Analysis of gene and protein regulation in *Xenopus*

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**Abstract**

In all animals, including humans, the germ cells which make sperm and egg cells require a set of genes that make proteins called Piwi proteins and small RNA molecules called piRNAs (Piwi-interacting RNAs). The function of Piwi proteins and piRNAs is not well understood, however current data suggest that PIWI proteins and piRNAs are responsible for silencing, or suppressing, junk genes called transposons that would otherwise cause damage to the genome of germ cells. The presence of the PIWI analog in *Xenopus*, Xiwi, was quantified at progressing stages of Xenopus oocytes. Additionally, the expressions of Tyrosinase, Pax-6, Rem2, and Gapdh were qualified and quantified in the *Xenopus tropicalis* genome. Xiwi was present in late stages of *X. tropicalis* embryos, as well as in Stage V and Stage VI *X. laevis* oocytes. Reverse transcription PCR was optimized using primers designed to target Tyrosinase, Pax-6, Rem2, and Gapdh, ultimately to determine the levels of their expression in developing *X. tropicalis* tadpoles. Future experiments will further optimize the RT-PCR reactions by varying conditions, and will assess the presence of Xiwi in early stage embryos more extensively.
Introduction

There are several notable aspects of gene regulation and control in *Xenopus*, African clawed frog. *X. tropicalis* and *X. laevis* prove to be model organisms due to their abundant eggs, easily-inducible fertilization, and large cellular cytoplasm, all allowing for a simple means of extracting and isolating cytoplasmic proteins. In this study, three specific genes and two proteins were analyzed in order to better understand gene expression and control in *Xenopus*.

The protein of interest, Xiwi, is a 100 kDa Piwi-analog of Xenopus. Three *Xenopus* members of the Piwi family were identified in the *Xenopus* genome, though Xiwi most closely relates to *Drosophila* Piwi (Lau et al. 2009). The *piwi* class of genes was originally identified as encoding regulatory proteins responsible for maintaining incomplete differentiation in stem cells and maintaining the stability of cell division rates in germ line cells (Cox, et al 2005). Piwi proteins are highly conserved across evolutionary lineages and are present in both plants and animals. In all vertebrates and some invertebrates, Piwi proteins are expressed in germ line cells and associate with a novel class of small RNAs, piwi-interacting RNAs (piRNAs). *Drosophila* with mutated Piwi genes lack piRNAs and are sterile, thus the expression and control of Piwi protein is essential for proper germ-line development (Lau, et al 2009).

Tyrosinase, a 558 bp gene, is one of the key enzymes essential for melanogenesis in vertebrates. It catalyzes melanin production in pigmented cells and play vital roles in determining animal coloration (Furguson, et. al 1997). The Tyrosinase enzyme converts the amino acid tyrosine to DOPAquinone in the initial step of the melanin-producing pathway, and further catalyzes the oxidation of DOPA to DOPAquinone (Kumasaka, et al. 2003). Several experiments have already showed that wild-type expression of Tyrosinase is contingent on the interaction of numerous elements including basal promoter elements that determine the site of transcription initiation for RNA polymerase, and DNA-binding factors that mediate melanocyte-specific expression of the *tyr* gene (Furguson et. al. 1997). Additionally, there is a correlation between increased Tyrosinase activity with increased levels of enzyme synthesis, thus the activity of Tyrosinase is primarily dependent on gene regulation (Kidson 2005).

The gene *pax 6* is a major player in proper eye development in *Xenopus*, as well as in all mammals and insects. Loss of Pax 6 function leads to an eyeless phenotype in mammals, and a defective or cyclopic phenotype in *Xenopus*. The 556-bp *pax 6* gene is believed to be a universal master control gene for eye morphogenesis (Onuma et al 2002). A loss-of-function mutation in *pax 6* renders an easily-noticeable phenotype, thus making *pax 6* an ideal gene for knockout experiments.

*Rem2* is a poorly understood, 278-bp gene found in animal organisms that is linked to stem cell function. A recent study in human embryonic stem cells report that the Rem2 GTPase, a suppressor of the *p53* pathway, is a major player in the maintenance of stem cell self-renewal and pluripotency. It was determined that Rem2 effects are mediated by suppressing the transcriptional activity of *p53* by preventing protein degradation during DNA damage (Edel et al. 2010). Primers for the *rem2* gene were used to qualify the gene's presence in the *Xenopus* genome.

Glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) is the enzyme that catalyses the sixth step in glycolysis, breaking down glucose for adenosine triphosphate and carbon molecules. It is a commonly-used “control” gene in gene expression experiments due to its stability, constitutive expression, and general abundance in many tissues. It is often referred to as a “housekeeping gene” for these purposes (Barber, et al 2005). The 493-bp *gapdh* gene was
employed to compare expression of the aforementioned genes in *X. tropicalis*, serving as a control.

In the following report I will elaborate on the experiments conducted to better understand gene regulation and control in *X. tropicalis* and *X. laevis*, focusing on the expression of Xiwi protein, and the genes tyr, pax 6, rem2, and gapdh.

**Results**

**Quantification of XIWI in progressing stages of *X. laevis* oocytes and *X. tropicalis* embryos**

Protein extracts from *X. laevis* oocytes and *X. tropicalis* embryos in different stages of development were separated by size using SDS-PAGE and, using Western blot analysis with anti-Xiwi antibody, the amounts of Xiwi protein in each stage was quantified. Xiwi, at 100 kDa, was found in Stage 5 and Stage 6 *X.laevis* oocytes. Additionally, Xiwi was found in *X. laevis* cytoplasmic extract (Figure 1). In *X. tropicalis*, Xiwi was found in oocytes, Stage 11 embryos, and Stage 17 embryos (Figure 2).

![Figure 1](image1.png)

*Figure 1* Xiwi expression during *Xenopus laevis* oogenesis, stages I-VI shown. Major bands at 100 kDa indicate presence of Xiwi protein in protein extracted from oocytes. Notably, these results suggest the presence of Xiwi at Stages V, VI, and in oocytes cytoplasm extract. The cytoplasm was dissected apart from the germinal vesicle.

![Figure 2](image2.png)

*Figure 2* Xiwi expression during *Xenopus tropicalis* development. Bands at 100 kDa indicate presence of Xiwi protein in protein extracted from embryos. Thus, Xiwi is present in the *X. tropicalis* oocytes, and Stage 11 and 17 embryos.
Optimization of RT-PCR using Tyrosinase, Pax 6, and Gapdh in X. tropicalis tadpole RNA

PCR conditions were tested in order to optimize the annealing of the selected primer to the template cDNA, originally deriving from X. tropicalis tadpole RNA. Parameters that were adjusted include primer annealing temperature, number of PCR cycles, and quantity of magnesium, a cofactor of Taq DNA polymerase. Figure 3 shows a reaction run with tyr and pax 6 primers for 30 cycles of PCR with an annealing temperature of 50°C (See Materials and Methods for reaction details). Figure 4 shows a reaction run with tyr, pax 6, and gapdh primers run for 30 cycles with an annealing temperature of 55°C. Figure 5 shows two reactions run with rem2 primers run for 25 cycles at annealing temperatures of 55°C and 60°C. In Figure 3, RNA samples were not originally treated with DNase, while in Figure 4, all RNA samples were treated with DNase (see Materials and Methods).
**Figure 3** 1.6% Agarose gel stained with ethidium bromide shows bands corresponding to *tyr* PCR product and *pax 6* PCR product. The gel was run for 35 minutes at a constant 100 volts. The inclusion or exclusion of reverse transcriptase (RT) in the RT-PCR reaction is shown above each lane. Annealing temperature for all reactions was 50°C. RNA samples were not originally treated with DNase.

**Figure 4** 1.6% Agarose gel stained with ethidium bromide shows bands corresponding to *tyr* PCR product, *pax 6* PCR product, and Gapdh PCR product. The gel was run for 40 minutes at a constant 100 volts. The inclusion or exclusion of reverse transcriptase (RT) in the RT-PCR reaction is shown above each lane. Annealing temperature for all reactions was 55°C. RNA samples were treated with DNase.
Figure 5 1.6% Agarose gel stained with ethidium bromide shows bands corresponding to rem2 PCR products at two annealing temperatures: 55°C and 60°C. Each gel was run for 35 minutes at a constant 100 volts. The inclusion or exclusion of reverse transcriptase (RT) in the RT-PCR reaction is shown above each lane. RNA samples were treated with DNase.

Quantitative RT-PCR with Gapdh in X. tropicalis RNA

To quantitatively measure primer binding and formation of the desired PCR product, gapdh, variable amounts of cDNA were used in the PCR reaction. The ratios listed indicate the relative amounts of total X. tropicalis RNA to Saccharomyces cerevisiae transfer RNA (trNA), each sample loaded contained a total amount of 1 μg RNA. Reactions were run at 25, 27, and 30 cycles to determine the saturation point of the specific PCR reaction (Figure 6).

Figure 6 1.5% Agarose gel stained with ethidium bromide shows bands corresponding to the 493 bp gapdh PCR product at decreasing amounts of X. tropicalis cDNA. The ratios listed indicate the relative amounts of total X. tropicalis RNA to Saccharomyces cerevisiae transfer RNA (trNA), each sample loaded contained a total amount of 1 μg RNA. The number of PCR cycles is indicated above each group
of lanes. The gel was run for 40 minutes at a constant 100 volts. The inclusion or exclusion of reverse transcriptase (RT) in the RT-PCR reaction is shown above each lane. Annealing temperature for all reactions was 55°C.

Discussion

There is much to be studied in *Xenopus* gene expression and control. Recent studies investigated the properties of Piwi protein and concluded that it plays a major role in the silencing of transposable elements in the genome, ultimately safeguarding the germ cell from "junk DNA" (Vagin, et al 2006). The Xiwi protein, the *Xenopus* analog of Piwi, may act similarly (Lau, et al 2007). To quantitatively determine the abundance of Xiwi protein in progressing stages of *X. laevis* oocytes and *X. tropicalis* embryos, protein extracts were sorted by size using SDS-PAGE and were treated with anti-Xiwi antibody. Xiwi, at 100 kDa, was found as hypothesized in *X. tropicalis* oocytes, Stage XI embryos, and Stage XVII embryos (Figure 2). In *X. laevis* oocytes, Xiwi was only found in Stage V and VI oocytes, contradicting previous studies (Lau, et al 2007). In *Xenopus*, it was found that expression of Xiwi is greatest in early stages of oocytes development (Lau et al 2007). A likely source of error is poor transferring of the SDS gel to the PVDF membrane, thus eliminating the presence of Xiwi bands in early stage oocytes. Future attempts should modify the transferring procedure and possibly increase the concentration of secondary antibody used to higher than 1:2000.

To optimize the reaction to obtain PCR products from *tyr*, *pax 6*, *rem2*, and *gapdh* in *Xenopus tropicalis* tadpole RNA, annealing temperatures and cycle repetitions were manipulated. At an annealing temperature of 50°C, multiple bands were found for both the *tyr* lane and *pax 6* lane (Figure 3). This is most likely due to non-specific binding of the primers to template cDNA. Given the absence of bands in the negative control (no reverse transcriptase), it can be deduced that there was no genomic DNA contamination in the PCR reaction tubes. When the annealing temperature was increased to 55°C, major improvements were shown for tyrosinase and gapdh (Figure 4). A clear, single band at 493 bp is visible in the gapdh lane, indicating relatively high abundance of the glyeraldehyde-3-phosphate dehydrogenase gene in *X. tropicalis* tadpoles. Likewise, a single band was found in the *tyr* lane. In the case of *rem2*, a 278 bp PCR product was identified after 25 cycles with annealing temperatures of 55°C and 60°C. However, a band of equal brightness can be seen above it, representing a genetic product of approximately 300 bp. This band can possibly represent an isoform of the Rem2 product with an extended C- or N- terminus, otherwise non-specific primer binding may have led to two PCR products. Further optimization should be executed in future experiments. Similarly, future experiments should optimize the *tyr* reaction further to obtain a brighter band, perhaps by increasing the concentration of RNA used in the reverse transcriptase reaction. Two bands are seen in the *pax 6* lane, indicating non-specific primer binding or DNA contamination (despite DNase treatment). Future experiments will further optimize the *pax 6* PCR reaction by altering number of cycles and/or annealing temperature.

For the quantitative RT-PCR assay, decreasing amounts of total *X. tropicalis* cDNA were combined with increasing amounts of cDNA from yeast transfer RNA. *Gapdh* primers were used
to quantify the amount of PCR product obtained from each cDNA sample. The number of PCR cycles was also varied in order to determine the point of saturation. No significant gapdh product was found when the PCR reaction was run for 25 or 27 cycles. The minimum number of cycles to produce a significant gapdh product is between 28 and 30 (Figure 6). As hypothesized, the band brightness decreases as the quantity of X. tropicalis cDNA decreases.

The aforementioned experiments served as an introduction to a bigger assessment of gene expression and control in X. tropicalis. In future experiments, we aim to utilize small piRNAs in the form of a small hairpin (shRNA) to silence, or turn off, genes in the Xenopus genome. A target gene we plan to silence is tyr, a gene that encodes one of the key enzymes essential for melanogenesis in vertebrates. Ultimately, by turning off the Tyrosinase enzyme using specifically designed piRNAs, we hope to create a transgenic, albino animal without pigmentation, an easy phenotype to identify. With the successful silencing of the tyr gene, many doors will open in the field of piRNA and Piwi analysis. In the long term, piRNAs may be developed to silence genes dangerous to the health and development of other organisms, perhaps even humans. Thus, future research in piRNA and Piwi protein has significant implications in developmental biology and human medicine.

Materials and Methods

Collection of Xenopus embryos
Natural matings were induced by injecting males and females with 10 units (100 μL) and 20 units (200 μL) respectively of Sigma human chorionic gonadotropin and allowed to mate overnight. Embryos were de-jellied in 2% cysteine solution in 0.1x modified Barth’s saline (MBS), pH 8.0, and sorted for quality. The embryos further developed in 0.1x MBS at 22°C.

Whole embryo protein extraction
Ten embryos were collected and frozen at -80°C. Embryos were lysed in 100 μL lysis buffer (100 mM NaCl, 50 mM Tris pH 7.5, 5 mM EDTA, 1% (v/v) NP40, 1% (w/v) Na Deoxycholate) and vortexed at high speed, 4°C for 15 minutes. The upper, protein-containing phase was transferred to a fresh tube. 1x protein-loading buffer and 12 μL β-mercaptoethanol were added before loading on protein gel.

SDS-PAGE western blot analysis
A SDS-PAGE discontinuous buffer system was used, consisting of a resolving gel and a stacking layer. Protein samples were denatured at 95°C for 5 minutes, and 20 μL of sample was loaded in each well. The system was run at a constant 100 volts for 75 minutes. The gel was transferred to a PVDF membrane using 1X CAPS buffer at a constant 500 mA for two hours. The membranes were blocked overnight at 4°C in 5% (w/v) nonfat milk and 1X PBS solution, and were later incubated overnight at the same temperature in a 1:1000 dilution of anti-Xiwi antibody; anti-Xiwi antibodies were generated by Lau et. al as described in Embo Journal 2009. After rinsing repeatedly with 1x PBS, the membranes were incubated in a 1:2000 dilution of blocking buffer.
and secondary anti-rabbit α-Xiwi, fifth bleed, for one hour at room temperature. After washing, electrochemiluminescence reagents were used according to protocol to visualize the blots.

**Embryo RNA extraction**

Ten *Xenopus* embryos or tadpoles were homogenized with 1 mL of Trizol reagent and stored for 5 minutes at room temperature. 0.1 mL 1-bromo-2-chloro-propane was mixed vigorously with the homogenate, stored for 15 minutes at room temperature, and the lower phase was discarded after centrifugation at 12,000 g for 15 minutes at 4°C. 0.2 mL of SEVAGS (24:1 chloroform: isoamyl alcohol) was mixed with the aqueous phase and centrifuged at 12,000 g for 15 minutes at 4°C. The aqueous phase was transferred into a new tube and 0.5 mL isopropanol was added; RNA was allowed to precipitate overnight at -20 °C. A pellet was formed after centrifugation at 12,000 g for 8 minutes at 23°C and was washed with 1 ml 75% ethanol. The pellet reformed after centrifugation at 7,500 g for 5 minutes at 23°C and was dissolved by pipetting in 50 µL of water after air-drying. The RNA sample was tested for quality (260/280 absorbance peak and concentration) using NanoDrop. Selected samples were further treated with TurboDNase according to TURBO DNA-free protocol.

**Reverse transcription and polymerase chain reaction**

cDNA was synthesized from RNA using SuperScript Reverse Transcriptase III. To 0.5-1 µg of total RNA, 0.5 µL of 100 mM random primers, 5 µL of 2 mM dNTP mix, and 2.5 µL of water were added. The reaction was heated at 65 °C for 5 minutes and incubated at 4 °C for 60 seconds. To the reaction, 4 µL of 5X first strand buffer, 1 µL 0.1M DTT, and 1 µl RNAse-OUT were added. 1 µL of Superscript RT III was then added, but omitted from "no-RT" control samples. The reaction tubes were heated to 50°C for 50 minutes, then 70 °C for 15 minutes. 1 µL of RNAse H was added and samples were incubated at 37°C for 20 minutes.

To a new tube, 10 µL of 10x PCR buffer + Mg, 10 µL 2 mM dNTP mix, 0.5 µL 100 mM forward primer, 0.5 µL 100 mM reverse primer (see below), 2 µL Taq polymerase, 2 µL cDNA (with or without RT), and variable amounts of MgCl₂ were added and the reaction was brought to 100 µL with water. The samples were denatured for 2 minutes at 94 °C, and then underwent 25-30 cycles of the following reaction sequence: 94°C for 20 seconds, 50-60 °C for 20 seconds, and 72 °C for 30 seconds. There was a final extension step for 2 minutes at 72 °C.

<table>
<thead>
<tr>
<th>Gene</th>
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<th>PCR Primer Sequence</th>
<th>Primer Length (nt)</th>
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<tr>
<td></td>
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<tr>
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<td>AGATCCACACACGGTGCTG</td>
<td>21</td>
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Visualizing PCR products on agarose gels
Visualization of PCR products was employed using 1.6% agarose gels in 1x TAE buffer with 5% ethidium bromide. Gels were run at 100 constant volts for 35 minutes. 10 μL of PCR sample was mixed with 4 μl 6x Agarose loading dye, and 1 kb ladder was used as a control.

References


