The *Drosophila* GOLPH3 homolog regulates the biosynthesis of heparan sulfate proteoglycans by modulating the retrograde trafficking of exostosins

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SUMMARY

The exostosin (EXT) genes encode glycosyltransferases required for glycosaminoglycan chain polymerization in the biosynthesis of heparan sulfate proteoglycans (HSPGs). Mutations in the tumor suppressor genes EXT1 and EXT2 disturb HSPG biosynthesis and cause multiple osteochondroma (MO). How EXT1 and EXT2 traffic within the Golgi complex is not clear. Here, we show that Rotini (Rti), the *Drosophila* GOLPH3, regulates the retrograde trafficking of EXTs. A reduction in Rti shifts the steady-state distribution of EXTs to the trans-Golgi. These accumulated EXTs tend to be degraded and their re-entrance towards the route for polymerizing GAG chains is disengaged. Conversely, EXTs are mislocalized towards the transitional endoplasmic reticulum/cis-Golgi when Rti is overexpressed. Both loss of function and overexpression of rti result in incomplete HSPGs and perturb Hedgehog signaling. Consistent with *Drosophila*, GOLPH3 modulates the dynamic retention and protein stability of EXT1/2 in mammalian species. Our data demonstrate that GOLPH3 modulates the activities of EXTs, thus implicating a putative role for GOLPH3 in the formation of MO.

KEY WORDS: EXT, GOLPH3, HSPGs

INTRODUCTION

Heparan sulfate proteoglycans (HSPGs) are extracellular matrix macromolecules that have been implicated in regulation of the signaling activities of secreted morphogens during development (Nybakken and Perrimon, 2002; Yan and Lin, 2009). HSPGs consist of a core protein decorated with heparan sulfate glycosaminoglycan (GAG) chains. The biosynthesis of GAGs is initiated when the tetrasaccharide linker tethers to the proteoglycan core protein and is followed by further addition of alternating β1-4-linked glucuronic acid (GlcA) and α1-4-linked N-acetylglucosamine (GlcNAc) disaccharides. The Golgi-resident type II transmembrane exostosin (EXT) glycosyltransferases catalyze the repetitive addition of GlcA-GlcNAc residues and generate GAG chains. As the polymer forms, one or more enzymes modify the GAG chains by subsequent epimerization and sulfation (Esko and Selleck, 2002).

Mutations of enzymes involved in HSPG biosynthesis have been demonstrated to cause developmental defects in model animals and to cause diseases in human (Nadanaka and Kitagawa, 2008). Genetic studies in *Drosophila* have shown that mutations of any one of the three EXT genes, *tout-velu* (*tv*)/EXT1, *sister of tout-velu* (*sotv*; now known as *Etv2*)/EXT2 and *brother of tout-velu* (*botv*)/EXTL3, reduce the distribution of the Wingless (Wg), Hedgehog (Hh) and Decapentaplegic (Dpp) morphogens and their subsequent signaling (Häcker et al., 2005; Nybakken and Perrimon, 2002; Yan and Lin, 2009). Although having overlapping GlcNAc transferase activities, each EXT is indispensable for the biosynthesis of full-length GAG chains and for the formation of morphogen gradients (Han et al., 2004). In human, the tumor suppressor genes EXT1 and EXT2 link genetically to multiple osteochondrosomas (MO, also known as hereditary multiple exostoses), an autosomal dominant skeletal disease characterized by the formation of multiple cartilaginous tumors (Bovée et al., 2010; Jennes et al., 2009). It has been proposed that the clonal expansion of EXT mutant chondrocytes would disturb HSPG biosynthesis and lead to a local perturbation in Indian hedgehog (IHH) diffusion, which eventually leads to extra bone formation (Duncan et al., 2001).

The synthesis of HSPGs requires sequential enzymatic modifications of glycoproteins in the Golgi. Efficient modification depends on the non-uniform distribution of different glycosylation enzymes within Golgi cisternae (Emr et al., 2009; Puthenveedu and Linstedt, 2005; Rabouille et al., 1995). The sub-compartmentalizing Golgi cisternae provide separate functional regions for different enzymes to ensure optimal processing conditions. The assembly line-like alignment of enzymes allows transiting proteins to encounter different enzymes in a sequential manner to accomplish glycosylation processing (Puthenveedu and Linstedt, 2005). Furthermore, the gradient-like distribution of a dynamic retained enzyme through the Golgi cisternae is an important factor for efficient glycosylation. To achieve the gradient-like distributions, accurate inter-cisternal vesicle transport mediated by coats and SNAREs is necessary (Puthenveedu and Linstedt, 2005). Coatamer protein complex I (COP I) and II (COP II) are two well-known coat proteins involved in vesicle-mediated transport. COP II mediates traffic from the endoplasmic reticulum (ER) to the Golgi, whereas COP I primarily acts to mediate transport from the Golgi to the ER and between Golgi cisternae (Lee et al., 2004). Currently, the regulators for the elaborate distribution of Golgi enzymes are not well characterized.

Golgi phosphoprotein 3 (GOLPH3; also known as GPP34/GMX33/MIDAS) is conserved from yeast to humans. Vps74p, the yeast homolog of GOLPH3, maintains the steady-state localization of Golgi mannosyltransferases dynamically through an interaction with COP1 coat protein (Schmitz et al., 2008; Tu et al., 2009).
2008). However, in mammalian cells, knockdown of GOLPH3 does not cause the mislocalization of mannosyltransferases (Dippold et al., 2009). Although GOLPH3 represents a new class of oncoenzyme acting on the mTOR (mammalian target of rapamycin) pathway (Scott et al., 2009), the actual effect(s) of GOLPH3 has not been identified.

Here, we report that Rotini (Rti), the Drosophila homolog of GOLPH3, determines the retrograde trafficking of fly EXTs in the Golgi complex. Both depletion and overexpression of rti result in impaired HSPGs and reduction of Hh signaling. This model is compatible with the function of GOLPH3 in regulating the dynamic retention of tumor suppressor proteins EXT1 and EXT2 in bone and cartilage cells. Based on our study, we propose that GOLPH3 is a new factor to fill a lacuna in the formation of MO.

**MATERIALS AND METHODS**

**Drosophila stocks and transgenics**

Fly stocks used were: UAS- hh-CFP, disp and UAS- hh-NH2 (Burke et al., 1999), inigo/igo (Bellaiche et al., 1998), UAS-tv-myc (The et al., 1999), UAS-sotv (Takai et al., 2004), hctl and UAS-Fringe-GT-myc (Brücker et al., 2000). Other stocks were provided by the Bloomington Stock Center. The UAS-rti (CG7085) and UAS-CG15338 are EcoRI/Xhol fragments of LD23816 and LP02710 directionally cloned into pUASp. The UASp-botv-v5/His is a KpnI/Eagl fragment cloned into pUASP. An rti RNAi construct was primers 5′-AATCTAGATCCTCAAGTGGGTCAACGAT-3′ and 5′-AATCTAGATGGTGTAGATGAGAGCGT-3′ and was cloned into pWIZ (Lee and Carthew, 2003).

**DNA constructs for cultured cells**

A fragment containing the full-length botv coding sequence from LD21192 was digested with KpnI and Xhol and ligated into pcAc5.1-V5/V5-His A. The C-terminus of the sotv cDNA sequence from GH02288 was digested with EcoRI and Xhol and ligated into pcAc5.1-V5/V5-His A. The N-terminus of sotv released from GH02288 by EcoRI digestion was ligated into EcoRI-digested pcAc5.1-sotv-V5-His A. The full-length tv coding sequence from D101920 and the GlcAT-1 coding sequence from GH05057 was digested with EcoRI and Xhol and ligated into pcAc5.1-V5/V5-His A. Donald L. Jarvis (University of Wyoming, Laramie, WY, USA) kindly provided pcAc5.1-GalT7-GFP.

The RNAi targeting the rti sequence was cloned into pGEM-3Z (Promega) using primers 5′-AAGAATTCTCTCTCAAGTGGTGTCAG-3′ and 5′-AAAAAGCTTTGTGCTATTGAGAGGAGCGT-3′ for in vitro transcription to generate dsRNA followed by transfection in Drosophila S2 cells.

For transfection of Myc-DDK-tagged EXT2 into mammalian cells, full-length EXT2 (IMAGE 3947503, from BCRC g1005062A11) was cloned into pCMV6 (ORIGENE) using SJII and MfuII.

**Antibody generation and immunohistochemistry**

For production of an Rti-specific antibody, the coding sequence of the full-length cDNA clone LD23816 was inserted into pET28a and expressed in Escherichia coli BL21. Three EXT cDNA sequences encoding amino acids 30-376 of Ttv (The et al., 1999), 53-308 of Sotv, and 148-572 of Botv were cloned into pET28a and expressed in E. coli BL21. The purified proteins were injected into either mice (Rti, Tv and Botv) or rats (Sotv) for antibody production. The resultant polyclonal antiserum was further purified by column affinity purification with the appropriately conjugated peptide.

Wing imaginal discs were dissected from third instar larvae in PBS and fixed for 30 minutes in fresh 4% paraformaldehyde. Treatment of discs to expose the epitope recognized by the 3G10 monoclonal antibody (Seikagaku) was carried out by incubating fixed discs with heparinase III (Sigma) for 16 hours at 37°C, and subsequent staining with the 3G10 antibody. Extracellular Hh staining was performed according to a protocol described for extracellular Wg (Strigini and Cohen, 2000).

Cultured cells were fixed in PBS containing 3.7% formaldehyde. Protein extracts were prepared from first to second instar larvae homogenized in immunoprecipitation (IP) buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM EDTA, 1 mM EGTA, 2 mM Na3VO4, 1 mM PMSF] without detergent. For co-immunoprecipitation, larval lysates were incubated with protein A/G (Santa Cruz Biotechnology) for 1 hour at 4°C. After addition of anti-V5 (Invitrogen) or anti-Myc (9E10) antibodies and incubation overnight at 4°C, the lysates were washed extensively and then boiled in SDS sample buffer.

Primary antibodies used were: mouse anti-Plc antibody (Capdevila et al., 1994), rabbit anti-Hh antibody [gift from C.-T. Chien (Academia Sinica, Taipei, Taiwan) and T. Tabata (University of Tokyo, Japan)], chicken anti- GFP (US Biological) or rabbit anti-GFP IgG (Chemical International), rabbit anti-Myc (9E10), rabbit anti-J-galactosidase antibody (Boulder, USA), mouse anti-Dip (Lum et al., 2003), 3G10 monoclonal antibody (Seikagaku), rabbit anti-Sil [gift from S. Goto (Yano et al., 2005)], rabbit anti-S11 [gift from U. Hacker (Lüders et al., 2003)], mouse anti-V5 (Invitrogen), goat anti-EXT2 (Santa Cruz Biotechnology), sheep anti TGN46 (Serotec), mouse KDEL (10C3, Santa Cruz Biotechnology), rat anti-HA, mouse anti-Tubulin, and mouse anti-Actin (Sigma). Alexa 488-, 546- and 633- (Molecular Probes), FITC-, Dylight 488- and 594-, and peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) were used.

**Immunoelectron microscopy**

Wing imaginal discs were dissected and fixed in a mixture of 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer for 3-4 hours. The individual discs were embedded with 12% gelatin in small blocks. Frozen sections were prepared by using an ultracytomicrotome (Reichert Ultracut S/Reichert FCS; Leica, Vienna, Austria). Ultrathin sections were collected and labeled with antibodies followed by Protein A-conjugated gold particles (Liou et al., 1996). The following antibodies were used: rabbit anti-mouse IgG and rabbit anti-rat IgG (Dako Cytomation, Glostrup, Denmark). The antibodies were detected with protein A conjugated to 10-nm or 15-nm gold particles (Cell Microscopy Center, Utrecht, The Netherlands). For double labeling, sections were incubated with mouse anti-Rti antibody followed by rabbit anti-mouse antibody and Protein A coupled to 10-nm gold particles. Sections were then fixed for 10 minutes in 1% glutaraldehyde and rabbit anti-GM130 antibody (abcam) was added, followed by Protein A coupled to 15-nm gold particles. Grids were examined using a Hitachi H-7650 transmission electron microscope.

**Immunogold particle quantification**

We examined two to three imaginal discs from flies with different genotypes; ~10-19 Golgi units were randomly chosen and counted. To quantify the distribution pattern of EXTs, the membrane density of EXT in each Golgi profile was counted by the point and intersection method for relating the number of gold particles to the surface of the Golgi structure in sections (Griffiths, 1993). Data were analyzed by one-way ANOVA, and the density of EXT in the trans-Golgi network (TGN) (or transitional ER (TER)) in three genetic backgrounds are grouped based on Duncan's multiple range test.

**Cell lines and transfection**

Drosophila Schneider 2 (S2) cells and cell lines examined were grown according to American Type Culture Collection guidelines (www.atcc.org). Cells were transfected with TransIT-LT1 transfection reagent (Mirus) according to the manufacturer’s protocol. Cells were treated with 10 μM MG132 (Z-Leu-Leu-Leu-al, Sigma) for 6 hours before cell harvesting. For immunofluorescence staining, the amount of GOLPH3 plasmid or siRNA was half of that used for western blotting.

For CHON-002, cells were infected with lentivirus-packaged GOLPH3 specific shRNAs [sh1: (TRCN0000150069) 5′-CAGTTAAGAATAATGCAGGGA-A-3′; sh2: (TRCN0000150176) 5′-GCTTGGTTCAATCATGGTTAT-3′] or plKO.AS3-w-GOLPH3 at a multiplicity of infection (M. O. I.) of 1 or 0.5.

**Yeast two-hybrid assay**

A Drosophila 0.24 hour embryonic cDNA library (a gift from C.T. Chien, Institute of Molecular Biology, Academia Sinica, Taipei 115, Taiwan) was screened using Y190 as host strain. The pAS2-1 and pGAD10 vectors were
used as nutritional selection markers. From several predicted candidates, 11 clones contained partial informative sequence. Among them, three clones corresponded to CG3279. Full-length rti was cloned into pAS2-1 and the CG3279 cDNA is carried by pGAD10. The yeast transformation exactly followed the Yeast protocols Handbook (PT3024-1, Clontech). A β-galactosidase colony-lift filter assay was used to examine the lacZ reporter gene expression.

GST pull-down assay
E. coli carrying different expression constructs were cultured, then IPTG was added (0.1 mM) to induce the desired proteins. The cultures were further incubated at specific temperatures for different times (37°C, 3 hours for GST-Dvt1, 25°C, 14 hours for His-Rti) to maximize the soluble fraction containing the target proteins. GST-Dvt1 was immobilized on GST-bound beads (Novagen 70541-3) at 4°C for 1 hour, incubated with His-Rti-containing cell lysate at 4°C for at least 3 hours, and then analyzed by western blotting using a mouse anti-His antibody.

Transfections were performed with Myc-DDK tag EXT1 (ORIGENE)/Myc-DDK tag EXT2 and pCMV-GOLPH3 (IMAGE 4999629, from BCRC gl004052E02) for overexpression or GOLPH3 siRNA (Invitrogen, Stealth RNAi siRNA Select RNAi, Catalog #1299003) for knockdown. The negative controls were scrambled sequences of 36% GC (low GC, Invitrogen Catalog #12935200) and 48% GC (medium GC, Invitrogen Catalog #12935300). The mock plasmid transfection for the GOLPH3 overexpression assay was performed with pCMV-GFP (Invitrogen).

RESULTS
Identification of the Drosophila GOLPH3 Golgi protein Rotini
In a germ line clone (GLC) screen based on the P transposase-insensitive clipped-FRT218 protocol (Lin et al., 2006), we identified the P[abar] induced lethal mutation 1164A4. In a wild-type embryo, the ventral surface of the larval cuticle bears rows of denticles at the anterior of each segment, alternating with the posterior naked regions (Fig. 1A). The embryos derived from 1164A4 GLCs showed a ‘lawn-of-denticles’ phenotype with some additional dorsализation and twisting (Fig. 1B).

The P[abar]1164A4 insertion resides 8 bp downstream of the translation start site of CG7085, causing a premature stop codon; from transgenic rescue experiments, it was confirmed that the 1164A4 mutant phenotype is due to the disruption of CG7085 (supplementary material Fig. S1A-E). The amino acid sequence of CG7085 is highly conserved with human GOLPH3. Because CG7085 is the only GOLPH3 homolog in the Drosophila genome and the mutant phenotype resembled a type of pasta, we refer to CG7085 as Rotini (Rti), the Drosophila GOLPH3 homolog. GOLPH3 has been proposed to be a Golgi matrix protein (Bell et al., 2001; Snyder et al., 2006; Wu et al., 2000). In Drosophila, Rti is ubiquitously expressed in the embryo and wing disc (data not shown) and partially colocalizes with the Golgi markers Fringe-GT-Myc and GM130 (supplementary material Fig. S1F-J).

The phenotypes of embryos derived from rti1164A4 GLCs crossed with a deficiency that covers the CG7085 locus (Fig. 1C) is comparable to that of embryos derived from rti1164A4 GLCs crossed with rti1164A4/CyO (Fig. 1B), suggesting that rti1164A4 behaves as a genetic null mutation. Western blot analysis of rti1164A4 GLC embryos failed to detect any protein (Fig. 1D). Therefore, we conclude that the rti1164A4 allele is a null mutation.

Rti is required for Hh signaling
The lawn-of-denticles phenotype of rti1164A4 GLC embryos resembles that of embryos mutant for hh, wg or genes involved in the biosynthesis of HSPGs. In the wild-type ventral embryonic ectoderm, Wg and Hh signaling are required for normal expression of engrailed (en) and wg (Hatini and DiNardo, 2001). In rti1164A4 GLC embryos, the level of En (Fig. 1E,F), Hh (data not shown) and Wg (Fig. 1G,H) are dramatically reduced. In the wild-type wing disc, Hh is produced in the posterior compartment. In rti clones located in the posterior compartment, Hh staining is dramatically reduced (Fig. 1L-N). Rti is required for Hh signal transduction.

Rti exerts its ultimate effect at the plasma membrane
To understand the point of Rti’s action, we traced the behavior of Hh. The significant reduction of Hh staining in rti mutant cells could...
be the result of the disruption of gene expression, protein processing or extracellular movement. It is shown that Rti affects neither the transcription of hh (supplementary material Fig. S2A-C) nor the Hh auto-cleavage processing (supplementary material Fig. S2D). After auto-cleavage, Hh moves to the plasma membrane and is released. Extracellular Hh can be visualized when discs are incubated with Hh antibody before fixation. Extracellular Hh is restricted to the posterior compartment and displays a limited posterior-to-anterior diffusion along the A/P boundary (Fig. 2A). In rti clones (without GFP) crossing almost the entire disc, extracellular Hh is detected in rti mutant cells in the posterior compartment and spreads extensively into the anterior compartment (Fig. 2B,C). This result indicates that, without Rti, Hh can still be transported to the plasma membrane and released for extracellular trafficking. We examined the behavior of Hh further in the absence of both Rti and the transmembrane protein Dispatch (Disp). Disp is required to release cholesterol-anchored Hh from Hh-producing cells, with accumulation of Hh in the plasma membrane in the disp<sup>+</sup> mutation (Burke et al., 1999). Consequently, if the rti mutation results in the failure of Hh transport to the membrane, Hh staining in the plasma membrane will be lost in the rti disp<sup>+</sup> double mutant. By analyzing the level of Hh staining in the rti clones generated in the wing disc of disp<sup>+</sup> homozygous larvae, we found that the staining of Hh in rti<sup>11644</sup>/disp<sup>+</sup> double mutant cells (Fig. 2D-F, without GFP) is indistinguishable from that in disp<sup>+</sup> mutant cells (Fig. 2D-F, with GFP). This indicates that, in the double-mutant cells, cholesterol-anchored Hh is transported to and retained at the plasma membrane, because lack of Disp prevents its release (Burke et al., 1999). As such, in rti mutant cells in the presence of wild-type Disp, Hh is still able to be transported to the membrane but is dissipated (Fig. 1J,K). Taken together, these data indicate that Rti is not directly necessary for Hh processing but exerts its ultimate effect at the membrane to trap the Hh released by Disp.

**Rti is required for proper HSPG expression**

Considering the similar Hh- and Ptc-like phenotypes in mutations of rti (Fig. 1I-N) and genes affecting the biosynthesis of HSPGs (Bellaiche et al., 1998; Takei et al., 2004), we explored the possibility that Rti might affect the expression of HPSGs. To detect the presence of HPSGs in vivo, we stained wing discs with 3G10, an antibody that recognizes an epitope following digestion of HSPGs with heparinase III (David et al., 1992). As has been reported in the case of mutations that perturb the biosynthesis of HSPGs (Takei et al., 2004), the 3G10 staining level in rti mutant cells is significantly reduced compared with that of nearby wild-type cells (Fig. 2G-I), indicating that Rti is required for HPSG expression. Surprisingly, overexpression of Rti also reduces 3G10 staining. Using the Gal4-UAS system (Brand and Perrimon, 1993), we find that UAS-rti driven by apterous-Gal4, ap > rti, targets Rti on the dorsal side of the wing disc and leads to a significant reduction in 3G10 staining compared with that of ventral wild-type cells (Fig. 2J). In addition to the posterior compartment, Hh is also distributed towards the anterior compartment but with a decrease in Hh staining in the dorsal wing disc (Fig. 2K). Using z-axis sections (supplementary material Fig. S2E-E<sup>+</sup>) and extracellular Hh staining (supplementary material Fig. S2F), we confirmed that Hh can indeed spread towards the anterior compartment.

The binding of secreted morphogen to GAG chains is required for HSPG function in cell signalling (Lin, 2004). In ttkv, sato and botv clones (Bellaiche et al., 1998; Han et al., 2004), the reduction of Hh can be explained by the hypothesis that released Hh is trapped by HSPGs produced from the surrounding wild-type cells. Nevertheless, owing to the comprehensive reduction of HSPGs on all cells overexpressing Rti in the entire dorsal wing disc (Fig. 2J) (i.e. the lack of wild-type HSPGs to trap Hh), Hh distributes anteriorly (Fig. 2B,C,K). This more broadly distributed and decreased level of Hh is not able to induce proper Ptc expression, as the Ptc staining is narrowed to a width of two cells in those regions where Rti is overexpressed (Fig. 2L).

**Rti regulates the stability of EXTs, the polymerases of HSPGs**

Quantitative or qualitative defects in either the HSPG core protein or the GAG chain can result in a similar reduction in 3G10 staining. To understand the effect of Rti on the HSPG core protein, we examined one of the core proteins, Dally-like protein (Dlp) (Khare and Baumgartner, 2000). Compared with extracts from wild-type, Dlp migrated much faster in extracts from both rti<sup>11644</sup> and ttkv<sup>1006109</sup> clones (without GFP) crossing almost the entire disc. (*A-C*) Loss of Rti does not prevent Hh protein from trafficking to the plasma membrane in rti clone cells. (*D-F*) Rti is not required for the expression of Disp to the plasma membrane. In wing imaginal disc derived from ywhd; rti<sup>11644</sup>; FRT<sup>90A</sup> (GFP, FRT<sup>95C</sup>; disp<sup>+</sup>/disp<sup>-</sup>) third instar larvae, the rti<sup>11644</sup> clones are marked by the absence of GFP. The Hh staining pattern in rti mutant cells is indistinguishable from that in wild-type cells. (*G-I*) Rti is involved in HSPG biosynthesis. The rti<sup>11644</sup> clones are marked by the absence of GFP. After treatment with heparinase III, the HSPG level shown by 3G10 staining is reduced in a rti mutant clone. (*J-L*) Overexpression of Rti reduces 3G10 staining (*J* and Hh (K) staining, and narrows the expression pattern of Ptc (L)). Rti is overexpressed on the dorsal side of the disc (ap > rti); the ventral side of the disc is used as control. The dashed lines represent the dorsal/ventral boundary of the imaginal disc. Scale bars: 30 μm.
embryos (Fig. 3A). It has previously been shown that the glypican core protein without GAG chains can still be appropriately expressed and delivered to the cell surface (Kirkpatrick et al., 2006). Similarly, Dlp can be delivered to the cell membrane when rti is mutant either in the embryo (Fig. 3B,C) or in clones in the imaginal disc (Fig. 3D-F). Conceivably, Rti affects glycosylation but not the core protein with respect to HSPG biosynthesis.

It is most likely that the loss of rti impairs the activity of enzymes required for GAG chain biosynthesis. We examined the protein level of enzymes that participate in the biosynthesis of GAGs (Esko and Selleck, 2002) (Fig. 3G, left panel). The protein lysates from Drosophila S2 cells co-transfected with pAc5.1-gene-tag and dsRNA against rti or a control gene were detected by western blotting with individual tags. In cells treated with RNAi against Rti, the protein levels of the two initiation enzymes \( \beta \text{GalT7} \) and glucuronosyltransferase (\( \beta \text{GalT7} \)) are reduced.

Fig. 3. Rti knockdown reduces fly EXT proteins. (A) Dlp western blot of embryonic proteins from wild-type (OreR) as well as \( ttv^{1001} / 109 \) and \( rti1164A4 \) GLC embryos without paternal function. (B-C) The core protein Dlp is transported to the plasma membrane (marked by Phalloidin) in the embryos derived from wild type (B) and \( rti1164A4 \) GLC (C). Scale bars: 20 \( \mu \text{m} \). (D-F) Rti does not affect Dlp staining. The \( rti1164A4 \) clones are marked by the absence of GFP and the clone boundaries are shown with dashed lines. Scale bars: 30 \( \mu \text{m} \). (G) Rti knockdown reduces EXT proteins. The left diagram shows the biosynthesis of HPSGs and the relevant enzymes. The right diagram shows a western blot with GFP and V5 tags and Rti antibodies from Drosophila S2 cells co-transfected with pAc5.1-gene-tag and dsRNA against rti or a control gene (con.).
(GlcAT1) are either not changed or are slightly reduced compared with cells treated with a control dsRNA. However, the protein levels of the three polymerization enzymes Ttv, Sotv, and Botv are significantly reduced. The protein level of Sulfateless (Sfl), an enzyme required for GAG sulfation that functions after the polymerases, is not changed (Fig. 3G, right panel). Consistent with this result, rti clones in the wing disc show reduced levels of these three EXTs (supplementary material Fig. S3A-I), but not of other GAG modification enzymes, such as Sfl and Slalom (Sll) (supplementary material Fig. S3J-O).

A pulse-chase experiment confirms that these EXTs are labile in the absence of Rti. rti clones were generated by a 37°C heat shock for 1 hour during the first larval instar stage. At the third larval instar stage, a second 37°C heat shock for 1 hour induced transient expression of UAS-EXT-Tag driven by heat shock-Gal4 (hs-Gal4). The life span of the EXT-Tag fusion protein was monitored at the specified time points by conventional fixation protocols (Fig. 4A). Without heat-shock treatment at third instar stage, Ttv-Myc is undetectable (Fig. 4B), indicating that the Ttv-Myc induced after the first heat-shock treatment cannot maintain its expression through the third larval instar. Half an hour after the third instar heat-shock treatment, the expression of Ttv-Myc is induced (Fig. 4C). Compared with its stability in nearby wild-type cells, Ttv-Myc is more labile at 5.5 hours (Fig. 4D) in the absence of Rti. Fifteen hours after heat shock, the transiently expressed Ttv-Myc has already diminished in both wild-type and mutant cells (Fig. 4E). Similarly, compared with its stability in nearby wild-type cells, Botv-V5 is more labile in rti clones (data not shown).

To confirm that Rti affects the stability of EXTs, the behavior of Ttv-Myc was monitored when its degradation is blocked by a mutation in Hrs, which encodes a protein that sorts cargo into multivesicular bodies destined for degradation in lysosomes (Bache et al., 2003). In an Hrs mutant, Ttv-Myc fusion protein is expected to be detectable because it accumulates in the multivesicular bodies. As expected, in Hrs clones Ttv-Myc could be detected at 5.5 hours (Fig. 4F) and until 19 hours after heat shock (Fig. 4G). Consistent with the prediction that rti mutation downregulates the stability of EXTs, in the rti Hrs double-mutant cells it is easy to detect the induced Ttv-Myc protein not only at 5.5 hours (Fig. 4H) but also for up to 19 hours (Fig. 4I), by which time Ttv-Myc has already partially decreased. Clearly, in the absence of Rti, Ttv becomes labile and enters the Hrs-mediated degradation pathway.

Rti mediates the retrograde transport of EXT enzymes within the Golgi complex

It is known that enzymes resident in the Golgi can cycle within the Golgi complex to maintain its appropriate role in post-translational modification (Pavelka et al., 2008). Given the effect of Rti on EXTs, we considered the possibility that Rti might function in the cycling of EXTs within the Golgi. Rti is detectable in all compartments of the Golgi complex, especially at the vesicles adjacent to the Golgi cisternae (supplementary material Fig. S1J,L). Moreover, based on a two-hybrid screen and a GST pull-down assay, a v-SNARE protein (CG3279), was found to interact physically with Rti (supplementary material Fig. S4), supporting the hypothesis that Rti functions in vesicle trafficking. Therefore, we assessed whether Rti is involved in either the anterograde or retrograde trafficking pathway by examining the steady-state distribution of EXTs in the Golgi complex in both reduction and overexpression of Rti. As rti10644 is a null mutation causing a significant reduction of EXTs (supplementary material Fig. S3A-I), it may not allow us to study their distribution. Hence, we used RNAi transgenic flies to knock down Rti. The wing phenotype in Rti knockdown flies can be suppressed when Rti is simultaneously overexpressed (supplementary material Table S1), suggesting that this dsRNA is specific for rti.

The Golgi of Drosophila wing discs from third instar larvae appears as peripheral ministacks rather than as a ribbon linked together to form sets of stacked cisternae. By their unique morphology and by specific Golgi markers, these ministacks can be divided into five regions: tER (transitional endoplasmic reticulum), cis-Golgi, middle-Golgi, trans-Golgi, and TGN (trans-Golgi networks) (Fig. 5A). In wild-type cells, the EXTs are localized mainly in the cis- and middle-Golgi stacks (Fig. 5B,E;
supplementary material Table S2). In the wing discs overexpressing *rti dsRNA*, the localization of EXTs is shifted to regions of the TGN (Fig. 5C,E; supplementary material Table S2), indicating that, without sufficient Rti, the retrograde trafficking of EXTs is blocked. Conversely, in cells overexpressing Rti, the distribution of EXTs is shifted back to the tER region (Fig. 5D,E; supplementary material Table S2), representing considerable backward transport. All of these data suggest that the function of Rti in EXTs is to maintain the dynamic retention of EXTs in the Golgi by the retrograde trafficking pathway. A role of Rti in modulating the retrograde trafficking of EXTs is further supported by the observation that Rti interacts with each individual EXT, and by a genetic interaction between Rti and coatamer protein complex I (COP I) (supplementary material Fig. S5).

**The quantity of GOLPH3 affects the amount and cellular distribution of EXT1 and EXT2**

As EXT1 and EXT2 are genetically linked to multiple osteochondroma, we analyzed the effect on EXTs by GOLPH3 in cells cultivated from bone tissue. We examined the change in EXTs by reducing GOLPH3 levels with three different GOLPH3-specific siRNA oligonucleotides and by increasing GOLPH3 with full-length GOLPH3 transfection. Western blot analysis of protein lysates from an osteosarcoma cell line (U2OS) shows a reduction of EXT1- and EXT2-Myc when GOLPH3 is knocked down (Fig. 6A), whereas overexpression of GOLPH3 causes EXT1- and EXT2-Myc reduction in a dosage-dependent manner (Fig. 6B). That EXTs are labile when GOLPH3 is knocked down or overexpressed is supported by the blockage of EXT1-Myc reduction by the proteasomal inhibitor MG132 (Fig. 6C). As the targets of GOLPH3, EXTs are sensitive to the quantity of GOLPH3 in osteosarcoma cells (U2OS, MG63), chondrosarcoma cells (SW1353), a chondrocyte cell line (CHON-002) and rhabdomyosarcoma (RD) cells (Fig. 6A,B; supplementary material Fig. S6A). GOLPH3 has been proposed to be involved in mTOR signaling based on studies of epidermis-derived carcinomas and cell lines (Scott et al., 2009). However, GOLPH3 does not affect EXTs in epidermal cell lines, including cervical (HeLa), kidney (HEK293T), lung (A549), breast (MCF7) and urinary bladder cells (J82) (supplementary material Fig. S6B).

As in *Drosophila*, EXT1 and EXT2 protein reduction can be explained by their mis-localization within the Golgi complex. We next examined the localization of EXTs and found that, without any treatment, the endogenous EXT2 partially colocalizes with both KDEL, an ER marker, and TGN46 (TGOLN2), a trans-Golgi marker (Fig. 6D-F). However, after GOLPH3 knockdown, the endogenous EXT2 tends to colocalize with TGN46 but not KDEL, indicating accumulation of EXT2 close to the TGN region (Fig. 6G-L). In addition, EXT2 tends to localize near the ER after GOLPH3 overexpression, as the staining pattern of EXT2 in this case is more similar to that of KDEL than of TGN46 (Fig. 6M-R). We conclude that GOLPH3 modulates the dynamic retention of EXTs in a retrograde manner.

**DISCUSSION**

**Rotini regulates the sub-compartmental distribution of EXTs in the Golgi**

Glycosyltransferases are enzymes that are dynamically retained in specific sub-compartments in the Golgi apparatus for efficient glycosylation processing. Here, we demonstrate that Rti mediates the distribution of EXTs in the Golgi complex. Based on our data, the role of Rti in modulating the retrograde trafficking of EXTs and
It has been reported that GPP34 is labeled at the periphery of stacked and flattened Golgi cisternae on both the cis- and trans sides from the purified Golgi fraction (Bell et al., 2001). GMx33α associates with electron-dense tails in the Golgi budding vesicles (Snyder et al., 2006). Here, we show that Rti localizes to all parts of the Golgi apparatus (supplementary material Fig. S1I,J). It appears that GOLPH3 proteins in different species show a more widespread Golgi distribution.

A role of GOLPH3 in the retrograde trafficking pathway has been reported in several studies. The targeting of yeast GOLPH3, Vps74p, to the Golgi apparatus by phosphatidylinositol-4-phosphate [PtdIns(4)P] is required for the dynamic retention of mannosyltransferase (Schmitz et al., 2008; Tu et al., 2008; Wood et al., 2009). In addition, mutations in Vps74p result in synthetic growth defects when combined with mutations in GET1/GET2 and RIC1/YPT6, components of retrograde transport to the cis-Golgi or ER (Pan et al., 2006; Tong et al., 2004). As seen with Vps74p, both Rti and GOLPH3 also function to maintain the proper retrograde transport of fly and human EXTs in the Golgi. Furthermore, Vps35, a highly conserved member of the retromer complex, physically interacts with GOLPH3, which suggests that GOLPH3 regulates the retrograde transport of proteins from the endosome to the TGN (Scott et al., 2009).

GOLPH3 proteins also play a role in the secretory pathway. It was found that GMx33α exits the Golgi associated with tubules and vesicles to facilitate cargo sorting from the Golgi (Snyder et al., 2006). GOLPH3 brings PtdIns(4)P and actomyosin 18A to shape the trans-Golgi architecture for efficient tubule and vesicle formation by
facilitating cargo trafficking from the Golgi to the plasma membrane (Dippold et al., 2009). All of these data suggest that GOLPH3 proteins may facilitate vesicle formation and sort cargo from the Golgi. Nevertheless, neither the core protein Dilp in flies (Fig. 3B-F) nor Gas1p in yeast (Tu et al., 2008) are targets carried by Rti and Vsp74p, respectively, from the Golgi to the plasma membrane, suggesting that GOLPH3 proteins may differentially transport selected cargos from the Golgi to the plasma membrane. All of these findings suggest that GOLPH3 family proteins carry out their general and dynamic functions in the endomembrane system, including the ER, Golgi, and plasma membrane.

Besides the lawn-of-denticles phenotype, rti GLC embryos also show a partially dorsalized and twisting mutant phenotype (Fig. 1B), which is not seen in mutations affecting HSPGs biosynthesis (Bornemann et al., 2008). Our preliminary results suggest that in follicle cells, Rti also influences the distribution pattern of Pipe, a putative sulfortransferase (Sen et al., 1998), in the Golgi complex (W.-L.C., P.-L. Chen, Y.-C. Lin, S.-C.C., Y.-Y.C., D. Stein, E. K. Lemosy and T.-B.C., unpublished). In addition, rti knockdown or overexpression disturbs wing (supplementary material Table S1) and eye development (C.H.C., unpublished). These diverse phenotypes displayed by rti mutation suggest that Rti may have additional targets.

### The putative role of GOLPH3 in multiple osteochondromas

The effect on EXTs by Rti/GOLPH3 in different cells is proposed to reflect the requirement for HSPGs. In Drosophila embryonic and imaginal disc cells, HSPGs are a prerequisite for Hh transport and signaling (Yan and Lin, 2009). For proper HSPG biosynthesis, it is likely that EXTs need to be recycled by Rti more frequently in order to reiterate the catalytic addition of carbohydrate to polymerize the GAG backbone. Compared with other GAG synthetic enzymes, EXTs are particularly sensitive to the activity of Rti. In human, during chondrogenesis, extracellular matrix proteoglycans are enriched in the surrounding environments. These enriched proteoglycans, including HSPGs, establish a supportive mechanical environment and modulate the concentration or activity of important growth factors (Quintana et al., 2009). The importance of HSPGs in chondrogenesis is emphasized by the disease MO. It is hypothesized that mutated EXTL and EXT2 in exostoses disturb HSPG biosynthesis and, consequently, IHH signaling (Duncan et al., 2001). Here, we demonstrate that EXTs are particularly sensitive to the activity of GOLPH3. It will be worthwhile to examine whether the failure of the proper retrograde trafficking of EXTs due to either depletion or overexpression of GOLPH3 can result in insufficient EXT activity and, consequently, an abnormal HSPG microenvironment for IHH transport.

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### Competing interests statement

The authors declare no competing financial interests.

### Author contributions

W.-L.C. designed and performed experiments, analysed data and wrote the paper; C.-W.C., C.-H.C. and K.-S.T. carried out yeast two-hybrid assays; C.-W.C. carried out the work shown in Fig. 3G and Figure S4A-F, C.-H.C. carried out the work shown in Fig. 1E-H, L-N; H.-H.S. isolated rti1644 and carried out the work shown in Fig. 1A-C, J-K and Fig. S2A-C; C.-S.C. produced Rti Ab and Fig. 1D; C.-W.H. carried out the work shown in Fig. S1F-H; M.-D.L. contributed Fig. S2E,F; T.-B.C. designed experiments, analysed data and wrote the paper.

### Supplementary material

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### References


**Fig. S1. The P(lArB)1164A4 insertion specifically affects CG7085, which is a member of Golgi-enriched proteins.** (A) The molecular map of the rti locus. rti1164A4 was recovered from the P(lArB)1164A4 insertion in a complex locus at 21A consisting of two genes in opposite orientations, CG7085 and CG15387, with a ~300 bp complementary region (yellow boxes) in their 5' TRs (white and yellow boxes). The P(lArB)1164A4 insertion resides 8 bp downstream of the translation start site of CG7085. Based on its phenotype (Fig. 1B), we refer to CG7085 as rotini (rti). The mutation caused by the P(lacW)i5379 insertion from the Bloomington Stock Center has an insertion within the 5' UTR of CG7085. Green boxes indicate exons. (B-E) The rti1164A4 insertion specifically affects CG7085 but not CG15387. The rti clone is created by the FRT/FLP technique and marked in the absence of Myc staining. Reintroduction of CG7085 (i.e. UAS-rti6-1) (B,C), but not CG15387 (D,E), to the rti clone disc driven by engrailed-Gal4 (enGal4) suppressed the phenotype of Hh reduction caused by rti mutation (Fig. 1I-K). Scale bars: 50 μm. (F-H) Wing imaginal disc immunostaining shows that the cytoplasmic Rti is highly colocalized with the Golgi protein Fringe-GT-Myc. Scale bars: 5 μm. (I,J) The subcellular localization of Rti in *Drosophila* wing imaginal disc cells under immunoelectron microscopy. Rti is labeled with 10-nm gold particles. (I) The localization of Rti is mainly in the Golgi complex. (J) Rti (arrows, 10-nm gold) is detectable in every part of the Golgi complex, including the cis-Golgi, which is marked by GM130 (arrowheads, 15-nm gold). Bracket indicates a Golgi unit. ER, endoplasmic reticulum; G, Golgi complex; M, mitochondria.
Fig. S2. Rti does not affect Hh transcription, auto-cleavage, or transportation to the plasma membrane. (A-C) Rti does not affect the transcription of hh. β-galactosidase expression, under the control of the hh promoter (hh-lacZ), is not affected within the rti1164A4 clone, which is marked by the absence of GFP. Scale bars: 30 μm. (D) Rti does not affect Hh autocleavage. Western blot analyses using an anti-HA antibody against en>Hh-FH and en>UAS-Hh-NHA embryos in wild type, and en>Hh-FH embryos in rti GLC without paternal function, are shown. The full-length Hh-HA (Hh-FH) is ~50 kDa, and the processed N-terminal fragment of Hh-NHA is ~30 kDa. OreR was used as a negative control. (E-E″) z-axis sections of wing discs. Overexpression of Rti (ap>rti) causes Hh to spread into the anterior compartment (arrowhead in E′) and lose its restriction to the posterior compartment (arrow in E″). The dashed lines indicate the site of the cross-section. Anterior is left, and dorsal is up. Scale bar: 30 μm. (F) Wing imaginal discs labeled with Hh antibody using an extracellular labeling protocol. Overexpression of Rti (ap>rti) causes Hh to spread into the anterior compartment. Anterior is left, and dorsal is up. Scale bar: 30 μm.
Fig. S3. rti mutation reduces the expression of EXTs but not of Sfl or Sll. (A-I) The expression of three endogenous EXTs, Botv (B), Ttv (E) and Sotv (H), is decreased in rti mutant clones. The rti1164A4 clones are marked by the absence of GFP and the clone boundaries are shown with dashed lines. Scale bars: 30 μm. (J-O) The expression of Sfl (K) and Sll (N) is not affected in rti mutant clones. The rti1164A4 clones are marked by the absence of GFP and the clone boundaries are shown with dashed lines. Scale bars: 30 μm.
Fig. S4. Rti interacts with a v-SNARE protein, dVti1. (A) CG3279 interacts with Rti in a two-hybrid assay. Clone 1: positive control (pTD1-1/pVA3-1); clone 2: pAS2-1-rti/pGAD10-CG3279; clones 3 and 4: negative controls: pAS2-1 rti/pGAD10 and pAS2-1/pGAD10-CG3279, respectively. (B) Rti physically interacts with dVti1 in a GST pull-down assay. His-Rti physically interacts with GST-dVti1, but not with GST. The input is indicated in the right-hand panel. (C) Amino acid sequence of CG3279 and alignment of identified homologs from yeast to human. The highest homology to CG3279 is with yeast Vti1p, showing 26% identity and 42% similarity. Specific residues conserved in all species are highlighted in green. (D-F) Endogenous Rti is partially colocalized with dVti1-V5 in S2 cells. (G-I) Endogenous Rti is also colocalized with dVti1-EYFP in wing imaginal disc cells (arrowheads).
Fig. S5. Rti physically interacts with EXTs and genetically interact with COP I. (A-C) Co-immunoprecipitation of Rti with Botv (A), Ttv (B) and Sotv (C). Protein extracts were isolated from transgenic third instar larvae carrying $hs\,>\,rti^{6-1};\,botv-v5$ or $hs\,>\,rti^{6-1};\,ttv-myc$ after 37°C heat shock for one hour to express V5-tagged Botv and Myc-tagged Ttv. For Sotv, protein extract was from Drosophila S2 cells co-transfected with pAc5.1-sotv-v5 and pAc 5.1-rti-GFP to express V5-tagged Sotv and GFP-tagged Rti. Cell lysates were immunoprecipitated and than analyzed by western blotting with the antibodies indicated. IP, immunoprecipitation; IB, immunoblot. Rti interacts with each individual EXT protein. (D-H) Rti interacts genetically with COP I. The whole mounts of adult wings are oriented proximal to the left, anterior up. (D) A wild-type wing. (E-H) Wings with overexpression of Rti on the dorsal side driven by $ap\,\text{-Gal4}$ ($ap\,>\,rti^{6-1}$). The swollen and bubbled phenotype caused by the overexpression of Rti (E) can be suppressed by the COP I mutation, $COP\alpha^{\text{EY09064}}$ (H), but not by the COP II mutation, $\text{Sec13}^{01031}$ (G), or by GFP control flies (F). See supplementary material Table S2 for a detailed quantitative comparison. (I) The staining pattern of 3G10 in the imaginal disc derived from flies with overexpression of Rti on the dorsal side ($ap\,>\,rti^{6-1}$) and mutation of one subunit of the COP I complex, $COP\alpha^{\text{EY09064}}$. Scale bar: 30 μm.
Fig. S6. EXTs are sensitive to the quantity of GOLPH3 in cartilage, bone and muscle cells. Western blot of EXT2 or EXT1- and EXT2-Myc in cell lysates. Rti antibody was used to recognize GOLPH3 based on their 82% sequence similarity. Actin or tubulin was used as an internal loading control. (A) The protein level of EXT1/2 is affected by GOLPH3 in cartilage, bone and muscle cells. In chondrosarcoma cells (SW1353), EXT2-Myc is reduced in cells treated with GOLPH3 siRNA. Lanes 1, 2 and 3 are three specific oligonucleotides with different GC% against GOLPH3. Control lanes 1 and 2 are two different scrambled sequences. EXT1- and EXT2-Myc are also decreased when each individual cell is transfected with GOLPH3 compared with cells transfected with GFP. In the chondrocyte cell line CHON-002, EXT2 is reduced in cells treated with GOLPH3 specific shRNA (sh1 and sh2 are two specific oligonucleotides against GOLPH3) as well as in cells that overexpress GOLPH3. The endogenous GOLPH3 protein level is shown in cell lysates transfected with GFP. In osteosarcoma cells (MG63), EXT1-Myc is reduced when cells are transfected with GOLPH3 compared to cells transfected with GFP. In rhabdomyosarcoma cells (RD), EXT1- and EXT2-Myc are reduced when cells are transfected with GOLPH3 compared to cells transfected with GFP. (B) The protein level of EXT1/2 is not affected by GOLPH3 in epidermal cells. In lung cells (A549), breast cells (MCF7) and urinary bladder cells (J82), the EXT1-Myc level does not change when cells are transfected with GOLPH3 compared with cells transfected with GFP. In cervix cells (HeLa) and kidney cells (HEK293T), the EXT1-Myc level does not change in cells treated with three specific oligonucleotides with different GC% against GOLPH3 (GOLPH3 siRNA lane 1, 2 and 3). Control lanes 1 and 2 show the results using two different scrambled sequences. Similarly, the level of EXT2-Myc does not change significantly when HeLa and kidney cells are treated with three specific oligonucleotides against GOLPH3 or with two scrambled sequences.