Published in final edited form as:

Cell Signal. 2014 August ; 26(8): 1717–1724. doi:10.1016/j.cellsig.2014.04.014.

The *Drosophila* Tankyrase regulates Wg Signaling depending on the concentration of Daxin

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Abstract

The canonical Wnt signaling pathway plays critical roles during development and homeostasis. Dysregulation of this pathway can lead to many human diseases, including cancers. A key process in this pathway consists of regulation of β -catenin concentration through an Axin-recruited destruction complex. Previous studies have demonstrated a role for Tankyrase (TNKS), a protein with poly (ADP-ribose) polymerase, in the regulation of Axin levels in human cancers. However, the role of TNKS in development is still unclear. Here, we have generated a *Drosophila tankyrase* (DTNKS) mutant and provided compelling evidence that DTNKS is involved in the degradation of *Drosophila* Axin (Daxin). We show that Daxin physically interacts with DTNKS, and its protein levels are elevated in the absence of DTNKS in eye discs. In S2 cells, DTNKS suppressed the levels of Daxin. Surprisingly, we found that Daxin in turn down-regulated DTNKS protein level. *In vivo* study showed that DTNKS regulated Wg signaling and wing patterning at a high Daxin protein level, but not at a normal level. Taken together, our findings identified a conserved role of DTNKS in regulating Daxin levels, and thereby Wg/Wnt signaling during development.

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Conflict of interest statement

The authors declared no conflict of interest.

Keywords

Tankyrase; Wg signaling; Daxin; *Drosophila*

1 Introduction

Wingless(Wg)/Wnt signaling is an evolutionarily conserved signaling pathway from invertebrates to vertebrates which plays critical roles during embryonic development, stem cell self-renewal, tissue homeostasis, adipogenesis, and neuronal maturation [1–3]. The key process in the canonical Wg/Wnt signaling pathway is the regulation of the concentration of β -catenin/Armadillo by a destruction complex composed of Axin, GSK3/Shaggy, APC and CKI [4–8]. In the absence of the Wg/Wnt ligand, the destruction complex associates with β -catenin/Armadillo and induces its degradation through the ubiquitin-proteasome mechanism [9–12]. When the ligand is present, β -catenin/Armadillo dissociates from the destruction complex and translocates to the nucleus. Within this destruction complex, Axin serves as a central scaffold protein and binds the components of the destruction complex through different domains [5–8, 13]. Previous study showed that Axin was present at a lower concentration than other components of the complex, and overexpression of Axin in cultured cells promoted degradation of β -catenin [14]. Thus, Axin is considered to be a limiting factor during the destruction complex formation, and its concentration is tightly regulated [14].

Tankyrases (TNKSs) are proteins with poly(ADP-ribose) polymerase activity and are evolutionarily conserved in human, mouse, rat, chicken, *C.elegans*, and *Drosophila* [15]. Two human TNKSs, hTNKS1 and hTNKS2, share an 85% amino acid identity [16–18]. Both hTNKSs contain an ankyrin repeat domain, a SAM domain, and a PARP domain [16–18]. hTNKSs have been shown to be essential in regulating telomere length. In human cells, hTNKS1 binds to the telomeric-repeat-binding-factor1 (TRF1), a negative regulator of telomere length maintenance [19], removes TRF1 from telomeres and further induces its ubiquitination and degradation [16, 20–23]. In addition, hTNKSs are also involved in GSV trafficking [24–26], spindle structure regulation [27], resolution of sister telomere association [28], and centrosome regulation and maturation [29, 30]. In human cells, hTNKSs mediate PARsylation of Axin1 and Axin2 to regulate the Axin protein at appropriate levels. Knocking-down TNKSs significantly increased the Axin protein levels, and thereby suppressed Wnt signaling [31]. A recent study showed that both human and *Drosophila* TNKS modulated the activity of the proteasome regulator PI31, and were involved in proteasome assembly [32].

It is less clear about the role of TNKS in development. Homozygous *TNKS1* mice and *TNKS2* mice are viable, but double mutant mice are embryonic lethal, suggesting that mouse TNKS1 and TNKS2 are functionally redundant [33]. Interestingly, *TNKS1*^{−/−} mice fail to show any effect on telomere maintenance, and appear to develop normally [33]. Similar results were obtained from *TNKS2*^{−/−} mice regarding telomere regulation [22, 34]. Thus, the role of TNKS during development remains largely unknown and needs to be further examined.

To elucidate the function of TNKS in development, we have generated a *Drosophila tankyrase (DTNKS)* mutant and examined its role in Wg signaling. We provide compelling evidence that DTNKS is involved in the degradation of Daxin. We show that Daxin interacts with DTNKS and suppresses its protein levels in S2 cells. Daxin levels are increased in the absence of TNKS in the eye disc. Importantly, we found that Daxin in turn down-regulated protein levels of DTNKS. *In vivo* study showed DTNKS regulated Wg signaling and wing patterning at a high Daxin protein level, but not at normal level. Taken together, our findings identified a conserved role of DTNKS in regulating axin levels, and thereby Wg/Wnt signaling during development.

2. Material and methods

2.1 *Drosophila* strains

All *Drosophila* stocks were maintained and crossed at 25°C according to standard procedures. The *En-gal4,UAS-GFP*, *ywhsflp;FRT^{82B}-ubiGFP*, *ywhsflp;FRT^{82B}-hsCD8GFP* and *SalE-gal4* lines were obtained from the Bloomington stock center. The *pW20-DTNKS-RNAi1*, *pW20-DTNKS-RNAi2* and *UAS-V5-DTNKS* transgenic fly lines were generated using the PhiC31 integrase-mediated site-specific transgenesis system. The *DTNKS^{m250}* and *DTNKS^{m23}* were generated from fly strain P{EPgHP37069 (BL#22129). The *SalE-gal4,UAS-Axin/TM6B* line was a gift from Dr H. Song's lab.

2.2 Generation of transgenic constructs

To generate N-terminal V5-tagged full-length and PARP-domain truncated DTNKS constructs, we amplified the *DTNKS* cDNA (DGRC #LD22548) by PCR and sub-cloned it into the UAST-attB-V5 vector with XhoI and XbaI sites. The primers were as follows:

Tank forward: 5'-CCGCTCGAGATGGCCAACAGCAGCCGAAG-3'

Tank reverse: 5'-GCTCTAGATCATCTTGTATCCTCCGTTCC-3'

Tank PARP forward: 5'-CCGCTCGAGATGGCCAACAGCAGCCGAAG-3'

Tank PARP reverse: 5'-GCTCTAGATCAATTCACGTTGTTACCAATGC-3'

To obtain the V5 tagged, ankyrin-domain truncated DTNKS construct, we amplified the cDNA fragments from the full-length cDNA by bridge PCR and sub-cloned it into the UAS-attB-V5 vector with XhoI and XbaI sites. The primers were as follows:

Tank ANK forward-1: 5'-CCGCTCGAGATGGCCAACAGCAGCCGAAG-3'

Tank ANK reverse-1: 5'-TCCC GCCGTATCCCTGGCGTTC-3'

Tank ANK forward-2: 5'-GATACGGCGGGA GAGGGGCAGA-3'

Tank ANK reverse-2: 5'-GCTCTAGATCATCTTGTATCCTCCGTTCC-3'

The PARP-domain truncated construct has a deletion of 961–1181aa, and the ankyrin-domain truncated construct has a deletion of 56–770aa.

A similar strategy was used to generate the UAS-Flag-Daxin and UAS-Flag-Daxin(19–27aa) constructs, which were sub-cloned into the UAS-Flag vector with BglII and XbaI sites. The primers were as follows:

Daxin forward: 5'-GAAGATCTGATGAGTGGCCATCCATCGGGAATC-3'

Daxin reverse: 5'-GCTCTAGATTA ATCGGATGGCTTGACAAGACC-3'

Daxin(19–27aa) forward: 5'-

GAAGATCTGATGAGTGGCCATCCATCGGGAATCCGGAACATGATGATAAT
GAGTGT GTTAAAAAGATGACCGAAGG-3'

Daxin(19–27aa) reverse: 5'-GCTCTAGATTA ATCGGATGGCTTGACAAGACC-3'

To generate DTNKS shRNA constructs, the following primers were annealed at 95°C for 5 min in annealing buffer (10mM Tris-HCl, pH7.5, 100mM NaCl, 1mM EDTA), and slowly cooled to room temperature. The oligos were sub-cloned into the pWALIU20 vector with NheI and EcoRI sites. The primers were as follows:

tank-RNAi-1 forward: 5'-CTAGCAGTCGTGCTGTGTCGAACCAAAGA
TAGTTATATTCAAGCATATCTTTGGTTCGACACAGCACGGCG-3'

tank-RNAi-1 reverse: 5'-AATTCGCCGTGCTGTGTCGAACCAAAGA
TATGCTTGAATATACTA TCTTTGGTTCGACACAGCACG ACTG-3'

tank-RNAi-2 forward: 5'-CTAGCAGTCGGAGTACTTGATAACCTACC
TAGTTATATTCAAGCATA GGTAGGTTATCAAGTACTCCG GCG-3'

tank-RNAi-2 reverse: 5'-AATTCGCCGGAGTACTTGATAACCTACC
TATGCTTGAATATACTA GGTAGGTTATCAAGTACTCCG ACTG-3'

2.3 Generation of *DTNKS* mutant clones

The *DTNKS* mutant clones were generated by the FLP-FRT method. The flies were heat shocked at 37°C for 1 hr at 1st and 2nd instar larval stages to induce mitotic clones.

2.4 *Drosophila* imaginal discs preparation, Immunostaining and microscopy

Drosophila imaginal discs were dissected from 3rd instar larvae in cold PBS solution, fixed in 4% formaldehyde for 15 min at room temperature and rinsed in PBS containing 0.1% Triton X-100 (PBST). For immunostaining experiments, imaginal discs were blocked in blocking buffer (PBST with 5% serum) for 15min and incubated with indicated antibodies. The imaginal discs were photographed using the Zeiss LSM710 Laser Scan Confocal Microscope. Antibodies used in this study were as follows: rabbit anti-Vg (1:25), mouse anti-Dll(1:500), guinea pig anti-Sens(1:200), goat anti-Daxin(dT-20, Santacruz, 1:10), mouse anti-Eya(DSHB, 1:20), and guinea pig anti-Daxin(1:500). Primary antibodies were detected by fluorescent-conjugated secondary antibodies(Invitrogen). The adult wing images were obtained using OLYMPUS BX41 microscope.

2.5 The total RNA isolation and Real-time quantitative PCR

Adult wild-type and *DTNKS^{m250}* flies were used to isolate RNA with RNAprep pure tissue kit (Tiangen). cDNAs were produced with the RT-PCR kit (K1005s, Promega) in a 10μL

reaction volume. Quantitative RT-PCR was performed using Applied Biosystems 7500 Real-Time PCR System. Three pairs of primers were used to detect DTNKS expression. The transcription levels of DTNKS were normalized to *Ribosomal protein L32 (rp49)*. The primers were as follows:

tank-1 forward: 5'-GTGTAGGACGGGCAGAGCAACT-3'

tank-1 reverse: 5'-CATGACCGCATCGAGATTAACG-3'

tank-2 forward: 5'-ATGGGCACTATGAGGTAACCGAACT-3'

tank-2 reverse: 5'-TGCAACATCGTGATCAGATTCCTTA-3'

tank-3 forward: 5'-CTATGCACATTGTTGGTGGACTT-3'

tank-3 reverse: 5'-GGACTACCGTGGAAGAGCATAC-3'

Rp49 forward: 5'-ATGCTAAGCTGTCGCACAAA-3'

Rp49 reverse: 5'-GTTCGATCCGTAACCGATGT-3'.

2.6 Luciferase assay

Drosophila Schneider 2 (S2) cells were seeded in 6-well plates, cotransfected with plasmids expressing DTNKS-shRNA, 12×dTCF Firefly luciferase, Dfz2, and Renilla luciferase by Effectene (Qiagen) or treated with XAV939 (Sigma Aldrich). After 36 hours transfection, medium containing secreted Wingless was added to the cultured cells, and luciferase assays were carried out 4 hours later. The activity of Wg signaling was determined by the ratio of Firefly/Renilla luciferase activity.

2.7 Cell culture and transfection

S2 cells were cultured in Schneider's medium supplemented with 1U/ml Penicillin and 1μg/ml Streptomycin at 25°C. Plasmid transfection was performed using Effectene (Qiagen) according to the manufactures' instructions.

2.8 Immunoblotting and immunoprecipitation

Cell lysates were prepared with RIPA buffer (Beyotime). Total proteins were resolved by SDS-PAGE, transferred onto PVDF membrane (BioRad), and probed with indicated antibodies. For co-immunoprecipitation experiments, cells were lysed in lysis buffer (20mM Tris-HCl, pH7.6, 150mM NaCl, 1mM EDTA, 0.1% NP-40, with 1mM protein inhibitor). Precleared cell lysates were incubated with the indicated antibodies and Protein G-sepharose beads (GE healthcare) for 4 hours at 4°C. Beads were washed with lysis buffer 3 times. The bound proteins were eluted by SDS buffer, and monitored by western immunoblotting. The results of western blots were analyzed by LICOR Odyssey imager. Antibodies used in this study were as follows: rabbit-anti-V5 (Sigma, 1:1000), mouse-anti-V5 (Invitrogen, 1:2000) mouse-anti-Flag (Sigma, 1:1000), mouse-anti-GFP (Invitrogen, 1:1000), and rabbit-anti-DTNKS (1:300).

3 Result

3.1 *Drosophila* tankyrase (DTNKS) is not required for viability

Drosophila CG4719 encodes the *Drosophila* tankyrase (DTNKS) (Fig. 1). The *DTNKS* gene is localized on the 3rd chromosome and encodes two polypeptides, one contains 1181 amino acids and the other contains 1520 amino acids. Both polypeptides contain three protein domains: an ankyrin repeat domain, a PARP domain, and a SAM domain. DTNKS shares high identity with hTNKS within these domains. The ankyrin repeat domain of DTNKS shares 71.6% identity with hTNKS1 (Fig. 1A). The PARP domain and SAM domain share 75% and 51.5% identity, respectively, with those of hTNKS (Fig 1A), indicating that DTNKS is an evolutionarily conserved protein.

To examine the functions of DTNKS in development, we generated *DTNKS* mutants through P element-mediated knockout technique. The P element, P{EPg}HP37069 (BL#22129), was inserted in the 5'-UTR of *DTNKS* gene. The imprecise excision of this P element generated two alleles of DTNKS: *DTNKS*^{m23} and *DTNKS*^{m250}. Segments of 1635 bp and 1924 bp of genomic DNA were deleted in *DTNKS*^{m23} and *DTNKS*^{m250}, respectively. Both deletions covered the first exon of DTNKS. Moreover, *DTNKS*^{m250} covered part of the regulatory region in front of the transcription starting site (Fig. 1B). Both alleles were homozygous viable. To determine whether our generated mutants are null, we performed real-time PCR to detect *DTNKS* mRNA transcription level in the *DTNKS*^{m250} mutant with three pairs of primer (tank-1, tank-2 and tank-3). The region covered by primer pair tank-1 was located at the deleted region, which served as a negative control. The regions covered by tank-2 and tank-3 were located at the non-deleted region, detecting the transcription level of *DTNKS*. The results from RT-PCR revealed that *DTNKS* mRNA level in *DTNKS*^{m250} mutant were as low as the negative control (Fig. 1C), suggesting that *DTNKS*^{m250} was a null allele. The *DTNKS*^{m250} homozygous mutant was fertile and had no obvious developmental defects, indicating that *DTNKS* was not required for viability.

3.2 DTNKS interacts with Daxin, and their protein levels are mutually interdependent

A previous study showed that hTNKS interacted with Axin and induced the degradation of Axin [31]. To explore whether DTNKS regulates Daxin in *Drosophila*, we examined the interaction between Daxin and DTNKS in *Drosophila* S2 cells. Using co-immunoprecipitation experiments, we found that DTNKS Co-IPed with Daxin, and vice versa (Fig. 2A). Axin contains an N-terminal tankyrase binding domain (TBD), which is conserved in different species and has been shown to mediate the interaction between hTNKS and Axin [31]. Deletion of this domain in Daxin (mapped to 17th–29th amino acids) also disrupted the interaction between DTNKS and Daxin (Fig. 2A, lane 2 and 5), confirming that Daxin binds to DTNKS via its N-terminal TBD. DTNKS contains an N-terminal ankyrin repeat domain, a middle SAM domain, and a C-terminal PARP domain. We next examined which domain in DTNKS mediated the interaction between DTNKS and Daxin. We generated two truncated DTNKS constructs, one deleting the PARP domain (961st–1181st amino acids) and the other deleting the ankyrin repeat domain (56th–770th amino acids) (Fig. 2E). Using co-immunoprecipitation experiment, we found that the ankyrin repeat domain, but not the PARP domain, was required for DTNKS binding to

Daxin (Fig. 2B). These data indicated that DTNKS physically interacted with Daxin, and this interaction was mediated via the TBD domain of Daxin, and the ankyrin repeat domain of DTNKS.

We found that the protein level of Daxin was much lower than that of Daxin (17–29aa) when co-expressed with DTNKS (Fig. 2A, lane 1 and 4 compared to lane 2 and 5), suggesting that the binding of DTNKS might affect Daxin protein levels. We then examined the Daxin protein level in the presence of increasing amounts of DTNKS. Co-transfection of DTNKS dramatically decreased protein levels of Daxin in a dosage-dependent manner (Fig. 2C), suggesting a conserved role of TNKS in the regulation of Axin protein levels.

Surprisingly, we found that the protein level of DTNKS was also reduced in the presence of co-expressed full-length Daxin, but not in the presence of Daxin (19–27aa) (Fig 2A, lane 1 compared with lane 2 and 3). Indeed, co-expression of increasing amounts of Daxin down-regulated DTNKS protein levels in a dosage-dependent manner (Fig. 2D). These data suggest that DTNKS and Daxin can regulate the protein levels of each other.

3.3 DTNKS regulates wing patterning depending on the concentration of Daxin

Daxin acts as a scaffold protein in the destruction complex to regulate the stability of Armadillo (β -catenin), and works as a negative regulator in Wg/Wnt signaling [5–8, 13, 35, 36]. The aforementioned experiments showed that DTNKS was involved in the degradation of Daxin in S2 cells. To determine whether DTNKS is required for Wg signaling, we generated two independent shRNAs targeting *DTNKS* to examine DTNKS' function in the Wg signaling pathway. We first examined the Wg-stimulated luciferase activity in S2 cells. The result showed that depletion of DTNKS using DTNKS-shRNA1 or DTNKS-shRNAi2 significantly reduced the Wg reporter activity ($p < 0.001$) (Fig. 3A). Similar result was also obtained after treated S2 cells with XAV939, a small molecule of hTNKS ($p < 0.001$) (Fig. 3B). These results indicate that DTNKS regulates Wg signaling in S2 cells. To confirm this finding *in vivo*, we generated transgenic flies expressing DTNKS-shRNAs and used genetic approaches to test the function of DTNKS during wing patterning. Wing margin bristles are a particularly sensitive indicator for proper levels of Wg signaling. Excessive Wg signaling causes ectopically expressed bristles, whereas low levels of Wg signaling leads to loss of bristles [37, 38]. However, we found that knocking-down DTNKS by DTNKS-shRNA using *SalE-gal4* or *En-gal4* (data not shown) had no obvious defects in wing patterning (Fig. 3C). Axin works as a negative regulator in Wg signaling, and a high concentration of Axin protein inhibits Wg signaling. Thus, we speculated that the elevated Daxin protein level in the absence of DTNKS was not high enough to influence Wg signaling. We tested this possibility by knocking-down DTNKS in a genetic background with high levels of Daxin. During wing patterning, ectopic expression of Daxin in the wing pouch by *SalE-gal4* generated wing-margin and bristles defects (Fig. 3D). This phenotype was enhanced when DTNKS was knocked-down simultaneously (Fig. 3E). We also found that ectopic expression of DTNKS in the wing significantly rescued the defect caused by overexpression of Daxin (Fig. 3F). Taken together, these results indicate that DTNKS regulates Daxin protein levels, and affects wing patterning under high concentrations of Daxin.

3.4 DTNKS is required for Daxin degradation, but has no obvious effect on Wg signaling

To understand further the function of DTNKS in Wg signaling *in vivo*, we examined Daxin protein levels and expression levels of Wg target genes through RNAi-mediated *DTNKS* knockdown and mosaic clonal analysis on *DTNKS* mutant in *Drosophila* imaginal discs. Consistent with our results in S2 cells, we found that Daxin protein levels were increased in *DTNKS* mutant cells (Fig. 4A–C). We then monitored Wg target genes in wing imaginal discs, including *senseless*(*Sens*), *distal-less*(*Dll*), and *vestigial*(*Vg*). Endogenous *Sens* protein is expressed in cells receiving high levels of Wg signaling (Fig. 5C). Knocking-down *DTNKS* in the posterior compartment of wing imaginal discs by *en-gal4* did not affect *sens* expression, when compared to control discs (Fig. 5C and 5D). *Dll* and *vg* are expressed in cells receiving both high and low levels of Wg signaling. Knocking down *DTNKS* also had no effect on their expression. (Fig. 5A and 5B).

Similar to the observations by shRNA-mediated *DTNKS* knockdown, expression of endogenous *sens*, *Dll*, and *vg* were not affected in *DTNKS* mutant clones cells (Fig. 5E and 5F). We also examined the role of *DTNKS* in the eye disc, another organ system in *Drosophila* where Wg signaling plays pivotal roles. In eye imaginal discs, Wg signaling functions to restrict the area of the eye field, which is dependent upon the activities of various transcription factors, including *eyeless* (*eye*), *eyes absent*(*eya*), *sine oculis*(*so*), and *dachshund* (*dac*) [39–44]. However, the expression of *eya*, an eye specification gene regulated by Wg signaling [44], was not affected in the absence of *DTNKS* (Fig. 5G). Taken together, these results suggest that DTNKS regulates Daxin protein levels, but has no obvious function in *Drosophila* wing and eye development under normal conditions.

3.5 DTNKS regulates expression of Wg target genes under high levels of Daxin

As DTNKS regulated Daxin protein levels and affected wing patterning at a high level of Daxin, we speculated that the regulation of Wg target genes by DTNKS also depended on the concentration of Daxin. To test this hypothesis, we examined expression of *Dll* and *vg* in wing discs in which Daxin was ectopically expressed. As expected, we found that expression of *Dll* and *vg* were decreased when Daxin was expressed by *Sal-gal4* in the wing imaginal discs (Fig. 6A). This reduction was dramatically enhanced when DTNKS was simultaneously knocked-down in wing imaginal discs (Fig. 6B). Taken together, these data suggest that DTNKS regulates Wg signaling, depending on the concentration of Daxin.

4 Discussion

4.1 The conserved role of DTNKS in the regulation of Daxin degradation

Wg/Wnt signaling is a conserved signaling pathway and plays important roles during many developmental processes and homeostasis. A tight regulation on the concentration of Axin is critical for Wg signaling transduction [14]. Although previous studies have demonstrated a role for TNKS in the regulation of Axin levels and Wnt signaling in human cancers, its functions during development remain largely unknown. By generating *Drosophila tankyrase* mutants, we demonstrated a role for *DTNKS* in the regulation of Daxin levels during imaginal disc development (Fig. 4A–C). In addition, we also show that DTNKS physically interacts with Daxin and regulates protein levels of Daxin in S2 cells (Fig. 2A, 2C). These

results indicate that the function of TNKS in regulating Axin protein levels is conserved among different species [31].

Surprisingly, we found that Daxin was also involved in the regulation of DTNKS protein levels (Fig. 2D). Coexpression of Daxin can dramatically reduce protein levels of DTNKS (Fig. 2D). With increasing amounts of Daxin, this effect on DTNKS was enhanced. Daxin is a scaffold protein without any known catalytic activity. Thus, we speculate that Daxin mediates degradation of DTNKS by recruiting other components and forming a destruction complex for DTNKS. A previous study suggests RNF146, one of the E3 ligases, forms a complex with Axin and TNKS and regulates the degradation of both Axin and TNKS in human cells [45, 46]. Therefore, we propose that degradation of DTNKS by Daxin may depend on the *Drosophila* RNF146-mediated ubiquitination pathway. Since endogenous levels of Daxin are low, the increased levels of Daxin may recruit more RNF146 and promote DTNKS degradation in S2 cells.

4.2 The role of DTNKS in Wg signaling

Our genetic studies showed that depletion of *DTNKS* has no obvious effect on wing patterning (Fig. 3C) or expression of Wg target genes (Fig. 5A–G'), suggesting the limited function of DTNKS for Wg signaling under normal developmental conditions. However, the luciferase assay showed that knock-down of DTNKS by shRNA, or suppression of DTNKS activity by XAV939 significantly reduces Wg reporter activity in S2 cells (Fig. 3A–B). Consistent with this finding, Knock-down of *DTNKS* caused dramatic defects in wing patterning (Fig. 3E) and reduction of Wg target gene expression under a genetic background with a high Daxin protein level (Fig. 6B–B''), indicating that DTNKS can regulate Wg signaling under certain conditions. A previous study showed that tubulin promoter-driven Daxin protein level was expressed ~4.3 fold higher than that of endogenous Daxin, and caused no obvious defect during wing patterning [47]. According to this result, a potential explanation for our findings is that the increase of Daxin caused by depletion of *DTNKS* is within the physiological range required for normal development, and is not enough to disrupt Wg signaling. Thus, we speculate that DTNKS plays important roles in Wg signaling in certain situations where Daxin protein levels reach a certain threshold.

4.3 The role of DTNKS in other developmental and cellular processes

A critical function of human Tankyrase (hTNKS) is the maintenance of telomere length. In human telomerase-positive cells, overexpression of hTNKS protects the telomere from degradation. hTNKS removes TRF1, a negative regulator of telomere length maintenance, from the telomere and subsequently triggers its ubiquitination and degradation [16, 20, 23, 48, 49]. However, knocking out mouse TNKS1 or TNKS2 showed no effect on telomere length maintenance [22, 33, 34]. Moreover, mouse TRF1 lacks a tankyrase binding consensus motif, RXXG/PDG, that is shared by all TNKS partners, and does not interact with mouse TNKS [15, 50–52]. As a result, TNKS does not remove mouse TRF1 from the telomere, or maintain the telomere length in mouse. Therefore, the function of TNKS on telomere length maintenance is not conserved between human and mouse. We propose that DTNKS is unlikely to be involved in regulation of *Drosophila* telomere length. This hypothesis is supported by two pieces of evidence. First, TRF1, the negative regulator of

telomere length maintenance, is not a conserved protein between human and *Drosophila*. We failed to identify a TRF1 homologue in *Drosophila*. Secondly, the structure of telomere and the maintenance of telomere length are different between human and *Drosophila*. In humans, telomeres are composed of TTAGGG tandemrepeat sequences. The maintenance of telomere length requires telomerase. *Drosophila* lacks telomerase and the maintenance of telomere length depends on transposition of three specialized retro-transposons *TART*, *HeT-A*, and *TAHRE*, rather than telomerase activity [53]. Previous study showed that the regulation of telomeres by TNKS only occurred in telomerase-positive cells [49], indicating that the regulation of telomere by TNKS requires telomerase activity. Therefore, we speculate that the function of TNKS in telomere length maintenance is not conserved in *Drosophila*.

Acknowledgments

We thank Y. Lin for fly injection assistance, Developmental Studies Hybridoma Bank (DSHB) for antibodies and Bloomington Stock Center for stocks. We thank the TRiP at Harvard Medical School (NIH/NIGMS R01-GM084947) for providing the pWALIUM20 vector. We gratefully acknowledge the comments on the manuscript by Dr T. Y. Belenkaya. This work is supported by grants the National Basic Research Program of China (2011CB943901 and 2011CB943802), National Natural Science Foundation of China (31030049), Research Foundation for Advanced Talents of Wenzhou Medical University (QTJ08012) and Wenzhou Medical University research grant (XNK07005), the Programme of Introducing Talents of Discipline to Universities (NO.B06016) and NIH grants (2R01 GM063891).

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Highlights

DTNKS interacts with Daxin and modulates its protein levels *in vivo* and in S2 cells.

Daxin in turn down-regulates DTNKS protein levels.

DTNKS regulates Wg target genes expression and wing patterning at high Daxin levels.

Figure 1. Generation of *DTNKS* mutant

(A) *DTNKS* is highly conserved with human *TNKS1*. It shares 71.6%, 51.5% and 75% identity with human *TNKS1* at the ankyrin repeat domain, the SAM domain, and the PARP domain, respectively. (B) Schematic diagram of the genomic region of *DTNKS* locus. Red boxes represent coding sequences and gray boxes indicate untranslated exon sequences. P{HP37069} indicates the insertion site of the P-element stock. Straight lines show intron sequences and broken lines indicate deleted sequences covering the first exon and ATG of *DTNKS*. (C) Quantitative RT-PCR using total RNAs from adult *DTNKS*^{m250} homozygotes or wild type flies. The expression level of *DTNKS* of each line was normalized to the control *rp49*. All three pairs of primers (tank-1, tank-2 and tank-3) show low expression of *DTNKS* in *DTNKS* mutant, as compared with wild type fly. Error bars represents standard error of the mean.

Figure 2. DTNKS interacts with Daxin and modulates protein levels of each other in S2 cells
 (A) DTNKS interacts with Daxin in S2 cells. V5-tagged DTNKS and Flag-tagged Daxin expression vectors were transfected into S2 cells (lane 1, 3, 4). V5-DTNKS and Flag-Daxin are co-precipitated with each other (lane 1, 3, 4). TBD of Daxin (mapped to 17th–29th amino acids) is required for Daxin to bind to DTNKS (lane 2, 5). A construct lacking this domain does not interact with DTNKS. (B) The ankyrin repeat domain but not the PARP domain of DTNKS is required for DTNKS binding to Daxin. Flag-Daxin does not bind to DTNKS lacking the ankyrin repeat domain. (C) Exogenous expression of DTNKS causes reduction

of Daxin protein levels. The protein level of Flag-Daxin was significantly reduced with increased amount of V5-DTNKS. (D) Exogenous expression of Daxin results in down-regulation of the DTNKS protein level. The protein level of V5-DTNKS was significantly reduced with increased amounts of Flag-Daxin. (E) The diagram of the DTNKS mutant constructs. The PARP-domain truncated construct DTNKS PARP has a deletion of 961–1181aa. The ankyrin repeat domain truncated construct DTNKS ANK has a deletion of 56–770aa.

Figure 3. DTNKS regulates Wg signaling and wing patterning

(A) Paracrine activation luciferase assay. The S2 cells were transfected with W20-DTNKS-shRNAs (or pWALIU20 vector), 12xdTCF firefly luciferase, Dfz2, and renilla luciferase. The medium containing secreted wingless was added to cultured cells. Knock-down of *DTNKS* by two independent shRNAs inhibits Wg reporter in S2 cells ($*P < 0.001$, $n=5$). (B) Paracrine activation luciferase assay as in figure A. Suppression of DTNKS activity using XAV939 inhibits Wg reporter in S2 cells ($*P < 0.001$, $n=6$). (C) Knock-down of *DTNKS* by shRNA driven by *SalE-gal4* has no effect on wing patterning. (D) Overexpression of Daxin driven by *SalE-gal4* led to wing margin defects. (E) The defect in (D) was enhanced when DTNKS shRNA was coexpressed using *SalE-gal4*. (F) The wing defect in (D) was rescued by ectopic expression of DTNKS.

Figure 4. Daxin is increased in *DTNKS* mutant clones

Protein levels of Daxin were examined in *DTNKS* mutant cells. (A) The absence of GFP signals labeled the *DTNKS* mutant cells. (B) Endogenous Daxin was increased in *DTNKS* mutant cells in eye discs.

Figure 5. Expression of Wg target gene is not affected in *DTNKS* mutant

All wing imaginal discs are oriented dorsal top-left, posterior top-right.

(A–D) Wing imaginal discs expressed UAS-GFP alone or together with W20-DTNKS shRNA by *en-gal4*. The target gene expression region was labeled by GFP signals. (A–A'') The expression pattern of Vg (A') and Dll (A'') in wing imaginal discs. (B–B'') The expression of Vg (B') and Dll (B'') was not affected when *DTNKS* was knocked-down by shRNA in the posterior compartment. (C–C') Expression pattern of Sens in wild-type wing imaginal discs. (D–D') Expression level of Sens was not affected when *DTNKS* was

knocked-down by shRNA in the posterior compartment. (E–E'') The wing imaginal disc containing *DTNKS* mutant cells was stained by anti-Vg (E') and anti-Dll (E'') antibodies. The absence of GFP signals labeled the mutant cells. Expression level of Vg and Dll was not affected in mutant cells. (F–F') The wing imaginal disc containing *DTNKS* mutant cells was stained by anti-Sens antibody. Expression level of Sens was not affected in mutant cells. (G–G') The eye imaginal disc containing *DTNKS* mutant cells was stained by anti-Eya antibody. Expression of Eya was not affected in *DTNKS* mutant cells.

Figure 6. Loss of DTNKS suppresses Wg signaling at a high level of Daxin

(A–B'') Wing imaginal discs expressed UAS-Daxin alone (A) or together with w20-DTNKS shRNA (B) by *SalE-gal4*. The target gene expression region was labeled by immunostaining of Daxin (green). (A–A'') Ectopic expression of Daxin by *SalE-gal4* caused reduction of Dll (A') and Vg (A'') in wing imaginal discs. (B–B'') Wing imaginal discs expressing UAS-Daxin with *DTNKS* by shRNA display further reduction of Dll (B') and Vg (B'').