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The Drosophila EGF domain protein Uninflatable sets the switch between wrapping glia growth and axon wrapping instructed by Notch

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eLife Assessment

This **important** study identifies a new key factor in orchestrating the process of glial wrapping of axons in Drosophila wandering larvae. The evidence supporting the claims of the authors is **convincing** and the EM studies are of outstanding quality. After the revision, the authors have addressed most of the concerns and the manuscript has been significantly improved. Both reviewers have agreed on the significance of the work. The work will be of interest to neuroscientists working on glial cell biology.

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Abstract

In the peripheral nervous system, sensory and motor axons are generally covered by wrapping glial cell processes. This neuron-glia interaction requires an intricate coordination of glial growth and differentiation. How this is controlled molecularly is largely unknown. At the example of Drosophila larval nerves, we show that glial growth, that occurs without any cell division, is initially triggered by the FGF-receptor tyrosine kinase Heartless (Htl). In a screen for genes acting downstream of activated FGF-receptor, we identified the large membrane protein Uninflatable (Uif), which supports the growth of excessive plasma membrane domains but does not support glial axon wrapping. Uif is also known to inhibit Notch. Surprisingly, we find that Notch signaling is required in postmitotic wrapping glia. While compromised *Notch* signaling results in a reduced wrapping efficiency, gain of *Notch* activity in wrapping glia leads to a hyperwrapping phenotype. Thus, Notch signaling is both necessary and sufficient for glial wrapping in Drosophila larvae. In addition, *Notch* suppresses both *uif* and *htl* function and thus stabilizes the switch between glial growth and glial axon wrapping. Given the general conservation of signaling mechanisms controlling glia development in mice and flies, similar mechanisms may act in the mammalian nervous system to control final glial differentiation.

Introduction

The generation of the many cell types during development is governed by both intrinsic as well as extrinsic regulatory mechanisms. Extrinsic information on the status of the immediate neighbors is often conveyed by the evolutionary well conserved transmembrane receptor Notch, that plays a decisive role in many developmental contexts (Henrique and Schweisguth, 2019; Ho et al., 2020; Sachan et al., 2023; Salazar et al., 2020). In addition, individual cell types often have to undergo discrete developmental steps once they have been specified. In the nervous system,

this can be illustrated by the example of axon-wrapping glial cells. These cells must first grow to a certain size before they can begin to differentiate. How such a switch is made, is not yet well understood.

Axon wrapping glial cells are generally very large cells. In the peripheral nervous system (PNS), myelin forming Schwann cells cover large segments of a single axon. Myelin formation is directed by the axon and only large diameter axons are wrapped with myelin. Small caliber axons, in contrast, are covered by a simple glial wrap in so-called Remak fibers. Here, a single non-myelinating Schwann cell covers many axons. In the peripheral nervous system, myelin formation is initiated by a Neuregulin-dependent activation of the EGF-receptor (Michailov et al., 2004; Taveggia et al., 2005). Interestingly, *neuregulin* mutants become normally myelinated within the CNS, indicating that oligodendrocytes have evolved an independent mechanism of myelination control (Brinkmann et al., 2008). An alternative pathway that might act in oligodendrocytes is initiated by the Notch receptor, that can be activated by neuronally expressed F3/Contactin, a GPI-linked membrane protein of the Ig-domain family (Hu et al., 2003).

Within the fly nervous system two main glial cell types are associated with axons (Bittern et al., 2021; Yildirim et al., 2022). Within the CNS, the ensheathing glia establishes a barrier around the CNS neuropil and also wraps axons that connect the neuropil with the periphery (Pogodalla et al., 2021). The ensheathing glial cells are formed during embryonic development through divisions of a single glioblast that also generates all astrocytes (Jacobs et al., 1989; Peco et al., 2016). The underlying asymmetric division of the glioblast is in part controlled by *Notch* (Peco *et al*., 2016). Together with the transcription factor Pointed, Notch directs the formation of astrocytes at the expense of the ensheathing glial lineage (Gabay et al., 1996; Klämbt, 1993; Peco *et al*., 2016). In glial cells, Pointed is a target of receptor tyrosine kinase (RTK) signaling (Klaes et al., 1994; Klämbt, 1993; Peco *et al*., 2016).

Within the Drosophila PNS, axon wrapping is mediated by the so-called wrapping glia (Kottmeier et al., 2020; Matzat et al., 2015; Stork et al., 2008). It follows a similar strategy as described for vertebrate Remak fibers. Wrapping glial cells are similarly large as their vertebrate counterparts and a single cell covers more than a millimeter of axon length (Matzat *et al*., 2015). In adult stages, excessive differentiation of glial processes can be observed around large caliber motor axons which eventually leads to the establishment of myelin-like structures (Rey et al., 2023).

The development of wrapping glial cells is in part controlled by RTK signaling, that is initiated by either the EGF-receptor, the FGF-receptor, or the Discoidin domain receptor (Corty et al., 2022; Franzdóttir et al., 2009; Kottmeier *et al*., 2020; Matzat *et al*., 2015; Shishido et al., 1997; Stork et al., 2014; Wu et al., 2017). In the developing adult visual system, the FGF-receptor Heartless initially controls proliferation and migration of the wrapping glial progenitor cells, which upon contact to axons stop their migration to then grow in size and differentiate (Franzdóttir *et al*., 2009; Sieglitz et al., 2013).

Glial cell development does not only depend on RTK-signaling. In Drosophila embryos, proper migration of postmitotic, peripheral glial cells requires *Notch* activity (Edenfeld et al., 2007). Additional post-mitotic *Notch* functions have been reported in the adult Drosophila CNS. *Notch* activity is needed in olfactory neurons innervating specific glomeruli for long-term memory formation, depending on presentation of Delta by projection neurons as well as on neuronal activity (Ge et al., 2004; Kidd et al., 2015; Lieber et al., 2011; Zhang et al., 2013; Zhang et al., 2015). Moreover, since *Notch* is prominently expressed by postmitotic glial cells in the adult Drosophila CNS, it may be involved in glial differentiation (Allen et al., 2020; Davie et al., 2018; Li et al., 2022; Seugnet et al., 2011).

We have previously shown that in peripheral larval nerves, the FGF-receptor Heartless controls glial growth (Kottmeier *et al*., 2020). To gain a deeper understanding on how differentiation of wrapping glial cells is regulated, we initiated a genetic screen looking for genes that act downstream of activated *heartless*. In Drosophila, such suppressor screens have been used successfully many times (Macagno et al., 2014; Rebay et al., 2000; Therrien et al., 2000). Our

screen led to the unexpected identification of the large transmembrane protein Uninflatable (Uif), which in epithelial cells localizes to the apical plasma membrane. Loss of *uninflatable* suppresses the phenotype caused by activated RTK signaling. In addition, we found that *uif* knockdown as well as *uif* knockout larvae show impaired glial growth. In contrast, an excess of Uninflatable leads to the formation of ectopic membrane processes that, however, fail to interact with axons. *uninflatable* is also known to inhibit *Notch* (Xie et al., 2012). Indeed, we could show that canonical Notch signaling is activated in wrapping glia by the unconventional ligand Contactin, where it is required and sufficient for axon wrapping. Moreover, *Notch* counteracts both *uninflatable* as well as *heartless* function. Thus, Uninflatable acts to switch the balance between glial growth induced by RTK signaling and wrapping of axons.

Results

The FGF-receptor tyrosine kinase Heartless triggers glial growth

The development of wrapping glial cells in Drosophila depends on the activity of several receptor tyrosine kinases (Corty *et al.*, 2022; Franzdóttir *et al.*, 2009; Kottmeier *et al.*, 2020; Matzat *et al.*, 2015; Wu *et al.*, 2017). Loss of the FGF-receptor Heartless specifically in wrapping glial cells causes a reduced complexity of wrapping glial cell processes in the peripheral nervous system of third instar larvae (Kottmeier *et al.*, 2020) (Figure 1A,B). This is reflected in a reduced wrapping index which indicates the percentage of individually wrapped axons or axon fascicles (Matzat *et al.*, 2015). While in control larvae the wrapping index is around 0.18, it drops to 0.07 when *heartless* is specifically silenced in wrapping glia (Kottmeier *et al.*, 2020).

In contrast, gain of *heartless* function that is caused by expression of *λhtl* (Michelson et al., 1998) specifically in the wrapping glia, results in exuberant glial growth but does not trigger cell division (Figure 1C). Segmental nerves are swollen in an area that is demarcated by the position of the wrapping glial cell nucleus. To determine axonal wrapping we prepared third instar larvae as open book filet preparations for electron microscopic analyses. This allowed to analyze the nerve ultrastructure at positions indicated (Figure 1C). Far from the nerve bulge, only little remnants of a poorly differentiated wrapping glial cell can be detected (Figure 2A,B). In positions closer to the bulged nerve area, the wrapping glia ramifies. However, in many cases, glial processes rather grow along each other than around axons, resulting in fascicles surrounded by extensive glial cell processes (Figure 2C). These glial membrane formations become more evident as the diameter of the nerve gets larger (Figure 2D-G). In the central area of the nerve bulge, liquid-filled areas are detected with only few thin glial cell processes (Figure 2H). In conclusion, expression of activated FGF-receptor Heartless triggers glial growth but does not instruct increased wrapping of axons.

Identification of genes acting downstream of *heartless*

In order to identify genes that are responsible for this excess in glial growth, we performed a wrapping glia specific RNA interference (RNAi)-based suppressor screen in animals that concomitantly express activated Heartless and double stranded RNA directed against individual genes (see Table S1 for all genes tested). For this we generated a stable stock that allows Gal4-based expression of activated *heartless* in wrapping glia and also allows easy scoring of the wrapping glial shape [*nrv2-Gal4/CyO^{weep}*; *UAS-λhtl, repo4.3-stinger::GFP/TM6*]. Virgins collected from this stock were crossed against males carrying the different *UAS-dsRNA* elements (Table S1). The offspring third instar larvae were assayed for the presence of nerve bulges under an UV-dissecting microscope (see Materials and Methods and Figure S1 for classification scheme). *repo4.3-stinger::GFP* directs expression of nuclear GFP in all glial nuclei. Among those, the wrapping glial nucleus can be easily identified based on the large size and typical position (Figure S1).

To test the efficacy of the above screening settings, we first silenced genes known to be involved in FGF-receptor signaling (Ashton-Beaucage and Therrien, 2017; Brunner et al., 1994; Klämbt, 1993; Vincent et al., 1998). The activated Heartless receptor tyrosine kinase recruits the adaptor protein Stumps which then signals via Son of sevenless (Sos), Ras85D, members of the

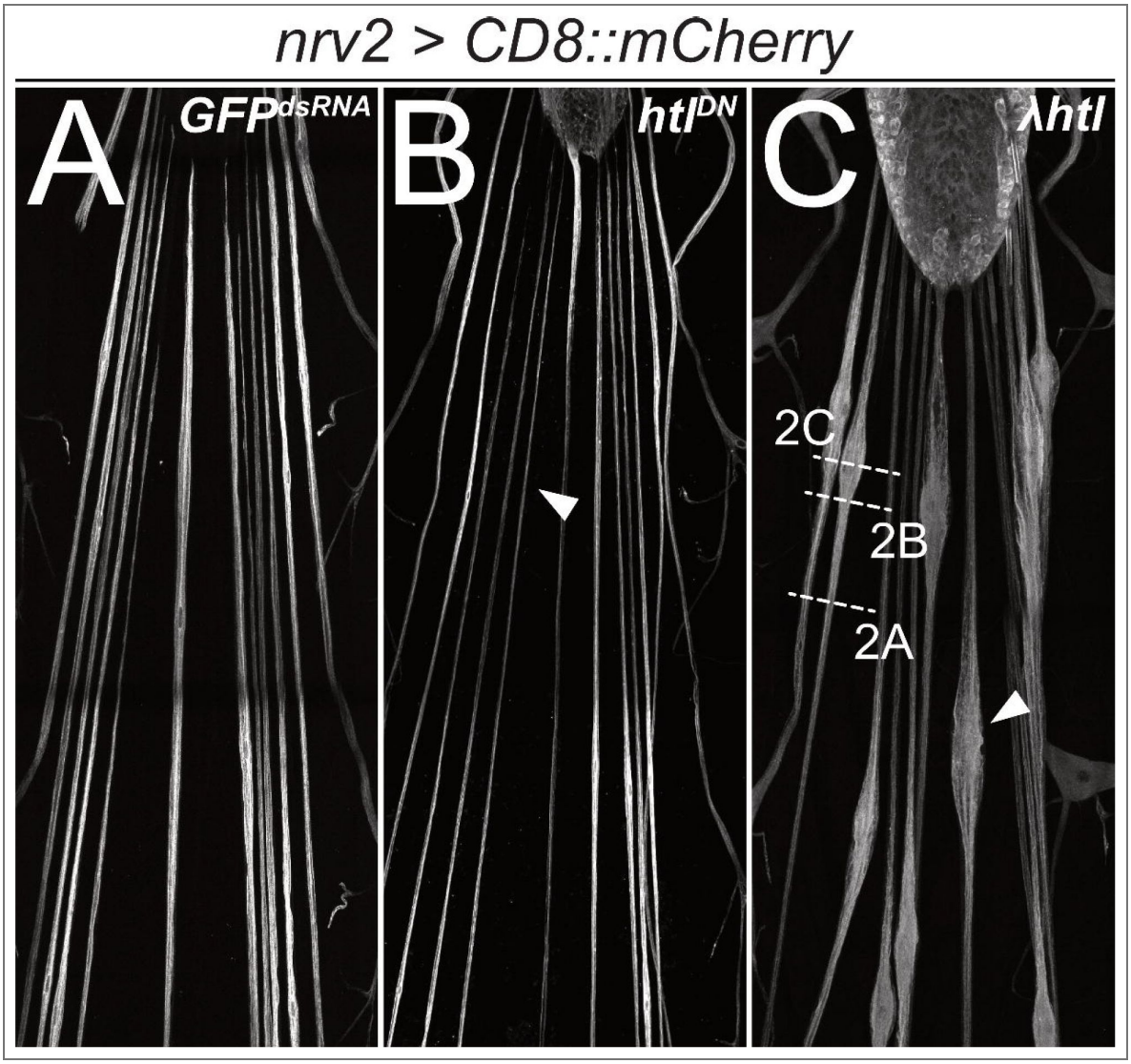

Figure 1. Heartless is required for wrapping glia development.

Confocal images of third instar larval filet preparations, stained for CD8::mCherry expression. The segmental nerves posterior to the ventral nerve cord are shown. Wrapping glial morphology is (**A**) not changed in control animals expressing (*nrv2-Gal4*) mock GFP^{dsRNA}. (**B**) Differentiation of wrapping glial cells is affected following expression of a dominant negative form of Htl and thin wrapping glial cells are detected (arrowhead). (**C**) Expression of a constitutively active form of Htl [*nrv2-Gal4; UAS-λhtl*] leads to nerve bulges around the wrapping glial nuclei (C, arrowhead). The white dashed lines indicate the level of sections shown in Figure 2.

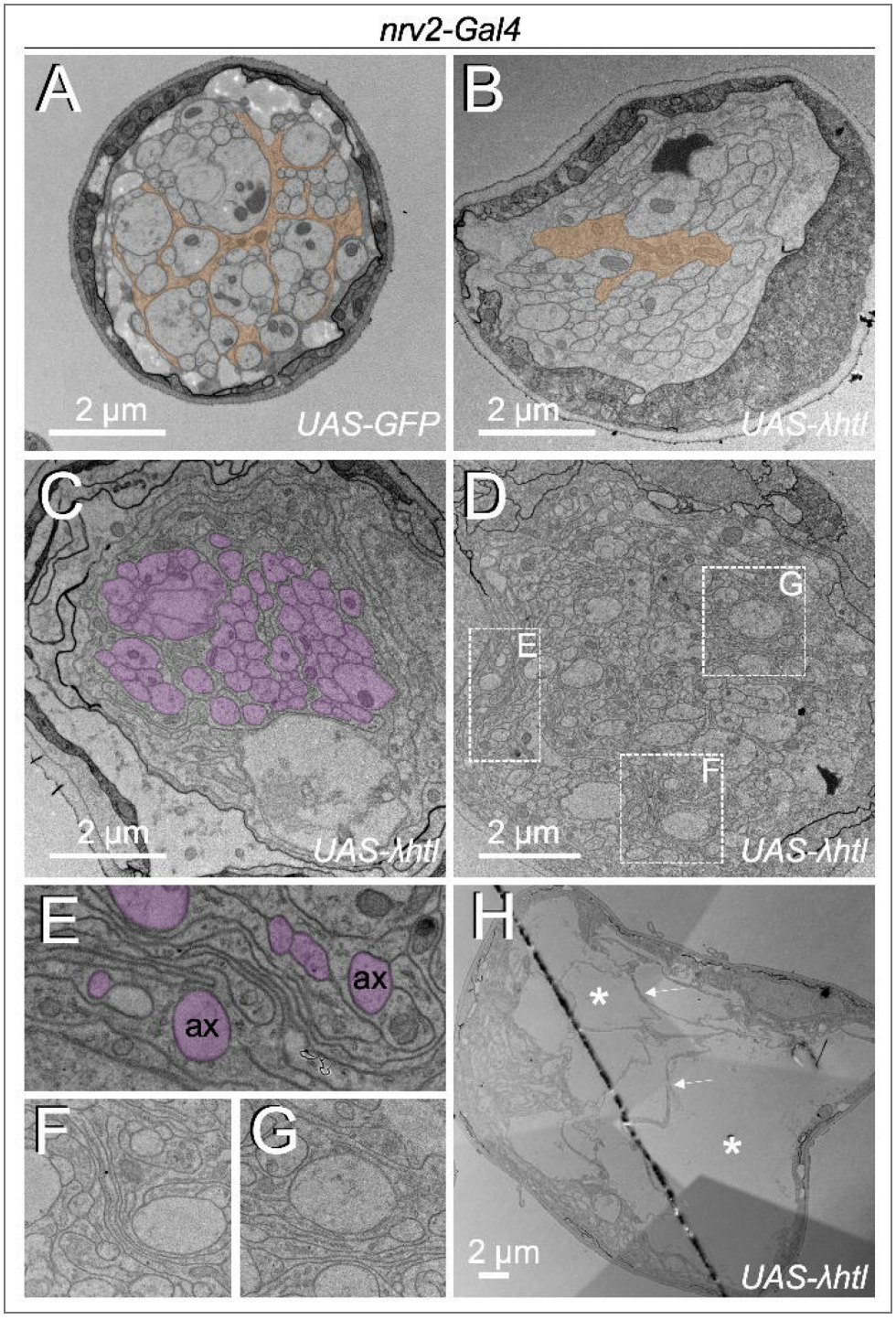

Figure 2. Heartless controls growth of wrapping glia cells.

Electron microscopic images of segmental nerves from wandering third instar larvae. (**A**) Nerve of a control larva expressing GFP in wrapping glia sectioned 150 µm posterior to the ventral nerve cord. Normally differentiated wrapping glia can be seen. (**B-H**) Nerves of larvae expressing activated Heartless in wrapping glia [*nrv2-Gal4; UAS-λhtl*] sectioned at the positions indicated in Figure 1C. (**B**) Note the poorly differentiated wrapping glial cells distant from the nerve bulge. (**C,D**) At the beginning of the nerve bulge, excessive differentiation of wrapping glial cell processes start to be detected that not always grow around axons (magenta). (**E-G**) Higher magnifications of the boxed areas in (D). Note the formation of wrapping glial cell processes that do not contact axons (ax, magenta) but rather contact glial cell processes. (**H**) In the central area of the nerve bulge, liquid-filled vacuolar structures (asterisks) can be detected. Thin wrapping glial cell processes (arrows) span the bulged area.

MAPK cascade, the MAPKKK raf, the MAPKK Downstream of raf1 (*Dsor1*), the MAPK rolled (rl), and the transcription factor Pointed that can be phosphorylated by Rl. Knockdown of all these genes, except *Raf*, either fully or almost fully rescued the nerve bulging phenotype (Table S1, Figure S1). Full rescue (classified as 4): *stumps, Ras85D, pnt*; almost full rescue (classified as 3): *Dsor1*, *rl, so*s. These findings indicate that the screening works efficiently.

We next selected 2,679 genes which we tested for their ability to suppress the *λhtl*-induced glial phenotype (Table S1). The different genes were chosen based on RNAseq data, glial expression and their involvement in major signaling pathways (Avet-Rochex et al., 2014), Petri & Klämbt, unpublished). The knockdown of 2,106 genes did not modify the nerve bulging phenotype, while knockdown of 105 genes caused a mild rescue and the knockdown of 97 genes a moderate rescue (classified as 1 or 2, respectively, see Figure S1; Table S1). Knockdown of 318 genes caused an almost complete or full rescue of the phenotype (classified as 3 or 4, respectively, see Figure S1; Table S1). Knockdown of the remaining 33 genes caused variable phenotypes or early lethality (Table S1).

We then tested additional UAS-dsRNA transgenes for the 318 genes whose knockdown efficiently rescued the nerve bulge phenotype in the initial screen. For 62 of these genes, we identified at least two independent UAS-dsRNA transgenes targeting independent regions of the mRNA that rescue the *λhtl* induced nerve bulging phenotype (Table S2). For additional 57 genes, a second but overlapping UAS-dsRNA construct was able to rescue the *λhtl* induced nerve bulging phenotype (Table S3).

The above 119 candidates are likely to act in the wrapping glia. We therefore performed wrapping glia-specific knockdown experiments using the following stock [*w*; *nrv2-Gal4/ nrv2-Gal4; nrv2-Gal4, UAS-CD8::mCherry/TM6*], where the presence of CD8::mCherry served as a proxy to determine the morphology of the wrapping glia. Indeed, knockdown of the majority of the 119 candidate genes caused wrapping glia differentiation defects (92 with phenotype, 7 no phenotype, 20 not tested, Tables S2,S3). In most cases, wrapping glial differentiation was impaired similar to what has been noted upon loss of *heartless* activity (Kottmeier *et al*., 2020). Moreover, pan-glial knockdown of most of these genes caused lethality (Tables S2, S3), further supporting the notion that we identified genes relevant for glial development. Among the group of candidate genes, most encode proteins involved in translation and protein stability (48/119), transcription and splicing (23/119), lipid metabolism and membrane dynamics (20/119). This further supports the notion that a main function of FGF-receptor signaling is to promote cellular growth of the wrapping glia.

Uninflatable is required for wrapping glial cell growth

uninflatable (*uif*) is among the 119 candidate genes that when silenced, suppresses the nerve bulging phenotype induced by activated *heartless* (Figure 3A-C). It encodes a large, single-pass transmembrane adhesion protein with eighteen EGF-like repeats in its extracellular domain that specifically localizes to the apical membrane domain of epithelial cells (Zhang and Ward, 2009) (Figure S2). Since *uif* null mutants die at late embryonic stages due to their inability to inflate their trachea properly (Zhang and Ward, 2009), we utilized CRISPR/Cas9 to generate *uif* deficient wrapping glial cells to independently verify the RNAi-based phenotype. Four different sgRNAs were generated, targeting different regions of the *uif* locus (Figure S2; see materials and methods for details). Assuming that Cas9-induced double strand breaks will generate deletions or indels that will cause the formation of a correspondingly shortened open reading frame, we anticipate that the sgRNA targeting the second exon generates an almost null situation. In contrast, the sgRNA targeting Cas9 to the presumed cleavage site $uif^{sgRNA\ CS}$ or the one targeting a sequence 5' to transmembrane domain might result in the formation of a secreted and not membrane bound Uif protein. Finally, the sgRNA that targets sequences coding for the short cytoplasmic domain might allow the formation of an almost intact Uif protein.

When Cas9 is expressed ubiquitously together with any of these four *sgRNA* constructs in *trans* to an *uif* deficiency, development is arrested during early larval stages with defects in tracheal inflation indicating the functionality of the *sgRNA* constructs ([*w; UAS-Cas9/ Df(2L)ED438; da-Gal4 /UAS-*$uif^{sgRNA\ X}$] data not shown).

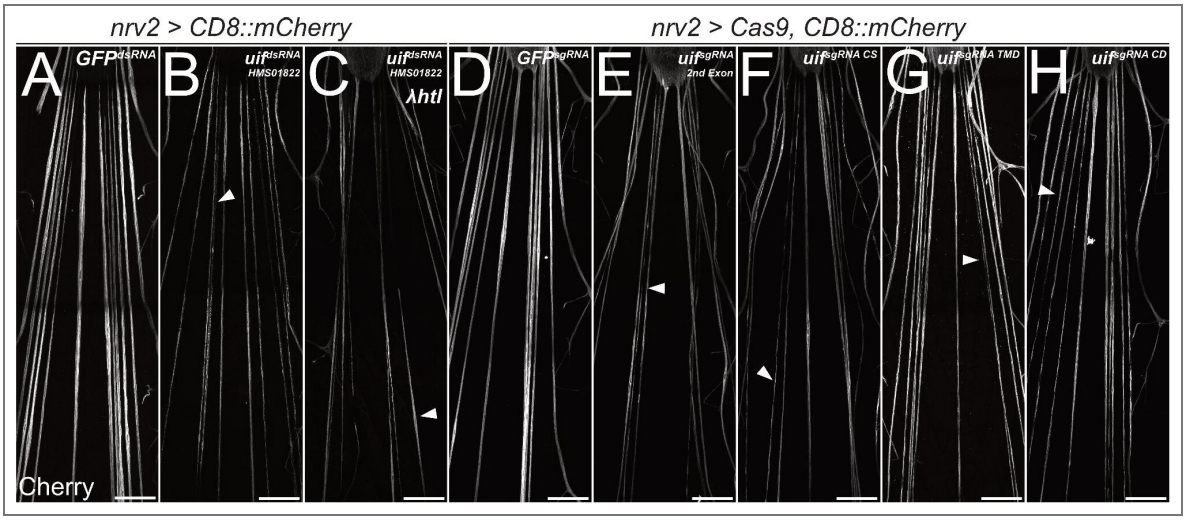

Figure 3. ***uninflatable*** **affects differentiation of wrapping glia.**

Confocal images of third instar larval filet preparations, stained for CD8::mCherry expression. The segmental nerves posterior to the ventral nerve cord are shown. (**A**) Filet preparation of a control third instar larva. (**B**) *uif* knockdown in wrapping glial cells [*nrv2-Gal4*; *UAS-uif*$^{dsRNA\text{-}HMS01822}$] impairs their development, which (**C**) cannot be rescued by co-expression of activated Htl. (**D-H**) Ubiquitous expression of (D) mock control *GFP*^{sgRNA}, (E) *uif*$^{sgRNA\ 2nd\ Exon}$, (F) *uif*$^{sgRNA\ CS}$, (G) *uif*$^{sgRNA\ TMD}$ or (H) *uif*$^{sgRNA\ CD}$ in *Cas9* expressing larvae. All *uif* sgRNAs disrupt wrapping glial cell development, wrapping glia appear thin and fragmented (arrowheads). n=5 larvae for all genotypes. Scale bars 100 µm.

When Cas9 is expressed specifically in wrapping glial cells together with any of the four *sgRNA* constructs, wrapping glial cells appear thin and patchy, similar to what we noted following silencing of *uif* expression by RNA interference ([+/+; *UAS-Cas9/+; nrv2-Gal4,UAS-CD8::mCherry/UAS-uif*$^{sgRNA\ X}$], Figure 3). These data indicate that all parts of the protein are required in wrapping glial development, as even induction of a C-terminal mutation impairs morphology.

To further analyze the poorly differentiated wrapping glial cells we initiated an electron microscopic analysis of nerves in third instar larval filet preparations. We took ultrathin sections 200 µm distal from the ventral nerve cord. As a control genotype we utilized animals that expressed double stranded RNA targeting GFP encoding mRNA in all wrapping glial cells (*UAS-GFP*^{dsRNA}). In cross sections we found severely reduced glial cell processes (Figure 4A,B). Upon knockdown of *uif*, the wrapping index drastically decreases from 0.17 to 0.03 (Figure S3, n=3 larvae, 5-9 nerves per specimen, p= 2.88x10^{-7}).

To test how *uif* affects wrapping glial development we performed overexpression studies. Gain of *uif* function in wrapping glia caused bulge formation around the wrapping glial nucleus (Figures 4C). Similar to what we had noted following expression of activated Heartless, more distal and proximal parts of the wrapping glia cell remained thin and do not fully develop (Figures 1C, 4C). Subsequent ultrastructural analysis revealed a reduced wrapping index outside of the bulge region where only little glial wrapping is observed and most axons are devoid of any glial cell contact (Figures 4D, S3A). Within the nerve bulge, an excess of wrapping glial membranes can be seen (Figure 4E,F). These processes fail to wrap individual axons which results in a significantly decreased wrapping index of 0.08 (Figure S3A, p=2.56x10^{-9}, n=3 larvae, 5-6 nerves per specimen). Interestingly, the excess glial cell processes form multilayered membrane stacks (Figure 4F). Thus, *heartless* appears to direct wrapping glial cell growth, while *uif* is needed for growth and stabilization of a specific, but still elusive, membrane compartment, possibly matching the apical domain of epithelial cells. To directly test this we stained for the distribution of PIP2 and PIP3 using specific PH-domain sensors, but in contrast to a differential distribution of PIP2 and PIP3 in ensheathing glia (Pogodalla *et al*., 2021), we found no specific localization of these membrane lipids in wrapping glia.

Notch is required for wrapping glial cell differentiation

Importantly, although *uif* is needed for wrapping glial cell growth, it is not sufficient to instruct the wrapping of axons. Thus, Uif might define the wrapping glial cell interface required to wrap axons, and Uif interacting proteins organize subsequent wrapping. One of these interacting proteins is Notch. Uif can bind Notch and antagonizes the canonical Notch signaling pathway (Loubéry et al., 2014; Xie *et al*., 2012). Likewise, it has been reported that *Notch* negatively regulates *uif* transcription (Djiane et al., 2013). Thus, the interaction of Uif and Notch might set a switch triggering glia-axon interaction leading to axon wrapping.

To determine a possible role of *Notch* signaling during wrapping glia differentiation, we first silenced *Notch* expression by RNAi in wrapping glia of otherwise normal animals. Knockdown of *Notch* expression using three different dsRNA constructs targeting different sequences of the *Notch* mRNA specifically in wrapping glia resulted in the appearance of thin wrapping glial cells in larval filet preparations (Figure 5A-C). A similar phenotype was also induced by removing *Notch* expression using conditional CRISPR/Cas9-based knockout (Figure 5D,E) or in mutant *Notch*^{ts1} animals (Shellenbarger and Mohler, 1975) that were kept at the restrictive temperature of 29°C during larval stages, only (Figure 5F,G). In contrast, upon expression of activated *Notch* (*N*^{ICD}) no significant changes of wrapping glial morphology could be detected using the confocal microscope (Figure S4).

Notch instructs axonal wrapping

To analyze the role of *Notch* signaling for wrapping glial differentiation in more detail, we performed an electron microscopic analysis. Upon expression of *Notch*^{dsRNA} the wrapping index drops significantly to 0.12 (Figure 6A,B,D, p=0.00079; n=5, larvae with 7-9 nerves per specimen).

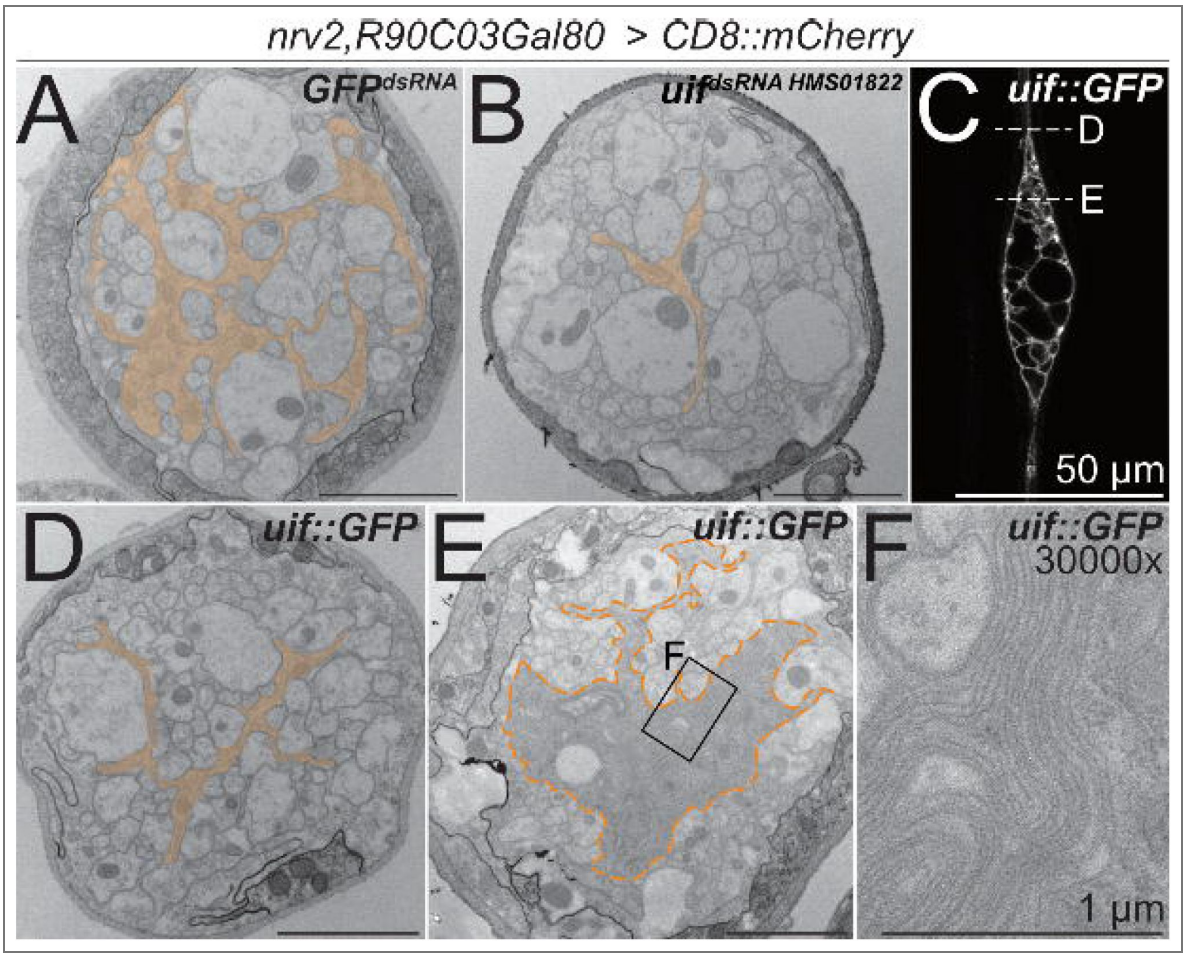

Figure 4. ***uif*** **affects axonal ensheathment of wrapping glia.**

(**A+B,D-F**) Electron microscopic cross sections of third instar larval abdominal peripheral nerves with wrapping glia specific expression [*nrv2-Gal4; R90C03-Gal80*] of (**A**) GFP^{dsRNA} mock control transgene, (**B**) $uif^{dsRNA\text{-}HMS01822}$. Upon knockdown of *uif*, wrapping glial cell complexity is reduced. (**C**) Single plane of a confocal image of a third instar larval nerve stained for CD8::mCherry expression. Expression of *uif::GFP* in wrapping glial cells causes bulge formation, while outside the bulge wrapping glia appear thin (arrowheads). The dashed lines indicate the plane of section in relation to the bulge of the images shown in (D,E). (**D**) Upon *uif::GFP* overexpression specifically in wrapping glia, glial morphology is reduced outside the bulge region. (**E**) Within the nerve bulge, wrapping glial membrane increases in size while most axons lack proper wrapping. (**F**) Close up of region indicated in (E). GFP^{dsRNA} n=4 larvae, 4-7 nerves per specimen; uif^{dsRNA} n=3 larvae, 5-9 nerves per specimen; *uif::GFP* n=3 larvae, 5-6 nerves per specimen. Scale bars 2 µm unless indicated otherwise.

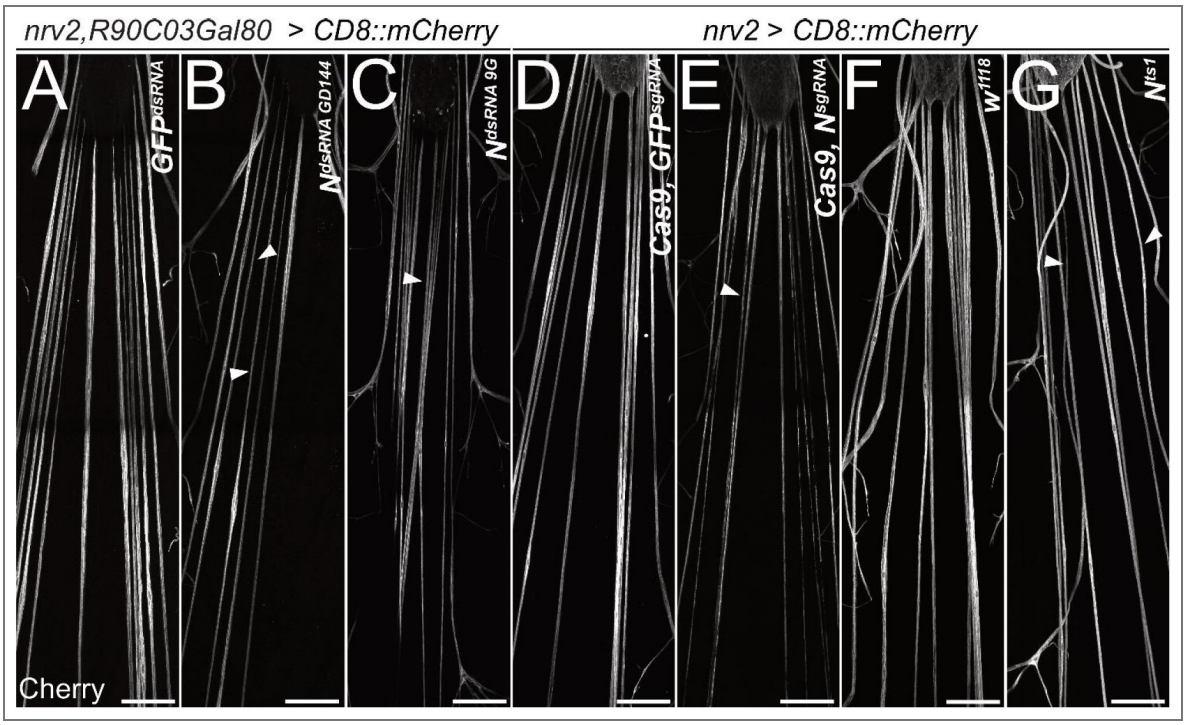

Figure 5. ***Notch*** **is required for wrapping glial development.**

Filet preparations of wandering third instar larvae stained for CD8::mCherry expression. The segmental nerves just posterior to the ventral nerve cord are shown. (**A**) Control larvae expressing the mock control GFP^{dsRNA} in wrapping glial cells specifically [*nrv2-Gal4; R90C03-Gal80*]. Upon expression of dsRNA targeting *Notch* mRNAs [(**B**) N^{GD144}, (**C**) N^{9G}. (**D**) Control larva expressing sgRNA directed against the GFP open reading frame in [*nrv2-Gal4; UAS-Cas9*]. (**E**) Conditional knockout of *Notch* leads to dramatically altered morphology of wrapping glial cells. (**F**) Control larva cultured at the same temperature regime as the N^{ts1} larva shown in (**G**). Wrapping glial cells appear smaller compared to the control. Scale bars are 100 µm.

In contrast, upon expression of the active form of *Notch*, $Notch^{ICD}$, we observed a dramatic and significant increase of the wrapping index to 0.28 (Figure 6C,D, p=0.014, n=5 larvae with 3-8 nerves per specimen). This indicates that the wrapping glia more efficiently enwraps axons compared to control larvae. Importantly, overexpression of *Notch* does not cause any bulge formation along the nerves as it is noted upon expression of the activated receptor tyrosine kinase Heartless (Figure 1C).

In summary, these data clearly demonstrate that *Notch* is not only required for development of wrapping glial cells but it is also sufficient to guide axon wrapping as gain of *Notch* function triggers extensive formation of glial processes resulting in a hyperwrapping phenotype.

Notch signaling is active in some adult wrapping glial cells

The above data suggest that *Notch* is expressed by differentiated wrapping glia in the PNS. To directly test this, we utilized a CRIMIC-based transposon insertion into the first coding intron of the *Notch* locus ($Notch^{CR00429\text{-}TG4.1}$) that carries a Trojan Gal4 element, which allows GAL4 expression under control of the endogenous *Notch* promoter (Diao et al., 2015; Lee et al., 2018). However, no Gal4 activity is associated with the $Notch^{CR00429\text{-}TG4.1}$ insertion. To alternatively detect *Notch* activity we utilized the common *Notch* activity reporter, *GbeSu(H)-lacZ*, where 3 copies of the Grainy head (Grh) protein binding element (Gbe) and 2 Suppressor of Hairless (Su(H)) protein binding sites drive *lacZ* reporter expression (Furriols and Bray, 2001). In larvae, *Notch* reporter activity was detected in neuroblasts of the brain lobes and the thoracic neuromeres but not in peripheral wrapping glia (Figure S5A-C). In adults, however, *Notch* activity could be detected in ensheathing glial cells as well as in peripheral wrapping glial cells (Figure S5D-F).

The above-mentioned data do not demonstrate whether canonical *Notch* signaling acts in larval wrapping glia. We therefore suppressed expression of the gene *Su(H)*, which encodes a transcription factor critically required for *Notch* dependent gene expression, specifically in wrapping glia. In such knockdown larvae, overall wrapping glial morphology appeared impaired when analyzed using a confocal microscope (Figure S6A,B). In an electron microscopic analysis, we could observe a clear reduction in the wrapping index (Figure 7A,B, Figure S3A, WI=0.075, $p=4.21x10^{-11}$, n=4 larvae with 6-8 nerves per specimen). This finding indicates an involvement of *Su(H)* in wrapping glia development. Moreover, when we silenced the gene *mastermind* (*mam*), that encodes a further transcription factor involved in *Notch* signaling (Henrique and Schweisguth, 2019), we noted a severe impairment of glial morphology at the confocal microscope. Wrapping glial cells accompanying the abdominal nerves appeared thin and not well differentiated (Figure S6C). Similarly, when looking at electron microscopic images, we noted a dramatic reduction in the wrapping index with a corresponding loss of glial complexity (Figure 7C, Figure S3, WI=0.07, $p=4.29x10^{-9}$,n=3 larvae with 5-9 nerves per specimen). In conclusion, these data indicate that canonical Notch signaling acts within larval wrapping glial cells to guide engulfment of axons.

Notch activation is not mediated by canonical ligands

Notch is generally activated by the transmembrane EGF-domain proteins encoded by *Delta* or *Serrate*. Both ligands, like the Notch receptor, are evolutionarily well conserved. To determine the expression of both genes we again utilized insertion of Trojan Gal4 elements in either Delta or Serrate. While Serrate does not appear to be expressed in neurons, we noted some expression of Delta in peripheral sensory neurons and in very few CNS neurons (Figure S7A,B). We then performed RNAi based knockdown experiments specifically in neurons using the nSyb-Gal4 driver and found no effect on wrapping glial morphology at the light microscopic and the electron microscopic level (Figure S7C-E, data not shown). These results were confirmed by a second dsRNA construct as well as by sgRNA / Cas9 mediated, cell type specific knockout experiments (Figure S8). In summary, these data suggest that during larval development of the peripheral wrapping glia, *Notch* is neither activated by Delta nor by Serrate.

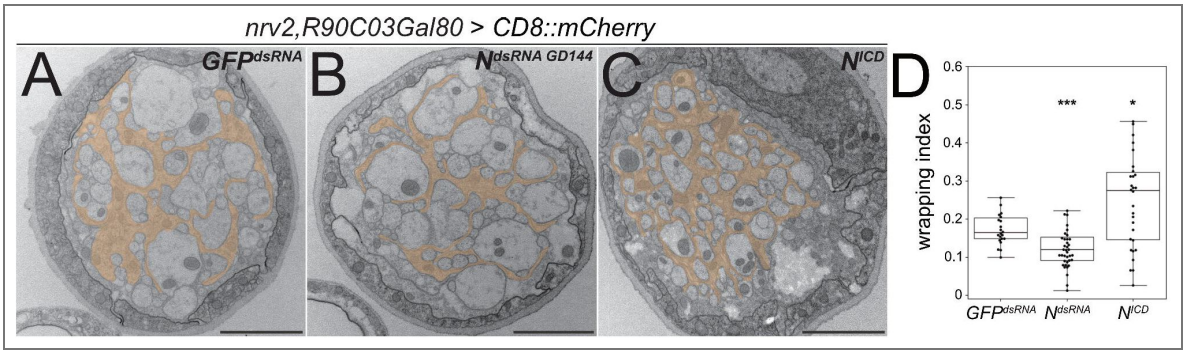

Figure 6. *Notch* function affects axonal wrapping.

Electron microscopic images of segmental nerves from wandering third instar larvae sectioned 100 µm posterior to the ventral nerve cord. (**A**) Control nerve of an animal expressing *dsRNA* directed against GFP [*nrv2-Gal4; R90C03-Gal80*]. Axons are wrapped by processes of the wrapping glia. The entire nerve is engulfed by the perineurial glia and the subperineurial glia. For quantification of glial wrapping see (D). (**B**) Upon expression of *dsRNA* targeting *Notch mRNA* (N^{GD144}) glial wrapping is reduced. (**C**) Upon expression of an activated form of Notch (N^{intra}) glial wrapping is increased. (**D**) Quantification of Wrapping Index (WI). While *Notch* knockdown significantly decreases the WI (from 0.17 to 0.12, p = 0.00079), activation of Notch signaling significantly increases glial wrapping and the WI (from 0.17 to 0.27, p = 0.014). For statistical analysis a t-test was performed for normally distributed data (Shapiro-test), Mann-Whitney-U test was performed for not normally distributed data. GFP^{dsRNA} n=4 larvae with 4-7 nerves each; N^{dsRNA} n=5, larvae with 7-9 nerves each; N^{ICD} n=5 larvae with 3-8 nerves each. α=0.05, * p ≤ 0.05, *** p ≤ 0.001. Scale bars 2 µm.

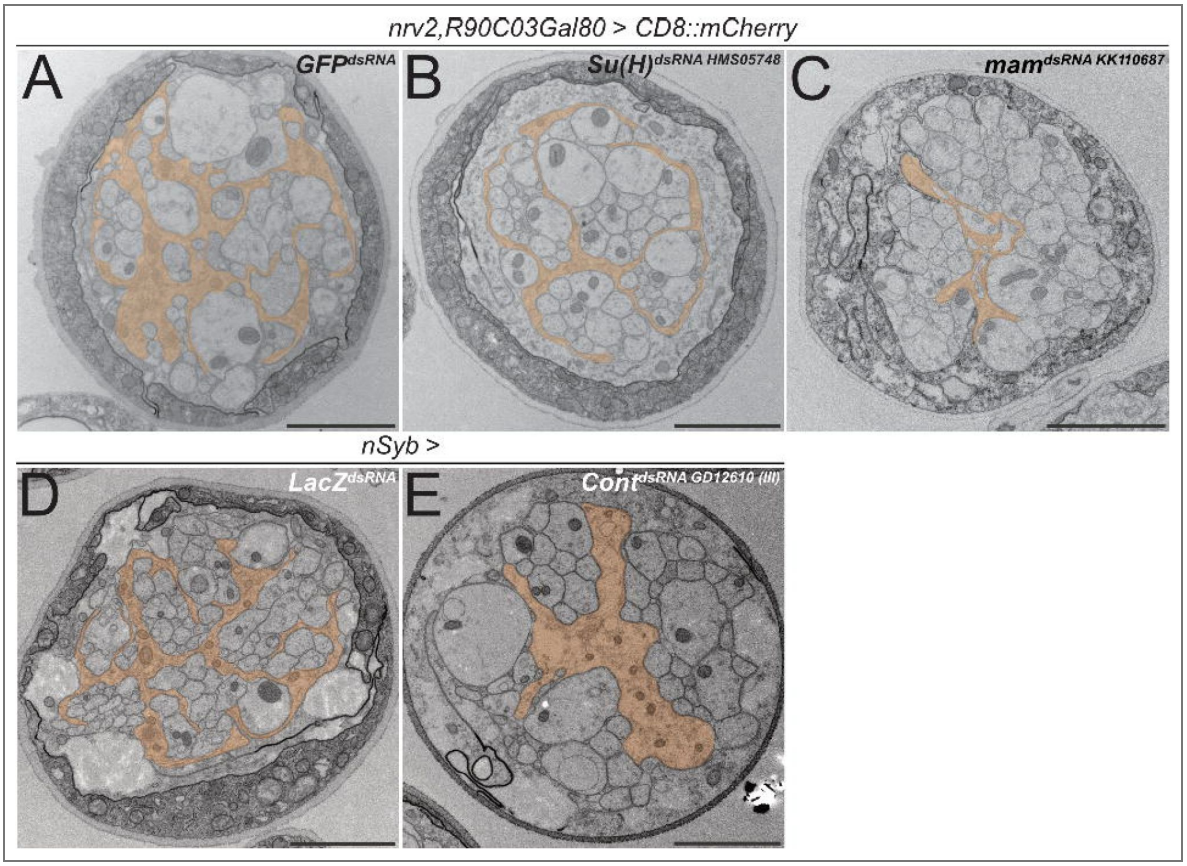

Figure 7. **Knockdown of *mam, Su(H)* and contactin impairs axonal wrapping.**

Electron microscopic cross sections of third instar larval abdominal peripheral nerves. Glial cell morphology is indicated in orange color. (**A-C**) Wrapping glia specific [*nrv2-Gal4; R90C03-Gal80*] expression of GFP^{dsRNA} as mock control, (B) *Suppressor of Hairless* (*Su(H)*) $dsRNA^{HMS05748}$ or (C) *mastermind* (*mam*) $dsRNA^{KK110687}$. Note reduced complexity of glial cell processes. (**D**) Neuron specific [*nSyb-Gal4*] expression of $LacZ^{dsRNA}$ as mock control and (**E**) *Contactin* (*Cont*) $dsRNA^{GD12610}$ inserted on the third Chromosome. Upon neuronal knockdown of *Cont*, glial wrapping of peripheral axons is impaired. Scale bars 2 µm.

In mice, F3/Contactin, a GPI-linked member of the Ig-domain superfamily, activates Notch during oligodendrocyte differentiation (Hu *et al*., 2003). F3/Contactin is well conserved during evolution and a homolog is encoded in the Drosophila genome. Drosophila Contactin binds Neuroglian and is an essential component of septate junctions which establish the occluding junctions in all epithelial cells and the glial blood-brain barrier (Faivre-Sarrailh et al., 2004; Izumi and Furuse, 2014; Peles and Salzer, 2000). In addition, single cell sequencing data indicate that *Contactin* is also expressed by neurons (Davie *et al*., 2018). We therefore silenced *Contactin* expression in all neurons using the neuron specific driver *nSyb-Gal4*. Such larvae survive and in third instar stage, a significant reduction in the wrapping index from 0.15 to 0.11 can be noted (Figure 7D,E, Figure S3B, p=0,006, n=5 larvae with 10 nerves per specimen for both RNAi lines). Wrapping glia morphology is impaired similar to what is noted upon knockdown of *Notch* and its downstream signaling components *Su(H)* and *mam* (Figures 6,7). This suggests that the non-canonical ligand Contactin acts in both flies and mammals to actively control wrapping glial differentiation and, moreover, indicates that additional ligands may exist to achieve full Notch activation.

Notch suppresses *heartless* and *uninflatable* during glial development

To further test whether Notch instructs glial axon wrapping downstream of *heartless* and *uninflatable*, we conducted epistasis experiments. Gain of *uif* function results in nerve bulges (Figure 4C). When we concomitantly silenced *Notch* using RNA interference, the nerve bulging phenotype is not changed (Figure 8A,B). However, when we co-expressed the intracellular domain of Notch, *UAS-N^{ICD}*, resembling the activated form of Notch, the bulging phenotype is rescued (Figure 8A,C). These data suggest that similar to the developing wing (Xie *et al*., 2012), Uninflatable suppresses Notch function.

Expression of a constitutively active FGF-receptor, *λhtl*, in wrapping glial cells results in prominent nerve bulging (Figure 8D). Upon co-expression of *Notch* dsRNA, the size of the *λhtl*-induced nerve bulges increases (Figure 8E). A similar enhancement of the bulging phenotype was noted when using the temperature-sensitive *Notch^{ts1}* allele and an appropriate temperature regime (Figure 8F). In contrast when we co-expressed N^{ICD} we noted a suppression of the severity of the nerve bulging phenotype (Figure 8G). Thus, *Notch* function appears to counteract both *uif* and *htl* during glial development.

Model underlying wrapping glia development

We conclude the following model underlying wrapping glia development in the Drosophila PNS (Figure 9). Initially, the activity of the FGF-receptor Heartless triggers wrapping glial cell growth. The large transmembrane protein Uif is present in the wrapping glia and is needed to stabilize the formation of a specific membrane domain capable to interact with axons. In addition, Uif suppresses Notch to inhibit precocious axon wrapping. As Heartless negatively regulates expression of Uif (Avet-Rochex *et al*., 2014), Notch becomes more and more active which also contributes to the silencing of *uif* and *htl*. This then sets the switch to initiate axon wrapping by the wrapping glia (Figure 9).

Discussion

Here we present a model underlying cell growth and subsequent wrapping of axons by the wrapping glia in the larval PNS of Drosophila. In a first step, wrapping glia shows an enormous increase in its size, which is regulated by the receptor tyrosine kinase Heartless. The large transmembrane protein Uninflatable, which harbors an array of EGF-domains in its extracellular domain, is needed to form excessive membrane domains capable of eventually wrapping axons. The last process is controlled by Notch whose activation then orchestrates axon-glia interaction.

Wrapping glial development is initiated by the activation of receptor tyrosine kinases (RTK) including the FGF-receptor Heartless (Htl). In perineurial glia, RTK activity will trigger cell division, whereas in the remaining glial cells RTK activity is used to support cell growth to

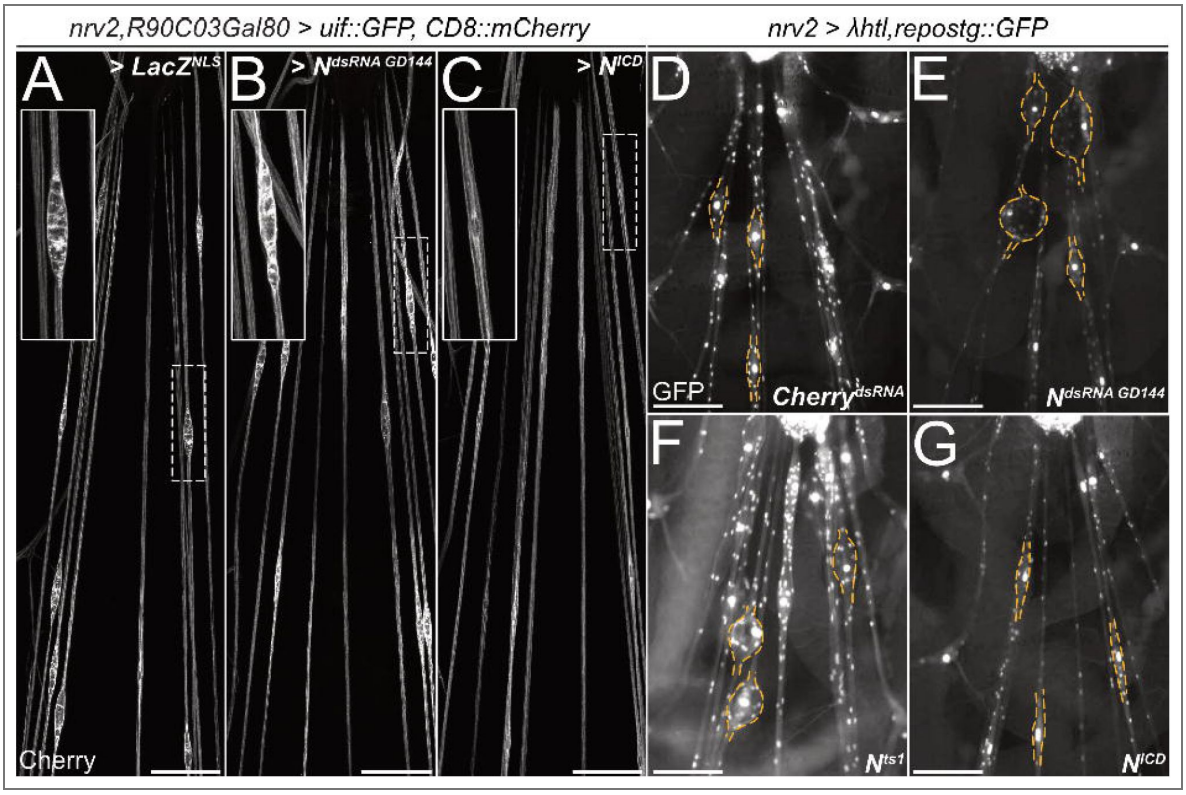

Figure 8. ***Notch*** **counteracts** ***heartless*** **and** ***uninflatable*** **function.**

Squeeze preparations of wandering third instar larvae. The segmental nerves posterior to the ventral nerve cord are shown. (**A-C**) Larvae expressing *uninflatable* and CD8::mCherry in the wrapping glia. The nerve bulge shown in the white dashed box is enlarged. (A) Control larvae co-expressing *LacZ*. Uif expression results in nerve bulges. (B) Upon co-expression of $Notch^{dsRNA}$ (*GD144*) the nerve bulging phenotype is not suppressed. (C) Upon activation of Notch signaling by expression of N^{ICD}, the nerve bulging phenotype is suppressed. (**D-G**) Larvae expressing an activated Heartless receptor (*λhtl*) in the wrapping glia [*nrv2-Gal4*] carrying a *repo3.4-stinger::GFP* element leading to a panglial nuclear GFP expression. (D) Control larvae co-expressing *dsRNA* directed against *mCherry*. Note the prominent nerve bulges. (E) Upon co-expression of $Notch^{dsRNA}$ the nerve bulging phenotype is enhanced. (F) Likewise, the nerve bulging phenotype is enhanced in a $Notch^{ts1}$ mutant background when the larvae are kept at the restrictive temperature. (G) Upon activation of Notch signaling by expression of N^{ICD}, the nerve bulging phenotype is significantly rescued. n=7 larvae for all genotypes.

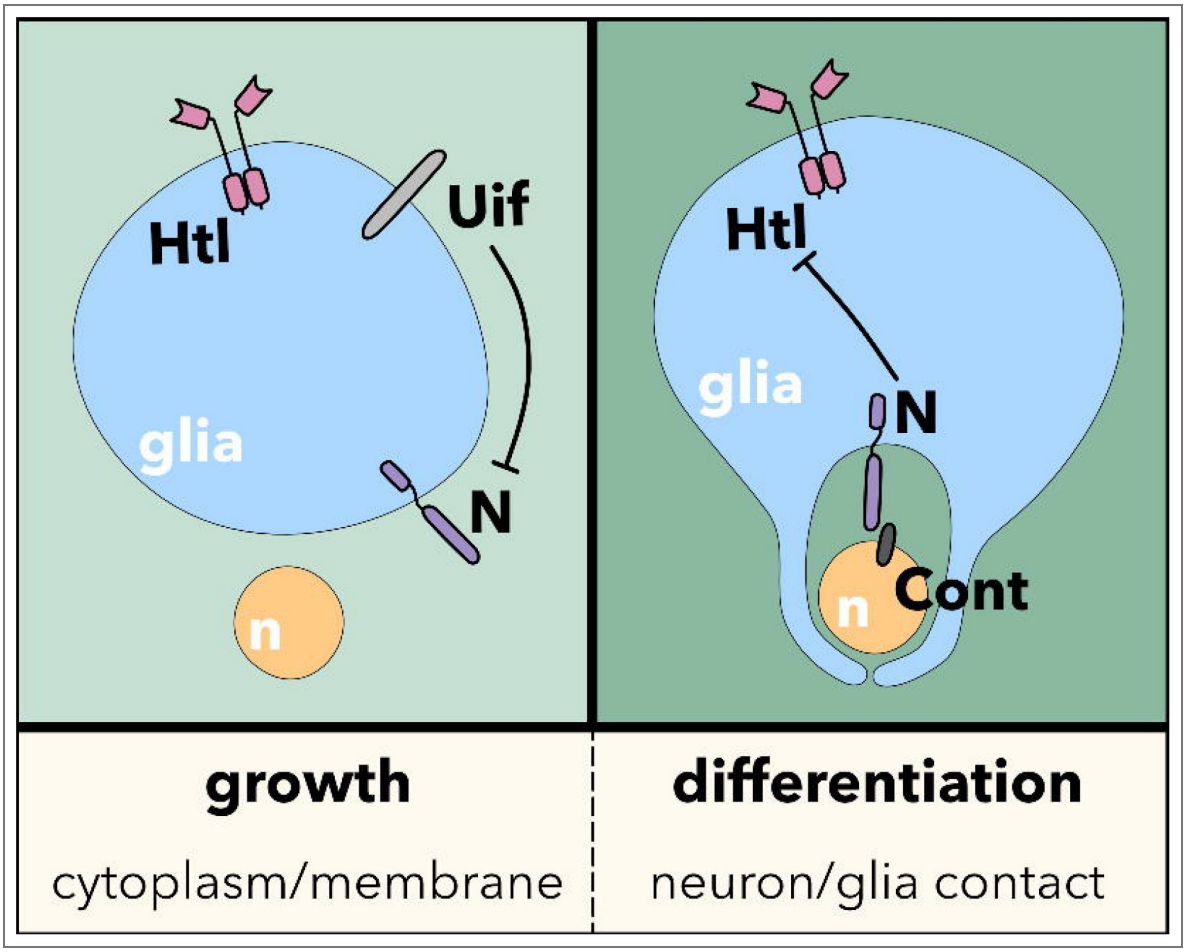

Figure 9. **Model**

The interplay of FGF-receptor, Uninflatable and Notch signaling controls the switch between glial cell growth and glial cell differentiation leading to extensive neuron-glia contact. For details please see the text.

subsequently allow differentiation (Avet-Rochex et al., 2012; Avet-Rochex *et al*., 2014; Franzdóttir *et al*., 2009; Read et al., 2009; Stork *et al*., 2014; Witte et al., 2009; Wu *et al*., 2017). This dual function of RTK signaling in controlling either proliferation, cell growth or differentiation is accompanied by a switch in activating ligands as well as the expression of additional regulator proteins that modulate RTK signaling strength (Franzdóttir *et al*., 2009; Ohm et al., 2024; Sieglitz *et al*., 2013).

Wrapping glial cells eventually have to cover all axons in the peripheral nerves to prevent degeneration of sensory axons (Kautzmann et al., 2025). In Drosophila larvae, wrapping glial cells can reach 2 mm in length and thus, have an enormous size (Matzat *et al*., 2015). To achieve this, they block mitosis and instead undergo endoreplication (Unhavaithaya and Orr-Weaver, 2012; Von Stetina et al., 2018; Zülbahar et al., 2018). Here we found that in larval peripheral wrapping glia, Htl supports this cell growth. This is corroborated by our suppressor screen, which identified many genes affecting translation. Similarly, we identified several genes that control metabolite transport across the wrapping glial membrane. Four of these genes are predicted to be involved in sugar/carbohydrate transport (*CG3409*, *CG4797*, *CG5078* and *CG6901*).

Initially, wrapping glial cells form only very thin processes that in peripheral nerves follow the axon bundles (Matzat *et al*., 2015). Starting from second instar larval stages onwards, glial cells extend processes that wrap around axon fascicles or single axons (Kautzmann *et al*., 2025; Matzat *et al*., 2015). This process requires the formation of membranes dedicated to axon-glia contact. Possibly, this is mediated by the large transmembrane protein Uninflatable that in epithelial cells is found specifically at the apical plasma membrane and in tracheal cells also can affect polyploidy (Zhang and Ward, 2009; Zhou et al., 2020).

Using existing antibodies (Zhang and Ward, 2009), Uninflatable cannot be detected in third instar larval nerves. Moreover, recent microarray data indicate that activated Heartless suppresses *uif* transcription (Avet-Rochex *et al*., 2014), which suggests that the amount of Uif present in wrapping glia decreases during larval development. Notably, Uif not only induces the generation of numerous membrane sheets, but it can also bind and inhibit Notch function (Loubéry *et al*., 2014; Xie *et al*., 2012). Thus, the inhibition of Notch by Uif is expected to gradually diminish during larval development. This antagonistic action of Uif will stabilize the switch from a glial cell growth phase to a subsequent phase with pronounced axon-glia interaction leading to axon wrapping.

During axon wrapping, Notch likely acts via its canonical signaling cascade. Although mutant analysis and RNAi-based knockdown studies clearly indicate a role of *Notch* in wrapping glia differentiation, we could detect Notch activity only in a subset of adult glial cells. This may be due to the fact that these glial cells form myelin-like structures (Rey *et al*., 2023) and thus require a different level of Notch activity.

Postmitotic functions of Notch have been reported in several instances. In the Drosophila nervous system, *Notch* is expressed strongly in axon-associated, postmitotic glial cells (Allen *et al*., 2020; Davie *et al*., 2018; Li *et al*., 2022; Seugnet *et al*., 2011). During embryogenesis, Notch is needed for the exact positioning of glial cells as they migrate along peripheral nerves (Edenfeld *et al*., 2007). Here we show that in larval wrapping glia *Notch* instructs axon wrapping. Thus, Notch promotes neuron-glia contact.

How could Notch signaling regulate axon-glia adhesion? The tight interaction of axons and glial cells calls for highly regulated adhesion of the two cell types. The transmembrane proteins of the Ig-domain superfamily Borderless and Turtle bind each other and mediate the differentiation of the wrapping glia in the developing eye (Cameron et al., 2013; Cameron et al., 2016; Chen et al., 2017). While Turtle is expressed on photoreceptor axons, Borderless is found on the ensheathing glia. Interestingly transcriptomic studies have indicated that in the developing eye, Borderless is downstream of Notch (Nfonsam et al., 2012).

Given our finding that Notch signaling is acting during axonal engulfment by the wrapping glia, we asked whether one of the canonical Notch ligands, Delta and Serrate (Henrique and Schweisguth, 2019), is responsible for Notch activation. However, neither Delta nor Serrate are

broadly expressed in neurons, and moreover, neuronal knockdown of neither gene did result in a glial wrapping phenotype. In addition to these canonical ligands, the GPI-linked F3/Contactin protein has been suggested to activate Notch during myelin formation in oligodendrocytes (Hu *et al*., 2003). Here, Notch activation leads to a gamma-secretase-dependent nuclear translocation of $Notch^{ICD}$ to upregulate expression of the myelin-related protein MAG and promote myelination. Quite similar, also in Drosophila, knockdown of *contactin* specifically in neurons causes a prominent defect in wrapping glial cell morphology. This surprising evolutionary conservation of the molecular control underlying the differentiation of wrapping glia suggests that Drosophila models may be useful to gain a deeper understanding of myelin biology.

STAR Methods

Experimental model and study participant details

All *Drosophila* work was conducted according to standard procedures. All fly stocks were kept at room temperature in plastic vials containing *Drosophila* standard food and dry yeast. Crosses were set up with male and virgin female flies in a ratio of 1:3 and kept at 25 °C. Induction of N^{ts1} allele was performed by placing Stage 16 embryos at 29°C.

Method details

sgRNA generation

To generate flies carrying sgRNAs targeted to different regions of the *uif* gene, sgRNA sequences specifically designed for the target gene region of interest were integrated into the pUAST-dU63gRNA vector carrying a ubiquitous U6:3 promoter. To do so, sense and anti-sense oligonucleotides containing the respective sgRNA template sequence ($uif^{2ndExon}$: TTCAATATCAAGCACTCGT; uif^{CS}: TGTTCTGCGTACCTCGGTAG; uif^{TMD}: CGCTGTGTGGGCTCCTTTAC; uif^{CD}: CTACAATGAAACGTACATGA) were phosphorylated, annealed and ligated into the vector. Flies were tested via single-fly PCR. The position of the different guide RNAs is indicated (Figure S2).

Heartless modifier screen

To test whether candidate genes can be linked to fibroblast growth factor (FGF) receptor signaling in wrapping glia, we utilized the nerve bulging phenotype caused by expression of a constitutively active Heartless (*UAS-λhtl*) in wrapping glia (*nrv2-Gal4*). The *repo4.3-stinger::GFP* reporter line labels all glial nuclei independent of *Gal4* while *nrv2-Gal4* was used to express *UAS-λhtl*. This strain was crossed against a collection of *UAS-dsRNA* lines. For screening, living larvae were mounted as live squeezed preparations, to ensure best signal to noise ratio (Kottmeier, 2013). Third instar wandering larvae were collected in ice-cold PBS. With their ventral side up, animals were transferred to a drop of silicon fat (KORALISON, medium viscosity, Kurt Obermeier GmbH) onto a microscope slide. To fix larvae in their position, they were covered with a cover slip and squeezed. Tracheae were oriented towards the microscope slide for visualization of the nervous system. About seven larvae per genotype were assessed using a Nikon fluorescence binocular (AZ-100).

Immunohistochemistry

For confocal analyses, at least six to ten animals including an equal ratio of both female an male animals were dissected. For experiments using the N^{ts1} allele, only hemizygous males were examined. For larval filet preparations third instar wandering larvae were collected in ice-cold PBS. The larvae were placed on a silicon pad with their dorsal side facing up and secured at both ends using needles. They were then carefully opened along the dorsal midline using dissection scissors and stretched out with four needles. Gut, fat body, and trachea were removed. For adult brain preparations, adult flies were anesthetized with CO_2 and briefly dipped in 70% ethanol. The head capsule was cut open with dissection scissors, and the tissue surrounding the brain was removed using forceps. Legs and wings were excised, and the thorax was opened dorsally. The ventral nerve cord was freed from the surrounding tissue to isolate the sample. After dissection,

the samples were fixed by covering them with Bouin's solution for 3 min at room temperature. This was followed by three quick buffer exchanges and three additional washes with PBT lasting 20 min each. Following blocking in 10% goat serum/PBT for one hour at room temperature primary antibodies were applied and incubated overnight at 4°C. Then samples were washed three times with PBT for 20 min each and then incubated with secondary antibodies for 3 h at room temperature. The tissues were covered with Vectashield mounting solution (Vector Laboratories) and stored at 4°C until imaging using an Zeiss LSM880 Fast-Airyscan microscope. Confocal images were analyzed using Fiji.

Electron microscopic analysis

For electron microscopy analyses larvae were dissected in 4% PFA and fixed as filet preparations for 45 min at room temperature, which was followed by fixation in 4% paraformaldehyde (PFA) and 0.5% glutaraldehyde in 0.1 M P-buffer at 4 °C overnight. The PFA was replaced by 2% OsO_4 in 0.1 M P-buffer for 1 hr on ice (dark). Uranyl acetate staining was performed en bloque using a 2% solution in H_2O for 30 min (dark). Following an EtOH series (50%, 70%, 80%, 90%, and 96%) on ice for 3 min each step, final dehydration was done at RT with 2×100% EtOH for 15 min and 2x propylene oxide for 15 min. Following slow epon infiltration specimens were embedded in flat molds and polymerized at 60°C for 2 days. After trimming, ultra-thin sections of segmental nerves about 150 µm distant from the tip of the ventral nerve cord were obtained using a 35° ultra knife (Diatome). Sections were collected on formvar-coated copper grids on which they were left to dry for at least 1 h prior to imaging, which was performed with a Zeiss TEM 900 at 80 kV in combination with a Morada camera (EMSIS).

Quantification and statistical analysis

Statistical details of every experiment can be found in the figure legends, with n representing the number of examined animals. Normal distribution of values was performed using the Shapiro Wilk test. To determine the level of significance the t-test was applied for normally distributed data, while the Man Whitney U test was applied for not normally distributed. Python was also used to generate all statistics and boxplots. For statistical analyses of EM data, the wrapping index was obtained by putting the number of individual wrapped axons or axon fascicles into relation to the number of all axons. A wrapping index of 1 implies that every single axon of the nerve is individually wrapped. All nerves that contained less than 76 or more than 82 axons were not included in the statistical analysis.

Data availability

Figure source data contain the numerical data used to generate Boxplot in Figure 6D, Figure S3, Figure S8.

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Additional files

Suplementary Tables Table S1: Summary of Heartless modifier screen, related to STAR Methods > Method details > Heartless modifier screenTable S2: Rescue of the Htl gain of function wrapping glial phenotype by two independent dsRNA transgenes, related to STAR Methods > Method details > Heartless modifier screen, Figure 3 Table S3: Rescue of Htl gain of function wrapping glial phenotype by two overlapping dsRNA transgenes, related to STAR Methods > Method details > Heartless modifier screen

Source data

Materials and Methods

Supplementary Figures

Additional information

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Peer reviews

Reviewer #1 (Public review):

Summary:

A central function of glial cells is the ensheathment of axons. Wrapping of larger-diameter axons involves myelin-forming glial classes (such as oligodendrocytes), whereas smaller axons are covered by non-myelin forming glial processes (such as olfactory ensheathing glia). While we have some insights into the underlying molecular mechanisms orchestrating myelination, our understanding of the signaling pathways at work in non-myelinating glia remains limited. As non-myelinating glial ensheathment of axons is highly conserved in both vertebrates and invertebrates, the nervous system of Drosophila melanogaster, and in particular the larval peripheral nerves, have emerged as powerful model to elucidate the regulation of axon ensheathment by a class of glia called wrapping glia. This study seeks to specifically address the question, as to which molecular mechanisms contribute to the regulation of the extent of glial ensheathment focusing on the interaction of wrapping glia with axons.

Strengths and Weaknesses:

For this purpose, the study combines state-of-the-art genetic approaches with high-resolution imaging, including classic electron microscopy. The genetic methods involve RNAi mediated knockdown, acute Crispr-Cas9 knock-outs and genetic epistasis approaches to manipulate gene function with the help of cell-type specific drivers. The successful use of acute Crispr-Cas9 mediated knockout tools (which required the generation of new genetic reagents for this study) will be of general interest to the Drosophila community.

The authors set out to identify new molecular determinants mediating the extent of axon wrapping in the peripheral nerves of third instar wandering Drosophila larvae. They could show that over-expressing a constitutive-active version of the Fibroblast growth factor receptor Heartless (Htl) causes an increase of wrapping glial branching, leading to the formation of swellings in nerves close to the cell body (named bulges). To identify new determinants involved in axon wrapping acting downstream of Htl, the authors next conducted an impressive large-scale genetic interaction screen (which has become rare, but remains a very powerful approach), and identified Uninflatable (Uif) in this way. Uif is a large single-pass transmembrane protein which contains a whole series of extracellular domains, including Epidermal growth factor-like domains. Linking this protein to glial branch formation is novel, as it has so far been mostly studied in the context of tracheal maturation and growth. Intriguingly, a knock-down or knock-out of uif reduces branch complexity and also suppresses htl over-expression defects. Importantly, uif over-expression causes the formation of excessive membrane stacks. Together these observations are in in line with the notion that htl may act upstream of uif.

Further epistasis experiments using this model implicated also the Notch signaling pathway as a crucial regulator of glial wrapping: reduction in Notch signaling reduces wrapping, whereas over-activation of the pathway increases axonal wrapping (but does not cause the formation of bulges). Importantly, defects caused by over-expression of uif can be suppressed by activated Notch signaling. Knock-down experiments in neurons suggest further that neither Delta nor Serrate act as neuronal ligands to activate Notch signaling in wrapping glia, whereas knock-down of Contactin, a GPI anchored Immunoglobulin domain containing

protein led to reduced axon wrapping by glia, and thus could act as an activating ligand in this context.

Based on these results the authors put forward a model proposing that Uif normally suppresses Notch signaling, and that activation of Notch by Contactin leads to suppression of Htl, to trigger the ensheathment of axons. While these are intriguing propositions, future experiments will need to conclusively address whether and how Uif could "stabilize" a specific membrane domain capable to interact with specific axons.

Moreover, to obtain evidence for Uif suppression by Notch to inhibit "precocious" axon wrapping and for a "gradual increase" of Notch signaling that silences uif and htl, (1) reporters for N and Htl signaling in larvae, (2) monitoring of different stages at a time point when branch extension begins, and (3) a reagent enabling the visualization of Uif expression could be important next tools/approaches. Considering the qualitatively different phenotypes of reduced branching, compared to excessive membrane stacks close to cell bodies, it would perhaps be worthwhile to explore more deeply how membrane formation in wrapping glia is orchestrated at the subcellular level by Uif.

However, the points raised above remain at present technically difficult to address because of the lack of appropriate genetic reagents. Also more detailed electron microscopy analyses of early developmental stages and comparisons of effects on cell bodies compared to branches will be very labor-intensive, and indeed may represent a new study.

In summary, in light of the importance of correct ensheathment of axons by glia for neuronal function, the proposed model for the interactions between Htl, Uif and N to control the correct extent of neuron and glial contacts will be of general interest to the glial biology community.

Comments on revisions:

The authors have addressed all my comments. However, the sgRNAs in the Star method table are still all for cleavage just before the transmembrane domain, while the Supplemental figure suggests different locations.

https://doi.org/10.7554/eLife.105759.2.sa2

Reviewer #2 (Public review):

The FGF receptor Heartless has previously been implicated in Drosophila peripheral glial growth and axonal wrapping. Here, the authors performed a large-scale screen of over 2,600 RNAi lines to identify factors regulating the downstream signaling of this process. They identified the transmembrane protein Uninflatable (Uif) as essential for the formation of plasma membrane domains. Furthermore, they found that Notch, a regulatory target of Uif, is required for glial wrapping. Interestingly, additional evidence implies that Notch reciprocally regulates uif and htl, suggesting a feedback loop. Consequently, the authors propose that Uif functions as a 'switch' to regulate the balance between glial growth and axonal wrapping.

Little is known about how glial cell properties are coordinated with axons, and the identification of Uif provides essential insight into this orchestration. The manuscript is well-written, and the experiments are generally well-controlled. The electron microscopy studies, in particular, are of outstanding quality and help mechanistically dissect the consequences of Uif and Notch signaling in the regulation of glial processes. Together, this important study provides convincing evidence of a new player coordinating the glial wrapping of axons.

Comments on revisions:

Overall, the authors have done an excellent job of responding to my substantive concerns in this significantly improved manuscript. In particular, the authors have provided important additional details about the design, prioritization, and outcomes of their screen, and relayed changes that strengthen and extend the impact of their study. I have revised my assessment accordingly, and I expect this study to be of high interest to a variety of researchers in the field.

https://doi.org/10.7554/eLife.105759.2.sa1

Author response:

The following is the authors' response to the current reviews.

We would like to proceed with this paper as a Version of Record but we will correct the mistake that we made in the Key resources table. As the reviewer noted we had added the wrong guide RNA sequence here. We are super thankful to the reviewer and apologize for the mistake.

The following is the authors' response to the original reviews.

> ***eLife Assessment***
>
> *This important study identifies a new key factor in orchestrating the process of glial wrapping of axons in Drosophila wandering larvae. The evidence supporting the claims of the authors is convincing and the EM studies are of outstanding quality.*

We are thankful for this kind and very positive judgment.

> *However, the quantification of the wrapping index, the role of Htl/Uif/Notch signaling in differentiation vs growth/wrapping, and the mechanism of how Uif "stabilizes" a specific membrane domain capable of interacting with specific axons might require further clarification or discussion.*

This is now addressed

> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *A central function of glial cells is the ensheathment of axons. Wrapping of larger-diameter axons involves myelin-forming glial classes (such as oligodendrocytes), whereas smaller axons are covered by non-myelin-forming glial processes (such as olfactory ensheathing glia). While we have some insights into the underlying molecular mechanisms orchestrating myelination, our understanding of the signaling pathways at work in non-myelinating glia remains limited. As non-myelinating glial ensheathment of axons is highly conserved in both vertebrates and invertebrates, the nervous system of Drosophila melanogaster, and in particular the larval peripheral nerves, have emerged as a powerful model to elucidate the regulation of axon ensheathment by a class of glia called wrapping glia. Using this model, this study seeks to specifically address the question, as to which molecular mechanisms contribute to the regulation of the extent of glial ensheathment focusing on the interaction of wrapping glia with axons.*
>
> *Strengths and Weaknesses:*
>
> *For this purpose, the study combines state-of-the-art genetic approaches with high-resolution imaging, including classic electron microscopy. The genetic methods involve RNAi-mediated knockdown, acute Crispr-Cas9 knock-outs, and genetic epistasis*

> *approaches to manipulate gene function with the help of cell-type specific drivers. The successful use of acute Crispr-Cas9 mediated knockout tools (which required the generation of new genetic reagents for this study) will be of general interest to the Drosophila community.*
>
> *The authors set out to identify new molecular determinants mediating the extent of axon wrapping in the peripheral nerves of third-instar wandering Drosophila larvae. They could show that over-expressing a constitutive-active version of the Fibroblast growth factor receptor Heartless (Htl) causes an increase in wrapping glial branching, leading to the formation of swellings in nerves close to the cell body (named bulges). To identify new determinants involved in axon wrapping acting downstream of Htl, the authors next conducted an impressive large-scale genetic interaction screen (which has become rare, but remains a very powerful approach), and identified Uninflatable (Uif) in this way. Uif is a large single-pass transmembrane protein that contains a whole series of extracellular domains, including Epidermal growth factor-like domains. Linking this protein to glial branch formation is novel, as it has so far been mostly studied in the context of tracheal maturation and growth. Intriguingly, a knock-down or knock-out of uif reduces branch complexity and also suppresses htl over-expression defects. Importantly, uif over-expression causes the formation of excessive membrane stacks. Together these observations are in in line with the notion that htl may act upstream of uif.*
>
> *Further epistasis experiments using this model implicated also the Notch signaling pathway as a crucial regulator of glial wrapping: reduction in Notch signaling reduces wrapping, whereas over-activation of the pathway increases axonal wrapping (but does not cause the formation of bulges). Importantly, defects caused by the over-expression of uif can be suppressed by activated Notch signaling. Knock-down experiments in neurons suggest further that neither Delta nor Serrate act as neuronal ligands to activate Notch signaling in wrapping glia, whereas knock-down of Contactin, a GPI anchored Immunoglobulin domain-containing protein led to reduced axon wrapping by glia, and thus could act as an activating ligand in this context.*
>
> *Based on these results the authors put forward a model proposing that Uif normally suppresses Notch signaling, and that activation of Notch by Contactin leads to suppression of Htl, to trigger the ensheathment of axons. While these are intriguing propositions, future experiments would need to conclusively address whether and how Uif could "stabilize" a specific membrane domain capable of interacting with specific axons.*

We absolutely agree with the reviewer that it would be fantastic to understand whether and how Uif could stabilize specific membrane domains that are capable of interacting with axons. To address this we need to be able to label such membrane domains and unfortunately we still cannot do so. We analyzed the distribution of PIP2/PIP3 but failed to detect any differences. Thus we still lack wrapping glial membrane markers that are able to label specific compartments.

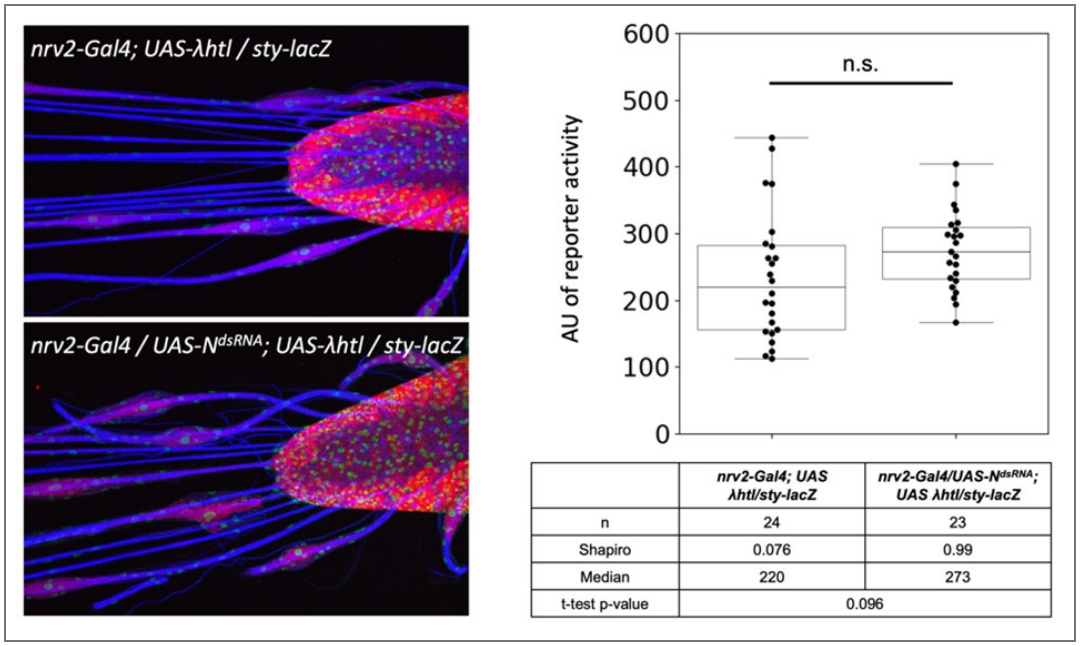
> *Moreover, to obtain evidence for Uif suppression by Notch to inhibit "precocious" axon wrapping and for a "gradual increase" of Notch signaling that silences uif and htl, (1) reporters for N and Htl signaling in larvae, (2) monitoring of different stages at a time point when branch extension begins, and (3) a reagent enabling to visualize Uif expression could be important next tools/approaches. Considering the qualitatively different phenotypes of reduced branching, compared to excessive membrane stacks close to cell bodies, it would perhaps be worthwhile to explore more deeply how membrane formation in wrapping glia is orchestrated at the subcellular level by Uif.*

In the revised version of the manuscript we have now included the use of Notch and RTK-signaling reporters.

> *(1) reporters for N and Htl signaling in larvae,*

We had already employed the classic reporter generated by the Bray lab: Gbe-Su(H)-lacZ. This unfortunately failed to detect any activity in larval wrapping glia nuclei but was able to detect Notch activity in the adult wrapping glia (Figure S5C,F).

We did, as requested, the analysis of a RTK signaling reporter. The activity of sty-lacZ that we had previously characterized in the lab (Sieglitz et al., 2013) increases by 22% when Notch is silenced. Given the normal distribution of the data points, this shows a trend which, however, is not in the significance range. We have not included this in the paper, but would be happy to do so, if requested.



	nrv2-Gal4; UAS λhtl/sty-lacZ	nrv2-Gal4/UAS-N^{dsRNA}; UAS λhtl/sty-lacZ
n	24	23
Shapiro	0.076	0.99
Median	220	273
t-test p-value	0.096	

Author response image 1.

> *(2) monitoring of different stages at a time point when branch extension begins,*

The reviewer asks for an important question; however, this is extremely difficult to tackle experimentally. It would require a detailed electron microscopic analysis of early larval stages which cannot be done in a reasonable amount of time. We have however added additional information on wrapping glia growth summarizing recently published work from the lab (Kautzmann et al., 2025).

> *(3) a reagent enabling to visualize Uif expression could be important next tools/approaches.*

The final comment of the reviewer also addresses an extremely relevant and important issue. We employed antibodies generated by the lab of R. Ward, but they did not allow detection of the protein in larval nerves. We also attempted to generate anti-Uif peptide antibodies but these antibodies unfortunately do not work in tissue. We are still trying to generate suitable reagents but for the current revision cannot offer any solution.

Lastly, we agree with the reviewer that it would be worthwhile to explore how Uif controls membrane formation at the subcellular level. This, however, is a completely new project and will require the identification of the binding partners of Uif in wrapping glia to start working on a link between Uif and membrane extension. The reduced branching phenotype might

well be a direct consequence of excessive membrane formation as it likely blocks recourses needed for efficient growth of glial processes.

> *Finally, in light of the importance of correct ensheathment of axons by glia for neuronal function, this study will be of general interest to the glial biology community.*

We are very grateful for this very positive comment.

> ***Reviewer #2 (Public review):***
>
> *The FGF receptor Heartless has previously been implicated in Drosophila peripheral glial growth and axonal wrapping. Here, the authors perform a large-scale screen of over 2600 RNAi lines to find factors that control the downstream signaling in this process. They identify a transmembrane protein Uninflatable to be necessary for the formation of plasma membrane domains. They further find that a Uif regulatory target, Notch, is necessary for glial wrapping. Interestingly, additional evidence suggests Notch itself regulates uif and htl, suggesting a feedback system. Together, they propose that Uif functions as a "switch" to regulate the balance between glial growl and wrapping of axons.*
>
> *Little is known about how glial cell properties are coordinated with axons, and the identification of Uif is a promising link to shed light on this orchestration. The manuscript is well-written, and the experiments are generally well-controlled. The EM studies in particular are of outstanding quality and really help to mechanistically dissect the consequences of Uif and Notch signaling in the regulation of glial processes. Together, this valuable study provides convincing evidence of a new player coordinating the interactions controlling the glial wrapping of axons.*
>
> ***Reviewer #1 (Recommendations for the authors):***
>
> *(1) To be reproducible and understandable, it would be important to provide detailed information about crosses and genotypes, as reagents are currently listed individually and genotypes are provided in rather simplified versions.*

We have added the requested information to the text.

> *(2) Neurons are inherently resistant to RNAi-mediated knockdown and it thus may be necessary to introduce the over-expression of UAS-dcr2 when assessing neuronal requirements and to specifically exclude Delta or Serrate as ligands.*

We agree with the reviewer and have repeated the knockdown experiments using UAS-dcr2 and obtained the same results. To use an RNAi independent approach we also employed sgRNA expression in the presence of Cas9. The neuron specific gene knockout also showed no glial wrapping phenotype. These results are now added to the manuscript.

> *(3) Throughout the manuscript, the authors use the terms "growth" and "differentiation" referring to the extent of branch formation versus axon wrapping. However glial differentiation and growth could have different meanings (for instance, growth could implicate changes in cell size or numbers, while differentiation could refer to a change from an immature precursor-like state to a mature cell identity). It may thus be useful to replace these general terms with more specific ones.*

This is a very good point. When we use the term “growth” we only infer on glial cell growth and thus, the increase in cell mass. Proliferation is excluded and this is now explicitly stated in the manuscript. The term “differentiation” is indeed difficult and therefore we changed it either directly addressing the morphology or to axon wrapping.

> *(4) Page 4. "remake" fibers should be Remak fibers.*

We have corrected this typo.

> *(5) Page 5. "Heartless controls glial growth but does promote axonal wrapping", this sentence is not clear in its message because of the "but".*

We have corrected this sentence.

> *(6) Generally, many gene names are used as abbreviations without introductions (e.g. Sos, Rl, Msk on page 7). These would require an introduction.*

All genetic elements are now introduced.

> *(7) Page 8. When Cas9 is expressed ubiquitously ... It would be helpful to add how this is done (nsyb-Gal4, nrv2-Gal4, or another Gal4 driver are used to express UAS-Cas9, as the listed Gal4 drivers seem to be specific to neurons or glia?).*

This now added. We used the following genotype for ubiquitous knockout using the four different uif specific sgRNAs (UAS-uif$^{sgRNA\ X}$): [w; UAS-Cas9/ Df(2L)ED438; da-Gal4 /UAS-uif$^{sgRNA\ X}$]. We used the following genotype for a glial knockout in wrapping glia ([+/+; UAS-Cas9/+; nrv2-Gal4,UAS-CD8::mCherry/UAS-uif$^{sgRNA\ X}$].

We had previously shown that nrv2-Gal4 is a wrapping glia specific driver in the larval PNS (Kottmeier et al., 2020).

> *Moreover, the authors mention that "This indicates that a putatively secreted version of Uif is not functional". This conclusion would need to be explained in detail.*
>
> *First, because it requires quite some detective work to understand the panels in Figure 1 on which this statement is based; second, since the acutely induced double-stranded breaks in the DNA and subsequent repair may cause variable defects, it may indeed be not certain what changes have been induced in each cell; and third considering that there is a putative cleavage site, would it be not be expected that the protein is not functional, when it is not cleaved, and there is no secreted extracellular part (unless the cleavage site is not required). The latter could probably only be addressed by rescue experiments with UAS transgenes with identified changes.*

We agree with the reviewer. The rescue experiments are unfortunately difficult, since even expression of a full length uif construct does not fully rescue the uif mutant phenotype (Loubéry et al., 2014). We therefore explained the conclusion taken from the different sgRNA knockout experiments better and also removed the statement that secreted Uif forms are non-functional.

> *In the Star Method reagent table, it is not clear, why all 8 oligonucleotides are for "uif cleavage just before transmembrane domain" despite targeting different locations.*

We are very sorry for this mistake and corrected it now. Thank you very much for spotting this.

> *(8) Page 13. However, we expressed activated Notch,... the word "when" seems to be missing, and it would be helpful to specify how this was done (over-expression of N[ICD].*

We corrected it now accordingly.

> *(9) To strengthen the point similarity of phenotypes caused by Htl pathway over-activation and Uif over-expression, it would be helpful to also show an EM electron micrograph of the former.*

We now added an extensive description of the phenotype caused by activated Heartless. This is shown as new Figure 2.

> *(10) Figure 4C, the larval nerve seems to be younger, as many extracellular spaces between axons are detected.*

This perception is a misunderstanding and we are sorry for not explaining this better. The third instar larvae are all age matched. The particular specimen in Figure 4C shows some fixation artifacts that result in the loss of material. Importantly, however, membranes are not affected. Similar loss of material is also seen in Figure 6C. For further examples please see a study on nerve anatomy by (Kautzmann et al., 2025).

> *(11) The model could be presented as a figure panel in the manuscript. To connect the recommendation section with the above public review, a step forward could be to adjust the model and the wording in the Result section and to move some of the less explored points and thoughts to the discussion.*

We are thankful for this advice and have moved an updated model figure to the end of the main text (now Figure 7).

> ***Reviewer #2 (Recommendations for the authors):***
>
> *(1) Screen and the interest in Uif: Out of the ~62 genes that came out of the RNAi screen, why did the authors prioritize and focus on Uif? What were the other genes that came out of the screen, and did any of those impinge on Notch signaling?*

We have now more thoroughly described the results of the screen. We selected Uif as it was the only transmembrane // adhesion protein identified and given the findings that Uif decorate apical membrane domains in epithelial cells, we hoped to identify a protein specific for a similar membrane domain in wrapping glia.

Notch as well as its downstream transcription factors were not included in the initial screen, and were only analyzed, once we had seen the contribution of Notch. Interestingly, here is one single hit in our screen linked to Notch signaling: Gp150. Here however, we have tested additional dsRNA expressing lines and were not able to reproduce the phenotype. This information is added to the discussion.

> *The authors performed a large-scale screen of 2600 RNAi lines, it seems more details about what came out of the screen and why the focus on Uif would benefit the manuscript.*

See above comment.

> *Relatedly, there would be a discussion of the limitations of the screen, and that it was really a screen looking to modify a gain-of-function phenotype from the activated Htl allele; it seems a screen of this design may lead to artifacts that may not reflect endogenous signaling.*

We have now added a short paragraph on suppressor screens, employing gain of function alleles to the introduction.

“In Drosophila, such suppressor screens have been used successfully many times (Macagno et al., 2014; Rebay et al., 2000; Therrien et al., 2000). Possibly, such screens also uncover genes that are not directly linked to the signaling pathway under study but this can be tested in further experiments. Our screen led to the unexpected identification of the large transmembrane protein Uninflatable, which in epithelial cells localizes to the apical plasma membrane. Loss of uninflatable suppresses the phenotype caused by activated RTK signaling.

In addition, we find that uif knockdown and uif knockout larvae show impaired glial growth while an excess of Uninflatable leads to the formation of ectopic wrapping membrane processes that, however, fail to interact with axons. uninflatable is also known to inhibit Notch. “

> *(2) In general this study relies on RNAi knockdown, and is generally well controlled in using multiple RNAi lines giving the same phenotype, and also controlled for by tissue-specific gene knockout. However, there is little in the way of antibody staining to directly confirm the target of interest is lost/reduced, which would obviously strengthen the study.*
>
> *Lacking the tools or ability to assess RNAi efficiency (qPCR, antibody staining), some conclusions need to be tempered. For example, in the experiments in Figure S6 regarding canonical Notch signaling, the authors do not find a phenotype by Delta or Serrate knockdown, but there are no experiments that show Delta or Serrate are lost. Thus, if the authors cannot directly test for RNAi efficiency, these conclusions should be tempered throughout the manuscript.*

We agree with the reviewer and now provide information on the use of Dicer in our RNAi experiments and conducted new sgRNA/Cas9 experiments. In addition we tempered our wording stating that Dl and or Ser are still possible ligands.

> *(3) More description is needed regarding how the authors are measuring and calculating the "wrapping index". In principle, the approach seems sound. However, are there cases where axons are "partially" wrapped of various magnitudes, and how are these cases treated in the analysis? Are there additional controls of previously characterized mutants to illustrate the dynamic range of the wrapping index in various conditions?*

This is now explained.

Further, can the authors quantify the phenotypes in the axonal "bulges" in Figures 1, 3, and 5?

This is a difficult question. Although we can easily quantify the number of bulges we cannot quantify the severity of the phenotype as this will require EM analysis. Sectioning nerves at a specific distance of the ventral nerve cord already requires very careful adjustments. Sectioning at the level of a bulge is way more difficult and it is not possible to get the number of sections needed to quantify the bulge phenotype.

The fact is that all wrapping glial cells develop swellings (bulges) at the position of the nucleus. As there are in general three wrapping glial cells per segmental nerve, the number of bulges is three.

> *(4) It seems difficult to clearly untangle the functions of Htl/Uif/Notch in differentiation itself vs subsequent steps in growth/wrapping. For example, if the differentiation steps are not properly coordinated, couldn't this give rise to some observed differences in growth or wrapping at later stages? I'm not sure of any obvious experiments to pursue here, but at least a brief discussion of these issues in the manuscript would be of use.*

We have discussed this in our discussion now more carefully. To discriminate the function of the three genes in either differentiation or in a stepwise mode of growth and differentiation.

When comparing the different loss of function phenotypes they al appear the same, which would argue all three genes act in a common process.

However, when we look at gain of function phenotypes, Htl and Uif behave different compared to Notch. This would favor for two distinct processes.

We have now added activity markers for RTK signaling to directly show that Notch silences RTK activity. Unfortunately we were not able to do a similar reciprocal experiment.

> *Minor:*
>
> *(1) The Introduction is too long, and would benefit from revisions to make it shorter and more concise.*

We have shortened the introduction and hopefully made it more concise.

> *(2) A schematic illustrating the model the authors propose about Htl, Uif, and Notch in glial differentiation, growth, and wrapping would benefit the clarity of this work.*

We had previously added the graphical abstract below that we updated and included as a Figure in the main text.

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