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ORIGINAL ARTICLE

Functional conservation of the human *EXT1* tumor suppressor gene and its *Drosophila* homolog *tout velu*

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Abstract Heparan sulfate proteoglycans play a vital role in signaling of various growth factors in both *Drosophila* and vertebrates. In *Drosophila*, mutations in the *tout velu* (*ttv*) gene, a homolog of the mammalian *EXT1* tumor suppressor gene, leads to abrogation of glycosaminoglycan (GAG) biosynthesis. This impairs distribution and signaling activities of various morphogens such as Hedgehog (Hh), Wingless (Wg), and Decapentaplegic (Dpp). Mutations in members of the exostosin (*EXT*) gene family lead to hereditary multiple exostosis in humans leading to bone outgrowths and tumors. In this study, we provide genetic and biochemical evidence that the human *EXT1* (*hEXT1*) gene is conserved through species and can functionally complement the *ttv* mutation in *Drosophila*. The *hEXT1* gene was able to rescue a *ttv* null mutant to adulthood and restore GAG biosynthesis.

Keywords *Drosophila* · HSPGs · EXT · Conservation · Evolution

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Introduction

Hereditary multiple exostosis (HME) is an autosomal dominant disorder that primarily affects endochondral bone growth (Wicklund et al. 1995) resulting in short stature and formation of benign cartilage-capped tumors (exostosis) in affected individuals (Solomon 1963). Although clinically limb length inequalities, skeletal deformities, and orthopedic complications are common characteristic features, in 2–5% of HME patients the benign exostosis transforms to malignant chondrosarcoma or osteosarcoma (Hennekam 1991; Wicklund et al. 1995). Hereditary and sporadic cases of HME have been linked to mutations in the *EXT1* and *EXT2* genes. The *EXT* genes encode for type II transmembrane glycoproteins that localize predominantly to the endoplasmic reticulum (ER) and golgi. They form a hetero-oligomeric complex in the golgi apparatus (Kobayashi et al. 2000; McCormick et al. 2000) and are involved in the synthesis of heparan sulfate glycosaminoglycans (HSGAGs; Lind et al. 1998; McCormick et al. 1998, 2000; Toyoda et al. 2000b; Wei et al. 2000). Heparan sulfate proteoglycans (HSPGs) consist of a glycosylated protein core, which can either be a transmembrane, glycosylphosphatidyl-inositol (GPI)-linked, or secreted protein, and they are abundant on the cell surface and extracellular matrix.

The *Drosophila* genome harbors three homologs of the mammalian *EXT* genes, namely, *tout velu* (*ttv*; Bellaiche et al. 1998), *sister of tout velu* (*sotv*; Bornemann et al. 2004; Han et al. 2004b), and *brother of tout velu* (*botv*; Han et al. 2004b). Mutations in either one of the three genes result in impaired HSPG biosynthesis (The et al. 1999; Han et al. 2004b). Ttv along with Sotv functions in vivo as a HSGAG copolymerase, similar to the vertebrate EXT1 and EXT2 as shown by biochemical and immunohistochemical studies (The et al. 1999; Toyoda et al. 2000a, b; Han et al. 2004b). Interestingly, HSPGs are implicated in shaping the gradient of several morphogens, such as Hedgehog (Hh), Wingless (Wg), and Decapentaplegic (Dpp) by affecting their distribution. In addition, the GPI-linked HSPG Dally has been shown to affect Hh signaling (Desbordes et al. 2003; Han et al. 2004a). Mutations in any of the three *Drosophila* *EXT* genes result in impaired Hh, Wg, and Dpp distribution and signaling (The et al. 1999; Bornemann et al. 2004; Han et al. 2004b; Takei et al. 2004).

Mice lacking *Ext1* fail to gastrulate and lack HS biosynthesis (Lin et al. 2000), while mice heterozygous for *Ext1* do not show exostoses and express about 50% HS (Lin et al. 2000). Homozygous *Ext2* mice also fail to gastrulate owing to defective HS biosynthesis, and a number of heterozygous animals showed exostoses as well (Stickens et al. 2005). Similar to Ttv, EXT1 has been implicated in Hh distribution and signaling mediated by HSPGs (Lin et al. 2000; Koziel et al. 2004). Interestingly, Indian Hedgehog (Ihh), a mammalian homolog of Hh, plays a pivotal role in controlling the rate of chondrocyte differentiation and bone development (Vortkamp et al. 1996; Zou et al. 1997; Koziel et al. 2004). Lin et al. (2000) have shown that Ihh is incapable of associating with the target cell surface in murine *Ext1*−/− embryos. Thus it appears that loss of *Ext1* leads to impaired Ihh binding and distribution.

To investigate whether human EXT1 (*hEXT1*) and Ttv are functionally conserved, we expressed the *hEXT1* gene, which is 56% identical to *ttv,* in *Drosophila*. We showed that hEXT1 localizes to the ER in *Drosophila* wing discs and that it interacts biochemically with Sotv in human cell lines to form a complex that functions as the active HSGAG copolymerase. The *hEXT1* transgene was able to rescue *Drosophila ttv* mutants to adulthood and could synthesize HSGAG chains in vivo. Our results highlight the functional conservation between the two orthologues, *Drosophila* Ttv and human EXT1.

Materials and methods

EXT construct

Human *EXT1-Green Fluorescent Protein* (*hEXT1-GFP*) was polymerase chain reaction amplified from pEXT1 GFP (McCormick et al. 2000; a gift from Tufaro lab, University of British Columbia), harboring GFP downstream of *EXT1* gene, using the primers 5′-GGA CTC AGA TCC CGC AGG ACA CAT-3′ and 5′-CCT CTA CAAATG TGG TAT GGC TGA TTATGA-3′ and cloned into pGEM-T-Easy (Invitrogen) and pUASp2 (a gift from Pernille Rorth) vectors at *Eco*RI site. The *sotv* complementary DNA (cDNA) cloned in pAC5.1/V5-His C construct (Han et al. 2004b, a gift from Xinhua Lin's lab). For transfecting human embryonic kidney (HEK) 293 cells *hEXT1* cDNA was cloned in p3XFLAG-myc-CMV-26 vector (Sigma) at *Eco*RI-*Xba*I site and *sotv* cDNA in pcDNA4/V5HisB (Invitrogen) at *Bam*HI-*Xho*I site.

Fly strains and genetics

Several *hEXT1-GFP* transgenic fly strains were generated of which the *hEXT1-GFP2.2*/*Cyo*; *Dp*/*TM3* (*hEXT1-GFP* transgene on the second chromosome) and *Sp*/*Cyo*; *hEXT1-GFP3.1* (*hEXT1-GFP* transgene on the third chromosome) were used. For rescue of homozygous $ttv^{l(2)00681}$ mutants, the *hEXT1-GFP2.2* transgene was recombined with the $ttv^{l(2)00681}$ mutation in the *FRT* $G^{13}ttv^{l(2)00681}$/*Cyo* mutant to generate *hEXT1-GFP2.2 FRT* $G^{13}ttv^{l(2)00681}$/*Cyo*; *Tubulin-Gal4*/*TM6B*. These flies were then homozygozed to check whether the homozygous $ttv^{l(2)00681}$ mutant could be rescued to adulthood. For ectopic expression of *hEXT1* transgene in the *engrailed* (*en*) domain, females with *en-GAL4* on the second chromosome were crossed to males of genotype *hEXT1-GFP2.2*/*Cyo*; *Dp*/*TM3.*

Females with germline clones (GLCs) lacking maternal and zygotic $ttv^{l(2)00681}$ activity were generated as described previously (Chou and Perrimon 1996). For expression of $ttv^{l(2)00681}$ and *hEXT1-GFP3.1* in the *hairy*-domain females with homozygous $ttv^{l(2)00681}$ germ-line clones having the genotype *y w* flp^{12}/+; *FRT* $G^{13}ttv^{l(2)00681}$/*FRT*$G^{13}P[ovo^{D1}]$; *hairy-Gal4*/+ were crossed to $ttv^{l(2)00681}$/*Cyo*; *UAS-ttv -myc14.1* or $ttv^{l(2)00681}$/*Cyo*; *UAS-hEXT1-GFP3.1* males. For sugar chain biochemistry, *hEXT1-GFP2.2 FRT* $G^{13}ttv^{l(2)00681}$/*Cyo*; *tubGal4*/*SM6 TM6B* was generated and non-Tubby larvae were used for sugar chain analysis. $ttv^{l(2)00681}$ mutant used in the study were maintained as a stock over *Cyo P*[*w+, ubq-GFP*], the second chromosome balancer marked with GFP and homozygous larvae were identified by the lack of GFP fluorescence detectable under a GFP dissecting microscope.

Antibody staining

Third instar larval imaginal discs were fixed for 20 min in 4% formaldehyde in phosphate-buffered saline with 0.1% Tween20 (PBT). Staining of larval imaginal discs were performed as described before (The et al. 1999). Stainings

were performed with FM4-64 membrane marker dye (Molecular Probe). Antibodies diluted in PBT include rat anti-Bip, 1:40 (Brabaham Institute, Cambridge, UK), rabbit anti-Lava Lamp, 1:5,000 (Sisson et al. 2000; a gift from John Sisson, University of Texas); mouse anti-HS 3G10, 1:100 (Seikagaku Corporation); mouse anti-V5, 1:5,000 (Invitrogen), and mouse anti-FLAG, 1:5,000 (Sigma). Fixation of embryo and HS GAG staining using 3G10 antibody were performed as described (The et al. 1999). Secondary antibodies for histochemical staining, Western blotting (WB), and fluorescent secondary antibodies were from Jackson Immunoresearch. Images of stained discs were taken with Leica TCS SP2 AOBS Confocal microscope. Images of stained embryos were taken with Zeiss Axioskop 2 Plus microscope. Camera exposure times were kept constant within an experiment.

Immunoprecipitaion and Western blotting

HEK 293 cells (1×10^7) were transfected with plasmids expressing Flag-tagged hEXT1, V5-tagged Sotv, or both as indicated, using Effectine (Qiagen) following the manufacturer's procedures. The cells were then lysed in 1.5 ml of lysis buffer [20 mM Tris–HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 5 mM ethylenediamine tetraacetic acid (EDTA), 150 µl Protease Inhibitor Cocktail (Sigma), and 75 µl phenylmethanesulphonylfluoride (PMSF) (Sigma; 10 mg/ml)] on ice for 20 min. The precleared lysate was used for immunoprecipitation using 5.0 µg of either anti-HA, anti-Flag, or anti-V5 antibody for 2 h at 4°C. Immunoprecipitates were washed five times with wash buffer [10 mM Tris–HCl (pH 7.5), 0.1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 10 µl/ml of Protease Inhibitor Cocktail (Sigma), and 5 µl/ml PMSF (10 mg/ml)] and analyzed by WB. WB was carried out as described (The et al. 1999). Antibodies used were mouse anti-V5, 1:5,000 (Invitrogen) and mouse anti-FLAG, 1:5,000 (Sigma).

Sugar chain biochemistry

Analyses of HS-derived disaccharides were performed as previously reported (Toyoda et al. 2000a) using 100 third instar larvae. As a wild type strain, Oregon R was used. Results shown are from two independent experiments.

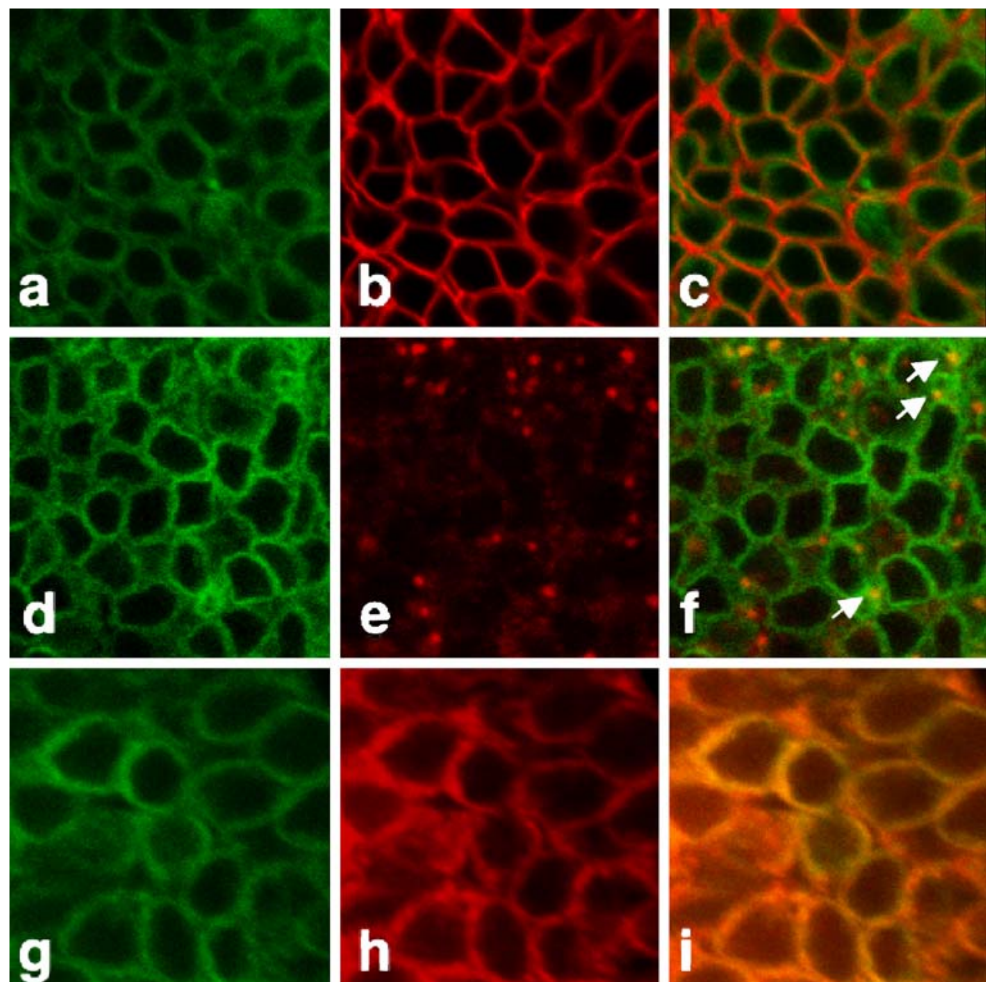

Fig. 1 hEXT1-GFP localizes to ER and golgi in *Drosophila melanogaster*. Wing imaginal disc cells, showing the expression of hEXT1-GFP indicated by presence of GFP (*green*) (**a**, **d**, and **g**), stained with membrane dye FM4-64 (*red*) (**b**), the golgi marker Lavalamp (*red*) (**e**), and ER marker Bip (*red*) (**h**). hEXT1-GFP does not colocalize with FM4-64 (**c**). Colocalization of hEXT1-GFP with Lavalamp (**f**) or Bip (**i**) is seen as *yellow*. *Arrows* indicate examples where hEXT1-GFP colocalization with golgi marker protein Lavalamp is detected (**f**)

Table 1 Rescue of a *ttv* mutant with *hEXT1-GFP* transgene

	Experiment number	Total number of flies	Total number of rescued flies	Percent of rescued flies
Control	1	42	0	0
	2	25	0	0
	3	49	0	0
Rescue	1	58	4	6.9
	2	37	2	5.4
	3	63	3	4.7

As control animals, *ttv* mutant flies that do not harbor the *hEXT1-GFP* were used, while in the rescue experiment, *ttv* mutant flies expressing the *hEXT1-GFP* were used. The percentage of rescued flies indicate the percentage of total animals rescued to adulthood.

Results

Subcellular localization of hEXT1 protein in *Drosophila*

In both *Drosophila* and mammals, EXT1 or EXT2 protein localizes mainly in the ER (McCormick et al. 1998, 2000; The et al. 1999; Han et al. 2004b; Kobayashi et al. 2000). However, when both the proteins were expressed in the same cell, the EXT proteins were found to be mostly relocated from the ER to the golgi network (McCormick et al. 2000) where polymerization and sulfation of HSPG occurs (Muckenthaler et al. 1998). To determine the subcellular localization of hEXT1-GFP in *Drosophila*, the wing imaginal discs expressing hEXT1-GFP under the control of *en-GAL4* were stained for colocalization with plasma membrane marker dye FM4-64 (Fig. 1a–c), ER protein Bip (Fig. 1d–f), and golgi protein Lava Lamp (Fig. 1g–i). hEXT1-GFP colocalized mostly with Bip (Fig. 1f) and partially with Lava Lamp (Fig. 1i) but not with membrane dye FM4-64 (Fig. 1c). These results show that hEXT1-GFP protein in *Drosophila* localizes mainly to the ER and partially to the golgi, as previously shown in case of EXT1 in human cells and Ttv in *Drosophila* (McCormick et al. 1998, 2000; The et al. 1999; Han et al. 2004b), indicating that the protein localizes as endogenous Ttv.

Rescue of homozygous *ttv* mutants by expression of *hEXT1*

Homozygous $ttv^{l(2)00681}$ null mutants (from here on *ttv*) die at the pupal stage, and animals depleted from maternal product are embryonic lethal. However, when the *hEXT1-*

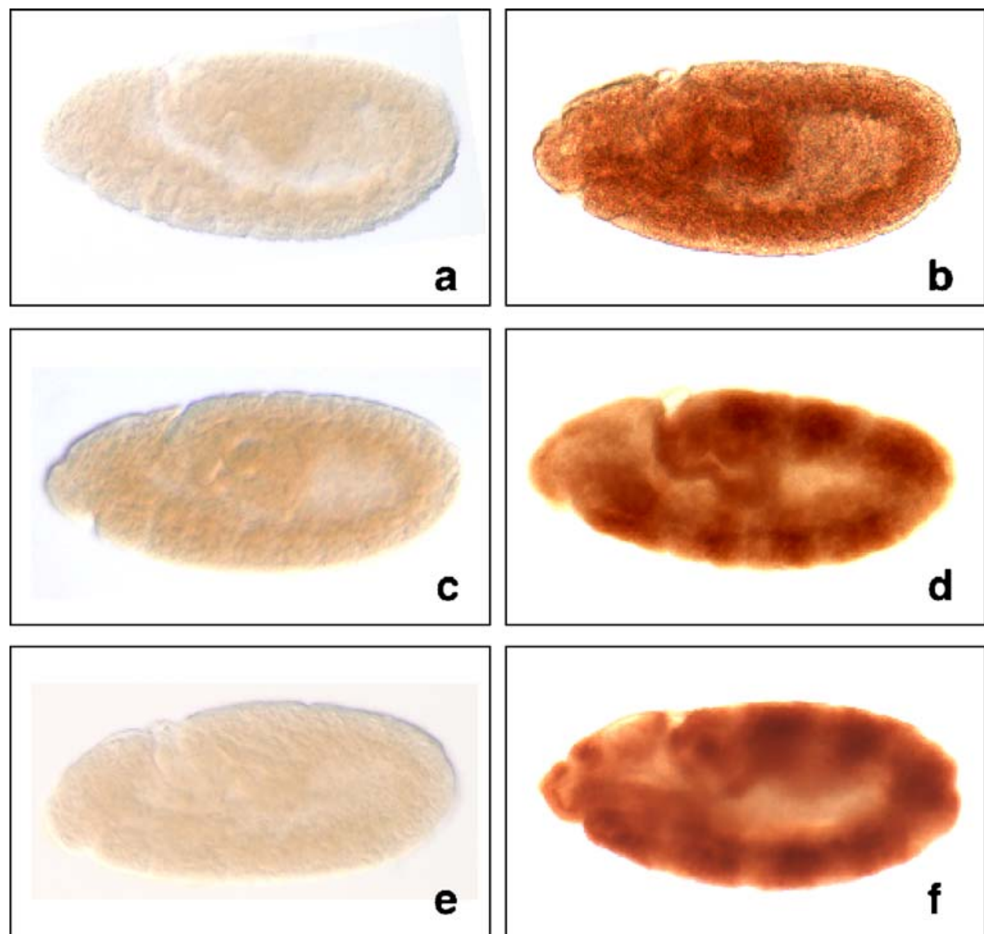

Fig. 2 HSPG biosynthesis in *ttv* mutant embryos is rescued by *hEXT1-GFP* expression in vivo. Staining of wild-type (WT) (**a**, **b**), *ttv* mutant (**c**), *ttv* mutant expressing *ttv-myc* transgene in the *hairy*-domain (**d**), and *ttv* mutant expressing *hEXT1-GFP* transgene in the *hairy*-domain (**e**, **f**) with 3G10 antibody. No staining is detected in the negative controls where WT embryos (**a**) or *ttv* mutants expressing *hEXT1-GFP* (**e**) were not treated with heparinase III. Anterior is to the *left* in all the panels

GFP transgene was expressed in homozygous *ttv* mutants using *tub-GAL4* as the driver, rescue of *ttv* mutants to adulthood was achieved. In three independent experiments (n=158), 5.7% of total animals developed to adult flies (Table 1). Interestingly, the rescued flies did not show any abnormal change in phenotype and are comparable to wild type control flies.

HS biosynthesis is restored in *ttv* germline clones by *hEXT1*

In *Drosophila* embryos, HSPG biosynthesis is abrogated in the absence of Ttv activity (The et al. 1999), and in vertebrates, the EXT proteins function similarly in the HSPG biosynthesis pathway (Lind et al. 1998; McCormick et al. 1998; Toyoda et al. 2000a, b). We asked whether the *hEXT1* transgene is able to effectively restore HSPG biosynthesis in homozygous *ttv* mutant embryos. To detect HSPGs in vivo, we stained embryos with an anti-HS antibody (3G10) that detects unsaturated glucoronate at the nonreducing ends of HS chains after digestion of HSPGs with heparinase III (David et al. 1992; The et al. 1999). Staining with 3G10 showed a uniform pattern in WT embryos (Fig. 2b), whereas no staining was detected in embryos that were not treated with heparinase III (Fig. 2a). When 3G10 staining was tested in the *ttv* mutant embryos, a strong reduction in staining intensity was observed (Fig. 2c) that was regained when wild type *ttv* activity was restored in the *hairy*-domain (Fig. 2d) as was previously shown (The et al. 1999). Interestingly, strong and specific staining was seen as well in embryos where the *ttv* activity was restored by *hEXT1-GFP* transgene expressed in the *hairy*-domain (Fig. 2f) indicating that the *hEXT1-GFP* transgene is functional in homozygous *ttv* embryos. Thus, the *hEXT1-GFP* transgene, as a true ortholog, can substitute the activity of the *ttv* gene and restore HS biosynthesis in *Drosophila*.

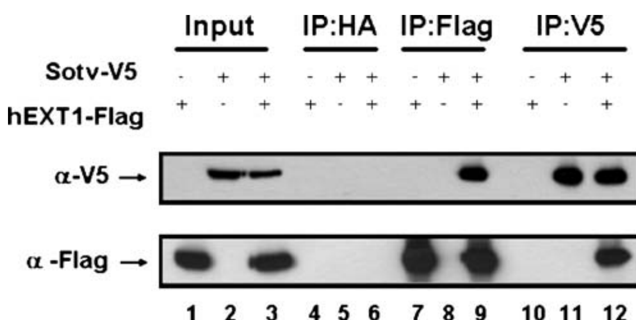

Fig. 3 Co-immunoprecipitation of hEXT1-GFP and Sotv. HEK 293 cells were transfected with plasmids expressing Flag-tagged hEXT1, V5-tagged Sotv, or both as indicated. The cell lysates were immunoprecipitated with either anti-HA as a negative control (*lane 4–6*), anti-Flag (*lane 7–9*), or anti-V5 (*lane 10–12*) antibody and then analyzed by WB with mouse anti-V5 (*α-V5*, *upper panel*) or mouse anti-Flag (*α-Flag*, *lower panel*)

Ttv protein interacts with hEXT1

Biochemical studies have shown that the vertebrate EXT1 and EXT2 can associate to form biologically functional hetero-oligomeric complex that exhibits glycosyltransferase activity in which both subunits are essential for full activity (Kobayashi et al. 2000; McCormick et al. 2000; Senay et al. 2000; Wei et al. 2000; Zak et al. 2002). Han et al. (2004b) have shown that Ttv and Sotv behave similarly in *Drosophila*. As hEXT1-GFP is able to restore HS biosynthesis in homozygous *ttv* mutants, we wanted to see whether it can interact biochemically with Sotv to reconstitute a functional HSGAG copolymerase. Therefore, we cotransfected FLAG tagged hEXT1-GFP and V5-tagged Sotv into HEK293 cells. Upon immunoprecipitation with anti-V5 or anti-FLAG antibody, we could detect Sotv interacting with hEXT1-GFP (Fig. 3). V5-tagged Sotv is seen to co-immunoprecipitate with hEXT1-Flag (lane 9, upper panel) and Flag-tagged hEXT1 with Sotv-V5 (lane 12, lower panel). This biochemical interaction shows that hEXT1-GFP is able to interact with Sotv in *Drosophila* and together they function as an active HSGAG polymerase. Similar interaction could also be reproduced in *Drosophila* Schneider's S2 cells transfected with hEXT1-GFP and V5-Sotv (data not shown). These data suggest that hEXT1 is capable of performing a similar function as Ttv, suggesting that HS biosynthesis in insects and vertebrates is conserved.

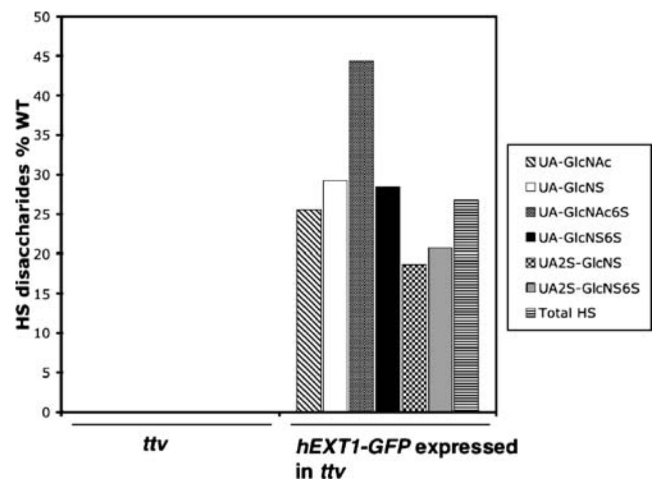

Fig. 4 Biosynthesis of HS-derived disaccharides is restored in *ttv* mutants expressing *hEXT1-GFP*. HS-derived disaccharides from *ttv* and *ttv* mutants expressing *hEXT1-GFP* transgene, normalized to the dry weight of the sample, are shown as a percentage of WT controls. The six different *bars* represent disaccharides profiled ΔUA-GlcNAc, ΔUA-GlcNS, ΔUA-GlcNA6S, ΔUA-GlcNS6S, ΔUA-2S-GlcNS, and ΔUA2S-GlcNs6S. The total HS content in the rescued *ttv* mutants is 26.6%

hEXT1-GFP interacts with Sotv and synthesizes HS in vivo

The *ttv* null mutants have undetectable levels of HS-derived disaccharides (Toyoda et al. 2000a, b). We next addressed whether hEXT1-GFP and Sotv can function as biologically active HS copolymerases to synthesize HS in homozygous *ttv* null mutants. Therefore, we evaluated the levels of HS-derived disaccharides in *ttv* mutant larvae expressing *hEXT1-GFP* transgene. We found that the *ttv* mutant animals expressing the *hEXT1-GFP* transgene could synthesize significant amounts of all the HS-derived disaccharides (25.5% ΔUA-GlcNAc, 29.2% ΔUA-GlcNS, 44.3% ΔUA-GlcNA6S, 28.4% ΔUA-GlcNS6S, 18.6% ΔUA-2S-GlcNS, and 20.7% ΔUA2S-GlcNs6S of wild type) in comparison to the *ttv* null mutants (Fig. 4), which showed no detectable traces of any of these HS-derived disaccharides. Thus, the h*EXT1* gene can effectively substitute the function of *ttv* in *Drosophila*, synthesizing 26.6% of total HS-derived disaccharides in vivo.

Discussion

Previous studies have shown that the *EXT* gene family is required for HSGAG biosynthesis in *Drosophila* and vertebrates. We were able to rescue the *ttv* null mutation in *Drosophila* using the human orthologue *EXT1*. The lower than expected percentage of rescued animals might be due to either a difference between the expression levels of the *hEXT1-GFP* transgene and wild type *ttv* or temporal differences in expression. The *ttv* gene is ubiquitously expressed in *Drosophila* embryos and larval tissues, therefore the tubulin promoter used to express the transgene is expected to mimic the expression pattern. However, we do not expect that the partial rescue will change the interpretation of the experiments as we could detect rescue of the HSPG synthesis in *ttv* mutant animals expressing the *hEXT1-GFP* transgene.

The hEXT1-GFP protein localizes in the ER in *Drosophila* wing imaginal disc cells as previously reported in human cell lines. In addition, we have been able to show interaction of hEXT1-GFP with Sotv, the EXT2 homologue, as previously shown for EXT1 in human cells and Ttv in *Drosophila*. The hEXT1-GFP and Sotv heterodimer most likely forms an active enzymatic complex as GAG synthesis of HSPGs is restored. Thus, we have demonstrated a functional conservation of HS copolymerase between insects and vertebrates.

HSPGs have been shown to affect distribution and signaling of several secreted growth factors, including FGF, Wnt, TGFβ, and Hh family members in *Drosophila* and vertebrates (Perrimon and Bernfield 2000; Lin and Perrimon 2002; Nybakken and Perrimon 2002). Studies carried out with *Ext1* and *Ext2* null mice show that HSPGs are required for developing embryos to survive and that haploinsufficiency of HS is the main cause of exostoses (Koziel et al. 2004; Stickens et al. 2005). Interestingly, Ihh and other growth factors affect bone development and Ihh protein is absent from the surface of *Ext1* deficient mice cells (Lin et al. 2000; Koziel et al. 2004).

We might therefore hypothesize that the absence of the Ttv orthologue EXT1 leads to an abrogation of HSGAG biosynthesis, and this would translate into altered signaling of Ihh and/or other growth factors. The growth factors are involved in chondrocyte differentiation and proliferation and alteration of their signaling could lead to formation of exostosis (Hopyan et al. 2002; Koziel et al. 2004). As EXT1 and EXT2 are copolymerases, HME arising from loss of EXT2 may result from the same molecular mechanism. The functional conservation of hEXT1 in *Drosophila* opens up possibilities of using *Drosophila* as an in vivo system to distinguish silent polymorphisms from inactivating mutations of the altered *EXT* genes from human HME patients.

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