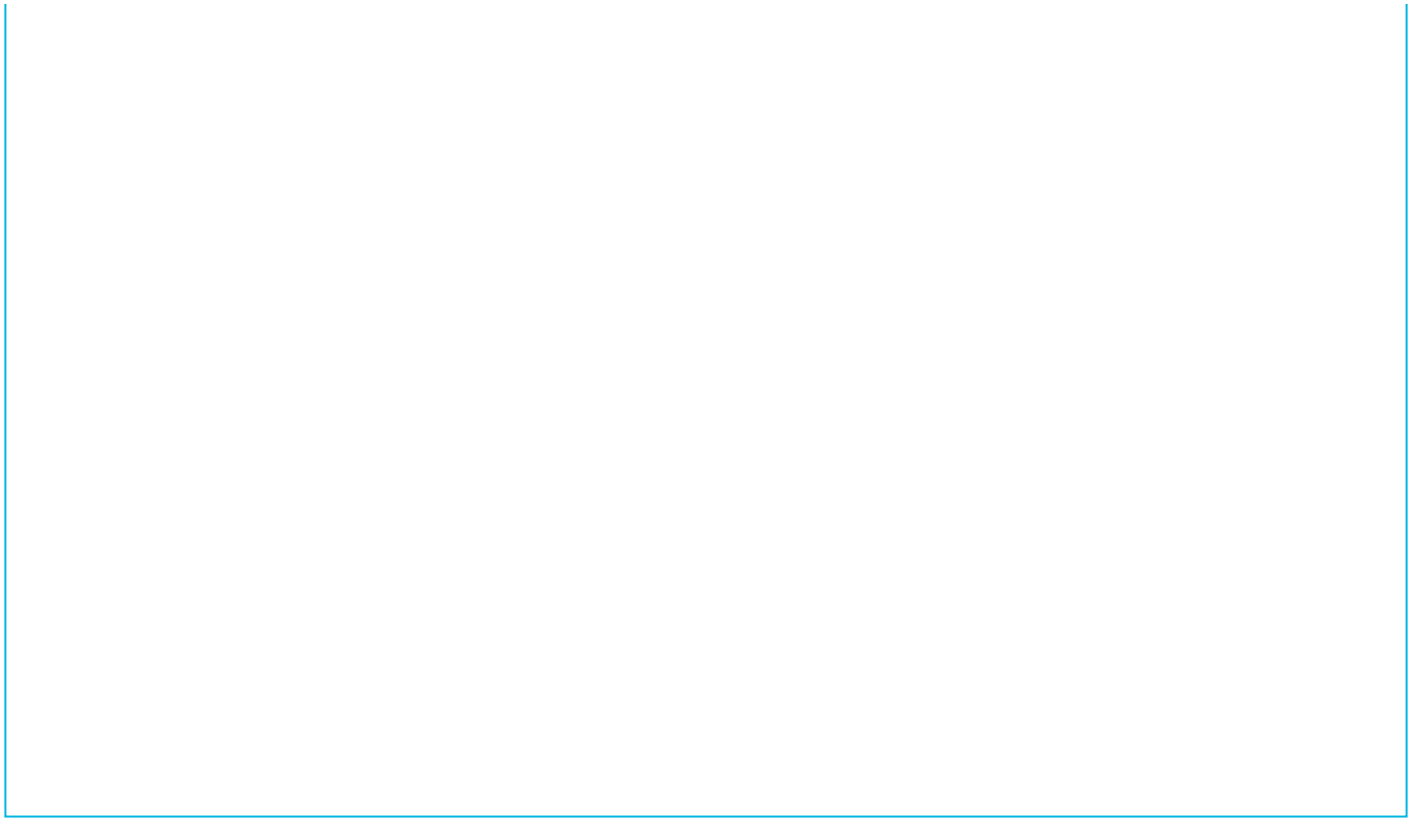

Fig. 7.

NDRG1 is a downstream target of PTPα/Akt signaling. WT and KO OPCs were differentiated on Lm2 substrate for 0, 3 and 5 days. (A) Cell lysates were probed with an antibody that recognizes phosphoserine/threonine within a conserved sequence motif targeted by arginine-directed (AGC-family) kinases, including Akt (Akt p-substrates). Arrowheads indicate bands that are upregulated upon OPC differentiation. (B) Treatment with Akt-specific inhibitor abolished phosphorylation of bands indicated by the arrowheads, demo an Akt-dependent effect. (C) Immunoblots of NDRG1 phosphorylation at T346 (pNDRG1^{T346}) and expression in differentiating W cultures, representative of three independent experiments. The (D) pNDRG1^{T346} bands and (E) NDRG1 bands were quantified pe actin from three blots of lysates of 5 day differentiated WT and KO cell cultures, and (F) the ratio of pNDRG1:NDRG1 determined, in each case with values from WT cells set at 1 and those from KO cells shown relative to this. (G) Immunoblot analysis of pNDRG1^{T346} and expression in the cortices of P7 and P14 WT and KO mice.

The identities of these Akt-targeted phosphoproteins are unknown, but other reports suggest that the 45 kDa band might be the anti-metastatic protein N-myc downstream-regulated gene-1 (NDRG1), a protein also implicated in CNS and PNS myelination (Heller et al., 2014; King et al., 2011; Okuda et al., 2004). To examine whether NDRG1 phosphorylation is indeed altered in our differentiating OPC cultures, an antibody that specifically recognizes NDRG1 phosphorylated at T346 was used to probe lysates of WT and KO OPC/OL cultures. The level of phosphorylated NDRG1^{T346} (pNDRG1^{T346}) clearly increased with OL differentiation, and this was reduced in OLs lacking PTPα (Fig. 7C,D).

compared to WT cells (Fig. 7C,E). After 5 days of differentiation, KO cells exhibited a significantly reduced (~60%) phosphorylation of NDRG1 per unit protein (Fig. 7F). Likewise, a PTPα-dependent reduction in NDRG1 phosphorylation and expression were observed in cortices of P14 KO mice, but a smaller reduction in NDRG1 phosphorylation and no difference in expression were detected in P7 KO brain cortices (Fig. 7G). Taken together, these results suggest that NDRG1 is a novel downstream target of PTPα, and that PTPα–Akt signaling may developmentally regulate the phosphorylation and expression of NDRG1 during OL maturation.

A Lm2–PTPα–Fyn signaling axis regulates Akt phosphorylation and OPC differentiation

To address whether Akt activity is dependent on engagement of Lm2 by the β1 integrin and/or DG receptors, differentiating WT OPCs on PDL/Lm2 were treated with function-blocking antibodies to β1 integrin and α-DG. Expression levels of β1 integrin and α-DG in OLs were not affected by the function-blocking antibodies (Fig. 8A). Fyn is a downstream effector of Lm2 signaling, and blocking Lm2 receptors prevents Fyn activation (Bechler et al., 2015; Laursen et al., 2009). We validated this in our WT cells by performing a Fyn kinase assay that showed that treatment with function-blocking antibodies against β1 integrin, α-DG or both block Fyn activation (Fig. 8B).The increased phosphorylation of Akt^{S473} in OLs as compared to OPCs (Fig. 5A) was impeded by the blocking β1 antibody and/or by the α-DG antibody but not by the non-specific IgM treatment control (Fig. 8A,C). Notably, $NDRG1^{T346}$ phosphorylation was not affected as greatly by the β1-blocking antibody as it was when the α-DG-blocking antibody was present (Fig. 8A,C). Taken together, these results suggest that both Lm2 receptors are functionally required for signaling to Fyn and Akt^{S473}, and that this stimulates $NDRG1^{T346}$ phosphorylation. Additionally, it appears that Lm2 can act through α-DG to stimulate partial $NDRG1^{T346}$ phosphorylation in a manner independent of β1 integrin, Fyn and $pAkt^{S473}$.

Fig. 8.

The Lm2–PTPα–Fyn–Akt signaling axis regulates NDRG1 phosphorylation during OPC differentiation. WT OPCs were differentiated for 5 days on PDL/Lm2-coated dishes with or without various antibodies or kinase inhibitors as detailed below. (A,B) Differentiation in the presence of non-specific IgM or neutralizing antibodies to β1 integrin, α-DG or both. Cell lysates were (A) probed for $pAkt^{S473}$ and $pNDRG1^{T346}$, and (B) Fyn immunoprecipitates were prepared and assayed for Fyn activity. (C) Quantification of Akt^{S473} and $NDRG1^{T346}$ phosphorylation per unit Akt and NDRG1, respectively. Function-blocking antibody treatment groups were normalized and compared to the IgM control group. **P*<0.05; ***P*<0.01 (one-way ANOVA followed by Tukey's post hoc test), *n*=3. (D–F) Differentiation in the presence of the SFK inhibitor SU6656 (1 µM). Cell lysates were (D) probed for MBP, $pAkt^{S473}$ and $pNDRG1^{T346}$. (E) Fyn immunoprecipitates were prepared and assayed for Fyn activity. (F) Akt activity was quantified as a measure of $pAkt^{S473}$ per unit Akt from blots as in D. (G) Differentiation in the presence of Akt inhibitor (120 nM). Cell lysates were probed for Olig2 and MBP as markers of differentiation status, and for $pSFK^{Y527}$ and $pNDRG1^{T386}$. Results in all graphs represent mean±s.d. from three independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001 (unpaired Student's *t*-test and one-way ANOVA followed by Tukey's post hoc test). (H) Schematic of the Lm2–PTPα–Fyn–Akt signaling pathway that targets NDRG1. Possible models of Lm2 binding to α6β1 integrin and DG individually and as a co-receptor complex are depicted at the top, corresponding to the boxed region of the signaling interactions below. These Lm2 receptors are complexed with PTPα and Fyn, and ligand engagement induces PTPα-catalyzed dephosphorylation and activation of Fyn. Lm2–DG engagement can also induce signaling (dashed lines) that is essential for OL differentiation.

To examine whether Akt activation requires Fyn signaling, WT OPCs differentiated in the presence of Lm2 were treated with the SFK inhibitor SU6656 or vehicle control. SU6656 treatment prevented OL differentiation as indicated by reduced MBP expression (Fig. 8D), confirming the known role of Fyn in OPC development. Fyn kinase assays confirmed that SU6656 treatment inhibited Fyn activity (Fig. 8E). Moreover, inhibition of Fyn reduced Akt^{S473} phosphorylation by ~2-fold (*P*<0.01) (Fig. 8D,F), indicating that Akt activation is dependent on Fyn during OPC differentiation. The phosphorylation of NDRG1 ($pNDRG1^{T346}$) was also reduced upon Fyn inhibition (Fig. 8D), revealing for the first time that NDRG1 phosphorylation correlates with Fyn activity in OLs.

Finally, to validate the important role of Akt in these signaling events and OL differentiation, WT OPCs were treated with Akt1/2 inhibitor or vehicle solution during differentiation. Inhibition of Akt did not affect Fyn activity, while reduc $pNDRG1^{T346}$ level and MBP expression (Fig. 8G). These findings show the connectivity between Lm2 receptor engagement, PTPα–Fyn signaling, Akt activation and NDRG1 phosphorylation (Fig. 8H).

DISCUSSION

This study identifies PTPα as an essential component of the OL differentiation program induced by the extrinsic ligand Lm2. This is in addition to the previously reported role of PTPα in promoting the intrinsic differentiation of OLs (Wang et al., 2009a), as confirmed in this study and discussed further below. Among numerous extracellular matrix proteins in the CNS, Lm2 has well-documented effects in promoting OPC differentiation, myelin membrane formation by OLs, and

show that OPCs devoid of PTPα survive comparably to WT OPCs but fail to differentiate in response to Lm2 as indicated by poor expression of the maturation markers MBP and CNP, unchanged expression of the progenitor marker PDGFRα and an inability to undergo the expansion in MBP-positive cell area that accompanies OL maturation. Thus, PTPα is required for Lm2-regulated gene expression and morphological changes that are hallmarks of OL differentiation.

Lm2 exerts its pro-differentiating effects on OPCs through binding to two receptors, α6β1 integrin and DG (Barros et al., 2009; Benninger et al., 2006; Buttery and ffrench-Constant, 1999; Colognato et al., 2007; Galvin et al., 2010). We found that PTPα forms a complex with each of these Lm2 receptors in OLs. Furthermore, α6 integrin immunoprecipitates contain not only PTPα, but also β-DG. This raises the possibility that a tripartite receptor complex of α6β1 integrin, PTPα and DG co-ordinates Lm2 binding and signaling, perhaps forming under particular conditions or existing in addition to functional complexes of PTPα partnered with either α6β1 integrin or DG (**Fig. 8**H). Whether such bi- or tri-receptor interactions result in different signaling strengths or outputs that modulate the rate or other aspects of OL maturation is an interesting question. Since α6β1 integrin and DG can each interact with additional receptors to regulate OL differentiation, for example α6β1 integrin binds with the L1 receptor contactin (Laursen et al., 2009), and DG modulates IGF-1 signaling and interacts with the IGF-1R substrate IRS-1 (Galvin et al., 2010), there is also the possibility that multi-receptor aggregates integrate or fine-tune laminin and other OL differentiation signals. PTPα has been shown to complex with the above Lm2 receptor partners in other cell types, with contactin in neurons and with IGF-1R in several types of cancer cell and fibroblasts (Khanna et al., 2015; Zeng et al., 1999), suggesting that it could do the same in OPCs and OLs. It should be noted that α-DG binds other ECM molecules besides laminin, and β-DG interacts with several molecules, including components of the dystrophin–glycoprotein complex (DGC) which is central to skeletal muscle function (Gawor and Prószyński, 2018). How these interactions may affect OL development and myelination, and if so, whether PTPα is involved, is unknown. Of interest, many DGC components are expressed in OLs including three dystrophin isoforms. Ablation of the latter delays OL maturation, in accordance with delayed CNS myelination in *mdx* mice (a model of Duchenne's muscular dystrophy) that lack an isoform of dystrophin (Aranmolate et al., 2017).

In Lm2-stimulated OPC differentiation in culture, Fyn is activated and required for MBP expression, cell branching complexity and myelin membrane formation (Colognato et al., 2004). PTPα is a well-characterized activator of SFKs including Fyn, catalyzing the dephosphorylation of the inhibitory phosphotyrosine site of SFKs to promote kinase activation (Pallen, 2003). In OPC/OL cultures differentiated on PDL or on PDL/Lm2 for 5 days, Fyn inhibitory phosphorylation was higher and the kinase activity of Fyn was significantly lower in PTPα-KO cells than in WT cells, demonstrating PTPα dependence. Despite this, Fyn was present in α6 integrin immunoprecipitates from both WT and KO cells. Thus, Fyn associates with α6β1 integrin and/or with the co-immunoprecipitated Lm2 receptor β-DG in a PTPα-independent manner, but PTPα is required for the activation and ensuing function of Fyn in Lm2-stimulated OL differentiation. PTPα-dependent differentiation of cells on PDL is also mediated via Fyn activation as shown here and previously (Wang et al., 2009a), and we postulate that this intrinsic signaling involves a pool of Fyn that is, at least temporally, not associated with Lm2 receptors. Notably, a specific effect of Lm2 was to induce elevated Fyn activity in proliferated, but as yet undifferentiated WT cultures (i.e. at 0 day), indicating that Lm2/PTPα-dependent activation of Fyn

Interestingly, Lm2, but not PDL, promotes the movement of α6β1 integrin into a subset of lipid rafts in newly differentiated OLs (Baron et al., 2003). A previous study of Lm-regulated OPC differentiation reported that the dephosphorylation of Fyn at its inhibitory phosphotyrosine site correlated with reduced levels of co-fractionating Csk (Colognato et al., 2004), the kinase responsible for phosphorylating this site to repress Fyn activity. Furthermore, this occurred in detergent-insoluble Fyn-enriched fractions, consistent with Csk exclusion from lipid rafts. Together with our findings, this indicates that the Lm2-regulated dephosphorylation of this critical tyrosine residue in Fyn may involve dual mechanisms – the engagement of the phosphatase activity of PTPα and the exclusion or displacement of the kinase activity of Csk from proximity with receptor-linked Fyn. Lm2-induced PTPα-dependent activation of Fyn occurs in undifferentiated OPCs and in differentiated OLs, whereas Csk levels are only downregulated in differentiated OLs (Colognato et al., 2004). We propose that PTPα directly dephosphorylates and activates Fyn to promote Lm2-mediated differentiation, and as differentiation proceeds, the Lm2-engaged α6β1–PTPα–Fyn complex is segregated into lipid rafts where Fyn activity is additionally regulated by the exclusion of Csk. The latter may also be PTPα dependent, since there is a precedent for PTPα-regulated Csk localization in thymocytes, where PTPα is found in rafts, and its expression indirectly regulates Csk recruitment to membrane rafts (Maksumova et al., 2005). Accordingly, the lack of Lm2-stimulated Fyn activation in PTPα-KO cells shows that a Csk-dependent mechanism of Fyn activation is not operative in the absence of PTPα. Alternatively, however, the defective maturation of the PTPα-KO OPCs likely prevents the formation of specialized membrane raft domains that occurs upon OL differentiation (Krämer et al., 1997) and thus precludes raft-dependent segregation of Fyn and Csk.

We show that Lm2 stimulates the activation of Akt and Erk1/2, as well as the PTPα-mediated activation of a Fyn–Akt–NDRG1 signaling axis. While Lm2 stimulates Erk1/2 activation and $Akt^{T308/S473}$ phosphorylation in differentiating OLs, only the activating phosphorylation of Akt at Ser473 is PTPα dependent. In the absence of PTPα, Erk1/2 activation and Akt^{T308} phosphorylation occur, but are insufficient to promote differentiation. We confirmed that phosphorylation of Akt^{S473} and the Akt substrate NDRG1 are also Fyn regulated, as they were inhibited by treating differentiating WT OPCs with the Src family kinase inhibitor SU6656. How PTPα–Fyn regulates Akt activation was not investigated in this study, but a potential mechanism could involve the PTPα–Fyn effector focal adhesion kinase (FAK) (Wang et al., 2009a) and FAK-mediated activation of PI3K (Demers et al., 2009; Xia et al., 2004), an upstream activator of Akt.

The use of Lm2 receptor function-blocking antibodies showed that DG was required for both MBP expression and cell spreading, whereas β1 integrin was required for cell spreading but not for full MBP expression. Since Lm2-stimulated MBP expression and cell spreading in differentiating OLs is PTPα dependent, this implies that, at least for Lm2-regulated MBP expression signaling, Lm2–β1 integrin signals to PTPα are redundant with or compensated for by Lm2–DG signals to PTPα. PTPα is required for Lm2-induced Fyn activity and Akt^{S473} phosphorylation, and Fyn and Akt activity are essential for Lm2-stimulated MBP expression. However, blocking either β1 integrin or DG function inhibited Fyn activation and Akt^{S473} phosphorylation, raising the question of how Lm2–DG–PTPα signaling can mediate Fyn-dependent MBP expression in the absence of β1 integrin action. In the model shown in **Fig. 8**H, Lm2 receptor engagement and signaling involves at least some α6β1 integrin–PTPα–Fyn and DG–PTPα–Fyn interactions, where each Lm2 receptor type can

preclude additional, potentially interdependent, α6β1 integrin–DG complexes that support PTPα-catalyzed Fyn activation. We have shown that Lm2-stimulated signaling by either α6β1 integrin or DG results in altered Fyn activation relative to that induced by both functional receptors, and propose that (1) this is sufficient for MBP expression but not cell spreading, and (2) that another signal generated by Lm2–DG is also required for MBP expression. Some possibilities concerning this latter DG-specific signal could be activation of a pool of Fyn activity in a particular subcellular location or modulation of some other critical signaling event/component.

We found NDRG1 to be a novel target of Lm2 signaling during OL differentiation, and that NDRG1 expression and phosphorylation are PTPα dependent. Blocking α-DG function greatly diminished NDRG1 phosphorylation while blocking β1 integrin function had less effect. This suggests that non-redundant DG signaling mediates a portion of Lm2-stimulated pNDRG1 expression and occurs independently of PTPα–Fyn–Akt signaling. This latter mechanism may involve the kinase Sgk1, which has been shown to phosphorylate NDRG1 under certain conditions in OLs and during Lm2-mediated phosphorylation of NDRG1^{T346} in myelinating Schwann cells (Heller et al., 2014; Miyata et al., 2011). It is not known if the DG-specific effect is manifested only under conditions when β1 integrin signaling is compromised, and if it is linked to the likewise non-redundant role of DG in promoting full MBP expression.

NDRG1 has been best studied as a suppressor of cancer progression and metastasis (Bae et al., 2013), and for the NDRG1 mutations that cause a type of hereditary severe neuropathy known as Charcot–Marie–Tooth disease type 4D (CMT4D) (Kalaydjieva et al., 2000). CMT4D is characterized by early onset peripheral nerve demyelination that is reproduced in mice deficient in NDRG1 expression (King et al., 2011; Okuda et al., 2004). Mild CNS abnormalities have been reported in a few CMT4D patients, although structural defects in the CNS have not been detected in NDGR1 mutant mice. Cell and animal studies support an important role of NDRG1 in intracellular trafficking (Heller et al., 2014; King et al., 2011; Li et al., 2017), consistent with endocytosis defects associated with Charcot–Marie–Tooth disease (Lee et al., 2012; Roberts et al., 2010; Stendel et al., 2010). However, its precise role(s) in OL development and CNS and PNS myelination are unclear, as are the effects of its phosphorylation on these processes. Strategies to promote NDRG1 expression have been successful in impeding cancer cell migration (Sun et al., 2013; Wangpu et al., 2016), and it would be interesting to investigate whether similar approaches to manipulate NDRG1 affect oligodendroglial developmen

MATERIALS AND METHODS

Antibodies

Primary rabbit antibodies were against: PTPα [1:2000 for blotting, 1:100 for immunoprecipitation, generated as previously described (Chen et al., 2006)], MBP (1:1000 for blotting, cat. #AB980, Millipore), Akt phospho-substrate antibody (1:1000 for blotting, cat. #9614, Cell Signaling Technology), pNDRG1^{T346} (1:1000 for blotting, cat. #5482, Cell Signaling Technology), NDRG1 (1:500 for blotting, cat. #ab124689, Abcam), pAkt^{T308} and pAkt^{S473} (1:1000 for blotting, 1:500 for staining, cat. #4060 and #13038, Cell Signaling Technology), Akt (1:1000 for blotting, cat. #4691, Cell Signaling Technology), pErk1/2 (1:1000 for blotting, cat. #9101, Cell Signaling Technology), Erk1/2 (1:1000 for blotting, cat. #9102,

for blotting, cat. #9234, Cell Signaling Technology), p70S6K (1:1000 for blotting, cat. #SC-230, Santa Cruz Biotechnology), α6 integrin (1:500 for blotting, cat. #3750, Cell Signaling Technology), PDGFRα (1:1000 for blotting, SC-338, Santa Cruz Biotechnology), Olig2 (1:500, cat. #AB9610, Millipore) and Fyn (1:1000 for blotting, SC-16, Santa Cruz Biotechnology). Primary mouse antibodies were against: β-actin clone AC15 (1:5000 for blotting, cat. #A5441, Sigma-Aldrich), CNP (1:1000 for blotting, cat. #C5922, Sigma-Aldrich), Fyn (1:200 for immunoprecipitation, cat. #610164, BD Transduction Labs); α-DG (1:1000 for blotting, 1:50 for FACS; cat. #05-593, Millipore), β-DG (1:1000 for blotting; cat. #SC-33702, Santa Cruz Biotechnology), β1 integrin (1:500 for blotting; cat. #I41720, BD Transduction Labs), α6β1 integrin (1:50 for FACS, cat. #MAB1410, Millipore) and anti-Sox10 (1:500 for immunofluorescence staining, cat. #MAB2864, R&D Systems). Other antibodies included rat anti-MBP (1:500 for immunofluorescence staining, cat. #MAB386, Chemicon) and goat anti-PDGFRα (1:200 for immunofluorescence staining, cat. #AF1062, R&D Systems).

Primary mouse neural stem cell-derived OPC cultures

Animal care and use followed the guidelines of The University of British Columbia (UBC) and the Canadian Council on Animal Care, and were reviewed and approved by UBC. PTPα-null mice (Ponniah et al., 1999) were backcrossed with C57BL/6 mice for ten generations. Heterozygous PTPα$^{+/-}$ C57/BL6 mice were bred to generate PTPα$^{+/+}$, PTPα$^{+/-}$ and PTPα$^{-/-}$ offspring. Homozygous breeding of PTPα$^{+/+}$ or PTPα$^{-/-}$ mice produced pups of a specific genotype as sources for the isolation of WT or PTPα KO neural stem cells and derivation of neurospheres and oligospheres as previously described (Shih et al., 2017).

OPC differentiation and inhibitor treatments

To differentiate OPCs, oligospheres (passage 1–4) were dissociated with Accutase and seeded at 4×10^4–5×10^4 cells/cm^2 in OPC proliferation medium on chamber slides or dishes coated with poly-D-lysine (PDL; 10 µg/ml, Sigma-Aldrich) with or without Lm2 (merosin) (10 µg/ml, Millipore). The dissociated OPCs were maintained in OPC proliferation medium for 5 days and then were cultured in differentiation medium [neural culture medium (DMEM/F12 from Hyclone, and B27 supplement, 1 mM L-glutamax, 1 mM sodium pyruvate; all from Life Technologies) containing 10 ng/ml ciliary neurotrophic factor (CNTF, Peprotech), 5 µg/ml N-acetyl-L-cysteine, and 50 nM triiodothyronine] for 5 days.

The SFK inhibitor SU6656 (Calbiochem), Akt1/2 inhibitor (Sigma-Aldrich) and mTOR inhibitor Torin (Cell Signaling Technology) were prepared in dimethylsulfoxide. WT OPCs were treated with 1 µM of SU6656, 120 nM of Akt1/2 inhibitor or 10 nM of Torin while differentiating for 5 days, prior to harvesting and preparation of cell lysates. The differentiation media containing the inhibitor or vehicle solution were changed every 2 days.

Lm2 receptor function-blocking experiments were performed with differentiating WT OPC cultures. The mouse monoclonal IgM against αDG (clone IIH6, 10 µg/ml, cat. #05-593, Millipore) was desalted by centrifugation (Amicon® Ultra-0.5 Filter Device, Millipore) to remove sodium azide prior to treatment. The Ha2/5 hamster monoclonal IgM antibody (10 µg/ml, cat. #555002, BD Pharmingen) was used to block β1 integrin. A non-specific hamster IgM antibody was used as a treatment control (cat. #553958, BD Pharmingen). Differentiation media containing fresh antibodies were

Immunohistochemistry and fluorescent microscopy

Primary OPCs were washed once with ice-cold PBS and fixed in 4% paraformaldehyde for 15 min. The fixed cells were then washed with PBS, permeabilized in PBS with 0.2% Triton X-100 (PBS-Tx) for 15 min, and blocked in 10% normal goat serum for 1 h at room temperature. Primary antibodies were diluted in blocking solution and incubated at 4°C overnight followed by three washes in PBS-Tx and incubation with the appropriate fluorophore-conjugated secondary antibodies. Stained cells were visualized using a Leica SP5 confocal microscope.

Immunoprecipitation

WT and KO cultures differentiated for 5 days on Lm2 were washed in PBS and incubated with the cross-linker dithiobis-succinimidyl propionate (Sigma, 2 mM in PBS) for 15 min, all at room temperature. The cells were washed once with ice-cold PBS and lysed in NP-40 lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 10 µg/ml aprotinin and 10 µg/ml leupeptin). The lysates were pre-cleared with Sepharose A/G beads followed by rotating incubation with anti-PTPα antibodies overnight at 4°C. Sepharose A/G beads were added and incubated at 4°C for 2 h with rotation. Beads were washed three times in lysis buffer and resuspended in 2× SDS-PAGE sample buffer. Samples were boiled for 5 min and resolved on 8% Tris-glycine gels.

Immunoblotting

Cells were washed twice with ice-cold PBS and lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 2 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM PMSF, 10 µg/ml aprotinin, and 10 µg/ml leupeptin. The cell lysates were transferred to microtubes and incubated for 30 min on ice, and centrifuged at 13,500 rpm for 15 min at 4°C. Proteins (25-50 µg cell lysate) were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane, which was then blocked for 1 h at room temperature in Odyssey blocking buffer diluted 1:1 with PBS. The membranes were probed overnight at 4°C with the relevant primary antibodies followed by washing three times with 0.1% Tween in PBS (PBST), and re-probing with species-specific secondary antibodies conjugated with fluorescent dyes. After a final three washes with PBST, the membranes were scanned and quantified using an Odyssey fluorescent scanner (LICOR Biosciences).

Fyn kinase assay

Fyn immunoprecipitates from OPC/OL cultures were assayed for kinase activity using the ELISA-based Universal Tyrosine Kinase Assay Kit (GenWay, San Diego, CA) according to the manufacturer's instructions.

Fluorescence-activated cell sorting analysis

Cells were detached with Accutase and re-suspended in FBS-containing PBS. Equal numbers of cells were stained for either α-DG or α6β1 surface expression under non-permeabilization conditions followed by fluorescently labeled secondary antibodies. Stained cells were analyzed using the BD Accuri C6 flow cytometer.

Statistical analyses

Data are presented as mean±s.d. Statistical analyses were performed using the Student's *t*-test or one-way ANOVA with a Tukey's post hoc test. Differences at $P<0.05$ were considered significant. All analyses were performed using Prism software (GraphPad).

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Footnotes

- **Competing interests**

  The authors declare no competing or financial interests.

- **Author contributions**

  Conceptualization: P.T.T.L. , C.J.P.; Methodology: P.T.T.L. , C.J.P.; Validation: P.T.T.L. , C.S., C.J.P.; Formal analysis: P.T.T.L. ; Investigation: P.T.T.L. , C.S.; Resources: C.J.P.; Data curation: P.T.T.L. ; Writing - original draft: P.T.T.L. , C.J.P.; Writing - review & editing: P.T.T.L. , C.J.P.; Visualization: P.T.T.L. , C.J.P.; Supervision: P.T.T.L. , C.J.P.; Project administration: P.T.T.L. , C.J.P.; Funding acquisition: C.J.P.

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- **Supplementary information**

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- Top
- Article
  - ABSTRACT
  - INTRODUCTION
  - RESULTS
  - DISCUSSION
  - MATERIALS AND METHODS
  - Acknowledgements
  - Footnotes
  - References
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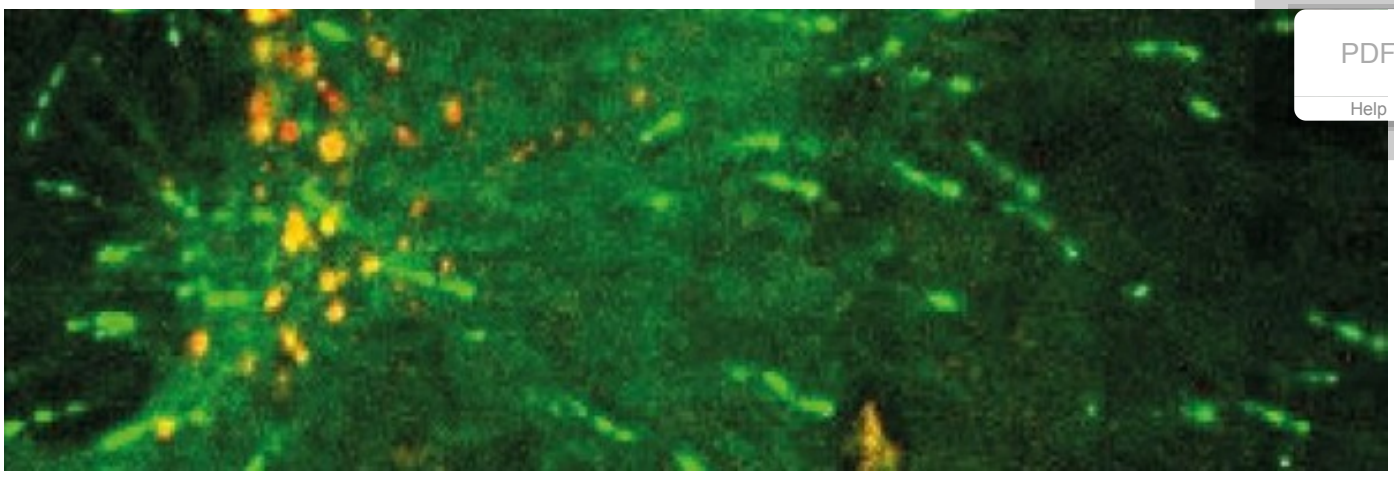

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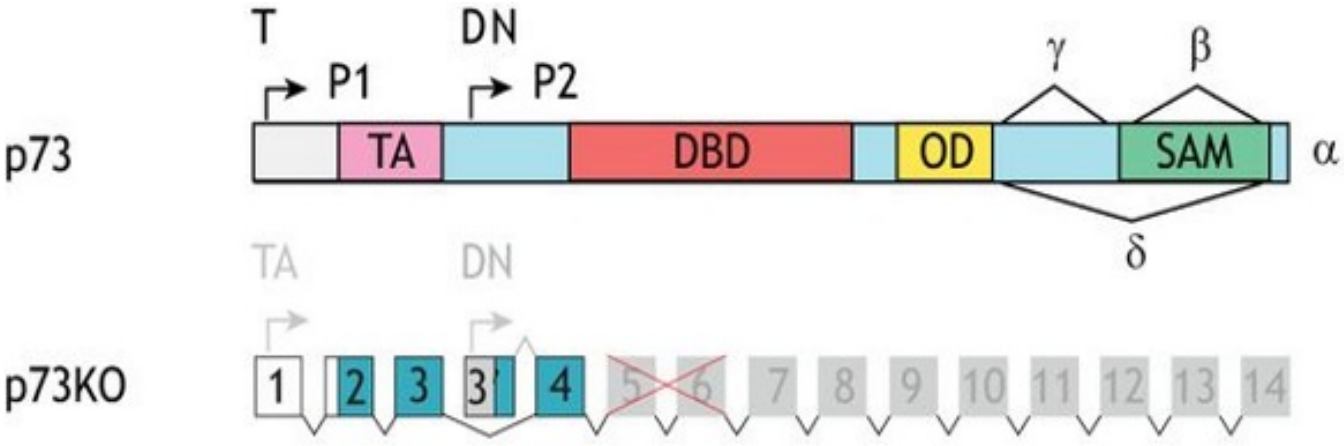

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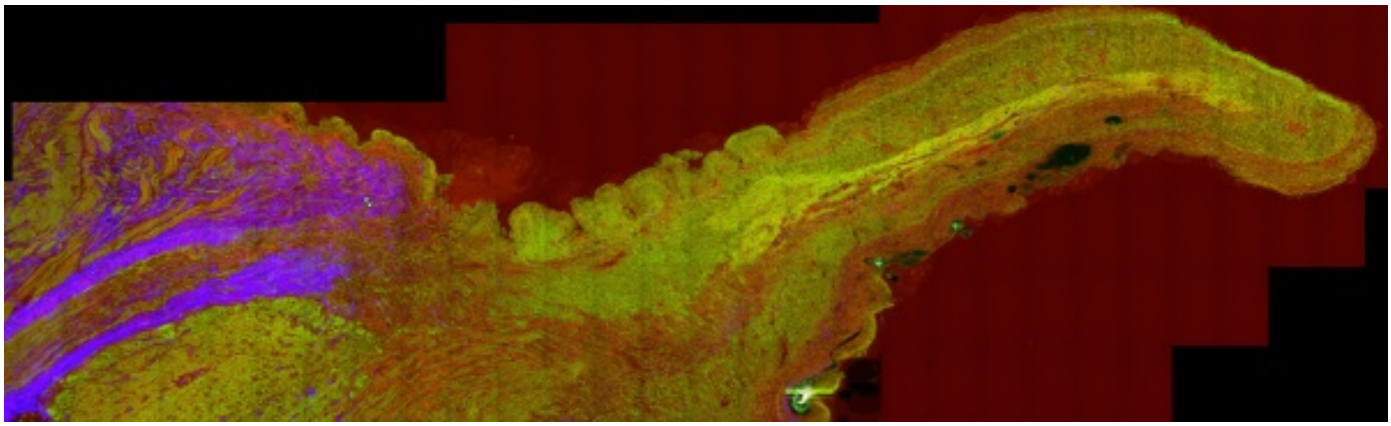

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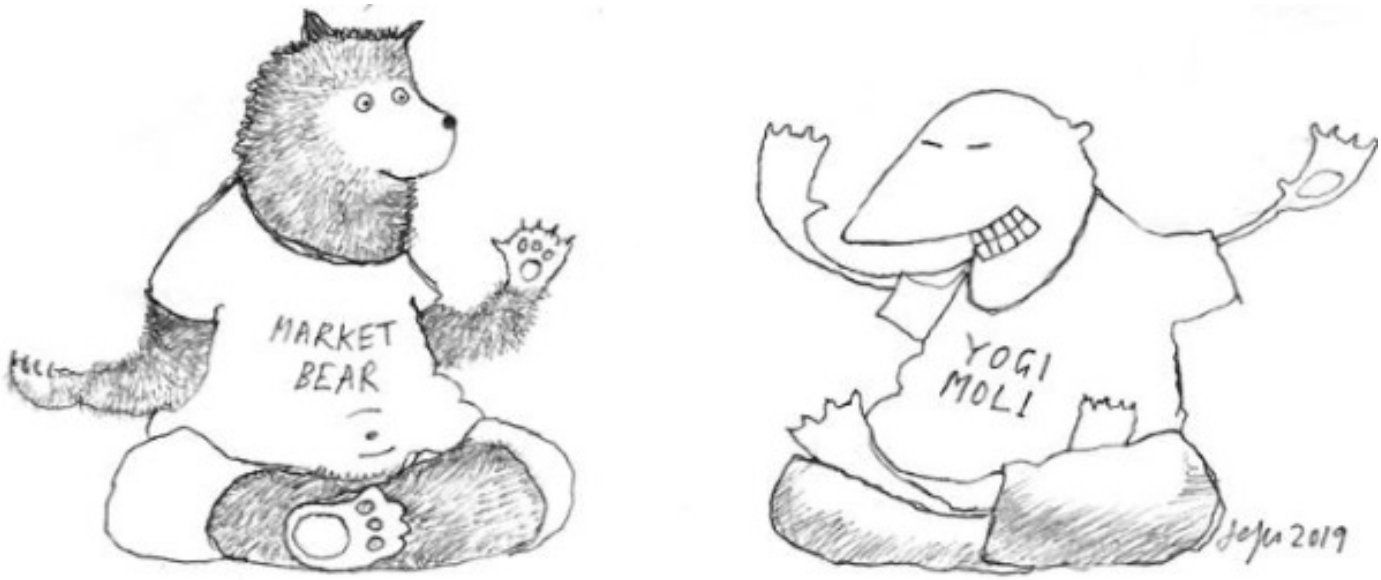

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