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# PTK7 proteolytic fragment proteins function during early *Xenopus* development

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ABSTRACT

Protein Tyrosine Kinase 7 (PTK7) is as a critical regulator of canonical and non-canonical Wnt-signaling during embryonic development and cancer cell formation. Disrupting PTK7 activity perturbs vertebrate nervous system development, and also promotes human cancer formation. Observations in different model systems suggest a complex cross-talk between PTK7 protein and Wnt signaling. During *Xenopus laevis* nervous system development, we previously showed that PTK7 protein positively regulates canonical Wnt signaling by maintaining optimal LRP6 protein levels, but PTK7 also acts in concert with LRP6 protein to repress non-canonical Wnt activity. PTK7 is a transmembrane protein, but studies in cancer cells showed that PTK7 undergoes "shedding" by metalloproteases to different proteolytic fragments. Some PTK7 proteolytic fragments are oncogenic, being localized to alternative cytoplasmic and nuclear cell compartments. In this study we examined the biological activity of two proteolytic carboxyl-terminal PTK7 proteolytic fragments, cPTK7 622–1070 and cPTK7 726–1070 during early *Xenopus* nervous system development. We found that these smaller PTK7 proteolytic fragments have similar activity to full-length PTK7 protein to promote canonical Wnt-signaling via regulation of LRP6 protein levels. In addition to cancer systems, this study shows in vivo proof that these smaller PTK7 proteolytic fragments can recapitulate full-length PTK7 protein activity in diverse systems, such as vertebrate nervous system development.

## 1. Introduction

Protein tyrosine kinase 7 (PTK7) is a transmembrane protein conserved in metazoans (Miller and Steele, 2000). Lacking, a functional catalytic tyrosine kinase activity, PTK7 belongs to the receptor tyrosine "pseudokinase" family of proteins. PTK7 contains an extracellular domain with seven immunoglobulin-like loops and a transmembrane domain. Numerous studies have connected PTK7 protein to the Wnt signaling network. PTK7 regulates cell-fate specification and morphogenesis during early vertebrate and invertebrate development, where disruption of its activity in vertebrates leads to neural tube defects/NTDs (Lu et al., 2004; Yen et al., 2009; Paudyal et al., 2010; Williams et al., 2014; Peradziryi et al., 2011; Puppo et al., 2011; Wehner et al., 2011; Lee et al., 2010; Hayes et al., 2013, 2014; Bin-Nun et al., 2014; Andreeva et al., 2014; Podleschny et al., 2015), including in humans (Wang et al., 2015).

PTK7 regulates both canonical and Planar-Cell-Polarity (PCP) Wnt activities in early development (Lu et al., 2004; Yen et al., 2009; Paudyal et al., 2010; Williams et al., 2014; Peradziryi et al., 2011; Puppo et al., 2011; Wehner et al., 2011; Lee et al., 2010; Hayes et al., 2013, 2014; Bin-Nun et al., 2014; Andreeva et al., 2014; Podleschny et al., 2015; Wang et al., 2015; Berger et al., 2017). PTK7 involvement in PCP was first shown in mouse and frog (*Xenopus laevis*) embryos (Lu et al., 2004; Yen et al., 2009; Paudyal et al., 2010; Williams et al., 2014). In PTK7 mutant mice, neural tube closure is perturbed, and stereociliary bundle orientation in the inner ear is disrupted (Lu et al., 2004; Yen et al., 2009; Paudyal et al., 2010; Williams et al., 2014; Lee et al., 2010). In *Xenopus*, PTK7 is expressed in the neural ectoderm during late gastrula and neurula stages and its knockdown, like in mice, disturbs neural tube closure and elongation (Lu et al., 2004). NTD phenotypes are a hallmark of PCP perturbation, suggesting that PTK7 regulates vertebrate embryo PCP activity. The mechanistic characterization of PCP component mutants is enigmatic, since both loss and gain of function give similar morphogenetic phenotypes (Wallingford et al., 2000; Djiane et al., 2000). Studies in

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*Xenopus* and *Drosophila* embryos and cell culture showed that PTK7 inhibits canonical Wnt signaling by binding and titrating out canonical Wnt ligands (Peradziryi et al., 2011). Other studies showed that PTK7 protein positively modulates canonical Wnt activity in both cell culture and *Xenopus* embryos (Puppo et al., 2011). We showed during *Xenopus* development that PTK7 knockdown inhibits canonical Wnt activity in the neural plate, phenocopying embryos depleted for canonical Wnt pathway components, such as Wnt3a and LRP6 (Bin-Nun et al., 2014). PTK7 protein depletion inhibits canonical Wnt signaling by strongly reducing LRP6 protein levels (Bin-Nun et al., 2014). PTK7 and LRP6 proteins physically interact, presumably via their transmembrane domains (Bin-Nun et al., 2014). In *Xenopus*, LRP6 protein positively modulates canonical Wnt but negatively modulates PCP activities (Tahinci et al., 2007; Bryja et al., 2009; et al., Andersson et al., 2010). Maintenance of high LRP6 protein levels by PTK7 suggests that PTK7 protein potentially acts as a positive modulator of LRP6 protein to inhibit Wnt-PCP activity, while simultaneously promoting canonical Wnt activity (Bin-Nun et al., 2014).

PTK7 protein also plays a role in cancer development and maintenance (Hayes et al., 2014; Andreeva et al., 2014). PTK7 expression is increased in different cancers, such as colon, gastric, esophageal and lung carcinomas, acute myeloid leukemia and liposarcoma (Mossie et al., 1995; Tian et al., 2016; Gorringe et al., 2005; Müller-Tidow et al., 2004; Gobble et al., 2011). PTK7 protein knockdown inhibits proliferation, invasion or anti-apoptotic activity in liposarcoma and colon cancer cells (Gobble et al., 2011; Meng et al., 2010). Expression of PTK7 in leukemia cells enhances cell migration and survival (Prebet et al., 2010). In esophageal carcinoma, PTK7 knockdown decreases phosphorylation of Akt, Erk, and FAK signaling molecules that promote proliferation, survival and migration (Shin et al., 2013, 2018). In contrast, PTK7 expression is decreased or lost in melanomas, and breast and renal cell carcinomas (Easty et al., 1997; Su et al., 2010; Behbahani et al., 2012). Aberrant canonical and PCP Wnt signaling plays a causative role in numerous human cancers (MacDonald et al., 2009; Wang, 2009). The nature of the PTK7 and Wnt pathway interactions in cancer is still unclear (Dunn and Tolwinski, 2016).

In cancer cells, full-length PTK7 undergoes proteolytic cleavage to form several smaller protein fragments (Golubkov et al., 2010, 2011, 2014; Na et al., 2012; Golubkov and Strongin, 2012, 2014). This "shedding" of the extracellular domain produces smaller PTK7 proteolytic fragments that localize to the extracellular space, plasma membrane, cytoplasmic and nuclear cellular compartments (Na et al., 2012; Golubkov and Strongin, 2012, 2014; Golubkov et al., 2014). These proteins are formed in a proteolytic process carried out by MT1-MMP, ADAM and γ-secretase proteases (Na et al., 2012; Golubkov and Strongin, 2012, 2014; Golubkov et al., 2014). These MT1-MMP induced proteolytic fragments are the N-terminal 1–621 soluble ectodomain fragment (sPTK7), and the C-terminal, cPTK7 622–1070 that is further processed by the γ-secretase to generate the cPTK7 722–1070 protein (Na et al., 2012; Golubkov and Strongin, 2012, 2014; Golubkov et al., 2014). The sPTK7 proteolytic fragment ends up in the cell membrane where it may interact with full-length PTK7 protein, and it appears to promote cancer cell migration (Na et al., 2012; Golubkov and Strongin, 2012, 2014; Golubkov et al., 2014). The cPTK7 622–1070 proteolytic fragment has a cytoplasmic and transmembrane domain (Fig. 1A). The cPTK7 722–1070 has a cytoplasmic domain, but no transmembrane domain; this protein is found in the nucleus (Fig. 1A). An additional PTK7 variant is generated in the *chuzhoi* (*chz*) mutation, in which there is an Ala-Asn-Pro tripeptide

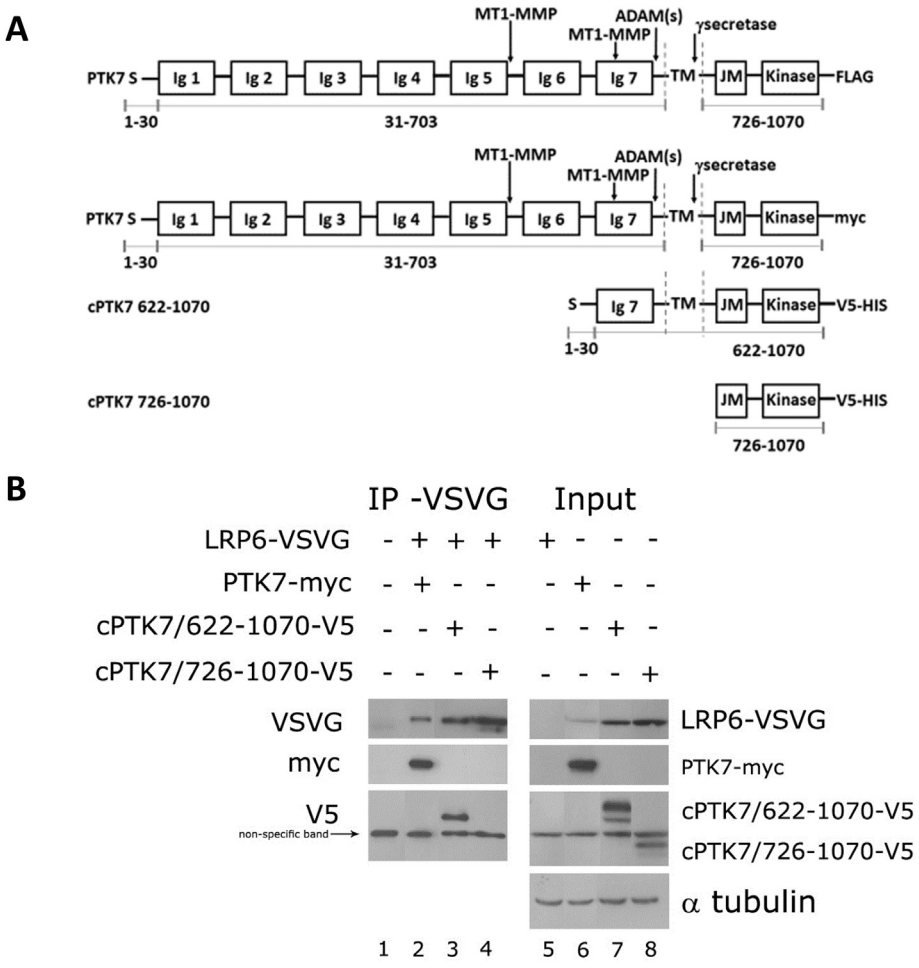


**Fig. 1. The cPTK7 622–1070 protein undergoes co-IP with LRP6 protein in HEK cells**

**A.** A schematic description of the PTK7 constructs used in this study: Full-length 1–1070 PTK7-Flag-tagged, full-length 1–1070 PTK7-Myc tagged, cPTK7 622–1070 V5/His-tagged and cPTK 726–1070-V5/His-tagged. S, signal peptide, Ig, immunoglobulin-like domain, TM, transmembrane domain, JM, juxtamembrane region, KINASE, the catalytically dead kinase domain. The amino acid residue numbering is shown below the construct structure. MT1-MMP, ADAM and γ-secretase cleavage sites are indicated by arrows. The full-length 1–1070 PTK7-Myc-tagged protein was used in the co-IP assays in HEK293 cell culture. The full-length 1–1070 PTK7-Flag-tagged protein was a more biologically active protein than the Myc-tagged protein in *Xenopus* embryos.

**B.** HEK293 cells (lane 1) were co-transfected with plasmids encoding the full length VSVG-LRP6 protein (lanes 2–4) and the full-length PTK7-Myc protein (lane 2) and the short PTK7 proteins, PTK7 622–1070-V5 (lane 3) and PTK7 726–1070-V5 (lane 4). Whole-cell extracts were immune-precipitated (left panel) by VSVG antibody (targeting LRP6 protein) on protein G Agarose beads. The co-IP proteins were blotted with VSVG, myc and V5 antibodies. Note that two proteins underwent co-IP with VSVG-LRP6 protein, full length PTK7 and PTK7 622–1070; the PTK7 726–1070 protein did not undergo co-IP with the VSVG-LRP6 proteins. The left panel shows the IP (lanes 1–4) and the right panel shows the total protein expression input (lanes 5–8) before co-IP. α-tubulin protein (right panel) serves as a positive control for protein loading in the protein input samples before co-IP.

insertion into the junction region between the fifth and sixth Ig-like domains in PTK7 (Paudyal et al., 2010; Golubkov et al., 2011). The *chz* mutation creates an additional MT1-MMP site between the fifth and sixth Ig-like domain region. This mutation causes NTDs and also stimulates cancer cell migration (Paudyal et al., 2010; Golubkov et al., 2011). Reducing levels of full-length protein versus PTK7 proteolytic fragments may alter both normal and oncogenic PTK7 activities (Na et al., 2012; Golubkov and Strongin, 2012, 2014; Golubkov et al., 2014).

In this study, we have developed functional assays in vivo to elucidate and compare how the recombinant cPTK7 622–1070 and cPTK7 726–1070 proteolytic fragment proteins regulate canonical Wnt-signaling and function during normal embryonic development. We have shown that these two proteolytic fragments can rescue PTK7 knock down phenotypes by promoting canonical Wnt activity. These smaller PTK7 proteins appear to have similar biological activity in comparison to the wild type full-length protein. This study shows a proof of concept that these small PTK7 proteolytic fragments can recapitulate in vivo activities of the wild type full-length PTK7 protein, and could indeed be mediators of PTK7 activity in different systems.

## 2. Materials and methods

### 2.1. Xenopus embryos

Ovulation, in vitro fertilization, culture, and explant dissections and treatments were as described (Aamar and Frank, 2004). All experiments in *Xenopus* were performed under the approval and guidelines of the Committee of the Ethics of Animal Experiments of the Technion - Israel Institute of Technology.

### 2.2. RNA and MO injections

Capped sense in-vitro transcribed mRNA encoding full length and proteolytic *ptk7* proteolytic fragments, full length *lrp6* and *lrp6ΔN* were injected into the animal hemisphere of one-cell stage embryos, as was the antisense *PTK7*-morpholino oligonucleotide (*PTK7-MO*; Gene Tools). *PTK7-MO* specificity was shown in previous studies using scrambled MOs; additionally, the PTK7 morphant embryonic phenotype is rescued by ectopic expression of full-length human PTK7 protein (Lu et al., 2004; Bin-Nun et al., 2014). Two-mixed *PTK7*-MOs (5-TGCATCGC GGCCTCTCCCCTCA-3; 5-TTCCTGCCCCGGATCCTCTCACTGC-3) are always co-injected (Lu et al., 2004; Bin-Nun et al., 2014). Further supporting *PTK7-MO* specificity parameters, the two mixed *PTK7-MOs* act additively at low concentrations (where individually each one separately has lower knockdown activity), and their target sequences do not significantly overlap on the *PTK7* mRNA (Lu et al., 2004; Bin-Nun et al., 2014).

### 2.3. Semi-quantitative (sq)RT-PCR analysis

SqRT-PCR was performed as described (Snir et al., 2006). In all sqRT-PCR experiments, three to six independent experimental repeats were performed. Samples are assayed at least twice for each marker. sqRT-PCR primers: *ef1a*, *odc*, *krox20*, *hoxB3*, *hoxB4*, *n-tub, hoxD1* and *gbx2* (Gutkovich et al., 2010; Polevoy et al., 2017); *siamois* and *xnr3* primer sequences (R. Harland Lab database). The sqRT-PCR reactions are simultaneously performed for all the different markers and all the samples in individual tubes. For each marker, all the samples are run on the same gel in adjacent wells, with identical exposure times. sqRT-PCR reactions were quantitated by phosphor-imager analysis.

### 2.4. Cell culture and transfection

Human embryonic kidney cells (HEK293), were grown in Dulbecco's modified Eagle medium, fetal calf serum, glutamine and antibiotics. Cells were transfected by the calcium phosphate procedure.

### 2.5. Immunoprecipitation (IP) in HEK293 cells

HEK293 cells were lysed on ice, 48 h after transfection, with 500 µl IP buffer. Cells were vortexed for 10 s, every 5 min, for 15 min. Supernatant was collected after spinning the tubes at 10,000 rpm, for 15 min. For IP, the relevant antibody was added to the lysates and rocked for 1 h, at 4 °C; recombinant protein G-Agarose beads (Invitrogen) were added and rocked overnight. Beads were spun down for 5 min at 2500 rpm, and washed four times with IP buffer. Electrophoresis loading buffer was added. Proteins were separated by SDS-polyacrylamide (10%) gel electrophoresis (reduced conditions).

### 2.6. Western analysis

Western analysis was performed as previously described (Bin-Nun et al., 2014; Zetser et al., 2001). Antibodies used were: α-Tubulin (Sigma T9026; mouse, 1:10,000), VSVG (Sigma V4888; rabbit, 1:2000), myc (Santa Cruz SC-789; rabbit, 1:1000), V5 (Sigma V8012; mouse, 1:1000). Secondary antibodies: goat anti-mouse (Thermo Scientific 31432; 1:4000), or goat anti-rabbit (Thermo Scientific 31460; 1:4000). α-Tubulin is the protein loading control for total protein.

## 3. Results

### 3.1. Physical interactions between PTK7 proteolytic fragments and LRP6 protein

The two PTK7 proteolytic fragments, cPTK7 622–1070 and cPTK7 726–1070 have different sub-cellular localizations (Na et al., 2012; Golubkov and Strongin, 2012, 2014; Golubkov et al., 2014), so we examined their interactions with full-length LRP6 protein by co-IP in HEK cells (Fig. 1A). The individual PTK7 proteolytic fragment could modulate Wnt signaling in embryos and HEK cells, perhaps by different mechanisms than the full-length PTK7 protein that interacts directly with LRP6 protein at the membrane (Bin-Nun et al., 2014). For each PTK7 proteolytic fragment protein, we performed co-IP in HEK cells with co-transfected LRP6 protein. The cPTK7 622–1070 protein is membrane bound, while cPTK7 726–1070 protein is not (Fig. 1A). The two PTK7 proteins that have the intracellular and transmembrane domains (full-length PTK7, cPTK7 622–1070) both physically interact with LRP6 protein in the co-IP assay (Fig. 1B, lanes 2–3). The shorter intracellular/nuclear cPTK7 726–1070 PTK7 protein that lacks a transmembrane domain does not interact with LRP6 protein in the co-IP assay (Fig. 1B, lane 4 versus 8).

### 3.2. In PTK7 knock down embryos, ectopically expressed PTK7 proteolytic fragments rescue neural marker expression and embryo morphology

We examined the function of the individual cPTK7 726–1070 and cPTK7 622–1070 proteins in PTK7 knockdown embryos. Previous studies showed that antisense *PTK7*-morpholino oligonucleotide (*PTK7-MO*) mediated protein knockdown in *Xenopus* embryos inhibits posterior neural cell fate formation and neural tube folding and elongation by depleting LRP6 protein levels (Lu et al., 2004; Bin-Nun et al., 2014). We also showed that ectopic expression of the human PTK7 protein in *Xenopus* rescued the PTK7 morphant phenotype (Bin-Nun et al., 2014). To determine PTK7 proteolytic fragment function in vivo, we co-expressed the cPTK7 622–1070 (Fig. 2A) and cPTK7 726–1070 proteins (Fig. 2B) into *PTK7* morphant embryos. Embryos were examined for morphological rescue at neurula (Fig. 2A–B, upper twelve panels) and tailbud (Fig. 2A–B, lower twelve panels). Control embryos (CE) are shown in the eight panels on the left side, upper (neurula stage) and lower (tailbud stage). At neurula stages, the embryos expressing either one of the proteolytic PTK7 fragment proteins and the *PTK7-MO* show a significant increase in neural folding versus almost none in the open neural plate PTK7 morphant embryos. The neural plate folding is more pronounced

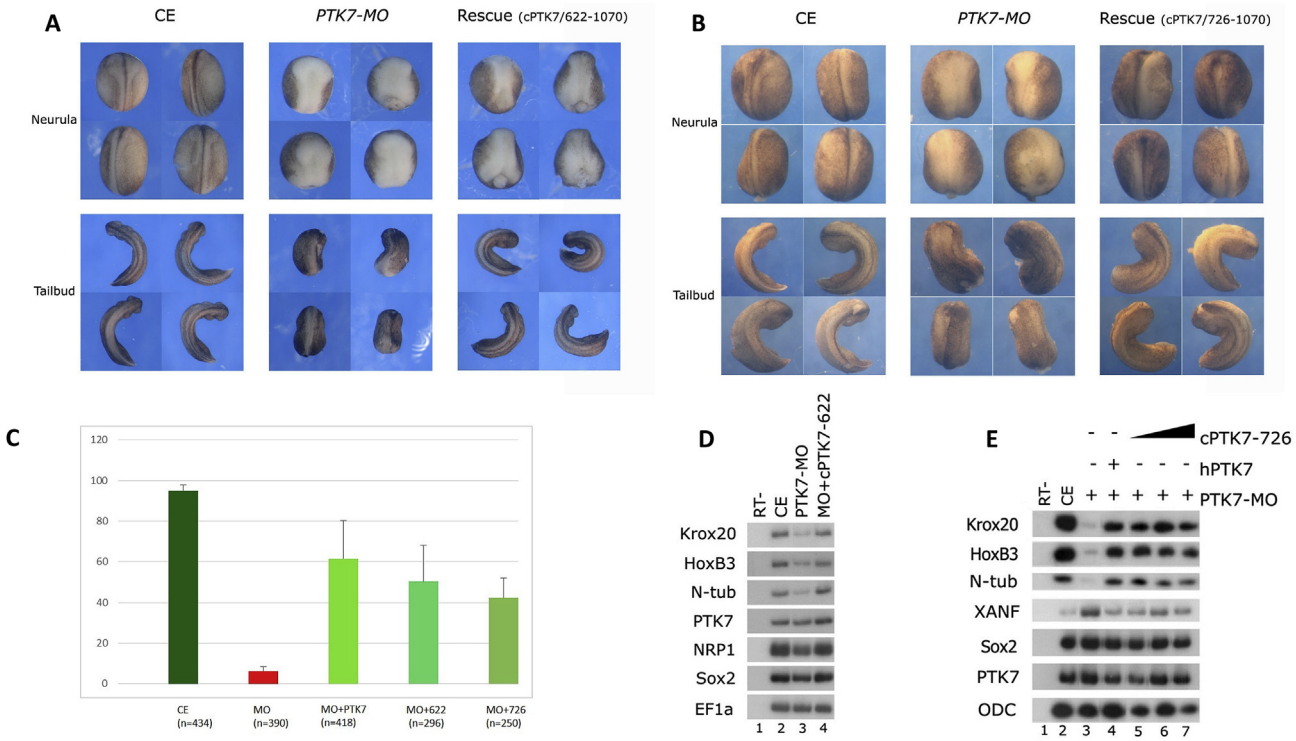


**Fig. 2. The cPTK7 622–1070 and cPTK 726–1070 proteins rescue NTDs and posterior neural cell fates in PTK7 knockdown/morphant embryos**

**A.** Embryos were examined for morphological rescue at neurula (stage 17–18, top two rows of panels) and tailbud (stage 24–26, lower two panels). Embryos were injected with 12 ng *PTK7-MO* and rescued by co-injection of 100–400 pg of *cPTK7 622–1070* protein encoding RNA. Four representative embryos are shown per injected group. The control embryos (CE) are the two panels of four on the left side (upper four: neurula stage and lower four: tailbud stage). The middle two panels of four are the *PTK7-MO* injected embryos. The two panels of four on the right are the PTK7-MO/*cPTK7 622–1070* co-injected rescued embryos. At neurula stages, stronger neural folding is observed in the *PTK7-MO/cPTK7 622–1070* co-injected embryos versus the *PTK7-MO* injected embryos (upper-right four panels, versus upper-center four panels). At tailbud stages the embryos are less squat and more elongated the in the *PTK7-MO/cPTK7 622–1070* co-injected embryos versus the *PTK7-MO* injected embryos (lower right four panels, versus lower center four panels). Rescue experiment statistics and data bar graphs are shown in Fig. 2C.

**B.** Embryos were examined for morphological rescue at neurula (stage 17–18, top two rows of panels) and tailbud (stage 24–26, lower two panels). Embryos were injected with 12 ng *PTK7-MO* and rescued by co-injection of 100–400 pg of *cPTK7 726–1070* protein encoding RNA. Four representative embryos are shown per injected group. The control embryos (CE) are the two panels of four on the left side (upper four: neurula stage and lower four: tailbud stage). The middle two panels of four are the *PTK7-MO* injected embryos. The two panels of four on the right are the PTK7-MO/*cPTK7 726–1070* co-injected rescued embryos. At neurula stages, stronger neural folding is observed in the *PTK7-MO/cPTK7 726–1070* co-injected embryos versus the *PTK7-MO* injected embryos (upper-right four panels, versus upper-center four panels). At tailbud stages the embryos are less squat and more elongated the in the *PTK7-MO/cPTK7 726–1070* co-injected embryos versus the *PTK7-MO* injected embryos (lower right four panels, versus lower center four panels). Rescue experiment statistics and data bar graphs are shown in Fig. 2C.

**C.** Statistical analysis of embryos shown in Fig. 2A–B. Embryos were scored at tailbud stage for rescue as described in the text. Control embryos (CE) n = 434, 95% normal neural plate folding; *PTK7-MO* injected embryos (MO) n = 390, 6% normal neural plate folding; *PTK7-MO*/PTK7 full-length protein co-injected embryos (MO + PTK7) n = 418, 61% normal neural plate folding; *PTK7-MO/cPTK7 622–1070* protein co-injected embryos (MO+622) n = 296, 50% normal neural plate folding; *cPTK7 726–1070* protein co-injected embryos (MO+726) n = 250, 42% normal neural plate folding;

**D.** Control embryos (lane 2) were injected at the one-cell stage with *PTK7-MO* (12 ng, lane 3) or the *PTK7-MO* and RNA encoding *cPTK7 622–1070* protein (400 pg, lane 4) Total RNA was isolated from pools of eight neurula (st.17) embryos from each group. Gene expression was analysed by Semi-quantitative (sq)RT-PCR analysis to: *krox20, hoxb3* (hindbrain), *n-tub* (primary neuron), *sox2* and *ptk7* (panneural). –RT was performed (lane 1) to total RNA from the control uninjected embryos. EF1α is as positive control for RNA levels. Markers are rescued by the cPTK7 622–1070 protein (lanes 2–4)

**E.** Control embryos (lane 2) were injected at the one-cell stage with *PTK7-MO* (12 ng, lane 3) or the *PTK7-MO* and RNA encoding the full-length human PTK7 protein (50 pg, lane 4), which serves as a positive control for rescue (ref. 11). In parallel, the *PTK7-MO* was co-injected with increasing concentrations of RNA encoding the cPTK7 726–1070 protein (100 pg, 200 pg, 400 pg, lanes 5–7). Total RNA was isolated from pools of eight neurula (st.17) embryos from each injected group and sqRT-PCR was performed to analyse expression of the *krox20, hoxb3* (hindbrain), *n-tub* (primary neuron), *xanf1* (forebrain), *sox2* and *ptk7* (panneural) genes. –RT was performed (lane 1) to total RNA from the control uninjected embryos. ODC serves as positive control for RNA levels. Markers are rescued by either the full-length PTK7 or cPTK7 726–1070 proteins (compare lane 3–4 to 5–7).

and the distance between the left and right folds is significantly shorter in the rescued embryos (Fig. 2A–B, upper panel, compare center four embryos to the four embryos on right side). At tailbud stages, the embryos are less squat and more elongated, as a result of the more efficient neural plate folding and elongation (Fig. 2A–B, lower panel, compare the center four embryos to the four embryos on the right side). In control embryos (n = 434), about 95% had normal neural folding (Fig. 2C), versus 6.3% in the PTK7 morphant (n = 390) group (Fig. 2C). The cPTK7 622–1070/*PTK7-MO* co-injected embryos (n = 296) had 8-fold higher neural folding versus the PTK7 morphants, while neural folding was almost 7-fold higher in the cPTK7 726–1070/*PTK7-MO* co-injected (n = 250) embryos (Fig. 2C). In comparison, full-length PTK7 protein rescued about 10-fold higher (n = 418, Fig. 2C), similar to our previous findings (Bin-Nun et al., 2014).

As shown in our previous studies (Bin-Nun et al., 2014), in *PTK7-MO* embryos, posterior neural marker expression is sharply decreased (Fig. 2D–E). Rescue activity by PTK7 proteolytic fragments in comparison to the full-length PTK7 was also assayed by the renewed embryonic expression of posterior neural markers (Fig. 2D–E). In the *PTK7-MO* embryos, ectopic cPTK7 622–1070 protein levels (Fig. 2D) rescued

inhibited expression levels of hindbrain (*krox20*, *hoxb3*) and primary neuron (*n-tub*) markers similar to the full-length protein (Bin-Nun et al., 2014, Fig. 2E). As shown previously for PTK7 morphant embryos, the loss of the posterior hindbrain cell fates is accompanied by an increase in anterior forebrain fates (Bin-Nun et al., 2014). Expression of a panneural marker (*sox2*) and the endogenous *ptk7* gene is normal in the PTK7 morphant embryos (Fig. 2D–E). Similarly, ectopic cPTK7 726–1070 protein levels (Fig. 2E) also rescued inhibited expression levels of hindbrain (*krox20*, *hoxb3*) and primary neuron (*n-tub*) markers protein (Fig. 2E, compare lanes 1–2 versus 5–7), similar to the full-length PTK7 protein (Bin-Nun et al., 2014, Fig. 2E, compare lanes 1–2 versus 4). These markers and their cell fates are strongly reduced in *Xenopus* embryos when canonical Wnt-signaling is perturbed (Bin-Nun et al., 2014; Elkouby et al., 2010). As shown previously, expression of the anterior forebrain marker, *xanf1*, increases in PTK7 morphant embryos and this expression was repressed in PTK7 rescued embryos (Bin-Nun et al., 2014). Expression of *xanf1* is very sensitive to the loss of embryonic Wnt-signaling, which triggers its increased and expanded expression (Elkouby et al., 2010). Like full-length PTK protein, the cPTK7 726–1070 protein also represses increased *xanf1* expression back to near normal levels (Fig. 2E). Thus by comparing both embryo morphology and expression of cell-fate specific neural markers, the cPTK7 622–1070 and cPTK7 726–1070 PTK7 proteins function similarly to full-length PTK7 protein (Bin-Nun et al., 2014) to pattern the developing posterior nervous system.

### 3.3. Regulation of canonical Wnt activity in Xenopus explants

We further addressed the function of the cPTK7 726–1070 and cPTK7 622–1070 proteolytic fragments in PTK7 knockdown and ectopic expression assays in *Xenopus* animal cap (AC) explants. Wnt pathway activation in AC explants triggers expression of endogenous *Xenopus* Wnt direct-target genes such as, *siamois*, *xnr3*, *gbx2* and *hoxd1* (Elkouby et al., 2010; Brannon et al., 1997; McKendry et al., 1997; Li et al., 2009, Fig. 3A). The activation of these markers in gastrula-stage ACs is a classic readout of Wnt-target genes in the *Xenopus* system, being essentially a reporter-like gene assay (Kühl and Pandur, 2008). The AC system is simpler, more accurate and more robust assay, in comparison to co-injecting another reporter gene DNA-component into the embryonic system. Our previous studies showed that *PTK7-MO* co-injection and subsequent depletion of LRP6 protein prevents activation of Wnt direct-target genes in the AC assay (Bin-Nun et al., 2014). To efficiently activate the canonical Wnt pathway in ACs, we ectopically expressed the LRP6ΔN Wnt ligand-independent activated-LRP6 protein (Bin-Nun et al., 2014; MacDonald et al., 2008). LRP6ΔN strongly activates Wnt direct-target genes in ACs, in a more efficient manner than the full-length LRP6 protein that is more dependent on endogenous Wnt activity that is lacking in the naïve AC explants. Like the full-length LRP6 protein, LRP6ΔN protein levels are also very sensitive to the loss of PTK7 protein activity (Bin-Nun et al., 2014). We co-injected the LRP6ΔN protein and the *PTK7-MO* in ACs alone or in combination with the full-length (human) or the two PTK7 fragments (cPTK7 726–1070, cPTK7 622–1070). Alone, LRP6ΔN protein induces expression of the *xnr3*, *siamois* and *hoxd1* genes in ACs (Fig. 3A–B, compare lanes 3–4), but co-injection of the *PTK7-MO* strongly reduces their expression (Fig. 3A–B, lanes 4 and 6). Ectopic co-expression of the full-length human PTK7 protein (Fig. 3A, compare lanes 4, 6–7) or the cPTK7 726–1070 or cPTK7 622–1070 proteins (Fig. 3B, compare lanes 4, 6–8) rescued expression of the Wnt direct-target genes in the AC explants. Relative expression rescue levels were similar, but the full-length protein appeared to be a bit less efficient (compare Fig. 3A versus Fig. 3B).

In addition to re-activating expression of Wnt target genes, we also observed that similar to wildtype PTK7 protein (Bin-Nun et al., 2014), the cPTK7 726–1070 protein rescues depleted LRP6ΔN protein levels triggered by the *PTK7-MO* in embryos (Fig. 4A). These results strongly suggest that the two small PTK7 proteins can physiologically support canonical Wnt-signaling in vivo, similar to the full-length protein (Bin-Nun et al., 2014).

### 3.4. Regulation of LRP6 protein and Wnt target gene expression levels in Xenopus embryos

We previously showed that depletion of PTK7 protein in *Xenopus* embryos reduces endogenous or exogenous LRP6 protein levels and this could be rescued by expression of ectopic human PTK7 protein (Bin-Nun et al., 2014). We determined if the cPTK7 726–1070 and cPTK7 622–1070 fragments could rescue LRP6 protein levels in PTK7 morphant embryos, similar to full-length PTK7 protein. We depleted endogenous

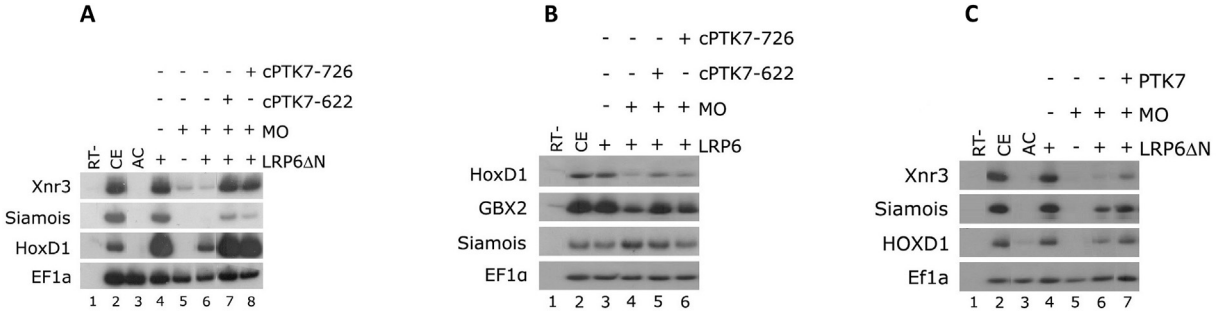


**Fig. 3. PTK7 cPTK7 622–1070 and 726–1070 proteins support LRP6 activation of Wnt direct-target genes**

**A.** Embryos were injected at the one-cell stage with *PTK7-MO* (10 ng, lanes 5–7). In parallel, these embryos co-injected animally with mRNA encoding the ligand-independent form of the LRP6 protein, LRP6ΔN (175 pg) that activates the Wnt pathway (lane 4), and mRNA encoding the full-length PTK7 protein (200 pg, lane 7). Animal caps (ACs) removed at the blastula stage were grown until gastrula st.10.5, when total RNA was isolated from five control embryos (lane 2) and 18 ACs from each group (lanes 3–7). Wnt direct-target gene expression was examined by sq (semiquantiative) RT-PCR: *xnr3*, *siamois* and *hoxd1*. *Ef1α* serves as positive control for RNA levels.

**B.** Embryos were injected at the one-cell stage with *PTK7-MO* (10 ng, lanes 5–8). In parallel, these embryos co-injected animally with mRNA encoding the ligand-independent form of the LRP6 protein, LRP6ΔN (175 pg) that activates the Wnt pathway (lane 4), and mRNA encoding the short PTK7 proteins cPTK7 622–1070 and cPTK7 726–1070 (200 pg, lanes 7–8). ACs removed at the blastula stage were grown until gastrula st.10.5, when total RNA was isolated from five control embryos (lane 2) and 18 ACs from each group (lanes 3–9). Wnt direct-target gene expression was examined by sqRT-PCR: *xnr3*, *siamois* and *hoxd1*. *Ef1α* serves as positive control for RNA levels

**C.** Embryos were injected at the one-cell stage with *PTK7-MO* (12 ng, lanes 4–6). In parallel, these embryos co-injected animally with mRNA encoding LRP6 protein (600 pg, lane 3), and mRNA encoding the short PTK7 proteins cPTK7 622–1070 and cPTK7 726–1070 (200 pg, lanes 5–6). Embryos were grown until gastrula st.10.5, when total RNA was isolated from five embryos in each group. Wnt direct-target gene expression was examined by sqRT-PCR: *hoxd1* and *gbx2*. *Siamois* expression is unchanged as described in text. *Ef1α* serves as positive control for RNA levels.

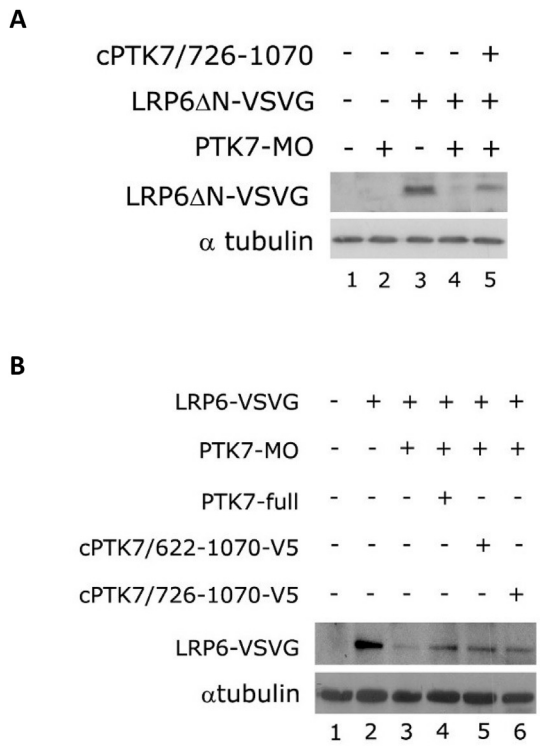


**Fig. 4. LRP6 protein levels are maintained by small PTK7 proteolytic fragments**

**A.** Control embryos (lane 1) were injected at the one-cell stage with *PTK7-MO* (12 ng, lane 2) and/or RNA encoding the activated LRP6ΔN-VSVG protein (200 pg, lane 3–4), and RNA encoding the human PTK7 726–1070 protein (lane 5). Protein was isolated for Western blot analysis at gastrula stages. Note the increased levels of LRP6ΔN-VSVG protein in lane 5 versus lane 4. α-tubulin protein serves as a positive control for protein loading.

**B.** Control embryos (lane 1) Embryos were animally injected at the one-cell stage with mRNA encoding the full-length LRP6 (600 pg, lanes 2–6). The *PTK7-MO* (12 ng, lanes 3–6) is co-injected parallel, along with mRNAs encoding the full-length PTK7 protein (200 pg, lane 4), mRNA encoding the cPTK7 622–1070 protein (200 pg, lane 5) or mRNA encoding the cPTK7 726–1070 protein (200 pg, lane 6). Embryos were grown to gastrula (stage 10.5). Total protein was isolated from ten embryos per group for Western analysis. The VSVG antibody detects the full-length LRP6 protein. α-tubulin protein serves as a positive control for protein loading.

PTK7 protein by injecting the *PTK7-MO*, and co-expressed low levels of tagged LRP6 protein along with the human full-length PTK7, cPTK7 726–1070 and cPTK7 622–1070 proteins. The *PTK7-MO* reduced levels of the ectopically expressed LRP6 protein (Fig. 4B, compare lanes 2–3). Co-expressing all the constructs (wild type human PTK7, cPTK7 726–1070, cPTK7 622–1070) significantly rescued LRP6 protein levels depleted in the PTK7 morphant embryos (Fig. 4B, compare lanes 2–6). In addition, we examined expression of the Wnt-target genes, *hoxd1* and *gbx2* in these same *PTK7* morphant embryos (Fig. 3B). In the embryo proper, *siamois* and *xnr3* gene expression is not perturbed by PTK7 knockdown. In vivo, unlike in ACs (that are solely dependent on externally provided Wnt-signaling), the activation of the *siamois* and *xnr3* genes is determined by maternal Wnt components that precede the zygotic Wnt-components. These zygotic Wnt-components include PTK7 protein that is required to activate *hoxd1* expression in the developing nervous system (Bin-Nun et al., 2014). Thus, in PTK7 morphant embryos, *hoxd1* expression was reduced but *siamois* and *xnr3* expression was normal (Bin-Nun et al., 2014). In morphant embryos, *hoxd1* and *gbx2* expression was reduced, and this was partially restored by the ectopic expression of the cPTK7 726–1070 or cPTK7 622–1070 proteins (Fig. 3B). As expected, *siamois* gene expression levels were not significantly altered by PTK7 knockdown (Bin-Nun et al., 2014, Fig. 3B). This result shows that the two small PTK7 proteolytic fragments can physiologically support canonical Wnt-signaling in vivo by maintaining LRP6 protein levels lost in the absence of endogenous PTK7 protein. These observations suggest that the smaller versions of PTK7 are acting similarly to full-length wild type protein in regulating canonical Wnt-signaling.

## 4. Discussion

Studies in cancer cells have found three proteolytic PTK7 fragments that stimulate cell invasion and motility and differentially regulate gene expression (Na et al., 2012; Golubkov and Strongin, 2012, 2014; Golubkov et al., 2014). Full-length PTK7 protein consists of seven extracellular immunoglobulin domains, a trans-membrane domain and an intercellular "pseudo" kinase domain (Na et al., 2012; Golubkov and Strongin, 2012, 2014; Golubkov et al., 2014). The full-length PTK7 is proteolytically cleaved by metalloproteinases to three natural fragments, sPTK7, cPTK7 622–1070 and cPTK7 726–1070 (Na et al., 2012; Golubkov and Strongin, 2012, 2014; Golubkov et al., 2014). In this study, we have examined the in vivo function and activities of the cPTK7 622–1070 and cPTK7 726–1070 proteolytic fragment proteins during *Xenopus laevis* nervous system development.

The cPTK7 622–1070 protein consists of one immunoglobulin domain, a transmembrane region and the kinase domain. The cPTK7 622–1070 protein is found in the cell membrane. In contrast, the shorter cPTK7 726–1070 protein consists of only the kinase intercellular domain and is found in the cell cytoplasm and nucleus (Na et al., 2012; Golubkov and Strongin, 2012, 2014; Golubkov et al., 2014). Based on cancer cell studies that describe the stimulation of cell invasion and motility, and differential regulation of gene expression, we hypothesized that the small proteolytic PTK7 fragments, cPTK7 622–1070 and cPTK7 726–1070 could be involved in regulating the canonical Wnt signaling pathway, perhaps like the full-length PTK7 proteins.

PTK7 and LRP6 proteins appear to physically interact via their transmembrane domains (Bin-Nun et al., 2014). Using a similar co-IP assay, we examined PTK7 proteolytic fragment interactions with LRP6 protein. We found that the longer cPTK7 622–1070 fragment that contains a transmembrane domain efficiently undergoes co-IP with LRP6 protein. The shorter cPTK7 726–1070 fragment does not undergo efficient co-IP with LRP6 protein, likely because it lacks a functional transmembrane domain.

To further address PTK7 proteolytic fragment activity in vivo, we knocked down PTK7 in *Xenopus* embryos and then re-introduced each small protein by ectopic embryonic expression to examine biological activity. PTK7 morphant embryos have poor neural folding accompanied by a loss of posterior neural cell fates (Bin-Nun et al., 2014). We found that both PTK7 proteolytic fragments (cPTK7 622–1070 and cPTK7 726–1070) rescued posterior neural convergence extension and neural folding that is dependent on both canonical and non-canonical/PCP Wnt activity (Elkouby et al., 2010; rev. in Komiya and Habas, 2008; Carron and Shi, 2016). Our previous studies showed that early canonical Wnt signaling and posterior neural cell fate induction is required for the later neural tube folding and elongation regulated by non-canonical/PCP Wnt activity (Bin-Nun et al., 2014; Elkouby et al., 2010). Furthermore, by examining the expression of posterior neural marker genes, we also determined that both PTK7 proteolytic fragments rescued posterior neural cell fates in PTK7 morphant embryos. Canonical Wnt signaling determines the different cell fates in the posterior regions of the *Xenopus* nervous system (Elkouby et al., 2010). Therefore, these results suggest that both of these PTK7 proteolytic fragments regulate canonical Wnt signaling, like full-length PTK7 protein.

We utilized AC explants to more directly assay canonical Wnt activity in *Xenopus* embryonic cells. Wnt direct-target genes (Elkouby et al., 2010; Brannon et al., 1997; McKendry et al., 1997; Li et al., 2009) are transcriptionally activated in this assay. In ACs, we exclusively activated canonical Wnt signaling via ectopic LRP6 protein expression. By

co-injecting the *PTK7-MO*, we knocked down endogenous PTK7 levels, subsequently depleting and inhibiting LRP6 protein activity. In this AC assay, we ectopically expressed the activated form of LRP6 protein (LRP6ΔN), which is very sensitive to PTK7 knockdown (Bin-Nun et al., 2014). In ACs, we simultaneously co-expressed LRP6ΔN and the full-length, or cPTK7 622–1070 or cPTK726-1070 on the *PTK7-MO* background. Co-expression of the human full-length PTK7 or either of the PTK7 proteolytic fragment proteins robustly rescued Wnt direct-target gene expression. We also showed that in embryos, the cPTK7 622–1070 or cPTK7 726–1070 proteins rescued reduced LRP6 protein levels and activity that were depleted by embryonic PTK7 knock down, similar to the full-length PTK7 protein. Thus in *Xenopus* explants and embryos, the small PTK7 proteolytic fragments support proper canonical Wnt signaling like the full-length protein.

It is interesting that both PTK proteolytic fragment proteins act in a similar manner in embryonic assays to rescue canonical Wnt signaling in *PTK7* morphant embryos and explants. By rescuing posterior neural cell fates, supporting activation of Wnt-target genes and maintaining LRP6 protein levels, the cPTK7 622–1070 or cPTK7 726–1070 proteins recapitulate activities of the full-length PTK7 protein. The cPTK7 622–1070 protein, while lacking an extracellular domain, still contains an intracellular domain and transmembrane domain that still physically interacts with LRP6 protein. This may suffice to replace the knocked down LRP6 protein levels in our various assays.

However, the cPTK7 726–1070 protein lacks a transmembrane domain. In cancer cells, biologically active cPTK7 726–1070 protein was detected in the cell cytoplasm and nucleus (Na et al., 2012; Golubkov and Strongin, 2012). The cPTK7 726–1070 protein also rescues the missing canonical Wnt activity in the embryo, similarly to the full-length and cPTK7 622–1070 proteins. While this protein does not directly interact with LRP6 protein by co-IP assays, this protein has significant biological activity and function in cancer cells, but the mechanism was not determined (Na et al., 2012; Golubkov and Strongin, 2012). Nevertheless, we show this protein strikingly rescues the lost Wnt signaling and depleted LRP6 protein levels, despite its apparent lack of direct interaction with LRP6 or its membrane localization. Potentially, the cPTK7 726–1070 protein could modulate canonical Wnt signaling and LRP6 protein levels via interactions with Dishevelled and/or β-catenin proteins. Future experiments will address this question.

In this study, we focused on the small PTK7 proteolytic fragment proteins, cPTK7 622–1070 and cPTK7 726–1070, based on the known role of the full-length PTK7 protein during neural tube formation. Clearly, to reach a deeper and fuller understanding of PTK7 protein in vivo, we need to understand the potential function of each of its individual proteolytic fragments, essentially the sums of all of the diverse PTK7 protein "parts". We still do not know which proteins are the bona fide biologically active molecules in vivo, the full-length PTK7 protein, its proteolytic fragments or a combination of all. This is still an open future question.

We found that the full-length PTK7 and its small proteolytic fragments stabilize LRP6 protein to promote canonical Wnt activity. We still have many unsolved questions when trying to understand the complex nature of Wnt pathway regulation in vivo during vertebrate embryonic development. Future research is required for identify which the small PTK7 proteolytic fragments actually exist during development and we will need to determine how these smaller PTK7 proteins directly interact with the all of the components of the Wnt signaling pathway. Whatever be the normal distribution of PTK7 proteolytic fragment proteins during normal embryo development or in cancer cells, these small PTK7 proteins are powerful molecules displaying strong Wnt regulatory activity.

## Author contribution

H.L and Y.C. were involved in the design and performance of all the experiments. H.L and Y.C. participated in writing of the manuscript. H.L. and Y.C. prepared Figs. 1–4.

N.B.N. was involved in the planning and design of experiments in the early part of this project. Her preliminary data enabled us to initially carry out this study.

V.G. designed and built the crucial PTK7 constructs/reagents used in this study. He gave us crucial scientific advice during the project. He participated in writing of the manuscript.

D.F. was involved in the design of all the experiments and the performance of a few of them. D.F. participated in writing the manuscript.

## Additional information

The authors declare no competing interests.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ydbio.2019.05.007.

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