Characterization of Tumor Protein p63 Regulated 1-Like Function in Xenopus laevis

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Characterization of Tumor Protein p63 Regulated 1-Like Function in *Xenopus laevis*

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Defense: April 11, 2017  

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Acknowledgements

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Abstract

Of the approximately 20,000 genes in the human genome, about 6,000 have unknown or poorly characterized function. Tumor protein p63 regulated 1-like (TPRG1l) is one of those genes with no functional data. TPRG1L is expressed during embryonic development and adulthood with strongest expression in the brain. It is implicated in carcinogenesis because its gene expression is activated by tumor regulators p63/p73. To elucidate TPRG1L function during embryogenesis, CRISPR-Cas9 genome editing system was utilized to induce Tprg1l mutations in Xenopus laevis. Embryos injected with Tprg1l sgRNA and Cas9 mRNA displayed axis formation and convergent extension defects suggesting that Tprg1l mutants may have dysregulated Wnt signaling. The Wnt signaling pathway is an evolutionarily conserved pathway that is critical for proper head development, dorsoventral and anteroposterior axis establishment, and planar cell polarization. Since Wnt responsive genes are repressed by p63/p73 factors that activate Tprg1l expression, I hypothesized that TPRG1L may also regulate Wnt signaling. Tprg1l mRNA overexpression displayed axis deformities as well as convergent extension defects consistent with disruption of Wnt signaling. TPRG1L has an inositol polyphosphate 5-phosphatase domain that may reduce inositol polyphosphates that are necessary for normal Wnt signaling. This observation together with the results of loss and gain of function experiments suggest that TPRG1L may be a novel inhibitor of the Wnt signaling pathway.

Keywords: TPRG1l, Mover, CRISPR-CAS9 mutagenesis, Wnt signaling, embryonic development, cancer, In situ hybridization
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Introduction

Tumor Protein p63 regulated 1-like

There is still much to discover about the functional role of gene products. This study aims to elucidate the functional role of tumor protein p63 regulated 1-like (TPRG1l) during early development in *Xenopus laevis*. TPRG1L was chosen because it has an unknown function and is implicated in tumor formation. Tumors can arise from a combination of rapid cell division and changes in cell differentiation. These aspects of cellular regulation are also common during development, thus characterization of TPRG1L function during embryogenesis may elucidate its function in tumor formation. Studies show TPRG1L is highly expressed in the brain and codes for a peripheral membrane pre-synaptic protein called Mover, which is found on synaptic vesicles in mice neurons (Kremer et al., 2007; Köber et al., 2015). Mover is a vertebrate specific homomorphic phospho-protein found in the hippocampus and calyx of held (Ahmed et al., 2012). While there have been studies on the function of Mover protein in the nervous system, there has been no studies on the functional role of TPRG1L during development.

A duplication event occurred during the divergence of vertebrates and invertebrates that resulted in the paralogs: tumor protein p63 regulated 1 (TPRG1) and TPRG1-like (TPRG1l). Both genes are regulated by tumor protein 63 (p63), a protein in the functional family of p53, which is an important regulator of normal cell cycle, programmed cell death and is commonly mutated in tumors. Similarities exist between the protein product of TPRG1L and TPRG1 but it has been shown that they do not form heterodimers with one another (Ahmed et al., 2013). In *Xenopus laevis*, TPRG1 is mainly expressed in the brain while TPRG1L is expressed in the brain, spinal cord, and foregut (Chen et al., 2015). The different expression profiles suggest that TPRG1 and TPRG1L may have different functions. However, the possibility of their functional roles being redundant to one another cannot be ruled out.

Tp63 Regulates Wnt Gene Expression and TPRG1l

The p53 family of transcription factors (p53/p63/p73) redundantly regulate Wnt responsive genes as well as TPRG1 and TPRG1L (Kurek et al., 2014; Wang et al., 2017). The Wnt signaling pathway is evolutionarily conserved as a key regulator of proper embryogenesis and also has a role in
carcinogenesis. Cell proliferation, cell differentiation, cell migration and body axis patterning in vertebrate embryos are all aspects of development regulated by the Wnt signaling pathway. After fertilization, the maternal canonical Wnt pathway is activated by the dorsal enrichment of Dishevelled (DSH) and GBP that stabilize and enrich beta-catenin on the dorsal side and the prospective organizer region. The organizer, a group of cells at the dorsal lip of the blastopore, initiates gastrulation and is essential for formation of the head structures and the central nervous system (Gilbert, Developmental Biology). Later in embryogenesis, Wnt signaling regulates anteroposterior axis formation. Wnt signaling also has non-canonical (beta-catenin and transcription independent) roles that regulate cell movement and tissue polarity, also known as planar cell polarity. Both canonical and non-canonical Wnt signaling are involved in cancer (Komiya and Habas, 2008).

The Wnt pathway has two opposing roles with regards to head formation. Early maternal Wnt signaling promotes formation of the organizer and the dorsoanterior structures and is therefore necessary for inducing head structures. The early pathway also regulates expression of Wnt antagonists. Later, zygotic Wnt signaling induces posterior fates and counteracts head formation (Kiecker et al., 2013; Hikasa and Sokol 2013). This dual activity often complicates analysis that rely on gain- and loss-of-function experiments, since the spatiotemporal experimental parameters need to be tightly manipulated and monitored. Cellular competence that allows the embryo to switch between early and late stage Wnt signaling is reliant on a change of nuclear factors, not a change in cellular pathways. The function of different Wnt molecules are difficult to elucidate since molecules in the Wnt may have different roles and work in different combinations to enact changes in throughout the embryo’s development.

**CRISPR-Cas9 Genome Editing and Mutagenesis**

The CRISPR-Cas9 genome editing system is an innovative, inexpensive, and efficient tool to alter an organism’s genome. This study used the CRISPR-Cas9 system to induce a knockout mutation of *Tprg1l* in *X. laevis* embryos during the one cell stage. Single stranded guide RNA (sgRNA) consists of a 20-nt sequence complementary to the genomic sequence of the gene of interest followed by a CRISPR sequence that provides a scaffold structure to bind with Cas9. For this study, two different sgRNAs targeting different parts of *Tprg1l* gene were used (see Methods and Results). Cas9 is an RNA guided
endonuclease that uses a sgRNA to find the gene and then induce a double stranded break (DSB) in the target gene. The cell’s DNA repair mechanisms would be called upon to fix the DSB via homologous recombination (HR) or non-homologous end joining (NHEJ). The latter method tends to frequently induce indel mutations that leave the gene non-functional (Cohen, 2015).

**Xenopus laevis as a Model Organism**

The use of *Xenopus laevis* has made significant contributions to our understanding of the functional role of genes involved in development. *X. laevis* embryos are easy to microinject with oligonucleotides, mRNA, and protein which are key methods in developmental biology research. Females can be easily induced to lay hundreds of eggs year round, and can be fertilized in vitro. This makes the animals easy to maintain and convenient to conduct experiments with. Embryos are robust enough to be manipulated via microsurgery or microinjection and eggs average 1.3mm in diameter, which is advantageous for researchers. Their short developmental time is also of benefit, because their major organ structure are formed within three days. They have been essential in understanding the mechanism behind tissue formation and body axis formation as well. The Wnt signaling pathway is well studied in *Xenopus laevis*, and the resources and tools needed to study it are easily accessible. Their genome is structurally similar to that of humans, so finding a gene’s functional role in *X. laevis* can provide insight onto the gene’s role in humans (Tadjuidje et al., 2010).
Methods

sgRNA design and synthesis

The CRISPR-direct website was screened to find target sequences for \textit{Tprg1l} gene sequence that would be compatible with the DR274 (Addgene 42250) cloning and expression vector (Hwang et al 2013). A sequence to target both long and short chromosome genes within the first exon was not found due to low similarity within the 5' region of the sequences. Therefore, one sgRNA to target the first exon of the copy of \textit{Tprg1l} on the long chromosome was chosen. A second target sequence that targets both long and short chromosome genes in the highly conserved last exon at the 3' of the sequence was also selected (Appendix). There was precaution to not target \textit{Tprg1}, which is a paralog of \textit{Tprg1l} (Figure. 1).

The sense and anti-sense oligomers for sgRNA constructs were ordered from Integrated DNA technologies, were then hybridized and phosphorylated, and then ligated into BsaI site of DR274 plasmid and cloned via bacterial transformation (cultured in LB with Kanamycin). Plasmid DNA was extracted from the colonies and screened for the presence of target sequence by PCR. Target sequence positive colonies were linearized with DraI and transcribed in-vitro using NEB HiScribe T7 kit.

Cas9 Mutagenesis via Microinjection

Two methods of Cas9 mutagenesis were attempted in this study. The first involved microinjection of Cas9 protein from PNABio that was prepared according to company recommendations. The protein was then coupled with synthesized sgRNA for an hour along with lineage tracer before injections (Square T et al 2015). The second involved microinjection of synthesized Cas9 mRNA, synthesized sgRNA, and lineage tracer. The Cas9 mRNA was synthesized using Cas9+pCS2 plasmid (Addgene 47322) after linearizing it with Acc651 and transcribing capped mRNA using SP6 mMessage kit (Ambion). Injection needles were pulled from glass capillary micropipettes. Microinjections were done while the embryos were at the 1-cell stage or 2-cell stage.
Cloning of full length *Tprg1l* CDS for gain of function studies

Full length coding sequences of long and short chromosome *Tprg1l* genes were amplified from Stage 11 cDNA by PCR using primers that included restriction enzymes site for efficient cloning (see Appendix). PCR products were purified, digested with BamHI and XbaI and cloned into BamHI and XbaI sites of pCS2+ expression vector. Diagnostic digests of plasmids using PvuII enzyme revealed the presence of either the long or the short chromosome CDS sequences. Plasmids were linearized with NotI and mRNAs were transcribed with SP6 mMessage kit (Ambion).

Embryos

Male *X. laevis* were sacrificed by lethal anesthesia and testes were harvested. A sperm suspension was prepared in 1x MMR by homogenizing a piece of a testis. Female *X. laevis* were induced to lay eggs by injection of hCG 12 hours before experiments. Females were gently squeezed and eggs were collected in a petri dish and were immediately fertilized by adding sperm suspension. Embryos were kept in 0.2x MMR. Fertilized eggs were dejellied using 2% L-Cystein solution (pH 8). 1 cell stage embryos were transferred into 5% Ficoll. Embryos were injected with sgRNA mixed with either Cas9 protein or Cas9 mRNA via microinjection. Some sibling embryos were kept as uninjected controls. Embryos were staged according to Nieuwkoop and Faber (*Nieuwkoop and Faber, 1967*).

Genotype analysis

Injected embryos were allowed to develop until the uninjected embryos reached tail-bud stage and observed for any obvious abnormal phenotypes. The animals were then frozen down for genomic DNA extraction using the DNeasy kit from Qiagen. The extracted genomic DNA was used as template to amplify *Tprg1l* genomic sequence via PCR using primers about 100bp downstream and about 100bp upstream of the *Tprg1l* sgRNA target sites. The PCR products were then run on a gel and purified using the Gel Extraction Kit from Qiagen and processed according to the directions from QuintaraBio for sequencing.
Whole-mount In situ hybridization

Embryos were fixed in 1X MEM salts with 4% formaldehyde at various stages and were preserved in methanol. To enhance probe penetration and increase signal detection, some embryos were fixed for 1 hour, hemisected with a clean razor blade and refixed for another hour. \textit{Tprg1l} CDS was amplified by PCR from stage 11 cDNA using a reverse primer that contained a T7 RNA polymerase binding site (Appendix). The PCR product was purified using Qiagen PCR purification kit and anti-sense probe was in vitro transcribed using T7 RNA Polymerase in the presence of Dig-UTP. Whole-mount in situ protocol was modified from Harland (Harland, 1991). As a control for in situ hybridization analysis, antisense and sense \textit{Xbra} probes were used (pXT-Xbra plasmid was linearized with EcoRV for antisense probe or with Smal for sense probe and transcribed with T7 or SP6, respectively). In situ were developed using BM Purple as substrate.
Results

**Cas9 mRNA was more efficient compared to Cas9 protein for mutagenesis.**

In order to mutate *Tprg1l* gene in *Xenopus laevis*, we designed two different sgRNAs and injected them individually into 1 or 2-cell stage embryos either with Cas9 protein or Cas9 mRNA. A control group was injected with *Tyrosinase (tyr)* sgRNA to assess the overall efficiency of mutagenesis. *Tyr* is a gene that regulates the production of melanin and tyr mutations cause lack of or complete absence of pigmentation. During this study, the batch of Cas9 protein we used proved to be less efficient than the one used by our collaborators Dr. Medeiros and Dr. Square to induce mutations with *tyr* or *Tprg1l* sgRNA as assessed by phenotypes. To resolve this issue, Cas9 mRNA was transcribed for microinjections. Embryos that were injected with the *tyr* sgRNA and Cas9 protein did not show any pigment defects or showed partial defects with low frequency (less than 10%). Cas9 mRNA (~400pg) injected embryos showed full or partial loss of pigments with high frequency (greater than 60%) (Fig 1). Uninjected embryos from the same batch of fertilization were used as a negative control for nonspecific developmental defects. They all show wild-type pigmentation pattern with dark eyes, heart, and pigmentation throughout the body (Fig 1a). Embryos injected with *tyr* sgRNA+Cas9 mRNA at the two-cell stage showed pigmet loss in one eye (Fig. 1b and Fig. 1d) consistent with the first cell division splitting the future left and right sides of embryos. Embryos injected with *tyr* sgRNA + Cas9 mRNA show considerable pigment loss throughout the body, indicating that *tyr* was successfully mutated in those animals (Fig 1b-d).

There are benefits to using Cas9 protein for mutagenesis over Cas9 mRNA, one of which is that Cas9 protein would be more readily available to cause a mutation right after injections. The Cas9 mRNA requires time for the cell to translate the mRNA into a functional protein. Embryos can divide during the time required for translation; so the stage of development that the mutagenesis occurs is dependent on the timing and efficiency of translation of the Cas9 mRNA. This may result in the different severity of mutant phenotypes, which could explain why the eye marked with a red asterisk has considerably more pigment loss than the eye marked with a blue asterisk on a different tadpole (Fig 1b and Fig 1d). The different severity of pigmentation loss in the eyes may also be caused by the different mutations between tadpole B and tadpole D. There are multiple causes that could explain why these tadpoles have different...
degrees of pigment loss. However, the different batches of Cas9 enzyme purchased from the same company may end up having different activities depending on several factors. To maintain consistency across experiments, Cas9 mRNA injections were preferred.

Figure 1. Embryos injected with tyrosinase (tyr) sgRNA and Cas9 mRNA showed reduced pigmentation.

A) The uninjected control which shows strong pigmentation in the eyes and throughout the body. (B-D) Embryos injected with tyr sgRNA and Cas9 mRNA at 1-2 cell stage. All show considerable pigment loss throughout the body and in the eyes. Tadpole B shows more pronounced pigment loss in the eye marked with a red asterisk. Tadpole D shows partial pigment loss in the eye marked with a blue asterisk.

Cas9 mutagenesis of Tprg1l caused head and axis deformities

Tprg1l sgRNA2 was designed to target both Tprg1l genes on the long and short chromosomes (Fig. 2). In two independent experiments, embryos injected with this Tprg1l sgRNA2 + Cas9 mRNA resulted in a mutant phenotype involving reduced head size and axis deformities. Embryo A has a double axis that resulted in two heads, which is evident by the two cement glands present (Fig. 3b). There is a boat-shaped body phenotype present in embryo A-C and E. This suggests that planar cell polarization (PCP) was interrupted during development. Embryos A-E show a reduction in head size, which indicates that anterior development was inhibited. Embryos A-E also lack proper pigmentation throughout the head and body, possibly due to absence of pigment cells derived from the neural crest in the head rather than pigment not being expressed.
In another experiment, injection of \textit{Tprg1l} sgRNA1 that only targets the long chromosome showed a similar phenotype to the \textit{Tprg1l} sgRNA2 that targets both long and short chromosome genes (data not shown). This may be due to the long chromosome gene having a more robust expression or activity than the short chromosome gene. In the process of cloning full length coding sequence (CDS) of \textit{Tprg1l} for gain of function studies, cDNA from Stage 11 embryos was used as template in PCR reactions. PCR product of \textit{Tprg1l} CDS was then cloned into an expression plasmid. 6 individual colonies were tested for presence of long vs short gene product, and only 1 out the 6 was short chromosome gene product and the remaining 5 were long chromosome gene products suggesting that at least at Stage 11, long chromosome \textit{Tprg1l} gene product is more abundant than the short chromosome product. This suggests that \textit{Tprg1l} sgRNA1 that only targets the long chromosome can still result in high frequency of mutant phenotypes.

\textbf{Figure 2. Comparison of the coding sequences of \textit{Tprg1l} on the long and short chromosomes in \textit{Xenopus laevis}.}

The long chromosome coding sequence is on the top row and the short chromosome coding sequence is on the bottom row. Gene specific sgRNA target sequences are highlighted in red followed by a PAM (5'-NGG) site highlighted in cyan. \textit{Tprg1l} sgRNA1 within the first exon only targets the long chromosome. Further down the coding sequence, within the last coding exon, is the \textit{Tprg1l} sgRNA2 that targets both the long and short chromosomes.
Figure 3. *Tprg1* sgRNA plus *Cas9* mRNA injected embryos have axis formation defects.  
(A) Two uninjected embryos at the tail-bud stage with wild-type phenotypes. (B-E) Embryos injected with *Tprg1* sgRNA and *Cas9* mRNA. Red arrows indicate cement glands. Embryos in panels B, C and E are at the same tailbud stage as controls. Embryo in D is at a later stage. All embryos display small heads and axis defects.
Genotyping results have been inconclusive to characterize the mutations induced by $Tprg1l$ sgRNA and Cas9 mRNA injections.

In order to characterize the mutations induced by $Tprg1l$ sgRNA and Cas9 mRNA injections, animals displaying abnormal phenotypes were frozen down and genomic DNA was extracted. PCR was carried out to amplify about 300bp genomic DNA sequence surrounding the sgRNA target site since majority of mutations induced by CRISPR-Cas9 systems are within the 100bp surrounding the target and PAM site. PCR products were purified and sequenced directly. Sequencing results were hard to interpret (even from uninjected control samples) due to sequence variations between the long and short chromosome gene sequences and possibility due to the mosaic nature of different mutations induced by CRISPR-Cas9 in experimental samples (data not shown). PCR products will need to be cloned and isolated individually before sequencing them to fully characterize the presence and types of mutations induced by sgRNA and Cas9 mRNA injections.
Tprg1l mRNA Overexpression Results in Body Axis Deformities

Tprg1l sgRNA injections showed axis defects and suggested that TPRG1L may regulate Wnt signaling. In order to test the function of TPRG1L, gain of function experiments were carried out by injecting Tprg1l mRNA into 1-2 cell stage embryos either at the animal or the vegetal pole. Embryos injected with a lower dose (300pg) of Tprg1l mRNA (300 picograms) showed a mild phenotype and embryos injected with a high-dose of Tprg1l mRNA (600pg) showed delay in gastrulation (Figure 5) and later body axis defects at tailbud stages (Figure 6). Uninjected control animals fertilized at the same time and maintained under the same conditions (at room temperature) appeared further along developmentally than the Tprg1l mRNA injected embryos. While uninjected controls were at Stage 12, Tprg1l mRNA injected embryos appeared to be at Stage 10.5 judging from the size of the blastopore (Figure 5). Later at tailbud stages, Tprg1l mRNA injected animals displayed shortened bodies and various axis defects (Figure 6). Gastrulation delay and axis defects are also consistent with Wnt signaling dysregulation.

Figure 5. Overexpression of Tprg1l mRNA results in gastrulation delay.

(A) An un.injected control embryo at Stage 12. (B) Embryos injected with Tprg1l mRNA vegetally (veg) at 1-2 cell stage show gastrulation delay. (C) Embryos injected with Tprg1l mRNA animally (an) at 1-2 cell stage also show gastrulation delay. Cartoon drawings of St12 and St 10.5 embryos are shown for reference.
Overexpression of Tprg1l mRNA results in axis defects in tailbuds.

Uninjected tailbud (top) displaying wildtype phenotypes (normal head size, axis formation, pigmentation pattern).

Embryos injected with Tprg1l mRNA at 1-2 cell stage develop axis defects later in tailbud stages.

Tprg1l mRNA is ubiquitously expressed in early embryos and is enriched in the head at tailbud stages

In order to determine whether Tprg1l mRNA shows specific localization in the early embryo, in situ hybridization experiments were conducted using an antisense probe for Tprg1l mRNA. Brachyury (Xbra) sense probe was used as a negative control, and show no specific binding as expected (Figure 7a). Xbra anti-sense probe was used as a positive control. Strong signal of the Xbra anti-sense probe is apparent in the mesoderm in gastrula stage embryos (stage 10.5) and in the notochord and tail during tail-bud stage (Figure 7b). Tprg1l antisense probe shows faint purple colorization all around the embryo at early cleavage stages and at stage 10.5, suggesting that Tprg1l mRNA is ubiquitously expressed in early development (7C and D). Embryos at the tail-bud stage show specific binding of the Tprg1l anti-sense probe in the head and near the cement gland (Figure 8) consistent with reports of expression in the brain in mice and frogs.
Figure 7. Tprg1l is expressed ubiquitously in early embryos.

(A) Xbra sense probe used as a negative control shows no specific binding. (B) Xbra anti-sense probe used as a positive control detects Xbra expression in the mesoderm of these stage 10.5 embryos. Left-side embryos are cross sections and right-side embryos are a full spherical embryo. (C) Tprg1l anti-sense probe was used to visualize Tprg1l mRNA in early cleavage stages. There is faint purple color in the top-left most embryo. There are also small dots of purple color, indicated by red arrows. (D) Hemisected Stage 10.5 embryos show strong Xbra signal in the mesoderm with antisense Xbra probe (top left) and no signal with sense Xbra probe (top right). Faint Tprg1l signal is observed ubiquitously in the four bottom embryos. Red arrows indicate where the TPRG1L signal is most intense in the animal pole.
Figure 8. *Tprg1l* is enriched in the brain at tail-bud stages.

(A) *Xbra* anti-sense probe shows expression in the notochord and tail tip during tail-bud stage. (B) *Xbra* sense probe shows no staining. (C-D) *Tprg1l* anti-sense probe shows *Tprg1l* expression in the brain.
Discussion

Current literature has no data about the functional role of TPRG1l, but it is known that p63 regulates Tprg1l expression (Antonini et al., 2008). p63 also regulates Wnt response elements in an inhibitory manner (Katoh et al., 2016). Wnt activity is critical for head formation early in development and dorsoventral axis establishment. It is also important for planar cell polarity (PCP) and cell migration through non-canonical Wnt signaling. Reduced head size and axis deformities result from interruption of the Wnt signaling pathway early in development (Itoh and Sokol, 1999). If the non-canonical Wnt pathway is disturbed, the embryo exhibits a curved boat-shaped body structure. The axis duplication and reduced head size phenotypes observed in Tprg1l mutants suggests involvement in canonical Wnt signaling (Fig. 3B-E, Fig. 4B-D). Deformities observed in Tprg1l mRNA overexpression experiments (Fig. 5C-D and Fig. 6) support the idea that the non-canonical Wnt pathway (planar cell polarity) is disrupted by TPRG1L. These results point to TPRG1L acting in an inhibitory manner in the Wnt signaling network (Figure 9).

Figure 9. Proposed Model of TPRG1L Function

TP63 \[\rightarrow\] Wnt regulated genes

TPRG1l

Nothing is currently known about the molecular function of TPRG1L protein; however, protein sequence analysis identifies inositol polyphosphate 5-phosphatase (IPP5P) as a conserved domain (Smart protein database; Figure 10).
Inositol polyphosphate 5-phosphatase (IPP5P) has an enzymatic role in regulating primary cilia function, which is important for signal transduction of multiple signaling pathways including the Wnt pathway (Conduit et al., 2012). Ciliary proteins regulate PCP signaling, which means a knocking out a regulator of ciliary proteins would result in an abnormal body shape (May-Simera and Kelley, 2012). Wnt signaling also requires the generation of phosphorylated inositol (Gao and Wang, 2007). TPRG1L’s IPP5P domain indicates that it may act an inositol phosphatase. It may remove the phosphate from inositol and block the accumulation of phosphorylated inositol. So, TPRG1L could possibly oppose Wnt activity. This
information along with the \textit{Tprg1l} loss and gain of function phenotypes lead to the hypothesis that TPRG1L protein may inhibit Wnt signaling by blocking accumulation of phosphorylated inositol.

It will also be interesting to characterize TPRG1 protein as a possible inositol phosphatase and the interactions of TPRG1L and TPRG1 to determine whether they have any redundant roles and whether they have any feedback regulation.

Further studies are required to elucidate the mechanism in which TPRG1L may be involved in regulating the Wnt signaling pathway. To determine whether TPRG1L regulates Wnt signaling, gene expression analysis of Wnt targets in \textit{Tprg1l} loss and gain of function embryos will be carried out. To determine whether TPRG1L is involved in noncanonical Wnt signaling, embryos will also be analyzed for convergent extension movements. Additional investigation is needed to study the functional role of IPP5P domain in the TPRG1L protein. Enzymatic activity of TPRG1L and IPP5P domain can be analyzed. Inositol polyphosphate levels can be measured in \textit{Tprg1l} loss and gain of function experiments.

If future experiments show that TPRG1L affects Wnt responsive gene expression or convergent extension movements by regulation of inositol polyphosphates, TPRG1L will be characterized as a novel Wnt regulator. This may have important implications in understanding its role in early development as well as tumorigenesis.
References


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https://doi.org/10.1016/j.febslet.2007.08.070


## Appendix

### Oligomers used in this study

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### Primers used in this study

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Plasmids used in this study

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<td>For sgRNA cloning and synthesis</td>
<td>Kanamycin resistant</td>
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<td><a href="https://www.addgene.org/42250/">https://www.addgene.org/42250/</a></td>
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<tr>
<td>pCS2+</td>
<td>For CDS and insitu probe construct cloning</td>
<td>Ampicillin resistant</td>
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<tr>
<td>Tyrosinase a/b sgRNA in DR274</td>
<td>As a positive control for sgRNA experiments</td>
<td>From Tyler Square and Dan Medeiros</td>
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<tr>
<td>Xbra in situ probe in pXT vector</td>
<td>As a positive control for in situ hybridization experiments</td>
<td></td>
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<tr>
<td>Cas9 CDS in pCS2+</td>
<td>To generate Cas9 mRNA for injections</td>
<td><a href="https://www.addgene.org/47322/">https://www.addgene.org/47322/</a></td>
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