

# Daam2 Regulates Myelin Structure and the Oligodendrocyte Actin Cytoskeleton through Rac1 and Gelsolin

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Myelin is essential to neuronal health and CNS function, and oligodendrocytes (OLs) undergo a complex process of cytoskeletal remodeling to form compact myelin sheaths. We previously discovered that a formin protein, Dishevelled associated activator of morphogenesis 2 (Daam2), suppresses OL differentiation through Wnt signaling; however, its role in cytoskeletal control remains unknown. To investigate this, we used OL-specific Daam2 conditional knockout (Daam2 cKO) mice of either sex and found myelin decompaction during an active period of myelination in postnatal development and motor coordination deficits in adulthood. Using primary OL cultures, we found Daam2-depleted OLs showed morphologic dysregulation during differentiation, suggesting that Daam2 regulates the OL cytoskeleton. *In vivo* screening identified the actin regulators Rac1 and Gelsolin as possible effectors in Daam2-deficient OL cytoskeletal regulation. Using gain-of-function and loss-of-function (LOF) experiments in primary OLs, we found that Rac1 and Gelsolin operate downstream of Daam2 in OL differentiation, with Gelsolin and Daam2 promoting and inhibiting membrane spreading during late differentiation, respectively. *In vivo* experiments using Daam2 cKO mice revealed increased protein levels of Gelsolin in the developing white matter with no change in RNA levels, suggesting that Daam2 acts in a posttranslational manner to suppress Gelsolin levels. *In vitro* biochemical studies show Daam2 induces Gelsolin ubiquitination and degradation in OLs. Together, our studies show Daam2 is essential for formation of functional myelin through modulation of Gelsolin levels to regulate the OL cytoskeleton. These findings further demonstrate the critical role of cytoskeletal dynamics in myelination and reveal novel avenues for treatment of a variety of white matter diseases.

**Key words:** actin cytoskeleton; Daam2; gelsolin; myelin; oligodendrocyte; Rac1

## Significance Statement

Proper myelin formation is essential to CNS function, and oligodendrocytes (OLs) require extensive changes in the actin cytoskeleton to form myelin sheaths. Here, we show that the formin protein Dishevelled associated activator of morphogenesis 2 (Daam2) is necessary for myelin compaction during development and motor learning in adulthood. Further, we demonstrate that Daam2 regulates OL differentiation and morphology through actin regulators Rac1 and Gelsolin. Lastly, we find that Daam2 may control myelin compaction by modulating the ubiquitination and degradation of Gelsolin through recruitment of the E3 ubiquitin ligase Nedd4. These findings reveal novel pathways for regulating myelin structure and function during white matter development.

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## Introduction

Myelin is essential for CNS function and plays a variety of roles in supporting neuronal health and activity (Bercury and Macklin, 2015; Mount and Monje, 2017) and is formed by fully differentiated oligodendrocytes (OLs) around axons (Nave and Werner, 2014; Stadelmann et al., 2019). Abnormalities in myelin sheaths can result in disrupted signal transduction, long-term axonal damage, and a variety of neurologic deficits, including cognitive decline and motor dysfunction (Fields, 2008; Nir and Barak, 2021). To form myelin, OLs undergo a differentiation process that requires extensive alterations in cell morphology (Richter-Landsberg, 2008; Bercury and Macklin, 2015), which are mediated by cytoskeletal remodeling proteins. Genetic variants in cytoskeleton-associated genes have been implicated in dysmyelinating and demyelinating diseases such as Charcot-Marie-Tooth disease (Fabrizi et al., 2007; Boyer et al., 2011), multiple sclerosis (Fischer et al., 2013), and severe hypomyelinating leukodystrophy (Al-Abdi et al., 2020). Animal models have also linked pathogenic variants in cytoskeletal proteins with defects in myelin structure (Brown and Macklin, 2020). These underscore a central role for the OL cytoskeleton in CNS function; however, the molecular mechanisms underlying its regulation remain poorly defined.

OL differentiation occurs in discrete stages: (1) process extension, (2) axon ensheathment, and (3) wrapping of the myelin sheath (Bercury and Macklin, 2015; Nawaz et al., 2015; Zuchero et al., 2015). Specific regulatory dynamics in the actin cytoskeleton are necessary for progression through each stage (Michalski and Kothary, 2015). Actin filament assembly is integral to process extension and ensheathment (Nawaz et al., 2015; Zuchero et al., 2015) and actin regulators such as Arp2/3, N-WASP, and Rho-GTPases are known to be crucial for the actin growth cone during this process (Thomason et al., 2020). The Rho GTPase Rac1 plays an important role during these stages: Rac1 overexpression promotes process extension and branching during early OL differentiation *in vitro* (Thurnherr et al., 2006), and deletion of Rac1 in OLs leads to irregular myelin outfolding (Thurnherr et al., 2006; Katanov et al., 2020). These studies suggest that proper regulation of Rac1 levels is required for actin polymerization during process extension and ensheathment.

As OLs reach the wrapping stage, actin depolymerization is necessary to drive the formation of compact myelin. In this stage, actin disassembly proteins such as Gelsolin are upregulated, facilitating membrane compaction and flattening (Nawaz et al., 2015; Zuchero et al., 2015). Human gelsolin variants have been associated with demyelination, slow nerve conduction, and prolonged distal motor latencies (Kiuru and Seppäläinen, 1994; Westberg et al., 1999; Kiuru-Enari et al., 2002), and Gelsolin knock-out mice show thinner myelin during postnatal development (Zuchero et al., 2015). These findings indicate the importance of both Rac1 and Gelsolin in the formation of normal myelin; however, the stage-specific expression dynamics of these proteins in OLs have not been examined *in vivo* and little is known about the mechanisms underlying their regulation in OLs.

Previously, we discovered that Dishevelled associated activator of morphogenesis 2 (Daam2), part of the formin family of actin-modulating proteins, suppresses OL differentiation by recruiting Wnt signalosome components to the cell membrane (Lee et al., 2015) and by facilitating ubiquitination through E3 ubiquitin ligase recruitment (Ding et al., 2020). Recent evidence has also suggested a role for Daam2 in actin-mediated membrane protrusion (Luo et al., 2019) and a functional relationship with Rac1 during early spinal cord development (Cristobal et al.,

2021). In addition, Daam2 modulates levels of phosphatidylinositol (4,5)-bisphosphate (PIP2) at the membrane, a potent regulator of actin-binding proteins in OLs (Lee et al., 2015). Together, these data suggest that Daam2 may play a crucial role in regulating the actin cytoskeleton during OL differentiation. Here, we use multiple mouse models and *in vitro* differentiation studies to delineate the role of Daam2 in control of OL morphology and myelin structure. Further, we identify epistatic, hierarchical relationships between Daam2 and the actin-modulating molecules Rac1 and Gelsolin in controlling OL development and myelin formation.

## Materials and Methods

### Animals

We generated Daam2 conditional OL-specific knock-out mice as previously described (Ding et al., 2020). Briefly, mice containing loxP sequences flanking exon 6 of Daam2 (Daam2<sup>fl/fl</sup>) were crossed with Sox10-cre (JAX 025807) mice to create Sox10-cre Daam2<sup>fl/fl</sup> (Daam2 cKO) mice. As previously described, recombination efficiency was confirmed using a Daam2 cKO-Tomato reporter line: 88% of tdTomato+ cells were colocalized with Olig2+ cells. Mice of either sex were used in all experiments. All procedures were approved by the Institutional Animal Care and Use Committee at the Baylor College of Medicine and adhere to the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals.

### Behavioral tests

For the open field assay, mice were placed in a clear, open Plexiglas box (40 × 40 × 30 cm) equipped with an overhead camera and photograph beams to record movements. Movement data were quantified over 10 min and quantified using ANYmaze (Stoelting). For the accelerating rotarod assay, mice were placed on the rotarod apparatus (Ugo Basile) as the rod accelerated from 5 rpm to 40 rpm over 5 min. Latency to fall was measured as instances when the mouse fell off the rod or rode the cylinder for two consecutive revolutions. The training day consisted of four trials with ~30 min of rest between each trial.

### Electron microscopy and myelin measurement

As described previously (Hooshmand et al., 2014), mice were perfused with saline solution (0.1 M PBS, pH 7.4) at ~10 ml/min for 5 min and then perfused with fixing solution (1% glutaraldehyde, 4% paraformaldehyde, 0.1 M sodium cacodylate) at the same rate and duration. Spinal cord and corpus callosum tissues were then dissected out, placed in scintillation vials on ice, and postfixed for 1 h in fixing solution while rotating at 4°C. Lipid fixation was then performed by transferring tissue into 1% osmium tetroxide, 1.5% KFeCN, 0.1 M cacodylate solution for 1 h while rotating at 4°C. After lipid fixation, the tissue was washed three times for 15 min each in 0.1 M cacodylate at 4°C and fixed in 2% glutaraldehyde in 0.1 M cacodylate overnight at 4°C. Tissues were then washed, dehydrated, infiltrated, embedded, and cured for 5 d before sectioning and electron microscopy. For g-ratio measurement, axons with compact myelin were selected and the corresponding inner and outer diameters were manually measured using ImageJ.

### Primary OL culture, gene overexpression and knock-down

Primary OL precursor cell (OPC) cultures were initiated as described previously (Ding et al., 2020). Briefly, neural stem cells (NSCs) were harvested from embryonic mouse brains at embryonic day (E)14.5 and cultured as neurospheres in DMEM/F12 (Invitrogen), 1 × N-2 supplement (Thermo Fisher Scientific), 1 × B-27 supplement (Thermo Fisher Scientific), 20 ng/ml EGF (Sigma), 20 ng/ml bFGF (R&D Systems), and 1% penicillin-streptomycin (Thermo Fisher Scientific). Neurospheres were dissociated using Accutase cell detachment solution (Sigma) for up to two times before seeding in poly-D-lysine-coated coverslips or plates for OPC specification. OPC specification was induced by seeding on DMEM/F12, 1 × B-27 supplement, 10 ng/ml bFGF, and 10 ng/ml PDGF-AA (PeproTech). For overexpression and knock-down experiments, cells were transfected before differentiation for at least 5 h using DNA

constructs and IMfectin Transfection Reagent (GenDepot). OL differentiation was induced by changing medium into basal chemically defined medium (BDM), 15 nM triiodothyronine (Sigma), 10 ng/ml CNTF (Peprotech), 5 mg/ml *N*-acetyl-L-cysteine (Sigma). For imaging experiments, coverslip-seeded cells were fixed with 4% PFA for 15 min and immunocytochemistry was performed using similar methods to tissue staining as described below. Antibodies used are as following: mouse anti-MBP (1:300, BioLegend), rabbit anti-Olig2 (1:500, Millipore), mouse anti-Rac1 (1:500, Millipore), rabbit anti-NG2 (1:200, Millipore), rabbit anti-Olig2 (1:500, Millipore), rabbit anti-Gelsolin (1:100, ProteinTech), rat anti-CD140a/PDGFR $\alpha$  (1:200, Thermo Fisher Scientific), mouse anti-MAG (1:500, Millipore).

#### Immunofluorescence staining and histology

Spinal cords and brains of mice were fixed in 4% paraformaldehyde overnight, dehydrated in 20% sucrose, and embedded in Tissue-Tek OCT Compound (Sakura Finetek USA). Embedded blocks were stored at  $-80^{\circ}\text{C}$ , sectioned at 15  $\mu\text{m}$ , and slides were stored at  $-80^{\circ}\text{C}$  before staining. For immunofluorescence staining, tissues were washed in PBS three times for 5 min each, permeabilized with PBS containing 0.3% Triton X-100 (PBST) and blocked with 10% goat serum in PBST (blocking solution) for 1 h at room temperature. Tissues were then incubated with primary antibodies in blocking solution overnight at  $4^{\circ}\text{C}$ . After primary antibody incubation, slides were washed with PBS three times for 5 min each, incubated in secondary antibodies in blocking solution for 1 h at room temperature, and washed with PBS three times for 5 min each. Nuclei were stained using DAPI and tissues were mounted with VectaShield anti-fade mounting medium. Antibodies used in staining tissue are as following: mouse anti-APC/CC-1 (1:500, Millipore), rabbit anti-Gelsolin (1:100, ProteinTech).

#### Cell line cultures and biochemical assays

Oli-Neu cells were cultured in DMEM medium (GenDepot), 1 $\times$  N-1 supplement (Sigma), 3% heat-inactivated horse serum (Invitrogen), and 1% penicillin-streptomycin (Thermo Fisher Scientific). Biochemical studies including co-immunoprecipitation (co-IP), degradation, and ubiquitination were performed using lysates from primary cell culture and Oli-Neu, as indicated in figure legends. Cell lysates were obtained using 1 $\times$  RIPA cell lysis buffer (GenDepot), 1 $\times$  Xpert Protease Inhibitor Cocktail Solution (GenDepot), and 1% SDS. For co-IP assays, cell lysates were incubated with protein A or protein G agarose beads (Thermo Fisher Scientific) and incubated with antibodies overnight at  $4^{\circ}\text{C}$ . Protein beads were washed three times with 1 $\times$  RIPA buffer, boiled with 2 $\times$  SDS sample buffer for 10 min at  $95^{\circ}\text{C}$ , and analyzed using western blotting. For Rac1 activation assays, GTP-Rac1 was measured using a Rac1 pull-down activation assay (Cytoskeleton) before western blotting. For degradation assays, transfected OLs were treated with 100  $\mu\text{g}/\text{ml}$  cycloheximide (Sigma) and harvested with lysis buffer at 0, 4, and 8 h. For ubiquitination assays, Oli-Neu cells were treated with 40  $\mu\text{g}/\text{ml}$  MG132 (Selleckchem) for 6 h before harvesting using lysis buffer. For western blotting, the antibodies used are as follows: mouse anti-Rac1 (1:3000, Millipore), mouse anti- $\beta$ -actin (1:3000, Millipore), rabbit anti-Gelsolin (1:2000, ProteinTech), mouse anti-Flag (1:3000, Sigma), mouse anti-c-Myc (1:3000, Santa Cruz Biotechnology), rabbit anti-GAPDH (1:5000, GeneTex), mouse anti-HA (1:3000, Sigma), Peroxidase Affinipure goat anti-mouse IgG H + L (1:10,000, Jackson ImmunoResearch), Peroxidase Affinipure goat anti-rabbit IgG H + L (1:10,000, Jackson ImmunoResearch).

#### Imaging and morphologic analysis

Immunofluorescent-stained tissue and fixed cells were imaged using a Zeiss Imager.M2m microscope equipped with ApoTome.2, AxioCam 506 mono, and AxioCam MRc. For cell count analysis, images were exported and analyzed using ImageJ's particle analysis and cell counter plugins. Sholl analysis and cellular surface area measurements were performed using the Filament and Surface packages for Imaris (Oxford Instruments), respectively.

#### Statistical analyses

All statistical analysis and quantitative graphs were plotted using Prism 9 (GraphPad). For comparisons between two groups, Student's *t* tests were used. One-way ANOVA with multiple comparison test was used for

analyses among three or more groups. Two-way ANOVA with Sidak's multiple comparison test was used for analyses across multiple groups and radii for Sholl analysis, and across multiple groups and time points for *in vivo* expression analysis.

## Results

### Daam2 is necessary for compact myelin structure and motor coordination

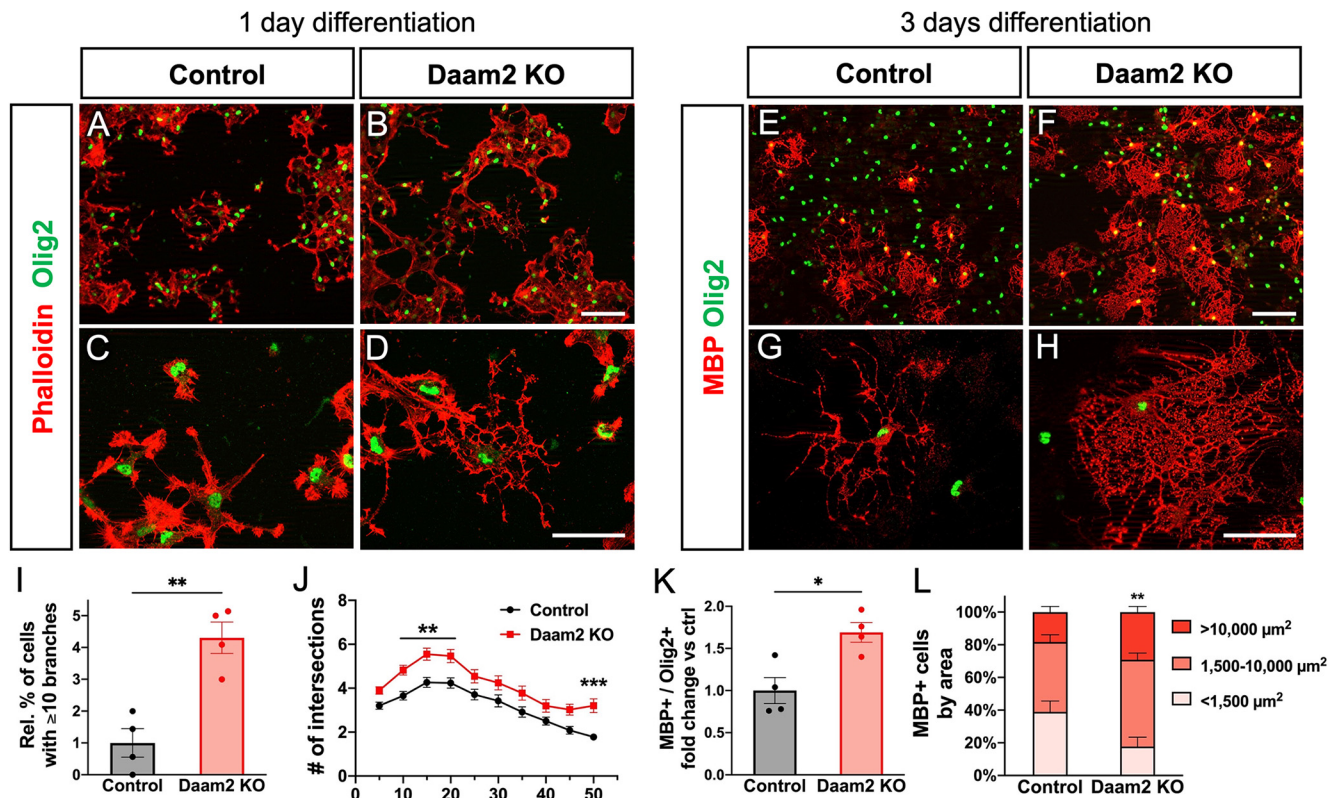
To investigate the role of Daam2 in myelin structure during development, we used Sox10<sup>cre</sup> Daam2<sup>fl/fl</sup> (Daam2 cKO) and Daam2<sup>fl/fl</sup> (control) mice of either sex to delete Daam2 specifically in the OL lineage and performed transmission electron microscopy (TEM) on major white matter tracts at postnatal day (P)21, an active period of myelination (Foran and Peterson, 1992; Korrell et al., 2019). At this stage, we found Daam2 cKO mice exhibited myelin decompaction in both the spinal cord and corpus callosum (Fig. 1A–I,M). In addition, we measured myelin thickness in axons that had compact myelin and found that Daam2 cKO mice had higher g-ratios compared with control mice, indicating slightly thinner myelin (Fig. 1J–L,N–P). To investigate whether Daam2 deletion led to persistent defects in myelin structure, we visualized myelin in P60 control and Daam2 cKO mice and found no significant differences in myelin structure at this stage (Extended Data Fig. 1–1). This suggests that Daam2 plays an important stage-specific role in the formation of compact myelin structure at a critical period of postnatal development.

Compact myelin is essential for rapid electrical conduction, and abnormalities in myelin structure are known to cause alterations in motor function (Arancibia-Carcamo and Attwell, 2014; Wang et al., 2018). Importantly, transient myelination defects have also been found to have persistent effects on motor and behavioral outcomes (Gika et al., 2010; Jackson et al., 2018). We thus investigated whether loss of Daam2 leads to motor deficits through a series of behavioral tasks. While adult Daam2 cKO mice did not exhibit significant changes in total distance traveled in the open field activity test (Fig. 1Q), we found that Daam2 cKO mice exhibited a slower rate of motor learning in the rotarod assay (Fig. 1R). Together, these suggest that OL-specific loss of Daam2 leads to structural abnormalities in myelin during a critical period postnatal development which subsequently results in persistent defects in motor coordination.

### Daam2 regulates branching and spreading in primary OL differentiation

The OL cytoskeleton plays a central role in forming compact, functional myelin (Nave and Werner, 2014; Bercury and Macklin, 2015). Although we previously found Daam2 regulates OL differentiation (Lee et al., 2015), the role of Daam2 in modulating the OL cytoskeleton remains unknown. To investigate this relationship at the cellular level, we performed *in vitro* OL differentiation assays using constitutive Daam2 KO and Daam2 heterozygous control cells. We investigated cellular morphology at two time points: 1 d postdifferentiation where OLs extend branches, and 3 d postdifferentiation, where OLs undergo actin breakdown and membrane spreading *in vitro* (Zuchero et al., 2015). During the branching stage of OL differentiation, we observed that Daam2-depleted OLs had more complex cellular morphologies compared with control OLs, as indicated by a 4-fold increase in the number of cells with 10 or more branches (Fig. 2A–D,I). We also performed Sholl analysis to measure branching complexity by counting the number of branches intersecting with concentric circles around the cell body. We found





**Figure 2.** Daam2 regulates OL morphology in early and late differentiation **A–H**. *In vitro* OL differentiation assays using cells from control and Daam2 KO mice at different stages of OL differentiation. **A–D**, **I**, Daam2 KO OLs exhibit more cells with highly branched morphologies compared with control cells at 1 d postdifferentiation; \*\* $p = 0.0025$  versus control by unpaired  $t$  test. **J**, Daam2 KO cells have a higher extent of branching compared with control cells at 1 d postdifferentiation; \*\* $p = 0.089$ , \*\* $p = 0.0024$ , \*\* $p = 0.0047$  by two-way ANOVA and Sidak's multiple comparison test. **E–H**, **K**, Daam2 KO cells exhibit a higher number of MBP+ cells compared with control cells at 3 d postdifferentiation; \* $p = 0.0118$  versus control by unpaired  $t$  test. **L**, MBP+ Daam2 KO cells exhibit a larger surface area compared with control cells at 3 d postdifferentiation; \*\* $p = 0.0015$  versus control by two-way ANOVA and Sidak's multiple comparison test. Scale bars: 100  $\mu\text{m}$  (**A**, **B**, **E**, **F**) and 50  $\mu\text{m}$  (**C**, **D**, **G**, **H**). All *in vitro* experiments and statistical tests were performed in four independent trials. All error bars indicate standard error of the mean.

that Daam2-depleted OLs had more than a 20% increase in Sholl intersections on average (Fig. 2*A–D*, *J*). During the membrane spreading stage at 3 d postdifferentiation, we found Daam2-depleted OLs exhibited precocious differentiation, confirming previous findings showing the inhibitory role of Daam2 in OL differentiation (Lee et al., 2015). We then measured the MBP+ surface area of each cell and found that Daam2-depleted cells had larger surface areas on average, with a 2-fold decrease in the fraction of cells with the smallest surface areas ( $<1500 \mu\text{m}^2$ ; Fig. 2*E–H*, *K–L*). These data suggest that Daam2 regulates both the process extension and the membrane wrapping stage in OL differentiation, which are driven in large part by remodeling of the actin cytoskeleton (Nawaz et al., 2015; Zuchero et al., 2015).

#### Daam2 associates with key cytoskeletal regulators Rac1 and gelsolin

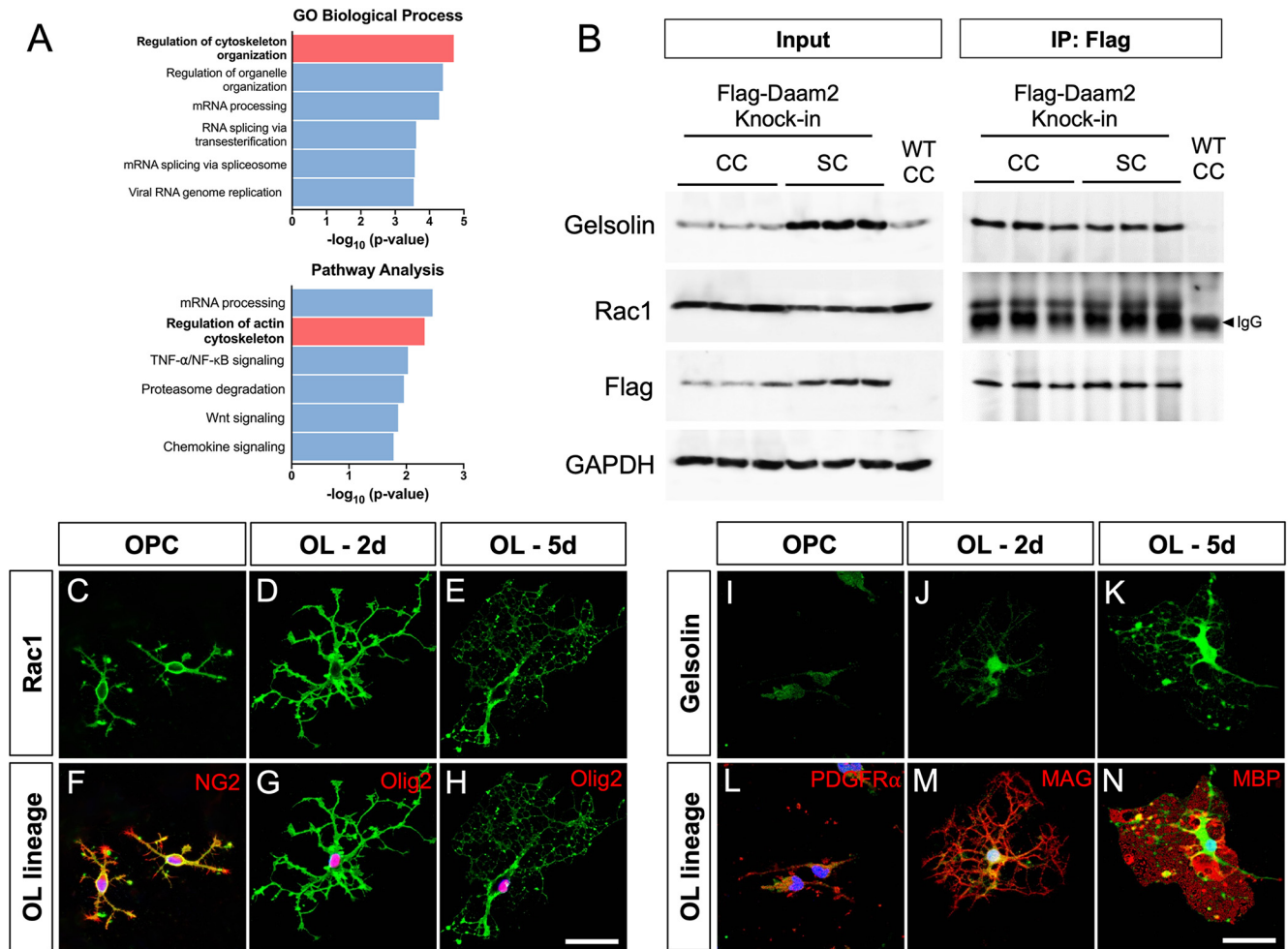
To identify potential mechanisms by which Daam2 may modulate OL morphology, we performed an *in vivo* Daam2 pull-down assay in mouse white matter tissue followed by mass spectrometry to identify candidate proteins interacting with Daam2. Gene set enrichment analysis (GSEA) showed many of the identified

proteins interacting with Daam2 regulate the actin cytoskeleton (Fig. 3*A*), which supports a major role for Daam2 in regulating morphologic differentiation of OLs. To further identify the most likely downstream interactors, we used a multi-pronged strategy considering developmental stage, lineage, and physical association with Daam2 (Ding et al., 2020). Using previous studies investigating gene expression in glial development (Deneen et al., 2006; Chaboub et al., 2016; John Lin et al., 2017), we identified 105 proteins in our screen that were expressed during early postnatal development in glial precursors and physically associated with Daam2. Of these, 12 candidates were expressed in the OL lineage (Marques et al., 2016; La Manno et al., 2021) and had GO terms relating to actin regulation (Extended Data Fig. 3-1). We chose to focus on two particular candidates because of their previously identified roles in regulating OL morphology: Rac1 in OL branching and Gelsolin in membrane spreading during myelination (Thurnherr et al., 2006; Zuchero et al., 2015). Although Rac1 and Gelsolin are known to modulate the actin cytoskeleton, little is known about their stage-specific expression levels and mechanisms controlling their expression and activity in OLs.

We first validated physical associations between Daam2, Rac1, and Gelsolin using *in vivo* co-IP in Flag-Daam2 knock-in mice. Briefly, we generated a mouse line inserting a Flag-tag sequence upstream of Daam2 and isolated the corpus callosum and spinal cord in P21 mice for co-IP experiments. We found Gelsolin and Rac1 both associate with Daam2 in white matter tracts *in vivo* (Fig. 3*B*), suggesting they may act along with Daam2 in regulating the OL cytoskeleton. We then investigated

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the open field test at eight weeks of age; ns = not significant by unpaired  $t$ -test;  $n = 11$ , 8 animals for control and Daam2 cKO, respectively. **R**, Daam2 cKO mice exhibit defects in motor coordination in the rotarod task at eight weeks of age; \*\* $p = 0.0029$ , \* $p = 0.0108$  by two-way ANOVA and Sidak's multiple comparison test;  $n = 8$  mice for control and 6 mice for Daam2 cKO mice. All error bars indicate standard error of the mean.



**Figure 3.** Rac1 and Gelsolin associate with Daam2 and are expressed in distinct stages of OL differentiation. **A, B**, GSEA of *in vivo* Daam2 pulldown mass spectrometry data shows enrichment of proteins involved in regulation of the actin cytoskeleton (see Extended Data Fig. 3–1 for list of genes found in mass spectrometry screen). **B**, *In vivo* co-IP in Flag-Daam2 knock-in animals showing association between Daam2 and two candidates Rac1 and Gelsolin. **C–H**, *In vitro* OL differentiation experiments showing temporal expression patterns of Rac1 and Gelsolin. **C–H**, Rac1 is expressed highly in the OPC and early differentiation stages *in vitro*. **I–N**, High Gelsolin expression is seen at the later stages of OL differentiation *in vitro*. Scale bars: 50  $\mu$ m. *In vitro* experiments performed in four independent trials, three replicates each.

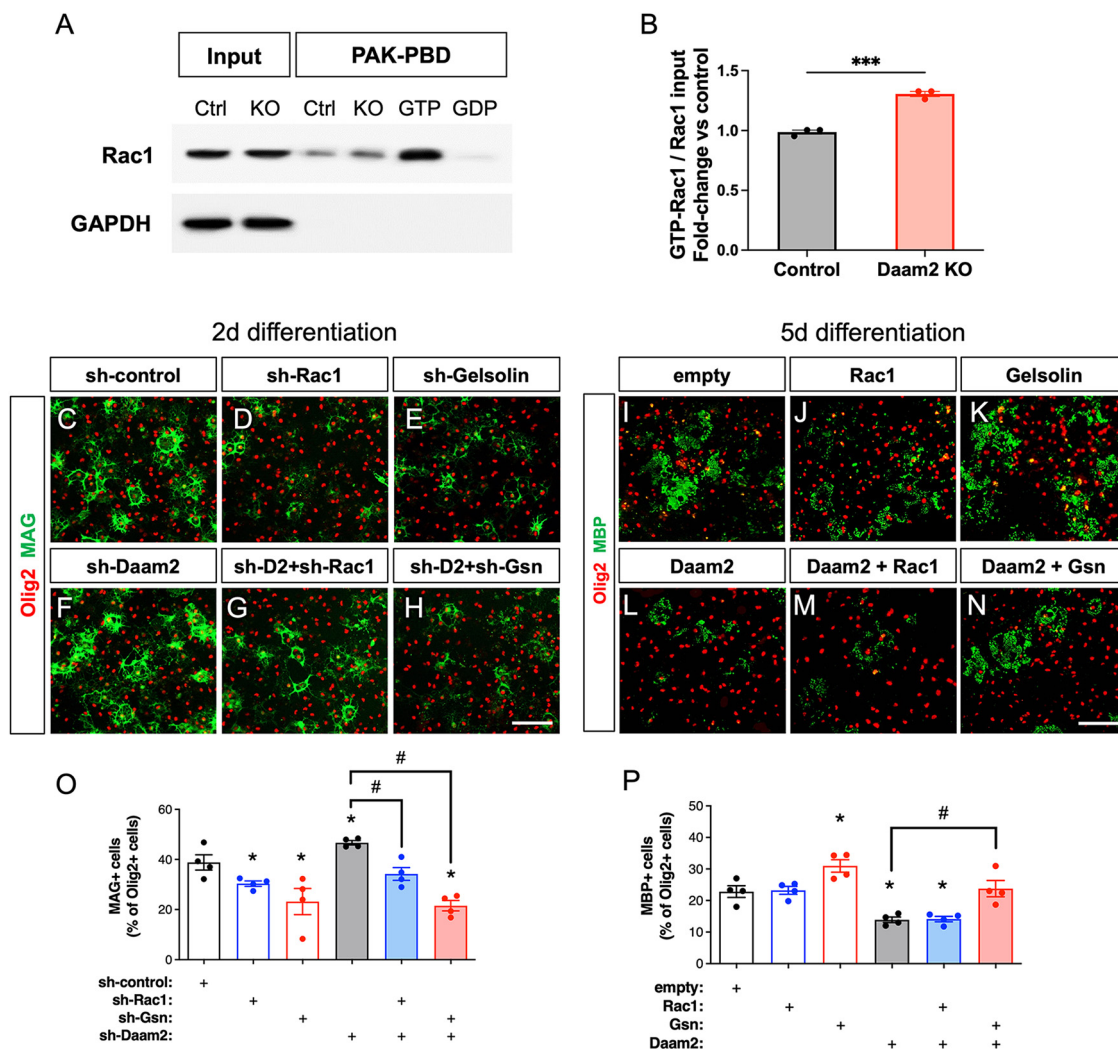
the stage-specific expression patterns and cellular localization of Rac1 and Gelsolin using *in vitro* differentiation assays. We found Rac1 is highly expressed throughout cellular processes in the OPCs and the branching stage of OL differentiation (Fig. 3C,D,F, G), while exhibiting a relative decrease in expression at the membrane spreading stage (Fig. 3E,H). On the other hand, we observed low levels of Gelsolin expression at the OPC stage (Fig. 3I,L) followed by a steady increase through the branching and membrane spreading stages (Fig. 3J,K,M,N). Together, these data suggest Daam2 may modulate OL morphology by cooperating with Rac1 at the branching stage of differentiation and with Gelsolin at the membrane spreading stage of differentiation.

#### Rac1 and gelsolin act downstream of Daam2 in regulating OL differentiation

While Rac1 and Gelsolin regulate key cytoskeletal processes in OL differentiation, little is known about upstream mechanisms regulating their action. We thus sought to examine possible epistatic relationships between these proteins and Daam2 in regulating OL differentiation. Given the observed increase in OL branch complexity on Daam2 deletion and the importance of Rac1 in process extension (Thurnherr et al., 2006), we sought to investigate the relationship between Daam2 and Rac1 in OLs. To

this end, we performed Rac1 pulldown activation assays in control and Daam2-depleted OLs at 2 d postdifferentiation. We found that the Daam2 KO cells had higher levels of activated GTP-bound Rac1 compared with control cells (Fig. 4A,B), suggesting that Daam2 inhibits Rac1 activation during differentiation. Further, complementary Rac1 overexpression experiments in primary OLs also induced higher branch complexity in early differentiation (Extended Data Fig. 4-1A,B,D,E,G) without an accompanying change in proliferation (Extended Data Fig. 4-1H, I,K). Together, these suggest that Daam2 regulates OL morphology at the early stages of differentiation through modulation of Rac1 activity.

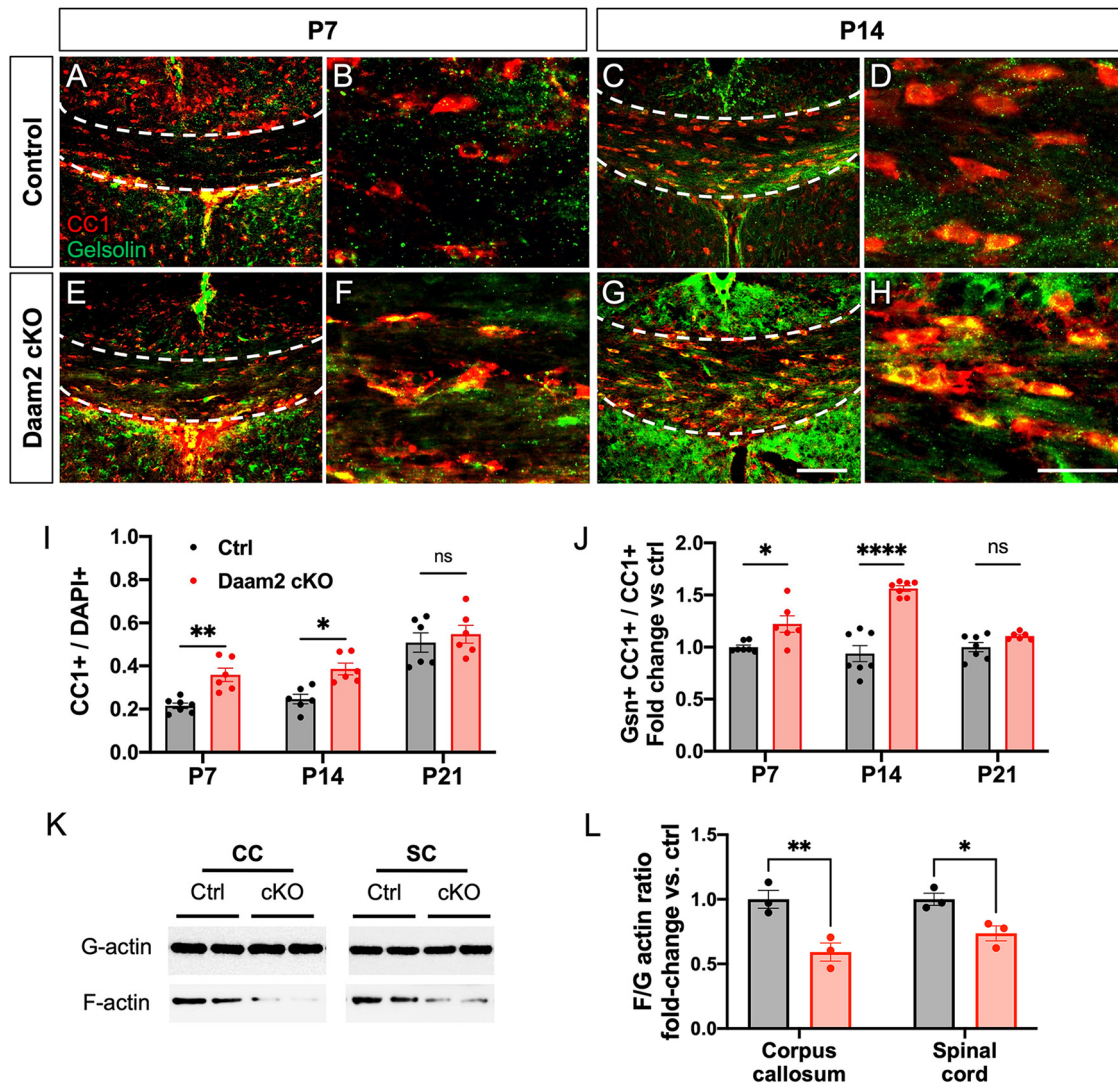
We then aimed to investigate the epistatic relationships between Daam2, Rac1, and Gelsolin in the progression of the OL developmental program. To address these questions, we performed single-loss-of-function (LOF) and double-LOF experiments using shRNAs targeting Rac1, Gelsolin and Daam2 and investigated their effects on OL maturation at 2 d postdifferentiation and stained for the mature OL marker myelin-associated glycoprotein (MAG). MAG is known to be expressed very early in the process of myelination, making it a more sensitive marker for investigating differentiation rates compared with MBP, which is expressed in later stages (Quarles, 2007; de Faria et al., 2019).



**Figure 4.** Rac1 and Gelsolin regulate OL differentiation downstream of Daam2. **A, B**, Rac1 pull-down activation assay from primary OL cultures 2 d postdifferentiation. Daam2 KO cells exhibit higher Rac1 activation compared with control cells;  $***p = 0.0003$  versus control by unpaired *t* test, with each point corresponding to an independent experiment. **C–H, O**, Single-knock-down and double-knock-down experiments in primary OL cultures 3 d postdifferentiation. **C–E, O**, Knock-down of Rac1 or Gelsolin result in a lower fraction of MAG+ cells compared with control. **F, O**, Knock-down of Daam2 results in a higher fraction of MAG+ cells compared with control. **G, H, O**, Knock-down of Rac1 or Gelsolin reverses the Daam2 knock-down phenotype;  $*p = 0.045$ ,  $p = 0.0097$ ,  $p = 0.0497$ ,  $p = 0.0003$  versus control,  $\#p = 0.0062$ ,  $p < 0.0001$  versus sh-Daam2 by one-way ANOVA with Tukey's multiple comparison test. **I–N, P**, Single-overexpression and double-overexpression experiments in primary OL cultures 5 d postdifferentiation. **J, K, P**, Overexpression of Gelsolin but not Rac1 leads to an increased number of MBP+ cells compared with control. **L, P**, Daam2 overexpression leads to a lower fraction of MBP+ cells compared with control. **M, N, P**, Gelsolin but not Rac1 overexpression reverses the Daam2 overexpression phenotype (see Extended Data Fig. 4-1 for analyses of morphology and proliferation);  $*p = 0.0106$ ,  $p = 0.003$ ,  $p = 0.0009$  versus control,  $\#p = 0.0003$  versus sh-Daam2 by one-way ANOVA with Tukey's multiple comparison test. Scale bars: 100  $\mu\text{m}$ . All *in vitro* experiments and statistical tests were performed in at least four independent trials, with each point corresponding to a single experiment. All error bars indicate standard error of the mean.

We found single knock-down of Rac1 or Gelsolin led to a significant decrease in MAG+ cells compared with control shRNA-treated cells (Fig. 4C–E,O), indicating both are necessary for OL differentiation. On the other hand, knock-down of Daam2 resulted in an increase in MAG+ cells compared with control, further supporting its role in slowing OL differentiation (Fig. 4C,F,O). Double-LOF experiments revealed knock-down of Rac1 reversed the Daam2 LOF inhibitory phenotype, returning the proportion of MAG+ cells to similar levels as control conditions (Fig. 4C, G,O). On the other hand, double-LOF experiments for Daam2 and Gelsolin resulted in partial rescue of the Daam2 LOF phenotype to levels below control conditions (Fig. 4C, H,O). Together, these LOF experiments suggest Rac1 and Gelsolin are necessary for OL differentiation and operate downstream of Daam2 in regulating this process.

We also performed complementary overexpression experiments to further investigate these relationships. Overexpression of Rac1 did not have significant effects on the relative number of MBP+ cells compared with control conditions at 5 d postdifferentiation (Fig. 4I,J,P). On the other hand, overexpression of Gelsolin led to a higher relative number of MBP+ cells compared with control (Fig. 4I,K,P). Together with single-LOF results, these results suggest that while Rac1 is essential for the early stages of OL differentiation, relative expression levels of Rac1 may not play a large role in later stages. On the other hand, our experiments show that Gelsolin is necessary for OL differentiation and that Gelsolin levels can directly influence later stages of differentiation. While overexpression of Daam2 inhibits OL differentiation (Fig. 4I,P), overexpression of Rac1 with Daam2 was not sufficient to reverse the Daam2 phenotype at this stage (Fig. 4I,M,P). On the other hand, overexpression of Gelsolin



with Daam2 reversed Daam2's inhibition of differentiation (Fig. 4*I,N,P*), suggesting that unlike Daam2, Gelsolin acts to promote OL differentiation and operates downstream of Daam2 in modulating this process.

#### Daam2 regulates gelsolin levels in developing white matter

Given the myelin decompaction observed in P21 Daam2 cKO mice, the known role of Gelsolin in myelin wrapping, and its epistatic relationship with Daam2 in OL differentiation, we proceeded to focus on the Daam2-Gelsolin axis in our subsequent studies. We examined expression of the mature OL marker CC1 and Gelsolin in the corpus callosum of Daam2 cKO and control mice. We found that Daam2 cKO mice had higher numbers of CC1+ cells in the corpus callosum at P7 and P14 compared with control and these numbers were similar in P21 mice (Fig. 5*A–I*). We also observed a relative increase in the number of CC1+ cells expressing Gelsolin at P7 and P14 (Fig. 5*J*), but not at P21. Further, we found Gelsolin mRNA levels in the P14 corpus

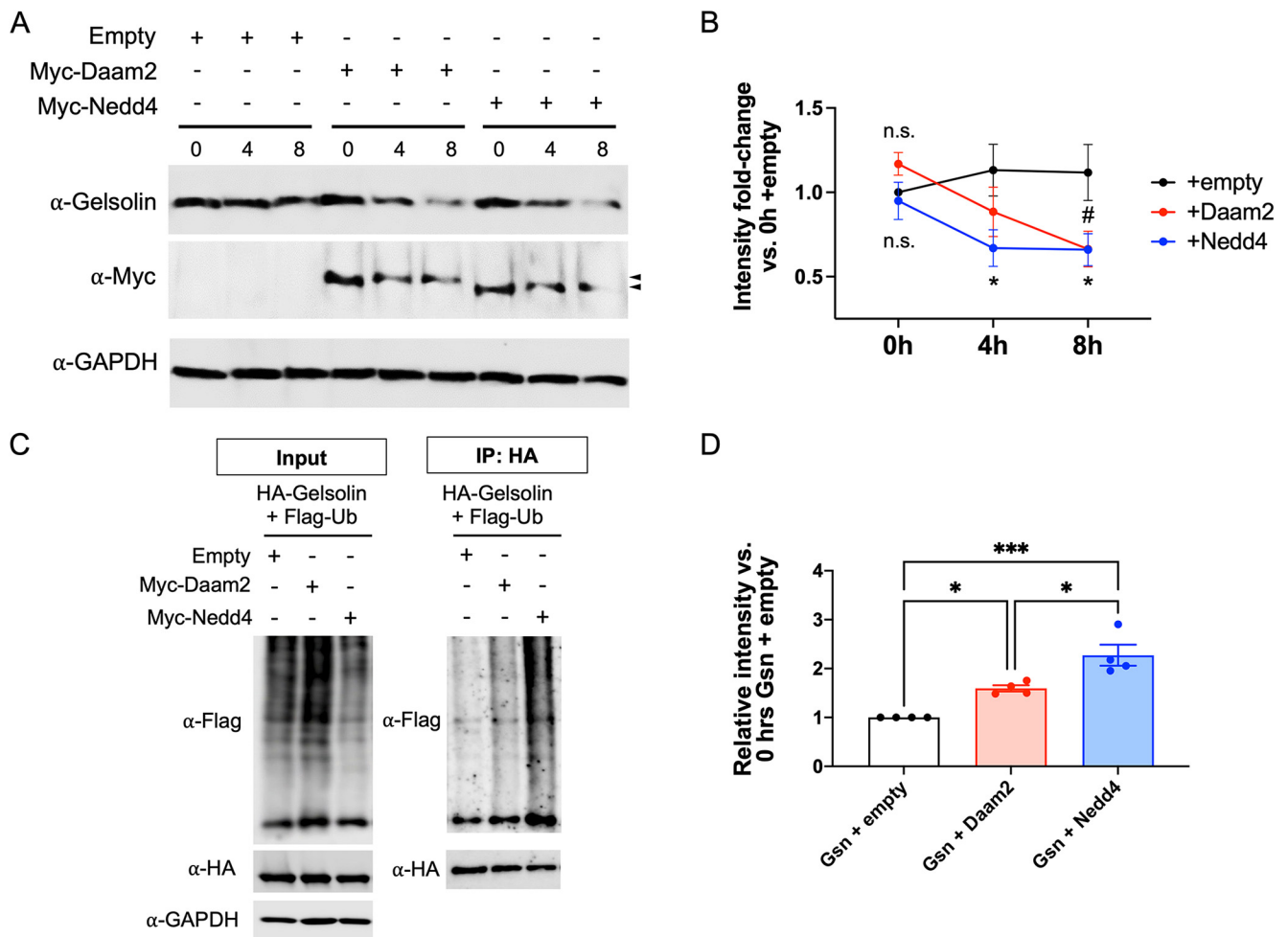
callosum remained the same on deletion of Daam2 (data not shown).

Because of the actin-severing activity of Gelsolin, we next investigated whether the upregulation of Gelsolin is associated with differences in relative ratios of F-actin and G-actin. We found Daam2 cKO mice exhibited lower F/G actin ratios in major white matter tracts relative to control mice (Fig. 5*K,L*), possibly through elevated levels of Gelsolin breaking down filamentous actin. These results suggest that the loss of Daam2 led to precocious differentiation of OLs in the corpus callosum and this effect may be because of dysregulation of Gelsolin and subsequently accelerated actin depolymerization.

#### Daam2 regulates gelsolin through the ubiquitin proteasome system (UPS)

Previous evidence has shown that Daam2 can modulate protein levels of specific targets through the UPS. Although Daam2 has no intrinsic ubiquitin ligase activity, it is known to associate with





**Figure 6.** Daam2 and Nedd4 induce Gelsolin degradation and ubiquitination. **A**, Gelsolin degradation accelerates in the presence of Daam2 or Nedd4 in primary OL cells over 8 h after cycloheximide treatment. **B**, Quantification of Gelsolin degradation in primary OLs after cycloheximide treatment. Degradation experiments were done in three independent trials, two to three replicates each; \* $p = 0.029$ ,  $p = 0.031$ , # $p = 0.0310$ , n.s. = not significant by two-way ANOVA and multiple comparisons test. **C**, Co-IP experiments show that Gelsolin ubiquitination increases in the presence of Daam2 or Nedd4 in Oli-Neu cells after 6 h of MG-132 treatment. **D**, Quantification of Gelsolin ubiquitination through co-IP. Ubiquitination experiments were done in at least four independent experiments, two to three replicates each; \* $p = 0.025$ ,  $p = 0.0125$ , \*\* $p = 0.0002$  by one-way ANOVA and multiple comparison test. All error bars indicate standard error of the mean.

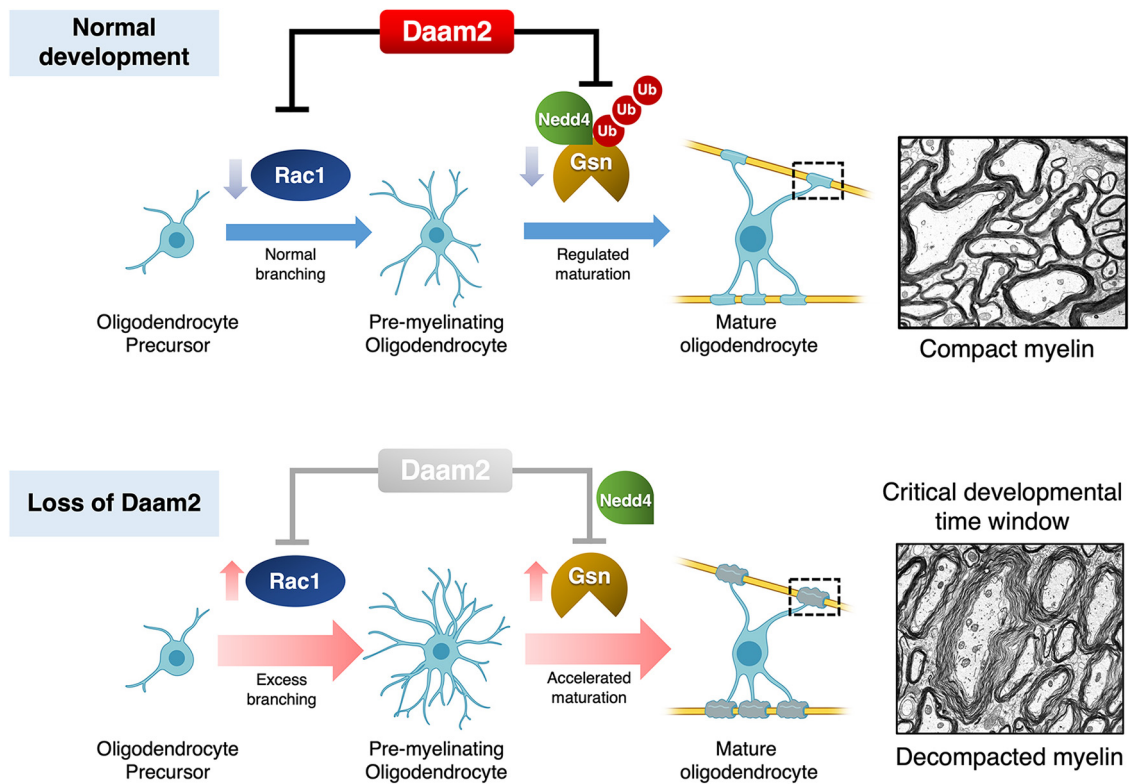
multiple UPS components and specifically regulate VHL ubiquitination in OLs through recruitment of the E3 ubiquitin ligase Nedd4 (Ding et al., 2020). In line with these observations, we sought to define whether Daam2 regulates Gelsolin levels via similar mechanisms and performed *in vitro* biochemical experiments overexpressing Gelsolin, Daam2, and Nedd4 in primary mouse OLs and Oli-Neu cells, an immortalized OL cell line. We examined the degradation rate of Gelsolin in primary OLs through the addition of cycloheximide, a small molecule that inhibits protein synthesis. In line with our *in vivo* results, we found that the presence of Daam2 led to accelerated degradation of Gelsolin over 8 h, with Nedd4 exerting a similar effect (Fig. 6A,C). We then investigated whether this phenomenon was mediated through the UPS by performing *in vitro* ubiquitination experiments in Oli-Neu cells and found Gelsolin was more highly ubiquitinated in the presence of Daam2 or Nedd4 (Fig. 6B,D). Together, these findings suggest Daam2 may recruit the E3 ligase Nedd4 to ubiquitinate Gelsolin proteins and tag them for subsequent degradation through the UPS (Fig. 7).

## Discussion

The current studies demonstrate that Daam2 is necessary for morphologic differentiation of OLs, subsequent myelin

compaction and motor learning. Specifically, Daam2 modulates the actin cytoskeleton during OL development by associating with Rac1 in early differentiation and by regulating Gelsolin levels in late differentiation. Daam2 induces the ubiquitination of Gelsolin, and this may be mediated through the recruitment of the E3 ubiquitin ligase Nedd4, leading to its subsequent degradation. While Gelsolin is required in myelin formation, our data suggest that premature upregulation of Gelsolin may lead to abnormalities in myelin sheath structure. This suggests that Daam2 plays an important role in modulating the timing of each stage of OL differentiation.

Myelin structure is crucial for proper CNS function. Decompacted myelin has been observed in human diseases such as Pelizaeus Merzbacher disease (Laukka et al., 2016) and a variety of animal models including PLP-mutant and NF1-mutant mice (Duncan and Radcliff, 2016; López-Juárez et al., 2017), leading to significant deficits in behavior and movement. Pathways involved in regulating OL morphology have also been implicated in psychiatric disorders including bipolar disease and schizophrenia (Tkachev et al., 2003; McIntosh et al., 2009; Gibson et al., 2018), and morphologic changes in OL lineage cells have been observed in experimental models of vanishing white matter disease (Lin et al., 2014),



**Figure 7.** Working model illustrating the role of Daam2 in regulating the OL cytoskeleton. Top, In normal development, Daam2 acts to regulate cytoskeletal remodeling proteins Rac1 and Gelsolin, potentially through recruitment of an unknown Rac-GAP and Nedd4, respectively. This regulation prevents excess branching and precocious maturation, leading to functional compact myelin during development. Bottom, When Daam2 is depleted, the rate of OL differentiation increases through increased Rac1 activity and higher Gelsolin protein levels. This leads to excess branching in the premyelinating stage, structural defects in myelin during a crucial period of development, and persistent motor deficits through adulthood.

multiple sclerosis (John et al., 2002), and Alzheimer's disease (Vanzulli et al., 2020). Developmental delays in myelination profoundly affect cognitive and motor function in humans (Pujol et al., 2004; Gika et al., 2010), and studies in mice have shown that defects in myelin and OL morphology early in development can result in persistent cognitive deficits (Makinodan et al., 2012; Jackson et al., 2018). Our data suggests that timely myelin compaction during development is necessary for motor function, and that Daam2 is a key modulator of this aspect in OLs.

The actin cytoskeleton plays a central role in mediating morphologic changes in OLs, but little is known to date about the upstream signaling pathways controlling actin regulation and the interplay between the cytoskeleton and gene expression changes during differentiation (Thomason et al., 2020). Notably, emerging evidence implicates OL cytoskeletal proteins as important regulators of gene expression programs at the transcriptional level, possibly through downstream mechanisms in the nucleus (Wang et al., 2008; Hernandez et al., 2016; Tsai and Casaccia, 2019). Daam2 is a known suppressor of OL differentiation via potentiation of upstream Wnt signaling (Lee and Deneen, 2012; Lee et al., 2015), but is expressed highly in mature stages of OL development (Marques et al., 2016; La Manno et al., 2021). This seemingly paradoxical role has been found for other Wnt-related proteins as well. Tcf4 is known to associate with  $\beta$ -catenin and inhibit OL differentiation (Fancy et al., 2009) while increasing in expression during myelination (Marques et al., 2016); however, its subsequent association with Kaiso and Sox10 reverses its role from a repressor into a pro-myelinating protein as the differentiation program progresses (Zhao et al., 2016). Similarly, GPR37 inhibits OL differentiation but increases in expression as myelination proceeds (Marques et al.,

2016; Yang et al., 2016). Because of the intrinsic ability of OLs to wrap around permissive substrates (Rosenberg et al., 2008; Lee et al., 2012) and their rapid differentiation process (Watkins et al., 2008; Czopka et al., 2013), a host of inhibitory signals have been found to be crucial for regulating proper myelination (Emery, 2010; Bergles and Richardson, 2015). Our study demonstrates that Daam2 plays an important role in regulating timely myelin compaction through its stage-specific modulation of key cytoskeletal proteins; however, the involvement of Wnt signaling in this process remains an open question and merits further investigation.

Formins are key regulators of the actin cytoskeleton and are traditionally viewed as downstream effectors of Rho GTPases through their GTPase binding domains (GBD; Goode and Eck, 2007). However, other mechanisms can modulate the structure and activity of formins, thereby modifying this relationship. For instance, the formin Daam1 binds to Wnt signaling components and recruits a guanine exchange factor (GEF) to induce activation of the Rho GTPase RhoA (Habas et al., 2001). This in turn generates polarized cytoskeleton remodeling through mechanisms downstream of RhoA such as ROCK kinase activation (Winter et al., 2001; Gao and Chen, 2010). Our previous work demonstrated that Rac1 operates downstream of Daam2 in activating Wnt signaling during early CNS development (Cristobal et al., 2021). The present study suggests this Daam2-Rac1 hierarchy is preserved in early OL differentiation, but that Rac1 may be negatively regulated by Daam2 in this context, possibly through recruitment of an unidentified GTPase-activating protein (GAP) inhibiting Rac1 activity. However, the exact mechanisms downstream of Daam2-Rac1 axis in OL differentiation remain to be discovered and will require further study.

As OLs reach the final stages of differentiation, they undergo massive disassembly of filamentous actin to enable lateral extension and myelin compaction (Nawaz et al., 2015; Zuchero et al., 2015). Actin-severing proteins such as Gelsolin are known to be central mediators of this process, and our data suggest Daam2 modulates Gelsolin levels to facilitate proper formation of compact myelin. Our present study shows Daam2 may recruit the E3 ubiquitin ligase Nedd4 to induce UPS-mediated degradation of Gelsolin. Higher actin depolymerization activity has not been implicated so far in myelin decompaction, and our current study offers the first evidence that precocious differentiation and higher levels of Gelsolin lead to defects in myelin compaction. Previous work has demonstrated that pharmacologically inducing actin depolymerization in the mouse spinal cord at P12 led to thicker myelin sheaths and induced myelin outfoldings, but not myelin decompaction (Zuchero et al., 2015). On the other hand, the myelin decompaction phenotype was previously observed in NF1-deficient mice (López-Juárez et al., 2017) and in mice with hyperactive HRas (Titus et al., 2017), and is thought to be because of dysregulation of the nitric oxide and Notch pathways and defects in junction protein expression and localization (López-Juárez et al., 2017; Titus et al., 2017). We hypothesize that the Daam2–Gelsolin axis may induce the decompaction phenotype through multiple pathways. MBP is known to mediate myelin compaction by localizing to the membrane and binding with membrane-bound PIP2 (Simons and Nave, 2015; Zuchero et al., 2015; Chang et al., 2016). Daam2 has been shown to promote PIP2 production at the membrane (Lee et al., 2015), and Gelsolin is known to compete with MBP for PIP2 binding (Zuchero et al., 2015). The deletion of Daam2 may act to both decrease the available levels of PIP2 and increase the competitive binding of Gelsolin, leading to mislocalization of MBP and the failure of myelin compaction. In addition, studies in epithelial cells have shown that excess actin depolymerization leads to disruption of tight junctions (Shen and Turner, 2005; Song et al., 2019), suggesting another potential mechanism by which Gelsolin dysregulation can lead to myelin decompaction.

This study demonstrates that Daam2 plays a role in regulating the actin-severing protein Gelsolin; however, the direct activity of Daam2 in actin polymerization remains unknown. As part of the formin family of proteins, Daam2 contains and Formin homology 2 (FH2) and FH3 domains (Lee and Deneen, 2012; Schneider et al., 2020); of these domains, FH2 is known to bind to effector proteins and even nucleate actin filaments directly (Liu et al., 2008; Jaiswal et al., 2013). In addition, its closest homologous protein, Daam1, cooperates with Rho GTPases (Habas et al., 2001) and actin-binding proteins such as profilin (Jaiswal et al., 2013) in a variety of contexts. Aside from Rac1 and Gelsolin, our mass-spec pulldown screen identified 10 other actin-related proteins that were also expressed in the OL lineage. ACAP2, a GAP, has been found to regulate OL development through the actin cytoskeleton (Miyamoto et al., 2014), while two candidates DNAJB6 and ALG2 have been implicated in neuromuscular disorders (Monies et al., 2014; Jonson et al., 2015; Ruggieri et al., 2016), although the mechanisms remain unclear. This suggests that apart from its previously known roles in Wnt signaling, Daam2 may directly actin nucleation and play a variety of yet unknown roles in the CNS.

Our previous work demonstrated Daam2 deletion leads to precocious OL differentiation during development. While this may be beneficial in inhibitory milieus such as those following injury or during disease progression, precocious differentiation during development is known to have deleterious effects,

including changes in cellular metabolism, depletion of progenitor cells and neurodevelopmental defects (Glickstein et al., 2009; Carosso et al., 2019). However, few studies have investigated the effects of precocious differentiation in the OL lineage specifically. Our present studies demonstrate that premature upregulation of factors which promote OL differentiation can lead to structural abnormalities and subsequent behavioral defects. The effect of these myelin abnormalities on neuronal and circuit activity of Daam2-deficient mice remain to be examined and understanding these will be crucial to gaining a clearer understanding of OLs' role in higher CNS function.

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