Microglial transglutaminase-2 drives myelination and myelin repair via GPR56/ADGRG1 in oligodendrocyte precursor cells

Stefanie Giera¹, Rong Luo¹, Yanqin Ying¹,⁵, Sarah D. Ackerman²,⁶, Sung-Jin Jeong¹,⁷, Hannah M. Stoveken³, Christopher J. Folts¹, Christina A. Welsh⁴, Gregory G. Tall³, Beth Stevens⁴, Kelly R. Monk²,⁸#, and Xianhua Piao¹#

¹F. M. Kirby Neurobiology Center, Children’s Hospital and Harvard Medical School, Boston, MA 02115 USA
²Department of Developmental Biology, Washington University School of Medicine, St. Louis, MO 63110, USA
³Department of Pharmacology, University of Michigan Medical Center, Ann Arbor, MI 48109, USA
⁴F. M. Kirby Neurobiology Center, Boston Children’s Hospital, Harvard Medical School, Boston, MA 02115 USA and Department of Neurology, Harvard Medical School, Boston, MA 02115, USA
⁵Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, 430030, P. R. China
⁶Present address: Institute of Neuroscience, University of Oregon, Eugene, OR 97403, USA
⁷Present address: Convergence Brain Research Department, Korea Brain Research Institute (KBRI), Daegu, 701-300, South Korea
⁸Present address: Vollum Institute, Oregon Health and Science University, Portland, OR 97239, USA
ABSTRACT

In the central nervous system (CNS), myelin formation and repair are regulated by oligodendrocyte (OL) lineage cells, which sense and integrate signals from their environment, including from other glial cells and the extracellular matrix (ECM). The signaling pathways that coordinate this complex communication, however, remain poorly understood. The adhesion G protein-coupled receptor ADGRG1 (also known as ADGRG1) is an evolutionarily conserved regulator of OL development in humans, mice, and zebrafish, although its activating ligand for OL lineage cells is unknown. Here, we report that microglia-derived transglutaminase-2 (TG2) signals to ADGRG1 on OL precursor cells (OPCs) in the presence of the ECM protein laminin and that TG2/laminin-dependent activation of ADGRG1 promotes OPC proliferation. Signaling by TG2/laminin to ADGRG1 on OPCs additionally improves remyelination in two murine models of demyelination. These findings identify a novel glia-to-glia signaling pathway that promotes myelin formation and repair, and suggest new strategies to enhance remyelination.

INTRODUCTION

Myelination of axons is essential for both efficient impulse conduction and for health of nerve fibers. During the development of the vertebrate central nervous system (CNS), myelin is produced and maintained by oligodendrocytes (OLs), which arise from
a lineage-restricted, proliferative pool of OL precursor cells (OPCs). OPCs are abundant
in the adult CNS, generating new oligodendrocytes and new myelin under conditions of
myelin damage, as is seen in demyelinating diseases and in rodent models of
demyelination. Unfortunately, the regenerative capacity of OPCs after injury is poor,
necessitating further investigation into the molecular mechanisms that guide both
developmental and post-injury induced OPC differentiation. A number of studies have
identified cell-autonomous regulators of OPC proliferation and terminal differentiation
into myelinating OLs, but our understanding of these processes remains incomplete
Furthermore, a comprehensive portrait that incorporates both cell-autonomous and -non-
autonomous factors, including OPC interactions with other cell types and with
extracellular matrix (ECM) proteins, is presently lacking (Mayoral and Chan, 2016,

Cell-cell interactions with other glial populations, in particular microglia,
influence OPC proliferation and maturation (Hagemeyer et al., 2017). While our
understanding of the molecular mechanisms underlying OPC-microglial interactions
during developmental myelination is limited, recent studies have offered some insight.
For example, the nonspecific suppression of microglial function with the anti-
inflammatory drug minocycline leads to a reduction in the numbers of neural progenitors
and OPCs during postnatal CNS development, suggesting that a cell-cell interaction is
critical to the maturation of the OL-lineage (Shigemoto-Mogami et al., 2014). By
comparison, crosstalk between OPCs and microglia has been widely reported in the
context of demyelinating insults (Miron et al., 2013), including in demyelinating diseases
such as toxin-induced murine models of demyelination in which clearance of damaged
myelin by microglia is necessary for OPC-dependent remyelination (Kotter et al., 2006). The molecular signaling pathways that mediate communication between microglia and oligodendrocyte lineage cells during development and repair, however, have not been fully delineated.

Similar gaps in knowledge exist for OPC communication with the ECM. The role of ECM proteins in modulating OPC proliferation and differentiation has been described (Jagielska et al., 2012, Urbanski et al., 2016). Nevertheless, the mechanisms through which OPCs communicate with the ECM and how these interactions are transduced into biologically- or therapeutically-relevant OPC behaviors remains to be elucidated.

To address the question of how OPCs integrate signals from the matrix and other glial cells, we focused on the family of adhesion G protein-coupled receptors (aGPCRs). Most aGPCRs possess a GPCR-Autoproteolysis-INducing (GAIN) domain that mediates autoproteolytic processing during protein maturation to generate an N- and a C-terminal fragment (NTF and CTF, respectively), which remain non-covalently associated on the cell surface (Langenhan et al., 2013, Hamann et al., 2015, Langenhan et al., 2016). These structural features make aGPCRs plausible transducers for cellular responses to ECM interactions, and specific aGPCRs have emerged as mechanical receptors and as transducers of cell-matrix interactions (Scholz et al., 2015, Petersen et al., 2015, Langenhan et al., 2016). Prior work has defined functions for several aGPCRs in the development of myelinating glial cells in both the peripheral and central nervous systems (Monk et al., 2009, Petersen et al., 2015, Ackerman et al., 2015, Giera et al., 2015, Langenhan et al., 2016, Shin et al., 2013, Ackerman et al., 2018). In particular, the
aGPCR ADGRG1 (also known as GPR56) has been shown to regulate OL development
and CNS myelination (Ackerman et al., 2015, Giera et al., 2015, Salzman et al., 2016).
ADGRG1 is an evolutionarily conserved regulator of OL development in
zebrafish, mice, and humans (Ackerman et al., 2015, Giera et al., 2015). Loss-of-function
mutations in ADGRG1 cause the devastating human brain malformation bilateral
frontoparietal polymicrogyria (BFPP), which is comprised of a constellation of structural
brain defects including CNS hypomyelination (Piao et al., 2004, Piao et al., 2005).
Conditional deletion of Adgrg1 in OL lineage cells results in CNS hypomyelination, and
this is specifically caused by deficiencies in ADGRG1 signaling in OPCs and immature
OLs (Giera et al., 2015). Loss of Adgrg1 in mice and zebrafish decreases OPC
proliferation, thereby leading to a reduced number of mature myelinating OLs and fewer
myelinated axons in the CNS (Ackerman et al., 2015, Giera et al., 2015). However, the
relevant ADGRG1 ligand during CNS myelination has not yet been defined.

In this study, we demonstrate that microglia-derived transglutaminase 2 (encoded
by Tgm2) is a novel ADGRG1 ligand for OPCs. Microglia-specific deletion of Tgm2
leads to reduced OPC cell division, fewer mature OLs, and hypomyelination during
postnatal CNS development, phenocopying the loss of Adgrg1. In vitro, we find that the
activation of ADGRG1 by TG2 requires the ECM protein laminin to promote OPC
proliferation. Moreover, we extend our analysis of the role ADGRG1-TG2 interactions
play in developmental myelination to mouse models of myelin damage. We provide
evidence that OPC-specific deletion of Adgrg1 impairs CNS remyelination after toxin-
induced demyelination, and that recombinant TG2 rescues remyelination failure in
organotypic cerebellar slices in a ADGRG1-dependent manner. Taken together, these
findings show that the tripartite signaling complex comprised of microglial TG2, extracellular laminin, and OPC ADGRG1 regulates OL development and myelin repair.

RESULTS

Putative ligands of ADGRG1 are expressed in microglia

aGPCR ligand binding is solely mediated by its N-terminal fragment (Hamann et al., 2015). To establish the distribution of an OPC-specific ADGRG1 ligand in the developing brain, we labeled putative ADGRG1 binding proteins in mouse corpus callosum (CC) tissue with a probe comprised of the ADGRG1 NTF fused to human immunoglobulin Fc fragment (ADGRG1\textsuperscript{N}-hFc; Figure 1A). These studies were performed at postnatal day 5 (P5) when OPCs are actively proliferating. To identify the lineage of ADGRG1 ligand-expressing cells, we performed a series of double IHC experiments with ADGRG1\textsuperscript{N}-hFc paired with Iba1 (to label microglia), GFAP (to label astrocytes), and PDGFR\(\alpha\) (to label OPCs). We observed robust and consistent putative ligand detection in microglia (Figure 1B and C), while no obvious putative ligand binding was detected in OPCs and only sparse signals were observed in astrocytes (Figure 1D-G). Quantitative analyses showed that ~ 80% Iba1+ microglia and ~20% of GFAP+ astrocytes express the putative ligand of ADGRG1 (Figure 1H).

To identify the ligand of ADGRG1 in OPCs, we employed an \textit{in vitro} proteomics approach. Biotinylated ADGRG1\textsuperscript{N}-hFc was incubated with protein lysates isolated from cultured primary glial cells that were enriched for microglia and astrocytes, and streptavidin beads were used to capture biotinylated ADGRG1 and its glial-derived candidate binding partner(s) (Figure 1-figure supplement 1). Purified complexes were then submitted for identification by mass spectrometry following digestion by trypsin.
Proteins with more than five identified tryptic peptides from three independent experiments are listed in Table 1.

**Tgm2 knockout mice display reduced myelination**

We first focused our attention on TG2 because it is a known binding partner of ADGRG1 in melanoma cells (Xu et al., 2006). To evaluate the candidacy of TG2 as the ligand of ADGRG1 in OPCs, we investigated whether deleting *Tgm2* phenocopies the myelination phenotype observed in *Adgrg1* knockout mice. Indeed, we observed significantly reduced numbers of *Plp*⁺ OLs in the CC of *Tgm2* knockout mice at both P14 and P28 compared to littermate controls (Figure 2A and B), with reductions in *Plp*⁺ OLs that are similar to reductions observed in *Adgrg1* knockout mice (Giera et al., 2015).

Similar to our analysis of callosal *Plp*⁺ OL density in adult *Adgrg1*-deficient mice (Giera et al., 2015) and consistent with a previous analysis of two-month-old *Tgm2*-deficient mice (Van Strien et al., 2011), four- to five-month-old *Tgm2* knockout mice showed normal levels of *Plp*⁺ OLs (Figure 2-figure supplement 1), indicating a gradual correction of OL numbers through adulthood.

Transmission electron micrographs of P28 CC showed significantly fewer myelinated axons in *Tgm2⁻/⁻* mice compared to littermate controls (Figure 2C and D), whereas myelin thickness corrected for axon caliber (g-ratio), total axon numbers, and axon diameters were comparable between the two groups of animals (Figure 2E-figure supplement 2). A gene dosage effect was not apparent, as we detected no difference in the density of *Plp*⁺ OLs or the percentage of myelinated axons in CC between *Tgm2*⁺⁺/⁺⁺ and *Tgm2*⁺⁻/⁻ mice (Figure 2-figure supplement 3). Taken together, these data support the finding that TG2 is the ligand of ADGRG1 in OPCs.
Microglia-specific ablation of Tgm2 reduces OL numbers

Recent transcriptomic studies revealed that Tgm2 is primarily expressed by microglia in postnatal murine CNS cells (Zhang et al., 2014), with expression detected in purified embryonic, early postnatal, and adult microglia (Matcovitch-Natan et al., 2016) (Figure 2-figure supplement 4). Consistent with these reports, our western blot analysis of freshly isolated, purified murine glial cells demonstrated selective enrichment in microglia, with no detectable TG2 protein in wildtype OPCs, OLs, astrocytes, or Tgm2−/− microglia (Figure 2F).

To test if microglial TG2 regulates developmental myelination, we studied the effect of conditionally deleting microglial Tgm2 on the production of mature OLs. Tgm2 floxed mice (Nanda et al., 2001) were crossed with Cx3cr1-Cre mice (Yona et al., 2013) where Cre recombinase expression is restricted to microglia. The numbers of Plp+ OLs in the CC of microglia-specific Tgm2 knockout mice (Tgm2fl/fl;Cx3cr1Cre+) were significantly reduced compared to littermate Tgm2fl/+;Cx3cr1Cre− controls (Figure 2G and H). Importantly, the reduction is comparable to what was observed in the constitutive Tgm2 knockout at P28 (Figure 2A and B), supporting the hypothesis that microglia are the primary source of TG2 during OL development.

TG2 promotes OPC proliferation and cell cycle progression

Next, we investigated whether TG2 regulates OPC proliferation in the developing white matter, a function that has been previously documented for ADGRG1. Indeed, numbers of Pdgfra+ OPCs in the CC of Tgm2−/− mice were significantly reduced compared to controls at P14 (Figure 3A and B). Our previous studies showed that loss of
Adgrg1 in the OL lineage causes premature cell cycle exit, resulting in a diminished size of Pdgfra+ OPC pools and fewer mature OLs (Giera et al., 2015). Therefore, we performed cell cycle exit assays (Figure 3C) and found significantly fewer BrdU+/Ki67+ double positive cells in the CC of Tgm2 knockouts compared to the controls (Figure 3D and E). Consistent with decreased OPC proliferation, we also observed an overall reduction in BrdU+ cells (Figure 3F) and fewer PDGFRα+/BrdU+ OPCs in Tgm2 knockout mice compared to littermate controls (Figure 3G and H). We conclude that OPCs prematurely exit the cell cycle in the absence of TG2, as we previously reported in Adgrg1-/- mice (Giera et al., 2015). Taken together, our data support a model in which microglial-derived TG2 promotes OPC proliferation via ADGRG1.

Other candidate ligands do not phenocopy loss of Adgrg1

We returned to our list of putative ADGRG1 ligands and asked if other candidates phenocopied the ablation of Adgrg1. Given that ADGRG1, like many aGPCRs, mediates cell-cell and cell-ECM interactions (Hamann et al., 2015, Langenhan et al., 2013), we prioritized the candidate binding partners of ADGRG1 by focusing on plasma membrane-associated and ECM proteins, and eliminated Atp1a1, Dars, and Macf1. Using zebrafish – a well-established model for studying myelination (Preston and Macklin, 2015, Ackerman and Monk, 2016), in which major classes of glia are present (Lyons and Talbot, 2014) and in which Adgrg1 function in OL development is conserved (Ackerman et al., 2015) – we screened the remaining candidates. We generated new mutant alleles of genes encoding the zebrafish orthologs of Gas6 (gas6), Mpp6 (mpp6a, mpp6b), and Plectin (pleca, plecb) using CRISPR/Cas9 genome editing (Figure 3-figure supplement 1A-E). Whole mount in situ hybridization (WISH) for myelin basic protein (mbp) at 5
days post-fertilization did not reveal CNS myelination phenotypes in any of the putative-ligand zebrafish mutants at this stage (Figure 3-figure supplement 1F-O), a time point at which adgrg1 zebrafish mutants exhibit reduced mbp expression (Ackerman et al., 2015). This lack of phenocopy suggested that Gas6, Mpp6, and Plectin do not function as ligands for ADGRG1 during OPC development. ADGRG1\(^N\) was used as the bait for the pull-down assay; therefore, it is not surprising that ADGRG1 was detected in the MS analysis. Additionally, ADGRG1 has been reported to undergo trans-trans homophilic interactions (i.e., interactions between ADGRG1 NTFs expressed on adjacent cells) (Paavola et al., 2011) and so it is also possible that ADGRG1\(^N\)-hFc probe immunoprecipitated ADGRG1 itself.

**TG2 functions together with laminin to stimulate OPC proliferation**

To establish conditions for mechanistic investigation of the function of TG2 on OPC-bound ADGRG1 during myelination, we performed in vitro analyses of wild-type (wt) and Adgrg1\(^{-/-}\) OPC cell expansion in the presence of recombinant TG2 (rTG2). To our surprise, rTG2 failed to enhance the proliferation of wt OPCs grown on poly-D-lysine (Figure 3I). TG2 is present in the extracellular milieu and is capable of cross-linking ECM proteins, including laminin-111 (Aeschlimann et al., 1992, Belkin, 2011). In light of this interaction, and the observation that laminins are broadly detected in the postnatal CC and in close proximity with OPCs in vivo (Relucio et al., 2012), we tested the need for laminin-111 in promoting OPC proliferation in a ADGRG1- and TG2-dependent manner. We found that OPC proliferation was significantly increased in the presence of both rTG2 and laminin-111, but not in cells exposed to either protein alone (Figure 3I). Fibronectin, another ECM protein expressed in the CNS and crosslinked by TG2 (Lorand
et al., 1988), did not enhance OPC proliferation in this assay (Figure 3I), suggesting specificity for laminin-111. These data support a model in which activated OPC ADGRG1 forms a signaling triad with TG2 and laminin-111 in order to support proliferation. To test this hypothesis, we generated an enzymatically dead TG2. TG2 can function as both GTPase intracellularly and crosslinking enzyme in the extracellular space (Lorand and Graham, 2003, Nakaoka et al., 1994). A single missense mutation, W241A, in the enzymatic core specifically abolishes its crosslinking enzymatic activity while preserving its GTPase function (Pinkas et al., 2007). Indeed, TG2-induced OPC proliferation in the presence of laminin-111 required the enzymatic crosslinking activity of TG2, as transamidation-inactive TG2-W241A mutant protein (Pinkas et al., 2007) failed to promote OPC proliferation (Figure 3J). To confirm that TG2 regulates OPC proliferation in a ADGRG1-dependent manner, we cultured OPCs derived from P5

**Adgrg1**+/+ or **Adgrg1**−/− mice in the presence of laminin-111, with or without rTG2. rTG2 did not promote proliferation of **Adgrg1**−/− OPCs (Figure 3K), further supporting a ADGRG1-dependent TG2 function on OPC proliferation.

**TG2 and ADGRG1 upregulate pro-mitogenic signaling proteins**

Next, we investigated intracellular mechanisms linking the interaction between ADGRG1 and TG2 with OPC cell division. Cyclin-dependent kinase 2 (CDK2) regulates OPC cell cycle progression (Belachew et al., 2002, Jablonska et al., 2007), and we previously reported reductions in CDK2 protein levels in **Adgrg1**-null OPCs (Giera et al., 2015). Therefore, we investigated whether loss of **Tgm2** caused similar reductions in CDK2. Indeed, western blot analysis of CDK2 in acutely isolated OPCs from P6 **Tgm2**−/−
mice showed significantly decreased levels of CDK2 protein compared to littermate controls (Figure 3L and M).

An additional regulator of cell cycle progression is RhoA (Chircop, 2014), which was recently reported to function downstream of ADGRG1 activation in OPCs (Ackerman et al., 2015, Giera et al., 2015). Using a pull-down assay to specifically isolate activated RhoA in corpus callosal tissue of Tgm2<sup>-/-</sup> mice and littermate controls, we found that active RhoA was significantly reduced in Tgm2<sup>-/-</sup> CC (Figure 3N and O). This suggests that ADGRG1 activates RhoA to regulate OPC proliferation in a TG2-dependent manner.

**ADGRG1 regulates remyelination in two murine models of demyelination**

Having found that the interaction of microglial TG2 with OPC-derived ADGRG1 sustained OPC proliferation during developmental myelination, we next asked if this interaction was relevant during myelin repair in the adult CNS using two established murine models of toxin-induced demyelination. The finding that Tgm2 is highly expressed in purified adult microglia, and that this expression is not diminished in a murine model of MS additionally motivated us (Wlodarczyk et al., 2017) (Figure 4-figure supplement 1A). Moreover, a previous report showed that Tgm2 knockout mice exhibit impaired remyelination following cuprizone exposure (Van Strien et al., 2011), leading us to hypothesize that the relevant OPC receptor for TG2 might be ADGRG1 in this remyelination model.

To study whether loss of OPC Adgrg1 impairs remyelination, we fed cuprizone to OPC-specific Adgrg1 knockout mice and littermate controls for 6 weeks and assessed remyelination status by analyzing myelin content after 0, 3, 7, and 10 days recovery.
Quantitative morphometry of Black-Gold-stained myelin revealed a significant decrease in remyelination at 7 and 10 DR in the CC of mice lacking Adgrg1 in OPCs compared with control littermates (Figure 4A and B). As we observed during CNS development, this reduction in myelin was accompanied by a reduction in the number of myelinating OLs (Figure 4C and Figure 4-figure supplement 1B). In the focal de-/re-myelination model using injections of lysophosphatidylcholine (LPC) into the external capsule of the CC (Hammond et al., 2015) to create lesions, mice lacking Adgrg1 in OPCs had reduced numbers of myelinating OLs in the lesion 14 dpl (Figure 4D and E). Additionally, in the same LPC injection model, microglia-specific Tgm2 knockout mice had fewer myelinated axons in the lesion compared to control mice (Figure 4F and G), while g-ratio was unaffected (Figure 4H).

To further evaluate whether TG2 promotes remyelination through OPC ADGRG1, we conducted studies using an ex vivo model of LPC-induced de-/re-myelination in organotypic cerebellar slices (Birgbauer et al., 2004), in which demyelination and repair mechanisms differ from those induced by cuprizone (Blakemore and Franklin, 2008). Demyelination was induced by a 24-hour exposure to LPC, and after four days recovery we observed robust remyelination in wt cerebellar slices. By comparison, this remyelination was significantly impaired in cerebellar slices derived from OPC-specific Adgrg1-null mice (Figure 5A-C). To test whether rTG2 could enhance remyelination, we added rTG2 to cerebellar slices during the recovery phase. Indeed, rTG2 increased myelination in both wt and Tgm2 single knockout cerebellar slices with 4 days of daily treatments (Figure 5D-F). Excitingly, this effect appeared to be ADGRG1-dependent as increased remyelination was not observed in Tgm2/Adgrg1 double knockout slices (Figure 5D-F). Taken together, the interaction between ADGRG1
and TG2 regulates efficient remyelination following cuprizone- and LPC-mediated injury.

DISCUSSION

Through a combined approach utilizing molecular, cellular, and developmental biology as well as unbiased proteomics, we demonstrate that microglial TG2 is the ligand of OPC-derived ADGRG1. Although the importance of OPC ADGRG1 in developmental CNS myelination has been previously demonstrated (Ackerman et al., 2015, Giera et al., 2015, Salzman et al., 2016), the relevant ligand remained undefined and the role of ADGRG1 in myelin repair was not addressed. Here, we demonstrate that microglia-derived TG2 provides essential ligand activity for OPC-derived ADGRG1 during CNS myelination and that this signaling pathway is implicated in remyelination. This de-orphanization is a mandatory first step in the therapeutic exploitation of this novel pathway.

This study elucidates a novel role for microglia-ECM-OPC interactions in developmental myelination as well as in remyelination. It is known that laminin regulates oligodendrogenesis and CNS myelination by interacting with integrins and dystroglycan (Colognato and Tzvetanova, 2011, Colognato et al., 2004, Colognato et al., 2007, Colognato et al., 2002) and promotion of OPC proliferation in vivo (Relucio et al., 2012). Here, we present a new tripartite signaling complex – microglial TG2, ECM laminin, and ADGRG1 on OPCs – that drives OL development.

TG2 is a member of the transglutaminase protein family; family members including TG2 function as crosslinking enzymes to ligate proteins between the ε-amino group of a lysine residue and the γ-carboxamide group of a glutamine residue (Fesus and
Unlike other members of the transglutaminase family, TG2 can be found both in the intracellular and the extracellular spaces of various types of tissues. The intracellular portion of TG2 is thought to predominantly act as a signaling molecule that requires its GTPase activity (Nakaoka et al., 1994), whereas the extracellular portion of TG2 binds to proteins of the ECM and exerts its crosslinking enzymatic activity (Belkin, 2011). A single missense mutation (W241A) in the catalytic core of TG2 specifically abolishes its crosslinking enzymatic activity with other functions preserved (Pinkas et al., 2007). Interestingly, the enzymatic dead TG2 failed to promote OPC proliferation \textit{in vitro} (Figure 3J), supporting the model that it functions as a crosslinking enzyme in regulating OL development and CNS myelin formation and repair.

It was previously reported that TG2 binds laminin-111 (Aeschlimann et al., 1992). In our study, we discovered that recombinant wt TG2, but not its enzymatic-dead counterpart, stimulates OPC proliferation via ADGRG1 in the presence of laminin-111. We speculate that TG2 interactions with ADGRG1 and laminin-111 modulate ECM properties including stiffness, thus promoting OPC proliferation (Jagielska et al., 2012). Although our study results support that ADGRG1-TG2-laminin-111 promotes OPC proliferation, it is possible that the same signaling triad could also regulates OPC differentiation. This model will be a subject of future investigation. It is worth noting that we focused our study in the white matter. Recent reports have described differences in gene expression, morphology, and metabolism of grey and white matter microglia and OPCs (Hagemeyer et al., 2017, Young et al., 2013). It will be an interesting research direction to evaluate whether there is a difference in microglial-ECM-OPC interactions in grey and white matter.
We show that TG2, contingent on its crosslinking activity and together with laminin-111, binds to the ADGRG1 NTF and initiates G-protein signaling (Figure 6).

Activation of ADGRG1 in OPCs activates the small GTPase RhoA, a known downstream signaling protein, and additionally promotes the accumulation of the critical pro-mitotic cell cycle regulator CDK2. The latter finding is novel and offers new avenues of investigation into aGPCR-dependent regulation of cell cycle progression, a necessary step in generating OLs during myelination or remyelination.

It is worth noting that loss of ADGRG1 does not cause as drastic OL development phenotypes compared to loss of other receptors such as GPR17 (Chen et al., 2009). Nevertheless, subtle fine-tuning of the developing brain is critically important for an organism as a whole: the complete loss of oligodendrocytes will be lethal, whereas dysregulation of myelin formation can cause diseases such as leukodystrophies, and subtle dyshomeostasis could certainly disrupt brain development (Sharon et al., 2016).

Although perturbations to the ADGRG1-TG2 pathway had mild effects on in vitro OPC proliferation, loss-of-function mutations in ADGRG1 manifest as CNS hypomyelination in humans (Giera et al., 2015, Piao et al., 2005, Piao et al., 2004), underscoring the importance of understanding ADGRG1 function in OL lineage cells.

Given the importance of myelin formation, maintenance, and repair in neurological diseases, these findings may be translated to clinical benefit in both developmental and acquired diseases of myelination. Pharmacological and biological modulators of ADGRG1 activity have recently been identified (Stoveken et al., 2016, Salzman et al., 2016). Furthermore, modulating TG2 expression and/or secretion, or developing peptidomimetic drugs could represent as viable therapeutic approaches in promoting remyelination. It will be important in future work to explore the intersection of...
therapeutic strategies to target aGPCRs with their cell-type-specific ligands, expression patterns, and contributions in human disease.

MATERIAL AND METHODS

Key resource table

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|-----------------------|--------|---------------------------------|----------|
| Sequence-Based Reagent | Adgrg1 common | | 5'- CGAGAAG ACTTCCGC TTCTG -3' | 20 µM |
| Sequence-Based Reagent | Adgrg1 wt | | 5'- AAAGTAG CTAAGATG CTCTCC -3' | 20 µM |
| Sequence-Based Reagent | Adgrg1 knockout | | 5'- GCAGCGC ATCGCCTT CTATC -3' | 20 µM |
| Sequence-Based Reagent | Adgrg10/+ common | | 5'- TGGTAGCT AACCTACT CCGAGC C -3' | 20 µM |
| Sequence-Based Reagent | Adgrg10/+ wt | | 5'- GGTGACTT TGTTGTTCC | 20 µM |</p>
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Mouse strains

All mice were treated according to the guidelines of the Animal Care and Use Committee at Boston Children's Hospital. The Adgrg1 knockout mice were obtained from Genentech/Lexicon Genetics. The mutant mice were originally created in a 129/BL6 background, but were derived into the FvB strain and bred into BALB/c strain resulting in a mixed genetic background of the mutant mice of 129/BL6/FvB/BALB/c (Li et al., 2008). Genotyping was performed by PCR using the following primers: A (5'-CGAGAAGACTCCGCTTCTG -3’), B (5’- AAAGTAGCTAAGATGCTCTCC -3’), and Neo (5’- GCAGCGCATCGCCTTCTATC -3’). Tgm2 knockout mice were provided by Dr. Gerry Melino, University of Rome, Italy (De Laurenzi and Melino, 2001). For Adgrg1/Tgm2 double knockout mice, Adgrg1 knockout mice were bred with Tgm2 knockout mice. Adgrg1^{fl/+} mice were generated at the Mouse Gene Manipulation Core at Boston Children’s Hospital (Giera et al., 2015). Genotyping was performed by PCR with the following primers: primer 1: 5’- TGGTAGCTAACCTACTCCAGGAGC -3’, primer 2: 5’- GGTGACTTTGGTGTTCTGCACGAC -3’ and primer 3: 5’-CACGAGACTAGTGAGACGTGCTAC -3’. Pdgfra^{Cre/ERT} mice in a C57BL/6 background were purchased from Jackson Laboratories (Cat# 018280) (Kang et al., 2010) and were crossed with Adgrg1^{flo/flo} mice to generate Adgrg1^{flo/flo};Pdgfra^{CreERT}+ mice and their littermate controls. Tgm2^{fl/+} mice were generously provided by Dr. Joan Cook-Mills (Northwestern University, Chicago, IL) (Nanda et al., 2001) and crossed with Cx3cr1^{Cre} mice purchased from Jackson Laboratories (Cat# 025524) to generate Tgm2^{flo/flo};Cx3cr1^{Cre+} mice and their littermate controls.

Antibodies
For IHC or western blot analyses, we used the following primary antibodies:
mouse anti-ADGRG1 (H11) (1:200) (Luo et al., 2011) and rabbit anti-ADGRG1 (199)
(1:200) (Li et al., 2008), rabbit anti-MBP (Millipore; Cat #AB980, 1:200), rabbit anti-
Iba1 (Wako, Cat# 019-19741, 1:400), rabbit anti-GFAP (Abcam, Cat# ab7260, 1:1000),
rabbit anti-NG2 (Millipore; Cat #AB5320, 1:200), rat anti-PDGFRα (BD Bioscience; Cat
#558774, 1:500), rabbit anti-PDGFRα (Cell Signaling Technologies; Cat #3164S, 1:500),
and rat anti-Ki67 (Affymetrix eBioscience; Cat #14-5698-80, 1:100), rat anti-BrdU
(Accurate Chemical and Scientific Corporation; Cat #OBT0030S, 1:100), mouse anti-
RhoA (Cytoskeleton, Cat# ARH03-A, 1:500), mouse anti-CDK2 (Santa Cruz; Cat #sc-
6248, 1:1000), mouse anti-β-actin (Sigma, Cat #A5044, 1:5000), rabbit anti-GSTπ (Enzo,
Cat#ADI-MSA-102-E, 1:500), and mouse anti-Ki67 (BD Bioscience; Cat #550609,
1:100). Secondary antibodies were goat anti-mouse or anti-rat conjugated with either
Alexa 488 (ThermoFisher, 1:1000) or Alexa 546 (ThermoFisher, 1:1000) and goat anti-
rabbit conjugated with Alexa 546 or 555 (ThermoFisher, 1:1000), goat anti mouse or
rabbit IgG-HRP (Sigma, Cat# A4416 or A6154, 1:3000).

**OPC cultures**

OPCs were isolated from mixed male and female P5-8 *Adgrg1*+/+ or *Adgrg1*−/−
mouse forebrains as previously described (Watkins et al., 2008, Wang et al., 2001).
Briefly, OPCs were purified by negatively selecting with mouse anti-Thy1.2 (Serotec,
Cat #MCA02R) and mouse anti-GalC (Millipore, Cat #MAB342), followed by mouse
anti-O4 (O4 hybridoma supernatant) for positive selection. After releasing OPCs from the
O4 plate by trypsinization, cells were resuspended in proliferation media containing
PDGF-AA and NT-3 (PreproTech). OPCs were plated on coverslips coated with poly-D-
lysine before coating with laminin I (R&D Systems, Cat#3400-010-01) or fibronectin (Millipore, Cat#341668) as previously described (Dugas et al., 2006). After 24 hr, TG2 (2 nM) was added to the cultures and incubated for an additional 24 hr before fixation with 4% PFA and staining for PDGFRα and Ki67.

**Mixed glia culture**

Mixed glia culture was isolated from P1 male and female wt forebrains, as previously described (Giera et al., 2015, O'Meara et al., 2011). Briefly, forebrains were dissociated and resulting cell suspension was cultured for 10 days in DMEM containing 10% FBS, 1% GlutaMax at 37°C and 8.5% CO₂. The culture was shaken for 20 hr at 250 rpm and 37°C to remove OPCs. Mixed glial cells were cultured for another 5-7 days before being washed twice with PBS and collected.

**Microglia isolation**

Microglia were isolated as previously described (Cardona et al., 2006). Briefly, male and female P7 pups were perfused with ice-cold HBSS, before isolating and mincing the brains. The tissue was homogenized with a dounce homogenizer containing RPMI media before being mixed with stock isotonic percoll (SIP) creating a 30% SIP solution. This cell/SIP mixture was layered on top of a 70% SIP solution before being centrifuged. The resulting interphase containing the microglia was collected and washed with HBSS and cells were counted before being lysed for western blot analysis.

**Cuprizone treatment**
Adgrg1^{fl/fl};Pdgfra^{CreERT-} and Adgrg1^{fl/fl};Pdgfra^{CreERT+} adult mice (8-10 weeks old) were injected with 100 mg/kg/day tamoxifen for 5 consecutive days. Mice were fed a diet containing 0.2% (w/w) cuprizone (Harlan Teklad) ad libitum for 6 weeks. A second pulse of tamoxifen was given at 4 weeks of cuprizone exposure, for 3 days with 100 mg/kg/day. After 6 weeks, mice were returned to normal feed for 3, 7 or 10 days of recovery (DR). Mice were perfused as described above at 0, 3, 7, 10 DR.

**Black-Gold myelin staining**

Coronal brain sections were stained with Black Gold myelin stain (Millipore; Cat# AG105) according to the manufacturer’s protocol. Sections were imaged using Nikon Eclipse 80i microscope (Nikon). Representative images were obtained with the same exposure setting for control and mutant. Images of the corpus callosum were analyzed using NIH Image J software. The percentage of remyelinated area of the total corpus callosum was determined using a threshold procedure.

**LPC injections**

Adgrg1^{fl/fl};Pdgfra^{CreERT-} and Adgrg1^{fl/fl};Pdgfra^{CreERT+} male mice (8-10 weeks old) were injected with 100 mg/kg/day tamoxifen for 5 consecutive days from -6 dpl to -1 dpl. Mice (Adgrg1^{fl/fl};Pdgfra^{CreERT-} and Adgrg1^{fl/fl};Pdgfra^{CreERT+} or Tgm2^{fl/fl};Cx3cr1^{Cre-} and Tgm2^{fl/fl};Cx3cr1^{Cre+}, 8-10 weeks old) were anesthetized using isoflurane (3%) before either lyssolecithin (LPC, 1% in saline, 2 µl, Millipore) or saline was injected bilaterally using a Hamilton syringe in a stereotaxic apparatus at the following coordinates 1 mm anterior to bregma, 1.5 mm posterior to bregma and 3.0 mm deep as previously described (Hammond et al., 2015). Day of injection was denoted as 0 dpl. Mice were left for 14 dpl.
and subsequently perfused for either immunohistological analysis or transmission electron microscopy.

**Cerebellum slice culture and LPC treatment**

Adgrg1\(^{fl/fl}\);Pdgfra\(^{CreERT+}\) and Adgrg1\(^{fl/fl}\);Pdgfra\(^{CreERT-}\) P7 pups were injected with tamoxifen (50 mg kg\(^{-1}\)) for 3 days before the cerebellar harvesting. All cerebella were harvested on P10 and placed in ice-cold Hank’s buffer (Fisher Scientific) before being cut into 300 µm thick sagittal sections with a vibratome (Leica Biosystems). Three slices were placed onto inserts (Millicell 0.4 µm, Millipore) in media containing 50% MEM, 25% Horse Serum, 25% Hank’s Buffer, 1% GlutaMax, 5 mg/ml Glucose. After 48 hr, the slices were incubated with 0.25 mg/ml lysolecithin (Sigma-Aldrich) for 24 hr. Afterward the slices were washed with media and remyelination was allowed to occur for 4 days, with or without 20 nM rTG2 protein as indicated in the figures by adding rTG2 to the media and slices. Slices were fixed with 4% PFA for 20 min, permeablized with 10% Triton-X 100 for 20 min and blocked with 10% goat serum, 1% bovine serum albumin (BSA) and 0.1% Triton X-100 in PBS for 1 hr at room temperature before being incubated with the primary antibody for neurofilament (mouse anti-NF200 antibody, SigmaAldrich; Cat#N0142 1:500) and myelin (rat anti-MBP, Abcam; Cat#ab7349, 1:200) overnight at 4°C. Primary antibodies were visualized by incubating the sections with the appropriate fluorophore-conjugated secondary antibody for 1 hr at room temperature followed by staining of the nuclei with Hoechst 33342 (1:2000, Life Technologies). After mounting the slices, images were taken with Zeiss LSM 700 confocal microscope. Quantification of myelinated fibers was performed using Image J
by subtracting background and applying threshold for both red and green channel, before measuring both myelinated and unmyelinated axon fibers.

**Histology analyses**

Mouse brains were harvested after perfusion, post-fixed with 4% PFA overnight, cryoprotected with 30% sucrose, and embedded in OCT. IHC was carried out as previously described (Jeong et al., 2012). Briefly, after antigen retrieval in Retrievagen A Solution (BD Pharmingen), brain sections were washed with 1x PBS, blocked with 10% goat serum, 1% bovine serum albumin (BSA) and 0.1% Triton X-100 in PBS for 1hr at room temperature before being incubated with the primary antibody overnight at 4°C. Primary antibodies were visualized by incubating the sections with the appropriate fluorophore-conjugated secondary antibody for 1 hr at room temperature followed by staining of the nuclei with Hoechst 33342 (1:2000, Life Technologies).

*In situ* hybridization was performed on 12 µm brain sections as previously described (Dugas et al., 2010, Bialas and Stevens, 2013). Probes targeting *Plp* (Addgene, Cat #22651) and *Pdgfra* (kind gift from Charles Stiles) were generated by digesting plasmids with EcoRI and HindIII, respectively. Sp6 and T7 polymerase were used to generate DIG-labeled RNA probes *in vitro* transcription (Roche Applied Science; DIG RNA labeling kit) as per manufacturer’s instructions. Hybridization occurred at 68°C and washes at 65°C. To detect the DIG-labeled probes, the TSA-Plus Cyanine 3 labeling system (Perkin Elmer) was used according to the manufacturer’s instructions.

Ligand binding *in situ* was performed on male and female P5 wt brains that were embedded into low-melting agarose and sectioned into 500 µm thick sections using a vibratome. Sections were washed with PBS before blocking for 30 min with 0.5% goat
serum and 0.1% BSA, incubating with ADGRG1N-hFc for 30 min, followed with the appropriate secondary antibody for 30 min. Sections were fixed with 4% PFA for 30 min before being blocked with 10% goat serum, 1% BSA and 0.1% Triton-X 100 for 1 hr. Tissue was incubated with a marker for co-staining overnight at 4°C followed with the appropriate secondary antibody for 30 min.

All images were captured using a confocal LSM 510 NLO system or a Nikon Eclipse Ti inverted microscope (Nikon). Representative images were obtained with the same exposure setting for control and mutant.

**Cell cycle exit assay**

Cell cycle exit assays were performed as previously described (Giera et al., 2015). Briefly, proliferating cells were labeled with BrdU (50mg kg\(^{-1}\)) by intraperitoneal injection of male and female P13 Tgm2\(^{-/-}\) pups and their littermate controls. Mouse brains were harvested 24 hr later, perfused, post-fixed with 4% PFA, cryoprotected with 30% sucrose, and embedded in OCT. Brain sections were processed for IHC with anti-BrdU and anti-Ki67 antibodies.

**Western Blot and GTP-Rho Pull-Down Assay**

The corpus callosa were dissected under a Leica stereo microscope (MZ 6; Leica™ Pte Ltd.), followed by washes in 1x PBS and lysis in ice-cold RIPA buffer (1% Nonidet P-40, 50 mM Tris pH 7.6, 120 mM NaCl, 1 mM EDTA) containing protease inhibitor cocktail set 1 (Calbiochem). The lysates were cleared of insoluble materials by centrifugation at 16,000 x \(g\) for 10 min at 4°C. Protein concentration was determined by a Bio-Rad protein assay method (Bio-Rad) according to the manufacturer's protocol, and
equal amounts of protein were used for SDS–PAGE and western blot analysis. The GTP-Rho pull-down assay was performed as previously described (Luo et al., 2011); in short, tissues were pulverized on liquid nitrogen, lysed in 300 µl of ice-cold RIPA buffer containing protease inhibitors with a cell disruptor for 10 minutes and homogenized with a 26 G syringe needle. Equal amounts of total protein were incubated with 60 µg GST-RBD beads (Cytoskeleton) at 4 °C for 90 min. The beads were washed twice with lysis buffer and once with TBS buffer. Bound Rho proteins were eluted by Laemmlli sample buffer and detected by western blot using mouse monoclonal anti-RhoA antibody (Cytoskeleton).

Purification of ADGRG1 Immunocomplexes

ADGRG1N and ADGRG1Ndel fusions proteins with either mFc or hFc tag were generated as previously described (Luo et al., 2011). BirA enzyme was used to in vitro biotinylate ADGRG1N-mFc-biotag protein as previously described (Stacey et al., 2002). Mixed glial cell lysate was incubated with biotinylated ADGRG1N protein in lysis buffer (20 mM Hepes [pH 7.3], 150 mM NaCl, and 5 mM MgCl₂) with protein inhibitor mixture (Roche Molecular Biochemicals) and 1% Brij 96 (Fluka). Streptavidin beads (Sigma) were used to affinity purify immunocomplexes. ADGRG1-associated proteins were eluted in 2x SDS loading buffer, subjected to SDS/PAGE. The whole lane was used for MS at the Taplin Biological Mass Spectrometry at Havard Medical School. Mass spectrometry data were searched against the mouse International Protein Index (IPI mouse 339) database using the protein identification software Mascot (v2.2.04, Matrix Sciences)(Luo et al., 2011).
Zebrafish stocks and rearing conditions

Zebrafish (Danio rerio) were maintained in the Washington University Zebrafish Consortium facility (http://zebrafish.wustl.edu/). All experiments were performed in compliance with Washington University's institutional animal protocols. Embryos were collected from pair-wise or harem matings and reared at 28.5 °C in egg water (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄). All zebrafish lines used in these studies were generated in the wt (AB) background, including: gas6<sup>stl228</sup>, mpp6a<sup>stl233</sup>, mpp6b<sup>stl234</sup>, pleca<sup>stl261</sup>, and plecb<sup>stl236</sup>.

Zebrafish mutant generation

Zebrafish guide RNAs (gRNAs) were designed and generated and cas9 RNA was synthesized at the Genome Engineering and iPSC center at Washington University School of Medicine (St. Louis, MO). gRNA sequences were as follows:

gas6: 5’- CAGAACCCGCAGAGCCAACCAGG -3’
mpp6a: 5’- GGCGGTTCAAAGTAATAACGTGG -3’
mpp6b: 5’- GCAGCAGGTGTTGGATAACC -3’
pleca: 5’- AAAACTAGGGAATAAGACTG -3’
plecb: 5’- TCCCTCCTGGAGGTCCTCTC -3’

Synthetic CRISPR guide mRNA was combined with cas9 synthetic mRNA and injected into 1-cell stage wt embryos (AB) at 100 pg quantities. To recover germ-line transmitted mutations, injected founders (F0s) were grown to adulthood, outcrossed to wt AB partners, and genomic DNA was extracted from individual F1 embryos for PCR amplification and restriction digest analysis of the targeted region. Genomic DNA from
F1 embryos that showed disruption of the target site was then cloned using the TOPO® TA Cloning® kit (Invitrogen) and Sanger sequenced.

Zebrfish genotyping

To genotype individual larvae for phenotypic analyses, we used the following primers to amplify a 505 bp fragment of gas6 from genomic DNA: 5’-CAGAAGAGCGAAAGTTTGAC -3’ and 5’-CACAGTGAACATCATCGAGT - 3’.

For gas6^{stl228/stl228}, we performed restriction digest analysis using SexAI, which cleaves wt into a 219 bp and a 286 bp fragment, but is unable to cut mutant (501 bp). To amplify mpp6a, we used the following primers to amplify a 317 bp fragment from genomic DNA:

5’- ACCCAGAGGCACCTTGATTA -3’ and 5’-GGTTCCCTCAGGATGTTAGA - 3’.

For mpp6a^{stl233/stl233}, we performed restriction digest analysis using HpyCH4IV, which cleaves wt into a 93 bp and a 224 bp fragment, but is unable to cut mutant (316 bp). To amplify mpp6b, we used the following primers to amplify a 400 bp fragment from genomic DNA: 5’- GGAAATGACCTCAGCAGAT -3’ and 5’- CATGCGTTTACCTCATTAGC - 3’.

For mpp6b^{stl234/stl234}, we performed restriction digest analysis using BstNI, which cleaves wt into a 153 bp and a 247 bp fragment, but is unable to cut mutant (394 bp). To amplify pleca, we used the following primers to amplify a 660 bp fragment from genomic DNA: 5’- GTGGCCTTACATGACATCTT -3’ and 5’- CTGAATGCTCACACAATC - 3’.

For pleca^{stl261/stl261}, we performed restriction digest analysis using DdeI, which cleaves wt into a 248 bp and a 412 bp fragment, but is unable to cut mutant (640 bp). To amplify plecb, we used the following primers to amplify a 18 β fragment from genomic DNA: 5’- ACTCATGGACTAACGTCTGC -3’ and 5’- GTTTTGACGTGGGATTAGAG - 3’.
plecb<sup>stl236/stl236</sup>, we performed restriction digest analysis using Hpy188III, which cleaves wt into a 66 bp and a 103 bp fragment, but is unable to cut mutant (169 bp).

**Whole mount in situ hybridization**

Whole-mount ISH was performed using standard protocols (Thisse and Thisse, 2008). In brief, embryos were fixed at 5 dpf in 4% PFA at 4° C overnight, and then washed into 100% methanol for dehydration. Following dehydration, embryos were washed in 0.2% PBS-Tween (PBSTw), permeabilized in proteinase K (20 mg/µl diluted 1:1000 in 0.2% PBSTw), and incubated with a Digoxygenin-labeled riboprobe again <i>mbp</i> (Lyons et al., 2005) overnight at 65° C in hybridization buffer (50% formamide). Following probe treatment, embryos were washed to remove formamide, blocked in 2% blocking medium supplemented with 10% normal sheep serum and 0.2% Triton, and incubated in primary antibody (anti-DIG, Fab fragments (1:2000), Product # 1121467001, Roche) overnight in block. Following primary antibody treatment, embryos were washed in maleic acid buffer with 0.2% triton, and developed by alkaline phosphatase treatment. After complete, embryos were post-fixed in 4% PFA and stored long-term in 70% glycerol. Embryos were mounted on slides and imaged at 10x with an AxioCam MRm on a light microscope (Zeiss AxioImager M2). Genotypes were obtained subsequent to larval analyses.

**Transmission electron microscopy**

For mice, postnatal male brains were fixed by perfusion followed by immersion in a mixture of 2% glutaraldehyde and 4% paraformaldehyde in 0.1M sodium cacodylate buffer, pH 7.4. After overnight fixation, brains were postfixed with 1% osmium
tetroxide/1.5% potassium ferrocyanide for 1 hr, and after washing samples, were
incubated in 1% aqueous uranyl acetate for 1hr followed by subsequent dehydration in
alcohol. The samples were incubated in propylene oxide for 1 hr and infiltrated overnight
in a 1:1 mixture of propylene oxide and TAAB Epon (Marivac Canada Inc. St. Laurent,
Canada). The next day, samples were embedded in TAAB Epon and polymerized at 60°C
for 48 hr. Ultrathin sections (~60 nm) were cut on a Reichert Ultracut-S microtome,
mounted on copper grids, stained with lead citrate, and examined with a JEOL 1200EX
transmission electron microscope. Images were recorded with an AMT 2k CCD camera.
The photographs were analyzed using Image J Software (http://rsb.info.nih.gov/ij/) to
calculate g-ratio and axon diameter. G-ratio was calculated as previously described (Roy
et al., 2007).

**Statistical Analysis**

For all studies, images were scored blinded to genotype and treatments prior to
quantifications. For mouse studies, data is represented as mean ± s.e.m and asterisks
indicate significance: ***P ≤ 0.001; **P ≤ 0.01; *P ≤ 0.05. GRAPHPAD Prism Software
(GraphPad Software, Inc.) was used to determine statistical significance between
genotypes and treatments using unpaired or paired Student’s t-tests, two-tailed and
unequal variance depending on animals either being paired prior to data collection or not.
For *in vitro* culture, animals were paired prior to isolation of OPCs. One-way ANOVA
followed by Tukey post-hoc was used to analyze multiple treatment condition
experiments. Sample size was not pre-determined by statistical methods, but was based
on similar studies in the field.
Acknowledgements

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Figures and Figure Legends

Figure 1: Microglia express the putative ligand of ADGRG1.

(A) Schema of ADGRG1 receptor. GAIN domain and ADGRG1\textsuperscript{N}-hFc are shown. (B, D, and F) Double labeling of ADGRG1\textsuperscript{N}-hFc (green) and Iba1 (B, red), GFAP (D, red), PDGFR\textalpha{} (F, red) in P5 wt corpus callosum. DAPI, blue. Scale bar, 25 µm. (C, E, and G) Higher magnification of the boxed region in C, E and G. Scale bar, 10 µm. Staining was repeated \(N = 3-4\) animals. (H) Quantification of double positive ADGRG1\textsuperscript{N}-hFc\textsuperscript{+} and Iba\textsuperscript{+} cells and ADGRG1\textsuperscript{N}-hFc\textsuperscript{+} and GFAP\textsuperscript{+} cells. \(N = 3-4\) per staining.

Figure 2: Loss of Tgm2 leads to fewer mature OLs and hypomyelination in the CC.

(A) Representative in situ hybridization (ISH) images of Plp (red) in the CC of P14 (top panel) and P28 (bottom panel) Tgm2\textsuperscript{+/-} and Tgm2\textsuperscript{-/-} mice. DAPI, blue. Scale bar, 100 µm. (B) Quantification of Plp\textsuperscript{+} oligodendrocytes from the CC of Tgm2\textsuperscript{+/-} and Tgm2\textsuperscript{-/-} P14 and P28 mice. * \(P = 0.0484; N = 5\) per genotype (P14); * \(P = 0.0378; N = 4\) per genotype (P28), unpaired \(t\)-test. (C) Representative TEM images from P28 CC of Tgm2\textsuperscript{+/-} and Tgm2\textsuperscript{-/-} mice. Scale bar, 1 µm. (D) Percentage of myelinated axons in the CC of control (Tgm2\textsuperscript{+/-} and Tgm2\textsuperscript{-/-}) and Tgm2\textsuperscript{-/-} mice. * \(P = 0.0299; N = 3\) per genotype; unpaired \(t\)-test. (E) Scatter plot displaying g-ratio values in the CC of control and Tgm2\textsuperscript{-/-} mice. (F) TG2 western blot on acutely isolated microglia, OPCs, astrocytes, and mature oligodendrocytes from the P7 neonatal brain. \(\beta\)-actin was used as loading control. (G) Representative ISH images of Plp (red) in the CC of P28 Tgm2\textsuperscript{fl/+};Cx3cr1Cre\textsuperscript{-} and Tgm2\textsuperscript{fl/fl};Cx3cr1Cre\textsuperscript{+} mice. DAPI, blue. Scale bar, 100 µm. (H) Quantification of Plp\textsuperscript{+}...
oligodendrocytes in the CC of P28 $Tgm2^{fl/+};Cx3cr1Cre^-$ and $Tgm2^{fl/fl};Cx3cr1Cre^+$ mice.

** $P = 0.00850; N = 4$ per genotype, unpaired t-test. Error bars are means ± s.e.m (B, D, H).

**Figure 3: TG2 regulates OPC proliferation through ADGRG1.**

(A) Representative ISH images of $Pdgfra$ (red) in the CC of P14 $Tgm2^{+/+}$ and $Tgm2^{-/-}$ mice. DAPI, blue. Scale bar, 100 µm. (B) Quantification of $Pdgfra^+$ OPCs in the CC of control and $Tgm2^{-/-}$ mice. * $P = 0.0320$, unpaired t-test, $N = 6$ per genotype. (C) Cartoon showing the cell cycle exit assay. (D) Representative images of double IHC of BrdU (red) and Ki67 (green) in the CC of P14 $Tgm2^{+/+}$ and $Tgm2^{-/-}$ mice that were pulsed with BrdU 24 h earlier (arrows mark double positive Ki67+/BrdU+ cells). DAPI, blue. Scale bar, 50 µm. (E) The density of Ki67+/BrdU+ cells in the CC is lower in $Tgm2^{-/-}$ mice. * $P = 0.0207$, unpaired t-test, $N = 5$ per genotype. (F) The density of BrdU+ cells in the CC is reduced in $Tgm2^{-/-}$ mice. ** $P = 0.0099$, unpaired t-test, $N = 5$ per genotype. (G) Representative images of double IHC of PDGFRα (red) and BrdU (green) in the CC of P14 $Tgm2^{+/+}$ and $Tgm2^{-/-}$ mice (arrows mark double positive PDGFRα+/BrdU+ cells). DAPI, blue. Scale bar, 50 µm. (H) The number of BrdU+/PDGFRα+ cells in the CC is reduced in $Tgm2^{-/-}$ mice. * $P = 0.0465$, unpaired t-test, $N = 4$ per genotype. Error bars are means ± s.e.m. (I) The effect of recombinant TG2 (rTG2) on OPC proliferation in basal condition, laminin-111 or fibronectin. rTG2 stimulates OPC proliferation only in the presence of laminin-111. * $P = 0.039$, paired t-test, $N = 3-6$ per group. (J) The effect of wild type TG2 and its enzymatic dead mutant TG2-W241A proteins on OPC proliferation. Only wild type TG2 stimulates OPC proliferation. ** $P = 0.0091$; One-way ANOVA followed by Tukey post-hoc test, F(2,14) = 6.7, * $P = 0.05$ (control vs. rTG2);
n.s. $P = 0.70$ (control vs. TG2-W241A); ** $P = 0.0088$ (TG2 vs. TG2-W241A); (K) 

$Adgrg1^{-/-}$ OPCs fail to respond to rTG2-enhanced proliferation. * $P = 0.0063$, paired t-test, $N = 5$ per genotype. (L) Western blot analyses of CDK2 in acutely isolated OPCs from P7 $Tgm2^{+/+}$ and $Tgm2^{-/-}$ brains. The bracket indicates CDK2 protein isoform bands. 

β-actin was used as loading control. (M) CDK2 protein levels are reduced in the $Tgm2^{-/-}$ mice. ** $P = 0.002$, unpaired t-test, $N = 3$ per genotype. (N) Western blot of active RhoA (top panel) and total RhoA (bottom panel) in the CC of $Tgm2^{+/+}$ and $Tgm2^{-/-}$ mice. (O) The relative level of active RhoA to total RhoA was diminished in the CC of $Tgm2^{-/-}$ mice compared to $Tgm2^{+/+}$ control mice. * $P = 0.0207$, unpaired t-test, $N = 3$ per genotype. Error bars are means ± s.e.m (B, E, F, H, I, J, K, M, O)

**Figure 4: ADGRG1 is required for remyelination in vivo.**

(A) Representative images of Black-Gold myelin staining of the corpus callosum of $Adgrg1^{fl/fl};PdgfraCreER^{-}$ and $Adgrg1^{fl/fl};PdgfraCreER^{+}$ mice after cuprizone feeding for 6 weeks followed by recovery for 3 d (3 DR), 7 d (7 DR) and 10 d (10 DR) (dotted line outlines quantified region). Scale bar, 250 µm. (B) Percentage of remyelinated corpus callosum displayed significant decrease in myelination at 7 DR and 10 DR between $Adgrg1^{fl/fl};PdgfraCreER^{-}$ and $Adgrg1^{fl/fl};PdgfraCreER^{+}$ mice. * $P = 0.0285$ (7 DR), $N = 4$ (cre$^{-}$); $N = 4$ (cre$^{+}$); * $P = 0.0416$ (10 DR), $N = 3$ (cre$^{-}$); $N = 4$ (cre$^{+}$), unpaired t-test. 

(C) Number of GST$\pi^{+}$ OLs are significantly decreased at 10 DR between $Adgrg1^{fl/fl};PdgfraCreER^{-}$ and $Adgrg1^{fl/fl};PdgfraCreER^{+}$ mice. * $P = 0.0457$ (10 DR), $N = 3$ (cre$^{-}$); $N = 3$ (cre$^{+}$), unpaired t-test. (D) Representative images of GST$\pi^{+}$ OLs in the corpus callosum of $Adgrg1^{fl/fl};PdgfraCreER^{-}$ and $Adgrg1^{fl/fl};PdgfraCreER^{+}$ mice 14 days
post-lesion (dotted line outlines quantified region). Scale bar, 200 µm. (E) Number of GST\(\pi^+\) OLs are significantly decreased 14 days post-lesion between

\[\text{Adgrg1}^{fl/fl};\text{PdgfraCreER}^- \text{ and Adgrg1}^{fl/fl};\text{PdgfraCreER}^+ \text{ mice. * } P = 0.0133, N = 3 \text{ (cre\(^-\))}; N = 3 \text{ (cre\(^+\))}, \text{ unpaired } t\text{-test.} (F) \text{ Representative TEM images from the CC of}\]

\[\text{Tgm2}^{fl/fl};\text{Cx3cr1Cre}^- \text{ and Tgm2}^{fl/fl};\text{Cx3cr1Cre}^+ \text{ mice. Scale bar, 1 µm. (D) Percentage of myelinated axons in the CC of Tgm2}^{fl/fl};\text{Cx3cr1Cre}^- \text{ and Tgm2}^{fl/fl};\text{Cx3cr1Cre}^+ \text{ mice. * } P = 0.0493; N \text{ = 4 per genotype; unpaired } t\text{-test.} (E) \text{ Scatter plot displaying g-ratio values in the CC of Tgm2}^{fl/fl};\text{Cx3cr1Cre}^- \text{ and Tgm2}^{fl/fl};\text{Cx3cr1Cre}^+ \text{ mice.}\]

\textbf{Figure 5: ADGRG1 and its ligand TG2 are required for remyelination.}

(A) Representative images of cerebellar slice cultures from P10 \text{ Adgrg1}^{fl/fl};\text{PdgfraCreER}^- \text{ and Adgrg1}^{fl/fl};\text{PdgfraCreER}^+ \text{ mouse cerebella that were demyelinated with LPC for 24 h followed by 4 days of recovery (4DR). Slices were labeled with NF200 (green) and MBP (red); myelinated fibers appear yellow in merged images. Scale bar, 100 µm. (B)}

Composite images created in Image J show myelinated axons (black) for quantification.

(C) Percentage of myelinated axons after remyelination. Remyelination is reduced in cerebellar slices that lack OPC-derived ADGRG1. * \(P = 0.0135\), paired \(t\text{-test}, N = 5\) per genotype. (D) Representative images of cerebellar slice cultures from P10 \text{ Adgrg1}^{+/+};\text{Tgm2}^{+/+}, \text{Adgrg1}^{+/+};\text{Tgm2}^{--} \text{ and Adgrg1}^{--};\text{Tgm2}^{--} \text{ mouse cerebella that were demyelinated with LPC for 24 h followed by 4 days of remyelination in the presence or absence of rTG2 (20 nM). Slices were immunostained with NF200 (green) and MBP (red); myelinated fibers appear yellow in merged images. Scale bar, 100 µm. (E)}

Composite images created in Image J show myelinated axons (black) for quantification.
(F) Percentage of myelinated axons. Recombinant TG2 promotes remyelination in
Adgrg1<sup>+/+</sup>;Tgm2<sup>+/+</sup> and Adgrg1<sup>+/+</sup>;Tgm2<sup>-/-</sup>, but not Adgrg1<sup>-/-</sup>;Tgm2<sup>-/-</sup> cerebellar slices. * P
= 0.0475 (Adgrg1<sup>+/+</sup>;Tgm2<sup>+/+</sup>), * P = 0.0197 (Adgrg1<sup>+/+</sup>;Tgm2<sup>-/-</sup>), paired t-test, N = 5-6
per genotype. Error bars are mean ± s.e.m (C, F).

Figure 6: Microglia promote OPC proliferation via ADGRG1 signaling.

TG2, secreted by microglia, binds ADGRG1 but fails to activate the receptor in the
absence of ECM protein laminin-111. The binding of TG2 and laminin-111 to ADGRG1
leads to the dissociation of the ADGRG1 NTF from its CTF, allowing the tethered
agonist to initiate G-protein signaling, culminating in activated RhoA, which promotes
OPC proliferation. GAIN, GPCR-Autoproteolysis-INducing.
Table 1: Summary of mass spectrometry results from three independent purifications.

Total number of peptides sequences in each individual experiment specific for binding to ADGRG1N.

<table>
<thead>
<tr>
<th>Protein name (Gene ID)</th>
<th>Peptide Number (Exp1 + Exp2 + Exp3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plectin (Plec)</td>
<td>21 + 22 + 23</td>
</tr>
<tr>
<td>Transglutaminase 2 (Tgm2)</td>
<td>19 + 20 + 17</td>
</tr>
<tr>
<td>GPR56/ADGRG1 (Gpr56/Adgrg1)</td>
<td>12 + 11 + 9</td>
</tr>
<tr>
<td>Growth arrest-specific 6 (Gas6)</td>
<td>11 + 9 + 11</td>
</tr>
<tr>
<td>Sodium/potassium-transporting ATPase subunit alpha-1(Atp1a1)</td>
<td>8 + 7 + 9</td>
</tr>
<tr>
<td>Aspartyl-tRNA synthetase (Dars)</td>
<td>8 + 3 + 8</td>
</tr>
<tr>
<td>Membrane protein, palmitoylated 6 (Mpp6)</td>
<td>8 + 5 + 11</td>
</tr>
<tr>
<td>Microtubule-actin crosslinking factor 1 (Macf1)</td>
<td>8 + 13 + 8</td>
</tr>
</tbody>
</table>
Figure 1-figure supplemental 1: Flow chart of the *in vitro* biotinylation/proteomic approach.

Biotinylated ADGRG1N-mFc was used to purify ADGRG1 binding partner(s) from mixed glial cell (enriched in microglia and astrocytes) lysate. Protein complexes were subjected to SDS PAGE and tryptic peptide mass spectrometry protein identification after one-step purification with immobilized streptavidin. The whole lane was excised and subjected to tryptic peptide mass spectrometry (MS) protein identification analysis.

Figure 2-figure supplemental 1: Oligodendrocyte number recovers by 5 month in *Tgm2* knockout mice.

(A) Representative images of ISH of *Plp* in the CC of 4-5 month old *Tgm2*+/− and *Tgm2*−/− mice. Scale bar, 100 µm. DAPI, blue. (B) Quantification of *Plp*+ oligodendrocytes in the CC reveals similar numbers of OLs in the CC of *Tgm2*+/− and *Tgm2*−/− mice. *P* = 0.807, unpaired t-test, *N* = 3 per genotype. Error bars are means ± s.e.m.

Figure 2-figure supplemental 2: Loss of *Tgm2* does not affect g-ratio, myelinated axon distribution, axon diameter or axon number.

(A) Average g-ratio of myelin sheath thickness was comparable in control and *Tgm2*−/− mice. *P* = 0.728, unpaired t-test, *N* = 3 per genotype. (B) The distribution of myelinated
axons with respect to the axon diameter in the CC of control and $Tgm2^{-/-}$ mice. $P = 0.8291$, two-way ANOVA, $P = 0.2524$, Gaussian non-linear curve fit, $N = 3$ per genotype. (C) Axon diameter in the CC is comparable in control and $Tgm2^{-/-}$ mice. Mean ± s.d.; $P = 0.8771$; unpaired $t$-test; $N = 3$ per genotype. (B) Number of total axons (myelinated and unmyelinated) is comparable in the CC of in control and $Tgm2^{-/-}$ mice at P28. $P = 0.7947$, unpaired $t$-test, $N = 3$ per genotype. Error bars are means ± s.e.m (A-D).

Figure 2-figure supplemental 3: Deleting one allele of $Tgm2$ has no effect on the number of $Plp^+$ cells and myelinated axons.

(A) Representative images of ISH of $Plp$ on P28 in the CC in $Tgm2^{+/+}$ and $Tgm2^{-/-}$ mice. Scale bar, 100 μm. DAPI, blue. (B) Quantification of $Plp^+$ oligodendrocytes in the CC reveals similar numbers of OLs in the CC of $Tgm2^{+/+}$ and $Tgm2^{-/-}$ mice. * $P = 0.6005$, unpaired t-test, $N = 3$ per genotype. (C) Representative TEM images from P28 CC of $Tgm2^{+/+}$ and $Tgm2^{-/-}$ mice. Scale bar, 1 μm. (D) Percentage of myelinated axons was quantified in the CC in $Tgm2^{+/+}$ and $Tgm2^{-/-}$ mice. $P = 0.5129$, unpaired t-test, $N = 3$ per genotype. Error bars are means ± s.e.m (B, D).

Figure 2-figure supplemental 4: Developmental expression of $Tgm2$.

Developmental expression of $Tgm2$ in murine microglia purified from whole brains (E10.5 through P) or brain regions (adult) at the indicated embryonic and postnatal ages. RNA-sequencing data was extracted from public database of (Matcovitch-Natan et al., 2016). Cx: cortex, Hp: hippocampus, SC: spinal cord.
Figure 3-figure supplemental 1: Mutations in gas6, mpp6, and plec do not affect CNS Mbp expression.

(A-E) CRISPR-Cas9 technology was used to generate mutations in zebrafish gas6 (A), mpp6a (B), mpp6b (C), pleca (D), and plecb (E). (A) A CRISPR/Cas9 system guide RNA (gRNA) designed to disrupt exon 2 (targeted sequence highlighted blue and SexAI restriction enzyme site used for genotyping denoted) of gas6 generated gas6<sup>stl228</sup>, a 4 bp deletion predicted to result in a frameshift and premature STOP. Alignments of wt and mutant nucleotide sequences (top), and predicted wt (706 aa) and mutant (78 aa) protein sequences (bottom) shown. (B) A gRNA designed to disrupt the 3<sup>rd</sup> coding exon 5 (targeted sequence highlighted purple, and HpyCH4IV restriction enzyme site used for genotyping denoted) of mpp6a generated mpp6a<sup>stl233</sup>, a 1 bp deletion predicted to result in a frameshift and premature STOP. Alignments of wt and mutant nucleotide sequences (top), and predicted wt (550 aa) and mutant (69 aa) protein sequences (bottom) shown. (C) A gRNA designed to disrupt exon 2 (targeted sequence highlighted green and BstNI restriction enzyme site used for genotyping denoted) of mpp6b generated mpp6b<sup>stl234</sup>, a 6 bp deletion predicted to result in a 2 amino acid deletion and a single amino acid change within the receptor targeting domain. Alignments of wt and mutant nucleotide sequences (top), and predicted wt (539 aa) and mutant (537 aa) protein sequences (bottom) shown. (D) A gRNA designed to disrupt exon 3 (targeted sequence highlighted orange, and DdeI restriction enzyme site used for genotyping denoted) of pleca generated pleca<sup>stl261</sup>, a 20 bp deletion predicted to result in a frameshift and premature STOP. Alignments of wt and mutant nucleotide sequences (top), and predicted wt (4752 aa) and mutant (103 aa)
protein sequences (bottom) shown. (E) A gRNA designed to disrupt the 3rd coding exon (targeted sequence highlighted yellow, and Hpy188III restriction enzyme site used for genotyping denoted) of plecb generated plecb<sup>stl236</sup>, a 13 bp deletion predicted to result in a frameshift and premature STOP. Alignments of wt and mutant nucleotide sequences (top), and predicted wt (4530 aa) and mutant (111 aa) protein sequences (bottom) shown. (F–O) Whole mount ISH showing mbp expression (CNS denoted by black arrows) at 5 days post-fertilization (dpf) in (F) gas6 control larva (wt and gas6<sup>stl228/+</sup>, N=11/12), (G) gas<sup>stl228/stl228</sup> mutant larva (N=5/8), (H) mpp6a control larva (wt and mpp6a<sup>stl233/+</sup>, N=17/18), (I) mpp6a<sup>stl233/stl233</sup> mutant larva (N=5/7), (J) mpp6b control larva (wt and mpp6b<sup>stl234/+</sup>, N=24/24), (K) mpp6b<sup>stl234/stl234</sup> (N=4/4), (L) pleca control larva (wt and pleca<sup>stl261/+</sup>, N=18/18), (M) pleca<sup>stl261/stl261</sup> mutant larva (N=4/4), (N) plecb control larva (wt and plecb<sup>stl236/+</sup>, N=36/38), and (O) plecb<sup>stl236/stl236</sup> mutant larva (N=10/10). Dorsal views are shown for all, anterior to the left. No gross changes in mbp expression were observed in putative ligand mutants relative to sibling controls.

**Figure 4-figure supplemental 1:** Loss of Adgrg1 in the OL lineage leads to reduced numbers of myelinating OLs.

(A) Expression of Tgm2 in murine microglia purified from naive or EAE brains. RNA-sequencing data was extracted from public database of (Wlodarczyk et al., 2017). (B) Representative images of GST<sup>+</sup> OLs of the corpus callosum of Adgrg1<sup>fl/fl;PdgfraCreER</sup> and Adgrg1<sup>fl/fl;PdgfraCreER</sup> mice after cuprizone feeding for 6 weeks followed by recovery for 3 d (3 DR), 7 d (7 DR) and 10 d (10 DR) (dotted line outlines quantified region). Scale bar, 100 µm.
Figure 1 - Piao

A

ADGRG1-hFc/DAPI

B

C

D

E

F

G

H

%ADGRG1-hFc cells

Iba⁺  GFAP⁺
ADGRG1^N-mFc-biotinylated

+ Mixed glial cells (astrocytes/microglia) lysate

+ Streptavidin beads

→ Elution of biotinylated protein complex

→ SDS-PAGE followed by Mass Spectrometry
Figure 2 - Piao

A  

Tgm2^{+/−}  

P14  
P28  

Plp/DAPI

B  

\( \%\text{Ctrl P28 Plp}^+ \text{ cells} \)

\( Tgm2^{+/−} \)  
\( Tgm2^{−/−} \)

\( P14 \)  
\( P28 \)

C  

Tgm2^{+/+}  

Tgm2^{−/−}  

\% Myelinated Axons

D  

Tgm2^{+/+}/Tgm2^{−/−}  

Tgm2^{−/−}

\% Myelinated Axons

E  

g-ratio  

Axon Diameter [μm]

Tgm2^{+/+}/Tgm2^{−/−}  

Tgm2^{−/−}

F  

Tgm2^{+/+};Cx3cr1Cre−  

Tgm2^{+/+};Cx3cr1Cre+

G  

Tgm2^{fl/fl};Cx3cr1Cre−  

Tgm2^{fl/fl};Cx3cr1Cre+

H  

\% Ctrl Plp^+ cells

Tgm2^{fl/fl}; Tgm2^{fl/fl}; Cx3cr1Cre−  

Cx3cr1Cre+

\( ** \)

\( * \)
Figure 3-figure supplement 1 - Piao

A

\[ \text{SexAl} \]

\[
\begin{align*}
\text{gas6}^{+/+} & : \text{CCCCACGTTTCTTCGGCAGAACCACGGGTTGCTCGTGCTCTCGCAGCA} \\
\text{gas6}^{stl228/stl228} & : \text{CCCCACGTTTCTTCGGCAGAACCACGGGTTGCTCGTGCTCTCGCAGCA} \\
\text{Gas6(WT)} & : \text{SVSSRQAQHQLRRTRRNQVFHEDKGEIREVKKEAREVFENDPETFAYYP} \\
\text{Gas6(stl228)} & : \text{SVSSRQAQHQLRRTRRNQVFHEDKGEIREVKKEAREVFENDPETFAYYP}
\end{align*}
\]

B

\[ \text{HpyCH4IV} \]

\[
\begin{align*}
\text{mpp6a}^{+/+} & : \text{AGGATGTTAAGCTGGAGCCGCTACAGCTAGGTGAGATGAGATCGCC} \\
\text{mpp6a}^{stl233} & : \text{AGGATGTTAAGCTGGAGCCGCTACAGCTAGGTGAGATGAGATCGCC} \\
\text{Mpp6a (WT)} & : \text{DVKLEAVQSNVELVSEILSDMLLIRDESAELNILEKHGFQSLLEAHDKVAS} \\
\text{Mpp6a (stl233)} & : \text{DVKLEAVQSNKG*}
\end{align*}
\]

C

\[ \text{BstNI} \]

\[
\begin{align*}
\text{mpp6b}^{+/+} & : \text{CTCTCTGTGTTTGTCAGCTATGCGAGCGGTGATTGGAAACCTCGTGGCC} \\
\text{mpp6b}^{stl234/stl234} & : \text{CTCTCTGTGTTTGTCAGCTATGCGAGCGGTGATTGGAAACCTCGTGGCC} \\
\text{Mpp6b (WT)} & : \text{MITAMQQVLNDNLGELSTUGAKIDLIFLKGAMESPIVRLAKHERLEDVKLEAVQE} \\
\text{Mpp6b (stl234)} & : \text{MITAMQQVL--EELSPSTUGAKIDLIFLKGAMESPIVRLAKHERLEDVKLEAVQE}
\end{align*}
\]

D

\[ \text{Ddel} \]

\[
\begin{align*}
\text{pleca}^{+/+} & : \text{GAAACATAAGAGAAGAGAAGAAAACGCGAGAAGACGCGAGCAGAGAAAG} \\
\text{pleca}^{stl261/stl261} & : \text{GAAACATAAGAGAAGAGAAGAAAACGCGAGAAGACGCGAGCAGAGAAAG} \\
\text{Pleca (WT)} & : \text{KHKKKKEKLKNKTEDEEKKEKKEKDEGKEEETITITVRKRKNKMLKVTIAPGTVSA} \\
\text{Pleca (stl261)} & : \text{KHKKK*}
\end{align*}
\]

E

\[ \text{Hpy188III} \]

\[
\begin{align*}
\text{plecb}^{+/+} & : \text{GATGGCACATAACCTCTGCTCTCGCTCTCGCTCTGAGAGAGAGCTCGTGTGAGT} \\
\text{plecb}^{stl236/stl236} & : \text{GATGGCACATAACCTCTGCTCTCGCTCTCGCTCTGAGAGAGAGCTCGTGTGAGT} \\
\text{Plecb (WT)} & : \text{DGHNLSLLEELVEQLPFTVRGRMRFHKLQMNLQALDFLHRHQVKLNIRNDDARCHN} \\
\text{Plecb (stl236)} & : \text{DGHNLSLLEERRW*}
\end{align*}
\]

F

\[ \text{5 dpf mbp} \]

G

\[ \text{gas6 (control) \ N=11/12} \]

\[ \text{mpp6a (control) \ N=17/18} \]

\[ \text{mpp6b (control) \ N=24/24} \]

\[ \text{pleca (control) \ N=18/18} \]

\[ \text{plecb (control) \ N=38/38} \]

H

I

J

K

L

M

N

\[ \text{gas6#228/stl228 \ N=5/6} \]

\[ \text{mpp6a#stl233/stl233 \ N=5/7} \]

\[ \text{mpp6b#stl234/stl234 \ N=4/4} \]

\[ \text{pleca#stl261/stl261 \ N=4/4} \]

\[ \text{plecb#stl236/stl236 \ N=10/10} \]