Sortilin is a Negative Regulator of Sonic Hedgehog Processing and Anterograde Trafficking in Neurons

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ABSTRACT

Sonic Hedgehog (SHH) is a secreted morphogen that is an essential regulator of patterning and growth. The SHH protein requires cleavage of its full-length precursor (SHHFL) for secretion of biologically active SHH (SHHNp). Mutations in SHH that affect SHH processing are associated with human disease, which highlights the importance of processing for patterning in vivo. We identified Sortilin (SORT1), a member of the VPS10P receptor family, as a novel SHH interacting protein. SORT1 preferentially associates with SHHFL and SORT1 levels correlate inversely with cleavage of SHHFL. Consistent with an antagonistic relationship between SORT1 and SHH processing, loss of SORT1 results in an increase in SHH levels in axons and a partial rescue of Hedgehog-associated patterning defects in a mouse model of deficient SHH processing. Finally, we demonstrate a functional requirement for SORT1-mediated trafficking on SHH-dependent signaling from axons in the developing visual system in vivo. Our findings identify a novel role for SORT1 in the regulation of SHH processing and trafficking.

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LIST OF MAJOR ABBREVIATIONS

BDNF = Brain Derived Neurotrophic Factor

CNS = Central Nervous System

CN = Cortical Neuron

coIP = co-immunoprecipitation

EDU = 5-ethynyl-2'-deoxyuridine

EE = Early Endosomes

ER = Endoplasmic Reticulum

GFP = Green Fluorescent Protein

GST = Glutathione-S-Transferase

HH = Hedgehog

HSPG = Heparin Sulfate Proteoglycan

MF = Morphogenetic Furrow

ON = Optic Nerve

PM = Plasma Membrane

PTCH = Patched

PTM = Post-translational Modification

RGC = Retinal Ganglion Cell

RPC = Retinal Progenitor Cell

RSP = Regulated Secretory Pathway

SHH = Sonic Hedgehog

SHHC = Sonic Hedgehog C Terminal Domain

SHHFL = Sonic Hedgehog Full Length Precursor Protein

SHHN = Sonic Hedgehog N terminal Domain

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SHHNp = Sonic Hedgehog Mature Ligand

SMO = Smoothened

SORT1 = Sortilin1

SORT1FM = Sortilin1 Furin Mutant

TGN = *Trans* Golgi Network

tSORT1 = Truncated Sortilin1

VPS10P = Vacuolar Protein Sorting 10 Protein

YFP = Yellow Fluorescent Protein

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INTRODUCTION

Hedgehogs (Hh) are secreted morphogens (Lee et al., 1992; Porter et al., 1995) that direct proliferation and patterning events in developing and adult tissues in a variety of organisms (Riddle et al., 1993; Ruiz i Altaba, 1998; Wallace, 1999; Briscoe et al., 2000; Wang et al., 2000; Motoyama et al., 2003; Ihrie et al., 2011). In *Drosophila* there is a single Hh morphogen, while vertebrates have numerous, context-specific family members, including the mammalian Sonic (SHH), Desert (DHH), and Indian (IHH) hedgehogs (Echelard et al., 1993). All Hh proteins are dually-lipidated with cholesterol and palmitate moieties, which modulate downstream secretion and signaling properties (Porter et al., 1996a; Porter et al., 1996b; Pepinsky et al., 1998; Dawber et al., 2005). Hh proteins influence their target cells via interaction with their primary receptor Patched (Ptch) (Nakano et al., 1989; Hooper and Scott, 1989; Ingham et al., 1991; Chen and Struhl, 1996; Nusse, 1996; Stone et al., 1996; Marigo et al., 1996a) and a number of co-receptors (Tenzen et al., 2006; Martinelli and Fan, 2007; Izzi et al., 2011; Allen et al., 2011), resulting in activation of the Hh signaling cascade through the signal transducer Smoothened (Smo) (Chen and Struhl, 1996), and induction of target genes (Alexandre et al., 1996; Marigo et al., 1996b). Deregulated signaling, both in Hh producing and target cells, can cause congenital disease and cancer in humans (Roessler et al., 1996; Chiang et al., 1996; Mo et al., 1997; Goodrich et al., 1997; Ding et al., 1998), and there is a growing body of work suggesting that disruptions in the Hh morphogen processing, trafficking, and release mechanisms are causal in congenital defects and disease (Roessler et al., 1996; Roessler et al., 1997; Maity et al., 2005). However, there are still many unknown mechanisms at play in this context, and thus our work was aimed at identifying novel interacting candidates of the SHH ligand, and determining their significance in SHH ligand production and release.

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Hedgehog Signaling Cascade

Hh was first discovered as a segment polarity gene in mutation screens in *Drosophila,* where Hh mutant larvae were embryonic lethal and exhibited a ventral lawn of denticles (reminiscent of hedgehog bristles), rather than the wild type alternation of denticle and naked cuticle segments (Nüsslein-Volhard and Wieschaus, 1980; Perrimon and Mahowald, 1987; Mohler, 1988; Patel et al., 1989; Hidalgo and Ingham, 1990; Forbes et al., 1993; Bejsovec and Wieschaus, 1993). These screens also identified several components in the Hh signaling pathway as key regulators of segment patterning, and additional work in *Drosophila* uncovered the requirement for Hh signaling in pattern formation in a number of tissues, including the wing imaginal disc (Tabata and Kornberg, 1994) and the visual system (Heberlein et al., 1993; Ma et al., 1993). Orthologues for Hh and its pathway components were subsequently discovered in vertebrates, and cloning experiments revealed that Hh pathway function is highly conserved across numerous species (Echelard et al., 1993; Krauss et al., 1993; Chang et al., 1994; Marigo et al., 1995**)**.

The Hh signal transduction machinery contains several highly conserved components common to flies and vertebrates (summarized in Fig. 1). All Hhs mediate their activity through interaction with Patched (Ptch) (Nakano et al., 1989; Hooper and Scott, 1989; Ingham et al., 1991; Chen and Struhl, 1996; Nusse, 1996; Stone et al., 1996; Marigo et al., 1996a) and a number of co-receptors (whose function can positively and negatively regulate Hh pathway activation) (Chuang and McMahon, 1999; Lum et al., 2003; Desbordes and Sanson, 2003; Spoelgen et al., 2005; Yao et al., 2006; Tenzen et al., 2006; Martinelli and Fan, 2007; Capurro et

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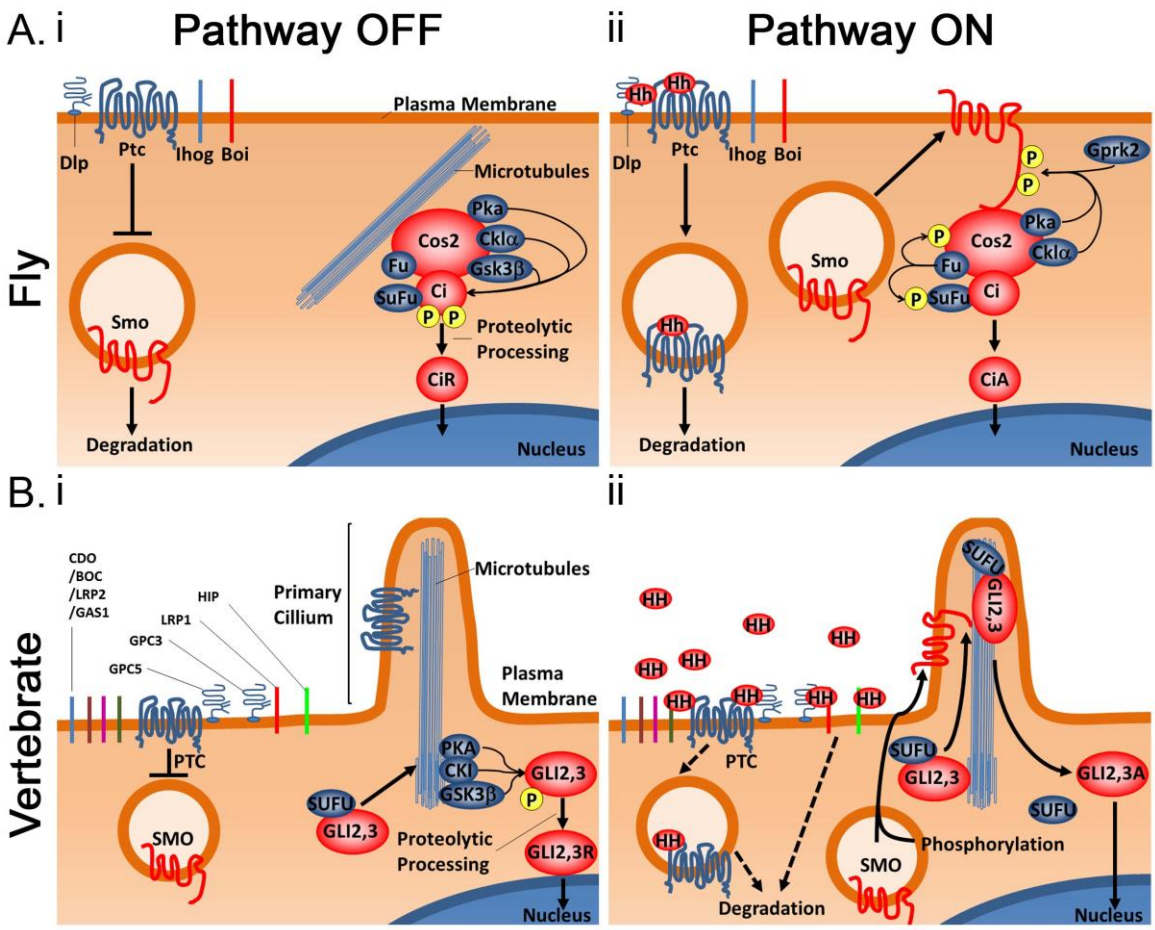
A. i
Pathway OFF
ii
Pathway ON
Fly
Plasma Membrane
Dlp
Ptc
Ihog
Boi
Microtubules
Pka
Cos2
Cklα
Fu
Gsk3β
SuFu
Ci
P
Proteolytic Processing
CiR
Smo
Degradation
Nucleus
Hh
Gprk2
CiA
B. i
ii
Vertebrate
CDO /BOC /LRP2 /GAS1
HIP
LRP1
GPC3
GPC5
Primary Cillium
PTC
SMO
PKA
CKI
GSK3β
SUFU
GLI2,3
GLI2,3R
GLI2,3A
HH
Phosphorylation

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Figure 1: Hedgehog Signal Transduction. Overview of Hh signal transduction in flies and vertebrates. **Fly OFF:** In the absence of Hh, Ptch inhibits Smo, which is targeted for degradation. The Hh signaling complex (Fu, SuFu, Cos2, and Ci) associates with microtubules, promoting the phosphorylation (by Pka, Cklα, and Gsk3β) and proteolytic processing of Ci to yield repressor Ci, which translocates to the nucleus to downregulate target genes. **Fly ON:** In the presence of Hh, Ptch binds Hh and, in concert with coreceptors Dlp, Ihog, and Boi, relieve Smo inhibition. Smo is targeted to the PM, where it is phosphorylated (by Gprk2, Pka, and Cklα) and undergoes a conformational change, whereby it interacts with the Hh signaling complex and promotes the phosphorylation of Cos2 and SuFu by Fu, culminating in the release of activator Ci, which translocates to the nucleus to upregulate target genes. **Vertebrate OFF:** In the absence of SHH, PTCH inhibits SMO. GLI proteins are phosphorylated at the base of the primary cilium in association with SUFU, leading to the production of repressor GLIs, which translocate to the nucleus to downregulate target genes. **Vertebrate ON:** In the presence of SHH, PTCH binds SHH and, in concert with the activity of co-receptors CDO, BOC, LRP2, GAS1, and GPC5, SMO inhibition is relieved. HH receptors GPC3, LRP1, and HIP antagonize HH pathway function by targeting HH for degradation. SMO is phosphorylated and is trafficked to the primary cilium, where it promotes the dissociation of activator GLIs from SUFU, and activator GLIs are subsequently translocated to the nucleus to upregulate target genes. Adapted by permission from Macmillan Publishers Ltd: [*Nat Rev Mol Cel Bio*], "The mechanisms of Hedgehog signalling and its roles in development and disease" by J. Briscoe and P. Therond, 2013, 14(7), 416-429.

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al., 2008; Capurro et al., 2012; Capurro et al., 2015). In the absence of Hh proteins, Ptch represses Smoothened (Smo) (Alcedo et al., 1996; Chen and Struhl, 1996; Stone et al., 1996); the binding of Hh to Ptch and the co-receptors results in a de-repression of Smo, which subsequently promotes an intracellular signaling cascade culminating in the nuclear translocation of the active transcription factors Cubitus Interruptus (Ci) in *Drosophila* and GLIs in vertebrates (Alexandre et al., 1996; Chen and Struhl, 1996; Marigo et al., 1996b), which bind consensus sequences in the promoter regions of target genes to alter gene expression (Kinzler and Vogelstein, 1990; Von Ohlen et al., 1997). Vertebrates additionally exhibit non-canonical HH signaling through PTCH via the activation of SRC-family kinases, whose activity culminates in cytoskeletal remodeling events involved in axon pathfinding and cell motility (Charron et al., 2003; Okada et al., 2006; Yam et al., 2009; Fabre et al., 2010; Yam et al., 2012, Hillman et al., 2012; Guo et al., 2012). Despite their similarities, there are some key differences between Hh signal transduction in flies and vertebrates, including in the specific components of the downstream signal transduction machinery; as well as the requirement in vertebrate Hh signaling for a specialized organelle, the primary cilium (Corbit et al., 2005).

In the absence of Hh in *Drosophila* (Fig. 1Ai), Ptch inhibits Smo by promoting Smo degradation (Nakano et al., 1989; Hooper and Scott, 1989; Ingham et al., 1991; Alcedo et al., 1996; Chen and Struhl, 1996). In the absence of Smo function, the Hh signaling complex (HSC), comprised of Costal 2 (Cos2), Fused (Fu), Suppressor of Fused (SuFu), and Ci, associates with microtubules and promotes the formation of the repressor form of Ci (Ci^R) through the activity of protein kinase A (PKA), casein kinase Iα (CKI), and glycogen synthase kinase 3β (GSK3β) (Forbes et al., 1993; Chen et al., 1998, Price and Kalderon, 2002; Jia et al., 2002). In the

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presence of Hh (Fig. 1Aii), Ptch binds Hh via a sterol sensing domain which, along with actions of co-receptors Heparin Sulfate Proteoglycans (HSPGs), Interference Hedgehog (Ihog), and Brother of Ihog (Boi), relieves Smo from inhibition (Alcedo et al., 1996; Chen and Struhl, 1996; Stone et al., 1996; Lum et al., 2003; Yao et al., 2006). Smo translocates to the plasma membrane (PM), where it is phosphorylated by PKA, CKIα, and G protein-coupled receptor kinase 2 (Gprk2), eliciting a conformational change in Smo which then interacts with the HSC to sequentially activate Cos2 and Fu, allowing for the release and activation of full length Ci^{A}, which is targeted to the nucleus to modulate target genes (see below) (Strutt et al., 1995; Alexandre et al., 1996; Alcedo et al., 1996; Molnar et al. 2007).

In vertebrates, PTCH proteins are enriched in and around the primary cilium, a microtubule extension of the PM that facilitates cellular response to a variety of extracellular stimuli and is essential for Hh signaling (Rohatgi et al., 2007). In the absence of HH (Fig. 1Bi), PTCH inhibits SMO via poorly understood mechanisms, resulting in phosphorylation of GLIs at the base of the cilium by PKA, CKI, and GSK3β, which in turn results in proteolytic cleavage and generation of repressor GLIs ($GLI2^{R}$ and $GLI3^{R}$) (Wang et al., 2000; Jacob et al., 2003; Pan et al., 2006). In the presence of HH (Fig. 1Bii), SMO inhibition is relieved by PTCH binding HH in concert with co-receptors CAM-related/downregulated by oncogenes (CDO), Brother of CDO (BOC), growth-arrest specific 1 (GAS1), Glypican-5 (GPC5), and low-density lipoprotein receptor related protein 2 (LRP2) (and in opposition of GPC3, LRP1, and Hedgehog Interacting Protein (HIP)) **(**Corbit et al., 2005; Spoelgen et al., 2005; Rohatgi et al., 2007; Martinelli and Fan, 2007; Allen et al., 2011; Li et al., 2011**).** SMO is phosphorylated by GPRK2 and CKI followed by trafficking to the primary cilium, where SMO increases the amount of time GLI-SUFU spends in the cilium and promotes the dissociation of the GLI-SUFU complex, leading to

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the release and translocation of activator GLIs to the nucleus (Marigo et al., 1996b; Rohatgi et al., 2007**)**.

In the absence of Hh proteins, Ci and GLIs are phosphorylated by PKA, CKI, and GSK3β in a Cos2 and Kif7-dependent fashion, which promotes the subsequent ubiquitination of Ci and GLIs by E3 ubiquitin ligase Slimb and β-transducin repeat-containing protein (βTrCP); ubiquitin tagged Ci and GLIs are then partially degraded by the proteasome, creating the truncated repressor transcription factors Ci^R and GLI^R (Chen et al., 1998; Wang et al., 2000; Price and Kalderon, 2002; Jia et al., 2002; Jacob et al., 2003; Pan et al., 2006; Bhatia et al., 2006). In the presence of Hh proteins and Smo activation, Ci and GLI protein truncation is avoided by inhibition of SUFU, Cos2/Kif7 activity, and activation of Fu in *Drosophila* (Forbes et al., 1993; Ding et al., 1999). Activator Ci and GLI proteins translocate to the nucleus, where they bind consensus sequences in the promoter regions of target genes and serve to activate or repress gene expression in a context-specific manner (Alexandre et al., 1996; Chen and Struhl, 1996; Marigo et al., 1996b). Through Ci and GLI the HH pathway alters the output of a number of genes involved in proliferation, differentiation, and self-renewal in numerous tissues, and thus is a key mechanism throughout development and disease (expanded below).

Sonic Hedgehog Signaling Directs Patterning of a Number of Progenitor Domains

In vertebrates, the majority of HH developmental functions are governed by SHH, which will be the focus of the rest of this thesis. SHH signaling is indispensable in a number of developing tissues, including the limb bud, skin structures, portions of the digestive system, and the central nervous system (CNS) (Laufer et al., 1994, Ekker et al., 1995, Ericson et al., 1995;

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Hahn et al., 1996; Oro et al., 1997; Apelqvist et al., 1997). The importance of SHH function in vertebrate development is highlighted by the observation that null mutations in SHH and pathway components are embryonic lethal due to improper patterning, proliferation, and differentiation events (Chiang et al., 2006). Furthermore, heterozygous deletion of the negative regulator PTCH is a causal mutation in Basal Cell Nevus Syndrome in humans, characterized by various developmental defects and diseases in SHH patterned tissues (Johnson et al., 1996; Hahn et al., 1996), highlighting the importance of maintaining normal pathway levels.

Hedgehog Signaling in Non-Neuronal Tissues

SHH signaling is responsible for the growth and patterning of a number of non-neuronal tissues, For example, in the tetrapod limb bud, SHH from cells in the zone of polarizing activity (ZPA) acts as a morphogen (conferring digit identity) and as a mitogen (controlling proliferation) to control digit specification (Riddle et al., 1993, Laufer et al., 1994), with deregulated SHH signaling resulting in polydactlyly, or extra digit formation (Masuya et al., 1995). Other tissues patterned by HH proteins are stem cell niches in developing and adult skin structures (Brownell et al., 2011), with aberrant signaling being associated with certain types of skin cancer, including basal cell carcinoma (Hahn et al., 1996; Oro et al., 1997). SHH also patterns various aspects of the developing digestive system, including the esophagus, lung, stomach, and pancreas (Apelqvist et al., 1997; Litingtung et al., 1998; Motoyama et al., 1998; Ramalho-Santos et al., 2000). Mutations that affect HH signaling in this context are associated with proliferation, neoplastic transformation, or progression of various types of cancer (Fujita et al., 1997; Berman et al., 2003; Thayer et al., 2003; Ma et al., 2006). Evidently, there are a number of non-neuronal

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tissues patterned by HH signaling. However, this thesis will primarily focus on the CNS, where SHH signaling notably patterns progenitor domains in midline tissues, the cerebellum, certain regions in the adult brain, and the visual system (see below).

Sonic Hedgehog Patterns Midline Tissues

One of the earliest functions of SHH signaling in CNS development is in patterning of midline tissues along the rostral-caudal axis (summarized in Fig. 2A). At the rostral midline (Fig. 2B), SHH from the prechordal plate induces ventral patterning and bilateral separation of a variety of telencephalic and diencephalic tissues, including the brain hemispheres and the eye fields (Ekker et al., 1995; Ericson et al., 1995; Chiang et al., 1996), and also is required for pattering of a number of craniofacial structures, including the palate (Mo et al., 1997). Disruptions in SHH signaling at the rostral midline, both genetic and chemical, result in a wide array of congenital defects, including a failure of the forebrain to divide in to two hemispheres, known as holoprosencephaly (HPE); a failure of eyefield bilateral separation, called cyclopia; and various craniofacial abnormalities, including cleft lip and cleft palate (Chiang et al., 1996; Belloni et al., 1996; Roessler et al., 1996; Roessler et al., 1997; Helms et al., 1997; Ahlgren et al., 1999). In caudal regions of the CNS (Fig. 2C), SHH is initially produced and secreted from the notochord to specify the formation of the floorplate, which subsequently expresses and secretes SHH; SHH from the notochord and floorplate forms a concentration gradient along the dorsoventral axis to pattern the developing neural tube by signaling to ventral neural progenitors, which differentiate in domains according to the concentration of SHH to which they are exposed

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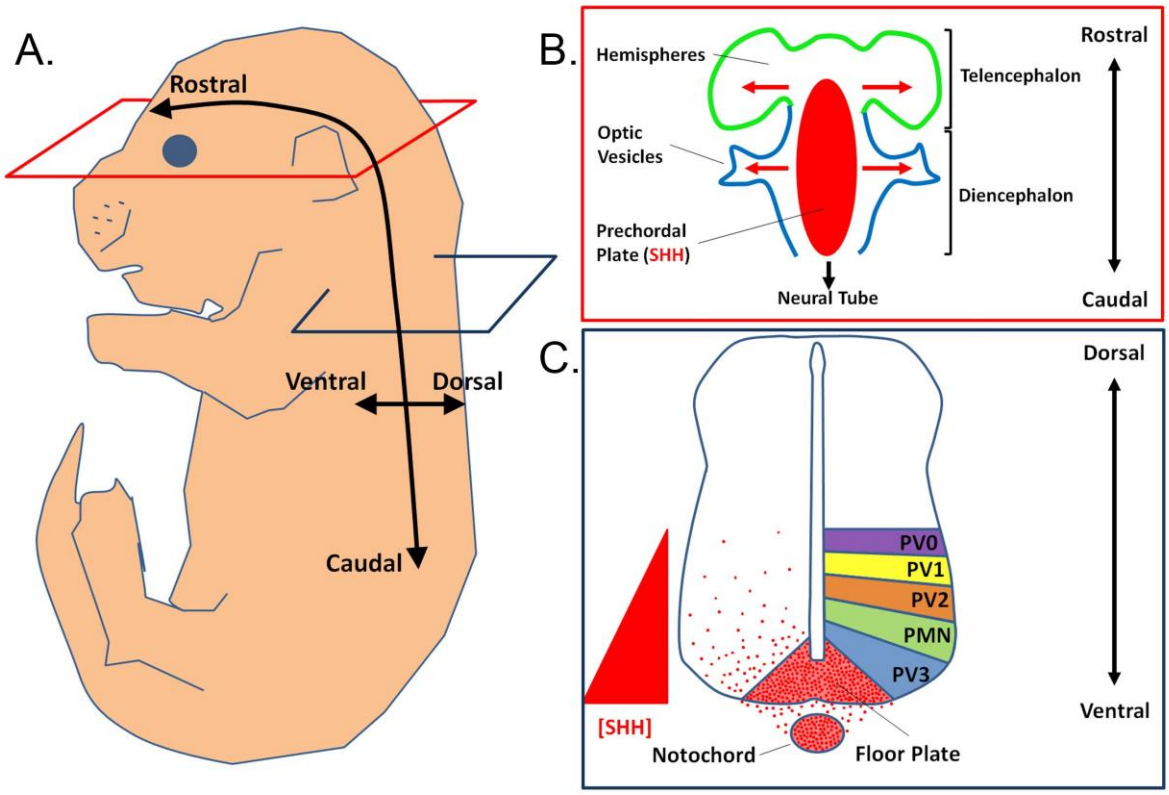
A.
Rostral
Ventral
Dorsal
Caudal
B.
Hemispheres
Optic
Vesicles
Prechordal
Plate (SHH)
Neural Tube
Telencephalon
Diencephalon
Rostral
Caudal
C.
Dorsal
PV0
PV1
PV2
PMN
PV3
[SHH]
Notochord
Floor Plate
Ventral

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Figure 2: Sonic Hedgehog Midline Patterning. Overview of SHH mediated patterning of midline CNS tissue. **A.** Schematic of embryonic mouse. Pertinent anatomical axes are represented – Rostral-Caudal (head to tail) and dorsoventral (back to belly). Square boxes represent anatomical planes depicted in A and B. Planes correspond to the matching coloured boxes. **B. Rostral Midline:** SHH is produced by cells in the prechordal plate (red oval), signaling to cells in the telencephalon to pattern bilateral separation of the hemispheres (green), and diencephalon to pattern bilateral separation of the optic vesicles (blue). Red arrows correspond to direction of bilateral separation. **C. Neural Tube:** SHH (red dots) is initially produced from the notochord, which induces the formation of the floorplate. SHH is subsequently produced from cells of the floorplate and is secreted in to the neural tube in a concentration gradient along the dorsal-ventral axis. Neural progenitor domains differentiate in response to the SHH gradient, with higher SHH concentrations eliciting ventral neural subtypes (PV3, PMN), and lower concentrations eliciting dorsal neural subtypes (PV0-2).

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(Echelard et al., 1993; Roelink et al., 1994; Johnson et al., 1994; Fan and Tessier-Lavigne, 1994; Fan et al., 1995; Ruiz i Altaba, 1998). Higher SHH concentrations elicit ventral V3 interneuron and motor neuron domains, and lower concentrations elicit dorsal V0-V2 interneuron domains (Briscoe et al., 2000). Loss of SHH signaling in the neural tube, such as by KO of SHH or SMO, results in a failure of floorplate specification and subsequent loss of ventral neuron subtypes (Chiang et al., 1996; Chen et al., 2001), while less severe mutations, including the SHH::GFP knock in allele**,** exhibit diminished ventral neuron specification (Chamberlain et al., 2008**)**. Conversely, over-activation of the pathway, such as by ectopic SHH expression or by homozygous KO of the PTCH1 allele, results in a ventralization of the neural tube and a failure in neural tube closure (Echelard et al., 1993, Johnson et al., 1994; Goodrich et al., 1997).

Sonic Hedgehog Patterns Discreet Regions of the Central Nervous System

In addition to its role in midline patterning, SHH is also involved in the development of discreet regions of the CNS. In this context, SHH is produced and secreted from several populations of neurons, whereby it mediates patterning events in adjacent progenitor cell populations. One such tissue patterned by SHH is the cerebellum (summarized in Fig. 3). Here, Purkinje cells (PCs) produce and secrete SHH, which signals to the overlying Granule Neuron Progenitor Cells (GNPs) located in the external granule layer (EGL) to promote their proliferation; reduction in SHH signaling, such as by blocking with an inhibitory antibody or by pharmacological inhibition, results in reduced proliferation of the GNPs (Weschler-Reya and Scott, 1999; Wallace and Raff, 1999; Dahmane and Ruiz i Altaba, 1999). In mice and humans,

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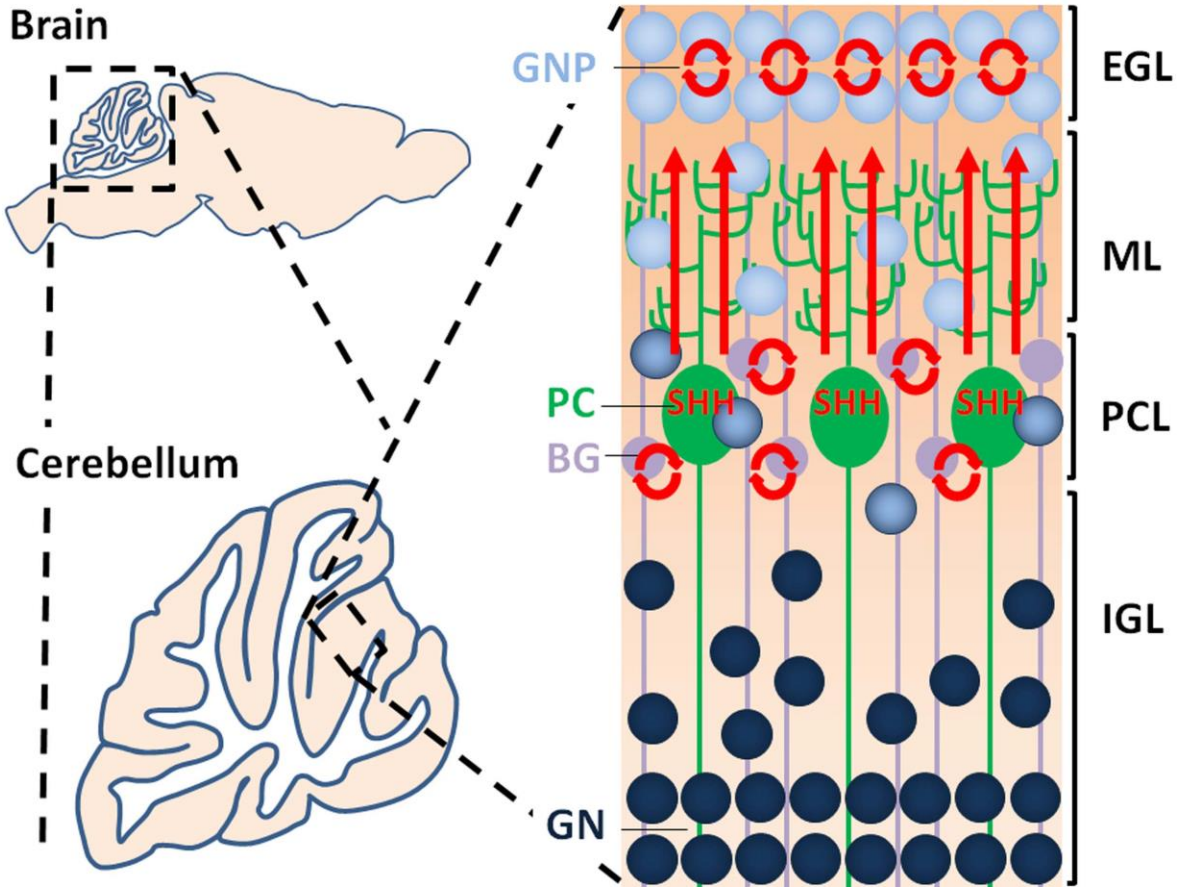
Brain
Cerebellum
GNP
EGL
ML
PC
SHH
SHH
SHH
PCL
BG
IGL
GN

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Figure 3: Sonic Hedgehog Promotes Proliferation in Granule Neuron Precursors in the Cerebellum. The cerebellum is located in the posterior of the brain, and contain a folded cortex comprised of layers of organized neurons. Within the cortex, Purkinje Cells (green) produce and secrete SHH (straight red arrows), which promotes proliferation (curcular red arrows) in overlying Granule Neuron Progenitors (light blue). Granule Neuron Progenitors migrate inwards along processes of radial Bergmann Glia (light purple) and differentiate in to Granule Neurons in the Inner Granule Layer (dark blue). EGL = External Granule Layer, ML = Molecular Layer, PCL – Purkinje Cell Layer, IGL = Inner Granule Layer. GNP = Granule Neuron Progenitor, PC = Purkinje Cell, BG = Bergmann Glia, GN = Granule Neuron.

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heterozygous deletion of the negative regulator PTCH results in ectopic proliferation in the EGL and the formation of tumors (Goodrich et al., 1997**;** Vorechovský et al., 1997), known as medulloblastoma, which is the most common pediatric solid tumor (reviewed by Remke et al., 2013).

Hh proteins are also involved in the maintenance of adult neural tissue. In the adult CNS, neurogenesis persists in the ventral subventricular zone (V-SVZ) along the walls of the lateral ventricles, and in the subgranular zone (SGZ) in the dentate gyrus of the hippocampus (reviewed by Braun and Jessberger, 2014), with various neural and glial subtypes arising from resident neural stem cells (NSCs) dependent on signaling from extrinsic cues, including SHH (Namaka et al., 2001; Machold et al., 2003; Palma et al., 2005; Ahn and Joyner, 2005). The importance for SHH signaling in this context is highlighted by the observation that reduction of the pathway via conditional deletion of SHH or SMO, or via pharmacological inhibition with cyclopamine, results in a reduction in proliferation of NSCs and changes in the distribution of resulting neurogenic niches (Balordi and Fishell, 2007; Ihrie et al., 2011). Conversely, increasing pathway activity by expression of a constitutively active SMO (SMOM2) or with SMO agonist, or by rescuing SHH pathway activation in SMO cKOs with GLI3 KO increases proliferation in these NSCs and restores positional identity of the neurogenic niches (Han et al., 2008; Bragina et al., 2010; Ihrie et al., 2011; Petrova et al., 2013).

Hedgehog and Visual System Development – Conservation from Flies to Vertebrates

Another tissue notably patterned by HH signaling is the developing visual system, the patterning of which by HH proteins is highly conserved across a number of species, including

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flies and vertebrates (Heberlein et al., 1993; Dominguez and Hafen, 1997; Jensen and Wallace, 1997; Levine et al., 1997; Wallace and Raff, 1999; and others). The overlying mechanism for all species is the production of HH proteins from a resident cellular source, which patterns proliferation and differentiation of pools of visual system progenitor cells (summarized in Fig. 4). In flies (Fig. 4A, top left of visual system diagram and inset), Hh is initially produced from differentiating photoreceptor cells located in the posterior region of the eye imaginal disc and promotes the differentiation of photoreceptors from anterior progenitors, which ultimately express Hh; these successive waves of posterior to anterior signalling drives the wave of differentiation that sweeps across the disc and is marked by the movement of the Morphogenetic Furrow (MF), a physical constriction of the disc caused by the basal movement of progenitor nuclei (Heberlein et al., 1993, Ma et al., 1993, Heberlein et al., 1995; Ma and Moses, 1995). Subsequently, Hh from the photoreceptors organizes surrounding uncommitted cells to differentiate in to several cell types to form the ommatidia, the repeating light sensing units that together form the *Drosophila* retina (Dominguez and Hafen, 1997).

In vertebrate visual systems, including in mouse, chick, frog, and zebrafish, SHH from the midline patterns the proper bilateral separation of the optic vesicles (Fig. 2B) (Ekker et al., 1995; Chiang et al., 1996; Pera and Kessel, 1997), and is required for formation of the optic stalk (MacDonald et al., 1995; Take-uchi et al., 2003; Dakubo et al., 2003). Within the eye itself (Fig. 4B, top left of visual system diagram and top right inset), SHH (and Tiggy Winkle Hh in zebrafish) is produced by the retinal ganglion cells (RGCs), the neurons that are the first to differentiate in the developing retina, and signals to adjacent retinal progenitor cells (RPCs) to regulate cell fate, proliferation, and self-renewal (Jensen and Wallace, 1997; Levine et al., 1997; Wallace and Raff, 1999; Neumann and Nuesslein-Volhard, 2000; Zhang and Yang, 2001; Wang

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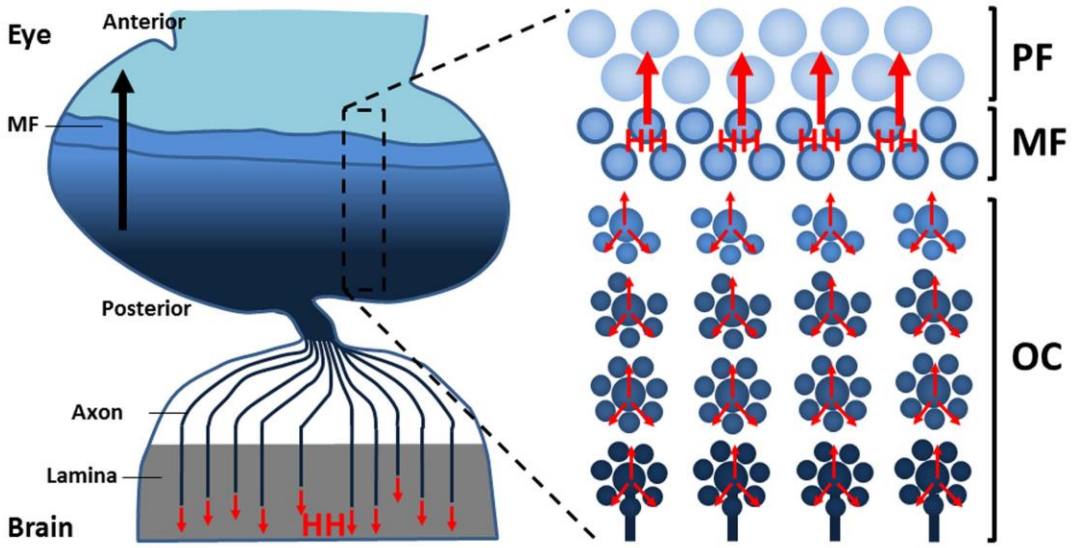
A. Fly



B. Vertebrate

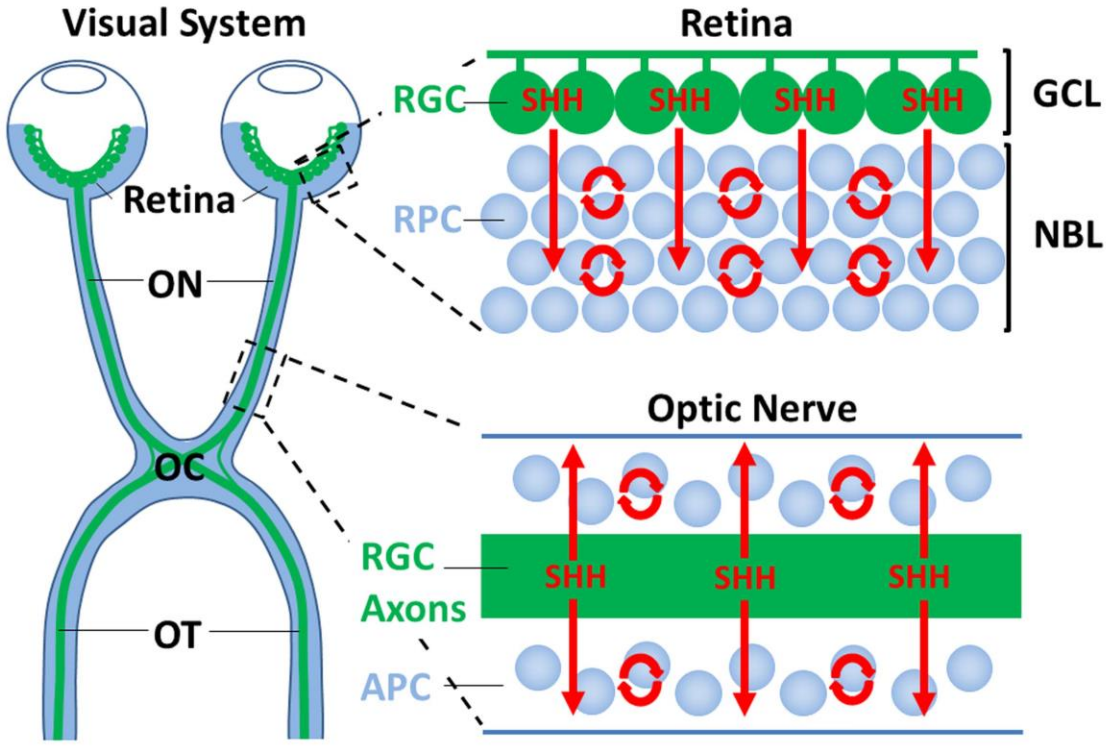


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Figure 4: Hedgehog Patterns the Visual System in Flies and Vertebrates. A. In the fly visual syste, Hh (red arrows) is initially produced in the posterior region, and signals to anterior cells (light blue) to differentiate in to photoreceptors (medium to dark blue), which also produce Hh to signal to cells anterior to themselves; this wave of differentiating photoreceptors is known as the morphogenetic furrow (medium blue with dark blue border). Photoreceptors in the wake of the morphogenetic furrow organize other photoreceptors in to sets of eight repeating units, known as ommatidia (dark blue clusters), which are the light sensing units of the drosophila eye. Hh is transmitted down ommatidia axons, through the optic stalk, and is targeted to laminar glial cell populations in the brain (grey). **B.** In vertebrates, SHH (straight red arrows) is produced by retinal ganglion cells (green), the first differentiated neurons in the retina, and is secreted to promote the proliferation (red circular arrows) and patterning of retinal progenitor cells (light blue) in the neuroblast layer. SHH is also transported down retinal ganglion cell axons, and is secreted in the optic nerve to promote proliferation of astrocyte precursor cells (light blue). MF = morphogenetic furrow, PF = prefurrow, OC = ommatidial clusters, RGC = retinal ganglion cell, RPC = retinal progenitor cell, GCL = ganglion cell layer, NBL = neuroblast layer, ON = optic nerve, OC = optic chiasm, OT = optic tract.

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et al., 2002; Wang et al., 2005; Yu et al., 2006). RPCs sequentially differentiate in to six neuronal cell types (RGCs, amacrine, horizontal, bipolar, cone photoreceptors, and rod photoreceptors) and one glial cell type (Muller glia) (Young, 1985), while HH signaling in the mouse retina promotes proliferation, biasing cycling RPCs away from differentiated fates, including the RGC fate (Zhang and Yang, 2001). Modulating SHH signaling in the retina, such as by conditional inactivation of SHH in RGCs, completely abrogates target gene expression, resulting in a decrease in proliferation in the RPC pool and a bias toward early cell fates, such as RGCs, at the expense of later cell types, including Bipolar Cells and Muller Glia **(**Wang et al., 2002).

In both flies and vertebrates, the Hh producing cells in the visual system are also the projection neurons that connect the neural retina to targets in the brain, with fly photoreceptor axons targeted to the lamina in the ganglion layer of the brain (Power, 1943; reviewed by Tsachaki and Sprecher, 2012), and RGC axons in vertebrates targeted to tissues in the midbrain (reviewed by Selhorst and Chen., 2009). Observations from both species are consistent a mechanism where Hh is transported from the cell bodies down the projecting axons, where it is secreted to signal to responsive cell populations (Huang and Kunes, 1996; Wallace and Raff, 1999). In flies (Fig. 4A lower left of visual system diagram), Hh from the photoreceptor axons stimulates the proliferation of glial populations in the lamina, with photoreceptor-specific deletion of Hh resulting in a loss in proliferation and target gene activation in these tissues (Huang and Kunes, 1996; Huang and Kunes, 1998). In the vertebrate visual system (Fig. 4B, lower left of visual system diagram and lower right inset), SHH from RGC axons signals in the optic nerve (ON), where it simulates the proliferation of resident astrocyte precursor cells, and also stimulates the migration of oligodendrocyte precursor cells (OPCs) from the optic chiasm

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(OC) (Wallace and Raff, 1999; Dakubo et al., 2003; Gao and Miller, 2006; Dakubo et al., 2008). Disruption of SHH signaling, such as by targeted deletion of SHH from RGCs or treatment with an inhibitory antibody, results in a downregulation of target genes and corresponding defects in target glial precursor populations in the ON (Wallace and Raff, 1999; Dakubo et al., 2003; Dakubo et al., 2008).

In addition to their roles in tissue patterning, SHH has been identified as a non-canonical cue for axon guidance for RGCs in the developing vertebrate ON, and for commissural neurons in the neural tube, where interactions of SHH with receptors in the extending growth cones elicit attractive and repulsive turning events, and mutations that affect the components of the SHH receptor complexes and intracellular machinery are associated with improper guidance events in target axons (Trousse et al., 2001; Charron et al., 2003; Kolpak et al., 2005; Okada et al., 2006; Sanchez-Comacho and Bovolenta, 2008; Yam et al., 2009; Fabre et al., 2010; Yam et al., 2012, Hillman et al., 2012; Guo et al., 2012).

Hedgehog Protein Production and Processing

All Hh proteins are produced as full length precursor proteins 45 kDa in size (Fig. 5), and enter the secretory pathway at the endoplasmic reticulum (ER) post signal-peptide cleavage (Bumcrot et al., 1995). Hh proteins then undergo endoproteolysis, whereby an intein domain in the 26 kDa Hh C-terminal (HhC) catalyzes a self-cleavage reaction, separating itself from the 19 kDa Hh N-terminal (HhN) domain and, at the same time, stimulating the covalent addition of cholesterol molecule to the now exposed C-terminus of HhN (Lee et al., 1992; Lee et al., 1994;

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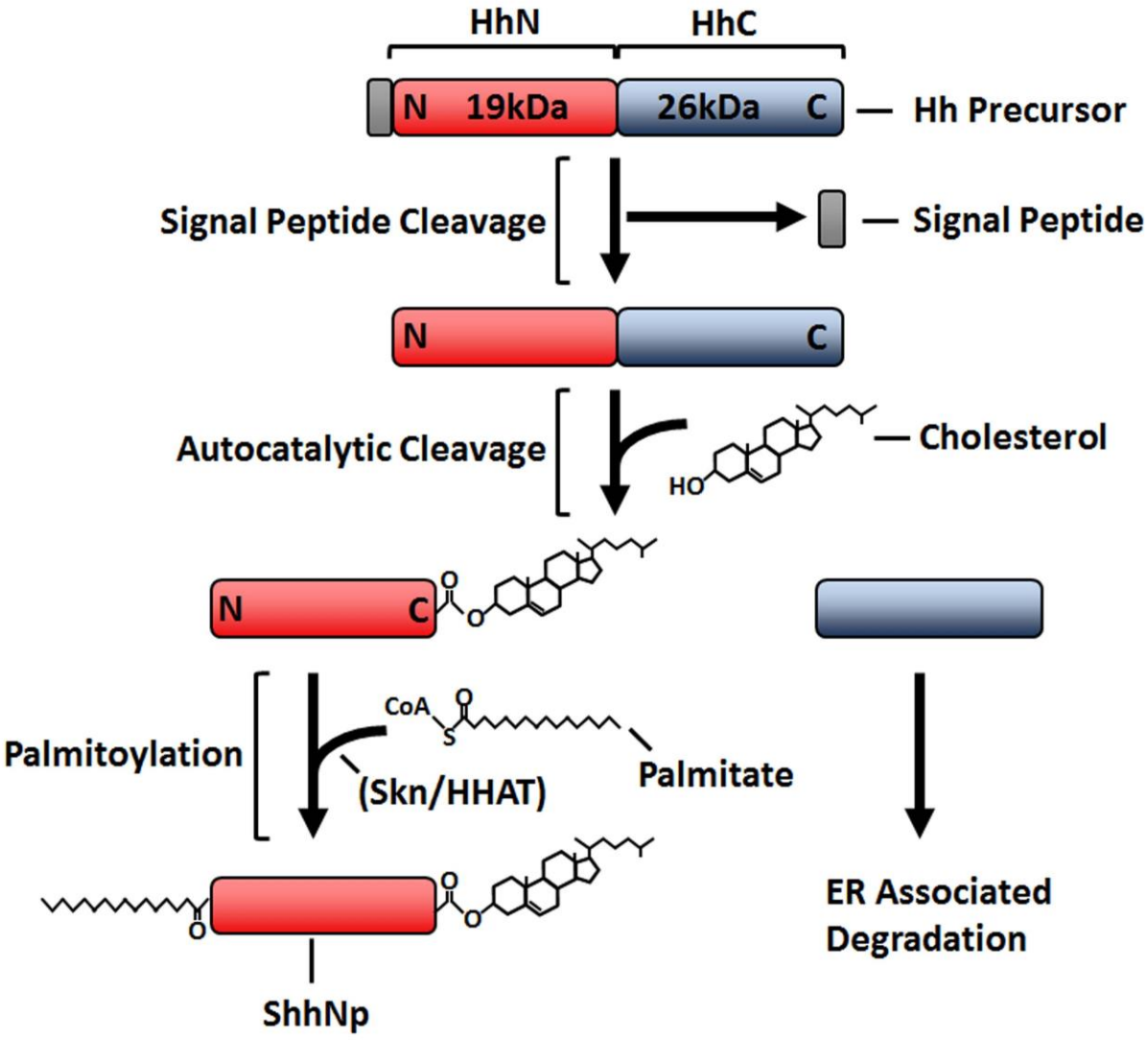
HhN
HhC
N
19kDa
26kDa
C
Hh Precursor
Signal Peptide Cleavage
Signal Peptide
N
C
Autocatalytic Cleavage
HO
Cholesterol
N
C
O
O
Palmitoylation
CoA
O
S
(Skn/HHAT)
Palmitate
O
O
O
ShhNp
ER Associated
Degradation

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Figure 5: Hedgehog Processing. Hh is produced as a 45kDa full length precursor, with a short ER signal peptide, a 19kDa N terminal domain, and a 26kDa C terminal domain. The signal peptide is cleaved upon ER entry, whereupon the HhC domain, via a conserved intein motif, catalyzes a self-cleavage reaction, separating itself from the HhN domain and stimulating the covalent addition of a cholesterol molecule to the exposed carboxy terminus of the HhN terminal domain. HhC is subsequently targeted for degradation via ER associated degradation. The HhN domain is further modified by the addition of a palmitate moiety by HHAT (or Skinny Hedgehog in flies) to the amino terminal end, forming the mature HhNp.

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Bumcrot et al., 1995; Porter et al., 1995; Fietz et al., 1995; Porter et al., 1996a; Porter et al., 1996b; Hall et al., 1997). HhC is subsequently targeted for destruction via the ER associated degradation (ERAD) pathway (Chen et al., 2011; Huang et al., 2013), and has also been reported to play a role in anterograde targeting of Hh in axons in *D. Melanogaster* (Chu et al., 2006). HhN is further modified by the addition of a palmitate moiety at the N-terminus by Skinny hedgehog (Skn, *D. Melanogaster*) (Chamoun et al., 2001) or Hh Acyltransferase (HHAT, vertebrates) (Pepinsky et al., 1998; Buglino and Resh, 2008), yielding the dually lipidated secreted HhN mature ligand (HhNp).These post-translational modifications (PTMs) serve to tether Hh proteins to the PM where they collect in lipid raft microdomains and are subsequently targeted for secretion (Karpen et al., 2001). While the vast majority of Hh proteins undergo these modifications, unmodified full length Hh proteins also mediate patterning in certain contexts (Fig. 6 (1), and see below) (Tokhunts et al., 2010; Pettigrew et al., 2014), hinting at complex and diverse mechanisms at play to facilitate Hh bioavailability from producing cells.

Mechanisms of Hedgehog Secretion

Hh PTMs play an important role in downstream signaling events, with mutant constructs that lack the PTMs exhibiting deficiencies in their ability to activate the downstream signaling cascade (Porter et al., 1996a; Porter et al., 1996b; Dawber et al., 2005). Interestingly, while these PTMs are essential for proper patterning, they render the fully modified Hh proteins insoluble in the aqueous extracellular milieu (Porter et al., 1996a; Porter et al., 1996b). Accordingly, various mechanisms exist to facilitate extracellular dispersal of insoluble Hh (summarized in Fig. 6 and described further below), reflecting the numerous roles Hhs play in tissue development.

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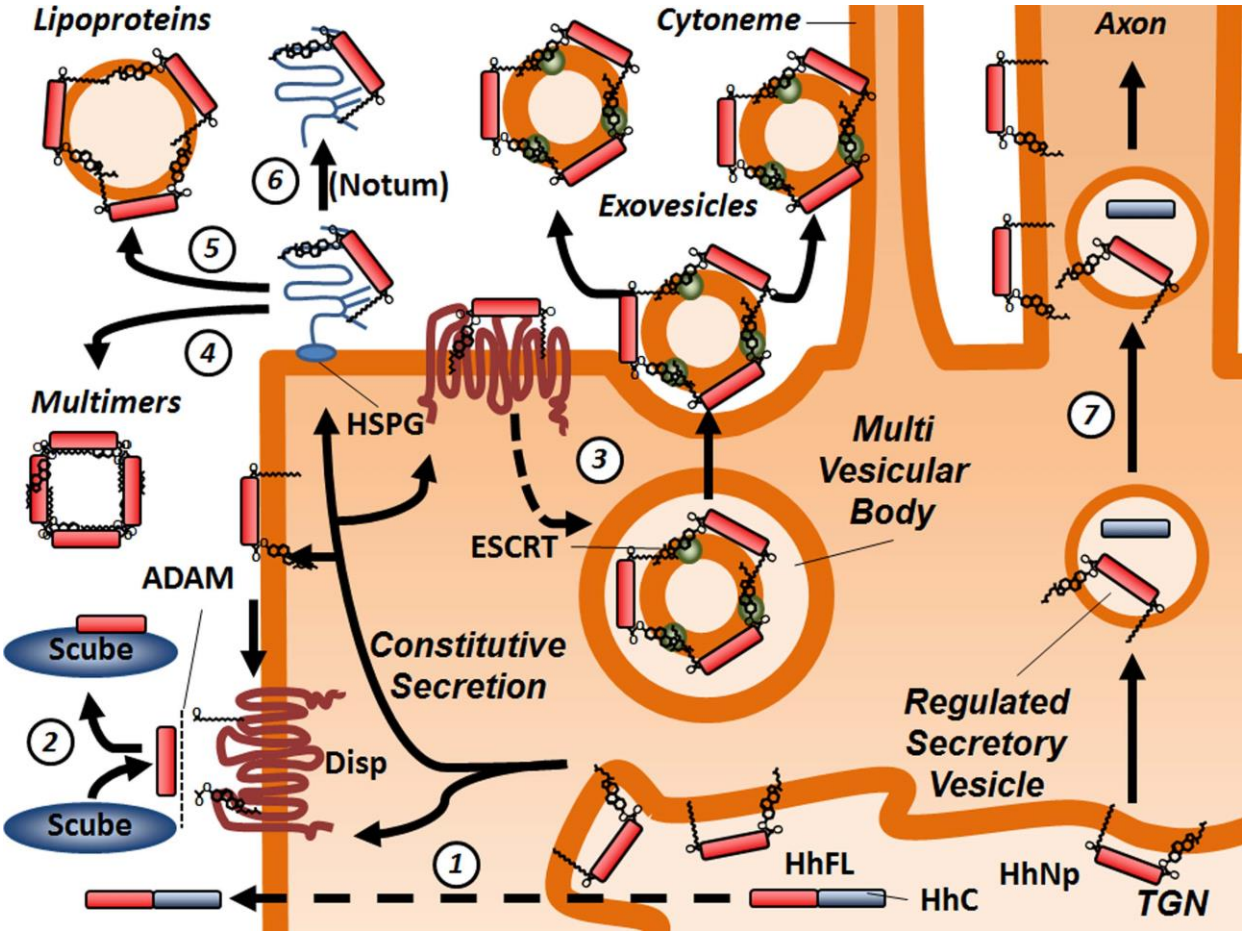
Lipoproteins
Cytoneme
Axon
6
(Notum)
Exovesicles
5
4
Multimers
HSPG
3
Multi
Vesicular
Body
7
ESCRT
ADAM
Scube
Constitutive
Secretion
2
Disp
Scube
Regulated
Secretory
Vesicle
1
HhFL
HhNp
HhC
TGN

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Figure 6: Mechanisms of Hedgehog Secretion. Hhs are targeted for secretion via a number of mechanisms. **1.** Full length Hh proteins can be targeted for secretion via an unknown mechanism. **2.** HhNp is targeted to the PM, where it interacts with Disp. Disp facilitates secretion of monomeric, unlipidated Hh proteins in concert with extracellular Scube2 and ADAM proteases. **3.** Monomeric Hh proteins are targeted to the PM, whereupon they are endocytosed via the activity of Disp and a number of co-receptors (not shown). Endocytosed Hh is repackaged, via the activity of the ESCRT pathway, in to exovesicles, which are targeted for release either via extracellular secretion (left arrow), or on cytonemes (right arrow). **4.** Hh interacts with HSPGs at the PM, which facilitates the creation of Hh multimers and oligomers, **5.** the inclusion of Hh proteins in to lipoprotein complexes, or **6.** release as a monomer along with the extracellular domain of the HSPG via Notum mediated cleavage of HSPGs from the membrane. **7.** HhNp and Hh C are targeted to neural axons in regulated secretory vesicles, and can undergo activity-dependent secretion.

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The simplest means of Hh secretion is via interaction with Dispatched (Disp), a multipass transmembrane receptor that interacts with Hh via a sterol-sensing domain, similar to the Hh receptor Ptch (Burke et al., 1999). In cell lines, cholesterol modified SHH interacts with DISP and is transferred to extracellular SCUBE2, a mechanism governed by shedding of the SHH lipid modifications by PM disintegrin and metalloprotease (ADAM) proteins (Ohlig et al., 2011; Ohlig et al., 2012; Creanga et al., 2012; Tukachinsky et al., 2012; Jakobs et al., 2014; Ortmann et al., 2015) (Fig. 6(2)). Disp also facilitates the secretion of Hh in more complex contexts: in the wing imaginal disc, Hh is produced exclusively by cells in the posterior compartment, and is initially targeted to the apical surface of the wing disc; Hh is then targeted for endocytosis by its producing cells via the activity of Disp, HSPGs (Dally, Dlp), Ihog, and Boi (Fig. 6 (3)) (Callejo et al., 2011; D'Angelo et al., 2015). Internalized Hh is then transcytosed to the basolateral surface via the endosomal sorting complex required for transport (ESCRT) and is secreted basolaterally in exovesicles with members of the ESCRT machinery where it forms a concentration gradient directionally towards the anterior compartment, reaching approximately 10 cell bodies from the Anterior/Posterior boundary (Matusek et al., 2014; Parchure et al., 2015). Recent work has also identified cytoskeletal extensions, termed cytonemes, as extracellular scaffolds on which this exovesicle-associated Hh can travel long range in the wing disc, and interact with the various modulators of Hh secretion (Fig. 6(5)), including Shifted/Drosophila WIF (Shf/DmWif), Dally, Boi, and Ihog (Rojas-Ríos et al., 2012, Bischoff et al., 2013, Gradilla et al., 2014),

Another major regulator of Hh secretion is the HSPG family (including Dally and Dlp in flies, and GPCs in vertebrates), who serve as platforms to facilitate Hh secretion via a number of mechanisms (Fig. 6 (3)-(6)). HSPGs contain negatively charged Heparin Sulfate (HS)

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glycosaminoglycan chains that interact with the Hh Carden-Weintraub (CW) motif, a series of positively charged amino acids in the HhN domain (Bellaiche et al., 1998; Desbordes and Sanson, 2003). As mentioned, HSPGs are involved in ESCRT mediated packaging of Hh in to exovesicles (Fig. 6(3)). HSPGs can also facilitate interactions between Hh monomers, leading to formation of large Hh oligomers (Fig. 6(4)) (Dierker et al., 2009a; Dierker et al., 2009b; Farshi et al., 2011). These Hh oligomers can be released from the PM, or can be further organized into higher-order multimers, covalently cross linked via tissue transglutaminase activity (Dierker et al., 2009a). HSPGs also facilitate Hh inclusion in to lipoprotein particles in the *Drosophila* wing imaginal disc and hemolymph, and also in human serum, dependent on the Hh lipid modifications (Fig. 6(5)) (Panáková et al., 2005, Palm et al., 2013). Finally, HSPGs can be cleaved from their glycophosphatidylinositol anchors by Notum at the PM and released, thus solubilizing monomeric Hhs in the extracellular milleux (Fig. 6(6)) (Traister et al., 2008; Ayers et al., 2010).

While the majority of studies have implicated solubilization and subsequent extracellular movement as the dominant mechanism for Hh spreading, an alternative pathway has been implicated in several projection neurons in different species, whereby Hh produced in neuronal cell bodies is trafficked along axons to signal to target cells (Fig. 6(6)). Consistent with an axonal targeting mechanism, SHH is localized to intracellular vesicles in hippocampal neurites (Petralia et al., 2011), and in neuronal cell lines can be found in puncta associated with markers for synaptic vesicles and dense core granules, and is secreted in response to neuronal depolarization (Beug et al., 2011). Furthermore, axonal targeting of Hh proteins is highly dependent on proper upstream processing events, as axonal targeting is altered in processing-deficient constructs in mammalian neurons (Beug et al., 2011); Interestingly, the HhC domain contains a motif that is

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required for targeting HhNp to fly photoreceptors, and targeted mutation of this motif results in a misrouting of HhNp away from the axon (Chu et al., 2006). Functionally, Hh axonal trafficking is, as discussed, notably prevalent in the projection neurons of the fly and vertebrate visual systems (Huang and Kunes, 1996; Wallace and Raff, 1999). This is confirmed by the observations that Hh puncta can be found in photoreceptor (fly) and RGC (vertebrate) axons (Huang and Kunes, 1996; Huang and Kunes, 1998; Traiffort et al., 2001; Beug et al., 2011); Hh target genes, but not Hh itself, are expressed in cells of the fly lamina, and likewise for SHH and SHH target genes in the vertebrate ON in a non-graded fashion (suggesting that they are not expressed in response to an extracellular concentration gradient of SHH from the retina) (Huang and Kunes, 1996; Huang and Kunes, 1998; Wallace and Raff, 1999); and RGC specific deletion of SHH is associated with a loss of SHH target gene induction and diminished proliferation of target cells in the ON (Dakubo et al., 2008). Outside of the visual system, axonal targeting is also mechanism for long range SHH signaling in stem cell niches in the upper telogen bulge of developing hair follicle, where target cells express target genes in response to SHH secreted from neural axons, with neural ablation of SHH resulting in a loss of SHH target gene expression and subsequent patterning defects (Brownell et al., 2011).

Identification of Novel Interacting Candidates of Sonic Hedgehog

Evidently, Hh proteins utilize a number of different mechanisms that allow for short and long range spread from their site of production to mediate patterning in development and disease, with additional mechanisms and interactors constantly emerging. Therefore, we sought to identify novel interacting candidates of SHH that may prove to be novel candidates in SHH

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mediated trafficking and secretion. Indeed, processing-deficient mutations that decrease the bioavailability of the SHH ligand in humans are causal in HPE (Roessler et al., 1997; Maity et al., 2005), while increased ligand availability promotes tumor growth and metastasis in prostate tumors (Li et al., 2014; Xie et al., 2015), underscoring the importance of further understanding the mechanisms that regulate SHH processing and dispersal. To identify novel SHH-interacting proteins, we performed a GST affinity purification screen using SHHN-GST and SHHC-GST as bait and rat brain microsomal fraction, in two different detergent preparations, as prey. Eluted proteins were resolved by SDS-PAGE, and unique bands were excised and analyzed by Mass Spectrometry (MS) (Beug, 2009) (summarized in Fig. 7A). We identified a number of novel interacting candidates, involved in diverse cellular functions, including intracellular sorting, extracellular reception, and protein maturation (Beug, 2009). The efficacy of the screen was confirmed by the identification of several known SHH interactors, including Low-density-lipoprotein Receptor-related Protein-1 (Capurro et al., 2001) and Glypican-5 (Gpc5) (Li et al., 2011). Interestingly, we identified two members of the Sortilin family, Sortilin 1 (SORT1) and sorting-protein related receptor with A-type repeats (SORLA), in SHHC and SHHN pulldowns, respectively (Beug, 2009). We prioritized SORT1 for further studies because of its known function in neuropeptide and receptor anterograde trafficking in neurons (Chen et al., 2005; Evans et al., 2011; Yang et al., 2011; Vaegter et al., 2011), and also because of the reported role for HhC in axonal trafficking (Chu et al., 2006). The interaction between SORT1 and SHH was confirmed in reciprocal co-immunoprecipitation (co-IP) experiments in cells expressing SORT1-myc-his and tagged SHHN (Fig. 7B) and SHHC (Fig. 7C), where SORT1-myc-his interacted with both SHH domains (Beug, 2009).

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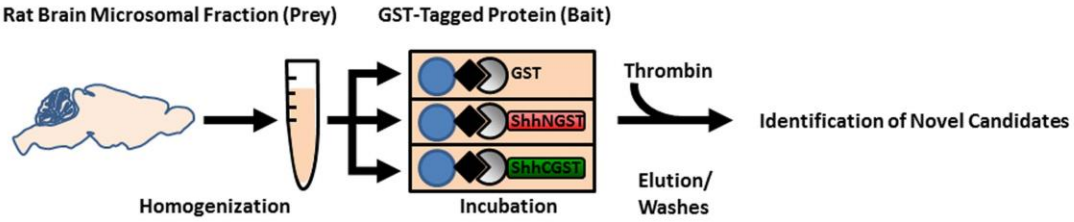
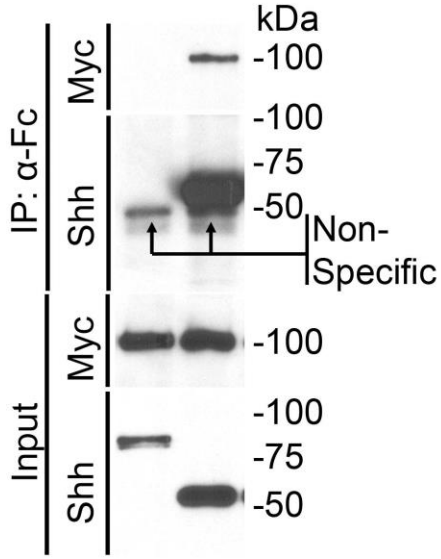
A.



B.

Sort1-Myc-His	+	+
ShhN::Fc	-	+
ShhN::AP	+	-



C.

pcDNA	+	+	-	-
Sort1-Myc-His	-	-	+	+
ShhNEYFP	-	+	-	+
ShhCECFP	+	-	+	-

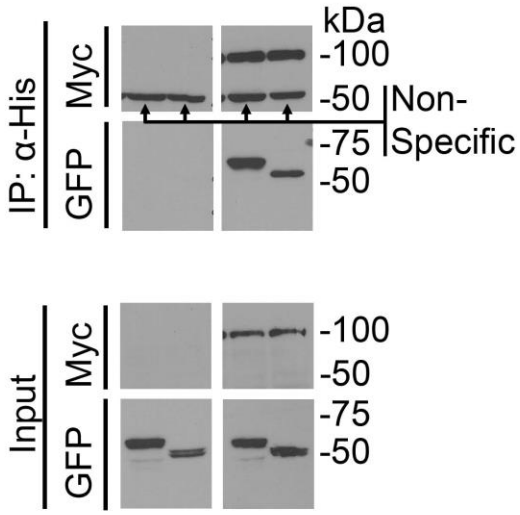


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Figure 7: Identification of Sortilin as a novel interacting candidate of Sonic Hedgehog. (A) Overview of GST screen. GST fusion constructs for SHHC and SHHN were coupled to sepharose beads to serve as bait, while homogonized rat brain was used as prey. Novel candidates were identified by mass spectrometry, including SORT1. **(B)** SORT1 and SHH co-IP. Lysates from COS cells expressing FC (right lane) or AP (left lane) tagged SHHN along with SORT1-myc-his were IPd with anti-FC beads. IP and input samples were analyzed by WB for presence of SHH constructs (bottom panels) or SORT1-myc-his (top panels). **(C)** co-IP of SORT1-myc-his from lysates of COS cells transfected with SHHCECFP (left lanes) or SHHNEYFP (right lanes) and with SORT1-myc-his. IP and input samples were analyzed by WB for SHH (bottom panels) or SORT1-myc-his (top panels). For B and C, labels indicate non-specific bands that correspond to heavy chain IgG present from the IPs. B and C adapted by permission from Shawn T Beug: [uOResearch], "Mechanisms of Morphogen Trafficking: Intraneuronal transport of SHH", 2009, (Doctoral Thesis). © Shawn Beug, 2009.

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Sortilins in Development and Disease

SORT1 is a multifunctional sorting receptor of the vacuolar protein sorting 10 protein (VPS10P) family, that includes SORT1, SORLA, and Sortilin-related receptor CNS expressed 1-3 (SORCS1-3) (Jacobsen et al., 1996; Petersen et al., 1996; Hampe et al., 2000; Rezgaoui et al., 2001; Hermey et al., 2003). SORT proteins are found primarily in developing and adult neuronal tissues, where they participate in diverse functions, but are also found in developing lung, kidney, liver, and glandular tissues, as well as being found in cells of the immune system (Musunuru et al., 2010; Pradbakaran et al., 2012; Wilson et al., 2014). While embryonic knockouts (KO) of SORT family members are viable (Zeng et al., 2009), KO animals have discrete phenotypes in various tissues, and these phenotypes have implicated SORTs in a variety of disease processes. For example, SORT1 is involved in the normal pruning of RGCs in perinatal retinae via activation of an apoptotic signaling cascade involving p75 neurotrophin receptor ($p75^{NTR}$) and proneurotrophins (proNTs), with SORT1 KO (*Sort1*$^{-/-}$) resulting in a significant decrease in RGC apoptosis (Nakamura et al., 2007). Furthermore, deregulation of the SORT1/$p75^{NTR}$/proNT apoptic axis causes a number of pathological neural apoptosis models, including lesioned spinal neurons and retinal ischemia (Volosin et al., 2006; Jansen et al., 2007; Wei et el., 2007). Another neuropathic disease model exacerbated by SORT1 KO is the $p75^{NTR}$ KO (*p75*$^{NTR-/-}$); in this case, SORT1 and $p75^{NTR}$ facilitate anterograde trafficking of tropomyosin kinase receptors (TRKs), which are major NT receptors at axon terminals (Vaegter et al., 2011). TRK activity is severely abrogated in *Sort1*$^{-/-}$ *x p75*$^{NTR-/-}$, leading to neuropathies associated with loss of NT signaling (Vaegter et al., 2011). SORT proteins have also been implicated in neurodegenerative diseases, including Alzheimer's disease (AD) and Frontotemporal Lobar Degeneration (FTLD) (Carlo et al., 2013, Lee et al., 2014). In AD pathology, Amyloid Precursor

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Protein (APP) is processed to Amyloid-β (Aβ), which forms neurotoxic oligomers that are cleared from neurons by apolipoprotein E (APOE) and SORT family members (Carlo et al., 2013), and SORT mutations are associated with increased Aβ levels in the brain and associated pathologies (Assareh et al., 2014). In FTLD pathology, Progranulin (PRG) levels in the brain are reduced, while SORT1 is responsible for endocytosis and degradation of PRG. Therefore, SORT1 depletion may be an effective targeted therapy for FTLD (Lee et al., 2014).

SORT family members also play a role in non-neuronal tissues. For example, SORT1 inactivation disrupts the sorting of Prosaposin in non-ciliated epidermal cells lining the efferent ducts (Zeng et al., 2009). Genome wide association studies (GWAS) have associated variants at the SORT1 gene locus (1p13.3) with increased plasma cholesterol levels and elevated risk for myocardial infarction (Munusuru et al., 2010). Finally, SORT1 has been associated with progression and invasion of various types of tumours, including breast (Demont et al., 2012), prostate (Tanimoto et al., 2015), ovarian (Ghaemimanesh et al., 2014), and colorectal (Akil et al., 2011). Evidently, members of the SORT family are increasingly being identified as important factors in a number of developing and diseased tissues, and further characterization of the diverse role for members of the SORT family could provide important insight into disease mechanisms.

Sortilin Family Structure

The VPS10P domain was first identified in yeast, where the VPS10P protein contains two of its namesake domains, and is responsible for sorting proteins to the yeast vacuole independent of the mannose-6-phosphate receptor pathway (Marcusson et al., 1994). All SORT family

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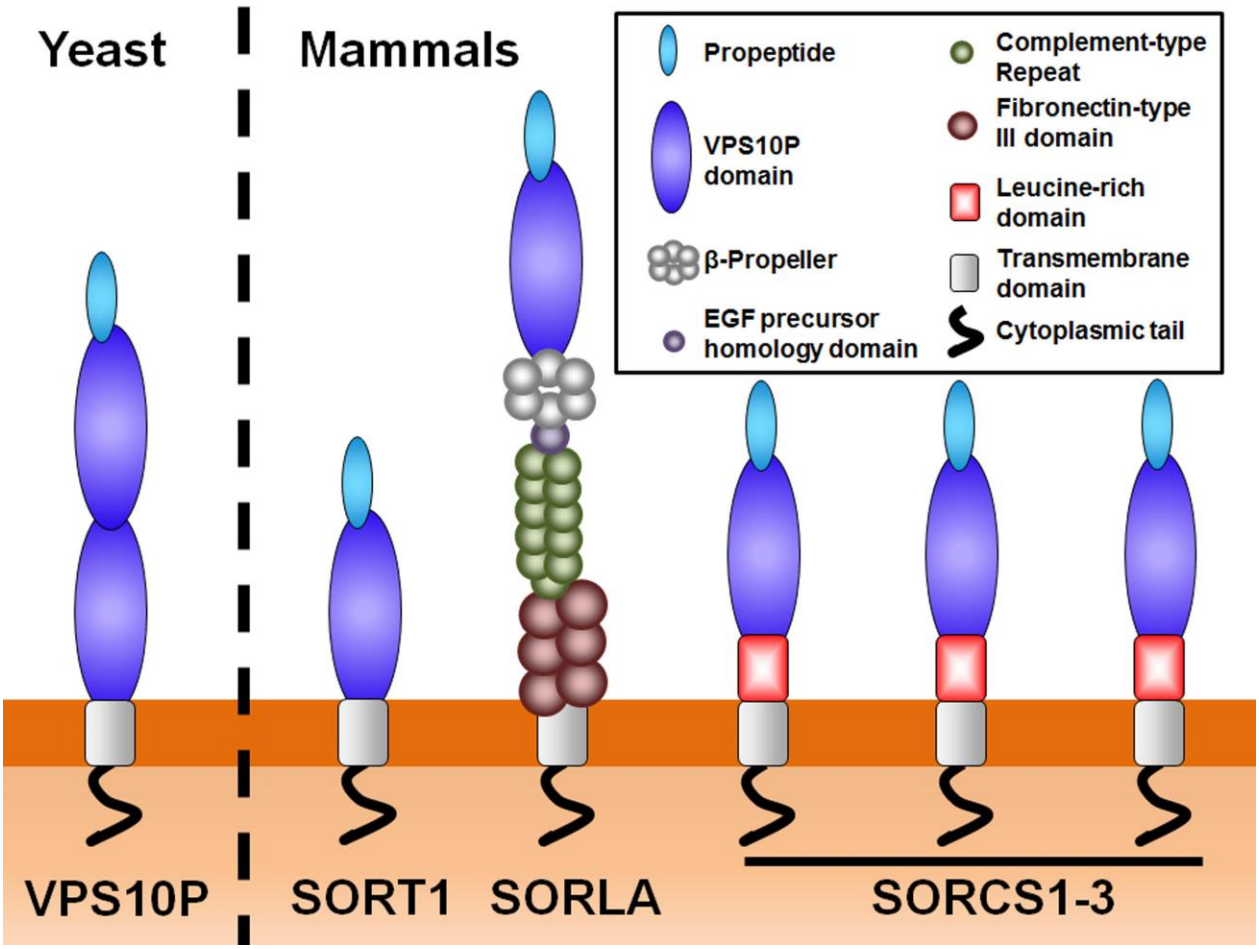
Yeast
Mammals
Propeptide
VPS10P domain
β-Propeller
EGF precursor homology domain
Complement-type Repeat
Fibronectin-type III domain
Leucine-rich domain
Transmembrane domain
Cytoplasmic tail
VPS10P
SORT1
SORLA
SORCS1-3

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Figure 8: Structure of VPS10P family. The yeast VPS10P receptor was the first family member characterized, and contains two of its namesake domains. Mammals have 5 VPS10P family receptors: SORT1, SORLA, and SORCS1-3. All family members contain a short cytoplasmic tail, a transmembrane region, the VPS10P domain, and a propeptide region, which comprises the entirety of the simplest member, SORT1. SORLA contains an additional β-propeller, an EGF precursor homology domain, a series of complement-type repeats, and Fibronectin type-III repeats; while SORCS1-3 have leucine rich domains. Adapted by permission from Macmillan Publishers Ltd: [*Nat Rev Neurosci*], "VPS10P-domain receptors — regulators of neuronal viability and function" by T. Willnow, C.M. Petersen, and A. Nykjaer, 2008, 9(12), 899-909.

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members (summarized in Fig. 8) contain large luminal ligand-binding domains of increasing complexity, unified by the inclusion of the VPS10P motif and an N-terminal propeptide that inhibits premature ligand binding to the VPS10P motif before furin mediated cleavage in the TGN (Munck Petersen et al., 1999). The SORT1 luminal domain consists entirely of the VPS10P motif (Petersen et al., 1997); SORLA contains the motif along with a β-propeller, an EGF precursor homology domain, a series of complement-type repeats, and Fibronectin type-III repeats (Jacobsen et al., 1996); and SORCS1-3 are highly homologous, containing the VPS10P motif and Leucine rich domains (Hermey et al., 1999). All members of the family are anchored to the PM via a transmembrane region, and contain a cytoplasmic tail that interacts with cytoplasmic effectors that target SORT proteins to their various subcellular pathways (Nielsen et al., 2001).

Intracellular Trafficking of Sortilin

SORT1 is largely localized intracellularly in vesicles and in the TGN, with a minority of the SORT1 pool existing on the PM (Petersen et al., 1997). SORT1 is initially trafficked through the *trans* Golgi network (TGN), where it is activated via propeptide cleavage by TGN resident Furin, a calcium-dependent serine endoprotease, which frees the ligand binding domains from competitive inhibition (Fig.9(1)) (Munck Petersen et al., 1999). SORT1 then interacts with a number of subcellular sorting complexes that facilitate movement to several different target pathways by acting as connectors between SORT1 and the cytoskeleton. SORT1 is targeted anterogradely to early endosomes (EEs) via interactions with Adaptor Protein 1 (AP-1) and

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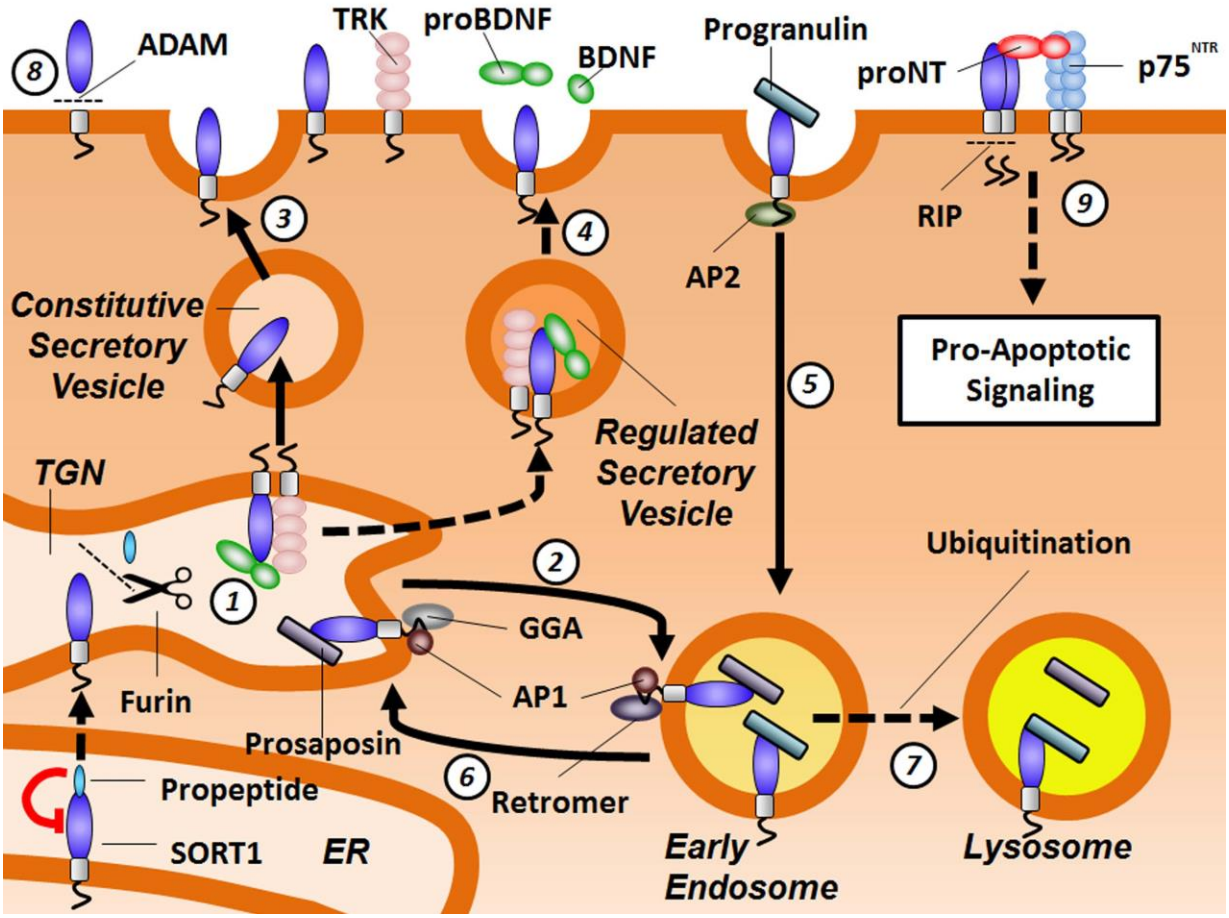
ADAM
TRK
proBDNF
BDNF
Progranulin
proNT
p75 NTR
RIP
AP2
Constitutive Secretory Vesicle
Regulated Secretory Vesicle
Pro-Apoptotic Signaling
TGN
Ubiquitination
GGA
AP1
Furin
Prosaposin
Propeptide
SORT1
ER
Retromer
Early Endosome
Lysosome
1
2
3
4
5
6
7
8
9

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Figure 9: Sortilin1 intracellular trafficking, ligands, and functions. 1. SORT1 ligand binding is inhibited by its propeptide domain, which is cleaved by furin upon entry into the TGN. **2.** SORT1 is targeted anterogradely along with various ligands from the TGN to a number of subcellular targets, including to early endosomes with prosaposin (and others) via cytoplasmic interaction with GGAs and AP1, and to the PM via **3.** the constitutive secretory pathway or **4.** the regulated secretory pathway with TRK receptors and proneurotrophins (and others). **5.** SORT1 at the PM is endocytosed and targeted to early endosomes with progranulin (and others) via cytoplasmic interaction with AP2. **6.** SORT1 dissociates from its ligands (which remain for lysosomal targeting) in early endosomes, and is recycled retrogradely to the TGN via interaction AP1 and the Retromer. **7.** SORT1 can be ubiquitinated by E3 ubiquitin ligases (not shown), and is subsequently targeted for degradation in the lysosome. **8.** SORT1 at the PM can be processed via VPS10P domain cleavage by ADAM proteases, or **9.** can act as a pro-apoptotic signaling receptor along with $p75^{NTR}$ via interaction with proneurotrophins after undergoing regulated intramembrane proteolysis (RIP) of the SORT1 cytoplasmic tail.

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Golgi localizing, γ-adaptin ear homology domain, ARF-interacting protein 1, 2 and 3 (GGA-1, -2, -3) (Fig. 9(2)) (Nielsen et al., 2001). SORT1 is also trafficked to the PM, either passively via the constitutive secretory pathway (Fig. 9(3)) (Petersen et al., 1997), or via the regulated secretory pathway in polarized cells (Fig. 9(4)) (Chen et al., 2005). SORT1 proteins at the PM are rapidly internalized into endosomes in clathrin-coated pits via AP-2 (Fig. 9(5)) (Nielsen et al., 2001), and internalized receptors are recycled from maturing lysosomes to the TGN via the retromer complex and AP-1 (Fig. 9(6)) (Canuel et al., 2007), and can by tagged for lysosomal degradation by E3 Ubiquitin Ligase Nedd4 (Fig. 9(7)) (Dumaresq-Doiron et al., 2013). At the PM, SORT1 can undergo ADAM mediated cleavage, to generate a soluble domain of unknown function (Fig. 9(7)) (Evans et al., 2011) and, as mentioned previously, SORT1 at the PM also participates in apoptotic signaling dependent on regulated intramembrane proteolysis (RIP) of the SORT1 cytoplasmic tail (Fig. 9(8)) (Skeldal et al., 2012).

Sortilin Ligands

SORT1 was first characterized as a sorting protein for the lysosome, to which it directs lipoprotein lipase, PRG, prosaposin, TGF-β, and ApoB100 (Fig. 9(2, 5)) (Zeng et al., 2009; Kwon and Christian, 2011; Chamberlain et al., 2013; Tanimoto et al., 2015). SORT1 and its family members additionally govern processing and trafficking of a number of secreted neuropeptides. For example, SORT1 is a major sorting receptor for NTs and their receptors (NTRs), including Brain Derived Neurotrophic Factor (BDNF) to regulated secretory vesicles (Fig. 9(4)), and disruption of SORT1 by acute knockdown (KD) or expression of a dominant negative mutant of SORT1 results in a misrouting of BDNF to the constitutive secretory pathway

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(Chen et al., 2015). SORT1 likewise facilitates anterograde trafficking of NT receptors TRK-A, TRK-B, and TRK-C to the regulated secretory pathway (Fig. 9(4)) (Vaegter et al., 2011). Additionally, SORT family member SORLA has been identified as a regulator of APP processing, which it achieves via binding in the TGN (Carlo et al., 2013). Finally, SORT family members are also PM level receptors, where they bind $p75^{NTR}$ to produce a receptor complex with a high affinity for proNTs that activates a pro-apoptotic signaling cascade (described previously and Fig. 9(9)) (Nykjaer et al., 2004; Teng et al., 2005).

Sortilin and Sonic Hedgehog are Novel Interacting Candidates

Our discovery of SORT1 as a novel interacting candidate with SHHC was fortuitous, as SORT1 is well established as a modulator of the intracellular trafficking of a variety of secreted proteins in neuronal tissues, including those trafficked to the axon (Chen et al., 2005; Evans et al., 2011; Yang et al., 2011; Vaegter et al., 2011), while we were looking for novel regulators of SHH axonal targeting and the Hh C-terminal domain has been shown to be crucial for Hh axonal targeting in flies (Huang and Kunes, 1996). Furthermore, SORT1 interacting specifically with SHHC was intriguing because SHHC is indispensable for the maturation and bioavailability of the SHH ligand (Hall et al., 1997), and mutations that affect the maturation of the SHH ligand are causal in congenital defects and cancer models (Roessler et al., 1997; Maity et al., 2005; Li et al., 2014; Xie et al., 2015). We therefore hypothesize that SORT1 interacts with SHH in neurons to influence the intracellular trafficking and bioavailablity of the mature SHH ligand, and subsequently influences downstream patterning events. We probed this interaction using *in vitro* expression of SHH along with various SORT1 perturbations in cell lines and primary neuron

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cultures, by examining genetic interactions using SORT1 deficient animals in a sensitized SHH system, and by in vivo manipulation of SORT1 function in SHH-dependent tissue patterning. Our results support a model where SORT1 binds the SHHFL precursor protein, thereby antagonizing its processing to SHHNp, and resulting in a reduction of SHHNp secretion and subsequent downstream signaling events.

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MATERIALS AND METHODS

DNA Constructs

SHH, SHHEYFP, SHHNEYFP, SPSHHCECFP, and YFP constructs were prepared as previously described (Beug et al, 2011). pEF, pEF SORT1, and pEF SORT1 furin mutant cDNAs were gifts from Dr. Peder Madsen (Aarhus University, Aarhus DM). SORT1-myc-his and GPC5-flag were generated as described (Beug, 2009). Tagged truncated SORT1 (tSORT1-myc) was a gift from Dr. Carlos Morales (McGill University, Montreal PQ) and was subcloned into the pCAGGS vector for the electroporation studies. BDNF-HA was a gift from Dr. Francis Lee (Cornell University, Ithica NY). Short hairpin constructs targeting SORT1 were obtained from OriGene. Renilla-Luciferase was a gift from Dr. Alan Mears (Children's Hospital of Eastern Ontario, Ottawa ON), GLI-Luciferase was a gift from Dr. H Sasaki (Osaka, Japan).

Cell Culture and Transfection

COS-1 (ATCC) and 3T3 (ATCC) fibroblasts were maintained in DMEM (Life Technologies) supplemented with 10% Fetal Bovine Serum (FBS). PC-12 cells (ATCC) were maintained in RPMI-1640 (Life Technologies), supplemented with 10% Horse Serum (HS), 5% FBS, 2 mM L-glutamine, and 1x Pen/Strep. For differentiation, PC-12 cells were plated on collagen-IV (Sigma) coated plates (20 $\mu g/cm^2$) in RPMI-1640 supplemented with 1% HS, 0.5% FBS, 2 mM L-glutamine, 1x Pen/Strep, and 50 ng/mL NGF2.5S (Millipore) and incubated for 5-7 days. Primary CNs were isolated by trypsinization (Life Technologies) of E14.5 mouse

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cortices and plated on poly-D-lysine (PDL) (Sigma) coated coverslips (Zeiss) in Neurobasal-A (Life Technologies) supplemented with 1x B-27 (Life Technologies), 1x Sato's, 0.5 mM L-glutamine, and Gentamicin (Life Technologies). RGCs were prepared and cultured as previously described (Barres and Chun, 1993). All cells were maintained at 37°C, 5% CO_2. COS-1 and PC12 cells, and primary neurons were transfected with Lipofectamine 2000 (Invitrogen), and 3T3 cells with Trans-IT (Mirus), as per manufacturer instructions. Stable transfectants were selected over two weeks in media containing either 1μg/mL Puromycin (Sigma) or 400 μg/mL Geneticin (Life Technologies). For SORT1 KD, four functional short hairpins and one scrambled control were tested, and efficiency of SORT1 KD was determined via WB. In CNs, sh-expressing neurons were identified by GFP expression and SORT1 KD confirmed via lack of SORT1 staining (via immunocytochemistry (ICC)).

Western Blotting and co-Immunoprecipitation

Cells were lysed in ice-cold RIPA buffer with protease inhibitors (Roche), and protein concentration was determined via a standard Bradford Assay. Lysates were subjected to SDS-PAGE, and proteins were transferred on to Hybond-C Extra membranes (Milipore). Membranes were incubated overnight at 4°C with primary antibodies (Appendix 1), followed by incubation with HRP conjugated secondary antibodies (Milipore) and developed with an ECL detection kit (Milipore). Densitometry was performed on bands using the blot analyzer function on ImageJ. For co-IP, transfected cells were lysed in ice cold buffer containing 10 mM HEPES-NaOH pH 7.4, 100 mM NaCl, 1% NP-40, 10% Glycerol, and protease inhibitors (Roche). Clarified lysates were pre-cleared with Protein A beads (Invitrogen), and incubated with primary antibodies

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(Appendix 1) or species-matched nonspecific IgG, then precipitated using fresh Protein A beads. Protein complexes were eluted from beads using SDS loading buffer, and analyzed via SDS-PAGE.

Analysis of Induced Secretion and Luciferase Assays

PC12 cells cultured in differentiation conditions were transfected with SHHFL, followed by incubation for 5-7 days. Cells were washed twice with pre-warmed basal medium (5.6 mM KCl, 145 mM NaCl, 2.2 mM CaCl2, 0.5 mM MgCl2, 5.6 mM glucose, 15 mM HEPES-KOH (pH 7.4) and 0.1 mg/mL BSA), and incubated for 60 minutes with basal medium or stimulating medium (56 mM KCl, 95 mM NaCl, 2.2 mM CaCl2, 0.5 mM MgCl2, 5.6 mM glucose, 15 mM HEPES-KOH (pH 7.4) and 0.1 mg/mL BSA) with 1 mg/mL Heparin. Cell culture supernatants were centrifuged at 10,000xg to remove cellular debris, and were analyzed via ELISA, as per manufacturer instructions (R&D Systems). For luciferase assays, 3T3 cells were transiently transfected with Renilla-Luciferase and Gli-Luciferase along with indicated plasmids, serum starved for 24h, and incubated with recombinant SHH (R&D Systems) for 24h, followed by measuring Luciferase and Renilla activity, as per manufacturer instructions (Promega).

Mouse Lines and In Utero *electroporation*

Animal husbandry was in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Wild type CD1 mice (Charles River) were used for the isolation of primary CNs. Perinatal

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Sprague Dawley rat pups (Charles River) were used for the isolation of primary RGCs. *Sort1*$^{-/-}$ mice (genotyped by PCR using the following primers: F- 5'CTCAGGAATGGCATTCTCAG3', R- 5'AGCCTTTACCTGGTGTCATC3') (Zeng et al., 2009) and SHH::GFP expressing mice (Jackson Labs) (genotyped as described (Chamberlain et al., 2008)), were maintained on a C57/Bl6J background (Charles River). F2 mice from *Sort1*$^{+/-}$ x *Shh*$^{+/GFP}$ matings were mated to generate *Sort1;Shh::GFP* double mutants and littermate controls at E11.5 and E14.5. Mice were mated in the afternoon, and the presence of a vaginal plug the next morning was considered embryonic day 0.5 (E0.5). *In utero* electroporation was performed by Jimmy Peng (IRCM), as previously described (Garcia-Frigola et al., 2007) at E13.5, and cells in S-phase were labelled in vivo via an intra-peritoneal injection of the dam with 5-ethynyl-2'-deoxyuridine (EdU) (Life Technologies) 1 hour prior to sacrifice at E15.5.

Immunocytochemistry, Immunohistochemistry, In Situ *Hybridization, and Hematoxylin & Eosin Staining*

Cells cultured on PDL-coated coverslips were fixed in 4% PFA for 5-20 minutes and permeabilized with 0.1% Triton-X 100 (as indicated). Fixed cells were incubated overnight at 4°C with primary antibodies (Appendix 1), followed by incubation with fluorophore-conjugated secondary antibodies (1:1,000, Life technologies). Nuclei were stained with Hoechst-33342 (1:25,000, Invitrogen). Coverslips were mounted on permafrost slides (Fisher) with fluorescent mounting medium (Dako). Embryonic tissue was fixed overnight in 4% PFA, cryoprotected in sucrose and frozen in 50:50 30% sucrose:OCT. *In situ* hybridization (ISH), was performed on frozen sections using digoxigenin-labeled antisense probes against *Vsx2, Gli1, Dlx2, and Nkx2.1.*

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For immunohistochemistry (IHC) frozen sections were stained with rabbit anti-GFP and goat anti-Pax2 (1:200; Covance), nuclei were stained with Hoechst-3342 and EdU was detected using the 543 nm fluorophore click-IT reaction kit (Life Technologies). For Hematoxylin & Eosin staining, frozen sections were rehydrated with PBS, stained with Hematoxylin, washed and dehydrated using an ethanol series, stained with Eosin and rehydrated prior to mounting.

Microscopy and Image Analysis

IHC and ICC samples were imaged using a Zeiss LSM510 META confocal microscope with 63x/1.4 Oil Plan-Appochromat or 20x/0.5 EC Plan-Neofluar objectives and the Zen 2009 software; or a Zeiss Axio Imager.M2 with 63x/1.4 Oil Plan-Appochromat or 20x/0.8 Ph2 Plan-Appochromat objectives and AxioVision Rel. 4.8 software. Only cells with an intact nucleus and low to medium expression of transfected constructs were imaged. In neuron images, axons were identified by length (longest process, minimum ten neuron body diameters in length), having few branches, and having positive Tau staining. For distribution analysis, 1nm optical sections of individual neurons were imaged using a constant gain and the pixel intensity was measured using ImageJ, with the mean ratio of signal in the axon compared to the soma calculated from measurements of the indicated number of neurons. Co-localization with organelle markers was quantified in 1nm sections of individual neurons using the Intensity Correlation Analysis plug-in on ImageJ, and mean Pearson's Correlation Coefficients were compared between conditions.

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RESULTS

Sortilin interacts preferentially with full-length Sonic Hedgehog

Our preliminary results indicated that, while SORT1 pulled down with SHHC in the affinity screen, both SHHN and SHHC domains could co-IP with SORT1 (Fig. 7B and C) (Beug, 2009). We next asked whether SORT1-myc-his could co-IP with a wild type SHH construct that undergoes processing from SHHFL (47 kDa band) to yield SHHNp (19 kDa band) (Fig. 10A). Surprisingly, the interaction between SORT 1 and SHHFL was stronger compared with SHHNp (Fig. 10A), suggesting that SORT1 interacts preferentially with SHHFL, rather than SHHN, and consistent with the SHHC pulldown. All subsequent full length SORT1 overexpression experiments were conducted with untagged SORT1.

SORT1 interacting with SHHC and SHHFL is consistent with the interaction occurring early in the secretory pathway, where the majority of SHHFL is processed (Chen et al., 2011, Aikin et al., 2012). To test this hypothesis, we compared SHH distribution in cells that were co-transfected with SHHFL and a truncated isoform of SORT1 (tSORT1), which lacks the cytoplasmic tail and trafficking domains required for proper intracellular trafficking (Nielsen et al., 2001), resulting in the accumulation of bound cargo proteins in the TGN (Chen et al., 2005). We confirmed that tSORT1 could redistribute known ligand BDNF to a perinuclear pattern (Fig. 11A), consistent with the interaction of these proteins in the TGN (Chen et al., 2005). Consistent with an intracellular interaction, tSORT1 co-expression resulted in an accumulation of SHH in a perinuclear pattern in permeabilized cells (Fig. 11B), which overlapped with a TGN marker (Fig.

A.

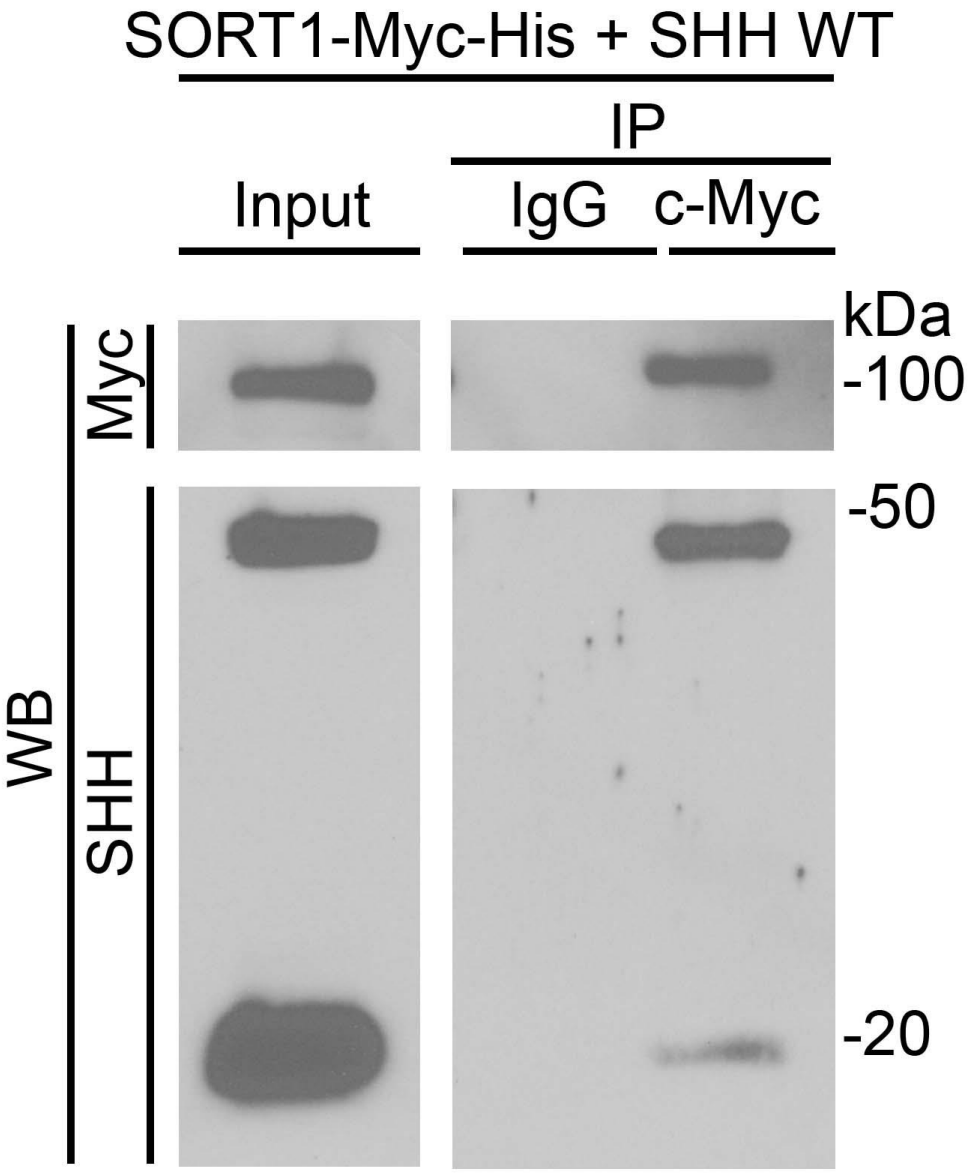

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Figure 10: SORT1-myc-his co-IPs with full length and N-terminal Sonic Hedgehog. (A) co-IP of SORT1-myc-his from lysates of COS cells transfected with SHHFL and SORT1-myc-his. Lysates IP'd with anti-myc (right panel, right lane) or species matched IgG (right panel, left lane). IP and input samples were analyzed by WB for SHH (bottom panels) or SORT1-myc-his (top panels). Representative blots from 3 independent experiments.

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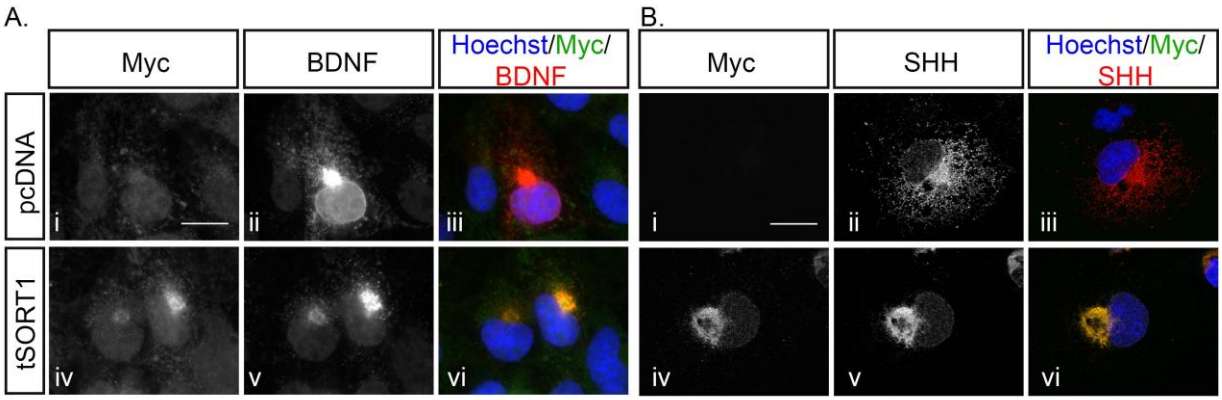
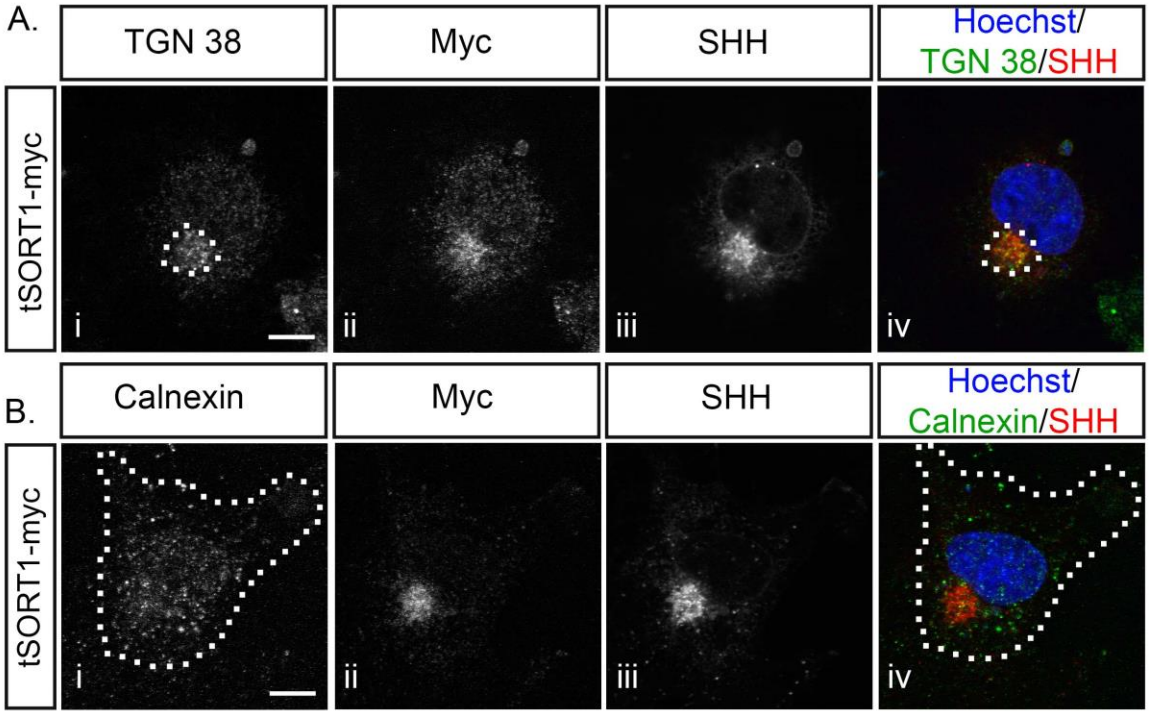

A.
Myc
BDNF
Hoechst/Myc/
BDNF
pcDNA
tSORT1
i
ii
iii
iv
v
vi
B.
Myc
SHH
Hoechst/Myc/
SHH
i
ii
iii
iv
v
vi

`

Figure 11: Truncated Sortilin sequesters BDNF-HA and Sonic Hedgehog early in the secretory pathway. (A, B) Effect of tSORT1 on BDNF-HA and SHH distribution. Representative ICC and Hoechst nuclear labeling on fixed and permeabilized COS cells expressing BDNF-HA (A) or SHHFL (B), with pcDNA (top rows), and tSORT1-myc (bottom rows). Images are representative from 3 independent experiments; scale bar, 10μm


A.
TGN 38
Myc
SHH
Hoechst/
TGN 38/SHH
tSORT1-myc
i
ii
iii
iv
B.
Calnexin
Myc
SHH
Hoechst/
Calnexin/SHH
tSORT1-myc
i
ii
iii
iv

`

Figure 12: Truncated Sortilin sequesters Sonic Hedgehog in the TGN, not the ER. (A, B) Representative ICC and Hoechst nuclear staining on fixed and permeabilized COS1 cells expressing SHHFL and tSORT1-myc. Dashed lines indicate extent of TGN (A, marked by TGN-38) or ER staining (B, marked by Calnexin). Images are representative from 3 independent experiments; scale bar, 10μm

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12A) but not with an ER marker (Fig. 12B). These results are consistent with the possibility that SORT1 interacts with SHH in the TGN.

In addition to intracellular protein sorting, a small pool of SORT1 localizes to the PM where it functions in neurotrophin signaling (Nykjaer et al., 2004; Teng et al., 2005). Thus, we investigated whether SORT1 expression in SHH-receiving cells would modulate SHH pathway activation. We transfected 3T3 cells with SORT1 or GPC5, a SHH-binding protein that potentiates SHH signaling (Li et al., 2011), and monitored activation of a GLI-luciferase SHH reporter in response to treatment with recombinant SHH. SORT1 expression had no effect on SHH signaling compared with controls, while expression of GPC5 strongly enhanced SHH signaling (Fig. 13A). Taken together, these results suggest that SORT1 interactions with SHH are more relevant to SHH trafficking or processing rather than the response of cells to SHH ligand.

Sortilin promotes the persistence of full-length Sonic Hedgehog

Based on the evidence that SORT1 interacts preferentially with SHHC and SHHFL, and that tSORT1 retains SHH in the TGN, we investigated whether this interaction affected intracellular levels of SHH. We transfected cells with SHH and SORT1 perturbation constructs, and after 6 hours of cycloheximide treatment (to block protein synthesis) measured SHHFL levels relative to the total SHH pool (SHHFL + SHHNp). We used the PC12 and 3T3 cell lines, which have been shown to process SHH efficiently (Vincent et al., 2003; Beug et al., 2011). Increasing SORT1 by SORT1 or tSORT1 transfection (constructs used summarized in Fig. 14A) increased the relative levels of SHHFL compared to vector control (Fig. 14B, B' center left and far right panels).

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A.

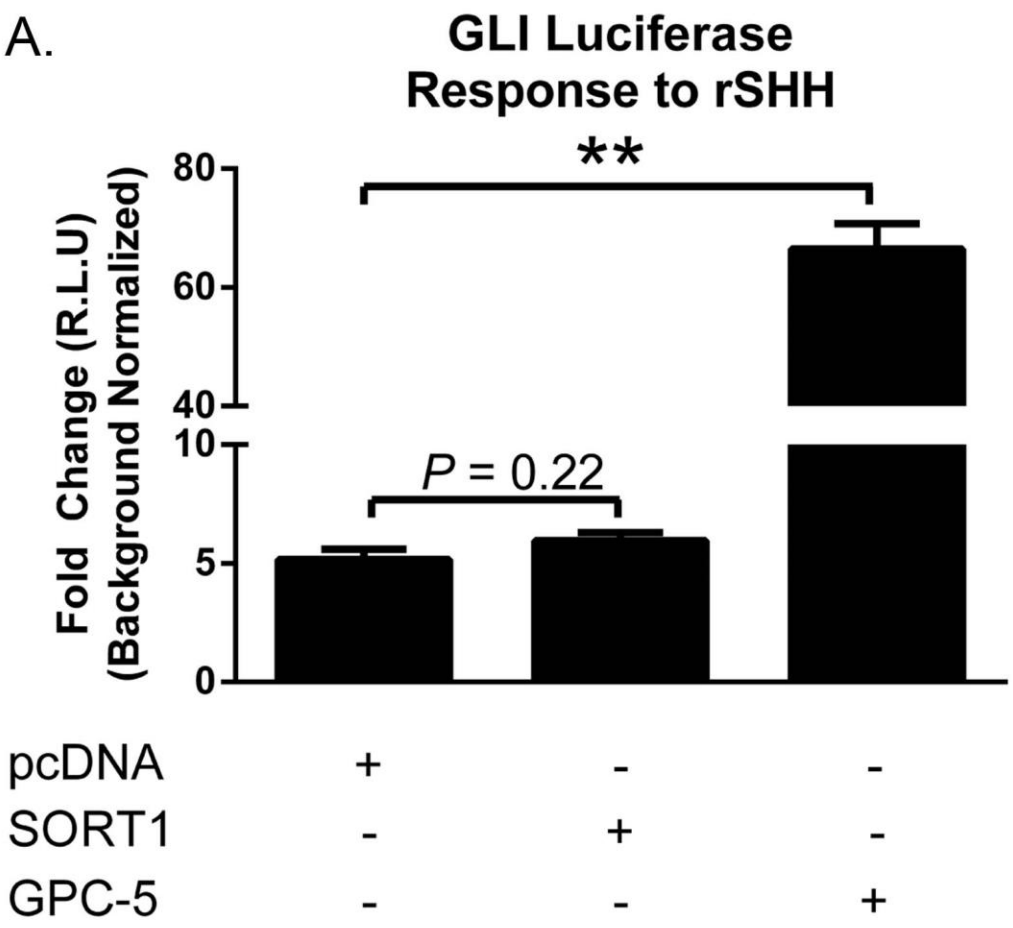
GLI Luciferase
Response to rSHH
**
Fold Change (R.L.U)
(Background Normalized)
80
60
40
10
5
0
P = 0.22
pcDNA + - -
SORT1 - + -
GPC-5 - - +

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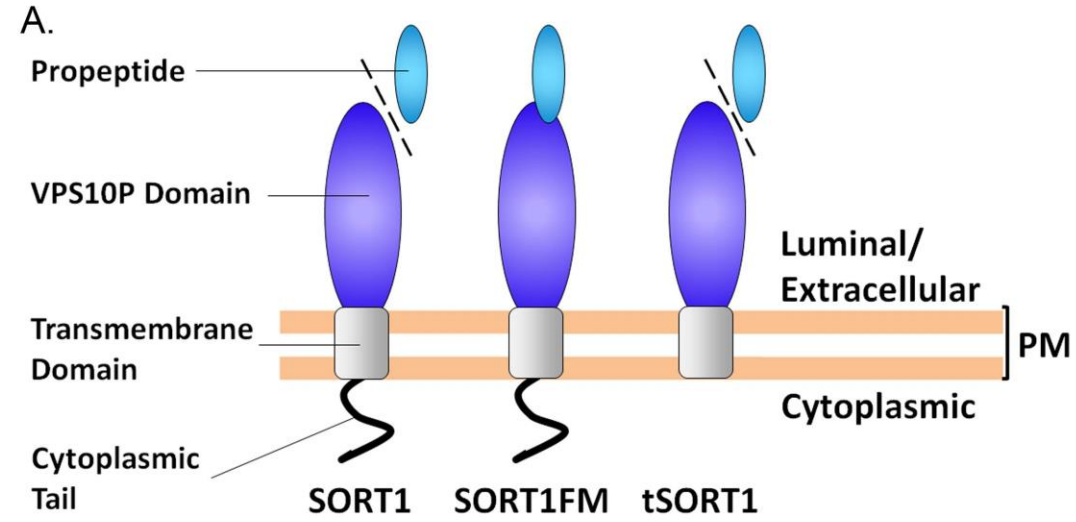
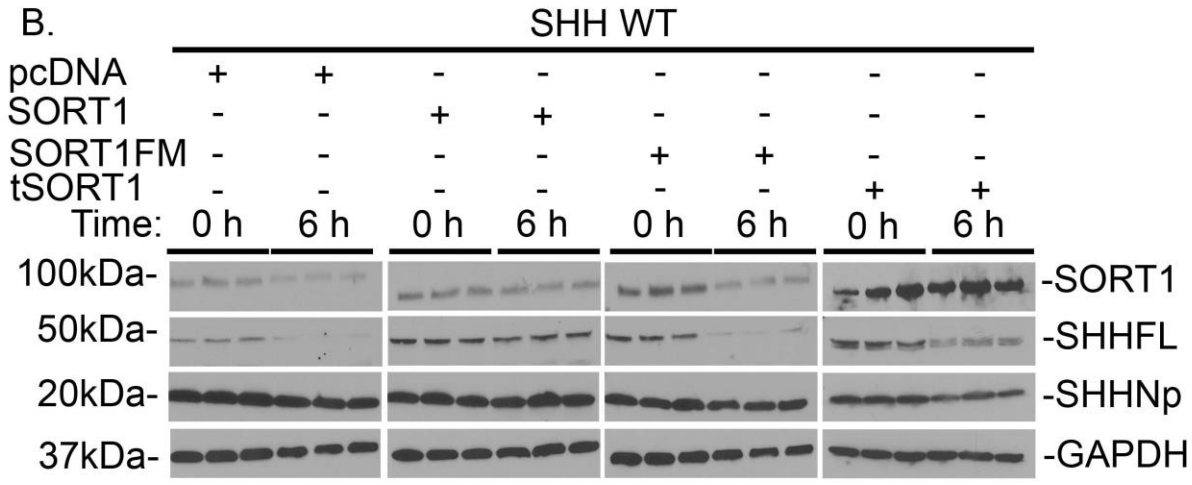
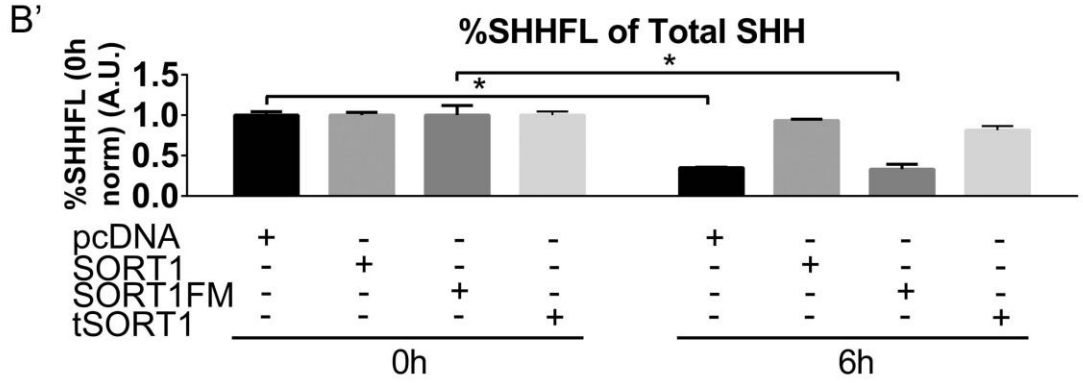
Figure 13: Sortilin1 perturbation does not alter Sonic Hedgehog target gene expression in receiving cells. (A) SORT1 overexpression in receiving cells does not affect signal transduction in response to exogenous SHH. Gli-luciferase activity in serum starved 3T3 cells transfected with the indicated expression vectors, along with GLI-dependent luciferase and constitutively active Renilla-luciferas, and treated with recombinant SHH for 24 hours. Bars indicate mean GLI-luciferase activity normalized to constitutive Renilla-luciferase (n = 3 independent experiments per condition) relative to untreated cells. Error bars represent SEM, ** $P < 0.01$, Student's *t*-test.


A.
Propeptide
VPS10P Domain
Transmembrane Domain
Cytoplasmic Tail
Luminal/ Extracellular
PM
Cytoplasmic
SORT1
SORT1FM
tSORT1


B.
SHH WT
pcDNA + + - - - - - -
SORT1 - - + + - - - -
SORT1FM - - - - + + - -
tSORT1 - - - - - - + +
Time: 0 h 6 h 0 h 6 h 0 h 6 h 0 h 6 h
100kDa- -SORT1
50kDa- -SHHFL
20kDa- -SHHNp
37kDa- -GAPDH


B'
%SHHFL of Total SHH
%SHHFL (0h norm) (A.U.)
1.5
1.0
0.5
0.0
*
*
pcDNA + - - - + - - -
SORT1 - + - - - + - -
SORT1FM - - + - - - + -
tSORT1 - - - + - - - +
0h
6h

`

Figure 14: Sortilin1 overexpression correlates with increased persistence of full length Sonic Hedgehog after cycloheximide treatment dependent on propeptide cleavage. (A) Schematic of SORT1 constructs used in this analysis. SORT1 is the wild type receptor; SORT1FM contains a mutation in its furin cleavage domain such that the propeptide cannot be cleaved, rendering the VPS10P domain inactive for ligand binding; tSORT1 lacks the cytoplasmic domain, and is this not actively trafficked to any SORT1 target organelles. **(B)** Western blot analysis of lysates from control (0 hours) and cycloheximide-treated (6 hours) PC12 cells transiently transfected with SHHFL and control, SORT1, SORT1FM, or tSORT expression vectors. Cell lysates were blotted for SORT1 constructs, SHH, and GAPDH as a loading control. Note the degradation of endogenous SORT1 (left), WT SORT1 (middle left), and SORT1FM (middle right) after six hours, indicative of ubiquitination and subsequent lysosomal degradation. tSORT1 is not targeted to endosomes, and therefore is not degraded (right). **(B')** SHH protein densitometry was determined using the Gels function on ImageJ. Bars indicate mean SHHFL levels from 3 independent experiments as a function of total SHH (SHHFL / (SHHFL + SHHNp)), normalized to t=0 untreated. Error bars represent SEM, * $P < 0.05$, Student's *t*-test.

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Conversely, short hairpin RNAi-mediated knockdown (KD) of SORT1 reduced relative levels of SHHFL (Fig. 15A, A'). These results are consistent with a role for SORT1 in SHH processing or stability.

A role for SORT1 in antagonizing SHH processing or regulating SHHFL stability is surprising, because SHH processing occurs primarily in the ER (Chen et al., 2011), while SORT1 is thought to only bind ligands after Furin-mediated cleavage of its pro-peptide in the TGN (Munck Petersen et al., 1999). To address whether SORT1 requires Furin cleavage in the TGN to mediate its effect on the levels of SHHFL, we co-expressed SHHFL with a mutant SORT1 that cannot be cleaved by Furin (SORT1FM) (Munck Petersen et al., 1999). In this instance, SORT1FM did not increase the levels of SHHFL protein (Fig. 14A, A' center right panels), indicating that Furin cleavage is required for SORT1 to promote SHHFL accumulation, and by extension that some degree of SHH cleavage that occurs in the TGN.

A positive correlation between SORT1 levels and SHHFL persistence could also occur if SHHFL was protected from lysosomal degradation, a known SORT1 target pathway (Hong et al., 1996; Lefrancois et al., 2003; Hassan et al., 2004; Ni and Morales, 2006; Canuel et al., 2008; Zeng et al., 2009; Evans et al., 2011; Yang et al., 2013). To test the lysosomal degradation hypothesis, we measured SHHFL stability in the presence or absence of the lysosomal inhibitor Bafilomycin A1 (BafA1), and observed no effect on SHHFL or SHHNp levels (Fig. 16A, A'). Moreover, if SORT1 is required for targeting SHHFL to the lysosome, either directly or indirectly via sorting of lysosome components, we would have predicted an increase in SHHFL in SORT1 KD cells. Taken together, these observations suggest that lysosomal targeting is not a major mechanism for controlling this pool of SHHFL in this context, and is therefore consistent

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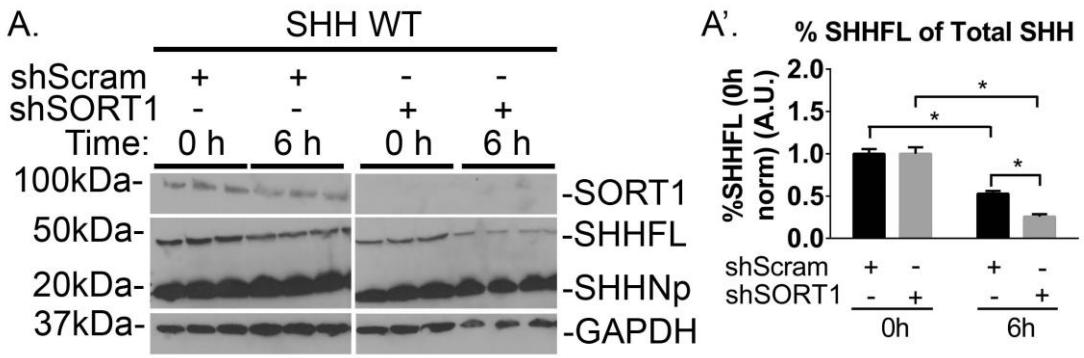
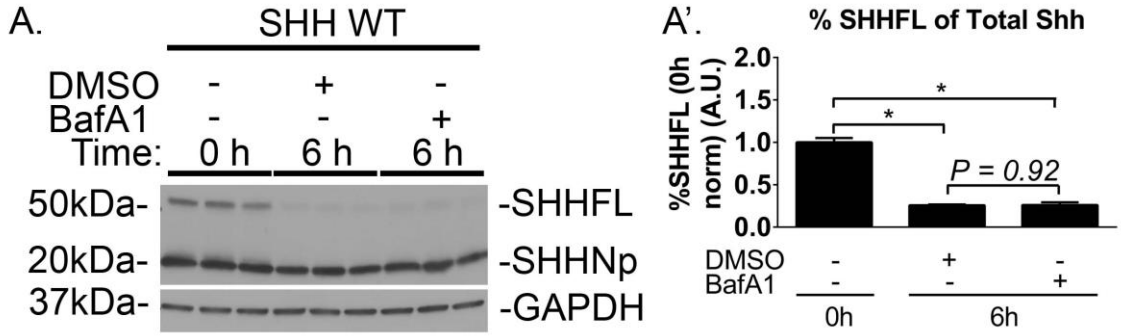

A.
SHH WT
shScram + + - -
shSORT1 - - + +
Time: 0 h 6 h 0 h 6 h
100kDa-
50kDa-
20kDa-
37kDa-
-SORT1
-SHHFL
-SHHNp
-GAPDH
A'.
% SHHFL of Total SHH
%SHHFL (0h norm) (A.U.)
2.0
1.5
1.0
0.5
0.0
*
*
*
shScram + - + -
shSORT1 - + - +
0h
6h

`

Figure 15: Sortilin1 knock down correlates with decreased persistence of full length Sonic Hedgehog after cycloheximide treatment. (A) Western blot analysis of lysates from control (0 hours) and cycloheximide-treated (6 hours) PC12 cells stably expressing shSORT1 or shScram and transiently transfected with SHHWT. Cell lysates were blotted for endogenous SORT1, SHH, and GAPDH as a loading control. Note that SORT1 is present in the shScram lane, but not in the shSORT1 lane. SHH blot shows a higher exposure than in Fig. 14 in order to better visualize the differences in SHHFL protein level between shScram and shSORT1 conditions at 6h. **(A')** SHH protein densitometry was determined using the Gels function on ImageJ. Bars indicate mean SHHFL levels from 3 independent experiments as a function of total SHH (SHHFL / (SHHFL + SHHNp)), normalized to t=0 untreated. Error bars represent SEM, * $P <$ 0.05, Student's *t*-test.


A.
SHH WT
DMSO - + -
BafA1 - - +
Time: 0 h 6 h 6 h
50kDa-
-SHHFL
20kDa-
-SHHNp
37kDa-
-GAPDH
A'.
% SHHFL of Total Shh
%SHHFL (0h norm) (A.U.)
2.0
1.5
1.0
0.5
0.0
*
*
P = 0.92
DMSO - + -
BafA1 - - +
0h
6h

`

Figure 16: Full length Sonic Hedgehog is not degraded in lysosomes in this context. (A) Effect of lysosome inhibitor BafA1 on SHH levels in PC12 cells. Western blot analysis of lysates from control (0 hours) and cycloheximide-treated plus DMSO or BafA1 (6 hours) PC12 cells transiently transfected with SHHFL. Cell lysates were blotted for endogenous SORT1, SHH, and GAPDH as a loading control. (A') SHH protein densitometry was determined using the Gels function on ImageJ. Bars indicate mean SHHFL levels from 3 independent experiments as a function of total SHH (SHHFL / (SHHFL + SHHNp)), normalized to t=0 untreated. Error bars represent SEM, * $P < 0.05$, Student's *t*-test.

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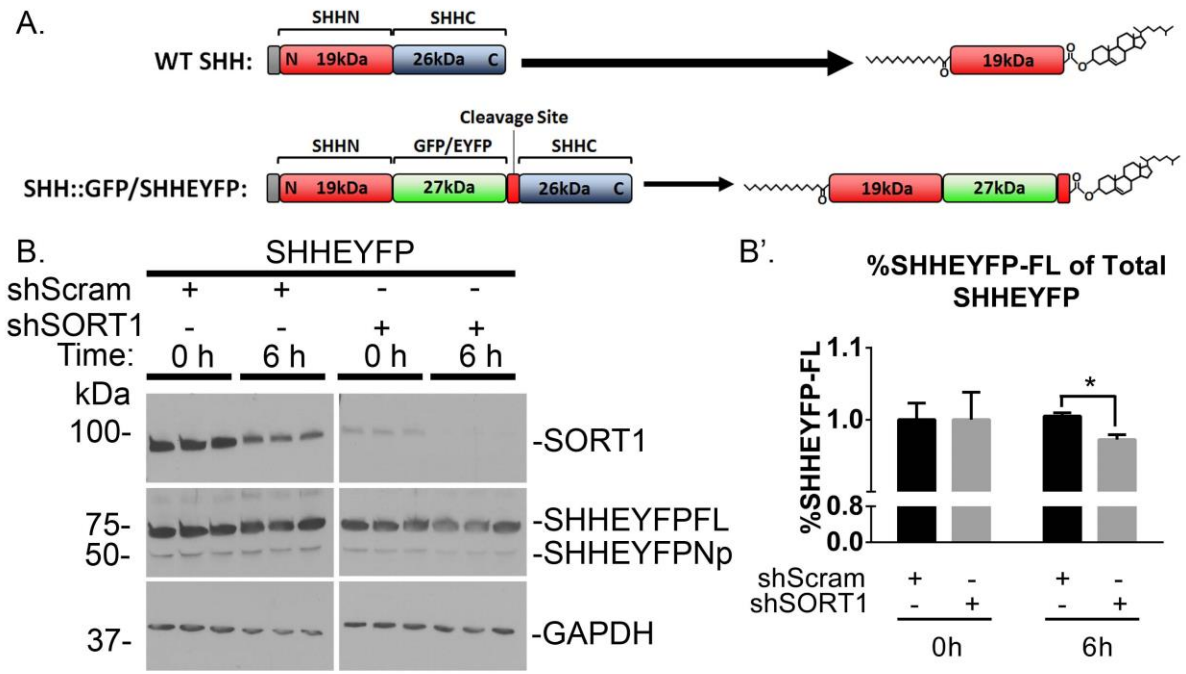
with a model where SORT1 associates with SHHFL in the TGN via its luminal ligand-binding domain, and inhibits processing.

Sortilin knockout can rescue midline defects in a Sonic Hedgehog processing mutant

Germline SORT1 knockout (*Sort1*$^{-/-}$) is not associated with developmental defects, including phenotypes associated with altered SHH signaling (Jansen et al., 2007; Zeng et al., 2009). Therefore, to examine whether SORT1 function impacts SHH processing *in vivo*, we examined its effect in a sensitized system using a processing deficient SHH mutant mouse model, the SHH::GFP homozygous knock-in (*Shh*$^{GFP/GFP}$) (Chamberlain et al, 2008). SHH::GFP, and our analogous SHHEYFP construct, is inefficiently processed due to the insertion of a fluorescent tag in the N terminal domain (summarized in Fig. 17A) (Chamberlain et al., 2008). Consistent with this processing defect, *Shh*$^{GFP/GFP}$ is embryonic lethal, and is characterized by craniofacial deformities and cyclopia due to loss of SHH function (Chamberlain et al., 2008). Previous work has shown that eye field defects observed in SHH deficient mice, including the *Shh*$^{GFP/GFP}$ model, can be rescued by increasing SHH signaling by reducing the gene dosage of SHH pathway antagonists (Litingtung and Chiang, 2000; Persson et al., 2002; Bai et al., 2004; Stamataki et al., 2005; Furimsky and Wallace, 2006; Chamberlain et al., 2008). Because SORT1 limits the rate of SHH processing in vitro, we reasoned that loss of function for SORT1 would ameliorate some of the Hh-associated defects in *Shh*$^{GFP/GFP}$ mice. We first confirmed that SORT1 could increase SHHEYFP processing *in vitro* (Fig. 17B, B'). Next we generated *Shh*$^{GFP/GFP}$ animals on wildtype or SORT1 mutant backgrounds (*Sort1*$^{+/+}$ versus *Sort1*$^{-/-}$) and compared the width of the eye field at E14.5, an age where bilateral separation of the eye field is


A.
SHHN
SHHC
WT SHH:
N 19kDa
26kDa C
19kDa
Cleavage Site
SHHN
GFP/EYFP
SHHC
SHH::GFP/SHHEYFP:
N 19kDa
27kDa
26kDa C
19kDa
27kDa
B.
SHHEYFP
shScram + + - -
shSORT1 - - + +
Time: 0 h 6 h 0 h 6 h
kDa
100-
-SORT1
75-
-SHHEYFPFL
50-
-SHHEYFPNp
37-
-GAPDH
B'.
%SHHEYFP-FL of Total SHHEYFP
%SHHEYFP-FL
1.1
1.0
0.8
0.0
*
shScram + - + -
shSORT1 - + - +
0h
6h

`

Figure 17: Sortilin1 knock down correlates with decreased cleavage of YFP tagged SHH after cycloheximide treatment. (A) Schematic of SHH::GFP and SHHEYFP constructs used in subsequent experiments. WT SHHFL (45 kDa) efficiently undergoes numerous posttranslational modifications, yielding SHHNp (19 kDa). SHHEYFP/GFPFL (72 kDa) have a fluorescent tag inserted in to the C-terminus of SHHN, followed by a cleavage site for the SHHC intein domain. Processing occurs as it does with WT SHH to yield SHHEYFP/GFPNp (46 kDa), albeit with reduced efficiency. **(B, B')** SHHEYFPFL processing is enhanced in the absence of SORT1. **(B)** Western blot analysis of control (0 hours) or cycloheximide (6 hours) treated PC12 cells stably expressing shSORT1 or shScram, and transiently transfected with SHHEYFP. Cell lysates were blotted for SORT1, SHH, and GAPDH as a loading control. Note that SHHEYFPFL persists in excess after 6h. (**A'**) SHH protein densitometry was performed using the Gels function on ImageJ. Bars indicate mean SHHEYFPNp levels from 3 independent experiments as a function of total SHHEYFP signal (SHHEYFPNp / (SHHEYFPFL + SHHEYFPNp)), normalized to t=0 untreated. Error bars represent SEM, * $P < 0.05$, Student's *t*-test.

`

readily apparent in wild type animals (Fig. 18Ai) (Marti et al., 1995, Lipinski et al., 2010). Remarkably, the optic field width (as confirmed by neural retina marker *Vsx2* expression (Fig. 18Aii)) was significantly increased in $Shh^{GFP/GFP}$; $Sort1^{-/-}$ mice compared with $Shh^{GFP/GFP}$; $Sort1^{+/+}$ controls (Fig. 18A iii, iv and A'). However, we did not observe full bilateral separation of the eye field comparable to $Shh^{+/GFP}$ levels (Fig. 18Ai and A'), indicating that there is not full rescue of SHH signaling in the compound mutants, which is also consistent with the failure to rescue the expression patterns at E11.5 of target genes *Nkx2.1* (Fig. 19, A-C) and *Dlx2* (Fig. 19, D-F), ventral midline markers known to be dependent on SHH signaling at this age (Rallu et al., 2002). This data is consistent with the interpretation that SORT1 exerts an inhibitory effect on SHH processing in vivo such that SORT1 deficiency results in a partial rescue of midline defects associated with defective SHH processing (summarized in Fig. 18C).

Sortilin reduces trafficking of Sonic Hedgehog to the regulated secretory pathway and activity dependent secretion

In some projection neurons of the CNS, SHH processing and lipid modification is required for targeting to the regulated secretory pathway (RSP) (Beug, 2009; Beug et al., 2011; Petralia et al., 2011), and activity dependent secretion (Beug, 2009; Beug et al., 2011). Because SORT1 inhibits SHH processing in non-neuronal cell lines, we next investigated how SORT1 would affect SHH targeting to the RSP in primary neurons. In retinal ganglion cells (RGCs), which express SHH and SORT1 endogenously (Fig. 20A), SHH co-localized extensively with SORT1 in the somatodendritic region, but not the axon (Fig. 20A and A'). We observed a similar co-localization pattern between endogenous SORT1 and transfected SHH in in primary cortical

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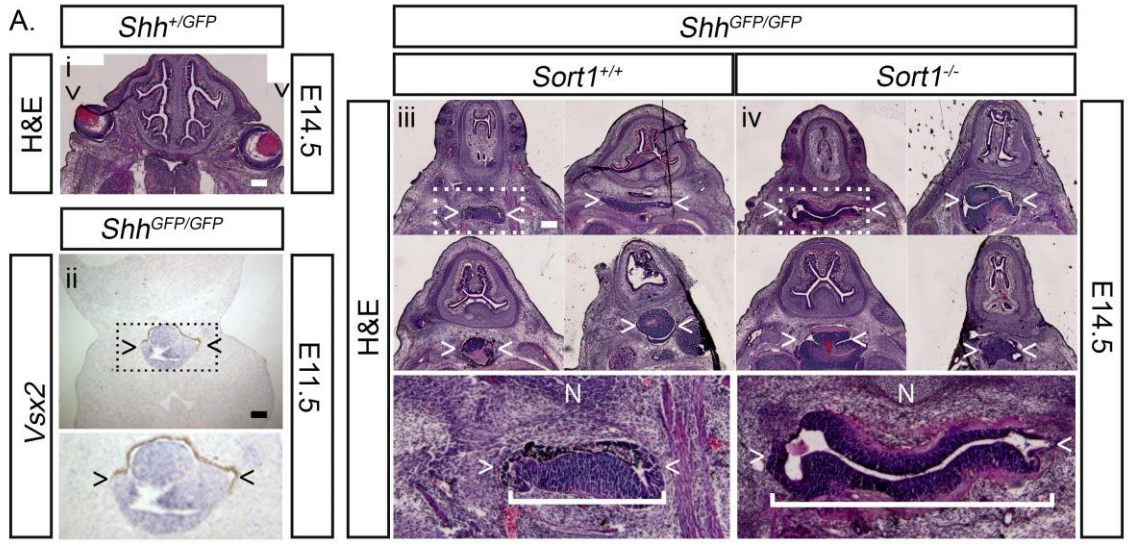
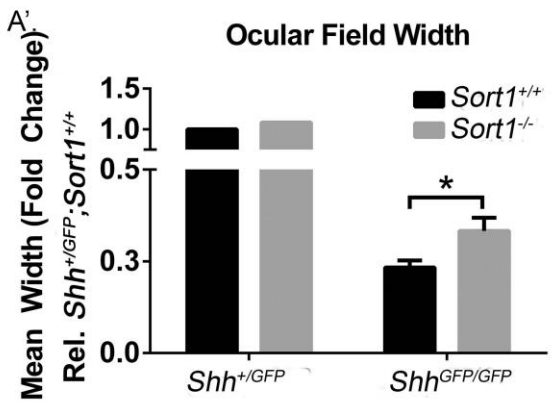
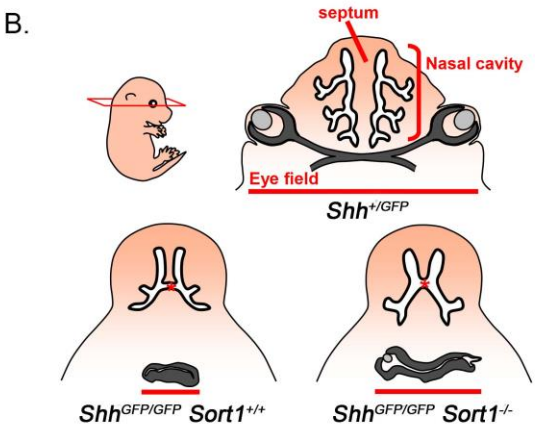

A.
Shh+/GFP
ShhGFP/GFP
Sort1+/+
Sort1-/-
H&E
E14.5
E11.5
Vsx2
i
ii
iii
iv
N


A'
Ocular Field Width
Mean Width (Fold Change)
Rel. Shh+/GFP;Sort1+/+
1.5
1.0
0.5
0.3
0.0
Sort1+/+
Sort1-/-
*
Shh+/GFP
ShhGFP/GFP


B.
septum
Nasal cavity
Eye field
Shh+/GFP
ShhGFP/GFP Sort1+/+
ShhGFP/GFP Sort1-/-

`

Figure 18: Impact of Sortilin1 knock out on patterning mediated by processing deficient Sonic Hedgehog mutants. (A, A') SORT1 KO is associated with an increase in eye field width in the $Shh^{GFP/GFP}$ homozygous mutant. H&E staining in horizontal (i, ii, iv) or coronal (ii) sections at the level of the eye field of mouse heads of $Shh^{GFP/GFP}$ mice on $Sort1^{+/+}$ and $Sort1^{-/-}$ backgrounds at E14.5. Wild type animals exhibit proper bilateral symmetry of the eyes (i). ISH in coronal sections of E11.5 $Shh^{GFP/GFP}$ brains confirms the location of a central *Vsx2+* single eye at the midline (ii). Examples from four mice per genotype are shown and lower panels are higher magnification images corresponding to the regions indicated in the dashed boxes (iii and iv). > <, indicates the location of eye field; N, nasal cavity to indicate the orientation of the tissue; ticked white line indicates the width of the eye field. (A'') Quantification of mean eye field width in compound $Shh^{+/GFP}$; $Sort1^{+/+}$ (n = 2), $Shh^{+/GFP}$; $Sort1^{-/-}$ (n = 2), $Shh^{GFP/GFP}$; $Sort1^{+/+}$ (n = 6) and $Shh^{GFP/GFP}$; $Sort1^{-/-}$ (n = 5) at E14.5. Serial sections were taken of the eye field for each genotype, and the eye field was measured at its widest point therein. Bars represent mean width of eye field, normalized to $Shh^{+/GFP}$;$Sort1^{+/+}$. Error bars represent SEM, * $P < 0.05$, Student's *t*-test. **(B)** Schematic showing plane of section and landmarks used for the analysis of H and E stained sections.

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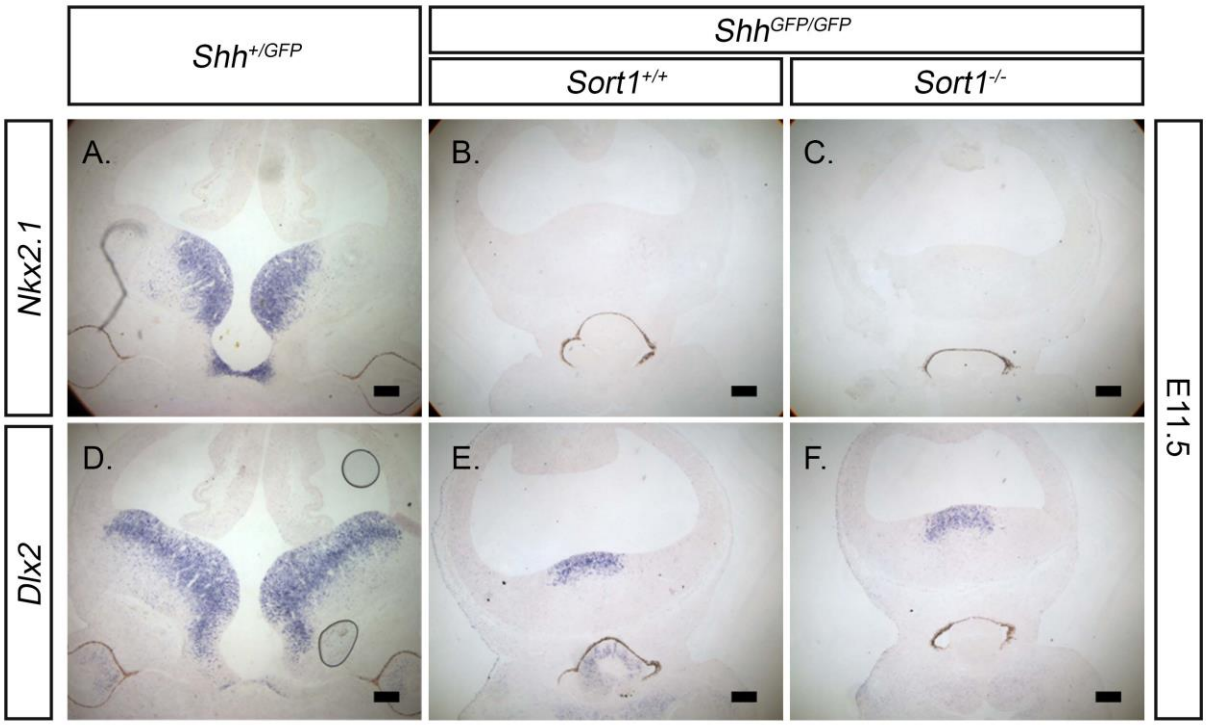
Shh+/GFP
ShhGFP/GFP
Sort1+/+
Sort1-/-
Nkx2.1
Dlx2
E11.5
A.
B.
C.
D.
E.
F.

`

Figure 19: Sortilin1 knock out does not rescue target gene expression in Sonic Hedgehog mutants as measured by in situ hybridization. (A-F) ISH in coronal sections of E11.5 $Shh^{+/GFP}$ (considered as the wild type condition) (A, D), $Shh^{GFP/GFP}$;$Sort1^{+/+}$ (B, E), or $Shh^{GFP/GFP}$;$Sort1^{-/-}$ for ventral midline marker *Nkx2.1* (A-C) or pan-ventral marker *Dlx2* (D-F). Images representative of four animals per genotype.

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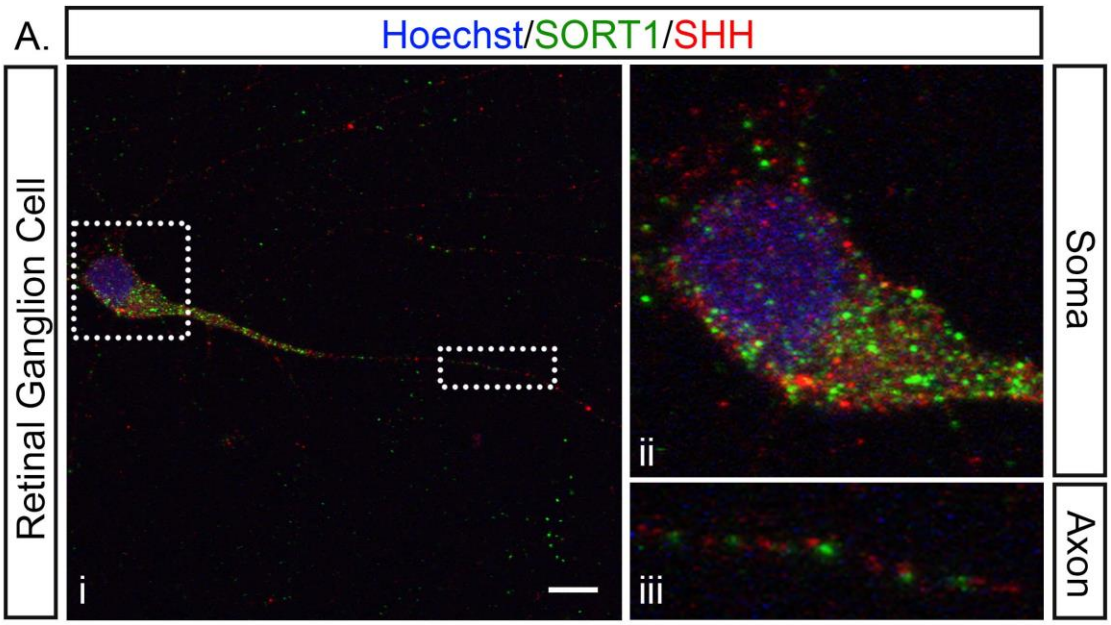
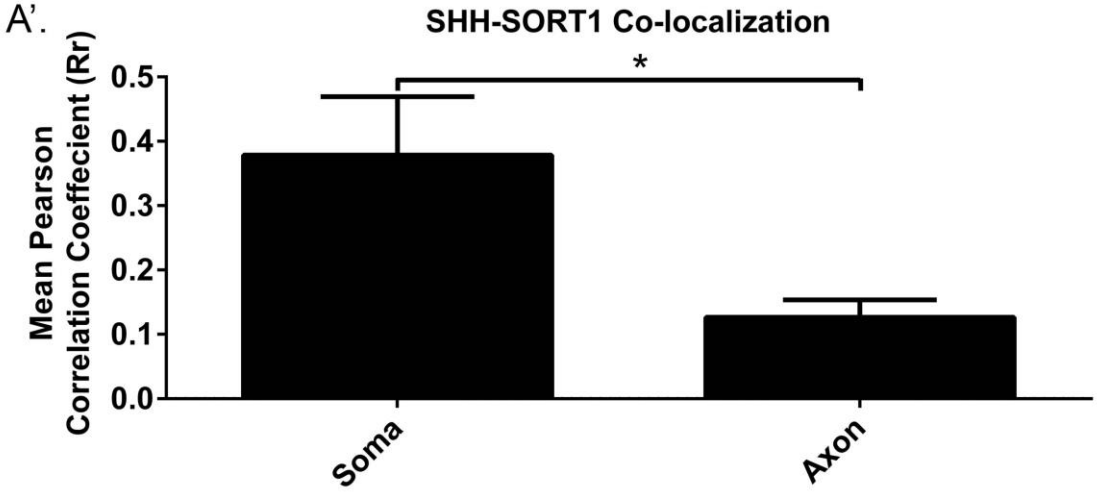

A.
Hoechst/SORT1/SHH
Retinal Ganglion Cell
i
ii
iii
Soma
Axon


A'.
SHH-SORT1 Co-localization
*
Mean Pearson Correlation Coeffecient (Rr)
0.5
0.4
0.3
0.2
0.1
0.0
Soma
Axon

`

Figure 20: Co-localization of endogenous Sonic Hedgehog and Sortilin1 in RGCs. (A, A') SHH and SORT1 co-localize extensively in the somatodendritic compartment, but not in axons, in primary RGCs. Representative ICC and Hoechst nuclear staining on fixed and permeabilized RGCs. Left panel shows low magnification view of the entire neuron (i), right panels show higher magnification images corresponding to the regions indicated in the dashed boxes (ii and iii). (A') Quantification of the co-localization of SHH and SORT1 in the indicated subcellular compartment in RGCs. Bars represent mean Pearson's Correlation Coeffecient (Rr) (n = 5 cells per condition). Error bars represent SEM, * $P < 0.05$, Student's *t*-test. Scale bars, 10μm.

`

neurons (CNs) (Fig. 21A and A'), confirming the efficacy of these neurons in subsequent SORT1 perturbation studies. We next examined the effect of SORT1 perturbations on SHH axonal targeting by examining two different pools of axonal SHH: extracellular (where it collects on lipid rafts) and intracellular (where it collects in regulated secretory vesicles), both of which require proper processing of SHH (Beug, 2009; Beug et al., 2011). When we compared the distribution of SHH in CNs as a function of SORT1 expression we found an inverse relationship between SORT1 expression and the levels of SHH in the axon. RNAi-mediated knockdown of SORT1 with two sh constructs in CNs (sh SORT1A and B, validation shown in Fig. 22) increased in axons intracellular SHH colocalization with RSP vesicles (marked by Synaptic Vesicle Protein 2 (SV2)) and extracellular SHH (Fig. 23A, B). Similarly, effects on SHH distribution were observed in CNs isolated from *Sort1*$^{-/-}$ mice (Fig. 24A, B). Re-introduction of SORT1 expression in *Sort1*$^{-/-}$ neurons significantly reduced SHH levels in the axons (Fig. 25B), and reduced, albeit not significantly, co-localization of SHH with SV2+ vesicles (Fig. 25A), suggesting that the effect was specific to changes in SORT1 levels. The effect of SORT1 on SHH distribution in the axon was specific to SHH, as SORT1 KD had the opposite effect on BDNF-HA distribution, abolishing axonal trafficking (Fig. 26A), consistent with SORT1 directing BDNF-HA to the axon (Chen et al, 2005). There was no apparent redirection of SHH from other subcellular compartments, because co-localization of SHH with lysosomal or endosomal markers was not changed in *Sort1*$^{-/-}$ neurons (Fig. 26B). Finally, SORT1 overexpression did not alter the total level of SV2+ vesicles in the axons (Fig. 26C), indicating that the effect of SORT1 on axonal SHH was not secondary to a general perturbation of biogenesis or trafficking of SV2+ vesicles in the axon. These results are consistent with SORT1 antagonizing SHH targeting to axons and axonal SV2+ vesicles, which is independent of

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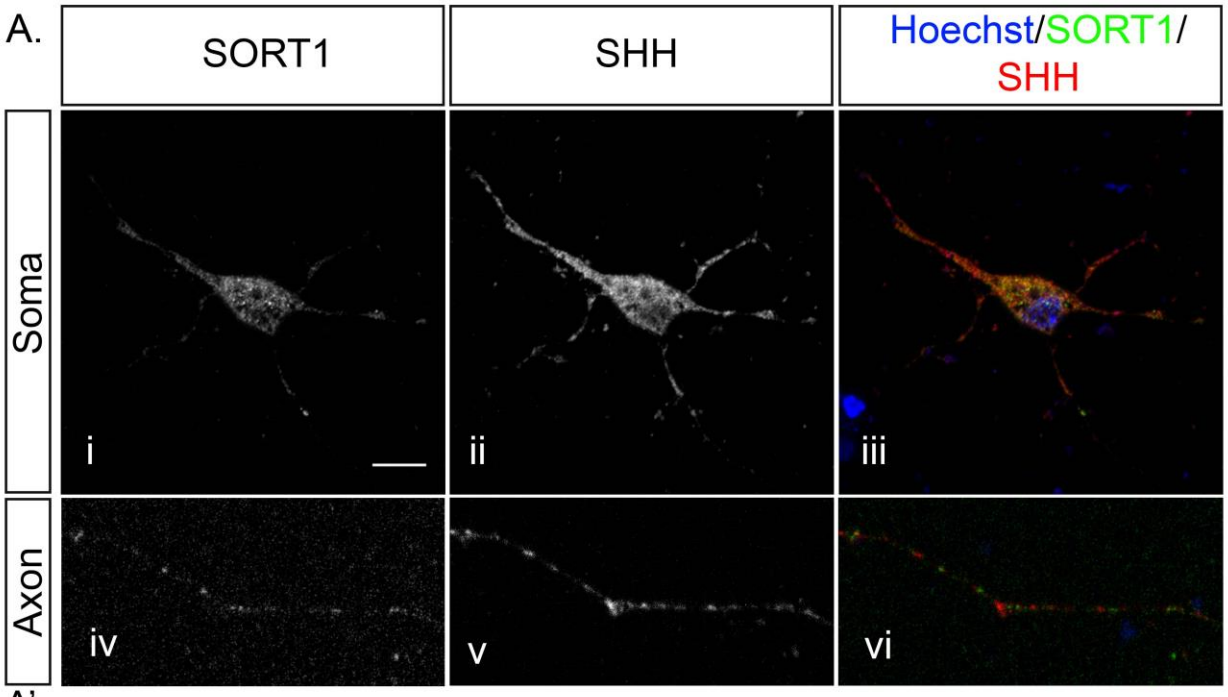
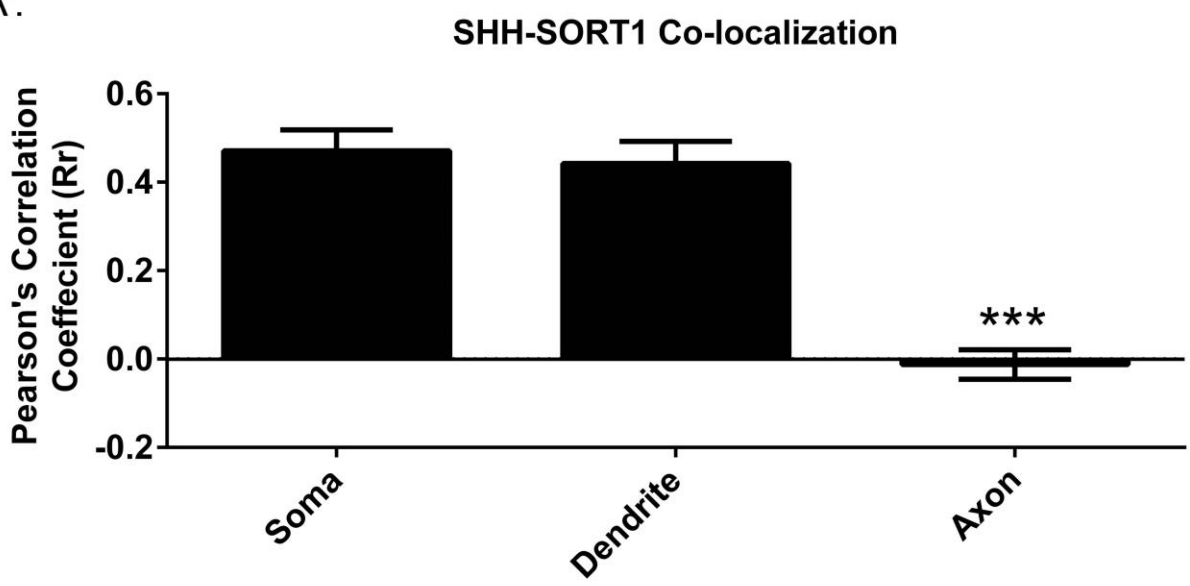
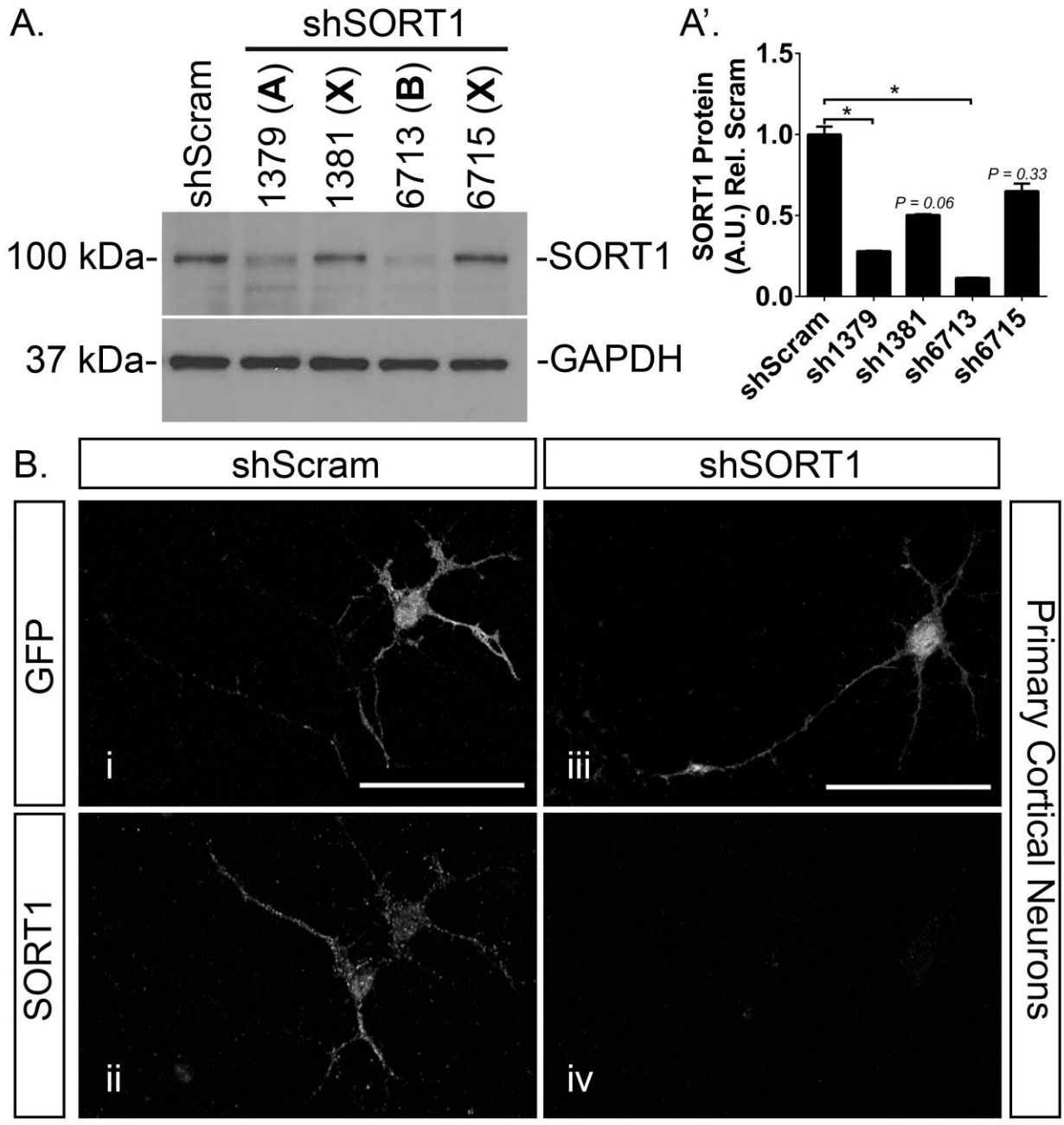

A.
SORT1
SHH
Hoechst/SORT1/
SHH
Soma
Axon
i
ii
iii
iv
v
vi

A'.


SHH-SORT1 Co-localization
Pearson's Correlation
Coeffecient (Rr)
0.6
0.4
0.2
0.0
-0.2
***
Soma
Dendrite
Axon

`

Figure 21: Co-localization of exogenous Sonic Hedgehog and endogenous Sortilin1 in primary CNs. (A) Representative ICC and Hoechst nuclear staining on fixed and permeabilized CNs. Panels show 1nm optic sections in the somatodendritic (upper), or axonal (lower) compartments. Scale bars, 10μm. **(A')** Quantification of co-localization of SHH and SORT1 in the indicated subcellular compartment in CNs from A. Bars represent mean Pearson's Correlation Coeffecient (Rr) (n = 20 neurons). Error bars represent SEM, *** $P < 0.001$, two tailed Student's t-test, relative to Soma.


A.
shSORT1
shScram
1379 (A)
1381 (X)
6713 (B)
6715 (X)
100 kDa-
-SORT1
37 kDa-
-GAPDH
A'.
SORT1 Protein (A.U.) Rel. Scram
1.5
1.0
0.5
0.0
*
*
P = 0.06
P = 0.33
shScram
sh1379
sh1381
sh6713
sh6715
B.
shScram
shSORT1
GFP
SORT1
i
ii
iii
iv
Primary Cortical Neurons

`

Figure 22: Validation of short hairpin-mediated Sortilin knockdown in primary neurons. (A, A') Representative western blots analysis of SORT1 levels in 3T3 cells transiently transfected with a control short hairpin (shScram), or the indicated shSORT1 constructs. GAPDH is used as a loading control. Functional short hairpin chosen for further analysis are indicated as "A" and "B", and indicated as shSORT1 A or B subsequently in the text. (A') SORT1 protein densitometry was performed on blots in A using the Gels function on ImageJ. Bars indicate mean SORT1 levels from 3 independent experiments, normalized to GAPDH, relative to scrambled control. Error bars represent SEM, * $P < 0.05$, Student's t-test. **(B)** Expression of functional SORT1 KD constructs correlates with a reduction of SORT1 signal in primary neurons. Representative ICC on fixed and permeabilized CNs. GFP indicates transfected cells (i, iii), SORT1 KD confirmed by staining with α-SORT1 antibody (ii, iv). Scale bars, 10um.

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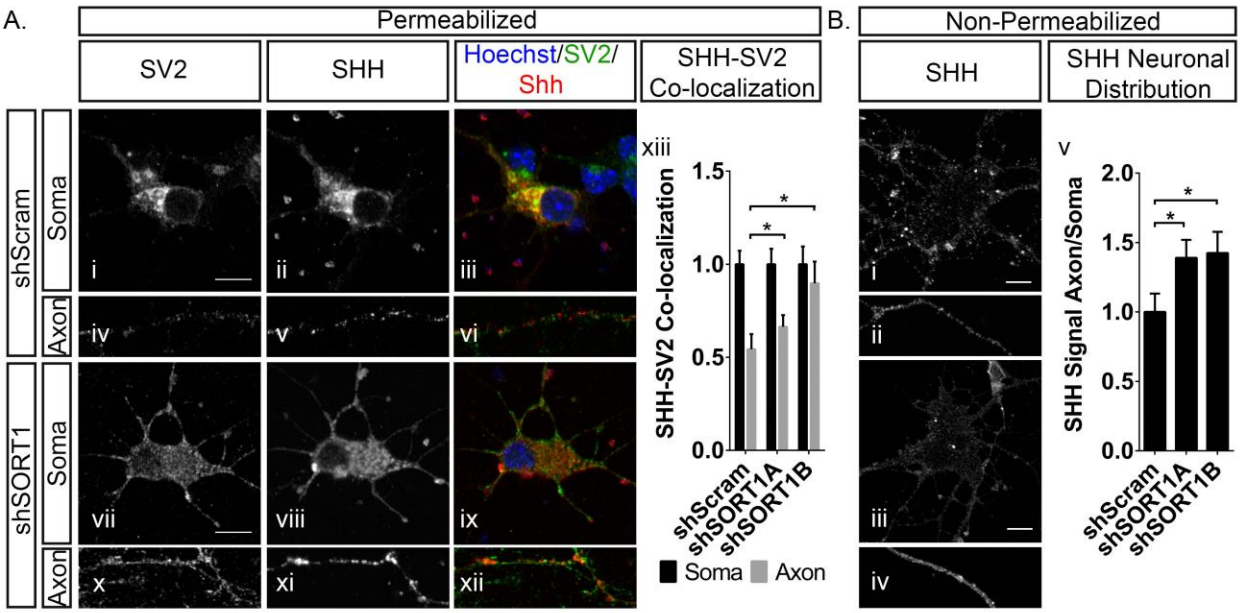
A.
Permeabilized
SV2
SHH
Hoechst/SV2/
Shh
SHH-SV2
Co-localization
shScram
Soma
Axon
shSORT1
Soma
Axon
i
ii
iii
iv
v
vi
vii
viii
ix
x
xi
xii
xiii
SHH-SV2 Co-localization
1.5
1.0
0.5
0.0
*
*
shScram
shSORT1A
shSORT1B
Soma
Axon
B.
Non-Permeabilized
SHH
SHH Neuronal
Distribution
i
ii
iii
iv
v
SHH Signal Axon/Soma
2.0
1.5
1.0
0.5
0.0
*
*
shScram
shSORT1A
shSORT1B

`

Figure 23: Sortilin1 knockdown is associated with an increase in surface axonal Sonic Hedgehog, and Sonic Hedgehog colocalization with axonal SV2. (A) SORT1 KD increased the co-localization of SHH with SV2. Representative ICC and Hoechst nuclear staining on fixed and permeabilized primary CNs expressing SHH and shScram (i-vi) or shSORT1 (vii-xii). Panels show 1nm optic sections in the somatodendritic (upper), or axonal (lower) compartments. Scale bars, 10μm. Co-localization of SHH and SV2 was quantified using the Intensity Correlation Analysis function in ImageJ (xiii). Bars indicate mean Pearson's Correlation Coeffecient (Rr) (n = 20 neurons per condition) normalized to control conditions. Error bars represent SEM, * $P < 0.05$, Student's t-test. **(B)** SORT1 KD correlates with increased ratio of SHH signal on the surface of the axon relative to the soma. Representative ICC on fixed, non-permeabilized primary CNs expressing SHH and shScram (i-ii) or shSORT1 (iii-iv). Panels show 1nm optic sections in the somatodendritic (upper), or axonal (lower) panels. Scale bars, 10μm. SHH distribution quantified as the ratio of SHH signal intensity in a distal region of the axon relative to signal intensity in the soma (v). Bars represent mean ratio of axon: soma SHH signal (n = 20 neurons per condition) normalized to control conditions. Error bars represent SEM, * $P < 0.05$, Student's t-test.

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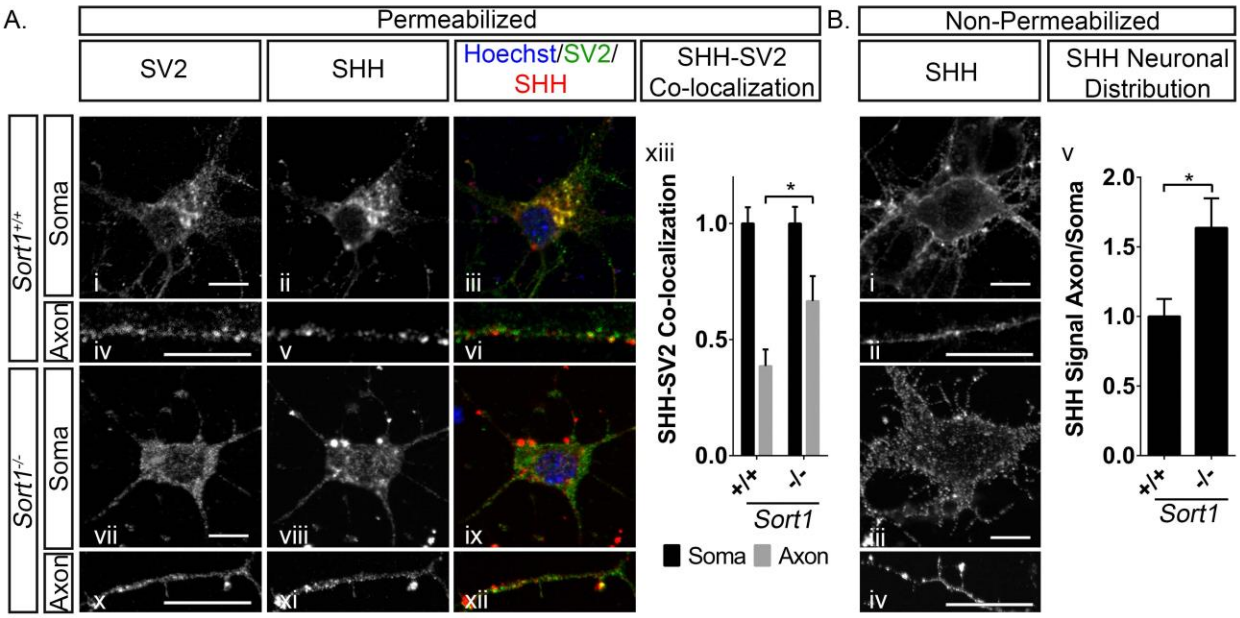
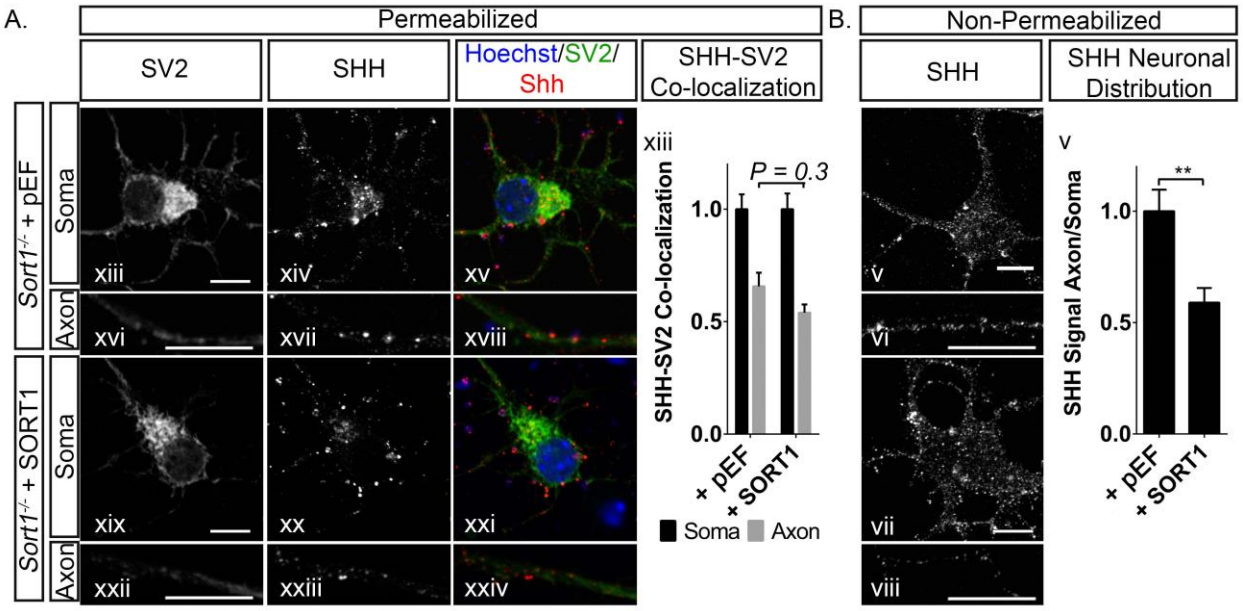

A.
Permeabilized
SV2
SHH
Hoechst/SV2/ SHH
SHH-SV2 Co-localization
B.
Non-Permeabilized
SHH
SHH Neuronal Distribution
Sort1+/+
Sort1-/-
Soma
Axon
i
ii
iii
iv
v
vi
vii
viii
ix
x
xi
xii
xiii
SHH-SV2 Co-localization
1.0
0.5
0.0
*
+/+
-/-
Sort1
Soma
Axon
v
SHH Signal Axon/Soma
2.0
1.5
1.0
0.5
0.0

`

Figure 24: Sortilin1 knockout is associated with an increase in surface axonal Sonic Hedgehog, and Sonic Hedgehog colocalization with axonal SV2. (A) SORT1 KO increased the co-localization of SHH with SV2. Representative ICC and Hoechst nuclear staining on fixed and permeabilized primary WT (i-vi) or *Sort1*$^{-/-}$ (vii-xii) CNs expressing SHH. Panels show 1nm optic sections in the somatodendritic (upper), or axonal (lower) compartments. Scale bars, 10μm. Co-localization of SHH and SV2 was quantified using the Intensity Correlation Analysis function in ImageJ (xiii). Bars indicate mean Pearson's Correlation Coeffecient (Rr) (n = 20 neurons per condition) normalized to control conditions. Error bars represent SEM, * $P < 0.05$, Student's t-test. **(B)** SORT1 KO correlates with increased ratio of SHH signal on the surface of the axon relative to the soma. Representative ICC on fixed, non-permeabilized primary *Sort1*$^{+/+}$ (i-ii) or *Sort1*$^{-/-}$ (iii-iv) CNs expressing SHH. Panels show 1nm optic sections in the somatodendritic (upper), or axonal (lower) panels. Scale bars, 10μm. SHH distribution quantified as the ratio of SHH signal intensity in a distal region of the axon relative to signal intensity in the soma (v). Bars represent mean ratio of axon: soma SHH signal (n = 20 neurons per condition) normalized to control conditions. Error bars represent SEM, * $P < 0.05$, Student's t-test.


A.
Permeabilized
B.
Non-Permeabilized
SV2
SHH
Hoechst/SV2/
Shh
SHH-SV2
Co-localization
SHH
SHH Neuronal
Distribution
Sort1-/- + pEF
Soma
Axon
Sort1-/- + SORT1
Soma
Axon
xiii
xiv
xv
xvi
xvii
xviii
xix
xx
xxi
xxii
xxiii
xxiv
v
vi
vii
viii
xiii
P = 0.3
SHH-SV2 Co-localization
1.0
0.5
0.0
+ pEF
+ SORT1
Soma
Axon
v
**
SHH Signal Axon/Soma
1.0
0.5
0.0
+ pEF
+ SORT1

`

Figure 25: Exogenous Sortilin1 rescues Sonic Hedgehog axonal phenotype in Sortilin1 KO neurons. (A) Exogenous SORT1 in *Sort1*$^{-/-}$ decreased the co-localization of SHH with SV2. Representative ICC and Hoechst nuclear staining on fixed and permeabilized primary *Sort1*$^{-/-}$ CNs expressing SHH and pEF (i-vi) or SORT1 (vii-xii). Panels show 1nm optic sections in the somatodendritic (upper), or axonal (lower) compartments. Scale bars, 10μm. Co-localization of SHH and SV2 was quantified using the Intensity Correlation Analysis function in ImageJ (xiii). Bars indicate mean Pearson's Correlation Coeffecient (Rr) (n = 20 neurons per condition) normalized to control conditions. Error bars represent SEM, * $P < 0.05$, Student's t-test. **(B)** Exogenous SORT1 in *Sort1*$^{-/-}$ correlates with decreased ratio of SHH signal on the surface of the axon relative to the soma. Representative ICC on fixed, non-permeabilized primary WT (i-ii) or *Sort1*$^{-/-}$ (iii-iv) CNs expressing SHH. Panels show 1nm optic sections in the somatodendritic (upper), or axonal (lower) panels. Scale bars, 10μm. SHH distribution quantified as the ratio of SHH signal intensity in a distal region of the axon relative to signal intensity in the soma (v). Bars represent mean ratio of axon: soma SHH signal (n = 20 neurons per condition) normalized to control conditions. Error bars represent SEM, * $P < 0.05$, Student's t-test.

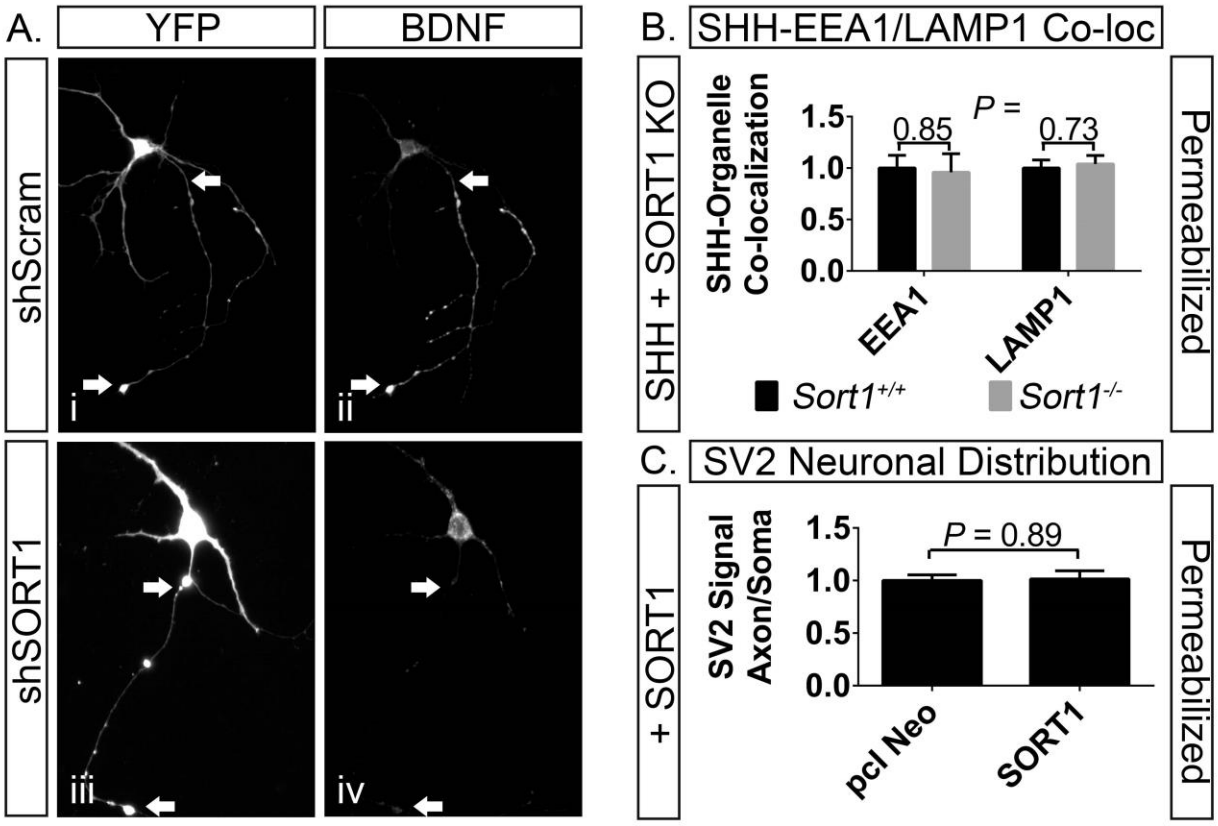

A.
YFP
BDNF
shScram
shSORT1
i
ii
iii
iv
B.
SHH-EEA1/LAMP1 Co-loc
SHH + SORT1 KO
Permeabilized
SHH-Organelle Co-localization
P =
0.85
0.73
1.5
1.0
0.5
0.0
EEA1
LAMP1
Sort1+/+
Sort1-/-
C.
SV2 Neuronal Distribution
+ SORT1
Permeabilized
SV2 Signal Axon/Soma
P = 0.89
1.5
1.0
0.5
0.0
pcI Neo
SORT1

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Figure 26: Sortilin1 perturbation reduces BDNF-HA targeting to the axon, does not affect Sonic Hedgehog endosomal or lysosomal targeting, and does not impair SV2+ vesicle distribution. (A) SORT1 KD reduced BDNF targeting to axons. Representative ICC on fixed, permeabilized primary cortical neurons transiently transfected with BDNF-HA and shScram (top panels) or shSORT1 (bottom panels). Panels show GFP (expressed through an IRES on the sh constructs) and BDNF-HA. Arrows denote approximate proximal and distal boundaries axonal compartments, as determined by morphology and YFP staining. Scale bars, 10um. **(B)** SORT1 expression does not correlate with changes in SHH co-localization with EEA1 or LAMP1 + vesicles. Co-localization of SHH and EEA1 or LAMP1 in *Sort1*$^{+/+}$ and *Sort1*$^{-/-}$ CNs, quantified using the Intensity Correlation Analysis function in ImageJ. Bars indicate mean Pearson's Correlation Coeffecient (Rr) (n = 20 neurons per condition) normalized to control conditions. Error bars represent SEM, * $P < 0.05$, two-tailed Student's t-test. **(C)** Sort1 overexpression does not correlate with a change in the ratio of SV2 signal in the axon relative to the soma. SV2 neuronal distribution quantified as the ratio of SV2 signal intensity in a distal region of the axon relative to signal intensity in the soma in CNs expressing pcDNA or SORT1-myc his. Bars indicate mean ratio of axon: soma SV2 signal (n = 20 neurons per condition) normalized to control conditions. Error bars represent SEM, * $P < 0.05$, two-tailed Student's t-test.

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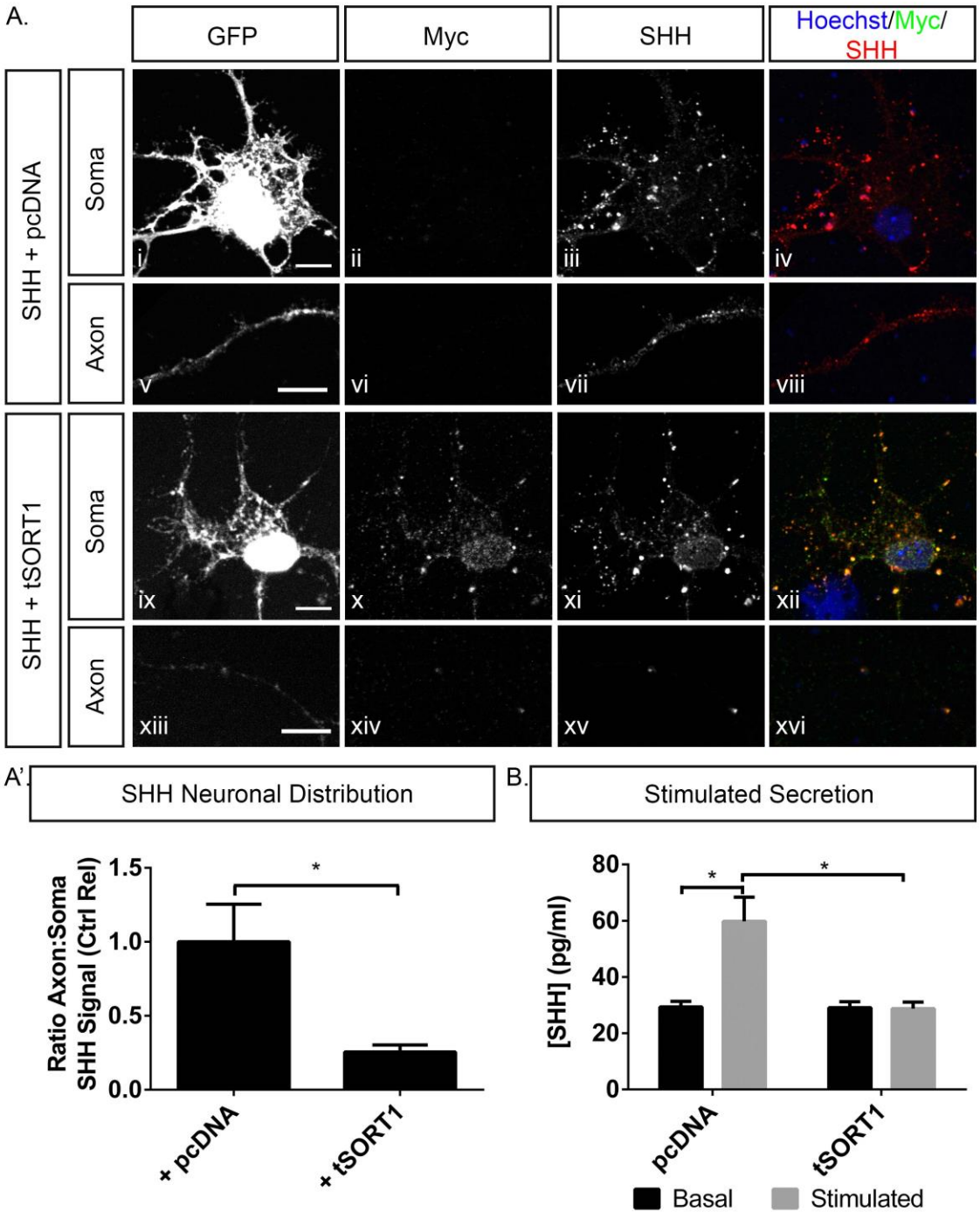
trafficking to the lysosome or endosome and is different from its role in BDNF-HA trafficking in axons.

Truncated Sortilin in the retina decreased astrocyte proliferation in the optic nerve

We next investigated the requirement for SORT1 trafficking in neuron-derived SHH signaling *in vivo*. We focused on RGCs, whose axons project from the retina through the optic nerve (ON) to targets in the brain. In addition to its short-range effects in the regulation of proliferation of adjacent neural progenitor cells in the retina (Wang et al, 2002; Wang et al., 2005), SHH is also transported down RGC axons, where it is released in the ON to stimulate astrocyte proliferation and oligodendrocyte precursor cell migration (Wallace and Raff, 1999, Dakubo et al., 2003; Soukkarieh et al, 2007; Dakubo et al., 2008). To perturb SORT1 trafficking, we expressed tSORT1 in neurons. In control experiments we determined that tSORT1 expression in CNs caused severe impairment of SHH axonal targeting (Fig. 27A, A'). Consistent with inhibiting RSP targeting, tSORT1 expression in differentiated PC12 cells (which differentiate into a neuronal phenotype that has neuron-like processes and activity-dependent RSP secretion of neuropeptides (Greene and Tischler, 1976)) resulted in a decrease in activity-dependent SHH secretion relative to control conditions (Fig. 27B), consistent with an antagonistic role for tSORT1 in stimulated SHH secretion.

We next asked whether tSORT1 expression in embryonic RGCs *in vivo* affected SHH-dependent proliferation in the ON and retina. Control or tSORT1 expression vectors were co-transfected with YFP into the eye at E13.5 by *in utero* electroporation, and the tissue was


A.
GFP
Myc
SHH
Hoechst/Myc/
SHH
SHH + pcDNA
Soma
Axon
SHH + tSORT1
Soma
Axon
i
ii
iii
iv
v
vi
vii
viii
ix
x
xi
xii
xiii
xiv
xv
xvi
A'
SHH Neuronal Distribution
Ratio Axon:Soma
SHH Signal (Ctrl Rel)
1.5
1.0
0.5
0.0
*
+ pcDNA
+ tSORT1
B.
Stimulated Secretion
[SHH] (pg/ml)
80
60
40
20
0
*
*
pcDNA
tSORT1
Basal
Stimulated

`

Figure 27: Truncated Sortilin reduces Sonic Hedgehog axonal targeting and stimulated secretion. (A) Representative ICC and Hoechst nuclear labeling of permeabilized primary CNs overexpressing SHH and pcDNA (i-vi) or tSORT1-myc (vii-xii), with YFP as a transfection control. Panels show 1nm optic sections in the somatodendritic (upper), or axonal (lower) compartments. Scale bar, 10μm. **(A')** SHH neuronal distribution quantified as the ratio of SHH signal intensity in a distal region of the axon relative to signal intensity in the soma (v). Bars indicate mean ratio of axon: soma SHH signal (n = 20 neurons per condition) normalized to control conditions. Error bars represent SEM, * $P < 0.05$, Student's t-test. **(B)** tSORT1-myc overexpression inhibits SHH stimulated secretion from PC12 cells. Differentiated PC12 cells stably overexpressing pcDNA or tSORT1-myc, and transiently transfected with SHHFL were exposed to basal or depolarizing conditions and the concentration of SHH in the medium was quantified by ELISA (n = 3 independent experiments per condition), error bars represent SEM, * $P < 0.05$, Student's *t*-test.

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harvested at E15.5 after a 2hr pulse with EdU to label cells in S-phase (schematic in Fig. 28A). Transfection of RGCs was confirmed by the presence of YFP in the cell bodies located in the retina and axons in the ON (Fig 29A). At this stage, the majority of the cells in the ON are Pax2+ astrocyte precursor cells (Fig. 29B), which are not electroporated in this procedure. Remarkably, the proportion of EdU positive nuclei in the ON was significantly reduced in tSORT1 electroporated conditions, relative to the control (Fig. 29A i-viii and A'). The proportion of EdU+ cells in the retina of tSORT1 electroporated animals was also reduced relative to control conditions, albeit not significantly (Fig. 29B ix-xvi and 29''). This effect of tSORT1 expression on proliferation was restricted to glial cells in the ON, as proliferation of mesenchymal cells located adjacent to the nerve was unaffected (Fig. 29A''). Because we observed YFP+ axons and Pax2+ glial cells in the ON (Fig. 29A and B), we ruled out RGC cell death and impaired astrocyte development as alternative interpretations for the tSORT1-dependent reduction in astrocyte proliferation. We were not able to correlate this increase in astrocyte proliferation with an increase in Gli expression via ISH (Fig. 30), but acknowledge that this is a low sensitivity method that may not detect subtle changes in target gene activation. Taken together, these findings indicate that tSORT1 expression in the retina exerts a non-cell autonomous effect distally in the ON. Since SHH is the only reported anterograde signal that controls astrocyte proliferation in the ON (reviewed in Tao and Zhang, 2014), our findings are also consistent with the interpretation that tSORT1 interferes with SHH signaling in the axon.

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A.

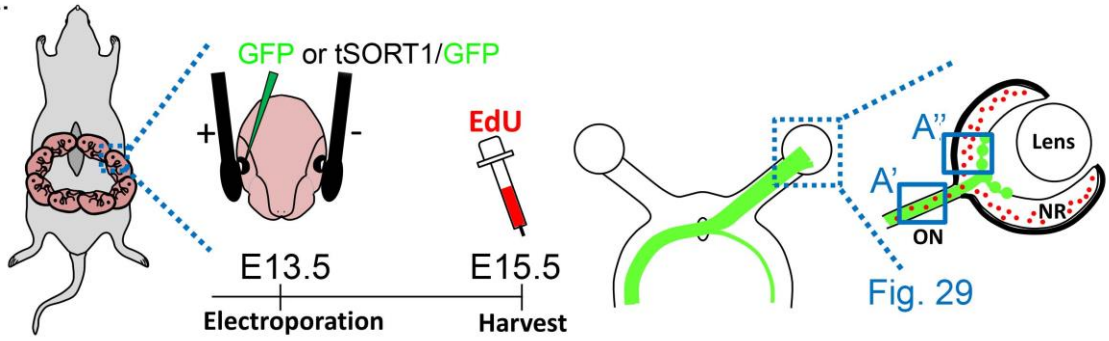
GFP or tSORT1/GFP
+
-
EdU
E13.5
E15.5
Electroporation
Harvest
A''
A'
Lens
NR
ON

Fig. 29

`

Figure 28: Schematic of *in utero* electroporation technique. Embryonic mice were electroporated in utero at E13.5 with YFP or YFP and tSORT1-myc, and harvested at E15.5 (with EdU S-phase labeling 1 hour before sacrifice). Horizontal sections of the head were used to image the retina and ON. Boxes represent the regions that were used for EdU quantification.

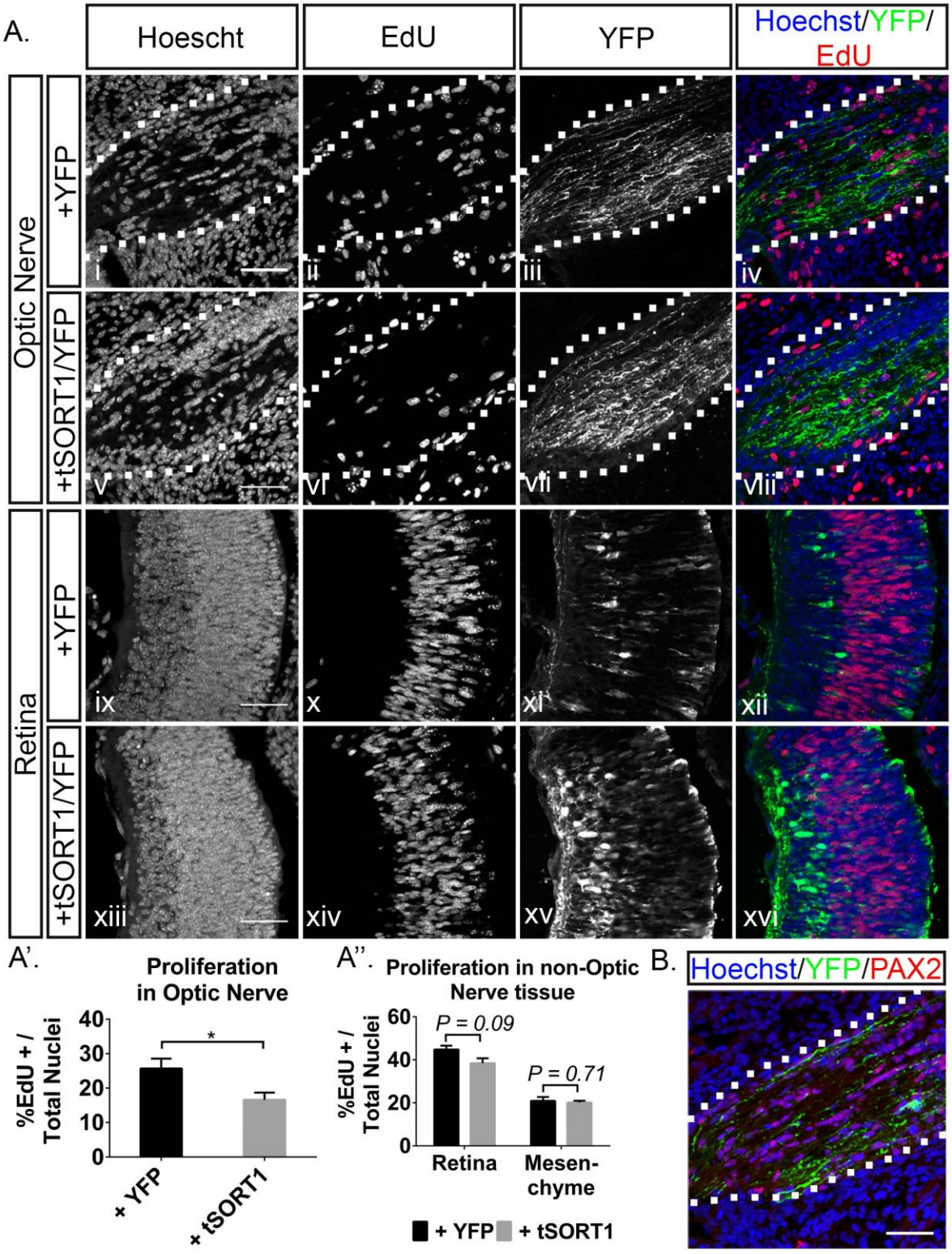
A.
Hoescht
EdU
YFP
Hoechst/YFP/
EdU
Optic Nerve
+YFP
+tSORT1/YFP
Retina
+YFP
+tSORT1/YFP
i
ii
iii
iv
v
vi
vii
viii
ix
x
xi
xii
xiii
xiv
xv
xvi
A'.
Proliferation
in Optic Nerve
%EdU + /
Total Nuclei
40
30
20
10
0
*
+ YFP
+ tSORT1
A''.
Proliferation in non-Optic
Nerve tissue
%EdU + /
Total Nuclei
60
40
20
0
P = 0.09
P = 0.71
Retina
Mesen-
chyme
+ YFP
+ tSORT1
B.
Hoechst/YFP/PAX2

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Figure 29: Truncated Sortilin expression in the retina affects astrocyte proliferation in the optic nerve. (A) Representative EdU staining, which marks cells in S-phase and Hoechst nuclear labeling in horizontal sections of the ON (outlined by dashed lines) (i – viii) and retina (ix – xvi) of transfected eyes from E13.5 mice electroporated in utero and EdU S-phase labeled, as described. YFP, a co-transfection marker, marks transfected cells in the retina and transfected axons in the ON. Scale bars, 50μm. **(A', A'')** Bars represent % of EdU+ nuclei as a function of total nuclei in the YFP+ region of the ON (A') or retina (B''), or in an adjacent mesenchymal region (A'') (YFP: n = 4 animals; tSORT1: n = 3 animals). Error bars represent SEM, * $P < 0.05$, Student's *t*-test. **(B)** IHC for ON *Pax2* (which marks astrocytes) and Hoechst. YFP marks axons of transfected RGCs and *Pax2* marks astrocyte precursor cells. Scale bar, 50μm.

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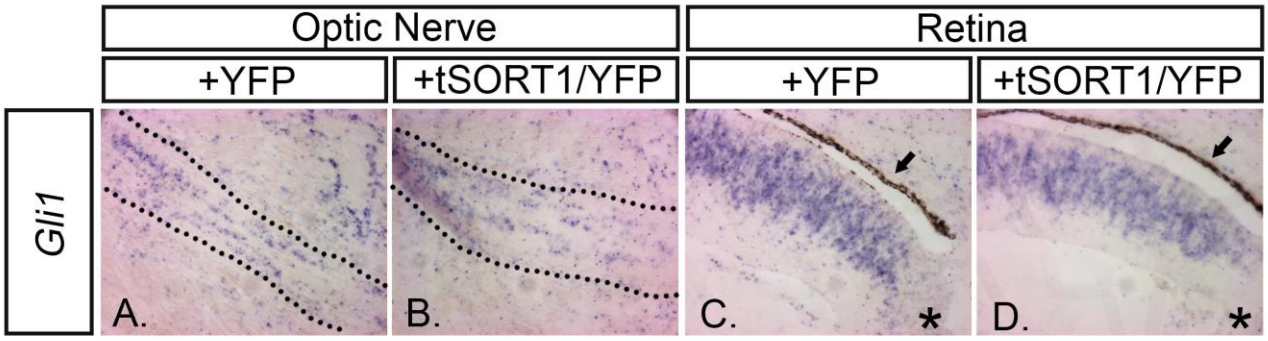
Optic Nerve
Retina
+YFP
+tSORT1/YFP
+YFP
+tSORT1/YFP
Gli1
A.
B.
C.
D.
*
*

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Figure 30: tSORT1 expression in the retina does not alter *Gli1* expression in the retina and optic nerve as measured by *in situ hybridization*. (A-D) Representative ISH hybridization for *Gli1* in horizontal sections of transfected ONs (A, B) and retinae (C, D) from E13.5 mice electroporated *in utero* as described with YFP (A, C) or YFP + tSORT1 (B, D). Dotted lines denote the ON (A, B), arrows the retinal pigment epithelium, * denotes optic disc.

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DISCUSSION

Our study provides the first evidence of a role for SORT1 in modulating the levels of processed SHH protein. SORT1 can interact with SHHFL, as well as the SHHN and SHHC domains, likely in the Golgi apparatus as evidenced by perturbation studies, with SORT1 levels inversely correlating with processing of the SHHFL protein. Consistent with this concept, loss of SORT1 was able to partially rescue SHH-dependent eye field defects in a SHH processing deficient mutant in vivo. Furthermore, SORT1 levels were inversely correlated with polarized trafficking and secretion of SHH in vitro, and SORT1 trafficking was required in RGCs for proper astrocyte proliferation in the ON, consistent with a role for SORT1 in Hh dependent signaling in axons. We therefore propose that SORT1 functions to modulate the rate of SHH processing and its subsequent downstream trafficking, release, and signaling activity (model in Fig. 31).

Identification of Sortilin as a novel interacting candidate of Sonic Hedgehog

Hh-mediated tissue patterning begins with the production and processing of the full length precursor protein (Lee et al., 1994, Porter et al., 1995, Porter et al., 1996, Pepinsky et al., 1998), whereupon mature HhNp is secreted via a number of mechanisms to affect target tissues at short and long range (Huang and Kunes, 1996, Feng et al., 2004, Panáková et al., 2005, Dierker et al., 2009, Creanga et al., 2012, Ayers et al., 2010, Rojas-Ríos et al., 2012). With new mechanisms for facilitating Hh secretion regularly emerging, the identification of novel intracellular interacting candidates provides interesting new targets for study. We used an

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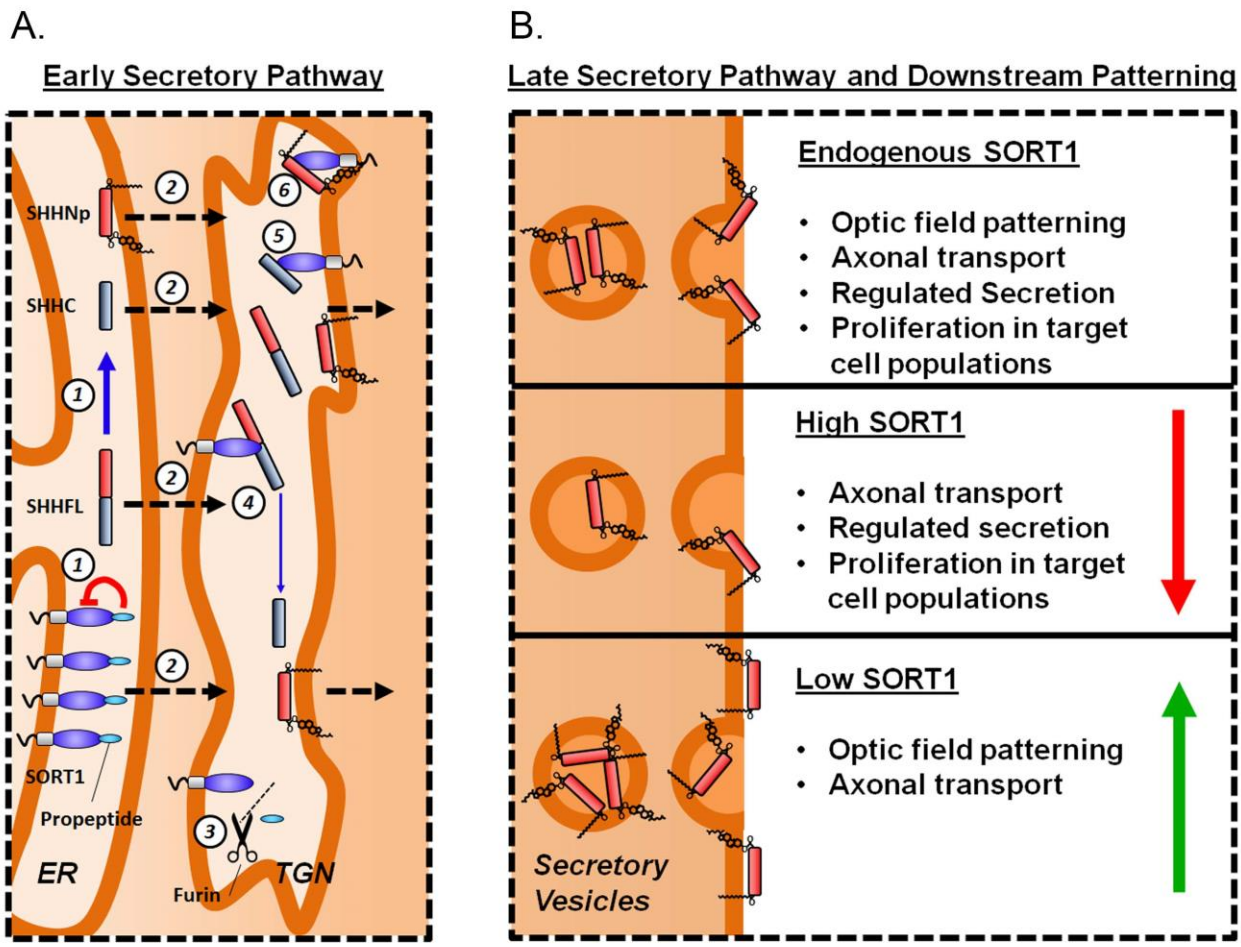
A.
Early Secretory Pathway
SHHNp
SHHC
SHHFL
SORT1
Propeptide
ER
TGN
Furin
B.
Late Secretory Pathway and Downstream Patterning
Endogenous SORT1
• Optic field patterning
• Axonal transport
• Regulated Secretion
• Proliferation in target cell populations
High SORT1
• Axonal transport
• Regulated secretion
• Proliferation in target cell populations
Low SORT1
• Optic field patterning
• Axonal transport
Secretory Vesicles

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Figure 31: Model for Sortilin1 function in Sonic Hedgehog processing and summary of downstream observations of the Sortilin1-Sonic Hedgehog interactions (A) SORT1 interacts with SHHFL and antagonizes processing. **(1)** SHH is processed in the ER to SHHNp, while SORT1 remains inactive due to the presence of its inhibitory propeptide. **(2)** SHHFL, SHHC, SHHN, and SORT1 are trafficked from the ER to the TGN. **(3)** In the TGN, SORT1 is activated by furin-mediated propeptide cleavage. **(4)** SORT1 binds SHHFL in the TGN and antagonizes processing. **(5)** SORT1 also is able to bind SHHC and **(6)** SHHN. **(B)** In endogenous conditions, SHH patterns the bilateral separation of the optic field, is targeted to the axon and for regulated secretion in neurons, and patterns the proliferation of ON astrocyte populations. In conditions with high SORT1 function, SHH axonal targeting and regulated secretion were diminished, as was the proliferation of SHH patterned astrocytes in the ON. In conditions with low SORT1 function, bilateral separation of the optic field was increased in a SHH sensitized system, as was targeting to the axon in neurons.

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affinity screen using purified, GST-tagged versions of both SHH domains, and via mass spectrometry identified a number of novel interacting candidates for SHH involved in various cellular processes (Beug, 2009). The identification of two SORT family members (SORT1 with SHHC, and SORLA with SHHN) was interesting, based on the reported roles of SORT family members in intracellular trafficking of a variety of proteins (Chen et al., 2005; Zeng et al., 2009; Vaegter et al., 2011; Kwon and Christian, 2011; Chamberlain et al., 2013; Tanimoto et al., 2015). We prioritized SORT1 for further analysis because of its role in targeting secreted neuropeptides and their receptors to the RSP in axons (Chen et al., 2005; Vaegter et al., 2011), which is also an important long range targeting mechanism for SHH proteins. Furthermore, the HhC domain in flies contains a conserved motif that regulates HhNp targeting to neuronal axons (Huang and Kunes, 2006), and thus proteins that interact with it in this context are interesting candidates for also regulating axonal targeting. Finally the intein domain in SHHC stimulates the catalytic cleavage of the SHH precursor protein (Hall et al., 1997), the first PTM to generate mature SHHNP, while SHH mutant constructs that are deficient in any of the PTMs display disrupted axonal targeting (Beug et al., 2011). Therefore, there are multiple mechanisms mediated by SHHC to govern SHHNp axonal trafficking that may involve SORT1 function. Our subsequent observation that SORT1 interacted with both SHHN and SHHC tagged proteins in co-IP experiments was interesting, as it raises the possibility that SORT1 may function at different points in SHH intracellular trafficking.

Identifying novel interacting by GST affinity purification has been successfully used to identify novel interacting candidates for a number of proteins (reviewed by Brymora et al., 2004). However, this screening approach does have a number of caveats. First, it was performed using tagged SHH proteins, which may affect binding of certain partners due to steric hindrance.

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Second, the SHH proteins examined were purified forms of separate SHH domains, which precludes any proteins that interact specifically with SHHFL or differentially lipid-modified forms of SHH. Third, detergent and other non-physiological components of the buffers could result in a change in protein structure that would expose otherwise hidden residues, resulting in non-physiological interactions. Fourth, the analysis was restricted to developing brain tissue, which would exclude potential interacting candidates from other spatial and temporal locations. Fifth, the screen was performed in a cell free system, which may result in the identification of non-physiological protein interactions. Finally, our validation of the screen was performed using overexpressed, tagged proteins, which could result in non-specific interactions. Despite these caveats, similar affinity-based screens have been used that identified interactors for SHH (Chuang and McMahon, 1999) and for SORT1 (Petersen et al., 1996), and our screen identified several known SHH interacting proteins, including GPC5 (Li et al., 2007) and LRP1 (Capurro et al., 2012), suggesting that physiologically relevant interactions were possible in this context.

Because the initial screen was based on examining proteins that interacted in a cell-free environment and the primary validations were performed using tagged SHH constructs, validating that the interaction was not an artifact of non-specific interactions was very important. Our observation that SORT1 could co-IP with untagged SHHFL and SHHNp suggests that the interaction was not an artifact of the protein tags. If this were indeed a non-specific interaction between SORT1 and SHH, we would expect SORT1FM to also bind SHH; however, in SHHFL stability assays, SORT1FM was not able to stabilize SHHFL as did SORT1, consistent with a specific interaction of SHHFL with the VPS10P domain. Furthermore, SORT1 depletion had the opposite effect on SHHFL stability, consistent with an interaction between ShhFL and endogenous SORT1. Finally, SORT1 depletion in neurons elicited different phenotypes in SHH

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and BDNF trafficking, consistent with divergent roles for SORT1 with respect to these cargo. Therefore, while we acknowledge that our initial screen and validation experiments could have issues with specificity, we believe that our subsequent approaches address these issues and are consistent with a specific interaction between SORT1 and SHH. Future experiments that could be performed to address the specificity issue would be co-IP endogenous SHH and SORT1; indeed, we did attempt this in neonatal rat cerebellum, but were not successful due to low SHH levels (and correspondingly lower levels of interactor proteins). One alternative to this would be to attempt the co-IP in SHH::GFP heterozygous mice, from which endogenous SHH::GFP has been purified and analyzed biochemically previously (Chamberlain et al., 2008).

An alternative approach to identifying novel interacting candidates would be to IP endogenous SHH from expressing tissues or cell populations in vivo followed by MS. However, endogenous SHH expression is low, and any interacting candidates would be present in much lower levels. Moreover, cell populations that express SHH are generally a minority of the total cell population of the tissue (Barres and Chun, 1993; Lee et al., 2009) and thus obtaining enough purified material to detect endogenous SHH as well as novel interacting candidates biochemically would be impractical. Another approach would be to IP endogenous SHH in expressing cell lines (Yuan et al., 2007; Maitah et al., 2011; Wei et al., 2011), followed by MS identification of interacting candidates. Alternatively, RNAi screens could be performed in these same SHH producing cells, coupled to a readout for SHH signaling, such as ELISA analysis of the media or induction of SHH target genes in reporter assays. Indeed, similar RNAi screens have been performed in *Drosophila*, which identified various novel regulators of lipid modified-Hh release (Aikin et al., 2012). A caveat to this approach is that it would not identify tissue-specific interactors, such as those that sort SHH to the regulated secretory pathway in axons.

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Sortilin interacts with Sonic Hedgehog in the TGN and promotes the persistence of the full length Sonic Hedgehog precursor

The majority of SHHFL processing occurs early in the secretory pathway (Chen et al., 2011). Therefore, we reasoned that an interaction between SHHFL and SORT1 would most likely also occur at some point early in the secretory pathway. tSORT1 provided the ideal tool to examine this hypothesis, because its subcellular targeting is abrogated due to the loss of sorting motifs contained in its cytoplasmic tail, causing retention in the TGN (Chen et al., 2005). Consistent with this interaction occurring early in the secretory pathway, tSORT1 restricted SHH distribution in a pattern that overlapped with a TGN marker, but not an ER marker. One potential consequence of SORT1 interacting with SHHFL early in the secretory pathway would be to modulate SHHFL processing. Consistent with this hypothesis, SORT1 overexpression antagonized SHHFL processing after cycloheximide treatment, with SORT1 depletion having the opposite effect. Together, these results are consistent with a model where SORT1 binds SHHFL early in the secretory pathway and inhibits SHHFL processing. However, this model is incongruent with the prevailing models of SHHFL processing and SORT1 ligand binding – namely, SHHFL processing occurs predominantly in the ER (Chen et al., 2011), while SORT1 can only bind its ligands in the TGN, once it undergoes furin-mediated cleavage of its propeptide (Munck Petersen et al., 1999). Our observation that SORT1FM did not antagonize SHHFL processing indicated that this interaction was not occurring before the TGN, because that is the earliest compartment that SORT1FM and WT SORT1 are structurally and functionally different (Munck Petersen et al., 1999). We also believe that the SHHFL and SORT1 interaction was not occurring after the TGN, as tSORT, which exists predominantly in the TGN, was also able to

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antagonize SHHFL processing. By extension, this indicates that a pool of SHHFL is able to escape the ER and interact with activated SORT1 in the TGN. However, the experiments that form the basis for the prevailing model that the ER is the site of ShhFL cleavage do not preclude the possibility of a pool of ShhFL escaping to the TGN. Briefly, SHH processing was examined in cells treated with high levels of Brefeldin A (an antibiotic that blocks transport from the ER to the Golgi apparatus), such that the Golgi apparatus was completely disrupted (Chen et al., 2011). In these conditions, SHHFL was completely processed in the absence of the Golgi apparatus (Chen et al., 2011). However, while these observations indicate that all SHHFL *can* be processed in the ER, they do not prove that all SHHFL *does* get processed in the ER. It is therefore possible that some SHHFL can be trafficked to the TGN, where it could interact with activated SORT1. Consistent with SHHFL trafficking to the TGN, uncleaved Hh proteins can be targeted for secretion in a variety of contexts, and are able to activate the Hh signaling cascade (Tokhunts et al., 2010). Also, SORT1 and its family members commonly interact with growth factor precursor proteins to affect intracellular processing and trafficking events (Chen et al., 2005; Gustafsen et al., 2013). Thus, the SORT family may play a diverse role in fine tuning morphogen and growth factor precursor processing and trafficking, with SHH being the latest in an ever growing list.

There are several unanswered questions in our model that warrant further investigation. While our observations support a model for SORT1 and SHHFL interacting in the TGN, we cannot rule out the interaction occurring in an alternative organelle. It is possible that SORT1 is binding SHHFL in the TGN and directing it to early endosomes, exosomes, or other secretory granules for an additional level of control of SHH secretion. Indeed, SORT1 has been implicated as a mediator of exosome release (Wilson et al., 2014), and exosomes are emerging as an alternative mechanism for SHH secretion (Gradilla et al., 2014). Furthermore, while our

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pharmacological inhibition and co-localization studies are not consistent with SHHFL being targeted to lysosomes, it is possible that this is a mechanism in a different context. To examine possible alternative target organelles for the SORT1-SHH interaction, a comprehensive biochemical subcellular analysis of SHH localization in expressing cells could be performed and changes compared between control and SORT1 expressing or knockdown conditions. To dissect the specific subcellular target organelles of SORT1, cells would be treated with pharmacological inhibitors targeting early and mid-stage endosomes, as well as exosomes and other secretory granules, and SHH processing, trafficking, and release would be assessed. Alternatively, we could assess SHH localization in cells expressing perturbation constructs for SORT1 cytoplasmic effectors. For example, we could express perturbation constructs that target the retromer, which aids in SORT1 retrograde targeting from EEs (Canuel et al., 2008). If SORT1 were interacting with SHH in EEs, we would expect to see a mislocalization of SHH toward EE compartments.

While SORT1 overexpression did not alter SHH target gene induction in expressing cells, we did not definitively rule out a role for SORT1 as a PM level receptor for SHH. One potential function for a SORT1-SHH PM interaction would be in the stimulation of a pro-apoptotic signaling cascade. SORT1 binds proNT and together with $p75^{NTR}$ initiates an apoptotic signaling cascade (Nykjaer et al., 2004; Teng et al., 2005). As SORT1 also appears to bind the precursor form of SHH, it could produce an analogous apoptotic signaling cascade. Furthermore, PTCH has been shown to function as a dependence receptor, stimulating apoptosis in the absence of SHHN, so there is a precedent for SHH receptors mediating apoptosis (Mille et al., 2009). To test this, purified SHH could be applied to SORT1 expressing cells, and metrics of apoptosis, such as TUNEL or cleaved caspase staining could be tested. A second function for a SORT1-SHH PM interaction could be as a modulator of SHH mediated axon guidance; SHH activates transcription

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independent remodeling of growth cones in RGCs and commissural neurons, acting as both a repellant and as an attractant in different contexts (Trousse et al., 2001; Charron et al., 2003; Kolpak et al., 2005; Okada et al., 2006; Sanchez-Comacho and Bovolenta, 2008; Yam et al., 2009; Fabre et al., 2010; Yam et al., 2012, Hillman et al., 2012; Guo et al., 2012). SORT1 has recently been identified as a mediator of proNGF-induced axon repulsion, and is known to collect at the growth cones of developing axons (Trigos et al., 2015). Therefore, it is possible that SORT1 could act as a co-receptor in this context. Indeed, there is a basis for SHH receptors functioning in multiple contexts of SHH signaling, including BOC, which mediates both transcription dependent and independent events (Okada et al., 2006; Izzi et al., 2011). To examine this, we could quantify axon turning events in response to a SHH gradient in neurons expressing SORT1 perturbation constructs versus controls. A third function for SORT1 as a PM receptor could be to target SHH for endocytosis, as it does for Progranulin (Tanimoto et al., 2015). There is a precedent in SHH reception for endocytosis, as GPC-3 and LRP2 cooperate to internalize extracellular SHH, serving to limit its potential to activate the signaling cascade (Capurro et al., 2012). Interestingly, while tSORT1 is primarily localized to the TGN, it also collects on the PM due to inclusion in constitutive secretory vesicles (which do not require specific targeting) and subsequent failure to be endocytosed (Finan et al, 2011). While we did not observe this PM level staining for tSORT1, or see evidence of SHH misrouted to the PM, the conditions in which we imaged (permeabilization resulting in disruption of the PM) were not ideal for visualizing this compartment and therefore cannot discount it as a possible location for co-localization. While we did not observe any changes in SHH and EEA1 colocalization in primary CNs as a function of SORT1 perturbation, we cannot discount endocytosis as a mechanism in another context. To test this, we could incubate cells expressing SORT1

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perturbation constructs with purified SHH protein, followed by surface biotinylation and analysis of the amount of SHH protein internalized in each condition.

Third, our initial screen and validation experiments identified SORT1 as interacting with the individual SHHN and SHHC domains, as well as SHHFL. We do not know if the SORT1 interaction with either of the individual SHH domains are important for inhibiting SHHFL cleavage, or whether they serve additional, unspecified functions. To determine if SORT1 interacting with the individual SHH domains is relevant for the SHHFL processing phenotype, we would use chimeric SHH proteins, consisting of the SHHC and relevant processing domains, attached to an unrelated domain, and examine processing in the context of SHH overexpression or knockdown. If the chimeric SHHC protein were still processed, we would then be able to rule out the SHHN-SORT1 reaction as relevant to SHH processing.

Fourth, while we do know that SORT1 binds SHHFL via the VPS10P domain, we do not know the specific residues on SHHN and SHHC required for binding. There is an intein motif in the SHHC domain that mediates processing (Hall et al., 1997), as well as a motif that participates in Hh axonal targeting (Huang and Kunes, 1996), while SHHN historically binds surface level HSPGs via the CW motif (Farshi et al., 2011). One could generate constructs that express the aforementioned fragments, and perform co-IPs with SORT1 proteins to determine the relevant binding regions

Fifth, as mentioned previously, we were careful to confirm the results of the screen with reciprocal co-IPs in cell lines. However, there is the possibility that the interaction was not direct, but rather occurring through another, unidentified binding partner. Future experiments to address this could involve Förster resonance energy transfer (FRET), using appropriately tagged versions

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of these constructs and imaging to confirm a direct interaction. Alternatively, co-IPs could be performed with purified proteins in a cell-free assay to remove potential intermediate binding partners.

Finally, our experiments were limited to analyzing SHHFL cleavage, while processing to SHHNp involves the cleavage reaction as well as dual lipidation of the SHHN terminal domain. SORT1 binding SHHFL, in addition to inhibiting the cleavage reaction, may extend to control additional aspects of SHH maturation, including cholesterol and palmitate addition. Furthermore, there are proteins known to be involved in SHH maturation, including HHAT, which interact with the precursor early in the secretory pathway (Buglino and Resh, 2008). Subsequent experiments could examine the lipid profile of mature SHH proteins in the context of SORT1 overexpression or knockdown, as well as potential interactions between SORT1 and HHAT. Furthermore, SHH IPs could be performed in the presence or absence of SORT1, and interaction profiles analyzed by MS to identify additional novel interactors of SHH dependent on the SORT1 interaction.

Sortilin1 knockout impacts Sonic Hedgehog Dependent patterning in a sensitized system

The novel role for SORT1 as an inhibitor of SHH processing led us to predict that SORT1 KO animals would exhibit SHH gain of function phenotypes, because of a relative overproduction of the secreted ligand. However, germline KO of SORT1 is not associated with gross phenotypic changes in SHH-dependent tissues. This is not unanticipated, as SORT1 KO is associated with subtle phenotypes that do not impact development (Vaegter et al., 2011); for example, *Sort1*$^{-/-}$ mice have no overt neurotrophic phenotypes despite its known roles in

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neurotrophin receptor trafficking. However, SORT1 KO aggravated neurotrophic phenotypes when crossed on to a sensitized background, p75NTR KO (Vaegter et al., 2011). Moreover, endogenous SHH responsive systems are sensitive to low concentrations of the ligand, as evidenced by the viability of the SHH heterozygous KO (Chiang et al., 1996). Finally, our screen identified SORLA as another potential novel interacting candidate of SHH; therefore there may be compensation by SORLA upon loss of SORT1. Thus, it is possible that any SHH-dependent phenotypes in *Sort1*$^{-/-}$ are masked by a combination of compensatory activity from SORT family members and the sensitivity of the system to a range of SHH ligand concentrations.

We therefore investigated genetic interaction between SORT1 and the SHH pathway using sensitized *in vivo* models of SHH signaling. Loss of SORT1 had no impact on the incidence or latency of medulloblastoma in *Ptch*$^{+/-}$ mice, which is dependent on SHH pathway over-activation (Appendix 2). Interestingly, a significantly higher percentage of *Sort1*$^{+/-}$ *x Ptch*$^{+/-}$ animals developed hydrocephalus, an enlargement of the ventricles in the brain which is known to be partially exacerbated in ciliopathy models by *Ptch*$^{+/-}$, relative to *Sort1*$^{+/+}$ or *Sort1*$^{-/-}$ *x Ptch*$^{+/-}$. However, reducing SORT1 levels in the context of *Shh*$^{GFP/GFP}$, characterized by midline defects due to deficient SHH processing, improved eye field patterning, a SHH-regulated process. The rescue was incomplete, as shown by the lack of rescue of expression of midline target genes that require the highest dose of SHH, indicating that SORT1 KO does not fully restore SHH signaling.

Midline patterning defects caused by a loss of SHH are well documented (Chiang et al., 1996). An interesting observation is that these defects can be rescued by a corresponding de-repression of the pathway in compound mutant animals (Furimsky and Wallace, 2006). For example, the severe cyclopic phenotype of the SHH KO mouse was rescued in increasing

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degrees when crossed on to GLI3 deficient backgrounds, depending on the dosage of GLI3 remaining (Furimsky and Wallace, 2006). Therefore, our observation that SORT1 KO could rescue midline defects in a SHH processing deficient system was consistent with a partial rescue in SHH mediated tissue patterning. Interestingly, there are known human mutations that affect SHH processing resulting in various midline patterning defects (Roessler et al., 1996; Roessler et al., 1997), and future experiments could target SORT1 for therapy in animals bearing this mutation

A major limitation to our analysis is that we were unable to correlate an improvement in eye field width with an improvement in SHH target gene expression by ISH. To detect changes in gene expression associated with a partial rescue in SHH patterning events, qPCR could be performed, which is much more sensitive than ISH. SHH dependent genes, such as *Gli1*, *CcnD1*, *Ptch*, and *Hes1* would be analyzed in whole embryos, as well as in tissues along the midline that are patterned by SHH. This analysis can be expanded to additional tissues as well, as there are numerous neuronal and non-neuronal tissues that display defects in reduced SHH models. For example, SHH is secreted from PCs in the developing cerebellum to pattern GNPs (Wallace, 1999), and SORT1 is also expressed in PCs (Sarret et al., 2003). Therefore, SHH target gene expression could be assessed in purified GNPs in *Sort1*$^{-/-}$ and littermate control animals. Finally, all of these experiments could be performed in SORT family member compound mutants, as subtle changes in gene expression may be masked by compensation of SORT family members.

The fact that hydrocephalus was observed in the heterozygous knockout animals, but not the homozygous, was interesting, as one would expect a complete SORT1 knockout to be more potent than a heterozygous knockout. One potential explanation for this is that, in *Sort1*$^{-/-}$, compensation mechanisms are enacted that temper the downstream effects of losing SORT1.

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However, in the *Sort1*$^{+/-}$, these compensatory mechanisms are not triggered, and thus an overall net loss of SORT1 function would be seen in *Sort1*$^{+/-}$ relative to *Sort1*$^{-/-}$. Consistent with this hypothesis is the observation that SORLA can interact with SHHN, and that SORT family members have highly overlapping functions in other systems, suggesting that SORLA may be a candidate compensatory receptor in *Sort1*$^{-/-}$ animals. To address this question, one could generate compound mutants for SORT1 and its family members, and examine patterning and disease progression in SHH-dependent systems. Another observation that merits further investigation is where in the pathway SORT1 is functioning to affect hydrocephaly, which has been linked to pathway defects in SHH in receiving (Gavino and Richards, 2011), but not producing cells. Loss of SORT1 function could indeed be affecting a pool of secreted SHH, which then leads to an over activation in the pathway in target cells. Alternatively, it is possible that SORT1 loss is affecting the receiving cells in a cell autonomous manner, altering response to SHH or another factor, ultimately exacerbating the hydrocephalus phenotype. Conditional SORT1 knockout animals in known SHH producing and responding tissues in the brain could be generated to pinpoint where in the pathway SORT1 is acting. These conditional mutants would have to be generated on KO backgrounds of other SORT family members, to remove any additional layers of compensation.

A genetic interaction of SORT1 and SHH could also be tested in non-neuronal target tissues, as SORT1 and SHH play intricate roles in many of the same tissues. For example, SORT1 targets PRG for lysosomal degradation in prostate cancer cells, which abrogates the autocrine PRG function of promoting proliferation and cell motility (Tanimoto et al., 2015). SHH, meanwhile, is produced in epithelial prostate cells and, much like PRG, functions as an autocrine as well as juxtacrine signaling molecule, signaling to stromal cells, with increased

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expression promoting tumor growth and metastasis (Karhadkar et al., 2004**;** Li et al, 2014; Pettigrew et al., 2014; Xie et al., 2015). Interestingly, increased processing of the SHH precursor protein has been implicated in tumorigenicity of prostate cancer cells (Xie et al., 2015), while SHHFL may be secreted here as an active signaling molecule as well (Pettigrew et al., 2014). Therefore, it would be interesting to see if the inhibitory function of SORT1 in SHH processing were a relevant process in this context.

Sortilin antagonizes Sonic Hedgehog Axonal Targeting

We have previously reported that constructs of SHH with mutations in processing residues show impaired RSP targeting in primary neurons (Beug et al., 2011). Our observation that SORT1 levels negatively correlated with SHH in the axon is consistent with the hypothesis that SORT1 regulates SHH processing, and provides further evidence that SHH processing is imperative for regulated secretory pathway targeting. This is notably different than SORT1-dependent anterograde trafficking of BDNF and TRK receptor to axons and the regulated secretory pathway (Chen et al., 2005; Evans et al., 2011; Yang et al., 2011; Vaegter et al., 2011) This suggests the possibility that, while SORT1 is the regulated secretory pathway receptor for BDNF and TRK receptors, there exists an additional, as yet unidentified, receptor that likewise regulates SHH targeting to the regulated secretory pathway.

Because of our observations that SORT1 controls SHH processing, we hypothesize that it is through this that SORT1 is limiting the amount of processed SHH exiting the TGN, and therefore causing a decrease in SHH being trafficked to the axon and secreted via the regulated secretory pathway. However, a decrease in SHH signal in the axon as a function of SORT1 could

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be caused by a number of different scenarios. One possible alternative is that SORT1 is causing a general retardation of proteins targeted to the regulated secretory pathway, or regulated secretory pathway proteins in general. We think that this is unlikely, as SORT1 had the opposite effect on known target BDNF-HA, and loss of SORT1 did not have any effect on SV2 axonal localization. Although our results suggest an interaction with SORT1 and SHH early in the secretory pathway, another alternative explanation to decreased SHH localization to the axon in response to SORT1 overexpression is an increase in retrograde trafficking of SHH away from the axon. To test this hypothesis, we could transfect a photoactivable SHH into cortical neurons, and activate the fluorophore in axonal and somatic compartments, and track the movement of the labeled bolus of SHH over time in live neurons. Alternatively, we could perform ligation assays in neurons expressing tagged SHH and SORT1 perturbation constructs, and examine whether there is a difference in SHH accumulation at either side of the ligation. Finally, it is possible that SORT1 overexpression could cause a reduction in SHH in the axon due to an increased rate of secretion from the axon. However, our secretion results in PC12s do not support this, as they indicate that there is in fact a decrease in SHH secretion in SORT1 overexpressing PC12s.

One potential pitfall to our analysis is the use of cortical neurons and PC12s, which do not endogenously express SHH, as a model system for studying SHH trafficking alterations as a function of SORT1. The most appropriate neuronal subtype would have been RGCs, which endogenously express SHH and SORT1 and have been a well characterized model of anterograde SHH targeting in vivo (Wallace and Raff, 1999; Dakubo et al., 2003; Dakubo et al., 2008). Unfortunately, RGCs, and indeed all SHH expressing projection neurons, are present in extremely small numbers, and need to be purified from large heterologous populations in the surrounding tissue. Thus, for large scale biological imaging experiments, as well as biochemical

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treatments, RGCs were not practical as a model neuron. However, we are confident in the efficacy of CNs to study SHH trafficking because they produce a morphologically distinct axon, employ many of the same mechanisms for regulated secretion of neuropeptides and neurotransmitters as RGCs (Lesuisse and Martin, 2002), and have been used extensively in the study of morphogen and growth factor trafficking, including for both SHH and SORT1 function (Chen et al., 2005; Beug et al., 2011). Furthermore, overexpressed SHH co-localizes with endogenous SORT1 in CNs in the same pattern as endogenous SHH in RGCs does with SORT1, suggesting that exogenous SHH in CNs is trafficked similarly to that in RGCs. Finally, while PC12s are a neuronal cell line and do not possess a true axon, they do possess a functioning regulated secretory pathway responsive to neuronal depolarization, are easy to manipulate on a large scale, and have been used extensively in *in vitro* studies of SORT1 and SHH neuronal function (Chen et al., 2005; Beug et al., 2011). For these reasons, and due to the impracticality of primary neurons for use in large-scale biochemical studies, we are confident that PC12s are an appropriate system to study the downstream SHH secretory events in response to SORT1 perturbation.

Sortilin modulates long range Sonic Hedgehog signaling in an in vivo model of Sonic Hedgehog axonal trafficking

Axons in the ON signal to resident glial cells in a number of ways: they promote the survival of oligodendrocytes (Barres et al., 1993), the proliferation and/or survival of oligodendrocyte precursors (Barres and Raff, 1993), and the proliferation of astrocytes (Burne and Raff, 1997). To date, the only known axon derived signal that controls astrocyte

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proliferation in the ON is SHH (Wallace and Raff, 1999; Dakubo et al., 2008). We show that ectopic expression of tSORT1 in RGCs induced a non-cell autonomous effect on astrocyte proliferation in the optic nerve, identifying SORT1 function as important in axon-glial cell communication.

One alternative explanation to a decrease in proliferation is an increase in astrocyte cell death. However, we think this is unlikely, as we quantified proliferation as a function of the total cell population, thus the changes in proliferation would be independent from loss of cells by apoptosis. Another alternative explanation is that the tSORT1 overexpression in RGCs exacerbated SORT1-mediated RGC apoptosis. We think this is unlikely as well, as we did not observe any apparent differences in RGC cell or axon staining, and also because SORT1 constructs lacking the cytoplasmic tail are not able to activate the pro-apoptotic signaling cascade due to the requirement for cytoplasmic tail cleavage in the process (Skeldal et al., 2012). However, we cannot definitively rule out RGC death as a result of the *in vivo* electroporation manipulations. To address this, we could generate a transgenic mouse that expresses tSORT1 on an RGC specific promoter. A third alternative explanation to our observation is that SORT1 perturbation is affecting the release of a target neurotrophin. However, to date SHH is the only known RGC derived morphogen to affect astrocyte proliferation in the ON, and thus is the most likely candidate for this axon-derived signal. To definitively answer this question, SHH target gene expression in responsive cells in the ON would be analyzed. While we did not see a change in Gli1 expression in our analysis, we only examined gene expression by ISH, an admittedly low sensitivity assay. ONs from treated embryos could be isolated, and target genes analyzed by the more sensitive qPCR for SHH specific changes.

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In addition to the changes in astrocyte proliferation, future experiments could expand the analysis to include other SHH dependent events in the ON, including oligodendrocyte precursor cell migration from the optic chiasm and subsequent myelination events. Additionally, one could look at SORT1 effects on an alternative function of RGC-derived SHH in the ON, which is the promotion of ipsilateral (IL) segregation of a subset of RGC axons from the opposite retina at the optic chiasm (OC). tSORT1 would be expressed in the ipsilateral retina at E13.5, an age of peak RGC axon outgrowth (Fabre et al., 2010) , while a tracer, such as DiI or GFP, would be used in the contralateral eye to mark axon guidance. The percentage of IL projecting axons from the contralateral eye would be assessed, and would be quantified relative to control treated animals, with a decrease in IL projection expected in response to a decrease in SHH axonal secretion. This analysis could also be expanded to systems where SHH promotes axon guidance, such as commissural neurons.

Conclusion

Taken together, we have identified a novel interaction between SORT1 and SHH, with SORT1 inhibiting cleavage of SHHFL, resulting in a reduction SHH processing, polarized trafficking, and secretion. This interaction has implications for SHH signaling in developing and diseased tissues, including in neuroepithelial cells at the CNS midline, and in polarized trafficking of the protein in projection neurons. Considering the ubiquitous expression of SORT1 in known SHH producing cells, it is possible that SORT1 could additionally regulate SHH mediated tissue patterning in additional tissues.

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APPENDIX I

List of antibodies used in this study

Antigen	Species	Antibody #	Source	Application	Dilution
Calnexin	Rabbit	ab22595	Abcam	ICC	1:200
c-Myc tag	Mouse	9E10 (sc-40)	Santa Cruz Biotech	WB/ICC	1:1000/1:200
EEA1	Rabbit	ab2900	Abcam	ICC	1:200
GAPDH	Mouse	6C5 (ab8245)	Abcam	WB	1:5000
GFP	Goat	600-101-215	Rockland Inc	WB	1:1000
GFP	Rabbit	A11122	Life Technologies	ICC/IHC	1:1000
HA tag	Rabbit	Y11 (sc-805)	Santa Cruz Biotech	ICC	1:200
Lamp1	Rabbit	ab24170	Abcam	ICC	1:200
Pax2	Goat	PRB-276D	Covance	IHC	1:200
ShhN	Rabbit	H-160 (sc-9024)	Santa Cruz Biotech	WB/ICC	1:1000/1:200
ShhN (mature)	Mouse	5E1	Dev. Studies Hybridoma Bank	ICC	1:5000
Sortilin	Rabbit	ab16640	Abcam	WB/ICC	1:1000/1:200
SV2	Rabbit	119002	Synaptic Systems	ICC	1:200
Tau	Rabbit	314002	Synaptic Systems	ICC	1:5000
TGN-38	Mouse	sc-271624	Santa Cruz Biotech	ICC	1:200

Appendix I: List of antibodies used in this study. Antibodies used in this study listed in alphabetical order of common name, with species, antibody #, source, and application also indicated. WB = western blot, ICC = immunocytochemistry, IHC = immunohistochemistry.

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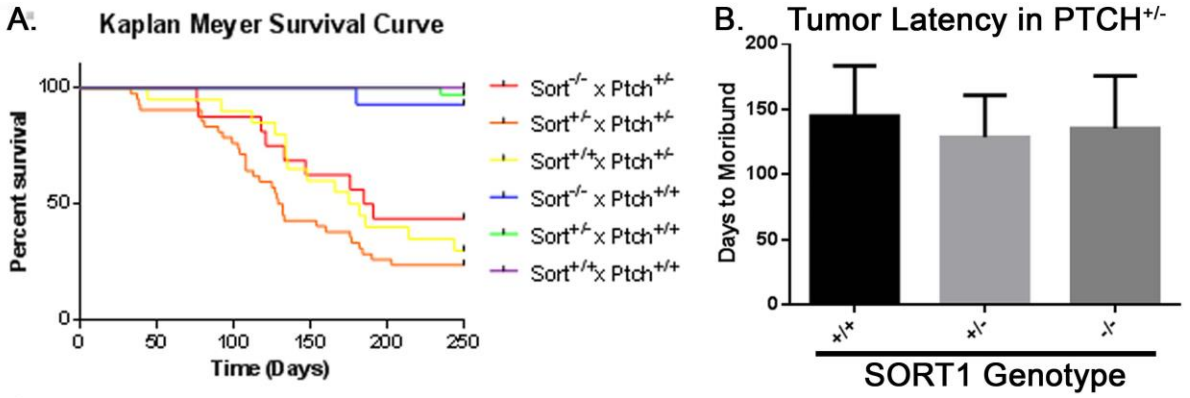
APPENDIX II

Sortilin perturbation did not affect tumor incidence or latency in Patched Heterozygous background.



C.

	Summary of Aging Study (# Animals (% of colony))					
Ptch		+/+			+/-	
Sort1	+/+	+/-	-/-	+/+	+/-	-/-
End of Study	19 (100)	42 (95)	14 (93)	10 (38)	11 (23)	6 (33)
Meduloblastoma	0 (0)	0 (0)	0 (0)	15 (58)	29 (60)	12 (67)
Hydrocephaly	0 (0)	0 (0)	0 (0)	1 (4)	6 (13)	0 (0)
Other	0 (0)	2 (5)	1 (7)	0 (0)	2 (4)	0 (0)
Total	19	44	15	26	48	18

Appendix II: Modulating Sortilin levels did not impact latency or incidence of medulloblastoma in Patched heterozygous animals. Results from aging study of SORT1 knockout animals crossed on to the *Ptch*$^{+/-}$ background. **A.** Kaplan-Meyer Survival curve depicting the dates of death (birth = day 0) of *Ptch*$^{+/+}$ or *Ptch*$^{+/-}$ littermates crossed on to *Sort*$^{+/+}$, *Sort*$^{+/-}$, or *Sort*$^{-/-}$. Y axis depicts % survival of the colony at a given day, while the X axis indicates the date from birth. Surviving animals were culled at end of study date, (birth + 250 days). **B.** Medulloblastoma tumor latency in indicated Ptch$^{+/-}$ crosses. Ptch$^{+/+}$ did not exhibit medulloblastoma, and were excluded from the analysis. Animals were euthanized when considered moribund (presence of domed skull, severe weight loss, disruption in motor skills), as per ACVS instruction. Bars show mean date to moribund, +/- S.E.M. **C.** Summary of all causes of death in the colony. End of study = 250 days; medulloblastoma determined as described; hydrocephaly determined as enlargement of the lateral ventricles, and presence of a significant amount of cerebrospinal fluid upon skull dissection; other causes include

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APPENDIX III

STATEMENT OF CONTRIBUTIONS:

Shawn Beug, Postdoctoral Fellow, CHEO Research Institute. Dr. Beug generated the SHHEYFP, SORT1-myc-his, and GPC5-flag constructs, and performed the GST affinity screen and co-IPs summarized in Fig. 7.

Chantal Mazerolle, Health and Safety Officer, OHRI. Ms. Mazerolle helped perform the co-IPs in Fig. 7B, C.

Jimmy Peng, Ph.D Student, ICRM Montreal. Mr. Peng performed the in utero electroporations and EdU injections seen in Fig. 29.

Erin Bassett, Postdoctoral Fellow, OHRI. Dr. Bassett generated the schematics in Fig. 18 and 28.

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APPENDIX IV

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