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

Wei-Ling Chang, Che-Wei Chang, Yu-Yun Chang, Hsin-Ho Sung, Ming-Der Lin, Shu-Chuan Chang, Chung-Hao Chen, Chia-Wei Huang, Kuei-Shu Tung and Tze-Bin Chou*

**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-acetylgalactosamine (GlcNAc) disaccharides. The Golgi-resident type II transmembrane exostosis (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 COPI coat protein (Schmitz et al., 2008; Tu et al., **Accepted 12 April 2013**

Institute of Molecular and Cellular Biology, National Taiwan University, No.1 Sec.4 Roosevelt Road, Taipei, 10617, Taiwan.

*Author for correspondence (tbchou@ntu.edu.tw)
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 effector(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-Fmo, disp and UAS-hh-Meta (Burke et al., 1999), In*DMelo*1 (Bellaiche et al., 1998), UAS-tv-mycc (The et al., 1999), UAS-sotv (Takei et al., 2004), hhMO2 and UAS-Fringe-GT-mycc (Brücker et al., 2000). Other stocks were provided by the Bloomington Stock Center. The UAS-rti (CG7085) and UAS-CG15387 are EcoRI/ Xhol fragments of LD23816 and LP02710 directionally cloned into pUAsp. The UAsp-botv- v5/His is a Kpn1/ Eag fragment cloned into pUA9Sp. An rti RNAi construct was primers 5′-AATCTAGATGTGCTATTGAGAGGAGCGT-3′ and 5′-AATCTAGATGCTTCAAGTGGGTCAACGAT-3′ for in vitro transcription to generate dsRNA followed by transfection into *Drosophila* S2 cells.

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 pAc5.1-V5-His A. The C-terminus of the *sotv* cDNA sequence from GH02288 was digested with EcoRI and *Xhol* and ligated into pAc5.1-V5-His A. The N-terminus of *sotv* released from GH02288 by EcoRI digestion was ligated into EcoRI-digested pAc5.1-V5-His A. The full-length *tv* coding sequence from LD10920 and the GlcAT-1 coding sequence from GH05057 was digested with EcoRI and *Xhol* and ligated into pAc5.1-V5-His A. Donald L. Jarvis (University of Wyoming, Laramie, WY, USA) kindly provided pAc5.1-GalT7-GFP.

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

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

Antibody generation and immunchemistry
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 were digested with *KpnI* and *AatII* and a fragment containing the full-length coding sequence from *LD23816* and *H11032* used primers 5′/His was a Rotini modulates EXT trafficking in *Escherichia coli* BL21. Three EXT cDNA sequences were used for production of an Rti-specific antibody, the coding sequence of the *dispp* stock and transgenic stocks and transgenics (Scott et al., 2009), the actual effector(s) of GOLPH3 has not been identified.

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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.

GSTM 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 g004052E02) for overexpression or GOLPH3 siRNA (Invitrogen, Stealth RNAi 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-FRT2L2R protocol (Lin et al., 2006), we identified the P[ArB] 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 dorso-lateral and twisting (Fig. 1B).

The P[ArB] 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/CyO 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. 1I-K). In the anterior compartment, Hh signaling results in the expression of Patched (Ptc) (Chen and Struhl, 1996), which can be detected as far as five cells away from the anterior-posterior (A/P) boundary. When an rti mutant clone is found in the anterior compartment abutting the A/P boundary, the Ptc expression pattern is narrowed to a width of two cells, compared with Ptc expression wild-type cells (Fig. 1L-N), suggesting that Rti is necessary 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
HSPGs. The Rti is involved in HSPG secretion (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′) 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 signaling (Lin, 2004). In tvv, sotv 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 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′) and extracellular Hh staining (supplementary material Fig. S2F), we confirmed that Hh can indeed spread towards the anterior compartment.

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**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 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 mutant (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 double mutant. By analyzing the level of Hh staining in the rti clones generated in the wing disc of disp homozygous larvae, we found that the staining of Hh in rti disp double mutant cells (Fig. 2D-F, without GFP) is indistinguishable from that in disp 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.

**Rotini modulates EXT trafficking**

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 around 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
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 β-galactosyltransferase (βGalT7) and glucuronosyltransferase (GlucAT1) are reduced (Fig. 3G, right panel).
Rotini modulates EXT trafficking

EXTs, in the reticulum, are involved in either the anterograde or retrograde trafficking functions in vesicle trafficking. Therefore, we assessed whether Rti (supplementary material Fig. S4), supporting the hypothesis that Rti protein (CG3279), was found to interact physically with Rti by a two-hybrid screen and a GST pull-down assay, a v-SNARE cisternae (supplementary material Fig. S1I,J). Moreover, based on the Golgi complex, especially at the vesicles adjacent to the Golgi of EXTs within the Golgi. Rti is detectable in all compartments of 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. S1LJ). 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 rti1164A4 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 parts: tER, cis-Golgi, middle-Golgi, trans-Golgi and TGN. The bracket indicates the Golgi unit. The distribution patterns of Botv in three different rti backgrounds. Arrows indicate Botv signal detected by 10-nm gold particles. The brackets indicate the Golgi units. (B) In wild type, Botv mainly localizes in the cis and middle-Golgi. (C) Rti depletion leads to Botv accumulation in TGN. (D) Rti overexpression causes Botv redistribution in tER. (E) Botv steady-state distributions. The number of Golgi examined and densities of gold particles calculated are listed in supplementary material Table S2. **P<0.05 in three genetic backgrounds.
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 coatomer 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 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.

Fig. 6. The quantity of GOLPH3 affects the amount and cellular distribution of EXT1 and EXT2. (A,B) GOLPH3 influences the amount of EXT1-Myc and EXT2-Myc in U2OS cell lysates. Rti antibody is used to recognize GOLPH3 based on sequence similarity (82%). (A) EXT1 and EXT2 are reduced in cells treated with GOLPH3 siRNA. Lanes 1, 2 and 3 are three specific oligonucleotides with different GC percentage against GOLPH3. Control lanes 1 and 2 are two different scrambled sequences. (B) EXT1 and EXT2 decrease in a dosage-dependent manner when cells are transfected with increasing amounts of GOLPH3. (C) The reduction of EXT1-Myc in cells transfected with GOLPH3 siRNA and with GOLPH3 is blocked after treatment with the proteasomal inhibitor MG132 but not with DMSO. (D-R) GOLPH3 modulates the cellular distribution of endogenous EXT2 in SW1353 cells. (D-F) EXT2 partially colocalizes with KDEL (an ER marker) and TGN (a trans-Golgi marker). (G-L) In GOLPH3 knockdown cells, EXT2 tends to colocalize with TGN46, but not with KDEL. (M-R) In cells overexpressing GOLPH3, the EXT2 distribution pattern is more similar to that of KDEL than to that of TGN46. Insets in E and F show enlargements.
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**Fig. 7. The role of Rti in modulating the retrograde trafficking of EXTs and its effect in morphogen signaling.** (A) In wild type, Rti cooperates with Vti1 and COP I to transport fly EXTs in a retrograde manner. The steady-state distribution of EXTs in cis- and middle-Golgi maintains the proper polymerization of HSPGs. The transport of extracellular Hh is mediated by HSPGs with full-length GAG side chains. (B) In the rti knockdown, the retrograde transport of EXTs is attenuated. The EXTs that accumulate at the TGN side in rti1164A4 cells tend to enter the Hrs-mediated MVB (multiple vesicle body)-lysosome degradation route. In this situation, HSPGs without long GAG chains can be transported to the plasma membrane. However, these impaired HSPGs affect morphogen signaling. (C) When Rti is overexpressed, EXTs are overwhelmed by the resulting retrograde trafficking. The inevitable result is the retention of EXTs at the cis-TER side of the Golgi, where EXTs cannot function efficiently. The resulting impaired HSPGs cause aberrant morphogen signaling.

its consequent effect on morphogen signaling is depicted (Fig. 7). In wild-type cells, Rti might cooperate, directly or indirectly, with v-SNARE and COP I to transport EXTs in a retrograde direction to prevent them from exiting the Golgi and to promote their enzymatic activity. The maintenance of the steady-state distribution of EXTs by Rti in the cis/middle-Golgi allows the proper polymerization of GAG chains (Fig. 7A). When Rti activity is partially reduced, the retrograde transport of EXTs is also reduced; this shifts the dynamic retention of EXTs to the trans-most side. In rti1164A4 cells, which completely lack rti function, EXTs cannot re-enter the route for polymerizing GAG chains by retrograde transport. These stalled EXTs tend to undergo Hrs-mediated degradation in the lysosome (Fig. 7B). Without EXTs, the impaired HSPGs are still transported to the membrane (Fig. 3B-F); however, morphogen signaling is impaired (Fig. 1L-N). When Rti is overexpressed, a quick or massive EXT retrograde recycling might not provide enough time for their catalytic reactions to occur. It is conceivable that, above a certain level, Rti activity forces EXTs to accumulate at the cis-TER side (Fig. 7C) where EXTs are not fully functional for GAG polymerization (Fig. 2J). We suspect that a labile pool of cis-TER-retained EXTs may be modified and targeted for degradation to maintain cellular stability, as previously described for ER-stressed proteins (Hampton, 2002). This produces an impaired HSPG environment, causing released Hh to move over a remote distance towards the anterior compartment (Fig. 2K) but fail to fully activate Ptc (Fig. 2L).

In conclusion, the proper function of EXTs depends not only on their enzymatic activities but also on their sub-compartmental distributions. As a Golgi coat protein, Rti dynamically retains EXTs in the Golgi to achieve sufficient activity for the biosynthesis of HSPGs, which then allows proper morphogen signaling.

**Distribution and functions of GOLPH3 family proteins**

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 actinomycin 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 Dlp 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 dorsализed 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 sulfotransferase (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 EXT1 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 HPSG 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-C, C.-H.C. carried out the work shown in Fig. S4G-J, Y.-Y.C. carried out the work shown in Figs. 1E-H-L; H.-S.S. isolated rti16444 and carried out the work shown in Fig. 1A-C, J.K and Fig. 2A-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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