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

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Key words: Drosophila, wrapping glia, axon-wrapping, RTK signaling, Uninflatable, Notch, differentiation

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 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 formation of plasma membrane domains but not 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 glial cells 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 growth and differentiation. 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

A bewildering number of different cell types is generated during development and it is a central question in developmental biology how this diversity is established. In principle, both intrinsic as well as extrinsic regulatory mechanisms can establish fate differences between individual cells. 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 vertebrates, myelin forming cells stretch over several 100 µm of axon length. In the central nervous system (CNS), oligodendrocytes wrap around axon segments of up to 15 neurons. In the peripheral nervous system, Schwann cells cover 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 (PNS), 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 of the Ig-domain family (Hu et al., 2003).

Within the fly nervous system two different types of glial cells are associated with axons and both can form myelin-like structures (Bittern et al., 2021; Yildirim et al., 2022). The development of these axon-wrapping glial cell types has been extensively analyzed. 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 PointedP1, 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, PointedP1 is a target of receptor tyrosine kinase (RTK) signaling (Klaes et al., 1994; Klämbt, 1993; Peco *et al.*, 2016).

Within the PNS, axon wrapping is mediated by the so-called wrapping glia (Kottmeier et al., 2020; Matzat et al., 2015; Stork et al., 2008). Wrapping of axons primarily occurs in Drosophila PNS. It follows a similar strategy as described for vertebrate remake fibers. Wrapping glial cells are similarly large as their vertebrate counterparts and a single cell covers more than a millimeter of axon length. 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 ensheathing and wrapping glial cells is in part controlled by RTK signaling, that is initiated by either the EGF-receptor, the FGF-receptor, or the Discoidin 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). The wrapping glial cells require both EGF-receptor and FGF-receptor activity (Kottmeier *et al.*, 2020; Matzat *et al.*, 2015). 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 migration not only depends on FGF-signaling. In Drosophila embryos, proper migration of postmitotic, peripheral glial cells require *Notch* activity (Edenfeld et al., 2007). Additional post-mitotic *Notch* functions have been reported in the adult Drosophila CNS. Here, *Notch* activity is needed in olfactory neurons innervating specific glomeruli. Depending on the presentation of the Delta ligand by projection neurons as well as on neuronal activity, *Notch* is needed for long-term memory formation (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 (Allen et al., 2020; Davie et al., 2018; Li et al., 2022; Seugnet et al., 2011), Notch may be involved in glial differentiation.

We have previously shown that the FGF-receptor Heartless controls glial growth but does promote axonal wrapping. To gain a deeper understand how differentiation of wrapping glial cells is regulated, we initiated a genetic screen looking for genes that act downstream of *heartless*. This led to the identification of the large transmembrane protein Uninflatable, which in epithelial cells localizes to the apical plasma membrane. Loss of *uninflatable* impairs glial differentiation while 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*. 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 the 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 differentiation 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, which is reflected in a reduced wrapping index (Kottmeier *et al.*, 2020) (**Figure 1A,B**). The wrapping index 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, is drops to 0.07 when heartless is silenced in wrapping glia (Kottmeier *et al.*, 2020).

In contrast, gain of *heartless* function specifically in the wrapping glia that is caused by expression of *λhtl*, results in exuberant growth (**Figure 1C**). Segmental nerves are swollen in an area that is demarcated by the position of the wrapping glial cell nucleus. Interestingly, however, the additional glial growth is not associated with an increased wrapping, rather cell growth is noted around the nucleus leading to characteristic nerve bulges (**Figure 1C**).

Identification of genes acting downstream of heartless

In order to identify genes that are responsible for this excess in glial growth, we performed an RNA interference (RNAi) based screen in animals that concomitantly express activated Heartless and double stranded RNA directed against individual genes specifically in wrapping glia.

Flies of the reporter strain [*nrv2Gal4; UAS-λhtl,repo-stgGFP*] were crossed against the desired *dsRNA* lines and the offspring third instar larvae were assayed for the presence of nerve bulges. We tested about 2,600 genes for their ability is able to suppress the *λhtl* phenotype (**Table S1**). The different genes were selected based on RNAseq data and their involvement in major signaling pathways (Avet-Rochex et al., 2014), Petri & Klämbt unpublished). Living third instar larvae were assessed under the UV-dissecting microscope according to the severity of the bulging phenotype. The knockdown of 2,106 genes did not modify the nerve bulging phenotype, while the knockdown of 318 genes caused an almost complete or full rescue of the phenotype. The knockdown of the remaining genes caused either a slight rescue, variable phenotypes or early lethality. Within the set of genes suppressing the nerve bulging phenotype, we identified genes encoding known downstream components of the FGF-receptor such as *Stumps*, *Sos*, *Rgl*, *Ras85D*, *Dsor1*, *Rl*, *Msk* and *MAPK-Ak2*. These expected findings indicate that the screening works efficiently. From the 318 genes efficiently suppressing the nerve bulge phenotype, 62 were identified with two independent dsRNA constructs targeting independent regions of the mRNA (**Table S2**). The knockdown of 58 of these genes in otherwise normal wrapping glia caused differentiation defects, while the knockdown of the remaining 4 genes had no apparent effect on wrapping glial cell morphology. In most cases, wrapping glial differentiation was impaired similar to what has been noted upon loss of *heartless* activity (Kottmeier *et al.*, 2020). The knockdown

of 47 of these 62 genes in a pan-glial manner caused lethality (**Table S2**), further supporting the notion that we identified genes relevant for glial development.

Uninflatable is required for wrapping glial cell growth

Among the above set of genes is *uninflatable* (*uif*), suggesting that *uif* is required for wrapping glial development downstream of *heartless* (**Figure 1A,D,E**). Uif encodes a large, single pass transmembrane 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). Since *uif* null mutants die at late embryonic stages due to their inability to inflate their trachea properly (Zhang and Ward, 2009), we utilized RNAi as well as CRISPR/Cas9 to generate *uif* deficient wrapping glial cells and to independently verify the RNAi-based phenotype. Four different sgRNAs were generated, targeting different regions of the *uif* locus (**Figure S1**; see materials and methods for details). 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 (data not shown).

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 (**Figure 1D,F-K**). This indicates that a putatively secreted version of Uif is not functional. Rather 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. We fixed and embedded third instar larvae as open book filet preparations in Epon. This allowed us to identify the position of the ventral nerve cord. We

then took ultrathin sections at a distance of 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 2A,B**). Upon knockdown of *uif*, the wrapping index drastically decreases from 0.17 to 0.03 (**Figure S2**, n=3 larvae, 5-9 nerves per specimen, p= 2.88×10^{-7}).

To test how *uif* affects wrapping glia differentiation we performed overexpression studies. Gain of *uif* function in wrapping glia caused bulge formation around the wrapping glial nucleus, similar to what we had noted following expression of activated heartless (**Figures 1C,2C**). Interestingly, more distal and proximal parts of the wrapping glia cell remained thin and do not fully develop (**Figure 2C**). 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 2D, S2**).

Within the nerve bulge, an excess of wrapping glial membranes can be seen (**Figure 2E,F**). These processes fail to wrap individual axons which results in a significantly decreased wrapping index of 0.08 (**Figure S2**, $p=2.56 \times 10^{-9}$, n=3 larvae, 5-6 nerves per specimen). Interestingly, the excess glial cell processes form multilayered membrane stacks (**Figure 2F**). Thus, *heartless* appears to direct wrapping glial cell growth, while *uif* is needed for growth and stabilization of a specific membrane compartment, possibly matching the apical domain of epithelial cells.

Notch **is required for wrapping glial cell differentiation**

Importantly, although *uif* is needed for wrapping glia differentiation, 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 wrapping glia differentiation.

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 a larval filet preparations (**Figure 3A-C**). A similar phenotype was also induced by removing *Notch* expression using conditional CRISPR/Cas9-based knockout (**Figure 3D,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 3F,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 S3**).

Notch **instructs wrapping glia differentiation and 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 4B,D**, p=0.00079; n=5, larvae with 7-9 nerves per specimen).

Interestingly, upon expression of the active form of *Notch*, $Notch^{ICD}$, we observed a dramatic and significant increase of the wrapping index to 0.28. This indicates that the wrapping glia more efficiently enwraps axons compared to control larvae (**Figure 4C,D**, p=0.014, n=5 larvae

with 3-8 nerves per specimen). Importantly, overexpression of *Notch* does not cause any bulge formation along the nerves as it is noted upon expression of an activated receptor tyrosine kinase such as λHtl (Kottmeier *et al.*, 2020; Matzat *et al.*, 2015) (**Figures 1C, S3**).

In summary, these data clearly demonstrate that *Notch* is not only required for differentiation of wrapping glial cells but it is also sufficient for wrapping glia differentiation 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). Unfortunately, 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 S4A-C**). In adults, however, *Notch* activity could be detected in ensheathing glial cells as well as in peripheral wrapping glial cells (**Figure S4D-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 glia differentiation appeared impaired when analyzed using a confocal microscope (**Figure S5B**). However, in an electron microscopic analysis we could observe a clear reduction in the wrapping index (**Figures 5A,B, S2**, 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 differentiation. Moreover, when we silenced the gene *mastermind*, that encodes a further transcription factor involved in *Notch* signaling (Henrique and Schweisguth, 2019), we noted an even more severe impairment of glial differentiation. In the confocal microscope, wrapping glial cells accompanying the abdominal nerves appeared thin and not well differentiated (**Figure S5C)**. Similarly, when looking at electron microscopic images, we noted a dramatic reduction in the wrapping index with a corresponding loss of glial complexity (**Figure 5C**, Figure S2, 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 during differentiation of the larval wrapping glial cells.

***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 are, as the Notch receptor, 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 have a neuronal expression, we noted some expression of Delta in peripheral sensory neurons (**Figure S6A,B**). We then performed RNAi based knockdown experiments specifically in neurons using nSyb-Gal4 driver and found no effect on wrapping glial morphology (**Figure S6C-F**). These data suggest that during differentiation of the peripheral wrapping glia of Drosophila *Notch* is neither activated by Delta nor by Serrate.

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 also 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 5D,E**, Figure S2, p=0,006, n=5 larvae with 10 nerves per specimen). Wrapping glia morphology is impaired similar to what is noted upon knockdown of *Notch* and its downstream signaling components *Su(H)* and *mam* (**Figures 4,5**). This suggests that the non-canonical ligand Contactin acts in both flies and mammals to actively control wrapping glia differentiation and moreover indicates that additional ligands may exist to achieve full Notch activation.

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

To further test whether Notch instructs glial differentiation downstream of *heartless* and *uninflatable*, we conducted epistasis experiments. Gain of *uif* function results in nerve bulges (**Figure 2C**). When we concomitantly silenced Notch using RNA interference, the nerve bulging phenotype is not changed (**Figures 3, 6A,B**). However, we expressed activated Notch the bulging phenotype is rescued (**Figure 6A,C**). These data suggest that Notch counteracts Uninflatable.

Expression of constitutively active FGF-receptor, *λhtl*, in wrapping glial cells results in prominent nerve bulging (**Figures 1, 6D**). Upon co-expression of *Notch* dsRNA, the size of the *λhtl* induced nerve bulges increases (**Figure 6E**). A similar enhancement of the bulging phenotype was noted when using the temperature sensitive $Notch^{ts1}$ allele and an appropriate temperature regime (**Figure 6F**). In contrast when we co-expressed N^{ICD} we noted a suppression of the severity of the nerve bulging phenotype (**Figure 6G**). Thus, *Notch* function appears to counteract both *uif* and *htl* during glial differentiation.

Model underlying wrapping glia differentiation

We conclude the following model underlying wrapping glia differentiation in the Drosophila PNS. 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 wrapping glia differentiation.

Discussion

Here we present a model underlying growth and differentiation of 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 to eventually wrap 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 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 or differentiation can be 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. For this either many small glial cells could be generated or few, but instead very large glial cells form. In Drosophila larvae, wrapping glial cells are of enormous size and can reach 2 mm in length (Matzat *et al.*, 2015). To reach this size, mitosis is blocked but endoreplication occurs so wrapping glial cells are highly polyploid (Unhavaithaya and Orr-Weaver, 2012; Von Stetina et al., 2018; Zülbahar et al., 2018). Here we found that Htl supports wrapping glial growth, which is also corroborated by the finding that many genes affecting translation were identified in our suppressor screen. Likewise, we found a number of genes involved in

controlling metabolite transport across the wrapping glial membrane, four of which 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 (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). To test whether a similar definition of apical-like and basolateral-like membrane domains exist in wrapping glia we stained for the distribution of PIP2 and PIP3 using specific PH-domain sensory, 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.

Using existing antibodies (Zhang and Ward, 2009), Uninflatable is expressed below detection level 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 growth. Interestingly, in addition to inducing the generation of super numerous membrane sheets, Uif binds and inhibits 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. Furthermore, *Notch* is able to suppress *uif* in glia but also in imaginal discs (Djiane *et al.*, 2013). This mutual antagonistic action of Uif and Notch will stabilize the switch from a glial growth phase to a subsequent differentiation phase with pronounced axon-glia interaction.

During differentiation, 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.

Post-mitotic 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 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). However, knockdown of *borderless* in wrapping glial cells did not cause a wrapping phenotype (Kautzmann et al., unpublished).

Given our finding that Notch signaling is acting during glial cell differentiation, 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 wrapping glial cell differentiation defect. 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.

Acknowledgements:

We are thankful to Robert Ward for sending antibodies, Thomas Klein, Stefanie Schirmeier and Marcos Gonzalez Gaitan for flies and DNA. We are grateful to Elena Rinne for help in generating guide RNA constructs and all our lab colleagues for support and discussions. This work was supported by the Deutsche Forschungsgemeinschaft through funds to C.K. (SFB 1348 B5).

The authors declare no competing interests.

Figure legends

Figure 1 Heartless and Uninflatable affect the development of wrapping glia. Confocal images of third instar larval filet preparations stained for CD8::mCherry localization showing segmental nerves posterior to the ventral nerve cord. The genotypes are indicated, arrowheads point to the wrapping glia. (**A**) Wrapping glial morphology is not changed in control animals expressing a mock *dsRNA.* (**B**) Upon expression of a dominant negative form of Htl wrapping glial morphology is impaired. (**C**) Upon expression of a constitutive active form of Htl, a prominent nerve bulging phenotype is apparent. (**D**) Wrapping glia specific knockdown of *uif* using RNA interference dramatically affects glial morphology. (**E**) Concomitant expression of activated Htl and dsRNA directed against *uif* results in a *uif* loss of function phenotype. (**F**) Wrapping glial morphology is not changed in control animals expressing a mock guide RNA. (**G-K**) Wrapping glia specific targeting of *uif* using different guide RNAs targeting different positions along the *uif* gene (see Figure S1 for details). All *uif* sgRNAs disrupt wrapping glial cell differentiation. n=5 larvae for all genotypes. Scale bars are 100 µm.

Figure 2 *uif* affects axonal ensheathment. (**A,B,D-F**) Electron microscopic images of cross sections of third instar larval abdominal peripheral nerves. Wrapping glial morphology is indicated, for wrapping indexes see Figure S2. The different genotypes are indicated. (**A**) Wrapping glial morphology is not changed in animals expressing a mock *dsRNA.* (**B**) Upon expression of dsRNA targeting the *uif* gene, wrapping glial morphology along the nerves is impaired. (**C**) Single plane of a confocal image stack showing a nerve bulge caused by *uif* overexpression in wrapping glia nerve stained for CD8::mCherry expression. The dashed white line indicates the section plane of the images shown in (D,E). Expression of *uif::GFP* in

wrapping glial cells causes bulge formation, while outside the bulge wrapping glia appear thin. (**D**) Upon expression of full length *uif* tagged with GFP, wrapping glial morphology along the nerves is impaired as observed following knockdown. (**E,F**) In nerve bulge areas caused by *uif* overexpression a dramatic increase of folded membrane stacks can be noted. 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 are 2 µm, except (C) 50 µm and (F) 1 µm.

Figure 3 *Notch* is required for wrapping glial development. Confocal images of third instar larval filet preparations stained for CD8::mCherry localization showing segmental nerves posterior to the ventral nerve cord. The genotypes are indicated, arrowheads point to the wrapping glia. (**A**) Wrapping glial morphology is not changed in control animals expressing a mock *dsRNA.* (**B,C**) Wrapping glia specific knockdown of *Notch* using RNA interference using two independent dsRNA expressing lines impairs glial morphology. (**D**) Wrapping glial morphology is not changed in control animals expressing a mock guide RNA. (**E**) Conditional targeting of *Notch* using wrapping glia specific *Cas9* and guide RNA expression leads to dramatically reduced differentiation of wrapping glial cells. (**F**) Wild type control for temperature shift experiment. (**G**) Wrapping glial differentiation is significantly impaired in N^{ts1} larvae that were cultured at the restrictive temperature from first instar larval stages onwards. n=5 larvae for all genotypes. Scale bars are 100 µm.

Figure 4 *Notch* function affects axonal wrapping. (**A-C**) Electron microscopic images of cross sections of segmental nerves from wandering third instar larvae sectioned 100 µm posterior to the ventral nerve cord. The different genotypes are indicated. (**A**) Control nerve of an animal expressing *dsRNA* directed against GFP. Axons are wrapped by processes of the

wrapping glia (orange shading). For wrapping index see (D). (**B**) Upon RNAi based knockdown of *Notch* glial wrapping is reduced. (**C**) Upon expression of activated *Notch* glial wrapping is increased. (**D**) Quantification of wrapping index. While *Notch* knockdown significantly decreases the wrapping index (from 0.17 to 0.12, $p = 0.00079$), activation of Notch signaling significantly increases the wrapping index (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, * $p \leq 0.05$, *** $p \leq 0.001$. Scale bars 2 µm.

Figure 5 Wrapping glia differentiation requires canonical *Notch* signaling. Electron microscopic images of cross sections of segmental nerves from wandering third instar larvae sectioned 100 µm posterior to the ventral nerve cord. The different genotypes are indicated. Glial cell morphology is indicated by orange shading. (**A**) Wrapping glia specific expression of GFP^{dsRNA} as mock control. (**B**) Knockdown of *Suppressor of Hairless* (*Su(H)*) or (**C**) *mastermind* (*mam*) results in a reduced complexity of wrapping glial cell processes. (**D**) Neuron specific expression of $LacZ^{dsRNA}$ as mock control does not affect glial morphology. (**E**) Upon neuronal knockdown of *Contactin* (*Cont*) glial wrapping of peripheral axons is strongly impaired. Scale bars 2 µm.

Figure 6 *Notch* counteracts *uif* and *heartless* function.

Confocal images of third instar larval filet preparations stained for CD8::mCherry localization showing segmental nerves posterior to the ventral nerve cord. All animal express Uif::GFP. The genotypes are indicated, the boxed area is shown in higher magnification as an inlay. (**A**)

Wrapping glial bulging is not affected by expression of a *lacZ.* (**B**) Concomitant knockdown of Notch does not affect the nerve bulges. (**C**) Concomitant expression of activated Notch suppresses the nerve bulging phenotype and restores normal development.

(D-G) Whole mount images of living wandering third instar larvae. The segmental nerves posterior to the ventral nerve cord are shown. All larvae express an activated Heartless receptor (*λhtl*) in the wrapping glia and nuclear GFP in all glial cells. The size of the nerve bulge is indicated by a dashed line. (**D**) Control larvae co-expressing *dsRNA* directed against *mCherry*. The nuclei of the wrapping glial cells are polyploid and thus larger than the perineurial glia nuclei. (**E**) Upon concomitant knockdown of *Notch,* the nerve bulging phenotype becomes more pronounced. (**D**) A similar increase in bulge size is noted when *λhtl* is expressed in $Notch^{ts1}$ mutant larvae kept at the restrictive temperature from first to third instar larval stages. (**G**) Concomitant expression of *Notch* rescues the nerve bulging phenotype caused by *λhtl*. n=5-7 larvae for each genotype. Scale bars are 100 µm.

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 first instar larvae 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-stg::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. About seven larvae

per genotype were assessed using a Nikon fluorescence binocular (AZ-100). Third instar wandering larvae were collected in ice-cold PBS. With their ventral side up, animals were transferred to a microscope slide containing a drop of *silicone fat* (KORALISON, medium viscosity, Kurt Obermeier GmbH). A cover slip was gently pressed on the larvae to fix them in their position.

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%) in P-buffer 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 30 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 100 µm distant from the tip of the ventral nerve cord were obtained using a 35° ultra knife (Diatome, Switzerland) and collected in 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.

Table S1: Heartless modifier screen results, related to STAR Methods > METHOD DETAILS > Heartless modifier screen, Excel data sheet.

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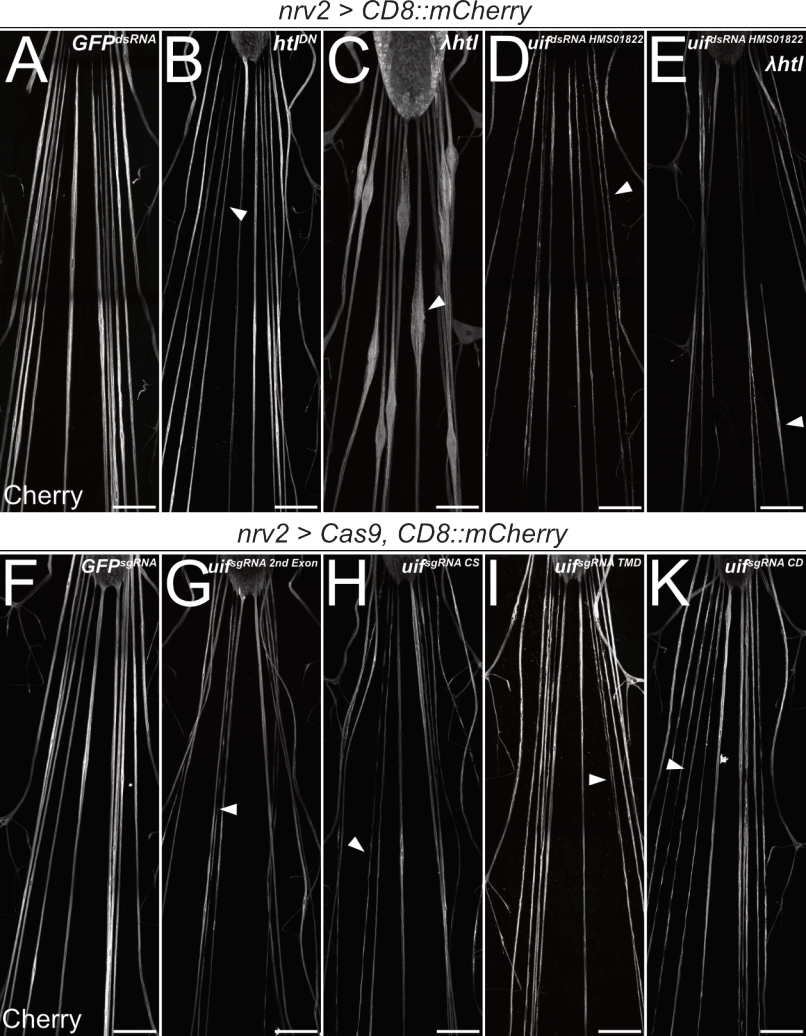
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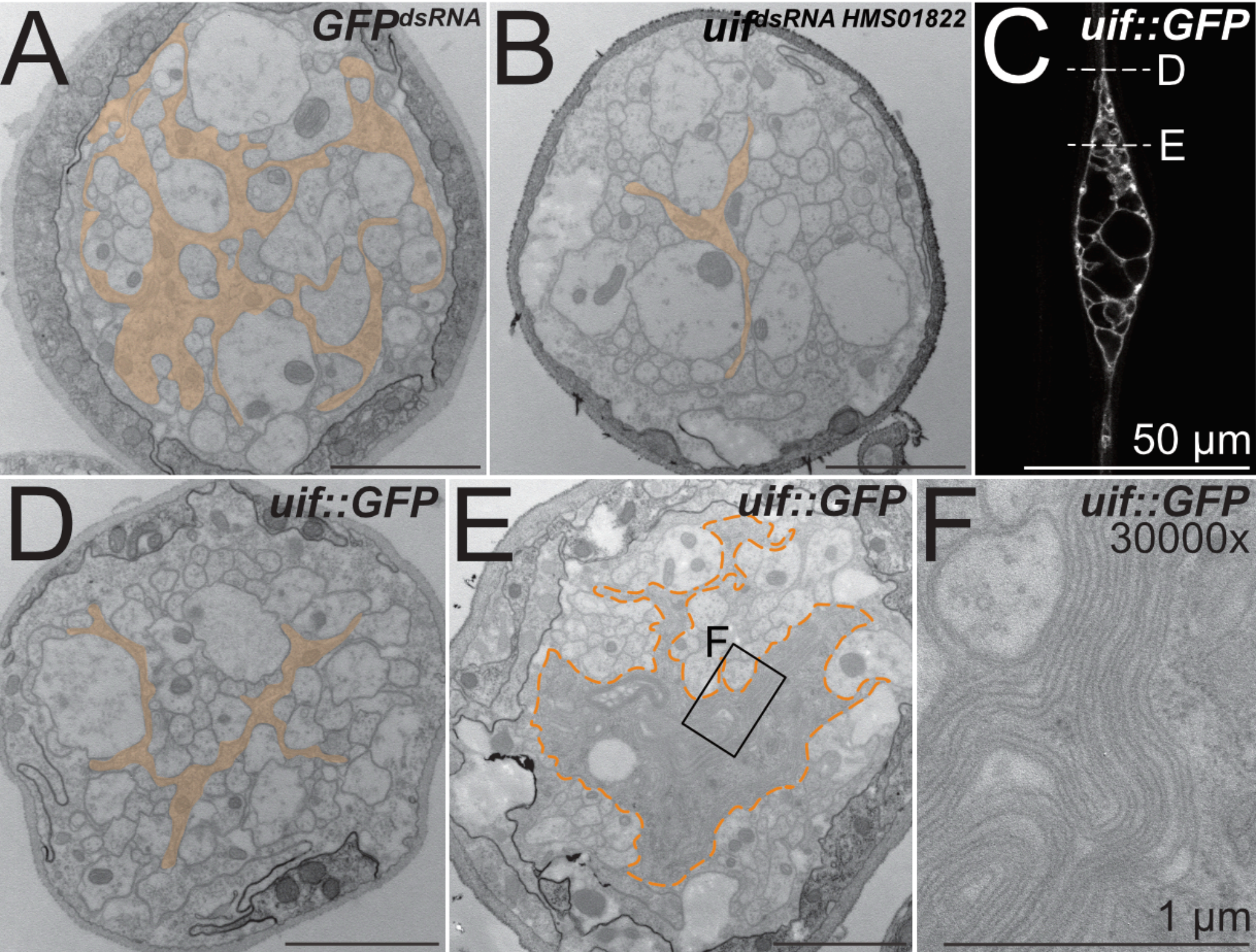

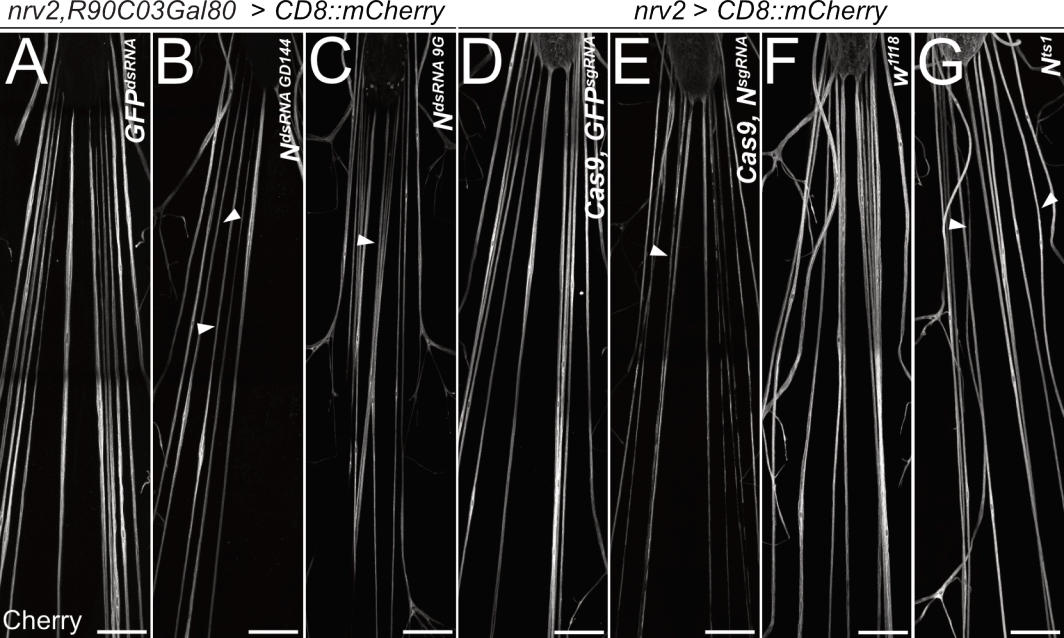
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nrv2 > CD8::mCherry
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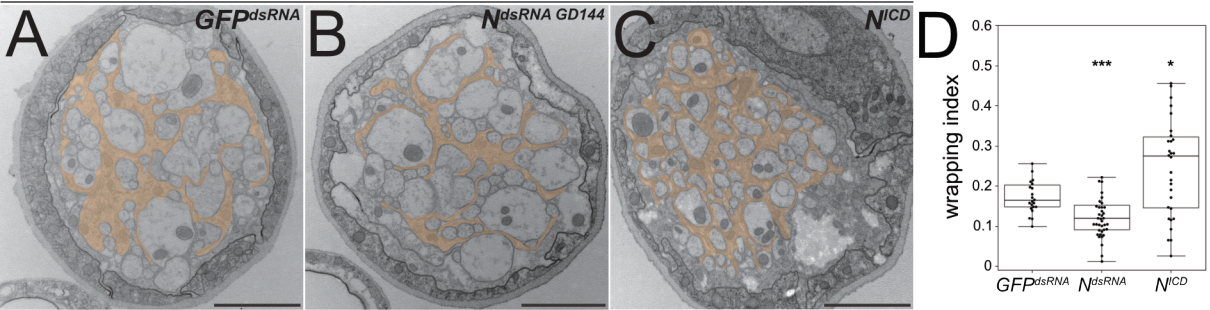
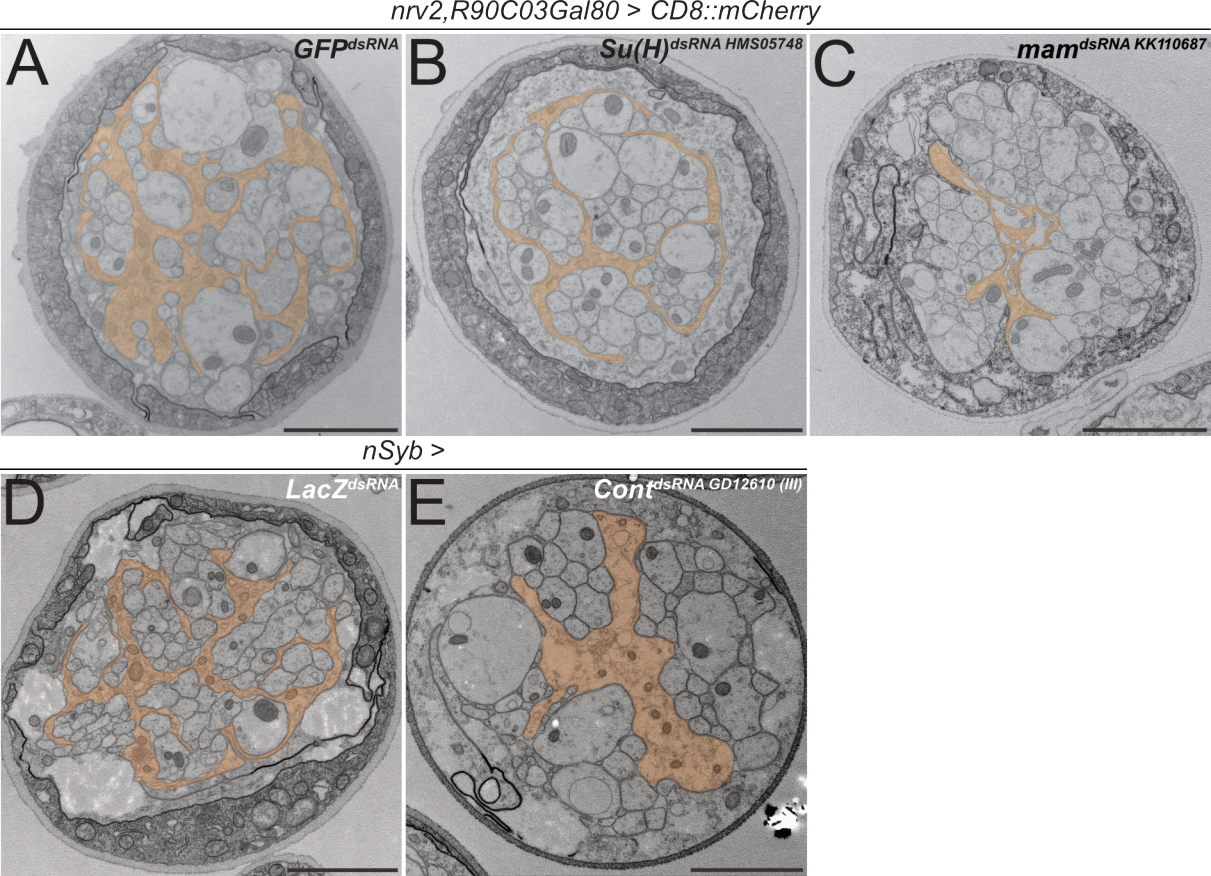
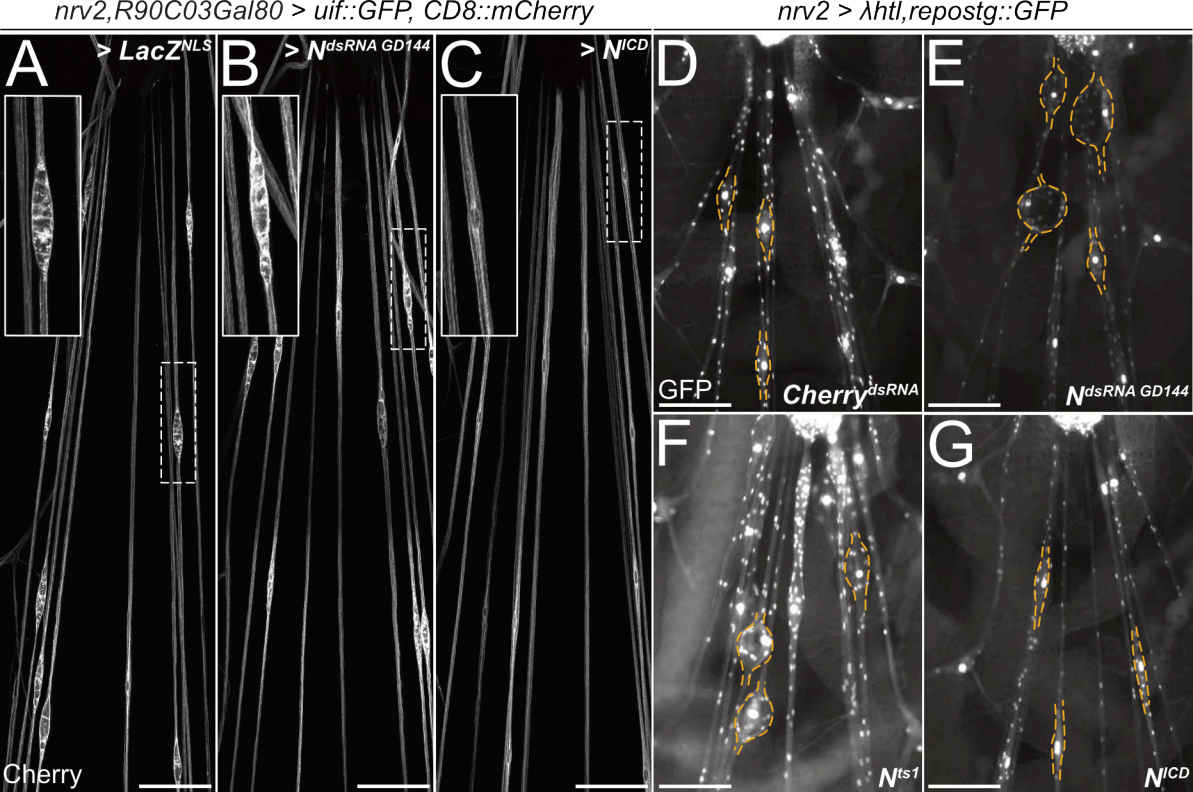



nrv2,R90C03Gal80 > CD8::mCherry
A
GFPdsRNA
B
Su(H)dsRNA HMS05748
C
mamdsRNA KK110687
nSyb >
D
LacZdsRNA
E
ContdsRNA GD12610 (III)


nrv2,R90C03Gal80 > uif::GFP, CD8::mCherry
nrv2 > λhtl,repostg::GFP
A
> LacZNLS
B
> NdsRNA GD144
C
> NICD
D
E
F
G
Cherry
GFP
CherrydsRNA
NdsRNA GD144
Nts1
NICD