

**Integrin-linked kinase functions as a cytoskeletal scaffold in oligodendrocyte migration, differentiation and central nervous system myelination**

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## **AUTHORIZATION**

All previously published content presented herein was obtained from articles where Ryan O'Meara was the primary author with the exception of Figure 1.2, which was obtained from:

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## ABSTRACT

In the central nervous system (CNS), oligodendrocytes (OLs) generate myelin to facilitate the rapid propagation of neuronal impulses. In multiple sclerosis (MS), chronic demyelination leads to irreversible neurodegeneration that eventually impairs physical and cognitive function. Much effort is directed at elucidating the mechanisms underlying OL development in hope to unveil therapeutic targets for promoting remyelination in MS. Many aspects of OL biology are regulated by the integrins, a large family of transmembrane extracellular matrix (ECM) receptors. ECM components such as laminin and fibronectin bind to OL integrin receptors and initiate downstream signaling cascades involved in survival, proliferation, differentiation/myelination and migration. Integrin-linked kinase (ILK), an adaptor protein that binds to integrin cytosolic tails, works to stabilize the ECM-integrin connection by indirectly targeting the actin cytoskeleton to ECM adhesion sites. We hypothesized that ILK played an important role in OL migration, differentiation and capacity to myelinate neuronal projections. To address this hypothesis, we developed three cell culture techniques to assess these cellular phenomena *in vitro*. Conditional knockout of *Ilk* compromised both the morphological and molecular differentiation of primary mouse OLs *in vitro*, and reduced their capacity to produce myelin-like membrane. ILK was required for proper OL ensheathment of neuronal extensions when co-cultured with primary neurons. Conditional ablation of *Ilk in vivo* produced a transient amyelination defect that was endogenously compensated for at later time points. Loss of ILK in primary OLs was associated with upregulated RhoA signaling, and pharmacological inhibition of the RhoA axis restored the morphology of a distinct subset of NG2<sup>+</sup> OPCs. ILK depletion in OL precursor cells (OPCs) resulted in a

substrate-dependent defect in migration velocity and migration initiation. Inhibition of the RhoA signaling pathway enhanced the migratory velocity of wild-type OPCs, an effect that was dependent on ILK expression. In sum, we established three primary mouse OL cell culture techniques, with which we defined roles for ILK in OL biology. Our work highlights the importance of integrin signaling in OLs and provides new experimental methods useful in MS research.

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## LIST OF ABBREVIATIONS

|                   |                                                         |
|-------------------|---------------------------------------------------------|
| An-1              | Anosmin-1                                               |
| ANOVA             | Analysis of variance                                    |
| ARD               | Ankyrin repeat domain                                   |
| Arp2/3            | Actin-related proteins 2 and 3                          |
| ATP               | Adenosine triphosphate                                  |
| BSA               | Bovine serum albumin                                    |
| BrdU              | 5-bromo-2'-deoxyuridine                                 |
| CCAC              | Canadian council of animal care                         |
| Cdc42             | Cell division control protein 42 homolog                |
| CG4               | Central glia-4                                          |
| CH                | Calponin homology                                       |
| cKO               | conditional knockout                                    |
| CNP               | 2',3'-cyclic nucleotide 3-phosphodiesterase             |
| CNS               | Central nervous system                                  |
| Cre               | Cre recombinase                                         |
| CSPG              | Chondroitin sulfate proteoglycan                        |
| CNTF              | Ciliary neurotrophic factor                             |
| CXCL1             | Chemokine (C-X-C motif) ligand 1                        |
| CXCR2             | Chemokine (C-X-C motif) receptor 2                      |
| DAPI              | 4',6-diamidino-2-phenylindole                           |
| DCC               | Deleted in colorectal cancer                            |
| dH <sub>2</sub> O | Distilled water                                         |
| DIV               | Day <i>in vitro</i>                                     |
| DMEM              | Dulbecco's modified eagle medium                        |
| DNA               | Deoxyribonucleic acid                                   |
| DRG               | Dorsal root ganglion                                    |
| DRGN              | Dorsal root ganglion neuron                             |
| ECM               | Extracellular matrix                                    |
| EGF               | Epidermal growth factor                                 |
| EGFP              | Enhanced green fluorescent protein                      |
| EGFR              | EGF receptor                                            |
| ERK               | Extracellular regulated kinase                          |
| ER <sup>T</sup>   | Ligand-binding domain mutated human estrogen receptor   |
| F-actin           | Filamentous actin                                       |
| FAK               | Focal adhesion kinase                                   |
| FBS               | Fetal bovine serum                                      |
| FGF/bFGF          | Fibroblast growth factor/basic fibroblast growth factor |
| fl                | Floxed                                                  |
| FOV               | Field of view                                           |
| Fn                | Fibronectin                                             |
| FuDR              | 5'-Fluoro-2'-deoxyuridine                               |
| g                 | Gravity                                                 |
| GAP               | GTPase-activating protein                               |
| GAPDH             | Glyceraldehyde 3-phosphate dehydrogenase                |



|                   |                                                                                  |
|-------------------|----------------------------------------------------------------------------------|
| GF                | Growth factor                                                                    |
| GFP               | Green fluorescent protein                                                        |
| GS                | Goat serum                                                                       |
| GSK3/GSK3 $\beta$ | Glycogen synthase kinase 3/ Glycogen synthase kinase 3 $\beta$                   |
| GTP               | Guanosine triphosphate                                                           |
| h                 | hour                                                                             |
| HBSS              | Hank's buffered saline solution                                                  |
| HRP               | Horseradish peroxidase                                                           |
| ILK               | Integrin-linked kinase                                                           |
| KD                | Kinase domain                                                                    |
| LINGO-1           | Leucine rich repeat and Ig domain containing NOGO receptor interacting protein 1 |
| Ln                | Laminin                                                                          |
| Ln-2/LN2          | Laminin-2                                                                        |
| MAG               | Myelin associated glycoprotein                                                   |
| MBP               | Myelin basic protein                                                             |
| MEM               | Minimum essential media                                                          |
| min               | Minute(s)                                                                        |
| mRNA              | messenger RNA                                                                    |
| MS                | Multiple Sclerosis                                                               |
| Myrf              | Myelin regulatory factor                                                         |
| NCAM              | Neural cell adhesion molecule                                                    |
| NG2               | NG2 chondroitin sulfate proteoglycan                                             |
| NGF               | Nerve growth factor                                                              |
| NF                | Neurofilament 200                                                                |
| NRG1              | Neuregulin-1                                                                     |
| N-WASP            | Neuronal Wiskott-Aldrich Syndrome protein                                        |
| OL                | Oligodendrocyte/Oligodendrocyte-lineage cell                                     |
| OPC               | Oligodendrocyte precursor cell                                                   |
| P(#)              | Post-natal day (#)                                                               |
| PBS               | Phosphate-buffered saline                                                        |
| PCR               | Polymerase chain reaction                                                        |
| PDL               | Poly-D-lysine                                                                    |
| PDVF              | Polyvinylidene fluoride                                                          |
| PDGF              | Platelet-derived growth factor                                                   |
| PDGFR- $\alpha$   | PDGF receptor- $\alpha$                                                          |
| Pen/Strep         | Penicillin/Streptomycin                                                          |
| PFA               | Paraformaldehyde                                                                 |
| PINCH             | Particularly interesting new cysteine-histidine rich protein                     |
| PLL               | Poly-L-lysine                                                                    |
| PLP               | Proteolipid protein                                                              |
| PMSF              | Phenylmethylsulfonyl fluoride                                                    |
| PPMS              | Primary progressive MS                                                           |
| PSA               | Polysialic acid                                                                  |
| pY397             | Phosphorylated tyrosine 397                                                      |
| p-Ser9            | Phospho-Serine 9                                                                 |

|          |                                                                                                |
|----------|------------------------------------------------------------------------------------------------|
| p-Ser473 | Phospho-Serine 473                                                                             |
| Rac1     | Ras-related C3 botulinum toxin substrate 1                                                     |
| RGD      | Arginine-Glycine-Aspartic acid                                                                 |
| RhoA     | Ras homolog gene family member A                                                               |
| RNAi     | Ribonucleic acid interference                                                                  |
| ROCK     | Rho kinase                                                                                     |
| rpm      | Revolutions per minute                                                                         |
| RRMS     | Relapsing remitting MS                                                                         |
| RT       | Room temperature                                                                               |
| SDS      | Sodium dodecyl sulfate                                                                         |
| SDS-PAGE | SDS-polyacrylamide gel electrophoresis                                                         |
| SEM      | Standard error of the mean                                                                     |
| SFK      | Src-family non-receptor tyrosine kinase                                                        |
| Shh      | Sonic Hedgehog                                                                                 |
| Sox10    | SRY-box containing gene 10                                                                     |
| SPMS     | Secondary progressive MS                                                                       |
| TAT-Cre  | His-TAT (trans-activator of transcription)-NLS (nuclear localization sequence)-Cre recombinase |
| TF       | Transcription factor                                                                           |
| Tn       | Tenascin                                                                                       |
| VEGF     | Vascular endothelial growth factor                                                             |
| Vn       | Vitronectin                                                                                    |
| WAVES    | Wiskott-Aldrich syndrome protein family verprolin-homologous proteins                          |
| WT       | Wild type                                                                                      |

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# **Chapter 1**

## **General Introduction**

## **Multiple Sclerosis**

Multiple Sclerosis (MS) is the most prevalent neurological disease affecting young adults in Canada. It tends to strike in early adulthood, and preferentially affects women over men in a ratio of 3:1 (Orton et al., 2006). Repeated attacks to the central nervous system (CNS) white matter leads to demyelination of fiber tracts, culminating in both physical and cognitive disability. While the brain has capacity to remyelinate, in chronic MS, the regenerative capacity is diminished and neurological deficits accumulate over time. While much advance has been made in the diagnosis and treatment over the last several decades, many facets of MS etiology and disease process remain enigmatic.

As the clinical signs/symptoms of MS are heterogeneous in nature, diagnosis is often not straightforward. Generally, MS first manifests as a transient episode of neurological impairment (*e.g.*, localized limb numbness or weakness) that can last days to months. Once the initial attack subsides, compromised function is most commonly regained. At this stage patients are sometimes classified as “probable MS”, with sure diagnosis confirmed at the occurrence of a second attack separated in time and space (*i.e.*, location of complaint/pathology). White matter lesions, oligoclonal banding in the cerebral spinal fluid and altered neurological function/perception are some examples of MS diagnostic criteria. Approximately 80% of MS patients will be initially diagnosed with relapsing remitting MS (RRMS), characterized by repeated attacks separated by periods of remission. Normally, no major dysfunction accumulates during this phase of the disease. After 10-30 years, approximately 60% of RRMS cases will convert to secondary progressive MS (SPMS; Wingerchuk and Carter, 2014), which is typified by gradual and consistent neurological impairment with no significant recovery. Some 10%

of MS cases will be the primary progressive type (PPMS), with continuous decline occurring from disease onset with no periods of remission.

### **Risk factors associated with Multiple Sclerosis**

The exact etiology of MS is unknown, but several risk factors have been identified. The positive correlation between MS prevalence and global latitude demonstrates geographical residence is a disease risk modifier (Simpson et al., 2011). As higher latitudes receive less direct sunlight than equatorial regions, this correlation is thought to be a function of vitamin D intake. Accordingly, low serum levels of 25-hydroxyvitamin D heighten MS risk in certain populations (Munger et al., 2006) and also predict poor MS prognosis (Ascherio et al., 2014). Interestingly, the risk of developing MS is higher for those born in May versus November (Willer et al., 2005), possibly resulting from reduced ultraviolet radiation exposure during winter pregnancies. Accordingly, May-born babies have lower serum 25-hydroxyvitamin D levels than November-born babies (Disanto et al., 2013), a possible explanation for this birth-month phenomenon. Certain viral infections and smoking habits are additional environmental factors known to impact MS risk (Ramagopalan and Sadovnick, 2011).

There is also an undeniable genetic component that modifies an individual's risk for developing MS over his/her lifetime. Having a first degree relative with MS significantly heightens the chance of disease acquisition (Nielsen et al., 2005), but coincidence among identical twins is roughly 18% (O'Gorman et al., 2013; Westerlind et al., 2014), indicating genes do not seal your fate. Genome-wide association studies have identified/confirmed 110 genetic variants that elevate MS risk (Sawcer et al., 2011;

Beecham et al., 2013), the majority of which are linked to the immune system. No single mutation is sufficient to cause the disease – an individual's risk is rather a function of their susceptibility variant burden (Gourraud et al., 2012) modified by environmental input.

### **Pathological features of Multiple Sclerosis**

CNS white matter focal demyelinated lesions are the pathological hallmark of MS, their development unequivocally mediated by the immune system. The initial step in lesion formation is the cryptic transit of circulating immune cells across the blood brain barrier into the brain parenchyma. Once penetrated, immune cells release cytokines and chemokines that act to propagate an immune response (Wu and Alvarez, 2011). This can cause oligodendrocytes (OLs) to express surface proteins that are recognized by immune cells, and thereby rendering themselves targets for attack (Patel and Balabanov, 2012). Immune cells can also indirectly damage OLs via release of reactive oxygen and nitrogen species (Patel and Balabanov, 2012), to which OLs are especially sensitive (Witherick et al., 2010). Antibody targeting of myelin also results in its degradation via complement aggregation and subsequent phagocytosis by macrophages (Brück, 2005). Paradoxically, some aspects of the immune response promote remyelination in early MS, but this reparative capacity diminishes with disease progression.

Myelin provides trophic support to neurons, and its disturbance can lead to axonal degeneration (Griffiths et al., 1998). Myelin's insulating property enhances transmission of neuronal impulses, demyelination thereby slowing axonal conductance (Rasminsky and Sears, 1972; Bostock and Sears, 1978). In MS lesions, sodium channels (normally



segregated at the nodes of Ranvier) are diffusely distributed along demyelinated axons (Craner et al., 2004), a phenomenon thought to contribute to reduced axonal conductance. Altered sodium flux can also overburden energy-dependent  $\text{Na}^+/\text{K}^+$  ATPase pumps (which maintain neuronal membrane potential), intact myelin negating this imposition by virtue of its insulating character (Trapp and Stys, 2009). Loss of myelin in MS lesions can therefore impose heightened energy demands on neurons, possibly further exacerbated by immune cell-mediated release of nitric oxide which interferes with ATP synthesis (Brorson et al., 1999; Smith and Lassmann, 2002; Nave, 2010). Lesion-associated immune cells are also thought to directly transect/damage demyelinated axons (Trapp et al., 1998; Medana et al., 2001; Sobottka et al., 2009), leading to degeneration. Collectively, neuronal death via these mechanisms is thought to underlie the permanent neurological decline seen in chronic MS.

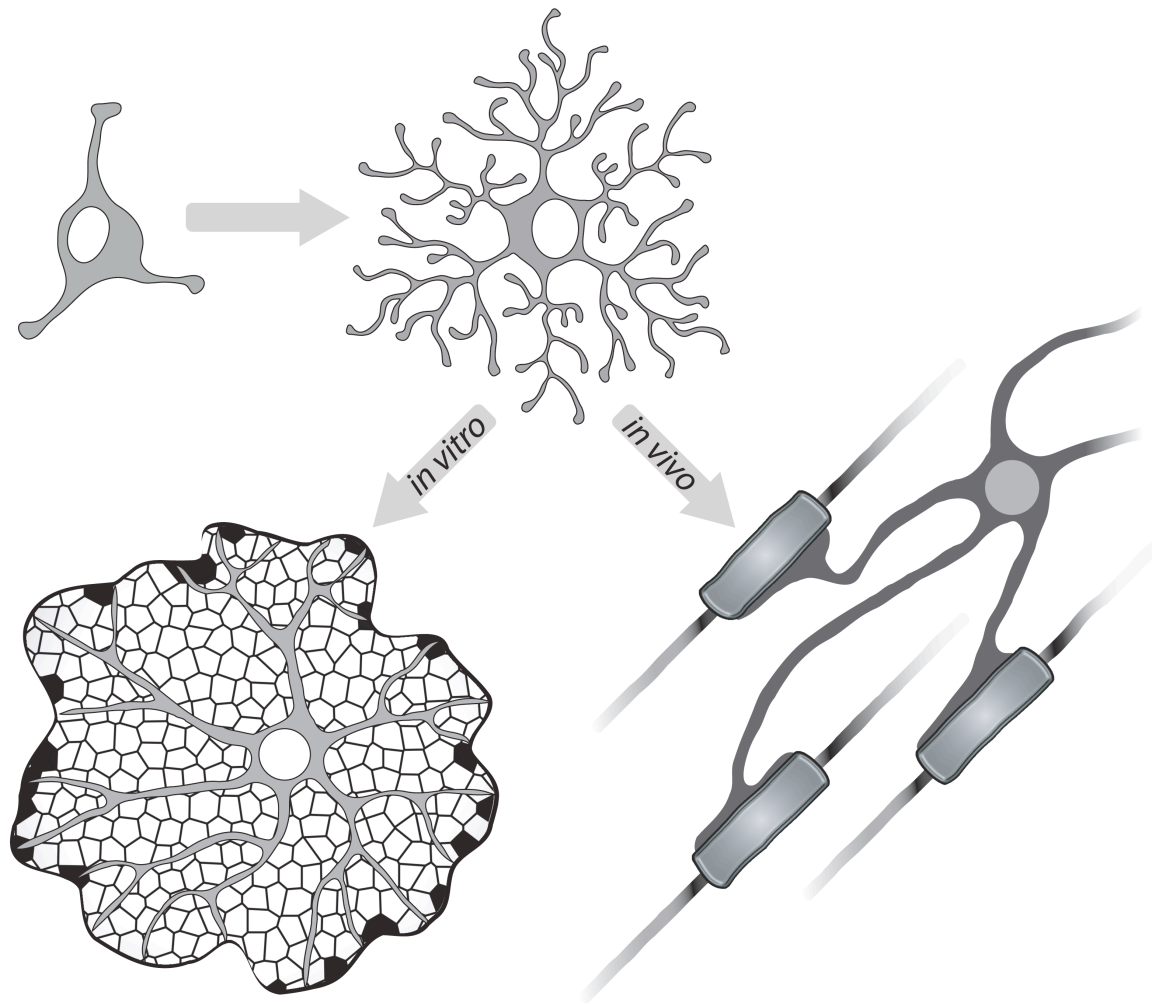
### **Therapeutic intervention in Multiple Sclerosis**

Several therapies useful for reducing the frequency and severity of relapses are currently available to MS patients (Goldenberg, 2012). As early stages of the disease are inflammation-mediated, existing drugs primarily act to suppress/modulate the immune system. They are less effective in later stages of the disease, where inflammatory demyelination subsides and progressive neurodegeneration drives neurological incapacitation. As such, ongoing research efforts are aimed at slowing/stopping disease progression and encouraging repair of injured tissue (Wingerchuk and Carter, 2014). One candidate approach for restoring axonal function and protecting against further damage is to promote remyelination by endogenous oligodendrocyte precursor cells (OPCs).

Notably, premyelinating OLs are present in the majority of chronic MS lesions, though they appear stalled in their ability to fully remyelinate denuded axons (Chang et al., 2002). OPCs also accumulate peripheral to early MS lesions (Kuhlmann et al., 2008), likely due to lesion-associated inhibitory factors that preclude their infiltration. These observations collectively suggest the lesion environment hinders OL migration and differentiation, consequently curtailing repair. Investigating OL signaling pathways that govern differentiation and migration is therefore a logical first step towards the ultimate goal of expediting remyelination in MS.

### **Oligodendrocytes**

OLs are responsible for generating myelin, a lipid-rich structure that envelops CNS axons allowing for saltatory conduction of action potentials. The notion of OLs being a homogeneous cell type was long-held, originating from a discrete region of the developing CNS and subsequently migrating extensively to populate the entire brain. It is now apparent however, that OPCs arise from several periventricular foci along the rostral-caudal axis of the developing neural tube (de Castro and Zalc, 2013). Newborn OPCs undergo a transient period of local proliferation followed by migration (Noll and Miller, 1993), their direction and extent governed by various patterning molecules. Once reached their final destination, OPCs differentiate by extending numerous processes that contact and wrap adjacent axons with multiple layers of lipid-rich myelin (Figure 1.1). The process of myelination is therefore dependent on both the capacity of OPCs to migrate and initiate differentiation.



**Figure 1.1. The morphological changes OLs undergo *in vitro* and *in vivo* during differentiation and myelination.** OPCs originate as cells with simple morphology, and as they differentiate, extend a complex network of branches. Once these processes contact axons *in vivo*, the OL initiates a myelinating event that involves the wrapping of the axon with multiple layers of membrane. In the 2-dimensional *in vitro* environment, the *in vivo* equivalent of myelinating OLs are thought to be those that exude MBP<sup>+</sup> myelin-like membrane between the gaps of the intricate arbor network.

### **Soluble cues in oligodendrocyte migration**

Over the past few decades, there has been much advance in the understanding of what factors direct OPC migration. Soluble signaling molecules can influence OPC migration by controlling the direction and/or speed of their migration. Ultimately, compounds that impart either attractive or repulsive cues concertedly act to precisely position OPCs prior to differentiation onset.

Arguably, the best-characterized soluble molecules that influence OPC migration are platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF). These proteins promote OPC migration in the direction of their increasing concentration gradient and migration velocity when uniformly distributed in solution (Noble et al., 1988; Armstrong et al., 1990; Milner et al., 1997; Zhang et al., 2004). In PDGF knockout mice, CNS regions more distant from periventricular OPC birth sites are hypomyelinated, consistent with the pro-migratory effect of PDGF (Fruttiger et al., 1999). Similarly, OPCs expressing a defective FGF receptor are impaired in their ability to infiltrate host tissue when transplanted into the brain of neonatal rats (Osterhout et al., 1997). Other growth factor signaling axes, such as those governed by vascular endothelial growth factor (VEGF) and the epidermal growth factor receptor (EGFR) are also known to enhance OPC migration (Ivkovic et al., 2008; Hayakawa et al., 2011).

The netrins and semaphorins, compounds originally identified as having roles in axonal guidance (Kennedy et al., 1994; Koncina et al., 2007), also control OPC motility. OPCs express the Deleted in Colorectal Cancer (DCC) receptor through which soluble Netrin-1 mediates a repulsive action *in vitro* (Jarjour et al., 2003; Bin et al., 2013), via upregulated RhoA signaling (Rajasekharan et al., 2010). A role for migration is also

evident *in vivo*, as knockout of either DCC or Netrin-1 decreases OPC dispersion in the developing spinal cord (Jarjour et al., 2003). Secreted factors semaphorin 3A and 3F, which bind to neuropilin receptors expressed by OLs (Cohen et al., 2003), have repulsive and attractive influences on OPCs respectively (Spassky et al., 2002; Piaton et al., 2011). Overexpression of semaphorin 3F in demyelinated lesions increases OPC infiltration, ultimately enhancing remyelination (Piaton et al., 2011). In contrast, OPC recruitment is reduced in lesions expressing semaphorin 3A (Piaton et al., 2011), altogether offering conflicting roles for these semaphorins in OPC migration.

Additional proteins including sonic hedgehog (Shh) and chemokine (C-X-C motif) ligand 1 and receptor 2 complex (CXCL1/CXCR2) can also modulate OPC migration. Shh, a morphogen with major roles in development, acts as an attractant for OPCs by enhancing their migration out of optic nerve explant cultures (Merchán et al., 2007; Ortega et al., 2012). CXCR2, the receptor for the diffusible CXCL1 ligand, is expressed by OPCs and works to negate the pro-migratory effect of PDGF (Tsai et al., 2002). In the developing spinal cord of CXCR2<sup>-/-</sup> mice, OPCs are distributed more dorsally than ventrally, consistent with lost sensitivity to the stop signal imparted by CXCL1 (Tsai et al., 2002). The above studies illustrate that soluble molecules collectively regulate various aspects of OPC migration to facilitate their distribution throughout the CNS. This migratory process is also influenced by immobilized guidance cues (in contrast to diffusible ones) that will be discussed in the following section.

### **Immobilized cues in oligodendrocyte migration**

OPC positioning is also controlled by immobilized stimuli, signals that arise through physical interactions between adjacent cells or conferred by the extracellular matrix (ECM). Together with soluble cues, bound ligands aid to disperse OPCs both prior to myelination and during reparative processes in the disease context.

Cell-cell contact can influence OPC migration by way of membrane-tethered protein interactions. This is exemplified by neural cell adhesion molecule (NCAM), a cell surface protein that homodimerizes with other NCAM molecules on neighbouring cells. NCAM can be post-translationally modified by addition of polysialic acid (PSA), this PSA-NCAM variant being strongly expressed at early stages of OPC development (Decker et al., 2000). PSA reduces NCAM's homodimerization capacity (Bonfanti, 2006), consequently lessening cellular adhesion. Depletion of PSA from NCAM-expressing OPCs reduces FGF and PDGF-driven migration (Decker et al., 2000; Zhang et al., 2004), as NCAM-mediated adhesion is consistent with lower migratory potential. Similarly *N*-Cadherin, which also forms homodimers with molecules on neighbouring cells, modulates the migration of immortalized Central glia-4 (CG4) OPCs (Schnädelbach et al., 2000). Blockade of *N*-Cadherin increases the migration of CG4 cells cultured on astrocytes, as the adhesive *N*-Cadherin interaction between both cell types is not conducive to CG4 migration. Cell-cell interactions are also mediated by receptor/ligand interactions illustrated by ephrin-expressing OPCs and Eph receptor-expressing retinal ganglion neuron axons in the ON. EphB receptor ligation by ephrinB imposes an inhibitory action on OPC migration (Prestoz et al., 2004), providing a

mechanism for control of OPC localization along the optic nerve prior to the onset of myelination.

OPC migration can also be governed by signals derived from the ECM, a 3-dimensional skeleton constructed of numerous adhesive proteins. The CNS ECM was traditionally viewed as a scaffold whose major function was to lend structural support to neural cells, however its roles in diverse functions including cell proliferation, differentiation and survival are being increasingly recognized (Colognato and Tzvetanova, 2011). During development, the ECM guides OPCs to their final destination via the reciprocal action of stimulatory and inhibitory motility cues. For example, a Tenascin-C (Tn-C) matrix imposes an inhibitory action on migrating OPCs *in vitro* (Frost et al., 1996; Kiernan et al., 1996), and negatively controls OPC migration rate along the optic nerve *in vivo* (Garcion et al., 2001). A similar effect is provided by the ECM protein Anosmin-1 (An-1), providing an inhibitory cue for OPC migration *in vitro* (Bribián et al., 2008). Since An-1 and Tn-C are expressed in the optic nerve (Bartsch et al., 1994; Bribián et al., 2006, 2008), they may act to oppose pro-migratory cues (such as Semaphorin 3F; Spassky et al., 2002), allowing precise control of OPC distribution along the length of the nerve. The ECM can also undergo remodeling as a consequence of pathology, exemplified by the deposition of chondroitin sulfate proteoglycans (CSPGs) upon CNS insult (Lau et al., 2013). CSPG accumulation is thought to preclude OPC-mediated repair of spinal cord injury by hindering migration, as enzymatic CSPG depletion enhances OPC recruitment into the lesion (Siebert et al., 2011).

Other ECM proteins such as Laminin (Ln), Fibronectin (Fn) and Vitronectin (Vn) transmit positive motility cues to OPCs. When cell culture surfaces are coated with these

matrices, OPC migration is enhanced over non-ECM control conditions (Frost et al., 1996; Milner et al., 1996; Schmidt et al., 1997; Niehaus et al., 1999). Ln appears to be especially mobilizing, as OPCs migrate roughly three times faster on Ln matrix as compared to on polylysine (a non-specific substrate) occasionally reaching speeds of over 120  $\mu\text{m}/\text{hour}$  (Schmidt et al., 1997). OPC migration on Ln, Fn and Vn operates in part via the integrins, a family of transmembrane ECM receptors (Milner et al., 1996). OL-lineage cells dynamically express the  $\alpha_v\beta_1$ ,  $\alpha_v\beta_3$ ,  $\alpha_v\beta_5$ ,  $\alpha_v\beta_8$  and  $\alpha_6\beta_1$  integrin receptors during development (Milner and Ffrench-Constant, 1994), each uniquely contributing to various aspects of OL biology. The migratory response of OPCs to Ln, Fn and Vn is likely mediated by distinct integrin receptors, as Ln and Fn-directed migration relies on  $\alpha_6\beta_1$  and  $\alpha_v\beta_3$  integrins respectively (Gudz et al., 2006). Of note, Ln is expressed in myelinating axon tracts (Colognato et al., 2002) and is upregulated after experimentally induced demyelination (Zhao et al., 2009), possibly guiding OPCs to sites of myelination/remyelination. In sum, the coordinated contribution of inhibitory/permissive cues presented by the ECM and neighboring cells governs OPC dispersal throughout the CNS to regulate both myelination and remyelination.

## **Myelin**

Myelin is a fundamental component of CNS axons that permits rapid communication between neurons. It is composed of concentric wrappings of lipid-rich OL membrane mostly devoid of cytoplasm and stabilized by myelin structural proteins (Snaidero et al., 2014). This fatty layer provides trophic support (Fünfschilling et al., 2012) and protection from noxious stimuli (Nave, 2010), while concurrently fulfilling its primary role as an



insulator that lowers the energy required for action potential propagation. OLs, the more developmentally mature counterpart of OPCs, generate myelin as they differentiate by extending multiple processes that contact adjacent axons (Fig 1.1). Once contacted, the OL process wraps the axon multiple times to form a myelin segment (internode), an individual OL capable of producing upwards of 50 internodes depending on CNS region (de Castro and Zalc, 2013). This segmented feature of myelin is what facilitates the saltatory mode of action potential conduction, where electrical charge “hops” across internodes over the length of an axon. Loss of myelin stemming from pathological circumstances can compromise the conductivity of axons (Rasminsky and Sears, 1972; Bostock and Sears, 1978), as well as render the underlying axon vulnerable to damage. As such, much effort has been invested in understanding what mechanisms regulate OL differentiation and myelination of CNS axons.

### **Oligodendrocyte differentiation**

OL-lineage cells undergo extensive molecular and morphological transformation while maturing from OPCs to myelinating OLs (Figure 1.1). Relative to their differentiated state, OPC morphology is rudimentary, cells bearing only a few processes. OPCs are highly motile, and can be identified by their expression of NG2 chondroitin sulfate proteoglycan (NG2), Olig2 and PDGF receptor- $\alpha$  (PDGFR- $\alpha$ ; Nishiyama et al., 2009). Differentiation involves process elongation and branching that renders the cell highly arborized, at which point the OL is relatively immotile. An OL possessing this morphology is generally thought of as intermediately differentiated, which coincides with the induction of myelin-associated glycoprotein (MAG) expression. Further maturation

leads to formation of membranous sheets that envelop axons *in vivo* to produce veritable myelin. At this stage, OLs are terminally differentiated, and express myelin basic protein (MBP) along with proteolipid protein (PLP). This morphological metamorphosis has been a primary research focus for several years, the molecular underpinnings of which are becoming increasingly known.

The machinery governing OL differentiation and myelin formation is initiated by intrinsic and extrinsic stimuli. Certain transcription factors (TFs) are considered intrinsic regulators of differentiation, whereas extrinsic stimuli include growth factors, hormones and receptor/ligand signaling. The following sections will provide a brief overview of some intrinsic and extrinsic mechanisms that control OL differentiation and subsequent CNS myelination.

### **Transcriptional regulation of oligodendrocyte maturation**

Certain TFs are viewed as intrinsic regulators of OL differentiation, Olig1 and Olig2 being prime examples (reviewed by Emery, 2010). Olig1/2 were discovered in a search for novel basic helix-loop-helix TFs (Lu et al., 2000; Zhou et al., 2000). These factors are highly expressed in CNS regions corresponding to OPC birth sites (Lu et al., 2000; Zhou et al., 2000) and play diverse regulatory roles in the OL-lineage (Mei et al., 2013). Olig2 knockout nullifies OPC generation in the spinal cord but not the brain (Lu et al., 2002), whereas double Olig1/2 knockout yields a CNS-wide ablation of OPC production, offering a region-specific compensatory function for Olig1 upon Olig2 loss (Lu et al., 2002; Zhou and Anderson, 2002). Olig1 also mediates remyelination (Arnett et al., 2004), but its role in developmental myelination is more controversial. Depending on the

knockout approach, *Olig1* loss produces either a severe neurological deficit paired with defective myelination (Xin et al., 2005) or only a mild delay in OL-lineage progression (Lu et al., 2002; Paes de Faria et al., 2014). Nonetheless, *Olig1/2* contribution to OL-lineage specification and CNS myelination is undeniable, functions they mediate in cooperation with other TFs.

Myelin regulatory factor (*Myrf*), SRY-box containing gene 10 (*Sox10*) and *Nkx2.2* are additional TFs that together with the *Oligs* constitute a transcriptional network that governs OL development. *Sox10* is glial-cell restricted (Kuhlbrodt et al., 1998) and its expression is driven by *Olig2* (Zhou et al., 2000) via direct binding to promoter regions (Küspert et al., 2011; Yu et al., 2013). *Sox10* regulates the expression of myelin genes such as *Mbp* and *Plp* (Stolt et al., 2002; Hornig et al., 2013) and its CNS-specific embryonic deletion bestows motor deficits upon juvenile mice, coupled with severe spinal cord hypomyelination (Hornig et al., 2013). *Sox10* can physically cooperate with other TFs such as *Myrf* to drive myelin gene expression (Li et al., 2007; Hornig et al., 2013), *Myrf* being another TF directly regulated by *Olig2* (Yu et al., 2013). Within myelin genes, *Myrf* occupancy sites highly overlap with those of *Sox10* and *Olig2* suggesting their membership to a transcriptional program that governs OL maturation (Bujalka et al., 2013). However, in contrast to *Olig2* and *Sox10*, *Myrf* expression is upregulated relatively late in OL development (Cahoy et al., 2008). This offers a preferential role for *Myrf* in myelin production over OL-lineage specification (Koenning et al., 2012), a theory backed by the fact that *Myrf* ablation arrests OLs at the premyelinating stage *in vivo* (Emery et al., 2009). Additionally, *Myrf* is required for myelin maintenance; its loss in adult mice produces a severe motor phenotype with

concomitant myelin gene downregulation and subsequent demyelination (Koenning et al., 2012). Nkx2.2, another member of the OL transcription regulatory network, can both positively and negatively regulate OL gene expression to encourage differentiation (Qi et al., 2001; Zhu et al., 2014). Ubiquitous knockout of Nkx2.2 delays the appearance of MBP and PLP-expressing cells in the embryonic spinal cord (Qi et al., 2001), culminating in early death and thereby hindering insight into the function of Nkx2.2 at later stages of myelination. To avoid this perinatal lethality, Zhu and colleagues (2014) used a CNP driver to restrict Nkx2.2 ablation to the OL-lineage, specifically at the precursor stage. These conditional Nkx2.2 knockout mice present with a transient delay in MBP and PLP expression, which is compensated for at post-natal day 21 (Zhu et al., 2014). This unique observation implies Nkx2.2 is not required for OPC maturation *per se*, but essential for timely initiation of differentiation (Zhu et al., 2014).

OPC differentiation is governed in part by cell-intrinsic mechanisms, numerous TFs being exemplary (Huang et al., 2013). This short section describes only the tip of the transcriptional iceberg that underlies OL differentiation, a topic worthy of a full review in its own right. The focus will now shift to the extrinsic cues that regulate OL differentiation and myelination of the CNS, specifically those derived from the ECM.

### **The ECM as an extrinsic modulator of oligodendrocyte development**

Historically regarded as an inanimate architectural framework for neural cells, the contribution of the ECM to CNS development and repair is becoming increasingly appreciated (Lau et al., 2013). The ECM provides a dynamic scaffold for the CNS parenchyma and participates in the control of cell migration, proliferation, survival and

differentiation. It is composed of adhesive proteins, proteoglycans and polysaccharides that bind cell surface receptors to modulate cellular behaviour through activation of signal transduction cascades. ECM-derived cues are viewed as extrinsic regulators of OL biology, their influence either promoting or deterring OL differentiation.

Several ECM molecules act to impede OL differentiation, the CSPGs being a prime example. In MS, high levels of CSPGs are present along the edges of active lesions (Sobel and Ahmed, 2001), such deposition thought to impede remyelination (reviewed by Lau et al., 2013; Harlow and Macklin, 2014), an idea backed by accumulating evidence. CSPG substrates impair OL process extension (Siebert and Osterhout, 2011; Lau et al., 2012; Pendleton et al., 2013), myelin-like membrane production (Siebert and Osterhout, 2011) and the capacity of OLs to myelinate neurons in co-culture (Pendleton et al., 2013). The plausibility that CSPGs may limit repair in disease is well illustrated by Lau and colleagues (2012) who not only observed CSPG deposition in experimentally induced demyelinated lesions, but demonstrated that blockade of their synthesis enhances remyelination.

Tn-C also imposes a negative influence on OPC differentiation, similar to its role in antagonizing OPC migration (Frost et al., 1996; Kiernan et al., 1996; Garcion et al., 2001). Tn-C substrates inhibit induction of MBP and reduce the formation of myelin-like membranes (Czopka et al., 2009, 2010). Juvenile Tn-C knockout animals present with increased numbers of MBP<sup>+</sup> OLs in the forebrain (Czopka et al., 2010) suggestive of accelerated OL differentiation. However, this elevation in mature OL numbers is not significant at later developmental stages (Czopka et al., 2010), and abnormal myelination is appropriately not seen in the adult Tn-C null mouse (Kiernan et al., 1999). Closely

related Tn-R also hinders OL morphological development, but paradoxically increases the proportion of MBP-expressing OLs when presented as a growth substrate (Pesheva et al., 1997; Czopka et al., 2009).

In contrast to the unambiguous impact of CSPGs and Tns on OL maturation, the function of Fn in this process is more convoluted. Early work suggested Fn supported OL morphological development, Fn substrates having proved permissive for OL myelin-like membrane extension (Buttery and Ffrench-Constant, 1999) and OL process outgrowth (Liang et al., 2004). Subsequent studies contradicted these original findings, as Fn matrices were reported to compromise OL process branching (Maier et al., 2005; Sisková et al., 2009; Lafrenaye and Fuss, 2010) and the production of membrane sheets (Maier et al., 2005; Sisková et al., 2006). However, limited conclusions can be drawn from these later studies as they lacked proper control conditions (Sisková et al., 2006, 2009; Lafrenaye and Fuss, 2010) and formal quantification (Maier et al., 2005). The recent finding that the molecular conformation of Fn may dictate its functional impact on OL development could provide a resolution to this controversy (Stoffels et al., 2013a). Experimentally induced demyelination increases Fn production by lesion-bound cells (Stoffels et al., 2013a), a phenomenon thought to promote the reparative response (Stoffels et al., 2013b), as OPCs upregulate  $\alpha_v$  integrin (a component of Fn integrin receptors; To and Midwood, 2011) post-demyelination (Zhao et al., 2009). Fn in its aggregated form however, which is present in demyelinated MS lesions (Stoffels et al., 2013a), prevents remyelination in toxin-induced demyelination paradigms. Accordingly, Stoffels and colleagues (2013a) go on to show that MS shadow plaques (remyelinated lesions) contain little aggregated Fn, consistent with its action in curbing regeneration.

In contrast to the above-described molecules that hinder OL development, the Ln family of ECM proteins is exemplarily permissive in this regard. Lns are adhesive heterotrimeric proteins composed of variable  $\alpha$ ,  $\beta$  and  $\gamma$  chains, the combination of which imparts identity onto each of the 16 Ln family members (Domogatskaya et al., 2012). Merosin, a name that encompasses all  $\alpha 2$ -chain containing Lns (*i.e.*, Ln-2, Ln-4 and Ln-12; Wewer and Engvall, 1996) is particularly important for proper OL development. Ln  $\alpha 2$ -chain deficient (*dy/dy*) juvenile mice transiently possess fewer mature OLs in the corpus callosum, offering an importance for Ln in timely OL development (Relucio et al., 2009). At the ultrastructural level, *dy/dy* mice suffer from region-specific hypomyelination (Chun et al., 2003; Relucio et al., 2009) and dysmyelination (Chun et al., 2003) of CNS white matter tracts coupled with decreased MBP expression. The importance of Ln in myelin production is further supported by its positive effect on OL differentiation when presented as a substrate *in vitro* (Buttery and Ffrench-Constant, 1999; Chun et al., 2003; Olsen and Ffrench-Constant, 2005; Relucio et al., 2009; Lafrenaye and Fuss, 2010; Eyermann et al., 2012), compounded by its axonal surface-like expression pattern during myelination (Colognato et al., 2002) and remyelination (Zhao et al., 2009).

These compelling observations have fuelled the search for cell-surface receptors that govern Ln-directed OL differentiation. This role can be fulfilled by dystroglycan, a transmembrane component of the dystrophin-glycoprotein complex best known for mediating the ECM-cytoskeleton linkage in skeletal muscle (Straub and Campbell, 1997). OLs express dystroglycan, and its perturbation hinders their morphological maturation (Eyermann et al., 2012) and ability to myelinate neuronal processes *in vitro* (Colognato et

al., 2007). Sulfatide, a lipid component of OL membranes also has laminin-receptive qualities, as evidenced by defective OL morphological elaboration consequent to blockade of sulfatide-Ln interactions (Baron et al., 2014).

The best-characterized receptor that governs Ln-mediated enhancement of OL differentiation is the  $\alpha 6\beta 1$  integrin. This transmembrane receptor is expressed by OLs (Milner and Ffrench-Constant, 1994) and acts to stabilize the connection between the ECM and the cytoskeleton. This occurs upon binding of Ln to the  $\alpha 6\beta 1$  integrin extracellular domain, initiating the recruitment of scaffolding and signaling proteins that guide the indirect linkage of the  $\beta$  integrin cytosolic tail to the cytoskeleton. Perturbation of the  $\beta 1$  integrin subunit consequently negates the morphology-enhancing effect of Ln on cultured OLs (Buttery and Ffrench-Constant, 1999; Relvas et al., 2001; Barros et al., 2009), precludes normal developmental myelination (Lee et al., 2006; Barros et al., 2009; Câmara et al., 2009) and compromises the capacity for remyelination in toxin-induced demyelination paradigms (Relvas et al., 2001; Lee et al., 2006). The unequivocal function of the  $\alpha 6\beta 1$  integrin in OL differentiation and CNS myelination has subsequently sparked investigation into how the growth permissive signal of Ln is propagated downstream of  $\beta 1$  integrin.

### **$\beta 1$ integrin signal transduction**

ECM engagement results in clustering of integrin receptors into specialized adhesive structures termed focal contacts. Integrin effectors are activated within these domains, in turn triggering their mobilization of downstream cytoskeletal-remodelers that effectuate matrix-cued cellular transformation.  $\beta 1$  integrin signal transduction is governed both by



enzymes (*e.g.*, kinases) and scaffold/adaptor molecules that facilitate protein-protein interactions. In OLs, enzymatic propagation of  $\beta 1$  integrin signaling is chiefly executed by Fyn and focal adhesion kinase (FAK), which by aid of their kinase activity, initiate molecular cascades that regulate OL development.

The enzymatic function of Fyn, a member of the Src non-receptor tyrosine kinase (SFK) family, is crucial for integrin-mediated OL maturation. Fyn activity is highest at the onset of CNS myelination (Umemori et al., 1994) and during OL differentiation, concordant with its expression level (Osterhout et al., 1999; Liang et al., 2004). Fyn interacts directly with  $\alpha 6\beta 1$  integrin in OLs (Colognato et al., 2004) where ECM- $\beta 1$  integrin engagement increases its activity (Liang et al., 2004). Fyn depletion precludes OL morphological development on Ln but not on a non  $\beta 1$ -integrin binding substrate (Colognato et al., 2004), highlighting Fyn's specific role in transducing  $\alpha 6\beta 1$  integrin signals. Appropriately then, Fyn-deficient mice suffer from reduced myelin content (Umemori et al., 1994; Sperber et al., 2001), myelin protein levels (Umemori et al., 1994; Sperber et al., 2001; Nakahara et al., 2003; Goto et al., 2008), number of mature OLs (Sperber et al., 2001; Nakahara et al., 2003) and myelinated axons (Sperber et al., 2001). These defects can be attributed to the loss of Fyn activity, as comparable myelin content deficiencies are seen in a kinase-dead Fyn mutant (Sperber et al., 2001). Inclusively, these findings showcase a critical function for Fyn in OL maturation and CNS myelination, roles played at least in part through signal transduction downstream of Ln-engaged  $\alpha 6\beta 1$  integrin.

Integrin engagement also activates FAK, a tyrosine kinase that fittingly colocalizes with integrins at ECM contact sites (reviewed by Guan, 1997). During Ln-

directed CG4 cell differentiation, FAK undergoes autophosphorylation at tyrosine 397 (pY397; indicative of its activation) concomitant with morphological elaboration (Hoshina et al., 2007). RNA interference (RNAi)-mediated FAK knockdown precludes this morphological enhancement (Hoshina et al., 2007; Lafrenaye and Fuss, 2010), which is reversed upon re-expression of RNAi-resistant wild type (WT) FAK but not autophosphorylation-defective FAK (Hoshina et al., 2007). Similar to Fyn, FAK activity (*i.e.*, pY397) is upregulated at the onset of myelination (Fox et al., 2004), and FAK conditional ablation results in transient hypomyelination that is resolved later in development (Câmara et al., 2009; Forrest et al., 2009). These findings collectively indicate that FAK contributes to signaling downstream of Ln-bound  $\alpha 6 \beta 1$  integrin to promote OL differentiation.

The extensive cellular transformation characteristic of OL morphological differentiation implies a requirement for cytoskeleton reorganization. The Rho GTPases Cdc42, Rac1 and RhoA act as signaling hubs in cytoskeletal remodeling, their activities regulated partly by Fyn and FAK. The concerted action of these GTPases confers dynamism onto neural cell architecture via governance of downstream effectors that nucleate, stabilize, extend or cleave the cytoskeletal network (Govek et al., 2005). OL differentiation coincides with downregulated RhoA activity (Liang et al., 2004), which is both necessary and sufficient for OL morphological elaboration (Wolf et al., 2001; Liang et al., 2004). Notably, Fyn interacts with p190RhoGTPase-activating protein (p190RhoGAP; Wolf et al., 2001), which acts to constrain RhoA activity (Govek et al., 2005). Pharmacological inhibition of Fyn-family kinases reduces p190RhoGAP activation (Wolf et al., 2001), whose impaired function yields elevated RhoA activity that

ultimately hinders OL process outgrowth (Liang et al., 2004). Fyn appears to also regulate Cdc42 and Rac1, as a Fyn-family inhibitor decreases their activity in cultured OLs (Liang et al., 2004). Active Cdc42 and Rac1 levels are also reduced in FAK-depleted CG4 cells (Hoshina et al., 2007), offering a similar role for FAK in their regulation. In contrast to RhoA, Cdc42 and Rac1 activation increases with OL differentiation (Liang et al., 2004). Cdc42 and Rac are expressed at the distal tips of OL extensions (Bacon et al., 2007), suggestive of a function in process growth. Accordingly, OL expression of constitutively active/dominant negative Cdc42 or Rac1 enhances/hinders morphological maturation respectively (Liang et al., 2004). OL-specific Cdc42/Rac1 knockout *in vivo* illustrates their importance in myelination, as both mouse lines suffer from a CNS dysmyelination phenotype that is exacerbated in double Cdc42/Rac1 mutants (Thurnherr et al., 2006). In sum, the collaborative influence of the RhoGTPases on cytoskeletal dynamism is critical for OL morphological development, their activities regulated in part by the Fyn and FAK signaling axes.

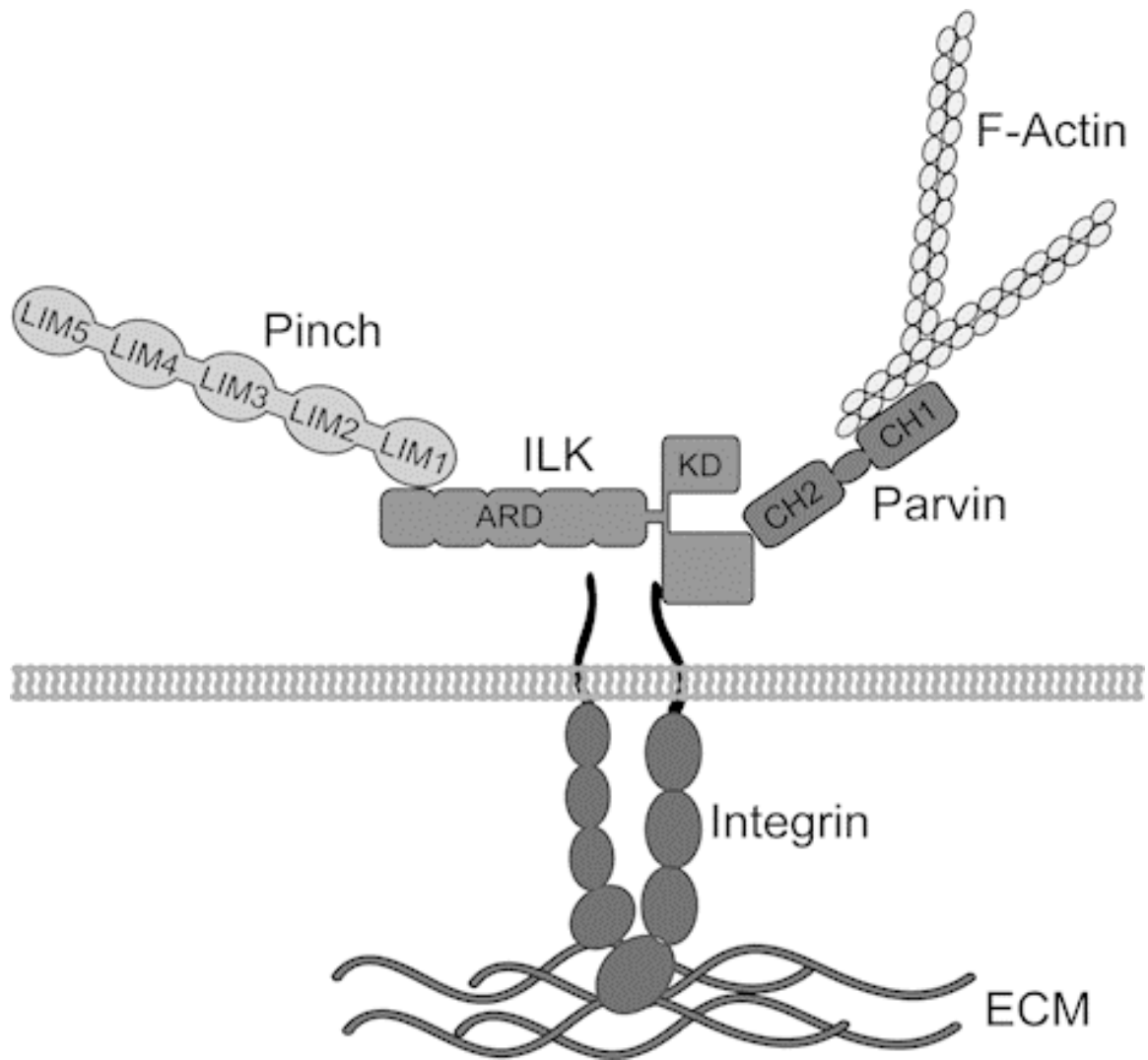
The preceding section aimed to highlight the importance of integrin effector molecules that, via their enzymatic activity, evoke cytoskeletal remodeling to facilitate OL metamorphosis. In the following section, we introduce integrin-linked kinase (ILK) as a candidate for an analogous function in OLs, albeit not through enzymatic function. Rather, the primary contribution of ILK in this process likely lies in its capacity to mediate protein-protein interactions, effectively closing the molecular distance between enzymes and substrates, in addition to providing integrin-ECM contact sites with structural reinforcement.

## **Integrin-linked kinase**

Signaling downstream of  $\beta 1$  integrin is also reliant on adaptor/scaffolding proteins with little to no intrinsic enzymatic function (Brakebusch and Fässler, 2003). These proteins provide a structural framework for compartmentalization of enzymatic effectors, thereby serving as signaling hubs. One such example is ILK, a ubiquitously expressed focal adhesion protein with roles in cellular differentiation, proliferation, survival and migration (Wickström et al., 2010). ILK was categorized as a serine/threonine kinase at its discovery (Hannigan et al., 1996), but more recent work suggests it is not capable of enzymatic catalysis, and rather uses its kinase-like domain for protein-protein interactions (Wickström et al., 2010; Fukuda et al., 2011; Widmaier et al., 2012).

Upon integrin engagement, ILK is recruited to nascent focal adhesions (Li et al., 1999) where it binds to the cytosolic C-terminal tails of  $\beta 1$  and  $\beta 3$  subunit-containing integrin receptors (Hannigan et al., 1996; Pasquet et al., 2002; Fukuda et al., 2009). Here, ILK lends structural support to cell-matrix adhesions and provides a platform for transduction of ECM-derived signals. ILK loss results in a variety of cellular consequences, notably reduced cell spreading, disorganized actin architecture and barred focal contact formation at the leading cellular edge (Elad et al., 2013). Structurally, ILK is composed of an N-terminal ankyrin repeat domain and a C-terminal kinase-like domain joined by a short 14 amino acid link region (Fukuda et al., 2009; Ghatak et al., 2013; Stiegler et al., 2013). ILK forms an obligate heterotrimeric complex (Figure 1.2; Fukuda et al., 2003; Stanchi et al., 2009) with PINCH (Particularly Interesting New Cysteine-Histidine rich protein; Tu et al., 1999) and parvin (Tu et al., 2001; Yamaji et al., 2001), each member essential for the proper functionality of the ternary complex (reviewed by

Brakebusch and Fässler, 2003). PINCH couples ILK to receptor tyrosine kinase signaling via NCK2 (Tu et al., 1998), whereas parvin links ILK to the cytoskeleton by way of its actin-binding calponin homology (CH) domains (Olski et al., 2001; Lange et al., 2009). In addition to its obligate binding partners, ILK interacts with over 700 other proteins (Dobrev et al., 2008), suggesting a function in compartmentalizing signal cascades. To this effect, ILK loss would deregulate signaling axes originating from integrin adhesions, offering an explanation for the compromised cytoskeletal integrity seen in ILK-deficient cells (Elad et al., 2013). In support of this theory, ILK perturbation leads to upregulated RhoA activity (Kogata et al., 2009; Blumbach et al., 2010), an event associated with cytoskeletal reorganization (Govek et al., 2005).



**Figure 1.2. ILK is a member of a complex that stabilizes cell-ECM contact sites.** ILK forms a heterotrimeric entity with proteins PINCH and parvin, which bind to ILK's ankyrin repeat domain (ARD) and kinase domain (KD) respectively. This complex localizes to  $\beta 1$  and  $\beta 3$ -integrin cytosolic tails where it couples filamentous actin (F-actin) to the ECM. Image obtained from (Ghatak et al., 2013).

## **Rationale**

The ECM protein Ln enhances OL membrane extrusion, OPC migration and is required for proper CNS myelination. Parallel functions exist for  $\beta 1$  integrin, a subunit of the  $\alpha 6\beta 1$  integrin Ln receptor expressed by OLs. The  $\beta 1$  integrin cytosolic tail is bound by ILK, a focal adhesion protein that stabilizes the linkage between the ECM and the cytoskeleton. Compared to  $\beta 1$  integrin, the role of ILK in OL biology is not well understood. We believe the current state of the literature, as highlighted above, provides adequate rationale for a thorough investigation of ILK's role in OL biology. Furthering our understanding of the mechanisms underlying OL differentiation, migration and OL-mediated CNS myelination will be useful when considering the development of therapeutics aimed at promoting regeneration in demyelinating diseases such as MS.

## **Hypothesis**

ILK is important for OL differentiation, OPC migration and CNS myelination.

## **Aims**

1. Devise methods to establish enriched mouse primary OL cultures and OL/dorsal root ganglion neuron co-cultures for the study of OL development and myelination.
2. Determine the impact of ILK loss on OL differentiation *in vitro*, and CNS myelination *in vivo*.
3. Investigate the role of ILK in OPC migration.

## **Chapter 2**

### **Derivation of Enriched Oligodendrocyte Cultures and Oligodendrocyte/Neuron Myelinating co-Cultures from Post- Natal Murine Tissues**



**Derivation of Enriched Oligodendrocyte Cultures and Oligodendrocyte/Neuron  
Myelinating co-Cultures from Post-Natal Murine Tissues**

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### **Author Contributions**

HC generously provided us with the rudimentary framework required to produce the finished product. RO and RK designed the protocol and analyzed results. SDR contributed expert cell culture advice. RO conducted experiments. RO wrote the protocol, RK edited the final version. RK oversaw the entirety of this work. RO produced the accompanied explanatory video available at:

<http://www.jove.com/video/3324/derivation-enriched-oligodendrocyte-cultures-oligodendrocyteneuron>

**Abstract**

This article describes methods to derive enriched populations of murine oligodendrocyte precursor cells (OPCs) in primary culture, which differentiate to produce mature oligodendrocytes (OLs). In addition, this report describes techniques to produce murine myelinating co-cultures by seeding mouse OPCs onto a neurite bed of mouse dorsal root ganglion neurons (DRGNs).

**Keywords**

Oligodendrocyte, myelination, *in vitro*, dorsal root ganglion neuron, co-culture, primary cells, mouse, neuroscience.

## Introduction

Identifying the molecular mechanisms underlying OL development is not only critical to furthering our knowledge of OL biology, but also has implications for understanding the pathogenesis of demyelinating diseases such as Multiple Sclerosis (MS). Cellular development is commonly studied with primary cell culture models. Primary cell culture facilitates the evaluation of a given cell type by providing a controlled environment, free of the extraneous variables that are present *in vivo*. While OL cultures derived from rats have provided a vast amount of insight into OL biology, similar efforts at establishing OL cultures from mice has been met with major obstacles. Developing methods to culture murine primary OLs is imperative in order to take advantage of the available transgenic mouse lines.

Multiple methods for extraction of OPCs from rodent tissue have been described, ranging from neurosphere derivation, differential adhesion purification and immunopurification (McCarthy and Vellis, 1980; Barres et al., 1992; Avellana-Adalid et al., 1996). While many methods offer success, most require extensive culture times and/or costly equipment/reagents. To circumvent this, purifying OPCs from murine tissue with an adaptation of the method originally described by McCarthy & de Vellis (1980) is preferred. This method involves physically separating OPCs from a mixed glial culture derived from neonatal rodent cortices. The result is a purified OPC population, which can be differentiated into an OL-enriched culture. This approach is appealing due to its relatively short culture time and the unnecessary requirement for growth factors or immunopanning antibodies.

While exploring the mechanisms of OL development in a purified culture is informative, it does not provide the most physiologically relevant environment for assessing myelin sheath formation. Co-culturing OLs with neurons would lend insight into the molecular underpinnings regulating OL-mediated myelination of axons. For many OL/neuron co-culture studies, dorsal root ganglion neurons (DRGNs) have proven to be the neuron type of choice. They are ideal for co-culture with OLs due to their ease of extraction, minimal amount of contaminating cells, and formation of dense neurite beds. While studies using rat/mouse myelinating xenocultures have been published (Chan et al., 2004; Ishibashi et al., 2006; Câmara et al., 2009), a method for the derivation of such OL/DRGN myelinating co-cultures from post-natal murine tissue has not been described. Here we present detailed methods on how to effectively produce such cultures, along with examples of expected results. These methods are useful for addressing questions relevant to OL development/myelinating function, and are useful tools in the field of neuroscience.

**Ethics Statement**

The mice used in this work were cared for according to Canadian Council on Animal Care (CCAC) guidelines. Ethical approval for experiments conducted was obtained from the University of Ottawa Animal Care Committee under protocol number OGH-119.

## Materials

**Table 2.1.** Reagent list.

| <b>Product Name</b>                                | <b>Company</b>    | <b>Product Number</b> |
|----------------------------------------------------|-------------------|-----------------------|
| Dulbecco's Modified Eagle Medium (DMEM)            | Multicell         | 319-005-CL            |
| Hank's Balanced Salt Solution (HBSS)               | Invitrogen        | 14170-112             |
| Minimum Essential Media (MEM)                      | GIBCO             | 12360-038             |
| Fetal Bovine Serum (FBS)                           | GIBCO             | 10091-148             |
| Penicillin-Streptomycin (Pen/Strep)                | GIBCO             | 15140-122             |
| GlutaMAX                                           | Invitrogen        | 35050-061             |
| Poly-l-lysine                                      | Sigma-Aldrich     | P2636                 |
| Bovine Serum Albumin (BSA)                         | Sigma-Aldrich     | A4503                 |
| Human merosin purified protein (LN2)               | Millipore         | CC085                 |
| Recombinant rat ciliary neurotrophic factor (CNTF) | PeproTech         | 450-50                |
| L-thyroxine                                        | Biochemika        | 89430                 |
| Holo-transferrin                                   | Sigma-Aldrich     | T0665                 |
| B27 supplement                                     | GIBCO             | 0080085-SA            |
| Bovine insulin                                     | Sigma-Aldrich     | I6634                 |
| 3,3',5-Triiodo-L-thyronine                         | Sigma-Aldrich     | I6634                 |
| Progesterone                                       | Sigma-Aldrich     | P8783                 |
| Putrescine                                         | Sigma-Aldrich     | P7505                 |
| Sodium Selenite                                    | Sigma-Aldrich     | S5261                 |
| 5-Fluoro-2'-deoxyuridine (FuDR)                    | Sigma-Aldrich     | F0503                 |
| Papain solution                                    | Worthington       | LS003126              |
| DNaseI                                             | ROCHE             | 1010159001            |
| L-cysteine                                         | Sigma-Aldrich     | C7352                 |
| 24-well tissue culture dishes                      | Cellstar          | 662-160               |
| T25-tissue culture flasks with vent cap            | Corning           | 430639                |
| 10 cm tissue culture dishes                        | Corning           | 430167                |
| 10 cm petri dish                                   | Fisher Scientific | 0875713               |
| Collagenase A                                      | ROCHE             | 103578                |
| CellTrics 50 $\mu$ m filter (optional)             | PARTEC            | 04-004-2327           |

**Table 2.2.** Recipe for 100X OL-Supplement\*

| <b>Ingredient</b>          | <b>Amount to add</b> |
|----------------------------|----------------------|
| DMEM                       | 100 mL               |
| BSA                        | 1.02 g               |
| Progesterone               | 0.6 mg               |
| Putrescine                 | 161 mg               |
| Sodium Selenite            | 0.05 mg              |
| 3,3',5-Triiodo-L-thyronine | 4 mg                 |

\*Store at -80°C in 250 µL aliquots

**Table 2.3.** Recipe for OL media.

| <b>Ingredient</b>                      | <b>Amount to add</b> |
|----------------------------------------|----------------------|
| DMEM                                   | 23.75 mL             |
| 100X OL-Supplement                     | 250 µL               |
| Bovine insulin (from 1 mg/mL stock)    | 125 µL               |
| GlutaMAX                               | 250 µL               |
| Holo-transferrin (from 33 mg/mL stock) | 37.5 µL              |
| B27 Supplement                         | 500 µL               |
| FBS                                    | 125 µL               |
| CNTF (from 50 ng/µL stock)             | 25 µL                |

**Table 2.4.** Recipe for mixed glial culture media (made up in DMEM).

| <b>Ingredient</b>            | <b>Final concentration</b>                       |
|------------------------------|--------------------------------------------------|
| FBS                          | 10%                                              |
| Pen/Strep (0.33% from stock) | 33 units/mL Penicillin and 33 µg/mL Streptomycin |
| GlutaMAX                     | 1%                                               |

**Table 2.5.** Recipe for DRGN media (made up in DMEM).

| <b>Ingredient</b>         | <b>Final concentration</b>                         |
|---------------------------|----------------------------------------------------|
| FBS                       | 10%                                                |
| Pen/Strep (1% from stock) | 100 units/mL Penicillin and 100 µg/mL Streptomycin |



**Table 2.6.** Recipe for OPC papain digestion solution (made up in MEM).

| <b>Ingredient</b> | <b>Final Concentration</b> |
|-------------------|----------------------------|
| Papain solution   | 1.54 mg/mL                 |
| L-cysteine        | 360 µg/mL                  |
| DNaseI            | 60 µg/mL                   |

**Table 2.7.** DRG papain digestion solution (made up in HBSS).

| <b>Ingredient</b> | <b>Final Concentration</b> |
|-------------------|----------------------------|
| Papain            | 1.54 mg/mL                 |
| L-cysteine        | 360 µg/mL                  |

**Table 2.8.** DRG Collagenase A digestion solution (made up in HBSS).

| <b>Ingredient</b> | <b>Final Concentration</b> |
|-------------------|----------------------------|
| Collagenase A     | 4 mg/mL                    |

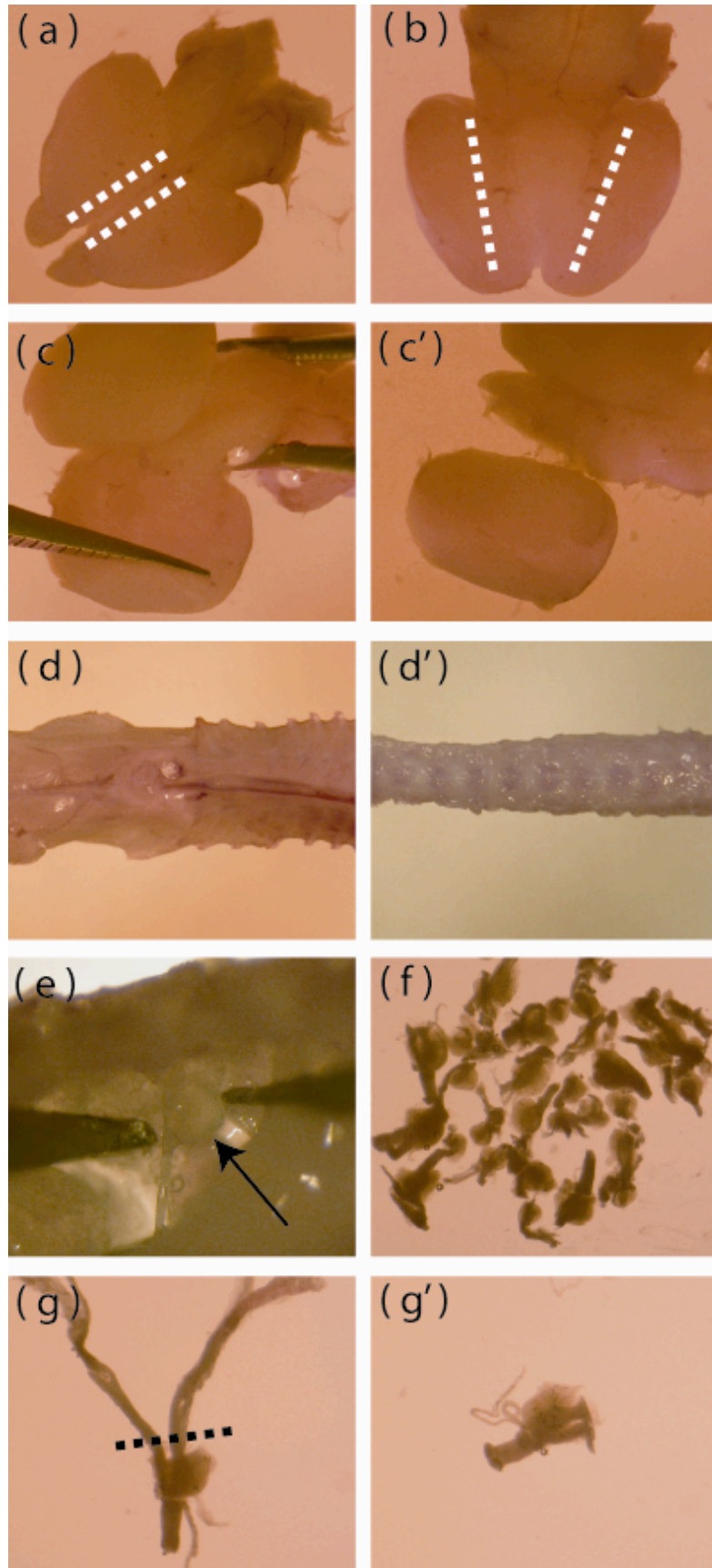
## **Methods**

### **[1] Dissection – neonatal mouse cortex for OPC extraction**

- 1.1) Sacrifice P0-P2 mouse according to institutional guidelines.
- 1.2) Dissect the brain and place in a Petri dish containing ice-cold MEM (antibiotic-free).
- 1.3) Transfer the dish to a dissection microscope.
- 1.4) Using a scalpel with the brain dorsal side up, make a shallow incision sagittally along the most medial edge of each cortex (Fig 2.1a). This incision should only pass through the meningeal layer in order to facilitate its removal.
- 1.5.) Use fine tipped forceps to peel off the meninges in a lateral fashion. If done carefully, this layer can be removed in one piece. During this step, remove the olfactory bulbs.
- 1.6) With the brain ventral side-up, make a deep sagittal incision where the cortex meets the ventral area of the diencephalon (Fig 2.1b).
- 1.7) With the brain dorsal side-up, separate the cortices from the midbrain by prying the tissue in a medial to lateral fashion (Fig 2.1c, c'). Remove any residual meninges at this step.

1.8) Dice each cortex into approximately 4 pieces and gently transfer to a 15 mL conical tube containing 350  $\mu$ L of MEM per mouse brain. Keep the tube on ice until all mice have been processed.

1.9) Repeat steps 1.1-1.8 for remaining mice.



**Figure 2.1. Dissection microscope images of particular aspects of neonatal mouse cortex and DRG isolation.** [a] Dorsal view of a freshly extracted neonatal mouse brain. The dotted lines indicate the area where incisions must be made to facilitate the removal of the meningeal layer. [b] Ventral view of brain, dotted lines indicate the area where the cortex meets the ventral diencephalon. Deep incisions must be made along the dotted lines to aid the isolation of the cortices. [c-c'] Visual depiction of how to pry the cortex away from the remainder of the brain. [d] A freshly isolated P5-P10 mouse spine prior to trimming away excess muscle and bone [d']. [e] Location of DRGs within the spinal column. [f] The approximate number of DRGs that should be isolated from one mouse. [g] A DRG with long roots that require trimming prior to enzymatic digestion. The dotted line indicates the region where the roots should be trimmed. [g'] DRG post root-trimming.

## **[2] Dissociation of neonatal cortices and maintenance of mixed glial cultures**

**Note:** The introduction of bubbles into the cell suspension should be avoided during all of the following steps.

2.1) Add the 15 mL conical tube containing the freshly dissected brains to a 37°C water bath for 3 min.

2.2) Transfer brains to a sterile tissue culture hood.

2.3) Gently pass diced cortices through a P1000 pipette tip to generate smaller fragments. Stop pipetting once there are no brain pieces large enough to disrupt a smooth flow of suspension through the pipette tip.

2.4) Add 75 µL of OPC papain solution per brain into the conical tube. The OPC papain solution must be pre-warmed at 37°C for 20 min prior to use.

2.5) Incubate in a 37°C water bath for 20 min. Approximately every 2 minutes, gently invert the tube to prevent tissue aggregation. During this time, add 5 mL of mixed glial culture media to each poly-L-lysine (PLL) coated (1 mg/mL) T25 flask (one flask per mouse brain), and place in a 37°C tissue culture incubator at 8.5% CO<sub>2</sub>.

2.6) After 20 min, return the tissue suspension to the sterile hood and add 2 mL of mixed glial culture media per brain to the tube. Let sit for 10 min at room temperature to allow inactivation of the OPC papain solution.

2.7) Aliquot the tissue suspension into 5 mL plastic tubes. The number of tubes should match the number of brains dissected, resulting in approximately 2.5 mL per tube.

2.8) Using a sterile flame-polished glass Pasteur pipette, gently triturate the tissue in each tube. Triturate slowly at first, and gradually increase speed as pieces dissociate. Triturate approximately 10-15 times, however this number may vary based on the efficacy of the digestion. Under-trituration will result in poor dissociation of the tissue, whereas over-trituration will negatively impact on cell viability. It is important to not introduce bubbles into the solution as this will severely impact on cell viability.

2.9) Once there are no visible tissue clumps remaining in the suspension, transfer to a 50 mL conical tube containing 4 mL of mixed glial culture media per brain (*i.e.*, 4 brains = 16 mL mixed glial culture media).

2.10) Gently invert the 50 mL conical tube and repeat for the remaining 5 mL tubes.

2.11) Aliquot the pooled cell suspension into 15 mL conical tubes (approximately 6.5 mL per 15 mL tube). The number of 15 mL tubes should match the number of brains dissected.

2.12) Centrifuge the tubes at 1200 rpm (~300 g) for 5 min.

2.13) Carefully aspirate the supernatant and add 1 mL of warm mixed glial culture media to each 15 mL conical tube.

2.14) Slowly resuspend the pellet with a P1000 pipette tip, being careful not to introduce bubbles. Add the cell suspension from each tube to a pre-equilibrated PLL-coated T25 flask, rendering the total volume of the culture media to 6 mL.

2.15) Place the flasks in a tissue culture incubator for 3-4 hours to allow the cells to attach to the PLL substrate. Then perform a full media change by pipetting out the media, and adding 6 mL of fresh mixed glial culture media to the flasks. This step removes much of the debris caused by the trituration, and promotes culture viability. If OL/DRGN co-cultures are desired, see section [3].

2.16) After 3 days of culture, perform a 2/3 media change by removing 4 mL of media, and replacing with 4 mL of fresh mixed glial culture media. At this point, an astrocyte monolayer should be forming on the base of the flasks.

2.17) On Day 6, perform another 2/3 media change and supplement the flasks with a final concentration of 5  $\mu\text{g/mL}$  insulin. At this point, an astrocyte monolayer should be clearly visible, on top of which OPCs will be proliferating.



### **[3] DRGN isolation**

**Note:** To produce OL/DRGNs co-cultures, DRGNs should be established the day after mixed glial culture generation. Both culture types are grown independently, and combined after 9-10 days.

3.1) Sacrifice P5-P10 mouse according to institutional guidelines.

3.2) Extract the spine, and transfer to a clean Petri dish.

3.3) Trim away as much muscle and bone from the spine as possible (Fig 2.1d, d'), as this will ease the dissection of the dorsal root ganglia (DRGs).

3.4) Transfer the trimmed spine to a new Petri dish ventral side-up. Using dissection scissors and starting caudally, cut medially through the spinal column in a longitudinal fashion.

3.5) Using two pairs of forceps, gently pry open the spinal column to expose the spinal cord.

3.6) DRGs can be found beneath and lateral to the spinal cord. Using fine tipped forceps, gently remove the DRGs while avoiding damage to the ganglia (Fig 2.1e).

3.7) Transfer the removed DRGs to ice cold Hank's buffered salt solution (HBSS, antibiotic-free) in a new Petri dish. The dissector should aim to extract 40 DRGs per mouse (Fig 2.1f).

3.8) Once the DRGs have been extracted, trim the DRGs of any excessively long roots (Fig 1g, g') to minimize introduction of contaminating cells into the culture (glial cells, fibroblasts).

3.9) Transfer the DRGs to a 1.5 mL centrifuge tube containing 500  $\mu$ L of ice cold HBSS.

3.10) Centrifuge at 1200 rpm ( $\sim$ 300 g) for 5 min at 4°C to pellet the DRGs.

3.11) Transfer the centrifuge tubes to a sterile tissue culture hood and remove the HBSS from the tubes.

3.12) Add 500  $\mu$ L of pre-warmed (20 min at 37°C) DRG papain solution, and incubate the tubes in a 37°C water bath for 10 min. Invert the tubes every 2 min to prevent tissue aggregation.

3.13) Repeat step 3.10.

3.14) Remove the DRG papain solution and add 500  $\mu$ L of pre-warmed (20 min at 37°C) Collagenase A solution. Incubate in a 37°C water bath for 10 min, inverting every 2 min.

3.15) Repeat step 3.10.

3.16) Remove supernatant and add 1 mL of DRGN media. Invert tube several times.

3.17) Repeat step 3.10.

3.18) Repeat step 3.16.

3.19) Coat a sterile flame-polished glass Pasteur pipette with bovine serum albumin (BSA) by pipetting a solution of 0.25% BSA in HBSS several times. The coating with BSA solution will prevent the DRGs from adhering to the walls of the glass pipette.

3.20) Triturate the DRGs with the BSA-coated pipette gently at first, and with increasing intensity once clumps begin dissociating. Triturate approximately 10-15 times, however this number is dependant on the degree of digestion, and the number of DRGs per tube.

3.21) Once dissociation is achieved, pass the suspension through a 50  $\mu\text{m}$  filter into a sterile Petri dish containing 7 mL of DRGN media. Filtration will eliminate much of the debris from the cell suspension, although this step is not critical.

3.22) Incubate the Petri dish at 8.5%  $\text{CO}_2$  for approximately 1.25 hours.

3.23) During this incubation time, coat several 12 mm coverslips with LN2 (10  $\mu$ g/mL in PBS) in a 24-well dish.

3.24) Once the incubation is finished, observe the Petri dish under bright field. DRGNs are identified as large bodied, phase dark cells. Apply a modest swirl to the Petri dish to lift any adhered DRGNs. Many contaminating cells will have strongly adhered to the Petri dish, thereby enriching your cell suspension for DRGNs.

3.25) Transfer the cell suspension to a 15 mL conical tube. Gently rinse the dish with 4 mL of DRGN media to collect any residual DRGNs. Transfer the additional 4 mL to the conical tube.

3.26) Centrifuge for 5 min at 1200 rpm (~300 g).

3.27) Aspirate supernatant and resuspend the pellet in 500  $\mu$ L of fresh DRGN media.

3.28) Using a hemocytometer, calculate the number of yielded DRGNs. Be sure to only count the DRGNs, and not other cell types. DRGNs can be identified by their large spherical cell bodies.

3.29) Seed 30,000 – 50,000 DRGNs to each LN2-coated coverslip in 1 mL of DRGN media, and place in a 37°C tissue culture incubator at 8.5% CO<sub>2</sub>.

3.30) The next morning, perform a full media change by replacing the DRGN media with OL media (minus CNTF) with a final concentration of 1% Pen/Strep and 10  $\mu$ M FuDR.

3.31) On Days 3 and 5, perform a 3/4 media change with the same media as in Step 3.30.

3.32) On Day 7, perform a full media change with OL media (minus CNTF, Pen/Strep, FuDR).

3.33) On Day 9, the DRGNs should have formed an extensive neurite bed, and are now ready to be co-cultured with OPCs.

**[4] Purification of OPCs from mixed glial cultures for establishment of OL-enriched cultures or OL/DRGN co-cultures**

4.1) On Day 9 of the mixed glial culture, transfer the flasks to an orbital shaker in a 5% CO<sub>2</sub> tissue culture incubator. Place the flasks on top of empty T25 flasks to prevent any heat generated from the orbital shaker from adversely affecting the mixed glial cultures. Allow the cultures to equilibrate to this new incubator for 1 hour.

4.2) Once the flasks have equilibrated, shake the flasks at 50 rpm for 45 min. The purpose of this shake is to remove any loosely adherent contaminating cells from the monolayer.

4.3) Once the shaking is completed, move cells to a tissue culture hood and remove all the media from the flasks. Replace with 4 mL of fresh mixed glial culture media supplemented with 5 µg/mL insulin.

4.4) Place the flasks back onto the shaker, and allow to equilibrate for approximately 3 hours.

4.5) Once the flasks are equilibrated, fasten them securely to the orbital shaker, and shake the flasks for approximately 16 hours at 220 rpm (overnight).

4.6) The next morning, if OLs are to be grown in the absence of DRGNs (*i.e.*, OL-enriched culture), coat several sterile 12 mm coverslips with LN2 (10 µg/mL in PBS) for 1 hour. Transfer the coverslips to 24-well dishes, wash with PBS followed by an OL media wash. Add 1 mL of OL media to each well and equilibrate at 8.5% CO<sub>2</sub>.

4.7) Equilibrate 10 cm tissue culture dishes at 5% CO<sub>2</sub> for 30 min. One dish will be required for every 2 flasks. These will be used for the differential adhesion-enrichment of the suspended OPCs.

4.8) Once the 30 min equilibration period has passed, transfer the media from the shaken flasks to the dishes. Each dish should receive media from 2 flasks, equaling approximately 8 mL of cell suspension per 10 cm dish.

4.9) Incubate the dishes at 5% CO<sub>2</sub> for 30 min, while providing a gentle nudge at the 15 min mark. This nudge will help prevent OPCs from adhering to the 10 cm dish.

4.10) Once the incubation is complete, examine the dishes under bright field. OPCs are identified as small cell clumps, typically of 3-5 cells but sometimes form large aggregates resembling neurospheres. On the base of the plate, many non-OL lineage cells should be firmly adhered. Gently swirl the plates to detach any loosely adhered OPCs, and transfer the cell suspension from each plate into a 15 mL conical tube.

4.11) Centrifuge at 1200 rpm (~300 g) for 5 min.

4.12) Resuspend the pellet in 1 mL of OL media with a P1000 pipette tip, followed by resuspension with a P200 pipette tip.

4.13) Count the cells using a hemocytometer.

4.14) For enriched-OL cultures, seed 25,000 - 50,000 OPCs to each 12 mm LN2-coated coverslip in a final volume of 1 mL OL media.

4.15) For OL/DRGN co-cultures, perform a full OL media (minus CNTF) change on the DRGNs from section [C], and gently add 50,000 cells from the OPC-enriched cell suspension. Take care to not disrupt the DRGN neurite bed during the addition of OPCs.

4.16) Place cultures in a 37°C tissue culture incubator at 8.5% CO<sub>2</sub>, and avoid removing until fixation. Murine OPCs are sensitive to changes in pH, and removal from the incubator will alter the pH of the OL media. Also of note, addition of dH<sub>2</sub>O to the empty wells surrounding the cell cultures will prevent evaporation of the culture media, thus minimizing fluctuations in the concentrations solutes within the OL media. This will provide a more consistent environment for the differentiation of the OPCs. Media changes are not required once OPCs have been seeded onto LN2 substrates or DRGN neurite beds, provided the experimental endpoint does not exceed 6 DIV.



## **[5] Processing of cultures for immunofluorescence microscopy**

5.1) Fix cultures with 100% methanol at -20°C for 10 min, or 3% paraformaldehyde at room temperature for 15 min.

5.2) Permeabilize coverslips with 0.1% Triton-X-100 for 10 min, wash with phosphate-buffered saline (PBS) and block for 1 hour in 10% goat serum.

5.3) Incubate coverslips with primary antibodies diluted in blocking solution overnight at 4°C.

5.4) Wash coverslips several times with PBS, and incubate with Alexa-fluor conjugated secondary antibodies (Invitrogen) diluted in blocking solution for 45 min.

5.5) Counterstain with 4',6-diamidino-2-phenylindole (DAPI) and wash coverslips several times with PBS.

5.6) Mount coverslips in DAKO fluorescent mounting medium.

5.7) Analyze slides via immunofluorescence microscopy. In this protocol, slides were analyzed with either a Zeiss Axiovert 200M inverted fluorescence microscope or a Zeiss LSM 510 META laser scanning confocal microscope.

## **[6] Whole cell protein extraction from OL-enriched cultures**

6.1) Remove 24-well cultures from incubator and cool on ice for 3 min.

6.2) Carefully remove media, and add 10-20  $\mu$ L of lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton-X-100, with 0.1% pepstatin, aprotinin, PMSF, leupeptin, sodium orthovanadate) to each well (A minimum of 8 wells per sample is suggested).

6.3) Scrape wells using a wide-bore P1000 pipette tip, and transfer the lysate to a 1.5 mL centrifuge tube.

6.4) Pass the lysate through a 30½-gauge syringe approximately 15 times, and chill on ice for 30 min.

6.5) Centrifuge tubes at 14,000 rpm (~20,000 g) for 15 min at 4°C.

6.6) Transfer supernatant to new centrifuge tubes, and store at -80°C.

## **[7] SDS-PAGE analysis on enriched-OL culture protein**

7.1) Resolve 30 µg of protein per sample in reducing buffer by SDS-PAGE on standard 12% poly-acrylamide gels.

7.2) Semi-dry transfer gels onto PVDF membranes.

7.3) Block membranes for 1 hour in 5% skim milk powder in TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Tween-20).

7.4) Incubate membranes with primary antibodies diluted in blocking solution for 1 hour.

7.5) Wash membranes several times with TBST.

7.6) Incubate the membranes with HRP-conjugated secondary antibodies for 45 min in blocking solution.

7.7) Wash membranes several times with TBST, and incubate with Amersham ECL Plus western blotting detection reagent (GE Healthcare) for 5 min.

7.8) Detect protein bands with standard scientific imaging film.

## Results

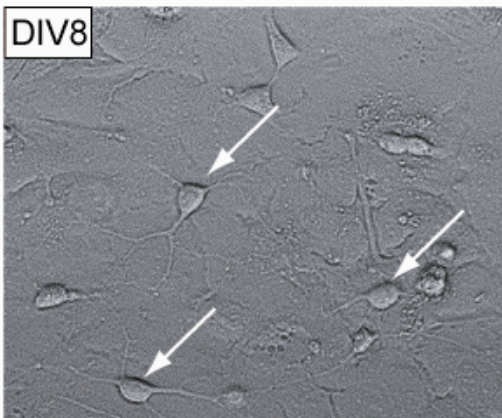
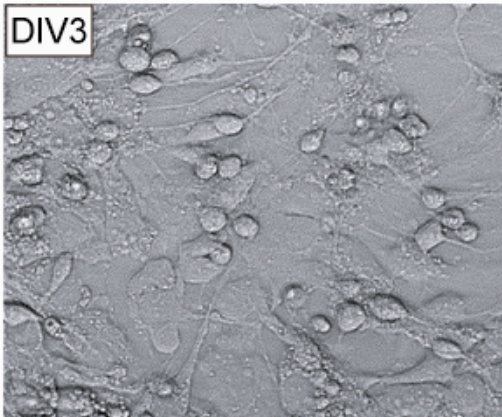
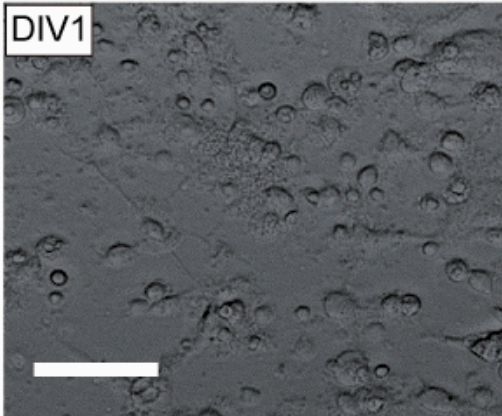
In this protocol, OPCs are expanded on an astrocyte monolayer within a mixed glial culture. This mixed glial culture is derived from P0-P2 neonatal mouse cortex. At day 1 *in vitro* (DIV1), the mixed glial culture contains cells with varying morphologies as seen by phase contrast microscopy (Fig 2.2a). At DIV3, an astrocyte monolayer begins to form on the base of the flask, and at DIV8, OPCs can be clearly observed on the monolayer surface. At DIV9, the proliferating OPCs have reached sufficient density to be purified by overnight high-speed orbital shaking. Once the purification process has been completed, the result is an OPC-enriched cell population. At DIV1-post purification, OPCs have simple morphology, extending few processes (Fig 2.2b). At DIV3 post purification, cells have extended a complex meshwork of processes, reminiscent of immature OLs. At DIV6 post purification, the purified OLs have flattened and projected leaflet-like membrane structures. This morphological development is typical of the *in vitro* maturation of OLs.

Immunofluorescence microscopy indicates the purified cells are of OL-lineage (Fig 2.3a). Seeded OPCs initially express chondroitin sulfate proteoglycan (NG2), and develop into myelin-associated glycoprotein (MAG) positive immature OLs within three days post seeding (Fig 2.3b). At DIV6, many OLs express myelin basic protein (MBP), and possess typical mature OL morphology. Percent OL-lineage cells were quantified at different time points to determine the purity of the OL-enriched cultures (Fig 2.3c). At DIV1 post purification, cultures are  $50 \pm 14\%$  NG2<sup>+</sup> OPCs, with no MAG<sup>+</sup> or MBP<sup>+</sup> OLs. This indicates the purified OL-lineage cells are in the precursor stage at seeding time, with negligible numbers of differentiated OLs. At DIV3, many OLs have

differentiated into MAG<sup>+ve</sup> cells (24±5.9%) while some retain the precursor phenotype, and remain NG2<sup>+</sup> (13±8.0%). At DIV3, a small proportion of MAG<sup>+ve</sup> cells (3.2±1.2%) are also expressing MBP. At DIV6, 20±5.9% of OLs are MAG<sup>+ve</sup> while 12±7.3% persist as NG2<sup>+ve</sup> OPCs. In addition, 21±9.3% of cells within the culture are MBP<sup>+ve</sup> OLs at this time point. SDS-PAGE analysis shows the graded expression of 2'3'-cyclic-nucleotide 3'-phosphodiesterase (CNP) and MBP over the 6 day culture period, further demonstrating the ability of OPCs in culture to terminally differentiate into mature OLs (Fig 2.3d). Collectively, these data establishes this method as a means of producing an OL-enriched culture system suitable for the study of OL maturation from OPCs.

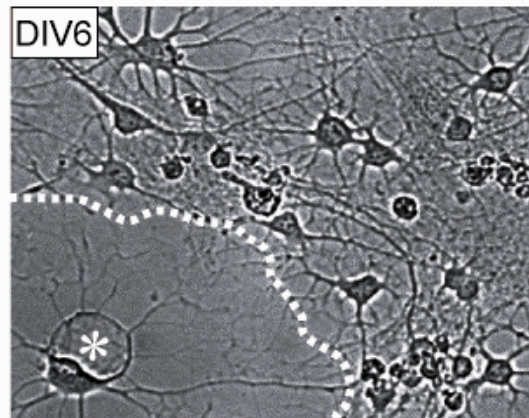
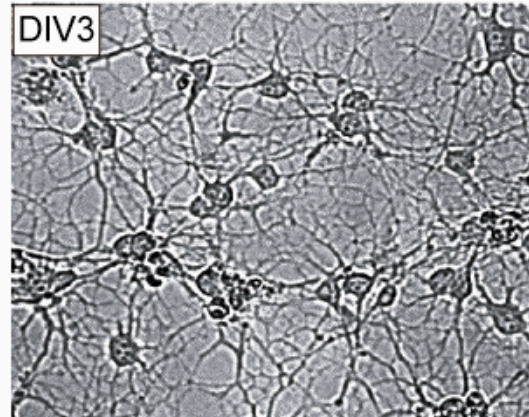
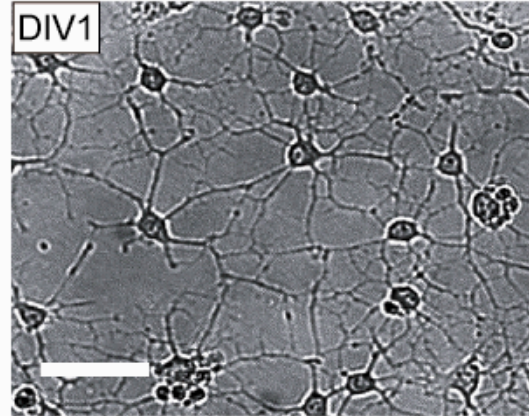
(a)

Mixed Glial Culture

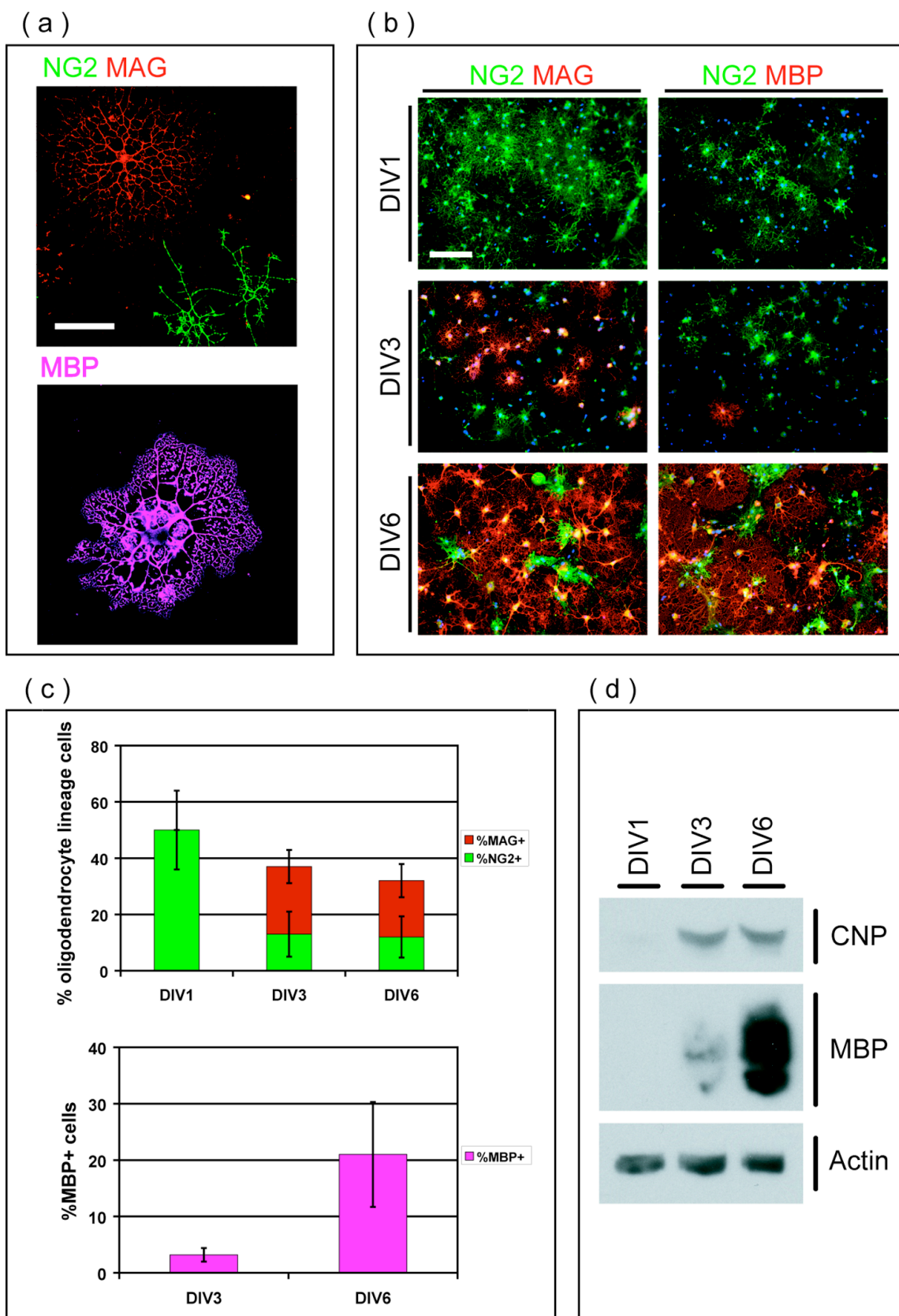


(b)

Oligodendrocyte-Enriched Culture



**Figure 2.2. OPCs are expanded within a mixed glial culture, purified, and subsequently differentiated as an OL-enriched culture.** [a] Phase contrast images of mixed glial cultures at different stages of development. At DIV1, cells appear round with few flattened cells. Stratification of mixed glial culture begins at DIV3, where astrocytes form a uniform monolayer at the base of the flask, upon which OPCs proliferate. Many OPCs are seen at DIV8 (arrows) adhered to the surface of the astrocyte monolayer. [b] Once purified from the mixed glial culture, DIV1 OPCs have extended only a few processes. At DIV3, cells have extended many processes, reminiscent of intermediate-stage OLs. At DIV6, flattened OLs (asterisk) appear to have produced membranous sheets (dashed line). Scale bars, 50  $\mu\text{m}$ .

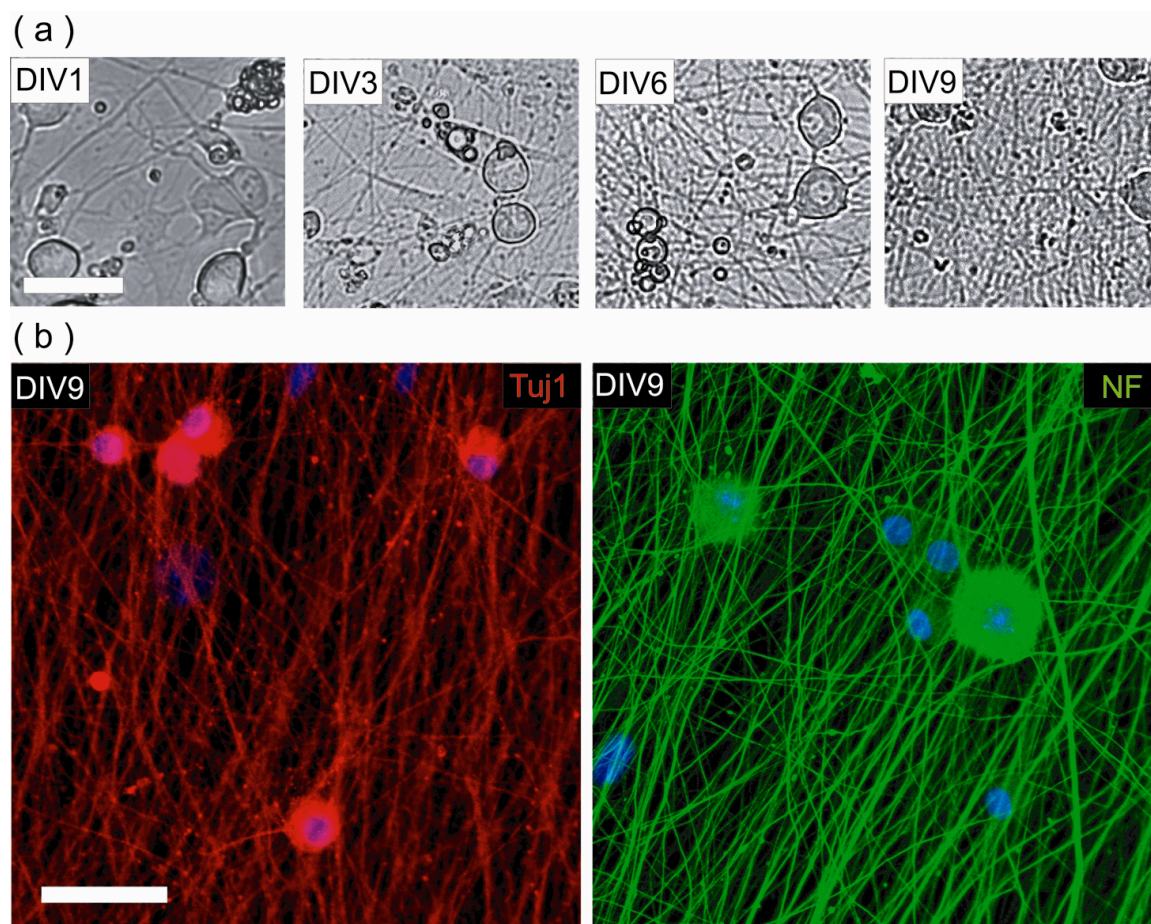




**Figure 2.3. Characterization of OL-enriched culture.** [a] Confocal images of isolated OLs at different stages of development. NG2<sup>+ve</sup> OPCs have simple morphology, whereas MAG<sup>+ve</sup> OLs possess multiple arborous processes. MBP<sup>+ve</sup> OLs have extended membranous myelin-like sheets. Scale bar, 50  $\mu$ m. [b] Purified OL-lineage cells originate as OPCs, and differentiate into MAG<sup>+ve</sup>, MBP<sup>+ve</sup> OLs over 6 DIV. At DIV1, all OPCs are NG2<sup>+ve</sup>, while none are MAG<sup>+ve</sup> or MBP<sup>+ve</sup>. At DIV3, MAG<sup>+ve</sup> and few MBP<sup>+ve</sup> OLs are now evident. The majority of OLs are MAG and MBP<sup>+ve</sup> at DIV6, with few remaining NG2<sup>+ve</sup> OPCs. Scale bar, 100  $\mu$ m. [c] Mean values  $\pm$  SD of the percent OL-lineage cells at different stages of development over 6 DIV. At DIV1, all OL-lineage cells are NG2<sup>+ve</sup>, accounting for 50 $\pm$ 14% of total cells within the culture. At DIV3 and DIV6, OL-lineage cells respectively account for 36 $\pm$ 6.8% and 32 $\pm$ 8.4% of total cells, consisting of varying proportions of NG2<sup>+ve</sup>, MAG<sup>+ve</sup> and MBP<sup>+ve</sup> OLs. [d] SDS-PAGE performed on protein derived from enriched OL-cultures demonstrating the graded expression of OL-markers CNP and MBP over the 6 DIV culture period.

This protocol also describes methods to establish OL/DRGN co-cultures using murine-only tissue sources. However, in order to produce the co-culture, DRGNs must first be cultured alone to produce an adequate neurite network. These post-natal murine neuron cultures are grown for 9 days in low serum media with 10  $\mu$ M FuDR supplementation to prevent the proliferation of contaminating fibroblasts and glial cells. Over the course of 9 days *in vitro*, isolated DRGNs produce a dense neurite bed (Fig 2.4a). This neurite bed is immunopositive for the neuronal markers neurofilament 200 (NF) and Tuj1 (Fig 2.4b). At this point, purified OPCs may be added to the neurite beds, and cultured for an additional 6 days to produce myelinating co-cultures.

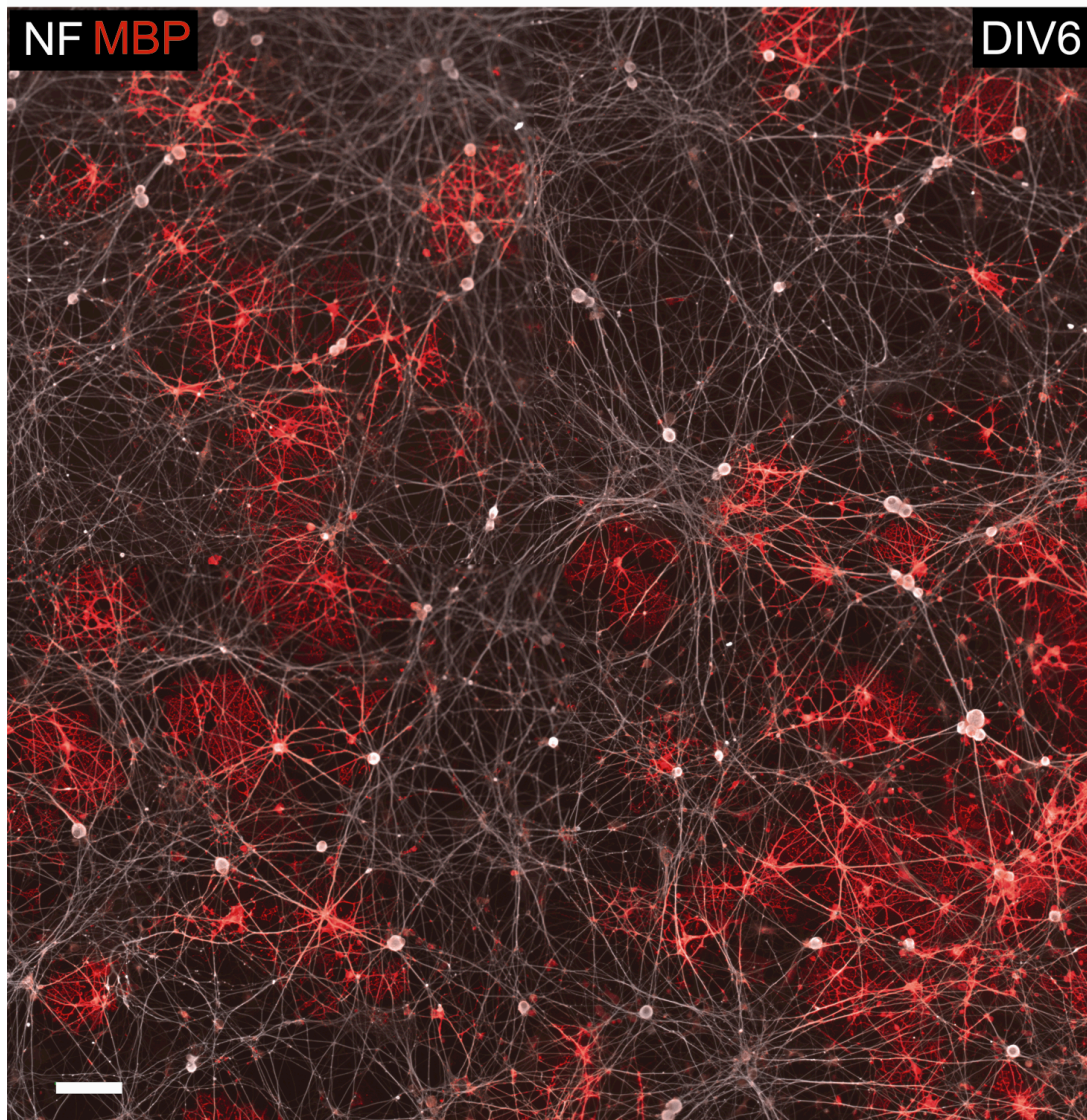
At DIV6 of OL/DRGN co-culture, many MBP<sup>+ve</sup> OLs can be observed among the NF<sup>+ve</sup> DRGN neurites (Fig 2.5a). Upon closer examination, OLs are evidenced to make contact with numerous DRGN neurites, often ensheathing them with an MBP<sup>+ve</sup> membrane (Fig 2.5b,c).



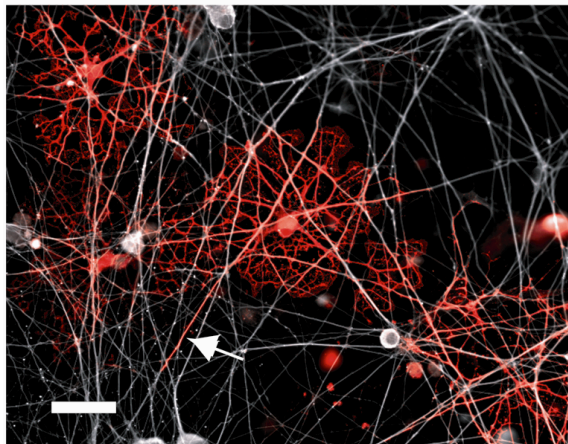
**Figure 2.4. Characterization of the DRGN culture pre-OPC seeding.** [a] Phase contrast images of DRGNs over the 9 DIV culture period pre-OPC seeding. DRGNs originate as large-bodied cells with few processes, and produce an increasingly complex neurite network. Scale bar, 100  $\mu\text{m}$ . [b] Confocal images of DRGN cultures fixed at DIV9 (pre-OPC seeding) and stained for neuron-specific markers Tuj1 and NF200. DRGNs have produced a neurite network upon which OPCs may be seeded to produce OL/DRGN myelinating co-cultures. Scale bar, 50  $\mu\text{m}$ .



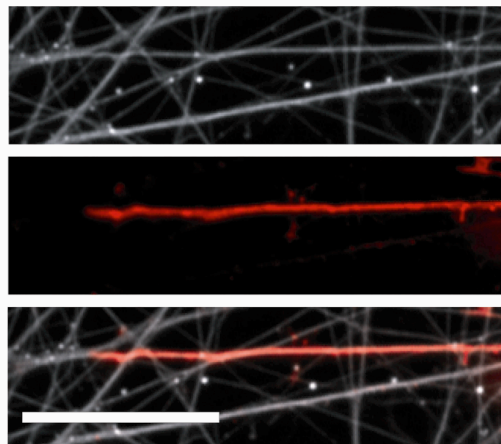
( a )



( b )



( c )



**Figure 2.5. OLs co-cultured with DRGNs result in OL-mediated wrapping of DRGN neurites with MBP<sup>+</sup> membrane.** [a] A 4-field confocal image montage of a DIV6 OL/DRGN co-culture. Many MBP<sup>+</sup> OLs can be seen interacting with the underlying DRGN neurite bed. Scale bar, 100  $\mu\text{m}$ . [b] A magnified confocal view of MBP<sup>+</sup> OLs wrapping multiple DRGN neurites. Scale bar, 50  $\mu\text{m}$ . [c] Digital magnification of the region denoted in [b] where a DRGN neurite is being wrapped with OL membrane. Scale bar, 25  $\mu\text{m}$ .

## Discussion

This report describes a method for isolating murine OPCs for differentiation in OL-enriched cultures or OL/DRGN co-cultures. When cultured alone, the OPCs differentiate into MBP<sup>+</sup>ve OLs, producing myelin-like membranous sheets. When added to DRGN neurite beds, OLs enwrap the DRGN neurites with MBP<sup>+</sup>ve membrane. This model benefits the investigation of the complex underpinnings governing OL-mediated axonal ensheathment.

While of great value, the establishment of such cultures is technically challenging. In particular, demanding aspects include efficient tissue digestion/dissociation, maintenance of balanced culture media pH, and DRGN media changes. It is important to consider that the length of digestion, amount of tissue being digested and the amount of trituration affects the efficacy and end result of the tissue dissociation. It is not unusual for experienced researchers to obtain low cellular yields from dissociated nervous system tissues. In addition, murine OPCs tend to be sensitive to changes in the pH of the culture media, particularly under alkaline conditions. The maintenance of cultures at 8.5% CO<sub>2</sub> aims to prevent this, since OPCs appear to better tolerate slightly acidic conditions over basic. With regards to feeding DRGNs, media changes must be performed quickly as to not desiccate the neurons, however, must be gentle as to not disrupt the developing neurite bed. Abrupt media changes may dislodge the neurite bed from the substrate, and likely result in its complete dissociation from the coverslip.

The potential merit of this model system greatly overshadows its technically demanding nature. One advantage of this system is the use of post-natal mice for cell culture derivation, circumventing the need to sacrifice breeding females to harvest

embryonic tissue. Another advantage is the lack of requirement for growth factors (GFs) for the expansion of OPCs. Mixed glial cultures provide an environment that supports the propagation of OPCs, presumably due to the presence of astrocyte-derived trophic factors. Other methods, such as derivation via neurospheres (Chen et al., 2007; Pedraza et al., 2008), rely on the mitogenic properties of GFs such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) for OPC expansion. Similarly, using postnatal (P5-10) mice for DRGNs avoids the requirement of supplementing the culture media with nerve growth factor (NGF), a neurotrophic factor required for the *in vitro* survival of embryonic DRGNs (Greene, 1977; Lewin et al., 1992). It is of interest to avoid using NGF as it negatively influences the myelinating capacity of OLs when cultured with DRGNs (Chan et al., 2004). Avoiding the use of GF-supplemented media also has economic benefits, as these reagents become costly when used on large scale.

Perhaps the most important benefit of this culture model is its derivation from mouse-only tissues, thus providing the opportunities to derive both OPCs and DRGNs from the wide variety of transgenic mouse lines. This allows for the study of both DRGN and/or OPC-specific properties that govern myelination. This will be especially important for elucidating the receptor/ligand interactions regulating OL-mediated myelination of axons. In all, this technique is of great value with regards to neuroscience research due to its applications towards understanding the molecular cues underlying myelination.

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**Disclosures**

The authors have nothing to disclose.



## **Chapter 3**

### **Integrin-Linked Kinase Regulates Process Extension in Oligodendrocytes via Control of Actin Cytoskeletal Dynamics**

**Integrin-linked kinase regulates process extension in oligodendrocytes via control of  
actin cytoskeletal dynamics**

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Running head: ILK regulates oligodendrocyte biology

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**Author Contributions**

RO, JPM and RK conceived and designed experiments. RO, JPM and RK analyzed and interpreted the results. JPM was the major contributor for figures 6, 7, 8 and part of 9. CA extensively contributed in a technical manner. KB helped with cloning, and PR's role aided in the acquisition and interpretation of electron micrographs. RO and JPM wrote the manuscript, RK provided feedback and edited the final product. RK oversaw the entirety of this work.

## Abstract

Integrin-linked kinase (ILK) is a major structural adaptor protein governing signaling complex formation and cytoskeletal dynamics. Here, through the use of conditional knockout mice, we demonstrate a requirement for ILK in oligodendrocyte differentiation and axonal myelination *in vivo*. In conjunction, ILK deficient primary oligodendrocytes are defined by a failure in process extension and an inability to form myelin membrane upon axonal contact. Surprisingly, phosphorylation of the canonical downstream targets Akt and GSK3 $\beta$  is unaffected following ILK loss. Rather, the defects are due in part to actin cytoskeleton dysregulation with a correspondent increase in active RhoA levels. Morphological rescue is possible following Rho kinase (ROCK) inhibition in an oligodendrocyte subset. Furthermore, phenotypic severity correlates with environmental complexity; oligodendrocytes are severely malformed *in vitro* (a relatively simple environment) but undergo phenotypic recovery in the context of the whole animal. Taken together, our work demonstrates ILK as necessary for normal oligodendrocyte development, reinforces its role as a bridge between the actin cytoskeleton and cell membrane, and highlights the overarching compensatory capacity of oligodendrocytes in response to cellular milieu.

## Introduction

During development, oligodendrocyte precursor cells (OPCs) differentiate into oligodendrocytes (OLs) that extend processes to contact multiple axons of central nervous system (CNS) neurons. The process of myelination is controlled in part by cell extrinsic factors derived from the extracellular matrix (ECM). Laminin-2 (Ln-2) is an ECM component with high relevance to OL-mediated myelination of the CNS (O'Meara et al., 2011a). Ln-2 is deposited in developing axonal tracts (Colognato et al., 2002), providing a ligand for the  $\alpha 6\beta 1$  integrin receptor expressed by OLs. The  $\beta 1$  integrin subunit was previously shown to regulate OL process extension *in vitro* (Buttery and Ffrench-Constant, 1999; Relvas et al., 2001; Liang et al., 2004; Barros et al., 2009) and myelination *in vivo* (Relvas et al., 2001; Lee et al., 2006; Barros et al., 2009; Câmara et al., 2009). In light of these findings, precisely how  $\beta 1$  integrin transduces ECM cues via signaling intermediates, and the mechanism by which this translates to cellular responses need to be addressed.

Integrin-linked kinase (ILK), a focal adhesion protein, binds to the cytosolic C-terminus of  $\beta 1$  integrin (Hannigan et al., 1996), and mediates signaling between the ECM and the cellular interior. ILK is composed of three domains, an N-terminal ankryin domain, a pleckstrin homology domain and a putative kinase domain. ILK forms an obligate heterotrimeric complex with PINCH and parvin, which together lend stability to the actin cytoskeleton at focal adhesions (Brakebusch and Fässler, 2003). This function is likely mediated through additional signaling proteins, such as paxillin and PIX, ultimately impacting on actin modulator proteins via Rho GTPases (Rosenberger et al., 2003).

In this study, we sought to investigate the role of ILK in OL differentiation and myelination of the CNS. By ablating ILK from cultured OPCs, we show that it is important for their molecular and morphological differentiation. Conditional ILK loss in post-natal mice results in amyelination of optic nerve axons, an effect that is compensated for at later time points. There is evidence of a bona fide kinase function for ILK (reviewed in Hannigan et al., 2011) however, we did not observe any changes in the phosphorylation status of its canonical targets GSK3 $\beta$  and Akt in ILK depleted OLs. Rather, we observe an increase in active RhoA levels as a result of ILK loss, a phenomenon associated with actin cytoskeletal defects. Interestingly, pharmacological manipulation of the RhoA pathway is able to rescue aberrant morphology of a distinct population of ILK-deficient OLs. Our data indicate an important role for ILK in OL development and myelination of the CNS, which, in turn, is linked to regulation of the actin cytoskeleton. Understanding OL differentiation will be important for rectifying or circumventing myelinating diseases such as Multiple Sclerosis, where demyelinated lesions are characterized by stalled premyelinating OLs (Chang et al., 2002).

## Materials and Methods

**Transgenic and reporter mice:** The mice used in this work were cared for according to Canadian Council on Animal Care (CCAC) guidelines. Ethical approval for experiments conducted was obtained from the University of Ottawa Animal Care Committee under protocol number OGH-119. *Ilk<sup>fl/fl</sup>* and *Plp-CreER<sup>T</sup>* mice were provided by Dr. Rene St-Arnaud (Terpstra et al., 2003) and Dr. Brian Popko (Doerflinger et al., 2003), respectively. Two mouse strains were primarily employed for experiments, *Ilk<sup>fl/fl</sup>;Plp-CreER<sup>T</sup>/+* mice (*Ilk cKO*), as well as *Ilk<sup>fl/fl</sup>;+/+* (WT) littermates controls. An injection of 0.375-0.5 mg of tamoxifen (7.5 or 10  $\mu$ L of a 50 mg/mL solution, respectively) was administered to P4 pups to induce recombination. For the low dose tamoxifen experiments, P4 mice were given a single 10  $\mu$ L injection from a 2 mg/mL tamoxifen solution to induce minimal recombination. *Ilk cKO* mice were crossed to a floxed stop tdTomato-EGFP (*mT/mG*) reporter line (Muzumdar et al., 2007) to generate *Ilk<sup>fl/fl</sup>;Plp-CreER<sup>T</sup>/+;mT/mG* (*Ilk cKO;mT/mG*) mice, which were bred to homozygosity for the reporter transgene. Mice of either sex were employed for all experiments.

**Cell culture:** OL cultures and OL/dorsal root ganglion neuron (DRGN) co-cultures were derived as previously described (O'Meara et al., 2011b). For ROCK inhibition, compound Y-27632 was added to each well at a final concentration of 10  $\mu$ M daily, for 6 days. For control wells, an equal volume of water (vehicle) was added.

**Immunohistochemistry:** Cell culture coverslips were fixed with 100% methanol at -20°C for 10 min or 3% paraformaldehyde (PFA) at room temperature for 15 min.

Coverslips were then washed with phosphate buffered saline (PBS), permeabilized with 0.1% Triton-X-100 solution, blocked with 10% goat serum (GS) and incubated with primary antibodies against myelin-associated glycoprotein (MAG; EMD Millipore), myelin basic protein (MBP; AbD Serotec), CC1 (Abcam), green fluorescent protein (GFP; Invitrogen), neurofilament-200 (NF-200; Sigma-Aldrich), cleaved caspase 3 (Cell Signaling [Asp175]), 5-bromo-2'-deoxyuridine (BrdU; BD Biosciences) and NG2 (EMD Millipore) in blocking solution at 4°C overnight. Coverslips were then washed with PBS, and incubated with Alexa fluor conjugated secondary antibodies (Alexa-488, Alexa-555, Alexa-647; Invitrogen) and/or rhodamine phalloidin (Invitrogen). Samples were then counterstained with 4',6-diamidino-2-phenylindole (DAPI) and mounted in DAKO (Dako North America, Inc.) mounting medium.

For immunohistochemistry of murine tissue, mice were anesthetized with tribromoethanol (Avertin) and perfused transcardially with 4% PFA. Optic nerves were dissected in PBS and post-fixed overnight in 4% PFA at 4°C followed by overnight cryopreservation in 30% sucrose/PBS at 4°C and then embedded in a 1:1 mixture of 30% sucrose/OCT (Sakura, CA). Briefly, 10 µm sections were washed with PBS followed by a 10-30 min incubation in citrate buffer (10 mM citric acid, 26 mM NaOH, pH 6) for certain antibodies. Sections were blocked in either 10% GS with 1% BSA and 0.2% Triton-X-100 or TBLS (0.5 mM Tris-HCl pH 7.4, 0.0085% NaCl, 0.01% BSA, 0.009% L-lysine, and 10% sodium azide) with 20% GS and 0.3% Triton-X-100 for 1 hour. Primary antibodies were diluted in either 1% GS with 1% BSA and 0.2% Triton-X-100 or TBLS with 20% GS and 0.3% Triton-X-100 and placed on sections overnight at 4°C. Sections were then washed and incubated with Alexa fluor conjugated secondary



antibodies (Invitrogen) in the same solution as primary antibodies. Sections were counterstained with DAPI and mounted in DAKO mounting medium.

**BrdU labeling:** DIV2 OL cultures were pulsed with approximately 20 nM of BrdU for 4 hours, and subsequently fixed with 3% PFA at room temperature for 15 min. Coverslips were washed in PBS, permeabilized with 0.1% Triton-X-100 solution, and treated with 2 N HCl for 20 min on a rotary shaker. Samples were then topped up with an equal volume of 0.1 M Tris-HCl pH 9.5 and incubated on the rotary shaker for an additional 20 min. Samples were then stained with anti-BrdU antibody using the same method as described above.

**Whole mount immunohistochemistry:** Mice were anesthetized with Avertin and sacrificed at either P14 or P28. The optic nerve was immediately removed and placed in a 4% PFA solution for 1 hour at room temperature with gentle agitation. The nerve was then washed in PBS followed by a 30 minute incubation in 2% Triton-X-100 and blocked in a 4% BSA, 1% Triton-X-100 solution. The optic nerve was then incubated overnight with an antibody against GFP in blocking solution. The next day, an Alexa fluor conjugated secondary antibody was applied in blocking solution for 4 hours, followed by washes in PBS. The optic nerves were finally counterstained with DAPI, given final washes in 0.1% Triton-X-100 and mounted in DAKO mounting medium. Images were acquired and processed as Z-stacks through the whole mount preparation to visualize OLs and associated internodes in their entirety.

**Electron microscopy:** Mice were anesthetized with Avertin and perfused transcardially with Karnovsky's fixative (4% PFA, 5% glutaraldehyde, 0.08 M sodium cacodylate, pH 7.4). The optic nerve was then removed and post-fixed in Karnovsky's fixative at 4°C. Fixed optic nerves were cut into ultrathin sections, stained with uranyl acetate and lead citrate, and analyzed by electron microscopy. Axon and myelin diameter was determined using the "analysis" tool in Image J set to a known scale. Number of myelinated fibers relative to total fibers was then determined and subdivided into groups by axon diameter. G-ratio was calculated by dividing the axon diameter by the axon plus myelin diameter.

**Western blot:** Mixed glial cultures were cooled on ice for 3 min, and washed with ice cold PBS. Cells were scraped off the dishes into protein lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40 [IGEPAL]) containing protease inhibitor cocktail (0.01 mg/mL pepstatin, 0.01 mg/mL aprotinin, 0.01 mg/mL leupeptin, 5 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM PMSF) and centrifuged at high speed to remove insoluble material. For optic nerves, protein was extracted through tissue homogenization in a lysis buffer solution (50 mM Tris pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 1% Triton-X-100) containing protease inhibitor cocktail plus 50 mM NaF. Optic nerves from three mice were pooled for each protein sample. For western blotting of proteins obtained from OL cultures, vessels were briefly cooled on ice before cells were lysed in a commercial lysis buffer (Cytoskeleton Inc.). Lysate was then centrifuged at high speed to remove insoluble material. Western blot exposure films were scanned with EPSON Perfection 2450 PHOTO scanner and images were imported into Image J. A box of standard dimensions was placed over each band of a given blot, and the mean gray value was measured for

each band. Levels of a given protein of interest were normalized to the GAPDH band intensity.

**G-LISA RhoA activation assay:** DIV3 vehicle and TAT-Cre treated primary OLs were scraped into RhoA G-LISA lysis buffer, and clarified lysates were flash frozen in liquid nitrogen. The G-LISA assay was conducted on the lysates according to the manufacturer's instructions (Cytoskeleton Inc.). Raw luminescence values were normalized to total RhoA levels as measured by western blotting.

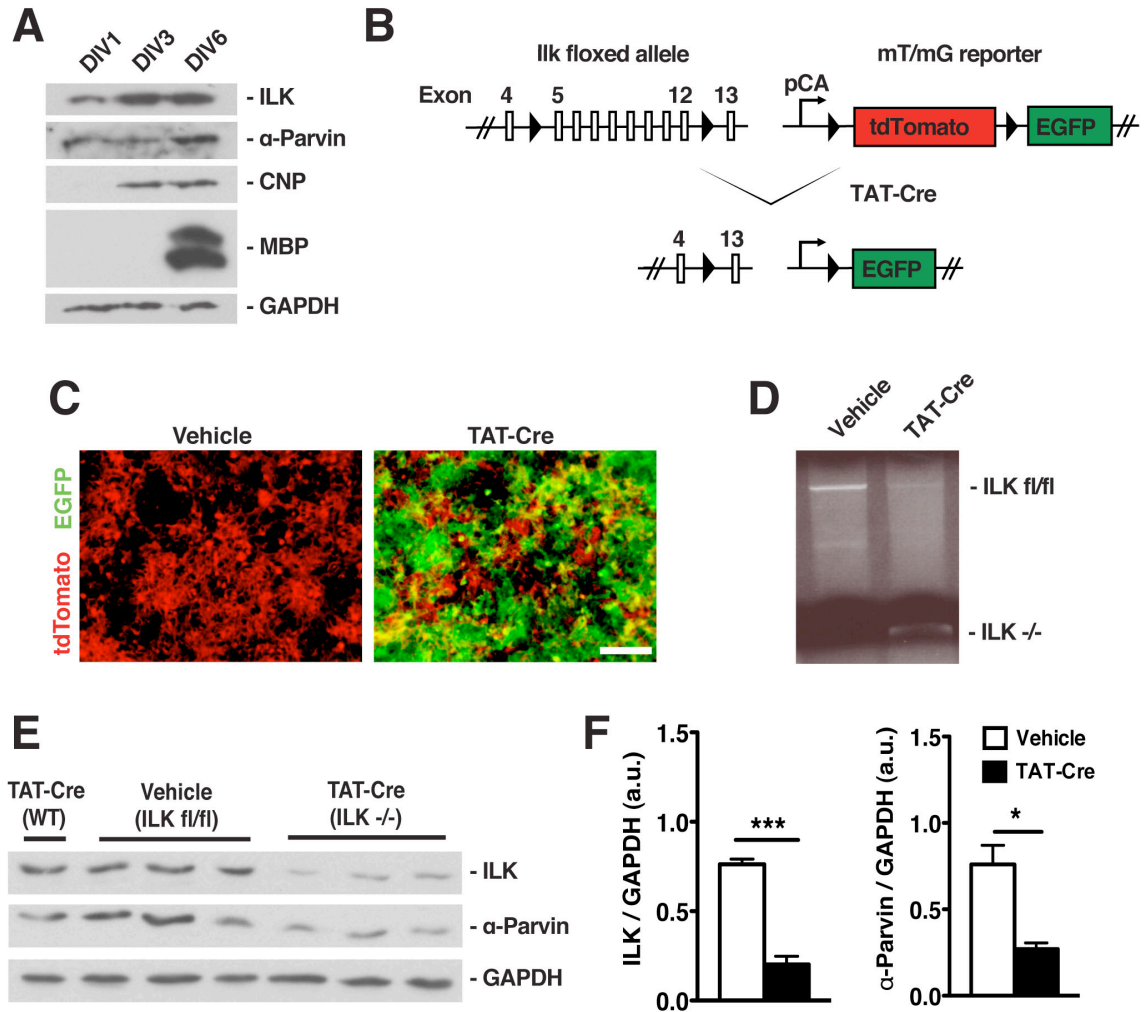
**His-TAT-NLS-Cre recombinase (TAT-Cre) treatment:** TAT-Cre (Excellgen) was added to mixed glial cultures at a concentration of approximately 2-5  $\mu$ M for 1-4 hours. The following day, the efficiency of the recombination was qualitatively assessed by EGFP fluorescence. OPCs were extracted from the mixed glial cultures and seeded onto Ln-2 substrate 72 hours post TAT-Cre administration as previously described (O'Meara et al., 2011b).

**Statistical analysis:** All statistical analyses were performed using Prism 5/6 GraphPad software. Unless stated otherwise, two-tailed Student's t-tests were employed for statistical analyses, with significance set at  $p < 0.05$ .

## Results

### ILK is required for primary OL differentiation

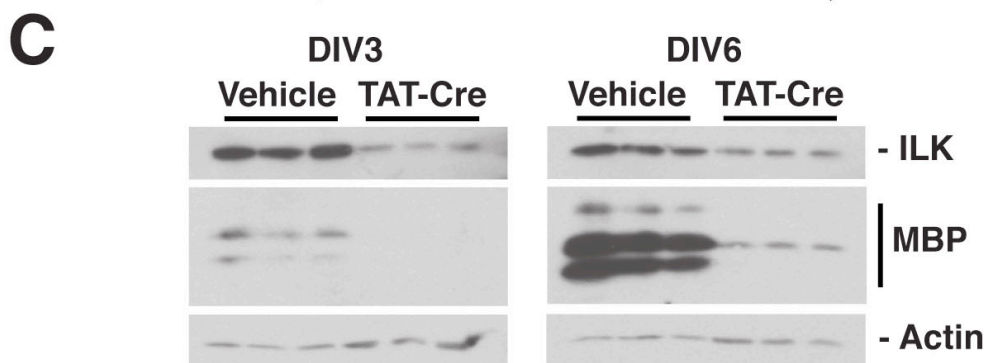
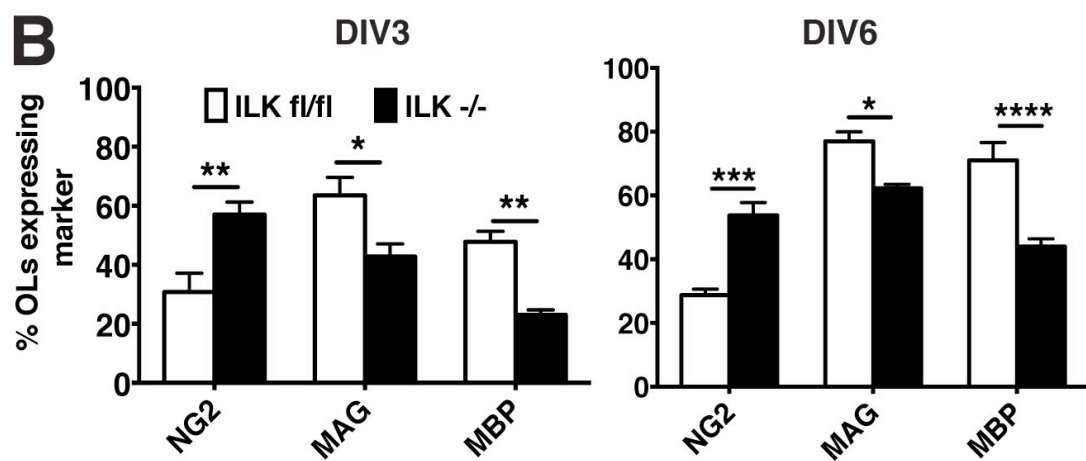
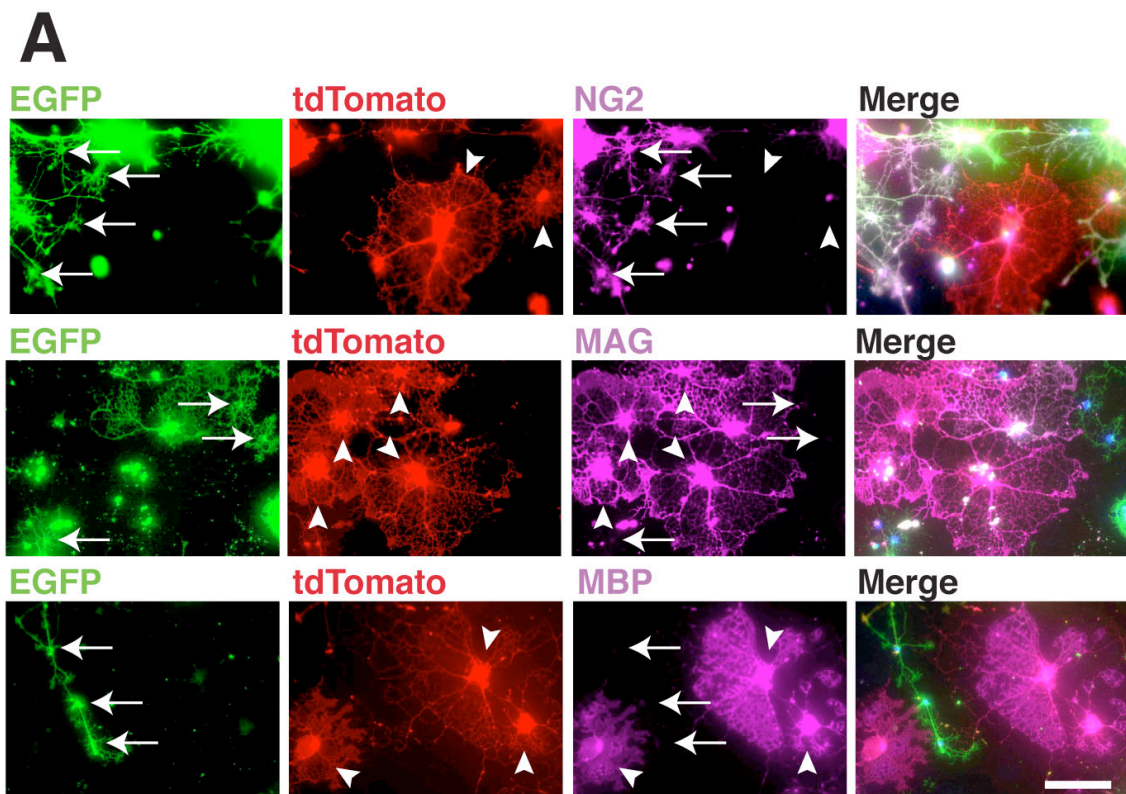
ILK is well known for its role in focal adhesion stabilization (Legate et al., 2006), a function highly dependent on its binding partner  $\alpha$ -parvin. Through  $\alpha$ -parvin, ILK is connected to the actin cytoskeleton, thus linking cellular interior and ECM. Both ILK and  $\alpha$ -parvin proteins were detected at all differentiation time points in cultured primary murine OLs (Figure 3.1A). Expression of 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNP) and MBP indicates the degree of OL differentiation in the cultures, as these are intermediate and late markers of OL differentiation, respectively (Figure 3.1A). To investigate the role of ILK in primary OL differentiation, we used a conditional knockout approach taking advantage of the *Ilk*<sup>fl/fl</sup> mutant mouse (Terpstra et al., 2003) (Figure 3.1B). In order to track which cells had undergone Cre-mediated recombination, *Ilk*<sup>fl/fl</sup> mice were crossed to the *mT/mG* reporter line (Muzumdar et al., 2007). Recombination was achieved in culture through the use of TAT-Cre (Peitz et al., 2002), resulting in cessation of tdTomato expression and induction of EGFP (Figure 3.1B, C) in conjunction with excision of the floxed region within the mouse *Ilk* gene (Figure 3.1D). Administration of TAT-Cre also significantly reduced ILK protein levels, and that of its obligate partner  $\alpha$ -parvin in transgenic mixed glial cultures (Figure 3.1E, F). OPCs were then derived from these TAT-Cre treated mixed glial cultures (as described in O'Meara et al., 2011b) and seeded on Ln-2 substrates as a purified population for further study.



**Figure 3.1. Genetic ablation of ILK from primary glial cells.** (A) Western blot analysis of ILK and  $\alpha$ -parvin in primary murine OLs at 3 time points of differentiation (DIV1, DIV3, DIV6). CNP and MBP are intermediate and late maturation markers, respectively. (B) Schematic showing the floxed *Ilk* allele, with loxP sites flanking the putative kinase domain (exons 5-12), and the *mT/mG* reporter allele. Cre recombination results in nullification of *Ilk*, concomitant with EGFP induction. (C) Immunofluorescence micrographs of mixed glial cultures derived from *Ilk<sup>fl/fl</sup>;mT/mG* mice treated with vehicle or TAT-Cre. (D) PCR performed on DNA isolated from TAT-Cre or vehicle treated *Ilk<sup>fl/fl</sup>* mixed glial cultures. (E) Western blot analysis for ILK and  $\alpha$ -parvin in *Ilk<sup>fl/fl</sup>;mT/mG*

mixed glial cultures treated with TAT-Cre or vehicle. Three independent samples of each are shown. TAT-Cre treated WT cells are shown as control for toxicity. **(F)** Densitometry analysis of western blots in (E) for ILK and  $\alpha$ -parvin between TAT-Cre and vehicle treated cultures. Data represents the mean  $\pm$  SEM (n=3). \* $p < 0.05$ , \*\*\* $p < 0.001$ ; Student's  $t$  test. Scale bar: 100  $\mu$ m.

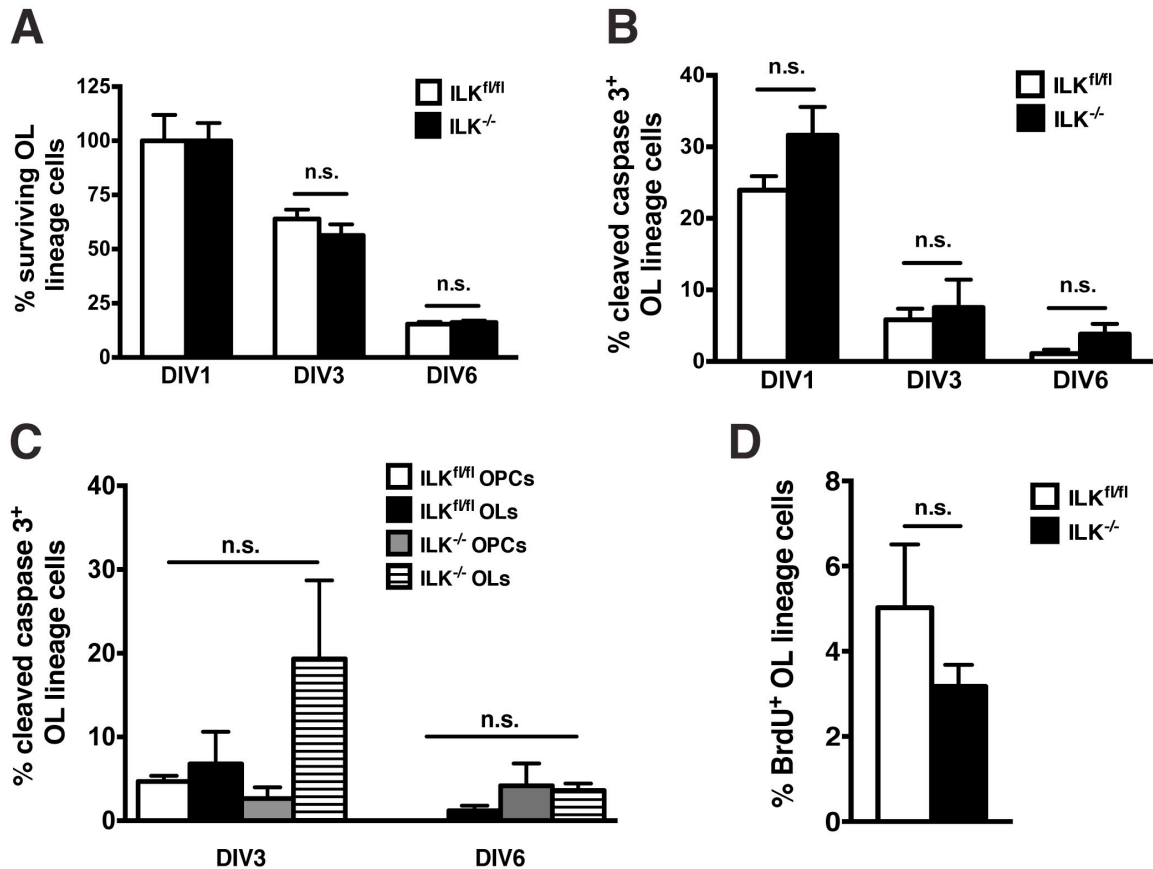
OPC differentiation requires both molecular and morphological cellular alterations. Molecular differentiation coincides with decreased expression of NG2 concomitant with MAG and MBP expression. Upon loss of ILK (*Ilk*<sup>-/-</sup>), a significant proportion of OPCs persisted as NG2<sup>+ve</sup> precursors at DIV3 and DIV6 of the differentiation time course (Figure 3.2A, B). Accordingly, fewer *Ilk*<sup>-/-</sup> OLs expressed MAG or MBP as compared to wild type (*Ilk*<sup>fl/fl</sup>) cells. However, a certain proportion of *Ilk*<sup>-/-</sup> OLs expressed MAG at DIV3 and DIV6, thus persistence of NG2<sup>+ve</sup> precursors was not a completely penetrant phenotype in response to ILK loss. Furthermore, MBP protein levels (as measured by western blot) were decreased in TAT-Cre treated cultures at both DIV3 and 6 (Figure 3.2C).





**Figure 3.2. Loss of ILK impacts the expression of developmental stage-specific OL markers.** (A) Immunostaining of TAT-Cre treated DIV6 OL cultures for NG2, MAG and MBP. Arrows and arrowheads indicate *Ilk*<sup>-/-</sup> and *Ilk*<sup>fl/fl</sup> OLs respectively. (B) Quantification of percent of *Ilk*<sup>-/-</sup> versus *Ilk*<sup>fl/fl</sup> OLs expressing NG2, MAG or MBP at DIV3 and DIV6. (C) Western blots of TAT-Cre and vehicle lysates probed for ILK and MBP, with actin serving as a loading control. Data represents the mean  $\pm$  SEM (n=4). \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001; two-way ANOVA followed by Bonferonni post-tests. Scale bar: 100  $\mu$ m.

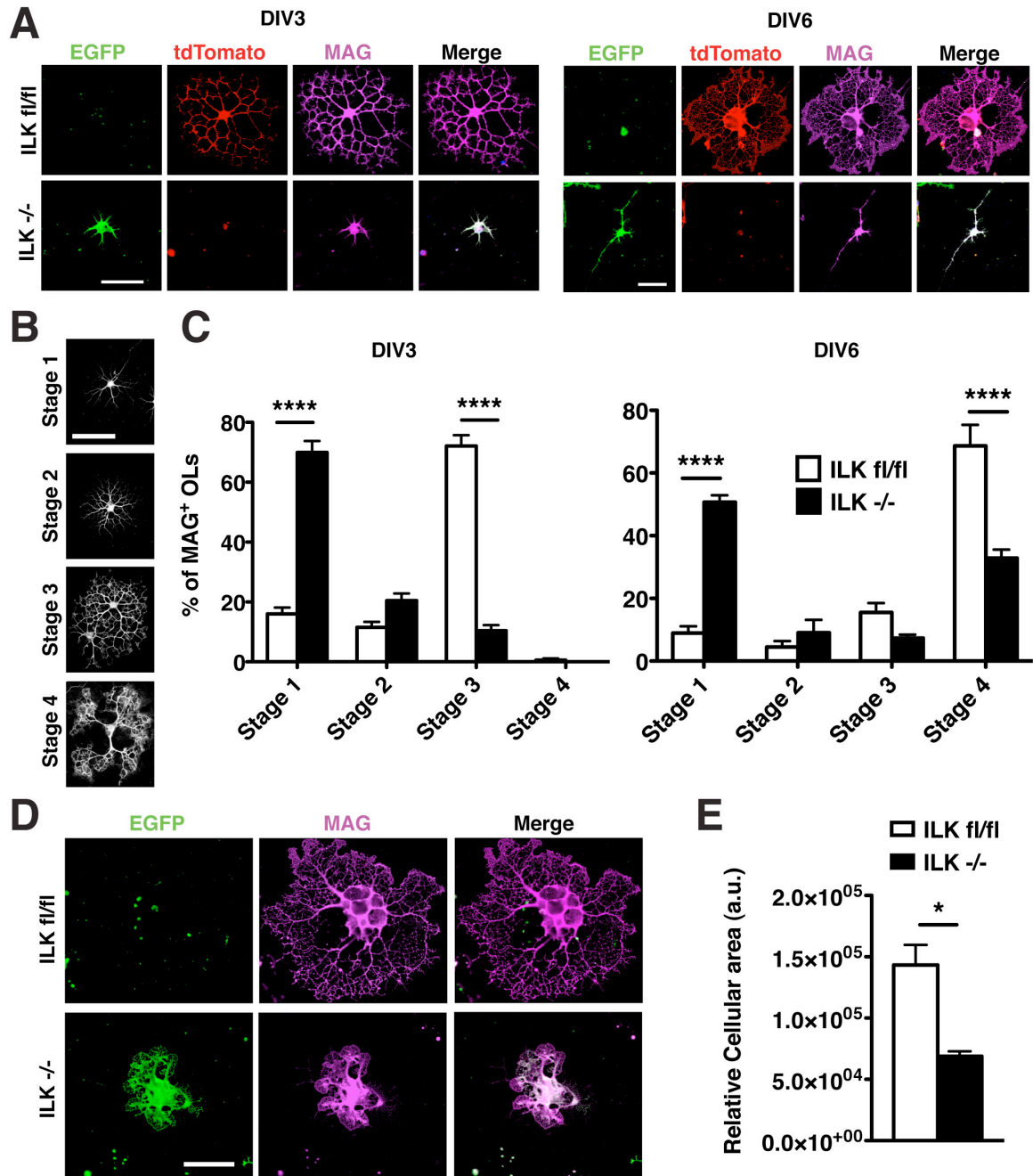
To investigate whether this apparent delay in lineage progression was an artifact of differential expansion/collapse of *Ilk*<sup>-/-</sup> or *Ilk*<sup>fl/fl</sup> OL populations, we conducted a series of cell death and proliferation assays. When total numbers of *Ilk*<sup>-/-</sup> and *Ilk*<sup>fl/fl</sup> OL-lineage cells were respectively considered as 100% at DIV1, there was no subsequent difference in cell counts between the genotypes at either DIV3 or DIV6 (Figure 3.3A). This suggests that loss of ILK does not translate to compromised survival of OL-lineage cells. In addition, there was no significant difference in the percentage of cleaved caspase 3<sup>+ve</sup> OL-lineage cells at DIV1, DIV3 or DIV6 (Figure 3.3B). When this same cleaved caspase 3 data was parsed to segregate mature and immature OL-lineage cells, we still did not observe any significant difference in cleaved caspase 3 activity between *Ilk*<sup>-/-</sup> and *Ilk*<sup>fl/fl</sup> populations (Figure 3.3C). Finally, we investigated whether there were differences in the rate of proliferation between *Ilk*<sup>-/-</sup> and *Ilk*<sup>fl/fl</sup> OPCs. We did not observe any difference in BrdU incorporation after a 4-hour pulse at DIV2 (Figure 3.3D). Thus, our data strongly suggest a role for ILK in the initiation of a molecular differentiation program triggering MAG and MBP expression. Simply stated, upon loss of ILK, OPCs tend to persist as NG2<sup>+ve</sup> precursors throughout the 6-day differentiation time course.



**Figure 3.3. No significant difference in OL survival, cleaved caspase 3-mediated death or proliferation upon ILK depletion. (A)** Percentage of surviving OL-lineage cells over the 6 day differentiation time course when total numbers of *Ilk*<sup>-/-</sup> and *Ilk*<sup>fl/fl</sup> cells were respectively considered as 100% at DIV1. **(B)** Percentage of cleaved caspase 3<sup>+</sup> OL-lineage cells at DIV1, DIV3 and DIV6 for *Ilk*<sup>-/-</sup> and *Ilk*<sup>fl/fl</sup> populations. **(C)** DIV3 and DIV6 cleaved caspase 3 data from panel [B] when immature OPCs and mature OLs are considered as distinct populations. **(D)** Percentage of BrdU<sup>+</sup> OLs after a 4-hour pulse on DIV2. Data represents the mean  $\pm$  SEM (n=4 [A]; n=3 [B,C,D]); n.s. = no significant difference. Two-way ANOVA followed by Bonferonni post-tests (A,B,C); Student's *t* test (D).

### Loss of ILK inhibits morphological maturation of OLs in primary culture

OL morphological differentiation is characterized by extension of multiple processes by OPCs, eventually generating myelin-like membranous structures. This morphological maturation coincides with the expression of MAG. At DIV3 of differentiation,  $MAG^{+ve} Ilk^{fl/fl}$  OLs had developed a complex arbor network, and at DIV6, these OLs had further differentiated to form myelin-like membranes (Figure 3.4A top row). Interestingly, most  $MAG^{+ve} Ilk^{-/-}$  OLs did not produce either an arbor network or myelin-like membranes (Figure 3.4A bottom row). Rather, they only extended a few primary arbors. To quantify this observation, a staging scheme was developed, from stage 1 to 4, where cells were binned based on their increasing morphological complexity (Figure 3.4B). At DIV3,  $MAG^{+ve} Ilk^{-/-}$  OLs were predominantly stage 1, whereas  $MAG^{+ve} Ilk^{fl/fl}$  OLs were largely stage 3 (Figure 3.4C). At DIV6, most  $MAG^{+ve} Ilk^{fl/fl}$  OLs were of stage 4 morphology, but the majority of  $MAG^{+ve} Ilk^{-/-}$  OLs persisted as stage 1 (Figure 3.4C). A subset of  $Ilk^{-/-}$  OLs attained stage 4 morphology at DIV6, however, their cellular area was significantly reduced as compared to stage 4  $MAG^{+ve} Ilk^{fl/fl}$  OLs (Figure 3.4D, E). The observed morphological deficits resulting from ILK loss were not a consequence of EGFP induction, as Cre-recombined  $Ilk^{+/+};mT/mG$  OLs displayed normal morphology (data not shown).



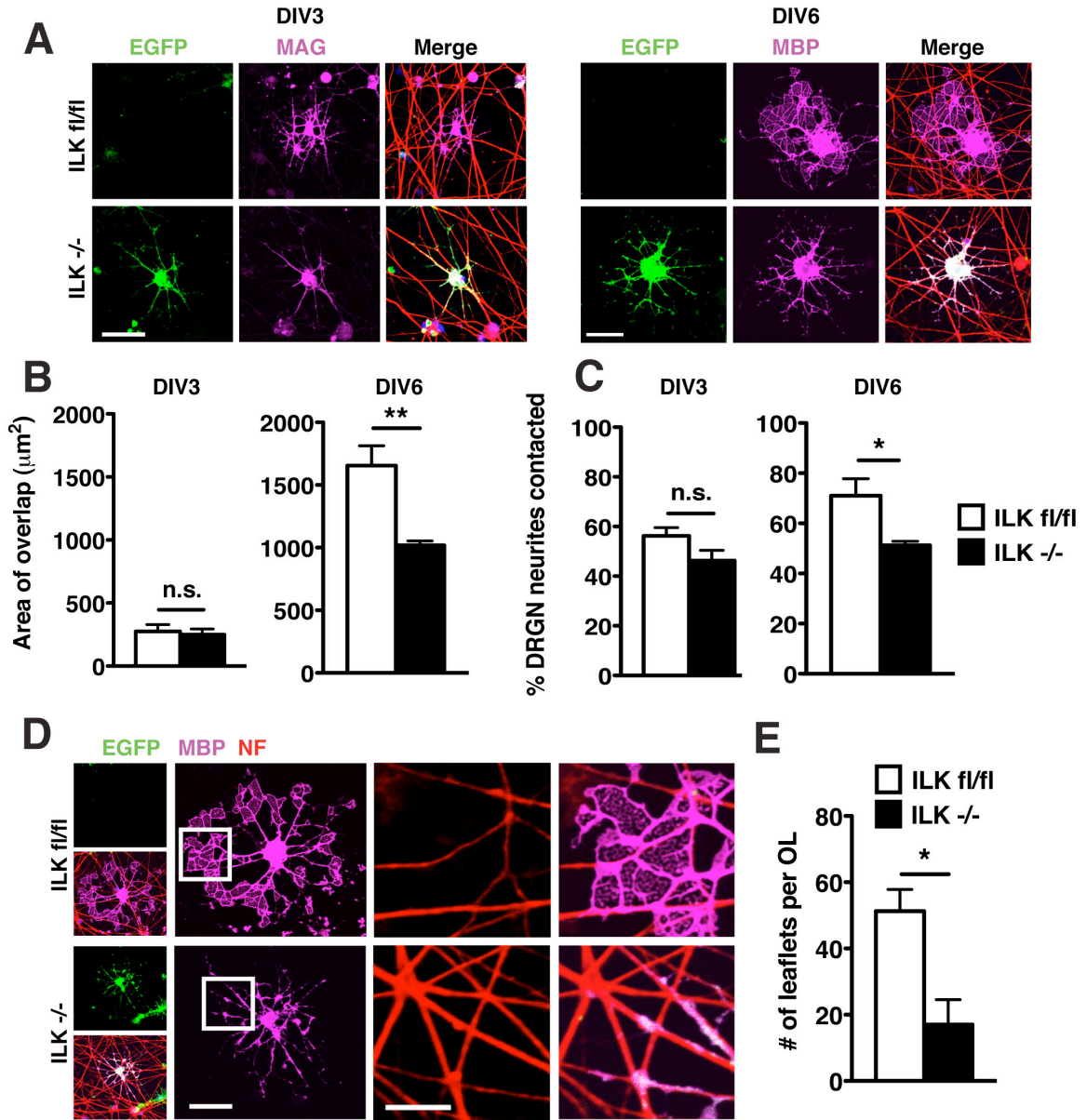
**Figure 3.4. Loss of ILK perturbs morphological maturation of OLs.** (A) Immunofluorescence micrographs depicting morphology of MAG<sup>+</sup> *Ilk*<sup>-/-</sup> and *Ilk*<sup>fl/fl</sup> at DIV3 and DIV6. (B) Staging scheme used to categorize the morphological complexity of MAG<sup>+</sup> OLs. (C) Quantification of percent of *Ilk*<sup>-/-</sup> and *Ilk*<sup>fl/fl</sup> MAG<sup>+</sup> OLs that fall into each of the 4 morphological stages at DIV3 and DIV6. (D) Immunofluorescence

micrographs showing the extent of membrane extension of stage 4 *Ilk*<sup>-/-</sup> and *Ilk*<sup>fl/fl</sup> OLs.

**(E)** Quantification of the average cellular area of stage 4 *Ilk*<sup>-/-</sup> and *Ilk*<sup>fl/fl</sup> OLs. Data represents the mean  $\pm$  SEM (n=4). \*p < 0.05, \*\*\*\*p < 0.0001; Student's *t* test (E) or two-way ANOVA followed by Bonferonni post-tests (C). Scale bars: 50  $\mu$ m.

### **Loss of ILK impacts *in vitro* myelinating capacity of OLs**

Due to the morphological deficits observed when *Ilk*<sup>-/-</sup> OLs are cultured on Ln-2, we hypothesized that this would translate to compromised myelinating capacity in an *in vitro* myelination paradigm. To this end, we utilized DRGNs in co-culture with primary OLs (as described in O'Meara et al., 2011b). At DIV3 of co-culture, there was no significant difference in the capacity for *Ilk*<sup>-/-</sup> OLs to contact and enwrap DRGN neurites as compared to *Ilk*<sup>fl/fl</sup> OLs (Figure 3.5A-C). However, at DIV6, *Ilk*<sup>-/-</sup> OLs contacted fewer DRGN neurites and had a reduced capacity to enwrap neighboring neurites with MBP<sup>+</sup> membrane (Figure 3.5A-C). The driving force underlying these deficits appeared to be an impaired ability of *Ilk*<sup>-/-</sup> OLs to form myelin leaflets (Figure 3.5D). These structures were observed solely when OLs were co-cultured with neurons, and may represent an *in vitro* version of early myelin sheaths. *Ilk*<sup>-/-</sup> OLs produced significantly fewer myelin leaflets per cell than *Ilk*<sup>fl/fl</sup> OLs (Figure 3.5E). These defects were not a result of ectopic EGFP expression, as *Ilk*<sup>+/+</sup>;mT/mG OLs myelinated DRGN neurites normally, and produced expected numbers of myelin leaflets (data not shown). In addition, we confirmed that there were no differences in the density of DRGN neurite bed underlying myelinating OLs (data not shown), suggesting the OLs themselves were solely responsible for the myelination phenotypes in response to ILK loss.



**Figure 3.5. Loss of ILK disrupts ability of OLs to myelinate axons *in vitro*.** (A) Immunofluorescence micrographs of *Ilk*<sup>-/-</sup> and *Ilk*<sup>fl/fl</sup> OLs myelinating DRGN neurites at DIV3 and DIV6 of co-culture. (B) Quantification of the area of overlap of myelin membrane with DRGN neurites by *Ilk*<sup>-/-</sup> and *Ilk*<sup>fl/fl</sup> OLs at DIV3 and DIV6. (C) Quantification of the percentage of neighbouring DRGN neurites contacted by *Ilk*<sup>-/-</sup> and *Ilk*<sup>fl/fl</sup> OLs. (D) Immunofluorescence micrographs depicting the structure of membrane extensions/leaflets for *Ilk*<sup>-/-</sup> and *Ilk*<sup>fl/fl</sup> OLs. (E) Quantification of the number of membrane

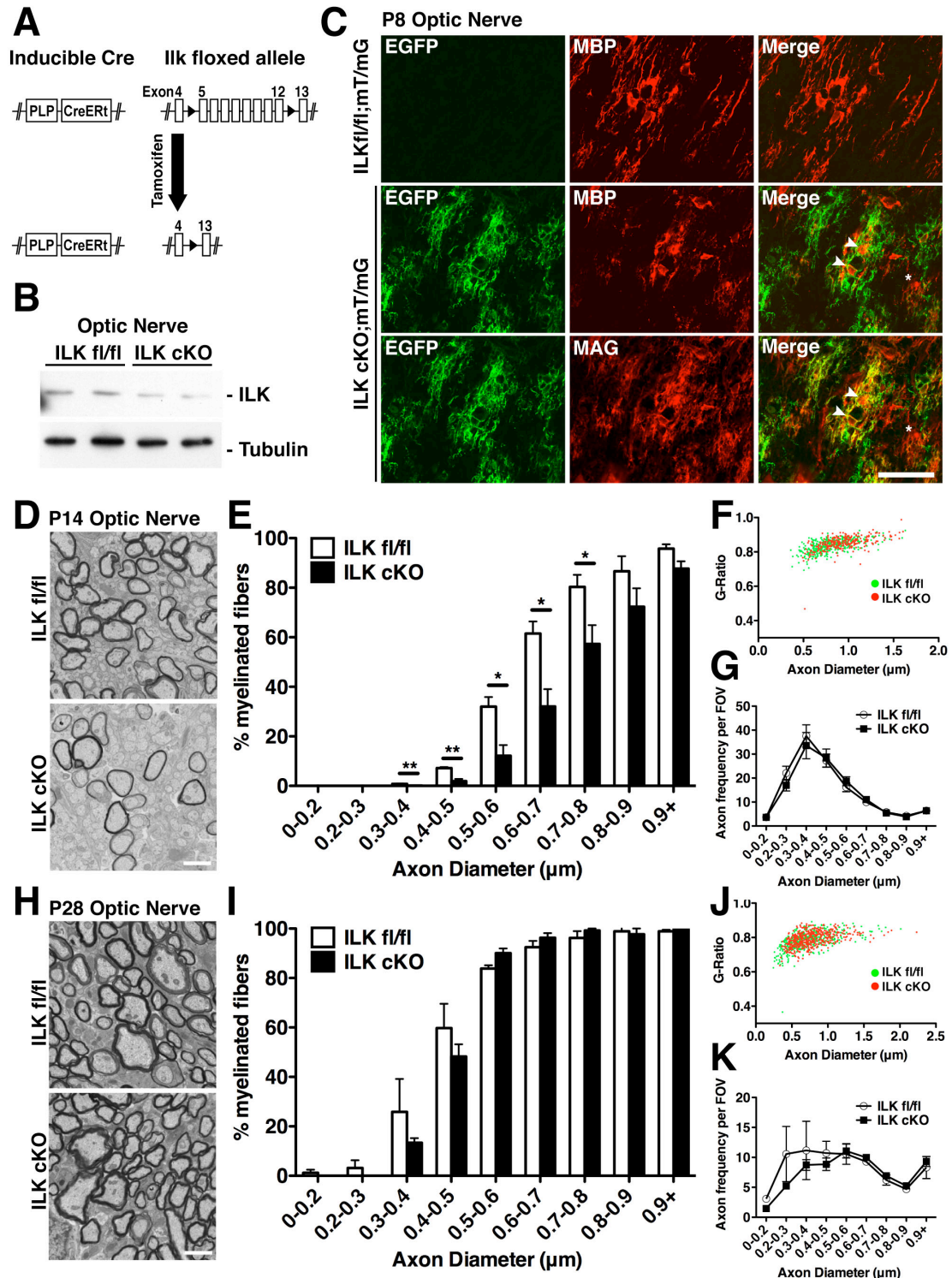


extensions produced per cell for  $Ilk^{-/-}$  and  $Ilk^{fl/fl}$  OLs. Data represents the mean  $\pm$  SEM (n=4). \*p < 0.05, \*\*p < 0.01; Student's *t* test. Scale bar: 50  $\mu$ m.

### **Generation of ILK conditional knockout mice for *in vivo* study**

To study the role of ILK in CNS myelination *in vivo* and avoid embryonic lethality associated with ILK deletion, we employed the Cre-loxP system. *Ilk<sup>fl/fl</sup>* mice were crossed to *Plp-CreER<sup>T</sup>* mice to generate *Ilk<sup>fl/fl</sup>;Plp-CreER<sup>T</sup>* conditional knockout mice (hereafter referred to as *Ilk cKO* mice). The *Plp-CreER<sup>T</sup>* line of mice allows for spatial and temporal control of floxed *Ilk* recombination; the Cre gene is placed under the control of the *proteolipid protein (Plp)* promoter, allowing for OL-specific expression (Doerflinger et al., 2003). As well, the Cre protein is fused to a mutated estrogen receptor, requiring tamoxifen administration for nuclear translocation (Figure 3.6A).

The optic nerve was chosen as a model system to study the impact of *Ilk* gene deletion on myelination, as it offers a relatively homogeneous population of cell types, with a large percentage of OLs, when compared to other CNS tissues. Since myelination of the optic nerve is initiated approximately 6 days post-natally (P6) (Thomson et al., 2005), *Ilk cKO* mice and WT littermates were administered tamoxifen at P4. Western blot analysis revealed a decrease in total ILK protein levels in the optic nerves of these mice following tamoxifen injection (Figure 3.6B). To verify specificity of the promoter to the OL lineage, *Ilk cKO* mice were crossed to the *mT/mG* reporter line (generating *Ilk cKO;mT/mG* mice). In these mice, EGFP is expressed predominantly in cells positive for the myelin markers MBP and MAG (Figure 3.6C), demonstrating the specificity of the *Plp* promoter to OLs.



**Figure 3.6.** Number of myelinated axons is decreased in the optic nerves of tamoxifen treated *Ilk cKO* mice at P14 but not at P28. (A) *Ilk cKO* mice were generated by crossing *Plp-CreER<sup>T</sup>* transgenic mice with *Ilk<sup>fl/fl</sup>* mice. Tamoxifen was

administered to induce Cre translocation into the nucleus whereupon the *Ilk* gene was excised. **(B)** Western blot analysis demonstrates reduction of ILK protein in the optic nerves of P4 tamoxifen injected *Ilk cKO* mice in comparison to WT controls. **(C)** Optic nerves from control *Ilk<sup>fl/fl</sup>;mT/mG* littermates at P8 contain MBP<sup>+ve</sup> OLs but no EGFP signal. Optic nerves from P4 tamoxifen injected *Ilk cKO;mT/mG* mice sacrificed at P8 show co-localization of MBP/MAG<sup>+ve</sup> OL cell bodies (both stains are on the same optic nerve section) with EGFP (arrowheads). There are also MBP/MAG<sup>+ve</sup> cell bodies that do not express EGFP (asterisk). Scale bar: 50  $\mu$ m. **(D,H)** Electron micrographs of optic nerves from P14 and P28 *Ilk cKO* and control mice given tamoxifen at P4. **(E,I)** Quantification for percentage myelinated axons at P14 and P28, further subdivided by axon caliber. **(F,J)** Representative scatter plot of G-ratio versus axon diameter at P14 and P28. **(G,K)** Frequency histogram for axons of varying diameter per field of view (FOV) (3000x magnification) at P14 and P28. Data represents the mean  $\pm$  SEM (n=3-4). \*p < 0.05, \*\*p < 0.01; Student's *t* test (E,I), two-way ANOVA with Bonferroni post-tests (G,K). Scale bars: 1.5  $\mu$ m.

### **Transient myelination following ILK ablation *in vivo***

Initial analysis of *Ilk cKO* mice revealed no overt signs of shaking, ataxia, tremor, or head wobbling following tamoxifen injection at P4. Mice were sacrificed at two developmental time points, P14 and P28, for ultrastructural analysis by electron microscopy. The optic nerves of P14 *Ilk cKO* mice displayed a significant reduction in total number of myelinated small caliber axons (0.3-0.8  $\mu\text{m}$ ) relative to WT littermate controls (Figure 3.6D, E). In contrast, there was no significant difference in number of myelinated large caliber axons (0.8  $\mu\text{m}$  and above) at this time point (Figure 3.6D, E). That small rather than large caliber axons are affected is in concordance with previous work done on OL-specific knockout mice for other members of the integrin-signaling pathway (Chun et al., 2003; Câmara et al., 2009; Forrest et al., 2009). To determine whether myelin thickness was also affected by ILK loss, we assessed the G-ratio (axon diameter/axon and myelin diameter) of axons in two separate groups; those  $< 0.8 \mu\text{m}$  and those  $\geq 0.8 \mu\text{m}$  in diameter (Figure 3.6F). There was no significant difference in the average G-ratio of axons either  $< 0.8 \mu\text{m}$  or those  $\geq 0.8 \mu\text{m}$ . As well, no difference was observed in axonal distribution (by diameter) between WT and *Ilk cKO* littermates (Figure 3.6G), excluding a shift in axon caliber frequency as responsible for the observed decrease in total number of myelinated small caliber axons.

Interestingly, when *Ilk cKO* mice were analyzed at P28, there was no observable difference in total number of myelinated fibers (at any given diameter) relative to control mice (Figure 3.6H, I). In addition, there was no significant difference in the average G-ratio of axons with diameters either  $< 0.8 \mu\text{m}$  or  $\geq 0.8 \mu\text{m}$  in P28 optic nerves (Figure 3.6J). Furthermore, no significant shift was observed between control and *Ilk cKO* mice

in axonal distribution by diameter (Figure 3.6K), discounting the possibility of axonal loss as explanation for the resurgence of small myelinated fibers by P28. Rather, our results imply gradual myelination recovery of P14 amyelinated axonal populations in the ILK depleted optic nerve.

The transient defects observed are due neither to a loss or a gain in total number of OLs as measured by NG2<sup>+</sup> OPCs and CC1<sup>+</sup> mature OLs at P14 or P28 (Figure 3.7A, B). To assess the possibility of OL turnover, we measured the number of cleaved caspase 3<sup>+</sup> cells in the optic nerves of P14 *Ilk cKO* and WT mice. No significant increase in the number of apoptotic cells was observed in ILK depleted optic nerves (Figure 3.7C). Furthermore, qualitative analysis for the abundance of recombined (EGFP<sup>+</sup>) *Ilk*<sup>-/-</sup> OLs in the optic nerves of *Ilk cKO;mT/mG* mice demonstrated a persistent EGFP<sup>+</sup> cell population capable of forming MBP<sup>+</sup> myelin tracts across multiple time points (data not shown). If *Ilk*<sup>-/-</sup> OLs were indeed being turned over, we would expect a progressive loss in EGFP signal as *Ilk*<sup>-/-</sup> cells were replaced by WT precursors.

In an effort to draw parallels between defects observed *in vitro* (morphological aberrations) and *in vivo* (loss of myelination), we employed a low dose tamoxifen regimen in our *Ilk cKO;mT/mG* and *PLPCreER<sup>T</sup>;mT/mG* control animals. Reducing tamoxifen levels allowed for spatial resolution of individual recombined OLs and associated myelin processes. Following administration of the drug, mice were sacrificed at P14 and the optic nerves were removed for whole mount preparations. A series of Z-stack images were taken so as to capture recombined OLs in their entirety. Strikingly, at a strictly qualitative level, many of the ILK depleted OLs displayed fully formed myelin

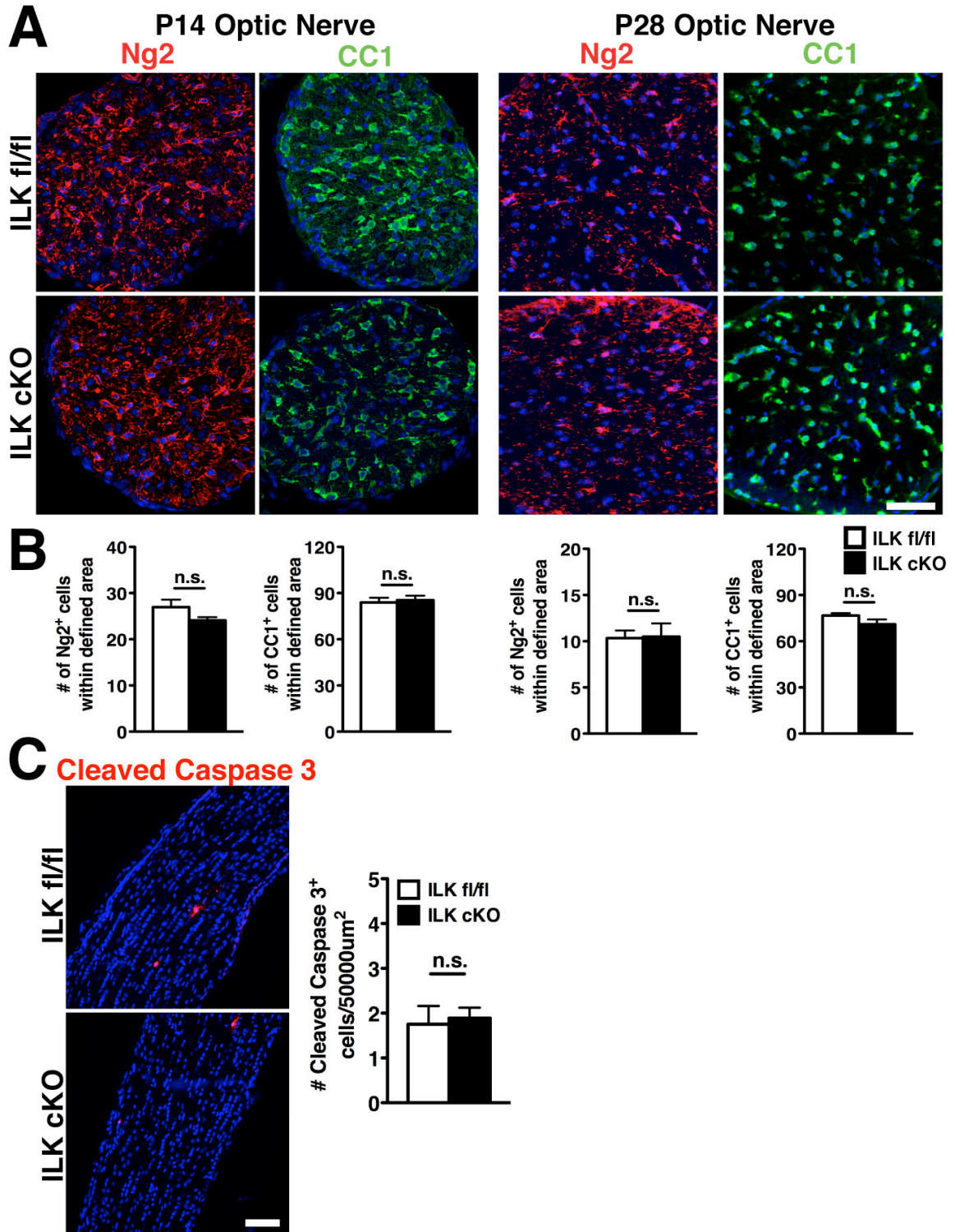
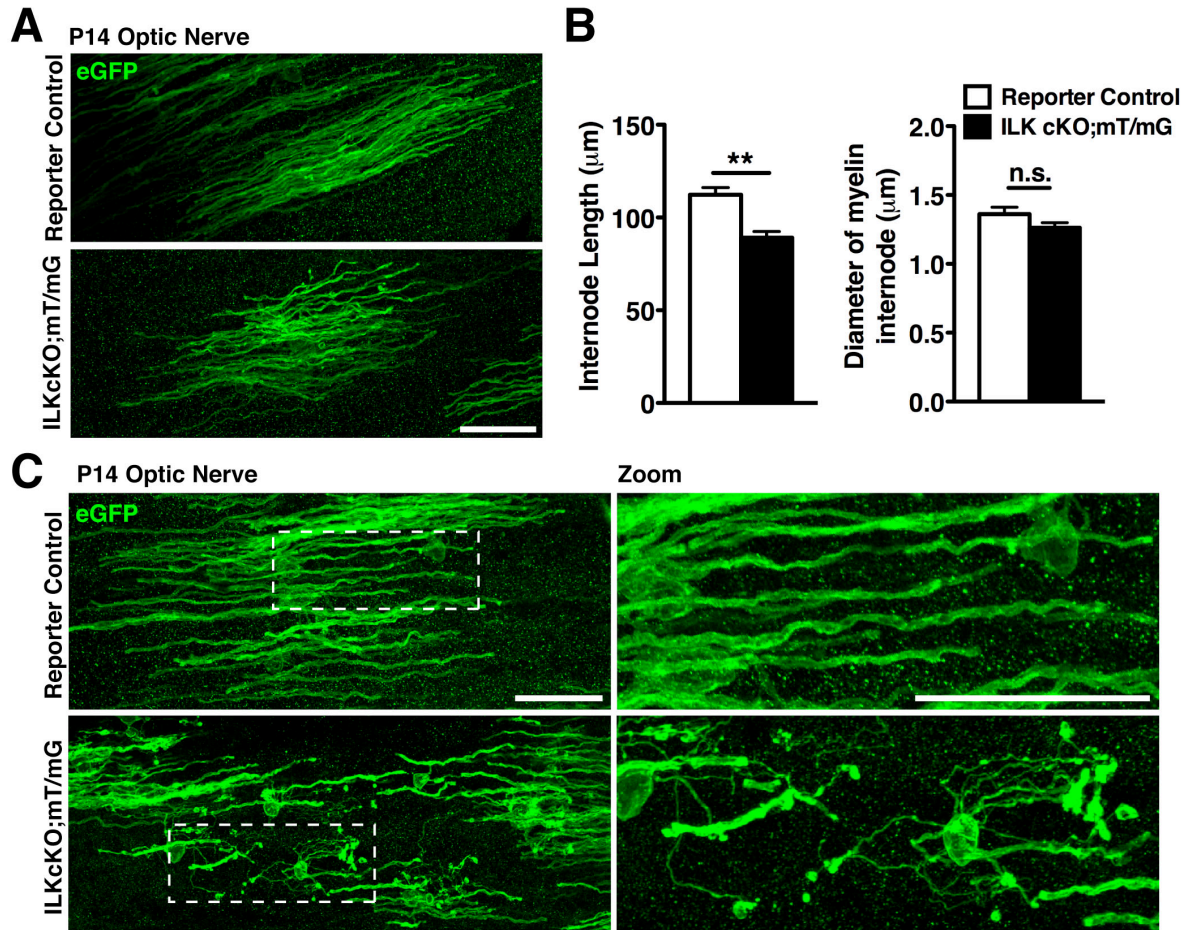


Figure 3.7. Number of precursor and mature OLs is unchanged in optic nerves of *Ilk cKO* mice. (A) Immunofluorescence micrographs of NG2<sup>+</sup> OPCs (red) and CC1<sup>+</sup>

mature OLs (green) counterstained with DAPI in the optic nerves of *Ilk cKO* and WT mice at P14 or P28. **(B)** Quantification of NG2<sup>+ve</sup> OPCs and CC1<sup>+ve</sup> mature OLs at P14 and P28. Respective cells were counted within a defined area of the optic nerve. **(C)** Immunofluorescence micrographs of cleaved-caspase 3<sup>+ve</sup> cells counterstained with DAPI along the length of an optic nerve in P14 *Ilk cKO* and WT mice. Quantification for total number of cleaved-caspase 3<sup>+ve</sup> cells within 50000  $\mu\text{m}^2$  of the P14 optic nerve. Data represents the mean  $\pm$  SEM (n=3), Student's *t* test. Scale bars: 50  $\mu\text{m}$ .



internodes similar to controls (Figure 3.8A). However, the average internode-length was less than that measured for reporter controls (Figure 3.8A, B), corroborating our *in vitro* data for a subset of mature ILK-depleted OLs which form smaller amounts of membrane. As well, loss in internode-length implies a defect in the growth of the process responsible, as the distance it is able to travel around the axon will determine length of the myelin segment. In conjunction we measured internode diameter to account for possible bias as a result of axon diameter (larger axons having longer myelin internodes and vice versa). No significant difference was observed between ILK-depleted and control OLs (Figure 3.8B) eliminating the variable as potential confounder. We did, however, observe ILK-depleted OLs whose processes failed to form myelin internodes (Figure 3.8C). While occasionally present in controls, the defect was more prevalent in the optic nerves of *Ilk cKO;mT/mG* mice, suggesting a role for ILK in directing myelin formation following axonal contact and/or process extension *in vivo*, defects observed in both purified and co-cultured OLs *in vitro*. Taken together, our data, both *in vitro* and *in vivo*, points to a role for ILK in the initial steps of myelination, and suggests that the observed amyelination is not due to a loss of OLs but rather to a defect in OL branching and membrane formation.

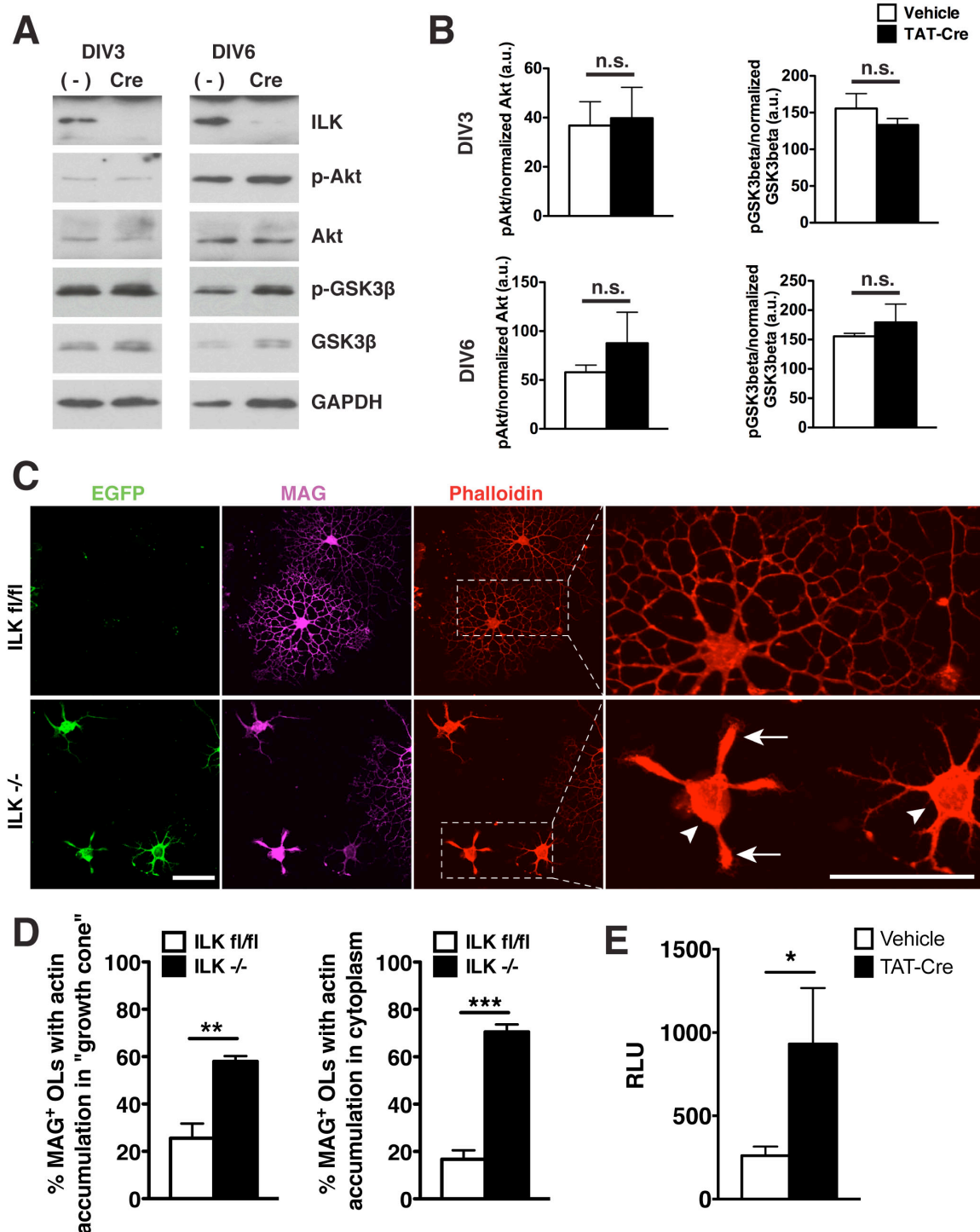


**Figure 3.8. ILK regulates internode length and triggers internode formation *in vivo*.**

(A) Compressed stack of immunofluorescence micrographs from the optic nerves of P14 *PLP**CreER*<sup>T</sup>;mT/mG control and *ILK cKO*;mT/mG mice. EGFP (green) denotes a recombination event and allows for tracing of reporter only (top panel) or ILK depleted internodes (bottom panel). (B) Quantification of internode length and diameter between control and ILK depleted mice. (C) Compressed stack of immunofluorescence micrographs from the optic nerves of P14 *PLP**CreER*<sup>T</sup>;mT/mG control and *ILK cKO*;mT/mG mice depicting abnormal morphology observed in a subset of ILK depleted OLs. Data represents the mean  $\pm$  SEM (n=4). \*\*p < 0.01; Student's *t* test. Scale Bar: 50  $\mu\text{m}$ .

**Morphological deficits of Ilk-deficient OLs are associated with abnormal actin accumulation, and are partially rescued through Rho kinase inhibition**

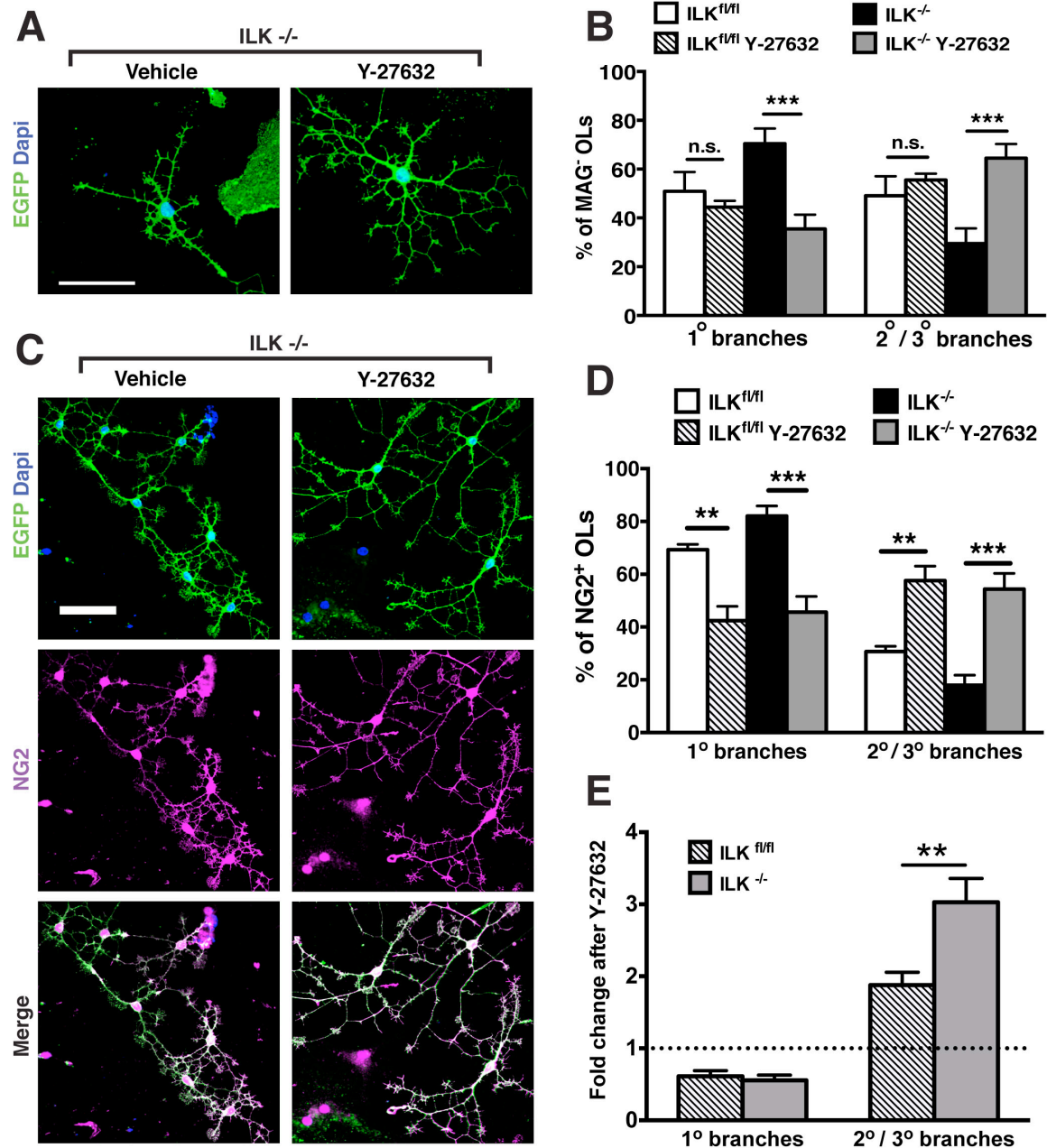
In our search for a mechanistic explanation for the aforementioned OL defects, we first explored the phosphorylation state of ILK's canonical downstream effectors Akt and GSK3 $\beta$ . To our surprise, no apparent change in Akt (Ser 473) or GSK3 $\beta$  (Ser 9) phosphorylation was observed in ILK depleted OL cultures at DIV3 or 6 (Figure 3.9A, B). We thus sought to investigate other possible mechanisms that could account for the observed morphological aberrations. ILK has previously been shown to co-localize with  $\beta$ 1 integrin and paxillin in OL focal adhesions (Chun et al., 2003). We further demonstrate ILK localization to the filamentous-actin (F-actin) rich leading edge or distal tips of outgrowing processes in the immortalized OL cell line OLi-neu (data not shown). When ILK is lost from primary OLs, there is an abnormal accumulation of F-actin in the cell body and sub-cortically in the developing process (Figure 3.9C, D). A well-known regulator of neural cell actin organization is the small RhoGTPase RhoA (Govek et al., 2005), and perturbation of RhoA results in morphologically aberrant OLs (Rajasekharan et al., 2009, 2010). As ILK is required for RhoA-dependent actin cytoskeleton reorganization (Graness et al., 2006), we hypothesized that the morphological deficits and actin accumulation in *Ilk*<sup>-/-</sup> OLs is caused by upregulation of the RhoA signaling pathway. Indeed, when we probed for active RhoA levels, an increase in GTP bound RhoA was detected in our TAT-Cre treated DIV3 OL cultures (Figure 3.9E).



**Figure 3.9. Abnormal actin accumulation is linked with *Ilk*<sup>-/-</sup> OL morphology.** (A) Western blots performed on DIV3 and DIV6 vehicle (-) or TAT-Cre (Cre) treated primary OL lysates. Membranes were probed for ILK, Akt (p-Ser 473 and total), GSK3β

(p-Ser 9 and total) and GAPDH as a loading control. **(B)** Densitometry analysis of relative band intensity for western blots depicted in panel (A). **(C)** Immunofluorescence micrographs of *Ilk*<sup>-/-</sup> and *Ilk*<sup>fl/fl</sup> OLs showing actin localization with rhodamine-phalloidin staining. Arrows denote filamentous actin accumulation in the process and “growth-cone” like structures while arrowheads denote cytoplasmic accumulation. **(D)** Quantification of the percent of MAG<sup>+ve</sup> *Ilk*<sup>-/-</sup> and *Ilk*<sup>fl/fl</sup> OLs with actin accumulation in the cytoplasm and/or in growth cone-like structures. **(E)** Results from a RhoA G-LISA activation assay measuring the relative amount of active (GTP-bound) RhoA between vehicle and TAT-Cre treated DIV3 OL cultures. Data represent the mean ± SEM (n=3-4). \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001; Student’s *t* test (B,D); one-tailed Mann Whitney U-test (E). Scale bars: 50 μm.

Stimulation of the RhoA pathway involves activation of ROCK, an event that is associated with destabilization of actin. The ROCK inhibitor Y-27632 is often used to rectify the effects of overactive RhoA signaling. We tested whether Y-27632 treatment would improve the morphological deficits of *Ilk*<sup>-/-</sup> OLs. Interestingly, this did not rescue the morphological deficits of MAG<sup>+ve</sup> *Ilk*<sup>-/-</sup> OLs (data not shown). Rather, Y-27632 had a specific impact on the morphology of immature (MAG<sup>-ve</sup>) *Ilk*<sup>-/-</sup> OLs (Figure 3.10A). Under normal conditions, MAG<sup>-ve</sup> *Ilk*<sup>-/-</sup> OLs mostly displayed primary (1°) branched morphology, whereas MAG<sup>-ve</sup> *Ilk*<sup>fl/fl</sup> OLs tended to have equal numbers of 1° and secondary/tertiary (2°/3°) branched cells (Figure 3.10B). Upon administration of Y-27632, quantification revealed a restoration of the proportion of 2°/3° branched MAG<sup>-ve</sup> *Ilk*<sup>-/-</sup> OLs to wild-type levels at DIV6 of culture (Figure 3.10B). It was unlikely that this phenomenon was a non-specific event independent of ILK loss, since application of Y-27632 to *Ilk*<sup>fl/fl</sup> OLs did not enhance MAG<sup>-ve</sup> OL morphology as compared to *Ilk*<sup>fl/fl</sup> vehicle treated cells.



**Figure 3.10. Administration of ROCK inhibitor Y-27632 rescues the morphology of a distinct *Ilk*<sup>-/-</sup> population of OL-lineage cells.** (A) Confocal micrographs depicting the morphology of DIV6 MAG<sup>-ve</sup> *Ilk*<sup>-/-</sup> OLs treated with Y-27632 or vehicle control. (B) Morphological quantification of MAG<sup>-ve</sup> *Ilk*<sup>-/-</sup> and *Ilk*<sup>fl/fl</sup> OLs treated with either vehicle or Y-27632. A morphological staging scheme adapted from the one depicted in Figure 4B was used for this experiment. (C) Confocal micrographs depicting the morphology of

DIV6 NG2<sup>+</sup> *Ilk*<sup>-/-</sup> OLs treated with ROCK inhibitor Y-27632 or vehicle. **(D)** Morphological quantification of NG2<sup>+</sup> *Ilk*<sup>-/-</sup> and *Ilk*<sup>fl/fl</sup> OLs treated with either vehicle or Y-27632. **(E)** Fold change in proportion of 1° and 2°/3° branched NG2<sup>+</sup> OPCs upon administration of Y-27632 to *Ilk*<sup>fl/fl</sup> and *Ilk*<sup>-/-</sup> populations. Data represent the mean ± SEM (n=4). n.s. = no significant difference; \*\*p < 0.01, \*\*\*p < 0.001; two-way ANOVA with Bonferroni post-tests. Scale bar: 50 μm.

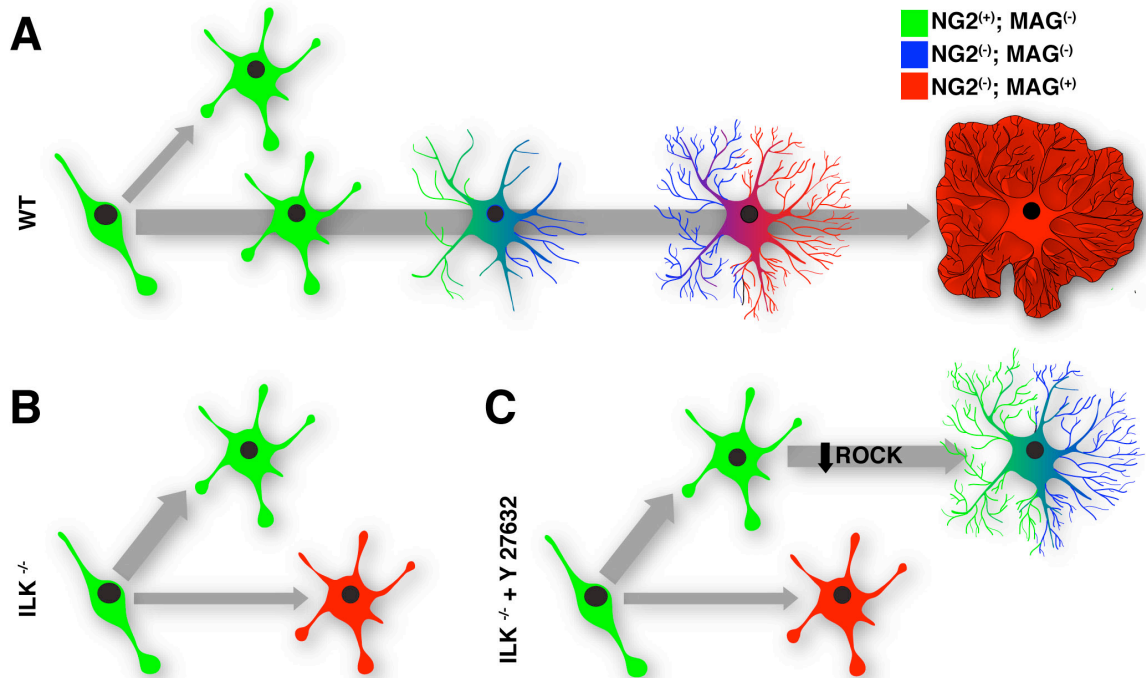


We were apparently enhancing the morphology of an immature (*i.e.* MAG<sup>-ve</sup>) population of *Ilk*<sup>-/-</sup> OL-lineage cells by inhibiting ROCK. We next sought to investigate whether these morphologically-rescued MAG<sup>-ve</sup> cells were in fact NG2<sup>+ve</sup> OPCs. To this end, we characterized the morphology of DIV6 NG2<sup>+ve</sup> *Ilk*<sup>-/-</sup> and *Ilk*<sup>fl/fl</sup> cells treated with either Y-27632 or vehicle. *Ilk*<sup>-/-</sup> NG2<sup>+ve</sup> OPCs responded in a similar fashion as did the MAG<sup>-ve</sup> OLs described above (Figure 3.10C). Y-27632 administration resulted in an increase from mainly NG2<sup>+ve</sup> 1<sup>o</sup> branched OPCs to predominantly 2<sup>o</sup>/3<sup>o</sup> branched cells (Figure 3.10D). Interestingly, in contrast to the above-described MAG<sup>-ve</sup> population, Y-27632 also enhanced the morphology of the NG2<sup>+ve</sup> *Ilk*<sup>fl/fl</sup> population. However, even when considering this apparent indirect (*i.e.* ILK independent) impact of Y-27632 on NG2<sup>+ve</sup> OPC morphology, ROCK inhibition enhanced the morphology of *Ilk*<sup>-/-</sup> NG2<sup>+ve</sup> OPCs to a greater extent than *Ilk*<sup>fl/fl</sup> OPCs (Figure 3.10E). Thus, RhoA regulation seems to be ILK-dependent during distinct phases of OL development. Specifically, ILK appears to depend on RhoA signaling at the point of differentiation when OPCs are transitioning from NG2<sup>+ve</sup> precursors to MAG<sup>-ve</sup> immature OLs.

## Discussion

### ILK regulates OL development

Following ILK loss in enriched primary OL cultures, two phenotypes were observed: (1) the persistence of NG2<sup>+ve</sup> OPCs coupled with a decrease in mature (MAG<sup>+ve</sup>) cells, and (2) an inability of the MAG<sup>+ve</sup> population to form arborizations or myelin-like membranes (Figure 3.11). At first glance, it is plausible to suspect a general delay in Ln-2 mediated differentiation as cause. While, to a degree, we believe this true, several observations suggest this interpretation as overly simplistic. First, the persistence of NG2<sup>+ve</sup> precursors in the *Ilk*<sup>-/-</sup> population remains static from DIV3 to 6, implying this OL subpopulation requires ILK signaling for both morphological and molecular differentiation. Second, there is the discord between morphology and maturation markers observed in a separate OL subpopulation; those capable of MAG expression upon ILK loss. Together, this suggests a functional OL heterogeneity exists with regard to ILK. Some cells necessitate ILK to initiate the entire differentiation gamut, while others require ILK for morphological maturation only. Alternatively, ILK's role could be linked to the exact maturation stage of the precursor cell at the time of its deletion. In either case, we would assume functional differences are an outcome of signaling alteration downstream of ILK, most likely through a shift in ILK complex composition at the  $\beta$ 1 integrin cytoplasmic tail. Elucidation of the mechanistic switch that defines the observed OL subgroups will be important as we move towards a fuller understanding of OL development.



**Figure 3.11. ILK differentially regulates both morphology and molecular marker activation in at least two OL subgroups. (A)** During normal OL development, OPCs give rise to two general populations when presented with a Ln-2 substrate; a smaller pool of NG2<sup>+</sup> cells which remain in a precursor state, and a second, larger group, which progresses through the various stages of morphological maturation with concomitant expression of molecular markers of differentiation (MAG). **(B)** Following ILK loss, an increased percentage of OPCs fail to commit to the maturing population, remaining as NG2<sup>+</sup> precursors. Simultaneously, a second pool of ILK-depleted OLs express MAG but remain morphologically stunted. This pool correlates with defects observed *in vivo*. **(C)** ROCK inhibition rescues a select group of immature OLs, restoring secondary and tertiary branching to normal levels. However, cells expressing MAG do not display a similar increase in morphological maturity, suggesting differential regulation of the Rho/ROCK pathway by ILK between varying OL subgroups.

Initial characterization of P14 *Ilk* *cKO* optic nerves revealed a decrease in total number of myelinated small caliber axons. Extrapolating from our *in vitro* (aberrant morphological differentiation) and *in vivo* (low dose tamoxifen) data, we suggest a dual explanation for the small axon phenotype; that *Ilk*<sup>-/-</sup> OLs suffer both from an inability to generate and extend the requisite network of processes as well as a deficiency in their capacity to initiate myelination following axonal contact. In regards to the former, OL morphological complexity is negatively correlated with the diameter of the axon(s) it myelinates. Individual OLs will either myelinate a large number of small axons or a small number of large axons (Butt and Berry, 2000; Almeida et al., 2011). Thus, following ILK loss, we would expect exacerbation of small axon amyelination as a result of the OLs inability to generate a high number of processes. The second mechanism, failure to initiate myelination following axonal contact, was first proposed by Câmara and colleagues in a dominant-negative model of  $\beta 1$  integrin (Câmara et al., 2009). The idea is as follows; once axonal contact is established, the OL process requires an axonal ligand “threshold” be broken to trigger a myelinating event. Larger axons are unaffected in this paradigm, as they provide excess surface ligand. Smaller axons, however, necessitate integrin-mediated signal amplification and, as such, are preferentially affected.

In contrast to our own model, Câmara et al. (2009) initially propose and then reject the possibility of morphological abnormalities as cause for small axon amyelination. We believe our *in vitro* data, together with the low dose tamoxifen experiments, imply morphological defects following ILK loss *in vivo*. However, our two models do not preclude one from the other. Rather, they highlight possible differences in functional outcome between receptor and effector disruption. Our findings are also

congruent with work published by others (Benninger et al., 2006), which controversially concluded no essential role for  $\beta 1$  integrin in CNS myelination. Here, the authors focused primarily on G-ratio as readout for myelination, which, similar to our own model, was unaffected. As well, the study was conducted primarily in older mice (2 months and above), by which time, full phenotypic recovery had occurred in our model. Coupled with our work, these findings strongly suggest the  $\beta 1$  integrin signaling pathway is critical for myelination during a small developmental window, with compensation occurring thereafter.

As alluded, our *in vivo* model demonstrates the capacity for compensation. When considering the transient nature of the amyelination phenotype, we are presented with two general possibilities, that death of *Ilk*<sup>-/-</sup> OLs permits WT cells to compensate, or that *Ilk*<sup>-/-</sup> OLs are developmentally delayed, requiring more time for myelination to proceed. We have eliminated the former, as there was no change in total OL number or an increase in apoptosis between the optic nerves of *Ilk* cKO and WT mice. The second scenario, a delay in morphological maturation, therefore seems the most likely explanation; that upon ILK loss compensation occurs through up-regulation of other integrin adhesion complexes such as those containing Fyn or FAK. Alternatively, compensation could arise through a switch in Ln-2 directed signaling from integrin to dystroglycan, another Ln-2 receptor expressed by OLs (Colognato et al., 2007).

However, given the nature of our *in vivo* model (a single tamoxifen injection), we must also consider a paradigm wherein non-recombined OLs outcompete *Ilk*<sup>-/-</sup> cells for bare axons. Two qualities lend credence to this theory. First, the myelination potential of any single OL is dependent on its ability to compete with neighboring OLs for axonal

space (Chong et al., 2012). Second, OLs are plastic, able to form myelin beyond what a permissive environment would normally allow (Almeida et al., 2011; Chong et al., 2012). It is therefore reasonable to postulate compensation as occurring through endogenous “WT” OLs, an intriguing possibility when considering future development of demyelinating disease therapies.

We must also consider the nature of the *Plp* promoter, as it defines two separate NG2<sup>+ve</sup> OPC populations; one that expresses PLP, and one that does not (Mallon et al., 2002). While it is unknown whether these two subpopulations differ functionally, we cannot exclude the possibility that ILK has a distinct role within each (recall, we observe phenotypic disparity between *Ilk*<sup>-/-</sup> OLs *in vitro*). As our *PLPCreER*<sup>T</sup> model would target one subpopulation (NG2<sup>+ve</sup>/PLP<sup>+ve</sup>) to the exclusion of the other, the question of whether we could exacerbate (or not) the amyelination phenotype by targeting all NG2<sup>+ve</sup> cells remains. If answered, it would provide some of the first insight into functional continuity, or lack thereof, for a member of the  $\beta$ 1 integrin signaling pathway between OL subgroups *in vivo*.

### **ILK regulates OL actin cytoskeleton dynamics in part through RhoA**

To our surprise, the phosphorylation status of the downstream effectors Akt and GSK3 $\beta$  were unchanged following ILK loss, prompting us to instead explore the actin cytoskeleton. ILK is known to provide a bridge between environmental cues and intracellular machinery, linking the ECM and integrins to the actin cytoskeleton across a multitude of cell types and species (McDonald et al., 2008). In the OL, dynamic remodeling of the actin cytoskeleton is necessary for filopodia and lamellipodia

formation, the engines driving arborization (Kim et al., 2006; Bacon et al., 2007). We therefore propose the morphological defects following ILK loss result from a breakdown in the connection between the ECM and actin cytoskeleton, ultimately leading to F-actin accumulation/disorganization and subsequent process outgrowth failure.

As ILK regulates actin dynamics indirectly through recruitment of focal adhesion complexes (Legate et al., 2006), the observed perturbation in F-actin is most likely an outcome of ILK complex dissolution. Under normal conditions, ILK-assembled complexes are capable of both direct F-actin binding and indirect actin remodeling, the latter an event largely regulated by the small Rho GTPase family (Wickström et al., 2010). With relevance to our work, ILK-directed focal adhesions act as checkpoints for RhoA and its effector ROCK, with loss of ILK or  $\alpha$ -parvin leading to increased pathway activity and subsequent F-actin disorganization (Kogata et al., 2009; Montanez et al., 2009; Pereira et al., 2009). Up-regulation of RhoA signaling in neurons manifests as cortical actin accumulation with concomitant inhibition of neurite extension (Yamaguchi et al., 2001), and in OLs as reduction in process outgrowth and branching (Liang et al., 2004; Rajasekharan et al., 2009, 2010; Wang et al., 2012). We observed an increase in active RhoA levels following ILK depletion in our OL cultures. When treated with the ROCK inhibitor Y-27632,  $MAG^{+ve} Ilk^{-/-}$  OLs did not respond as expected, with no increase in morphological complexity. Instead,  $NG2^{+ve}/MAG^{-ve} Ilk^{-/-}$  OLs displayed a surge in arbor sprouting. The results suggest ILK regulation of the RhoA/ROCK pathway is limited to a distinct OL subpopulation, specifically, that which is transitioning from a precursor to immature OL state.

It was surprising to note no change in morphological complexity of  $MAG^{+ve} Ilk^{-/-}$

OLs upon ROCK inhibition. The controlled manipulation of the actin cytoskeleton by ILK within this cell population must occur through pathways independent of Rho/ROCK signaling. One possibility is de-activation of the ILK regulated N-WASP/Arp2/3 complex (mediators of actin polymerization), as N-WASP disruption negatively impacts OL process outgrowth (Bacon et al., 2007). In addition, ILK is required for  $\alpha$ -parvin/ $\beta$ -parvin stabilization and paxillin localization, both of which bind F-actin directly (Attwell et al., 2003; Legate et al., 2006). Therefore, loss of ILK could lead to F-actin/integrin/ECM uncoupling, which may explain the observed OL morphological defects. Additionally, *Ilk*<sup>-/-</sup> OLs are characterized by an inability to extend myelin-like membranes. While this is possibly due to cytoskeletal defects, our results following ROCK inhibition (improved branching but not membrane formation) suggest other pathways are at play, implying ILK's governance of OL process outgrowth and myelin membrane formation occur through separate mechanisms.

Through our work, we have begun to dissect ILK-directed signaling pathways overseeing unique aspects of OL development. Precisely which downstream targets potentiate ILK's role in cytoskeletal reorganization, and how their actions differ at varying stages of OL differentiation remains an intriguing question for the future.



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## **Chapter 4**

### **Integrin-Linked Kinase Regulates Oligodendrocyte Precursor Cell Migration in a Substrate-Specific Manner and is Required for RhoA-Pathway Mediated Directionally Persistent Migration**

**Integrin-linked kinase regulates oligodendrocyte precursor cell migration in a substrate-specific manner and is required for RhoA-pathway mediated directionally persistent migration**

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**Author Contributions**

RO and RK conceived and designed experiments. RO and RK analyzed and interpreted the results. SEC contributed to the quantification of the data presented in Figure 4.5. Both SEC and JPM significantly contributed by means of cell culture. RO wrote the manuscript and RK edited the final version. RK was the primary supervisor of this work.

## **Abstract**

The decline of remyelination in chronic multiple sclerosis is in part attributed to inadequate oligodendrocyte precursor cell (OPC) migration, a process governed by the extracellular matrix (ECM). Cellular adhesions to the ECM are stabilized by integrin-linked kinase (ILK), a focal adhesion protein that mediates the linkage between matrix-bound integrin receptors and the cytoskeleton. To assess the role of ILK in OPC motility, we designed a novel oligosphere method that permits the measurement of several migration parameters and accommodates the sensitive nature of mouse OPCs. This technique is applicable towards conditional knockout genetics as ILK could be effectively depleted from oligospheres via administration of Cre-recombinase. By measuring the emergence of OPCs from oligospheres, we identified ILK as a substrate-specific regulator of OPC migration. ILK loss yielded an early but transient deficit in OPC net directional velocity on laminin matrix. Both the net directional velocity and the initiation of migration on fibronectin were heavily reliant on ILK, while parallel defects were observed on a non-integrin binding polylysine substrate, which additionally offers a function for ILK in OPC motility that is independent of integrin interaction with the ECM. We (and others) have previously implicated ILK in the regulation of RhoA signaling. Pharmacological inhibition of RhoA signaling promoted OPC net directional velocity on fibronectin in an ILK-dependent manner. Inclusive, our work provides a novel tool for studying mouse OPC migration and highlights the wide-ranging role of ILK in regulating disparate signaling pathways that collectively contribute to OPC migration.

## Introduction

Oligodendrocyte precursor cells (OPCs) migrate extensively prior to the onset of central nervous system (CNS) myelination (de Castro et al., 2013), a process governed in part by the extracellular matrix (ECM) during both normal development and remyelination of demyelinated lesions (Lau et al., 2013). Endogenous lesion repair in multiple sclerosis (MS) decreases with disease progression, a consequence of reduced OPC proliferation, differentiation and/or migration (Keough and Yong, 2013). Of note, OPCs accumulate at the periphery of early MS lesions (Kuhlmann et al., 2008), which is thought to be a result of lesion-derived inhibitory factors impeding OPC recruitment inwards (Sobel and Ahmed, 2001; Williams et al., 2007; Clemente et al., 2011; Bin et al., 2013; Boyd et al., 2013). Elucidating the molecular mechanisms that govern OPC migration will therefore facilitate the design of therapeutics aimed at promoting OPC infiltration into MS lesions.

OPC migration is regulated in large part by the integrins (Milner et al., 1996; Gudz et al., 2006), a family of transmembrane receptors that mediate adhesion to the ECM. During migration, integrin receptors cluster in discrete foci at the leading edge of lamellipodia, forming cell/matrix contacts that are used as anchor points for the actin cytoskeleton. Integrins are heterodimers composed of an  $\alpha$  and a  $\beta$  subunit, and oligodendrocyte (OL)-lineage cells express  $\alpha_v\beta_1$ ,  $\alpha_v\beta_3$ ,  $\alpha_v\beta_5$ ,  $\alpha_v\beta_8$  and  $\alpha_6\beta_1$  integrins inclusively (Milner and Ffrench-Constant, 1994). Bound to the intracellular tails of  $\beta_1$  and  $\beta_3$  integrin subunits is integrin-linked kinase (ILK; Hannigan et al., 1996; Pasquet et al., 2002), a focal adhesion protein that plays both signaling and scaffolding roles at actin-integrin connections (Wickström et al., 2010; Ghatak et al., 2013). ILK elicits these functions with help from binding partners PINCH and parvin, which respectively link

ILK to receptor tyrosine kinases and the actin cytoskeleton (Legate et al., 2006). With specific relevance to the OL lineage, ILK mediates cellular morphology by way of regulating actin architecture and the activity of the cytoskeletal remodeling protein RhoA (O'Meara et al., 2013). As cellular motility equivalently relies on Rho-GTPase signaling, cytoskeletal dynamics and morphological pliability, we hypothesized that ILK is required for ECM-directed OPC migration.

To address this hypothesis, we developed a novel *in vitro* assay useful for assessing various elements of mouse OPC migration. Combined with a conditional knockout approach, we demonstrated that ILK could be selectively deleted from OPCs to yield substrate-specific migratory consequences. ILK-deficient OPCs were severely impaired at migrating on fibronectin, but suffered from only a transient migration defect on laminin. Intriguingly, ILK-depleted OPCs also suffered from compromised migration on poly-D-lysine via a mechanism that was apparently independent of veritable integrin engagement. Inhibition of the RhoA pathway promoted directionally persistent OPC migration, in an ILK-dependent manner. Inclusively, our work identifies ILK as a key node in distinct signaling cascades that cumulatively regulate OPC migration and provides a new tool for the study of mouse OPC motility *in vitro*.

## Materials and Methods

**Transgenic animals:** Animals used for this work were cared for according to the Canadian Council on Animal Care guidelines under University of Ottawa Animal Care Committee protocol number OGH-130. The floxed *Ilk* mouse line (*Ilk<sup>fl/fl</sup>*; Terpstra et al., 2003) was graciously provided by Dr. René St-Arnaud (McGill University, Montreal, Canada). *Ilk<sup>fl/fl</sup>* mice were subsequently bred to homozygosity with the *mT/mG* reporter mouse strain (Muzumdar et al., 2007) to yield the *Ilk<sup>fl/fl</sup>;mT/mG* line.

**Cell culture:** Oligospheres were generated as a byproduct of the mouse OPC purification protocol described by O'Meara and colleagues (2011). Neonatal mouse cortices (P0-P3) were dissociated and seeded into poly-L-lysine coated T25 flasks in mixed glial culture media. Once cultures had achieved appropriate density, the flasks were shaken at high-speed overnight generating suspended clumps of OPCs (oligospheres). The media was passed through a 40 µm cell strainer and the oligospheres were subsequently backwashed onto sterile Petri dishes. Oligospheres were picked from the Petri dish with a P20 pipette by aid of a dissection microscope, and seeded individually onto substrate-coated coverslips in migration or differentiation media (see below). Coverslips were coated with either human placental merosin (laminin; 10 µg/mL; Millipore), fibronectin (50 µg/mL; Calbiochem) or >300,000 molecular weight poly-D-lysine (0.1 mg/mL; Sigma-Aldrich).

**Cell culture media:** Mixed glial culture media was Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% GlutaMAX (Life Technologies), 10% fetal bovine



serum (FBS), 0.33% Penicillin-Streptomycin (P/S). From culture day 6 and onwards, the mixed glial culture media was supplemented with 5 µg/mL insulin.

Migration media was composed of 48-72 h conditioned mixed glial culture media (0.22 µm filtered), supplemented with 2% B27 (Gibco), 100 µg/mL bovine serum albumin (BSA), 5 µg/mL insulin, 0.5 µg/mL Holo-transferrin, 60 ng/mL progesterone, 400 ng/mL 3,3',5-triiodo-L-thyronine, 400 ng/mL L-thyroxine, 16 µg/mL putrescine, 5 ng/mL sodium selenite, 10 ng/µL platelet-derived growth factor (PDGF-AA; PeproTech), 50 ng/µL ciliary neurotrophic factor (CNTF; Peprotech) and 1 µg/mL aphidicolin (Sigma-Aldrich). For Rho-kinase (ROCK) inhibition, migration media was supplemented with 10 µM Y-27632 (EMD Millipore).

Differentiation media was DMEM supplemented with 0.5% FBS, 1% GlutaMAX, 2% B27, 0.33% P/S, 100 µg/mL BSA, 5 µg/mL insulin, 0.5 µg/mL Holo-transferrin, 60 ng/mL progesterone, 400 ng/mL 3,3',5-triiodo-L-thyronine, 400 ng/mL L-thyroxine, 16 µg/mL putrescine, 5 ng/mL sodium selenite and 50 ng/µL CNTF.

**Conditional genetic ablation:** *In vitro* recombination of the *Ilk<sup>fl/fl</sup>* and *mT/mG* alleles was achieved by treating mixed glial cultures with recombinant His-TAT (trans-activator of transcription)-NLS (nuclear localization sequence)-Cre recombinase (hereafter called TAT-Cre; Excellgen Incorporated). Briefly, the media was removed and the monolayers were washed with PBS. TAT-Cre (or the corresponding vehicle-only control) was administered at 10 µL/mL in DMEM with 2% B27 and incubated on monolayers for 1-2 h. This treatment was performed twice for 48-72 h prior to oligosphere isolation to promote a high level of recombination.

**Immunostaining and microscopy:** Oligospheres were fixed with 3% paraformaldehyde (PFA) for 15 min at room temperature (RT) or overnight at 4°C. Oligospheres were then either processed for indirect immunofluorescence microscopy, or mounted onto slides in DAKO medium after staining with Hoechst.

For indirect immunofluorescence, fixed oligospheres were permeabilized with 0.1% Triton X-100 for 10 min at RT, and washed with PBS. Oligospheres were blocked with 10% goat serum, and incubated overnight at 4°C with primary antibodies against NG2 (EMD Millipore), MAG (EMD Millipore), MBP (AbDSerotec), Olig2 (EMD Millipore), PDGFR- $\alpha$  (provided by Dr. William Stallcup, Sanford Burnham Medical Research Institute, La Jolla, California), or ILK (Cell Signaling). The following day, the samples were washed with PBS and incubated with Alexa fluor-conjugated secondary antibodies (Invitrogen) for 45 min to 1 h at RT. Coverslips were subsequently washed with PBS, counterstained with Hoechst and mounted onto slides in DAKO medium.

Standard epifluorescent images were acquired with an Axio Imager M1 microscope equipped with an AxioCamHR HRm Rev.2 camera using EX Plan-Neofluar 10x/0.3 Ph1 or Fluar 5x/0.25 M27 objectives and Axiovision 4.8.2 software. Confocal microscopy was conducted with a Zeiss LSM 510 Meta DuoScan microscope using EC Plan-Neofluar 10x/0.3 M27, 20x/0.5 M27, 40x/1.3 Oil DIC M27 or Plan-Apochromat 63x/1.4 Oil DIC M27 objectives and Zen 8.0 software.

Phase contrast microscopy was performed with an Axiovert 200M microscope fitted with an AxioCamHR HRm Rev.2 camera using LD Plan Neofluar 20x/0.4 Korr Ph2 or EC Plan-Neofluar 10x/0.25 Ph1 objectives and Axiovision 4.6 software.

**Migration quantification:** Phase contrast images of oligospheres were acquired at time zero to record their original diameter. Migration assays were fixed at 4, 10 or 24 h and imaged by fluorescence microscopy. Using Photoshop, “exclusion zones” were digitally overlaid onto the residual sphere core, their purpose being to define the starting point of migration; only cells beyond the exclusion zone were considered to have truly migrated. For oligospheres seeded on laminin and fibronectin substrates, the size of the exclusion zone was 1.5x the sphere diameter at time zero. For the poly-D-lysine substrate, the exclusion zone was 1.75x, 2.0x and 2.75x the time zero sphere diameter for 4, 10 and 24 h time points, respectively.

Concentric rings were digitally laid around the exclusion zone, their diameter increment being 50, 100 and 200  $\mu\text{m}$  for 4, 10 and 24 h time points for assays conducted on laminin. For the fibronectin substrate, the ring diameter increment was 20, 40 and 80  $\mu\text{m}$  for the 4, 10 and 24 h time points, respectively. For the poly-D-lysine substrate, the ring increment for the 4, 10 and 24 h time points was 10, 20 and 40  $\mu\text{m}$ , respectively. The number of OPCs migrated to each concentric ring was quantified and represented as the percentage of OPCs in each ring. This can be considered a measure of migration distance or net directional velocity, when assay duration is taken into account. Migration may also be represented as the total number of OPCs migrated beyond the exclusion zone, regardless of distance. We interpret this parameter as a conveyance of the capacity to initiate migration and/or polarize.

**Immunoblot:** Mixed glial cultures were cooled on ice for 3 min, rinsed with ice cold PBS, and scraped into a commercial RIPA lysis buffer (Cell Signaling) supplemented with 2 mM PMSF. Lysates were left on ice for approximately 2 min, and centrifuged at 15,000 rpm for 5 min. Clarified lysates were transferred to new microfuge tubes and stored at -80°C.

For SDS-PAGE, 30 µg of cell lysate per sample was resolved on a polyacrylamide gel, and transferred to a PDVF membrane at 0.25 amps for 70 min. Membranes were blocked for approximately 1 h at RT with 5% skim milk powder in TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Tween-20). Primary antibodies against ILK (Cell Signaling) and GAPDH (Abcam) were incubated overnight in blocking buffer. Membranes were washed with TBST and probed with HRP-conjugated secondary antibodies for 45 min. Membranes were then washed with PBS, and treated with ECL (Thermo Scientific). Resulting films were scanned with an EPSON Perfection 2450 PHOTO scanner, imported into ImageJ, and a box of standard dimensions was placed over each band to measure the mean gray value. Densitometric values for ILK were normalized to GAPDH as a loading control.

**Statistical Analysis:** One “n” (*i.e.*, experiment) was considered as data obtained from pooled biological material (*i.e.*, cells) from mouse pups (usually 4-6) of a distinct litter. Differences in migration distance were assessed using two-way repeated measures ANOVA paired with Bonferroni multiple comparisons tests. All other data was tested using either unpaired two-tailed Student’s *t*-tests or one-way ANOVA paired with Bonferroni multiple comparisons tests. For all statistical tests, “n” was equal to or greater

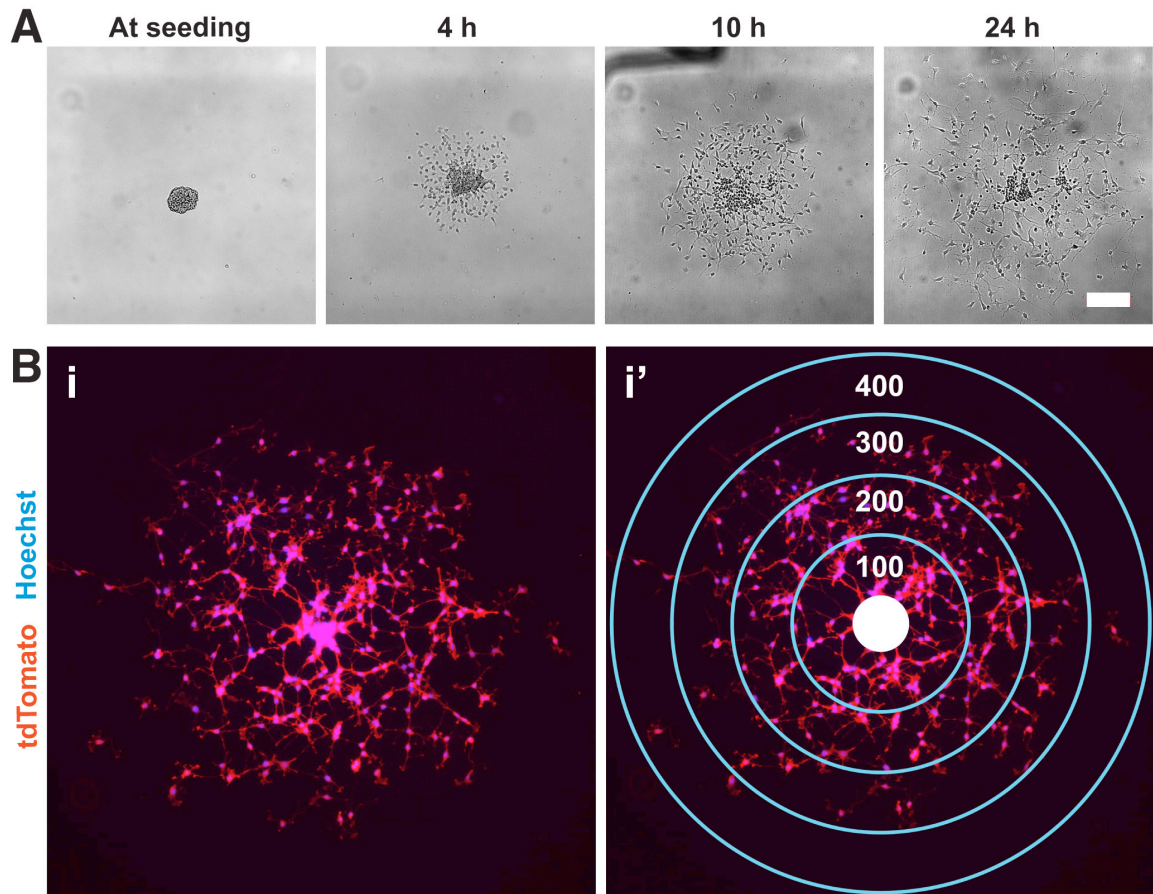
than 3, and differences in the mean were considered significant when  $p < 0.05$ . Experiments where no statistical analysis was performed and only qualitative observations were described,  $n=1$ .

## Results

### A novel assay for investigating OPC migration *in vitro*

The transwell and “agarose drop” assays are two methods commonly used to assess the *in vitro* migration of rat-derived OPCs (Frost et al., 2000). The transwell assay (Boyden, 1962) measures migration through a porous membrane, whereas the agarose drop assay (Varani et al., 1978) measures radial migration out of a drop of low-melting temperature agarose. While effective, the agarose drop assay calls for assay durations on the order of days, whereas interpretation of distance/velocity with the transwell method may be challenging. Both protocols also tend to require a large number of OPCs, which are difficult to obtain from mice (Chen et al., 2007).

Therefore, we sought to devise a rapid method for assessing multiple parameters of mouse OPC migration that required relatively few cells. We previously established a protocol for isolating mouse OPCs (O’Meara et al., 2011b), and as a byproduct of this isolation process (see Materials and Methods) OPCs tend to form suspended aggregates (here termed “oligospheres”) that could be further purified by filtration through cell strainers. When seeded onto laminin-coated coverslips, large numbers of OPCs migrated radially out of oligospheres over a 24 h period (Figure 4.1A). After a set experiment duration (*e.g.* 10 h) oligospheres were processed for immunofluorescence microscopy, and concentric rings (centered on the oligosphere core) were digitally overlaid on the acquired images (Figure 4.1B). When the number of OPCs in each concentric ring was counted, distributions were produced that described the distance traveled by the cells over

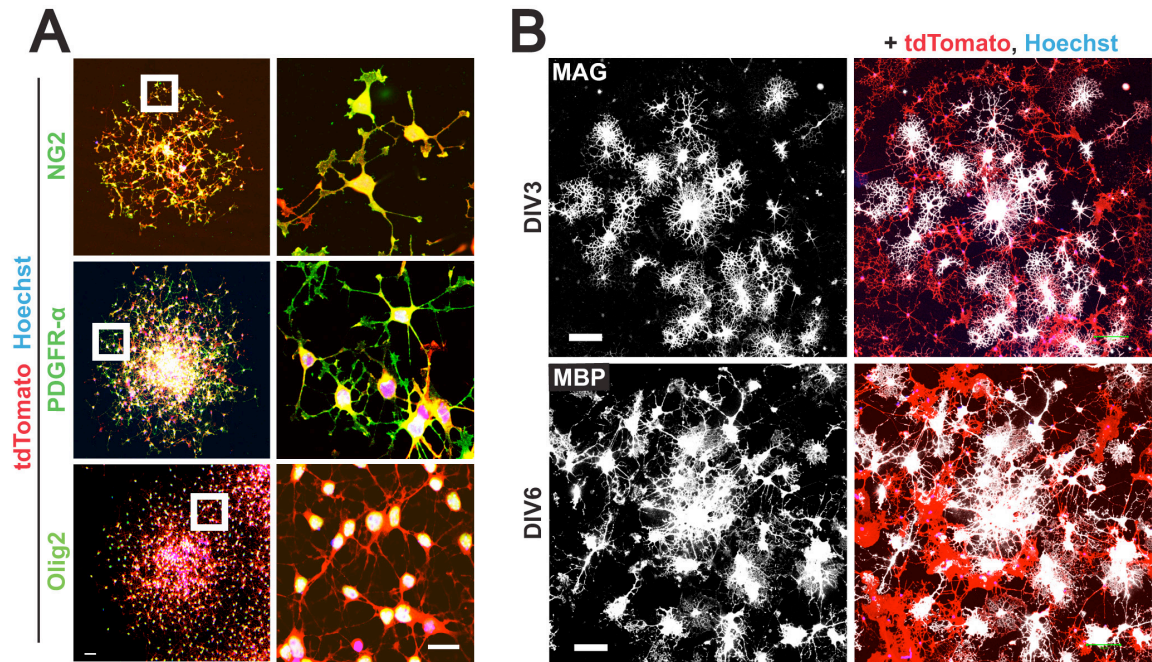


**Figure 4.1. Seeding oligospheres on migration-permissive substrates yields a quantitative means to assess OPC motility.** [A] Time-lapse phase contrast microscopy of an oligosphere from which OPCs emerge radially over a 24 h period. [B] A 10-hour migrated oligosphere before (i) and after (i') superimposing concentric rings (100  $\mu\text{m}$  increments) over the residual sphere core (solid white circle). Migration distance can be conveyed as the proportion of cells in each ring or the total number of migrated cells regardless of distance. Membrane-targeted tdTomato (red) facilitates visualization of cell bodies. Scale bar, [A] 50  $\mu\text{m}$ .

a given timeframe. This in turn could be considered a measure of net directional velocity, as velocity is a function of distance with respect to time. Reduced directional velocity would manifest as an accumulation of cells in rings proximal to the sphere core, whereas enhanced directional velocity would increase the proportion of cells in the distal rings. This data could also be portrayed as the total number of migrated OPCs (regardless of distance), which we interpret as a measure of migration initiation. A reduction in the total number of migrating OPCs subsequent to an experimental manipulation could be interpreted as compromised migration initiation.

To ensure the migratory cells were in fact OPCs, oligospheres were labeled with antibodies against the OPC markers platelet-derived growth factor receptor- $\alpha$  (PDGFR- $\alpha$ ), NG2 chondroitin sulfate proteoglycan (NG2) and Olig2 (reviewed by Nishiyama et al., 2009). As the majority of cells were positive for these markers (Figure 4.2A), we offer that the cells emanating from oligospheres are largely of early OL-lineage. Next, we differentiated the oligospheres for 3 and 6 days and stained for myelin-associated glycoprotein (MAG) and myelin basic protein (MBP), which mark intermediately and terminally differentiated OLs, respectively. A large proportion of the oligosphere-derived cells were MAG-positive at DIV3, and most of the remaining MAG-negative cells possessed highly branched morphologies typical of early-differentiated OLs (Figure 4.2B). At DIV6, the majority of oligosphere-derived cells were MBP-positive, many of which had produced structures reminiscent of myelin membranes. A minority of cells at DIV3 and DIV6 possessed astrocytic and microglial-like morphologies, suggesting oligospheres may be lowly contaminated with these cell types. Overall, our observations indicated the majority of cells migrating from oligospheres were OPCs, thereby





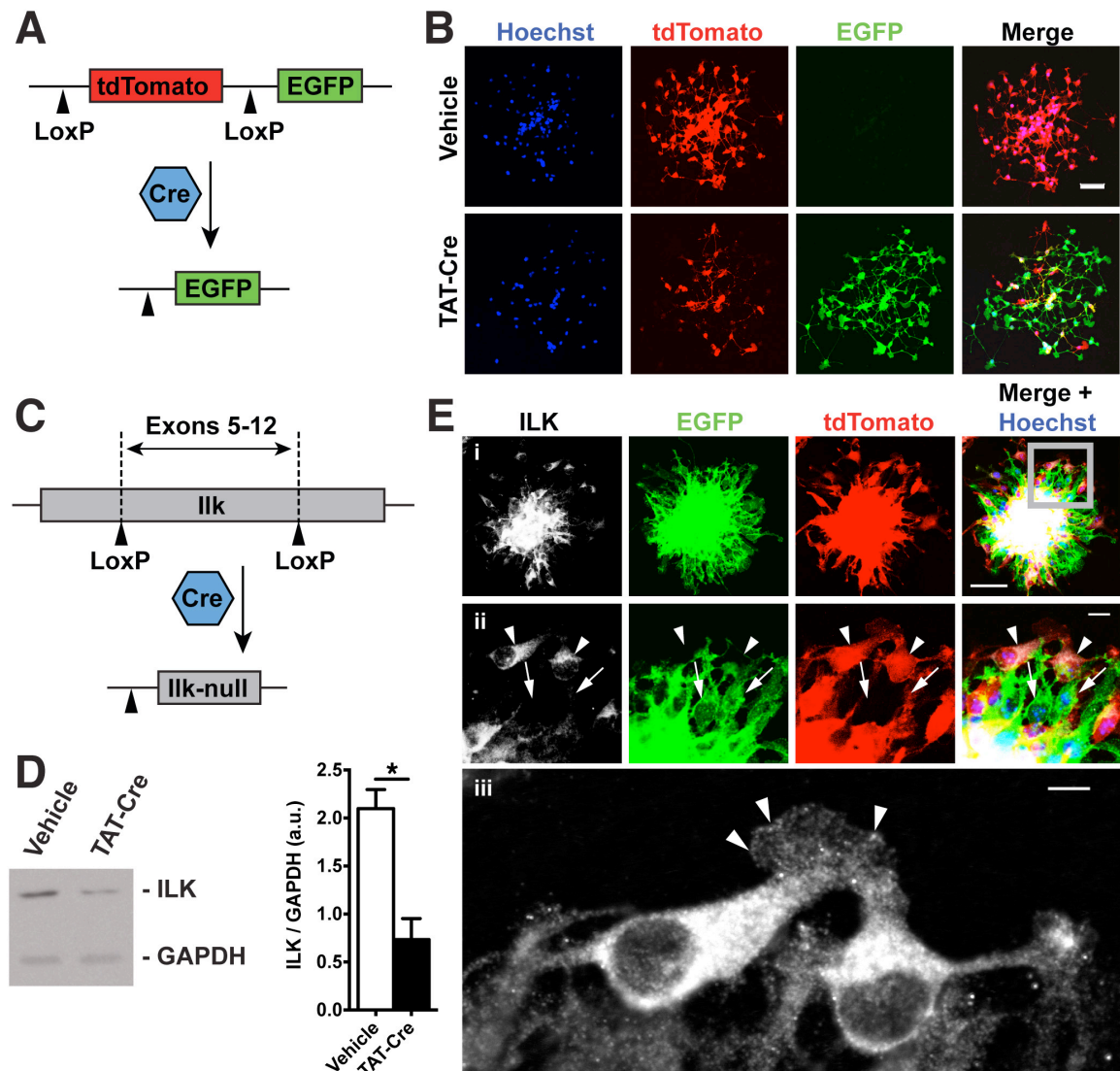
**Figure 4.2. Oligospheres are predominantly composed of OPCs.** [A] 10-hour migrated oligospheres showing the majority of migratory cells are positive for the OPC markers NG2, PDGFR- $\alpha$  and Olig2. [B] Differentiated oligospheres showing many of the emergent cells are MAG-positive at DIV3 and MBP-positive at DIV6 (intermediate and late markers of OL differentiation, respectively). Scale bars, [A] 50  $\mu\text{m}$  and 20  $\mu\text{m}$  (inset); [B] 100  $\mu\text{m}$ .

validating the utility of this assay for studying OPC migration.

### **Conditional ablation of ILK from oligospheres**

A practical application of the OPC migration assay is its use in conditional gene knockout studies. As proof of principle, we generated oligospheres from *mT/mG* mice, a reporter line harboring a locus coding for near-ubiquitous expression of the fluorescent protein tdTomato (Muzumdar et al., 2007). The action of Cre recombinase at this locus ablates tdTomato while concomitantly inducing EGFP (Figure 4.3A), thereby permitting identification of Cre-recombined cells. To induce recombination, mixed glial cultures from *mT/mG* mice were treated with membrane-permeable TAT-Cre or vehicle (control) prior to oligosphere isolation. After 10 h of migration, the majority of OPCs emerging from TAT-Cre treated oligospheres were EGFP-positive (Figure 4.3B). Of note, EGFP induction in itself did not appear to impact the migration of OPCs.

We next crossed the *mT/mG* strain with *Ilk<sup>fl/fl</sup>* mice to generate the *Ilk<sup>fl/fl</sup>;mT/mG* line. Recombination of the *Ilk<sup>fl/fl</sup>* locus results in the excision of exons 5-12 of the endogenous *Ilk* gene, presumably producing null alleles (Figure 4.3C; Terpstra et al., 2003). Accordingly, TAT-Cre administration to *Ilk<sup>fl/fl</sup>;mT/mG* mixed glial cultures reduced total ILK protein as compared to vehicle treated control cultures (Figure 4.3D). Confocal microscopy revealed TAT-Cre recombined *Ilk<sup>fl/fl</sup>;mT/mG* oligospheres (hereafter called *Ilk<sup>-/-</sup>* oligospheres) were composed of a mixture of EGFP-positive and tdTomato-positive (wild type; *Ilk<sup>fl/fl</sup>*) OPCs (Figure 4.3E<sub>i</sub>). ILK immunoreactivity in EGFP-positive OPCs was largely reduced (Figure 4.3E<sub>ii</sub>), suggesting that EGFP expression coincided with genetic ablation of *Ilk*. In *Ilk<sup>fl/fl</sup>* OPCs,



**Figure 4.3. Conditional genetic ablation of *Ilk* from oligospheres.** [A] Schematic representation of the *mT/mG* reporter locus (Muzumdar et al., 2007). Cre-recombination at the LoxP sites excises *tdTomato* and induces EGFP expression. [B] Oligospheres isolated from TAT-Cre and vehicle-treated *mT/mG* cultures, showing TAT-Cre treated oligospheres consist of both recombined (EGFP-positive) and non-recombined (*tdTomato*-positive) OPCs. Notably, EGFP induction in itself does not appear to impact the migratory capacity of OPCs. [C] Schematic representation of the murine *Ilk*<sup>*fl/fl*</sup> locus, where Cre recombination excises exons 5-12 of the *Ilk* gene (Terpstra et al., 2003). [D]

Western blot showing TAT-Cre reduces ILK protein when administered to *Ilk<sup>fl/fl</sup>;mT/mG* cultures, significance confirmed by densitometric analysis. [E<sub>i-iii</sub>] Confocal images of an anti-ILK labeled Cre-recombined oligosphere derived from *Ilk<sup>fl/fl</sup>;mT/mG* cultures at low (E<sub>i</sub>) and high (E<sub>ii</sub>) magnification (of inset). EGFP-positive OPCs (*Ilk<sup>-/-</sup>*; arrows) have reduced ILK immunoreactivity as compared to tdTomato-positive OPCs (*Ilk<sup>fl/fl</sup>*; arrow heads). ILK localization appears restricted to the cytoplasm and leading edge of cellular processes in *Ilk<sup>fl/fl</sup>* cells (E<sub>iii</sub>). For [D], data represent the mean  $\pm$  SEM (n=3), \*p<0.05 (Student's *t* test). Scale bars, 50  $\mu$ m for [B] and [E<sub>i</sub>]; 10 and 5  $\mu$ m for [E<sub>ii</sub>] and [E<sub>iii</sub>], respectively.

ILK localized mostly to the cytoplasm and discrete punctae at the leading edge of cellular protrusions, reminiscent of cell-matrix contacts (Figure 4.3E<sub>iii</sub>). As TAT-Cre treated oligospheres contained ILK-depleted OPCs, this provided a useful system for assessing the role of ILK in OPC migration.

### **Loss of ILK leads to a transient defect in OPC migration on laminin**

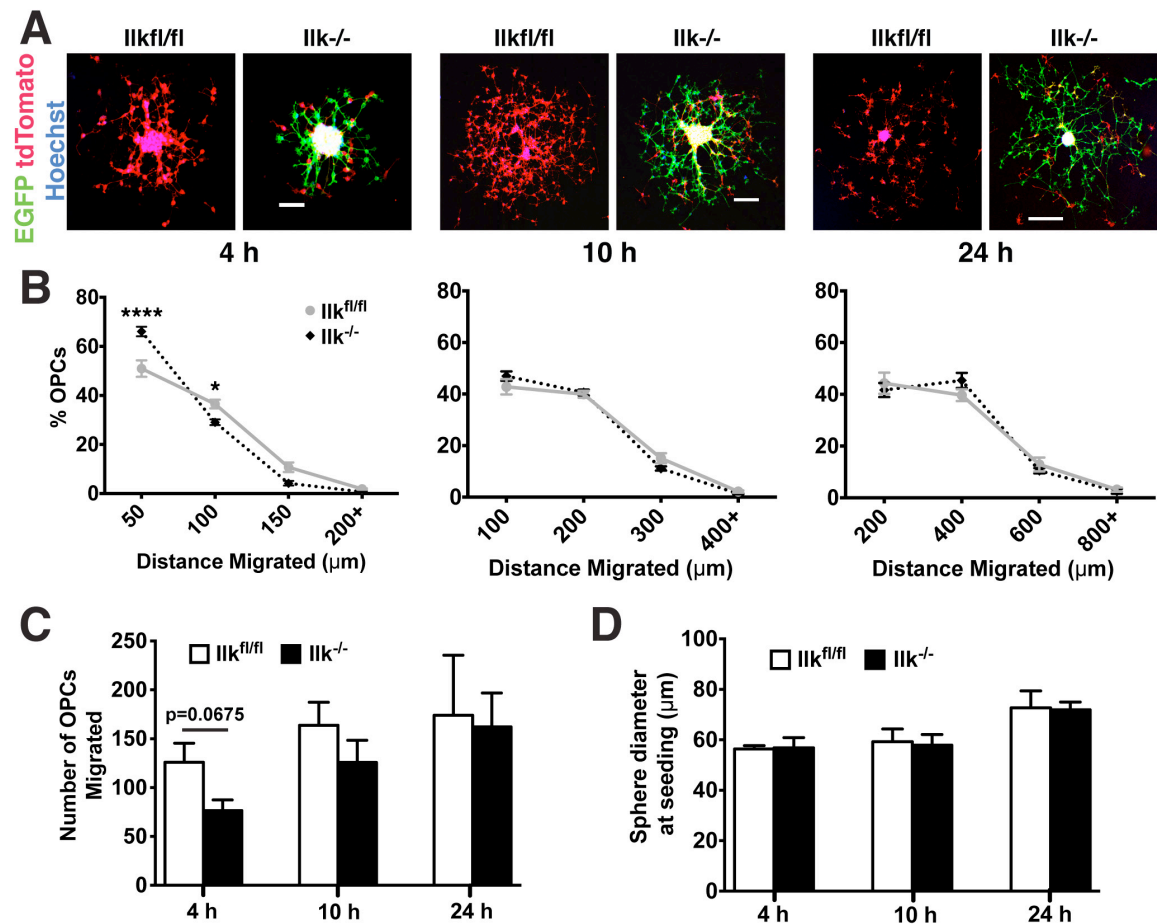
Merosin, a blanket term for  $\alpha_2$ -chain containing laminin (Ln) proteins (Ln-2, Ln-4 and Ln-13; Wewer and Engvall, 1996) influences various aspects of OL biology including growth factor-mediated survival (Decker and French-Constant, 2004; Laursen et al., 2009), morphological development (Buttery and French-Constant, 1999), and CNS myelination (Chun et al., 2003). Notably, myelinating CNS white matter tracts express the Ln  $\alpha_2$ -chain (Morissette and Carbonetto, 1995; Colognato et al., 2002), which is also upregulated after experimentally induced demyelination (Zhao et al., 2009; reviewed by Colognato and Tzvetanova, 2011). In addition, Ln promotes OPC migration (Frost et al., 1996; Schmidt et al., 1997; Niehaus et al., 1999), rendering Ln signaling pathways appealing pharmacological targets for overcoming the inhibitory barriers impeding OPC infiltration into MS lesions.

Ln binds the  $\alpha_6\beta_1$  integrin expressed by OLs (Buttery and French-Constant, 1999), triggering ILK recruitment to  $\beta_1$ -cytosolic tails (Legate et al., 2006), yielding Ln- $\alpha_6\beta_1$ -ILK complexes to which the actin cytoskeleton is stabilized. These adhesions presumably act as ECM anchor points that transmit cytoskeletal contractile forces during cellular migration. We therefore hypothesized that ILK loss would impede OPC migration on Ln due to actin-integrin disassociation. To this end, *Ilk*<sup>-/-</sup> and *Ilk*<sup>*fl/fl*</sup>

oligospheres were seeded onto Ln substrate, allowing radial migration to occur for 4, 10 and 24 h. Using concentric ring quantification, we found that ILK loss caused more OPCs to accumulate in the 50  $\mu$ m proximal ring after 4 h compared to control cells. Additionally, there was a correspondent decreased proportion of *Ilk*<sup>-/-</sup> OPCs that had attained the more distal 100  $\mu$ m ring, ultimately indicating a reduction in migration distance (Figure 4.4A,B). This in turn implies compromised net directional velocity of *Ilk*<sup>-/-</sup> OPCs, as velocity is a function of distance versus time. Interestingly, this deficit was not evident at 10 or 24 h. There was also a near-significant trend for fewer total migrated *Ilk*<sup>-/-</sup> OPCs at the 4 h time point ( $p = 0.0675$ ; Figure 4.4C), an indication of reduced capacity to initiate migration. Importantly, there was no difference between the diameters of *Ilk*<sup>-/-</sup> and *Ilk*<sup>fl/fl</sup> oligospheres at seeding, discounting size as a confounding variable in our observations (Figure 4.4D). Rather, we attribute our findings to a role for ILK in the initiation of Ln-directed migration and maintenance of directional velocity on Ln.

### **ILK loss severely impedes OPC migration on fibronectin**

Fibronectin (Fn) is a migration-permissive substrate for OPCs (Frost et al., 1996; Milner et al., 1996), but evidence suggests Fn aggregation impedes remyelination in MS (Stoffels et al., 2013a). This underscores the importance of elucidating the signaling pathways that govern OPC migration on Fn, as their pharmacological manipulation may influence the degree to which OPCs infiltrate MS lesions. OL-lineage cells express the  $\alpha_v\beta_1$ ,  $\alpha_v\beta_3$ ,  $\alpha_v\beta_5$  and  $\alpha_v\beta_8$  integrin receptors which recognize the Arg-Gly-Asp (RGD) integrin binding sequence intrinsic to Fn and other ECM molecules



**Figure 4.4. Transient impairment of net directional migration velocity of ILK deficient OPCs on laminin.** [A] Representative confocal images of *Ilk<sup>-/-</sup>* and *Ilk<sup>fl/fl</sup>* oligospheres migrated on Ln for 4, 10 and 24 h. [B] Quantification of OPC migration using the concentric ring method (see Materials and Methods). At the 4 h time point, *Ilk<sup>-/-</sup>* OPCs accumulated in the most proximal concentric ring (50  $\mu$ m) and fewer attained the more distant 100  $\mu$ m ring as compared to *Ilk<sup>fl/fl</sup>* control cells. Interestingly however, *Ilk<sup>-/-</sup>* and *Ilk<sup>fl/fl</sup>* OPCs migrated to the same extent at the 10 and 24 h time points. [C] No significant difference between the total number of migrated *Ilk<sup>-/-</sup>* and *Ilk<sup>fl/fl</sup>* OPCs was found at any time point. [D] There was no significant difference in initial diameter of *Ilk<sup>-/-</sup>* and *Ilk<sup>fl/fl</sup>* oligospheres. Data represent the mean  $\pm$  SEM (n=5, 5 and 4 for the 4, 10 and

24 h time points, respectively). For [B], \* $p < 0.05$ ; \*\*\*\* $p < 0.0001$  (repeated measures two-way ANOVA followed by Bonferroni post-tests). For [C] and [D], data was tested with Student's  $t$  test. Scale bars, 50, 100 and 200  $\mu\text{m}$  for 4, 10 and 24 h time points, respectively.



(Milner et al., 1996; Gudź et al., 2006). As ILK is recruited to  $\beta_1$  and  $\beta_3$  integrin tails to stabilize actin at cell-matrix contacts, we hypothesized that ILK-depleted OPCs would suffer from defective migration on Fn substrates.

*Ilk*<sup>-/-</sup> and *Ilk* <sup>$\eta/\eta$</sup>  oligospheres were seeded onto Fn-coated coverslips, and migration was assessed after 4, 10 and 24 h. In contrast to migration on Ln, there was no substantial OPC migration of either genotype on Fn at 4 h. Not surprisingly then, we noted no migration differences between *Ilk*<sup>-/-</sup> and *Ilk* <sup>$\eta/\eta$</sup>  OPCs at this time point (Figure 4.5A,B). Rather, we observed significantly reduced migration distance of *Ilk*<sup>-/-</sup> OPCs at 10 and 24 h. At 10 h, *Ilk*<sup>-/-</sup> OPCs accumulated in the most proximal concentric ring (40  $\mu$ m), with correspondingly fewer cells reaching the more distal ring (80  $\mu$ m). This defect persisted at 24 h, indicating an importance for ILK in the net directional velocity of OPCs on Fn. The total number of migrated *Ilk*<sup>-/-</sup> OPCs was also significantly reduced at 10 and 24 h (Figure 4.5C), implying a role for ILK in initiating migration on Fn. We observed no significant difference in the size of *Ilk* <sup>$\eta/\eta$</sup>  and *Ilk*<sup>-/-</sup> oligospheres at seeding time (Figure 4.5D), excluding sphere size as a factor in our analysis. Our data suggest OPC migration on Fn heavily relies on ILK, as its loss compromises both net directional velocity and migration initiation.

### **ILK is required for an integrin-independent mode of OPC migration**

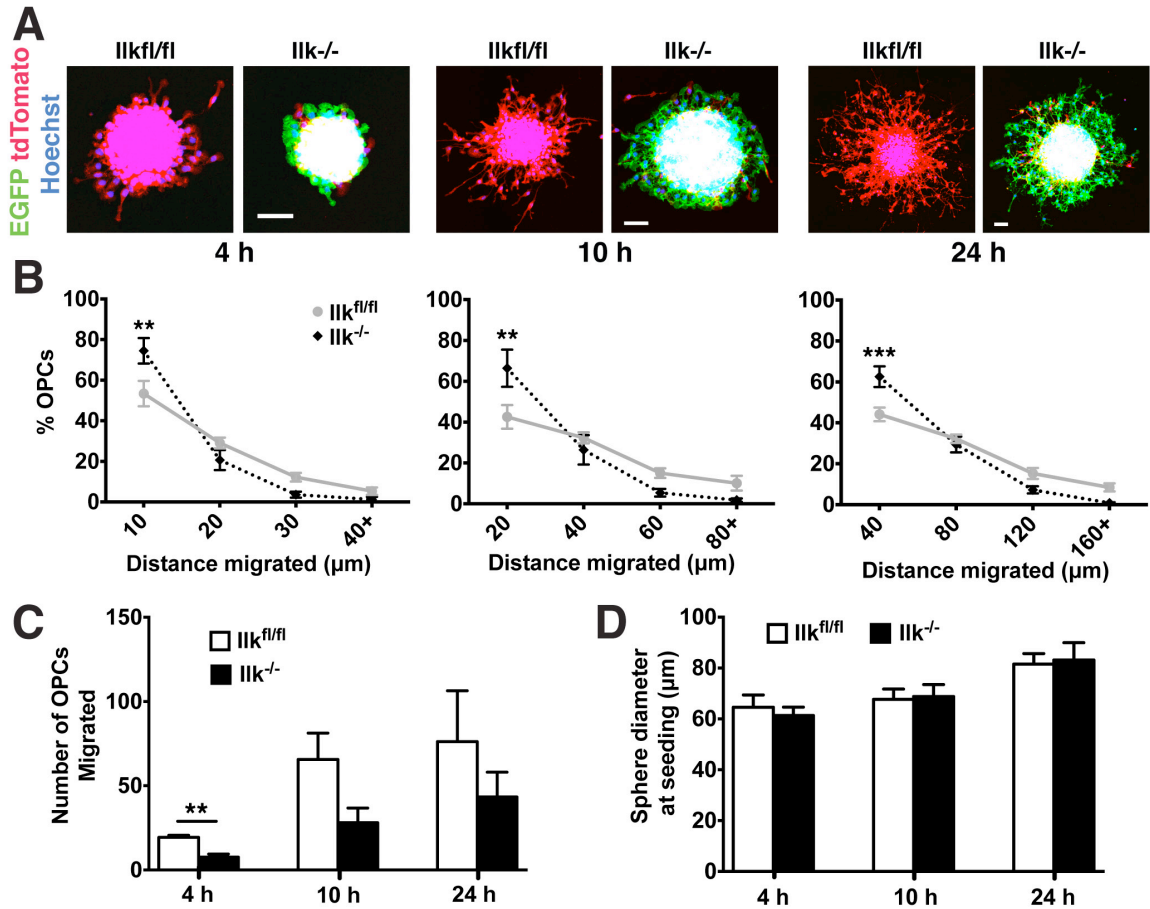
Presumably, defective migration of *Ilk*<sup>-/-</sup> OPCs on Ln and Fn stems from reduced integrin-actin stability at cell-matrix adhesion sites. Interestingly, recent studies have implicated ILK in more diverse cellular processes such as maintenance of nuclear integrity



significantly different for any of the time intervals investigated. Data represent the mean  $\pm$  SEM (n=3). For [B], \*p<0.05; \*\*p<0.01; \*\*\*p<0.001; \*\*\*\*p<0.0001 (repeated measures two-way ANOVA followed by Bonferroni post-tests). For [C] and [D], \*p<0.05 (Student's *t* test). Scale bars, 50  $\mu$ m for 4 h, 100  $\mu$ m for 10 and 24 h.

(Acconcia et al., 2007) and mRNA expression (Judah et al., 2012). A role for ILK in these processes could in turn impact OPC migration in a fashion that may not rely on canonical integrin adhesion/signaling. To test this hypothesis, we seeded oligospheres onto poly-D-lysine (PDL), which carries a net positive charge and interacts with anionic membrane domains to mediate adhesion (Mazia et al., 1975). Polylysine is therefore referred to as an integrin-independent substrate, as it does not mediate cell adhesion via the integrins (Machesky and Hall, 1997).

When migrated on PDL, *Ilk*<sup>-/-</sup> OPCs accumulated more so in the most proximal concentric rings at the 4, 10 and 24 h time points as compared to *Ilk*<sup>fl/fl</sup> cells (Figure 4.6A,B). These observations suggest ILK mediates OPC net directional velocity independent of integrin-ECM engagement. ILK depletion also yielded fewer total migrated OPCs at 4 hours, although no significant difference was observed at 10 and 24 h (Figure 4.6C). This early lag in emergence from the oligosphere offers a role for ILK in the initiation of OPC migration on PDL, which does not persist at later time points. Importantly, no difference was observed in the initial oligosphere diameters between genotypes (Figure 4.6D), excluding size as a confounding factor in our analysis. Instead, our data support an intriguing function for ILK in the initiation and net directional velocity of OPC migration independent of integrin engagement.



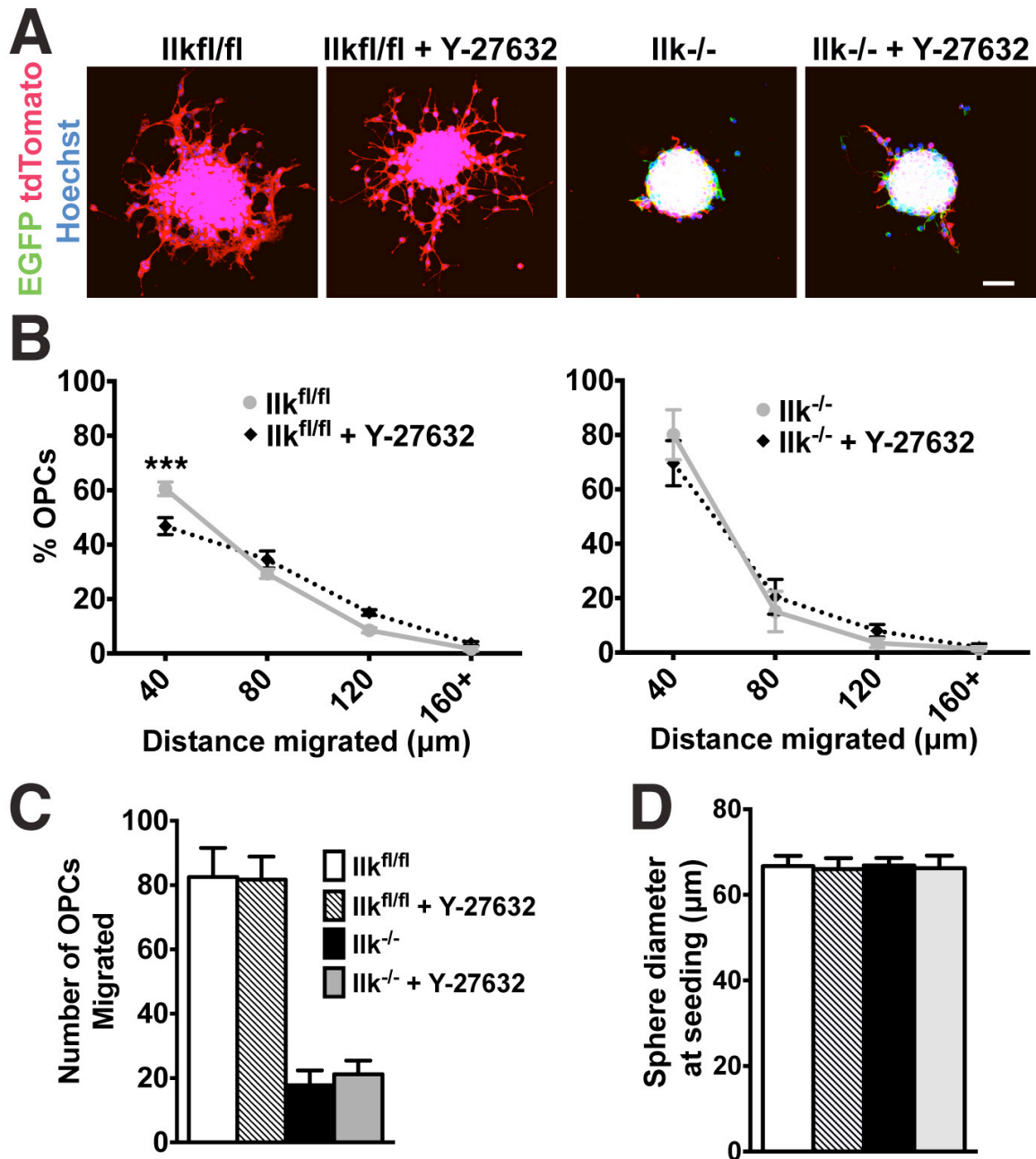
**Figure 4.6. ILK mediates the initiation and net directional velocity of OPC migration on PDL.** [A] Confocal images of *Ilk*<sup>-/-</sup> and *Ilk*<sup>fl/fl</sup> oligospheres migrated on PDL for 4, 10 and 24 h. [B] As compared to *Ilk*<sup>fl/fl</sup> OPCs, *Ilk*<sup>-/-</sup> OPCs were more concentrated in the 10, 20 and 40  $\mu$ m proximal concentric rings at 4, 10 and 24 h respectively. [C] No difference in the total number of migrated *Ilk*<sup>-/-</sup> versus *Ilk*<sup>fl/fl</sup> OPCs was observed at 10 or 24 h, although significantly fewer *Ilk*<sup>-/-</sup> OPCs had migrated at the 4 h time point. [D] There was no difference in the diameters of *Ilk*<sup>-/-</sup> versus *Ilk*<sup>fl/fl</sup> oligospheres prior to commencement of the assay for any of the time points assessed. Data represent the mean  $\pm$  SEM (n=4). For [B], \*\*p<0.01; \*\*\*p<0.001 (repeated measures two-way ANOVA followed by Bonferroni post-tests). For [C] and [D], \*\*p<0.01 (Student's *t* test). Scale bars, 50  $\mu$ m.

## **ROCK inhibition promotes fibronectin-mediated OPC migration in an ILK-dependent manner**

Previous work has shown that ILK is involved in the regulation of RhoA (Kogata et al., 2009; Blumbach et al., 2010; reviewed by Wickström et al., 2010 and Ghatak et al., 2013), in that ILK loss upregulates RhoA activity, and concomitantly reduces cell-matrix adhesions (Elad et al., 2013). Accordingly, we previously found ILK loss increased RhoA activity in OLs, a phenomenon that coincided with abnormal actin distribution (O'Meara et al., 2013). Conversely, reducing RhoA activity promotes reorganization of focal contacts (Lavelin et al., 2013), and diminishes actomyosin contractility to facilitate migration (reviewed by Lauffenburger and Horwitz, 1996). We therefore hypothesized that *Ilk*<sup>-/-</sup> OPCs suffered from heightened RhoA activity that hindered migration on Fn, and that pharmacological downregulation of RhoA would restore migration capacity.

Overactive RhoA signaling can be rectified by experimentally inhibiting ROCK, a RhoA effector. We therefore seeded oligospheres onto Fn-coated coverslips in the presence of Y-27632 (ROCK inhibitor) and allowed cells to migrate for 10 h. Y-27632 enhanced the net directional velocity of *Ilk*<sup>fl/fl</sup> OPCs as evidenced by an increased proportion of cells in more distal concentric quantification rings as compared to vehicle treated *Ilk*<sup>fl/fl</sup> control cells (Figure 4.7A,B). Migration initiation was not affected by Y-27632, as the total number of migrated *Ilk*<sup>fl/fl</sup> OPCs was unchanged (Figure 4.7C). In contrast to its effect on *Ilk*<sup>fl/fl</sup> OPCs, Y-27632 did not enhance the net directional velocity of *Ilk*<sup>-/-</sup> OPCs (Figure 4.7A,B), indicating ILK is required for this pro-migratory phenomenon. Similarly, application of Y-27632 did not affect the number of migrated *Ilk*<sup>-/-</sup> OPCs with respect to vehicle treated *Ilk*<sup>-/-</sup> control cells, revealing no influence on

migration initiation. There was no difference in oligosphere diameter across all treatment groups (Figure 4.7D), indicating our observations were not attributable to sphere size. Our data rather point to a role for Y-27632 in promoting the net directional velocity of wild type ( $Ilk^{fl/fl}$ ) OPCs, a phenomenon that requires ILK to occur.



**Figure 4.7. ROCK inhibition enhances OPC net directional migration velocity on fibronectin in an ILK-dependent manner.** [A] Confocal images of *Ilk*<sup>-/-</sup> and *Ilk*<sup>fl/fl</sup> Fn-migrated oligospheres at 10 h time point treated with either a ROCK inhibitor (Y-27632) or vehicle control (H<sub>2</sub>O). [B] *Ilk*<sup>fl/fl</sup> OPCs treated with Y-27632 were less concentrated in the ring most proximal to the oligosphere core (40  $\mu\text{m}$ ) as compared to vehicle treated



*Ilk<sup>fl/fl</sup>* control conditions. Y-27632 treatment had no significant impact on the distance distribution of *Ilk<sup>-/-</sup>* OPCs compared to vehicle treated controls. **[C]** No difference in the total number of migrated OPCs existed between Y-27632 treated *Ilk<sup>fl/fl</sup>* and vehicle treated *Ilk<sup>fl/fl</sup>* OPCs, or Y-27632 treated *Ilk<sup>-/-</sup>* and vehicle treated *Ilk<sup>-/-</sup>* OPCs. **[D]** The starting oligosphere size was not different for any of the groups. Data represent the mean  $\pm$  SEM (n=4). For [B], \*\*\*p<0.001 (repeated measures two-way ANOVA followed by Bonferroni post-tests). For [C] and [D], data was tested with one-way ANOVA followed by Bonferroni post-tests. Scale bar, 50  $\mu$ m.

## Discussion

### A novel assay to study mouse OPC migration *in vitro*

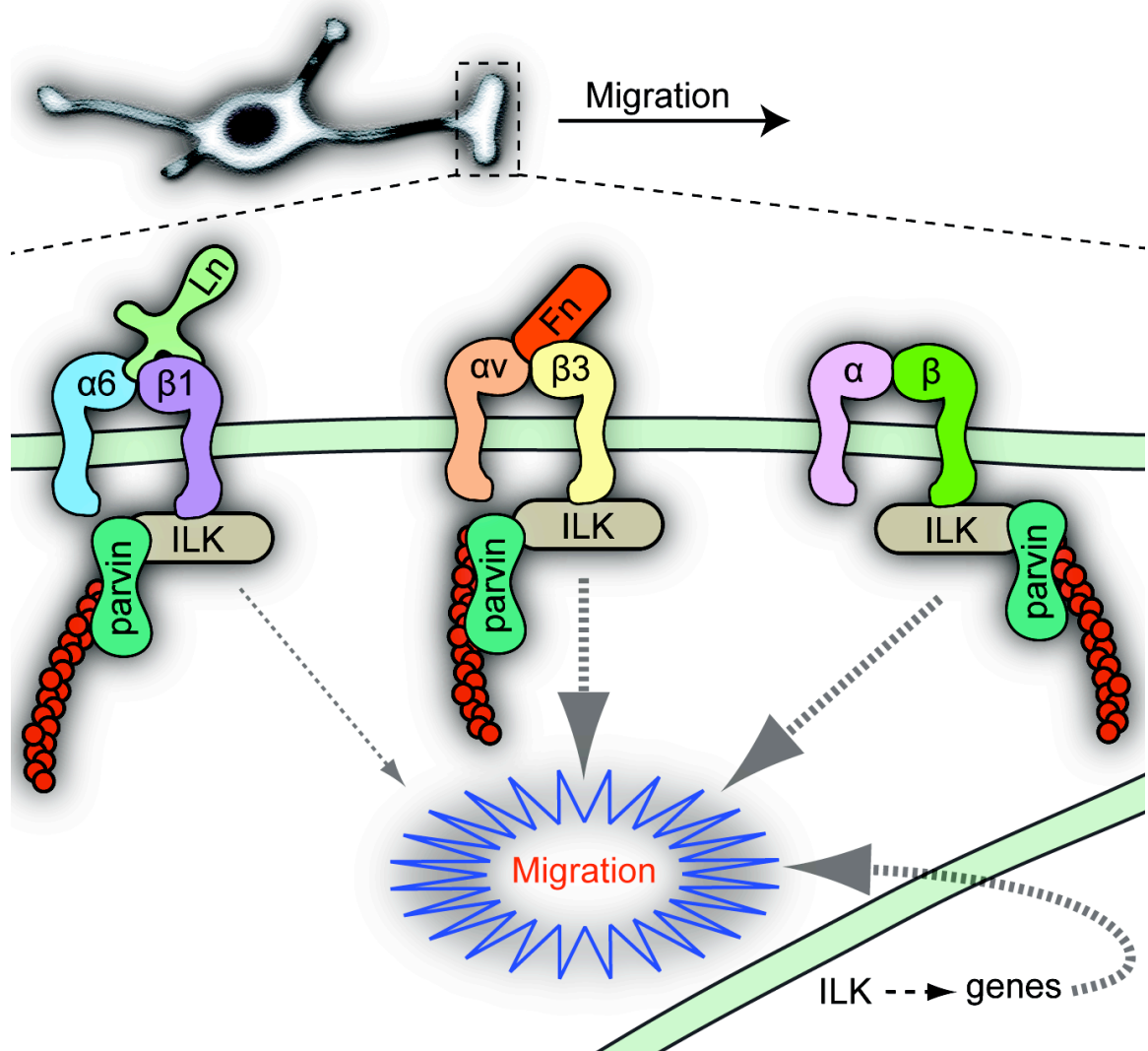
The use of mouse OPCs in basic research is becoming increasingly popular, a trend reflecting the availability of transgenic lines. However, the utility of mouse OPCs is limited, as they are more difficult to isolate (Chen et al., 2007) and do not survive as well in culture as their rat counterparts (Horiuchi et al., 2010). This imposes constraints on the commonly used methods for studying OPC migration *in vitro*, emphasizing the need for assays that accommodate the shortcomings of mouse OPCs by minimizing both assay duration and number of required cells. We estimate that an oligosphere is composed of approximately 300-1000 cells, and a typical preparation yields roughly 50-100 oligospheres. Additionally, migration can be measured in as little as 4 h, thereby providing a dually suitable technique for studying mouse OPC motility.

One limitation of the oligosphere method is the inability to quantitatively follow migration from a single sphere in real time. This assay is restricted in that oligospheres must be fixed (*i.e.* experiment terminated) to accurately quantify migration, a drawback potentially overcome by its application in live-imaging microscopy. In our hands however, we have experienced phototoxicity as a result of visualizing fluorescent proteins (*e.g.* EGFP, tdTomato) in live OPCs. In addition, live imaging may not be a suitable approach when assessing the effect of photosensitive compounds (*e.g.* Y-27632; Claassen et al., 2009) on OPC migration.

### **ILK governs OPC migration in a substrate-dependent manner**

OPCs express the  $\alpha_6\beta_1$  integrin Ln receptor (Milner and Ffrench-Constant, 1994), to which ILK binds at the  $\beta_1$  cytoplasmic tail (Hannigan et al., 1996). The compromised migratory potential of *Ilk*<sup>-/-</sup> OPCs on Ln presumably results from a disconnection between Ln- $\alpha_6\beta_1$  complexes and the actin cytoskeleton. Interestingly however, ILK loss yielded only a transient deficit in OPC migration on Ln. This is in agreement with previous studies that demonstrated  $\alpha_6\beta_1$  integrin is required for the early phases of OPC migration on Ln (Gudz et al., 2006), with no significant contribution in the long term (Milner et al., 1996). These data insinuate  $\alpha_6\beta_1$  integrin plays a more significant role in other aspects of OL biology, as it is the major  $\beta_1$ -type receptor expressed by OLs, and is consistently expressed throughout OL development (Milner and Ffrench-Constant, 1994). Accordingly, extension of OL myelin-like membrane (Buttery and Ffrench-Constant, 1999), and CNS myelination (Lee et al., 2006; Câmara et al., 2009) are regulated by Ln- $\alpha_6\beta_1$  interactions, and similar functions have been identified for ILK (Chun et al., 2003; O'Meara et al., 2013). The Ln- $\alpha_6\beta_1$ -ILK complex may therefore be more relevant in post-migratory OL development, while only mildly contributing to Ln-based motility (Figure 4.8). Notably, in the absence of ILK, we still observed extensive OPC migration on Ln suggesting alternate signaling mechanisms predominantly drive migration on this substrate.

We offer the possibility that OPCs express additional ILK-independent receptors that contribute to OPC migration on Ln. Existence of such receptors could explain how *Ilk*<sup>-/-</sup> OPCs are only mildly incapacitated in their migration on



**Figure 4.8. Hypothetical representation of how ILK differentially contributes to OPC migration through disparate signaling pathways.** The degree of dependence on ILK in the mediation of OPC migration is pathway-specific, indicated by the weight of the gray arrows. For example, ILK only modestly regulates OPC migration on Ln through the  $\alpha_6\beta_1$ , but it strongly regulates migration on Fn, likely via  $\alpha_v\beta_3$  (Gudz et al., 2006). ILK can also regulate OPC migration independent of integrin engagement (far right), which may be a result of ILK's putative role in regulating the expression of migration-related genes. In an additional/alternative hypothesis, the actin cytoskeleton

could be somewhat stabilized by an ECM-unbound integrin receptor via the ILK-parvin complex. This unengaged integrin would act as a relatively weak (albeit perhaps partially functional) cytoskeletal anchor that would be less effective at mediating OPC migration due to the absence of extracellular integrin ligands needed for transmission of force.

Ln, and ultimately travel to the same degree as control cells. One candidate is dystroglycan, a Ln binding protein expressed by OL-lineage cells (Colognato et al., 2007), with a function in OL process outgrowth (Eyermann et al., 2012). Dystroglycan localizes to OL adhesion contact sites, and co-precipitates with focal adhesion kinase (Eyermann et al., 2012). Recent work has also shown that nestin-Cre driven deletion of dystroglycan negatively impacts cerebellar granule neuron migration (Nguyen et al., 2013), offering a parallel function for dystroglycan in OPCs, as neurons and OLs share much of the same migratory mechanisms (de Castro and Bribián, 2005). The role of dystroglycan (and/or other Ln adhesion molecules) in OPC migration remains an open question for investigation, and may provide an explanation for the highly migration-permissive character of Ln.

In contrast to Ln, for which  $\alpha_6\beta_1$  is the exclusive integrin receptor, OPCs express the Fn-sensitive  $\alpha_v\beta_1$ ,  $\alpha_v\beta_3$ ,  $\alpha_v\beta_5$  and  $\alpha_v\beta_8$  integrins (Milner and French-Constant, 1994). With regard to our work,  $\alpha_v\beta_3$  and  $\alpha_v\beta_1$  are most relevant, as ILK exclusively binds  $\beta_1$  and  $\beta_3$  cytosolic tails (Hannigan et al., 1996; Pasquet et al., 2002). Gudz and colleagues (2006) previously demonstrated a role for  $\alpha_v\beta_3$  in OPC migration on Fn, while  $\alpha_v\beta_1$  was contrarily determined dispensable. A role for  $\beta_1$  integrin in OPC migration on Fn was suggested by Tiwari-Woodruff and group in 2001, although in their study, migration was assessed after 4 days *in vitro*, a point at which OPC proliferation may confound observations. Rather, in line with Gudz et al. (2006), we favour the concept that the migratory phenotype manifested by *Ilk*<sup>-/-</sup> OPCs on Fn is primarily a product of perturbed signaling/stability at Fn- $\alpha_v\beta_3$ -actin connections (Figure 4.8). Importantly, this does not discount a role for  $\alpha_v\beta_1$  integrin in OPC migration in a broader context, as this receptor

significantly mediates OPC migration on astrocyte-produced ECM (Milner et al., 1996), a substrate surely composed of various integrin ligands. *Ilk*<sup>-/-</sup> OPCs were not completely ablated in their ability to migrate on Fn, suggesting that receptors other than  $\alpha_v\beta_3$  and  $\alpha_v\beta_1$  may be at play. Of note, Gudz and colleagues (2006) previously found no role for  $\alpha_v\beta_5$  in OPC migration on Fn, leaving the less-characterized  $\alpha_v\beta_8$  integrin receptor with a possible function in this process (Milner et al., 1996).

### **ILK regulates OPC migration independent of integrin engagement**

The adhesion of cells to PDL is mediated by charge interactions between anionic membrane domains and the positively charged nature of polylysine (Mazia et al., 1975). As polylysine does not engage the integrins, the impaired migration of *Ilk*<sup>-/-</sup> OPCs cannot be a result of ILK's canonical role in stabilizing actin to ECM-bound integrin receptors. Rather, it's possible this reduced migration capacity subsequent to ILK loss is caused by the altered expression of genes required for membrane protrusion and/or cytoskeletal reorganization (Figure 4.8). In support of this idea, ILK has been found to localize to the nucleus where it has roles in maintaining nuclear integrity (Acconcia et al., 2007) and DNA synthesis (Nakrieko et al., 2008). ILK perturbation alters the expression of cytoskeletal genes (Judah et al., 2012), exemplified by reduced RhoE mRNA in ILK depleted peripheral nerves (Pereira et al., 2009). It is thus plausible that the migratory defects suffered by *Ilk*<sup>-/-</sup> OPCs in the absence of integrin engagement results in part from dysregulation of migration dependent genes.

The impaired migration of *Ilk*<sup>-/-</sup> OPCs on polylysine could also be caused by the disconnection of actin from ECM-unengaged integrin receptors that still retain some

capacity to stabilize the cytoskeleton. Upon binding to immobilized ECM, integrin receptors cluster into focal contact points (Cluzel et al., 2005), structures within the leading lamellipodium that promote cellular migration (Beningo et al., 2001; Zaidel-Bar et al., 2004). As cellular adhesion to polylysine is independent of integrin engagement, focal contacts do not form on this substrate (Machesky and Hall, 1997). Nonetheless, lamellipodia still contain polymerizing actin that colocalizes with vinculin (a focal contact protein) on polylysine, albeit in a diffuse pattern (Machesky and Hall, 1997). ILK has a role in the formation of focal contacts (Elad et al., 2013), and may behave similar to vinculin on polylysine, where it still mediates integrin-actin connections, but not at distinct focal contacts. In this scenario, the cytoskeletal machinery driving migration would presumably be compromised due to the lack of discrete integrin-actin anchor points. Consistently, we observed that PDL substrates resulted in slower OPC migration velocities as compared to Ln and Fn matrices. Therefore, in an alternative/additional explanation for the defective migration of *Ilk*<sup>-/-</sup> OPCs on polylysine, ILK loss could exacerbate the already compromised integrin-actin linkage, further hindering migration. Another possibility is that integrin receptors still cluster into focal contacts on polylysine, but since the ECM does not bind them, they are inherently less stable. Upon ILK loss in this context, the ECM-unengaged “focal contacts” (which presumably cannot withstand the cytoskeletal forces required for proper migration) would be further incapacitated due to a disconnection from actin, yielding impaired migration.

The ECM is altered in MS lesions (Sobel, 1998), and as such, a large research effort has been focused on understanding how the ECM modulates OL function. Our work advances the knowledge on how the ECM impacts OPC migration by identifying



ILK as a signaling intermediate in migration on Ln and Fn (Figure 4.8), two molecules present in demyelinated lesions (Zhao et al., 2009; Stoffels et al., 2013a). We also show that ILK can regulate OPC migration independent of veritable ECM engagement. As impaired OPC infiltration into MS lesions is thought to partly underlie the reduced regeneration seen in chronic MS, elucidation of the mechanics controlling OPC migration is an important objective in the pursuit of remyelination therapeutics.

### **RhoA pathway control of OPC migration on fibronectin necessitates ILK**

Our data indicate that downregulation of RhoA signaling enhances OPC migration velocity on Fn, an effect that requires ILK. Migration of OPCs on Fn is predominantly mediated by  $\alpha_v\beta_3$  integrin (Gudz et al., 2006), to which ILK associates at cytosolic  $\beta_3$  tails (Pasquet et al., 2002). Fn-ligation of  $\alpha_v\beta_3$  integrin promotes persistent directional migration, a phenomenon associated with relatively low RhoA-GTP levels (Danen et al., 2005). We can therefore speculate that, in wild type OPCs, Fn- $\alpha_v\beta_3$  ligation encourages persistent OPC migration through RhoA downregulation. Application of Y-27632 would further suppress RhoA signaling, thereby tipping the balance more in favour of Fn- $\alpha_v\beta_3$  integrin-mediated directionally persistent migration. This positive effect would depend on ILK's scaffolding role at Fn- $\alpha_v\beta_3$  contact sites, as its loss would disconnect the actin-matrix bond required for cellular traction. This provides an explanation for why Y-27632 treatment did not enhance the net migration velocity of *Ilk*<sup>-/-</sup> OPCs on Fn.

The RhoA signaling axis is becoming of increasing interest with regard to MS therapeutics. Other proteins such as Netrin-1, Slit2 and NG2 mediate OPC migration through modulation of RhoA signaling (Rajasekharan et al., 2010; Liu et al., 2012;

Binamé et al., 2013). RhoA pathway downregulation by pharmacological means enhances morphological development of OL-lineage cells (Wolf et al., 2001; Liang et al., 2004; O'Meara et al., 2013), even in the presence of OL-inhibitory factors (Baer et al., 2009; Siebert and Osterhout, 2011). Blockade of LINGO-1, an upstream regulator of RhoA (reviewed by Mi et al., 2013), not only facilitates OL differentiation *in vitro* (Mi et al., 2005) but also enhances remyelination *in vivo* (Mi et al., 2009). As multiple signaling pathways converge on RhoA to regulate OL biology, and the fact that RhoA-ROCK pathway inhibitors are currently in use for other CNS ailments (Chen et al., 2013), their efficacy in promoting remyelination in MS disease models remains an appealing direction for future investigation.

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# **Chapter 5**

## **General Discussion**

### **Developing cell culture models for studying mouse OLs *in vitro***

The primary research goal of this thesis was to elucidate the role of ILK in OL migration, differentiation and myelination. Early in this endeavour, we experienced significant difficulty in culturing primary mouse OLs by simply employing techniques that had originally been developed for rat-sourced cells. The possession of a *Ilk* cKO mouse prompted us to develop our own methods suitable for mouse OLs; an ambition achieved by modifying rat culture techniques (McCarthy and Vellis, 1980; Frost et al., 2000). Extensive experimentation led to the establishment of a reliable and valid method for isolating purified populations of neonatal mouse cortical OPCs (NG2<sup>+</sup>) and differentiating them into mature (MAG<sup>+</sup>/MBP<sup>+</sup>) OLs *in vitro*. Additionally, we adapted the rat OL/DRG neuron co-culture technique (Chan et al., 2004) for use with mouse cells to produce myelinating co-cultures. Finally, we designed an *in vitro* migration assay where mouse OPCs radially emerge from spherical aggregates (oligospheres) in response to immobilized matrix.

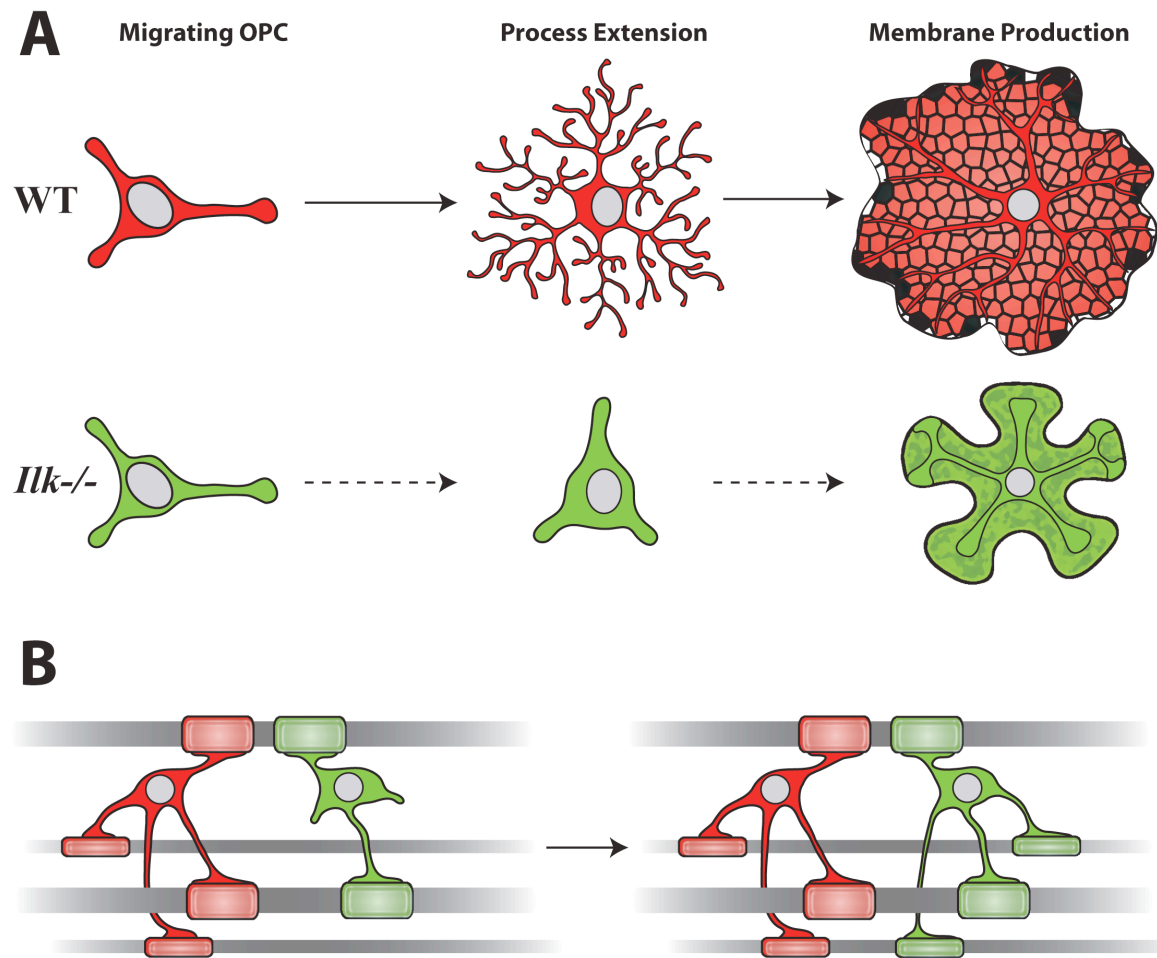
The significance of having pursued the development of these methods is twofold. First, it allowed us to use our conditional mouse model to study the function of ILK in the maturation, myelination and migration of primary OLs. Second, by publishing our methods we provided the field with new OL research tools, and accordingly, these techniques were subsequently implemented in several laboratories worldwide (Bin et al., 2012; Soyal et al., 2012; Giampetruzzi et al., 2013).

### **The function of ILK in OLs: A brief recapitulation**

Using the above-described methods, we identified a requirement for ILK in OPC migration, the extension of OL processes, formation of membrane sheets, ensheathment of neuronal processes, and timely expression of OL maturity markers (Figure 5.1). *In vivo*, ILK-depleted OLs produce shorter myelin internodes and transiently myelinate fewer small caliber optic nerve axons. Loss of ILK is accompanied by actin disorganization and heightened RhoA activity, and ectopic downregulation of RhoA signaling reinstates the morphology of a distinct population of *Ilk*<sup>-/-</sup> OPCs. Overall, our findings indicate ILK is required for proper OL function, with phenotypic severity (following ILK depletion) inversely correlated with cellular environmental complexity. Equivalently, OLs can functionally compensate for ILK-loss, the degree to which being positively associated with the richness of the cellular environment.

### ***Ilk* cKO mice recover from amyelination**

One theme evident herein is the ability of ILK-devoid cells to compensate for loss of function, best highlighted by the eventual recovery from the small axon amyelination phenotype. Our analysis favours the concept of developmental delay, *Ilk*<sup>-/-</sup> OLs seemingly able to “catch up” to WT OLs with time. This conclusion is supported by the fact that perturbation of other integrin signaling pathway members yields similar outcomes. Expressing dominant-negative  $\beta 1$  integrin in OLs results in small caliber axon amyelination with subsequent recovery (Câmara et al., 2009), phenocopying our *Ilk* cKO model. Likewise, knockout of FAK (functionally related to ILK) produces transient amyelination (Câmara et al., 2009; Forrest et al., 2009) whereas juvenile Ln deficient



**Figure 5.1. ILK regulates several aspects of OL development.** [A] WT OLs (red) originate as motile precursors, which extend a network of branches as they differentiate into immature OLs. Terminal OL differentiation is characterized by myelin-like membrane production *in vitro*. In ILK-depleted OLs (green), these biological processes are impaired (indicated by the dashed arrow). [B] *In vivo*, myelinating WT OLs contact and enwrap neighbouring axons with multiple layers of myelin membrane. Loss of ILK hinders the OL's ability to myelinate small diameter axons (at P14), likely a result of impaired process outgrowth. Given sufficient time however, *Ilk*<sup>-/-</sup> OLs regain the capacity to myelinate this axonal cohort, rendering the myelin of the *Ilk* cKO optic nerve indistinguishable from WT at P28.

mice (*dy/dy*) temporarily possess fewer mature OLs in several CNS regions with ensuing acquisition of normalcy (Relucio et al., 2009). These findings underscore the remarkable developmental plasticity of OLs; a trait we believe facilitates phenotypic recovery post ILK-loss.

### **Compensation following ILK loss: *In vivo* mechanistic considerations**

The question of how *Ilk* cKO ONs recover from small-caliber axon myelination is thought provoking. We previously put forth dystroglycan, another Ln-binding cell adhesion molecule, as a possible mediator of *Ilk*<sup>-/-</sup> OL functional recovery (see discussions of Chapter 3 and 4). While this hypothesis is viable, exclusively considering Ln pathways to explain phenotypic improvement would be shortsighted, as there are ample other axoglial systems that contribute to CNS myelination. This is well illustrated by the Neuregulin-1 (NRG1)/ErbB receptor-ligand complex that positively regulates CNS myelination. NRG1 isoform III is an axonal-surface bound ligand for OL ErbB receptors (Nave and Salzer, 2006), where perturbation of NRG1/ErbB signaling yields thinner myelin (Makinodan et al., 2012) as well as reduced action potential velocity (Roy et al., 2007). Neuronal-specific overexpression of NRG1 isoform I, a variant that is shed from the axonal surface, induces hypermyelination of small caliber axons (Brinkmann et al., 2008). This specific impact on small diameter axons provides a putative mechanism for recovery of this axonal cohort in *Ilk* cKO mice.

Alternatively (or additionally), repressing negative regulators of myelination could confer compensatory ability. Consider LINGO-1 (Leucine-rich repeat and Ig domain containing Nogo receptor interacting protein 1), a transmembrane protein known



for inhibiting axonal outgrowth in response to myelin-derived inhibitory factors (Mi et al., 2004). Mechanistically, OL-bound LINGO-1 is thought to self-associate with axonal-surface tethered LINGO-1 to inhibit OL differentiation and CNS myelination (Lee et al., 2007; Jepson et al., 2012). It's possible that compensatory downregulation of OL and/or axonal LINGO-1 could offset the loss of pro-myelin signaling in our *Ilk* cKO model.

We speculate that the recovery of small diameter axon myelination in *Ilk* cKO mice occurs via upregulation and/or downregulation of positive and negative regulators of myelination, respectively. This hypothesis is supported by the decrease in *Ilk*<sup>-/-</sup> OL phenotypic severity with increasing environmental complexity. Isolated *Ilk*<sup>-/-</sup> OLs suffer from major morphological deficits in purified cultures, whereas in the presence of neurons (co-culture), defects are less dramatic. *In vivo*, the most complex cellular context, ILK loss only yields transient myelination with no apparent long-lasting consequence. Neurons and other support cells therefore appear to offset the burden of ILK-depletion, possibly by employing additional axoglial systems that govern myelination.

### **Compensation following ILK loss: *In vitro* mechanistic considerations**

While the contribution of Ln-independent axoglial signaling satisfies the capacity for functional recovery *in vivo*, how can we explain compensation *in vitro* when only Ln substrate is present? This is best demonstrated by the fact that at DIV3, isolated *Ilk*<sup>-/-</sup> OLs are morphologically aberrant but by DIV6 many are paradoxically capable of producing myelin-like membranes (but to a lesser degree than WT). This morphological compensation must be restricted to Ln-specific mechanisms, as the axoglial support provided *in vivo* is absent. Dystroglycan could assume this role, but other

receptors/adhesion molecules such as membrane-associated carbohydrates and lipids also possess Ln affinity (Mecham, 1991). This is true for sulfatide (Mecham, 1991), a sulfated member of the glycosphingolipid family that comprises much of OL membranes (Jackman et al., 2009). Blockade of OL sulfatide-Ln interactions *in vitro* hinders the generation of mature OLs and the production of myelin-like membranes (Baron et al., 2014). Mice devoid of sulfatide develop progressive motor deficiencies (Honke et al., 2002) with concordant spinal cord hypomyelination (Marcus et al., 2006) possibly caused by reduced OL process extension *in vivo* (Shroff et al., 2009). Sulfatide-deficient mice are also plagued with myelin abnormalities including vacuolation (Honke et al., 2002; Marcus et al., 2006), lateral loop detachment (Honke et al., 2002; Marcus et al., 2006), uncompacted myelin (Marcus et al., 2006) and decreased ion channel segregation at the nodes of Ranvier (Ishibashi et al., 2002). Considering these facts, it is appealing to speculate that Ln-sulfatide signaling facilitates *Ilk*<sup>-/-</sup> OL functional compensation to allow production of myelin membrane in isolated culture. Accordingly, Ln-sulfatide interactions are known to activate SFKs and FAK (Li et al., 2005), two signaling mechanisms involved in CNS myelination (Sperber et al., 2001; Forrest et al., 2009). Also consistent with our hypothesis, the expression of sulfatide coincides with the onset of OPC differentiation (Jackman et al., 2009), a point only after which we see *Ilk*<sup>-/-</sup> OL morphological improvement.

Collectively, our work strongly supports the concept of functionally redundant signaling pathways that govern OL maturation. Our observations also provide evidence for the plasticity of myelination, capable of overcoming physiological setbacks perhaps by fine-tuning parallel axoglial myelination systems.

### **ILK: A kinase or a scaffold?**

Having identified a role for ILK in OL development, the question of its exact mechanism of function remains. At its discovery, ILK was classified as a kinase due to its observed capacity to phosphorylate the generic MBP substrate *in vitro* (Hannigan et al., 1996). Thereafter, several *bona fide* ILK targets were proposed, most notably glycogen synthase kinase 3 (GSK3) and Akt (Delcommenne et al., 1998; reviewed by Hannigan et al., 2011). Concurrently, the field was struggling to reconcile how ILK, with significant sequence divergence from typical serine/threonine kinases, could possess veritable activity (Hannigan et al., 1996; Legate et al., 2006; Wickström et al., 2010). While ILK retains select residues and motifs involved in ATP binding and substrate recognition, several highly conserved sequences required for catalysis are missing/altered (Ghatak et al., 2013).

Much structural and biochemical investigation has been conducted in an effort to shed light on the true catalytic nature of ILK's so-called kinase domain. Crystal structure analysis has revealed that the overall architecture of the ILK kinase domain is incompatible with typical kinase function (Fukuda et al., 2009). Additionally, the CH2 domain of parvin binds the active site of the ILK kinase domain (Fukuda et al., 2009), likely blocking any enzymatic functionality (Fukuda et al., 2011). Biochemically, activity for ILK is improbable as it is unable to phosphorylate any targets in an unbiased whole-cell lysate substrate screen (Fukuda et al., 2011). What, then, about the ILK point mutants purported to render the kinase inactive or hyperactive? When these mutations are introduced *in vivo*, mice are either indistinguishable from WT, or possess a phenotype attributable to reduced ILK affinity for parvin (Lange et al., 2009; Wickström et al.,

2010). Introducing point mutations into the ILK kinase domain also reduces its stability (Fukuda et al., 2011; Moik et al., 2013), likely by disrupting intramolecular bonding within the domain core (Fukuda et al., 2011). For these reasons, ILK is officially branded a pseudokinase, meaning it may have possessed catalytic activity earlier in evolution, but now uses the kinase domain for protein associations (Wickström et al., 2010). Conclusively, prevailing opinion is in support of ILK's primary function as a structural platform for protein interactions (Widmaier et al., 2012), a role consistent with its extensive interactome (Dobrev et al., 2008).

Given there were no changes in Akt or GSK3- $\beta$  phosphorylation in *Ilk*<sup>-/-</sup> OL cultures, we favour the view of ILK as a scaffold. Appropriately, *Ilk* deletion also caused  $\alpha$ -parvin destabilization and disorganized actin, findings remarkably similar to those of Lange and colleagues (2009) who used an ILK mutant possessing reduced affinity for  $\alpha$ -parvin. As  $\alpha$ -parvin provides a link between ILK and the actin cytoskeleton, we offer that the defects suffered by *Ilk*<sup>-/-</sup> OLs result from destabilized actin architecture rather than lost kinase function.

### **Functional mechanism of ILK in OLs**

In OLs, ILK appears to act as an adaptor that mediates the interaction of cytoskeletal proteins to stabilize process outgrowth. Consistently, other scaffolding molecules contribute to OL biology in a similar fashion, such as the Wiskott-Aldrich syndrome protein family verprolin-homologous proteins (WAVEs). WAVEs act as adaptors for actin monomers and the cytoskeleton extension factor Arp2/3 (Actin related proteins 2/3) to facilitate actin polymerization and subsequent membrane protrusion (Takenawa and

Suetsugu, 2007). Both molecular and morphological maturation are hindered in *Wave1*<sup>-/-</sup> OLs (Kim et al., 2006), mirroring the consequence of ILK loss. Also similar to our *Ilk* cKO mutants, *Wave1*<sup>-/-</sup> mice suffer from optic nerve amyelination. Interestingly, the WAVE2 isoform is required for PDGF-driven OPC migration (Miyamoto et al., 2008). In addition to ILK then, WAVES further demonstrate the importance of scaffolds in OL function, roles fulfilled in the absence of intrinsic kinase activity.

### **ILK-dependent signaling in OLs**

Although we disfavor the notion of ILK as possessing intrinsic activity, our work suggests it does in fact contribute to OL signaling indirectly, exhibited by RhoA hyperactivity in *Ilk*<sup>-/-</sup> cultures. The increased RhoA activity was positively correlated with the aberrant morphology of MAG<sup>+</sup> *Ilk*<sup>-/-</sup> OLs, thus we hypothesized that pharmacological downregulation of RhoA signaling would restore their morphology. Curiously, RhoA pathway inhibition did not rescue the defective arborization that was characteristic of MAG<sup>+</sup> *Ilk*<sup>-/-</sup> OLs, or ameliorate their capacity to produce myelin membrane. Instead, RhoA pathway suppression improved the morphology of a subpopulation of NG2<sup>+</sup>/MAG<sup>-</sup> *Ilk*<sup>-/-</sup> OPCs. These observations imply a partial role for ILK in governing OL morphology via RhoA during an early developmental time window, but not after the acquisition of maturity marker MAG<sup>+</sup>. Nonetheless, MAG<sup>+</sup> *Ilk*<sup>-/-</sup> OLs possess morphology suggestive of Rho GTPase dysregulation, highlighting a possible role for ILK in regulating other members of this signaling family.

Two candidates are Rac1 and Cdc42, Rho GTPases respectively responsible for generating cellular lamellipodia and filopodia (Govek et al., 2005) such that their

exogenous deactivation inhibits OL process elaboration (Liang et al., 2004). Given the persistent stunted morphology of  $\text{MAG}^+ \text{Ilk}^{-/-}$  OLs upon RhoA pathway depression, we can alternatively envision ILK as a regulator of Cdc42 and Rac1 to control late OL morphogenesis. Consistently, ILK depletion in SCs (myelinating cell of the peripheral nervous system) deregulates Cdc42 and Rac1 activity, along with a shortening of SC processes and profound sciatic nerve amyelination (Pereira et al., 2009). Counterintuitively however, Cdc42 and Rac1 activities are upregulated in  $\text{Ilk}^{-/-}$  sciatic nerves (Pereira et al., 2009), phenomena positively correlated with OL morphological enhancement (Liang et al., 2004). This contradiction may be reconciled by the suggestion that certain Rho GTPase signaling pathways have opposing developmental roles in OLs and SCs. For example, inhibition of non-muscle myosin II (an effector of RhoA activity) hinders the *in vitro* myelination capacity of SCs but paradoxically enhances both OL membrane production and myelinating ability (Wang et al., 2008). Pereira and colleagues (2009) nonetheless provide evidence for ILK as a regulator of Cdc42 and Rac1 in a cell type functionally related to OLs. The questions of whether these alternative signaling axes contribute to  $\text{MAG}^+$  OL process extension in an ILK-dependent manner, and whether their manipulation can rescue the morphology of this OL subpopulation upon ILK depletion remain.

Pharmacological downregulation of RhoA signaling enhanced the Fn-directed migration velocity of WT but not  $\text{Ilk}^{-/-}$  OPCs. This insensitivity of ILK-devoid OPCs to ROCK inhibition may stem from impaired capacity to rapidly form/turnover focal contacts (Elad et al., 2013), further exacerbated by ECM-cytoskeleton disconnect. When considering alternate ILK-dependent mechanisms that regulate motility, it is notable that

Cdc42 in itself is dispensable for OPC migration (Thurnherr et al., 2006) in contrast to its function in OL morphogenesis (Liang et al., 2004). This underscores the potential functional dichotomy of the Rho GTPases with regard to distinct OL biological processes. The role of Rac1 signaling in OPC migration is currently unknown, and ILK's possible regulation of Rac1-dependent motility remains a query worthy of future investigation.

### **Concluding remarks**

Our efforts have led to the generation of three new cell culture techniques useful for assessing OL differentiation, myelin protein expression, proliferation, survival/apoptosis, migration and myelination. We subsequently used these models as means to identify ILK as an important player in several of these processes *in vitro*. Additionally, we confirmed the relevance of our *in vitro* observations by determining a role for ILK in CNS myelination. Our work adds to the ever-growing understanding and appreciation of the outside-in signaling mechanisms that positively regulate OL development and myelination.

The fact that many MS lesions contain stalled premyelinating OLs or peripherally accumulated OPCs marks the importance of studying the machinery regulating OPC migration, differentiation and myelination. As future MS therapies are targeted at promoting regeneration, continued elucidation of OL signaling will expectedly reveal pathways useful in attaining this ultimate objective.

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