Role of Williams Syndrome Transcription Factor in heart development in *Xenopus*

Abstract:
Williams Syndrome Transcription factor (WSTF) was first identified in the deletion of several genes on chromosome 7. This heterozygous deletion results in Williams syndrome (WS), a developmental disorder. Patients with WS display a number of systemic defects, such as heart defects, a characteristic facial appearance, growth deficiency, and cognitive disability. WSTF is a subunit of several different ATP-dependent remodeling complexes and these remodeling complexes use ATP hydrolysis energy to rearrange chromatin along DNA to initiate gene transcription or repression, especially during development. Knockouts of WSTF in mice exhibit a wide spectrum of cardiac defects similar to those seen in WS patients. However, more studies on the correlation between the function of WSTF and early stages of heart development still need to be done. I therefore propose to use *Xenopus laevis* embryos to see if WSTF is involved in early stages of heart development. I will knock down the expression of WSTF in *Xenopus* embryos via microinjection of an antisense WSTF morpholino (a stable oligonucleotide analog). I will then examine the expression of cardiac marker genes such as Nkx2-5 and Cardiac $\alpha$-actin in intact embryos by in situ hybridization. To further characterize the details of any morphological changes in heart development in the WSTF knockdown embryos, I will also perform histological sectioning. The studies of WSTF in this proposal will provide us an opportunity to evaluate the early impacts on heart development in a critical gene missing in patients with Williams Syndrome.

Specific Aims:
Aim#1: To determine if WSTF plays a major role in early stages of heart development in *Xenopus laevis*
WSTF deletions in mice result in heart defects, and WSTF knockdowns in *Xenopus laevis* have been shown in our laboratory to recapitulate a number of the neural defects present in WS patients. We will test the hypothesis that WSTF also plays a role in heart development in *Xenopus*. To identify if WSTF plays a major role in early stages of heart development, an antisense WSTF morpholino will be injected into one cell of two-cell stage embryos to knock down WSTF expression in one half of the embryo only. As a negative control for injection, a negative control morpholino will be used. Unilateral injection is useful for studies of early heart development because the uninjected side of embryo will be observed as a stage-match control for comparison. Several early stages of embryos will be collected in WSTF knockdowns and also in controls. To look at heart development at the molecular level, expression of cardiac marker genes, such as Nkx2-5 and Cardiac $\alpha$-actin will be detected in knockdown WSTF whole mount embryos by in situ hybridization, comparing the expression of these markers to the uninjected side of embryos and also in whole mount control embryos. Nkx2-5 is expressed throughout the developing heart and pharyngeal endoderm. Nkx2-5 is one of the earliest genes expressed in heart precursor cells (Warkman and Krieg, 2007), and its expression will allow me to follow early stages of heart development in *Xenopus laevis*. Cardiac $\alpha$-actin, a cardiac muscle marker, is normally expressed in WSTF knockout mice (Yoshimura et al., 2009). I expect to see Cardiac $\alpha$-actin normally expressed in knockdown WSTF embryos in *Xenopus laevis* as well. To further analyze the details of morphological events involved in heart development in the knockdown WSTF embryos, I will also perform histological sectioning.
Introduction:
ATP-dependent remodeling complexes use ATP hydrolysis energy to rearrange chromatin along DNA so cellular processes, such as gene transcription or repression, DNA repair, or DNA replication, can occur. This is especially critical during development. Williams syndrome transcription factor (WSTF) is a subunit of at least three different ATP-dependent remodeling complexes: WINAC, WICH, and B-WICH. These three complexes have different functions but their roles are still poorly understood, particularly during embryogenesis (reviewed in Barnett et al., 2011). So far, only the WICH complex has been biochemically characterized in *Xenopus laevis*. Current studies in Dr. Krebs’ lab are also addressing whether WINAC and B-WICH complexes are present in *Xenopus laevis*.

WSTF is 1 of ~26 genes deleted on chromosome 7 in the genome of patients with Williams syndrome. Patients with WS display an array of issues including friendly and uninhibited personalities, elfin-like facial features, intellectual disabilities (most common of which are visuospatial construction defects and mental retardation), and cardiovascular problems including hypertension and supravalvular aortic stenosis (SVAS) (Bhattacharjee, 2005).

Work in Dr. Krebs’ lab has characterized the phenotypic defects in *WSTF* knockdown embryos in *Xenopus laevis*. These knockdown embryos display phenotypic defects reminiscent of those shown in Williams Syndrome patients. This work has shown that WSTF may facilitate the proper migration or survival of neural crest cells (Barnett et al., submitted). Neural crest cells are a class of migratory pluripotent stem cells that give rise to many diverse tissues, including parts of the heart structure, such as musculo-connective tissue of the large arteries, and part of the septum dividing the pulmonary circulation from the aorta (Gilbert, 2003). Work by Yoshimura et al. (2009) shows that WSTF knockouts in mice have an array of severe heart defects at the embryonic stage including atrial septal defects and ventricular septal defects. These abnormalities are also similar to cardiovascular complications found in WS patients (Pasqua et al. 2009). In fact, the WSTF knockout mice have phenotypes far more similar to WS patients than mice lacking a copy of the elastin gene, another gene deleted in WS patients that has also been suggested to lead to heart defects (Goergen et al., 2011). However, more studies on the correlation between the function of WSTF and early stages of heart development still need to be done.

WSTF has showed to play an important role in the regulation of several cardiac genes *in vivo* in WSTF knockout mice (Yoshimura et al., 2009). However, what kind of role WSTF plays in early stages of heart development still needs to be investigated. The research I propose here will give us more details on how WSTF is involved in early heart development, and will help us to understand whether the heart defects in WS patients may be due to a neural crest defect due to WSTF deletion.
Experimental design and methods:
Aim#1: To determine if WSTF plays a major role in heart development in *Xenopus laevis*

**Generation of fertilized *Xenopus* embryos**
Female frogs will be induced to ovulate by injecting 600 µl of human chorionic gonadotropin (HCG) into their dorsal lymph sac. Testes are obtained surgically from male frogs and testes are used to fertilize eggs *in vitro*. Fertilized embryos are then stripped of their jelly coat by exposure to 2% L-cysteine for 3-5 mins. 2-cell stage of fertilized embryos will be used for microinjection. All handling of adult frogs will be performed by Krebs lab personnel approved on the laboratory IACUC. My work will begin when the fertilized embryos are provided.

**Microinjection of morpholino oligonucleotides**
Customized single-stranded oligonucleotides (morpholinos) were generated by Gene Tools, Inc. These morpholinos are complementary to the 5’ untranslated region of *WSTF* mRNA to prevent *WSTF* mRNA from being translated. Thus, injection of the morpholino prevents the production of WSTF protein. As a negative control for injection, I will also use an inverse WSTF sequence morpholino. The WSTF MO has a 3’ carboxyfluorescin label, which will allow me to track which side of the embryos are injected by observing them under a fluorescence microscope. Embryos will be sorted into left- and right-side injected batches to keep track.

Several early stages of embryos will be collected in *WSTF* knockdowns and also in controls, primarily during neurulation, when primordial heart tissue can first be easily detected, and through early and late tailbud stages, when the heart is undergoing rapid development. These embryos will be stained and sectioned as described below.

**In situ hybridization**
I will detect the expression of *Nkx2-5* and Cardiac α-actin in knockdown *WSTF* whole mount (intact) embryos by *in situ* hybridization, comparing the expression of these markers to the uninjected side of embryos and also in whole mount control embryos. Briefly, *in situ* hybridization involves synthesizing an RNA probe *in vitro* that is complementary (i.e. will bind directly to) the mRNA I wish to detect. This *in vitro* transcribed RNA includes a modified ribonucleotide, digoxigenin-UTP, which I will be able to detect with an antibody. The antibody in turn is attached to an enzyme, which reacts with a colorless substrate to create a colored (purple) stain. The net result is that anywhere in the embryo that my gene of interest is expressed, I will develop a purple stain that reveals the location in which the gene is expressed, and how strongly it is expressed.

Cardiac α-actin has been provided by Dr. Tim Hinterburger (UAA). The construct containing *Nkx2-5* DNA sequence will be purchased. I will linearize the DNA templates with restriction enzymes, I will transcribe the digoxigenin-labeled sense (control) and antisense RNA probes *in vitro* using Ambion Megascript *in vitro* transcription kits (Megascript T7™, Megascript Sp6™, Ambion). Embryos are collected at desired stages and fixed in MEMFA, a formaldehyde-based buffer.
Histological sectioning
To look more closely at the structure of the heart in WSTF knockdown embryos after staining by in situ hybridization, I will also perform histological sectioning. Briefly, this entails rehydrating the fixed, stained embryos, embedding them in gelatin blocks (15% gelatin; Bloom-300, Sigma), and slicing through the embedded embryos using a vibratome (Leica VT1000P), a device that uses a razor blade to cut extremely thin sections. I will collect multiple sections through each embryo and examine them under the microscope to identify sections that reveal the fine structure of the heart.

Anticipated Results:
The development of heart starts out with two bilateral patches of specified mesoderm on the dorsal side of the embryo in the beginning of gastrulation. During gastrulation, these two patches move dorso-anteriorly. Then, during neurulation, the heart progenitors migrate ventrally until the heart patches meet in the anterior region of the embryos at the ventral midline. At this point the heart patches fuse to form a linear tube, then subsequently undergoing looping and remodeling to form the functional heart, with the mature heart formed at tadpole stage (stage 46; numerical stages are based on standardized staging tables for Xenopus) (Warkman and Krieg 2007). As described above, I will collect embryos at selected points in these stages from neurulation (14-22), and later tailbud stages when heart tube formation and looping occur (stages 31-36).

The lab is particularly interested in the activity of WSTF during neurulation, which is when prior work has shown defects in neural development and neural crest in WSTF knockdowns, so I will focus my initial attention at this stage. If WSTF is involved in the ventral movement of the two heart patches during neurulation, I will expect to see Nkx2-5 expression pattern disrupted in the WSTF morpholino-injected side compared to uninjected side by in situ hybridization. This disruption could take the form of reduction or elimination of Nkx2-5 expression, which could reflect a role of WSTF in expression of this critical cardiac gene, or it could result in misplaced expression, consistent with a role for WSTF in facilitating the proper migration of this tissue.

However, it is also possible that WSTF will not affect heart development at these earlier stages, but instead will affect the later stages when the tissue undergoes changes that lead first to looping, and later to formation of separate heart chambers and valves. To evaluate these types of changes at later stages (throughout the tailbud stages), I will perform histological sections, in both Nkx2-5 and cardiac actin-stained embryos. Both of these genes are normally expressed at these later stages. Sectioning will allow me to both determine whether these genes are expressed normally at these later stages, as well as to determine whether there are any structural defects in the developing heart. Unilateral embryos injected with the negative control morpholino are expected to show no differences in gene expression or structure between the injected side and uninjected side. However, defects in expression or structure in the WSTF knockdowns should show clear differences between the two sides. All structures will be compared to Mohun et al. (2000) for reference.

It seems most likely that WSTF will have effects on the heart during neurulation or later, given that major errors during gastrulation would be likely to result in a devastating loss of heart tissue. In addition to the defective (but present) heart in the mouse WSTF knockout, other work in our
laboratory has noted what appears to be heart structures persisting into tailbud stages (though that work did not use heart-specific markers). This would also be more consistent with WS, as these patients have a suite of specific heart defects but the bulk of the heart structure is intact. However, if we find a more dramatic, early defect in *Xenopus*, we can select additional heart markers (such as *tbx5* or other members of the *Nkx* family), to follow specific events in early heart stage development.

**Future impacts:**
In Dr. Krebs’ lab, it has been shown that WSTF-deficient embryos have eye and brain defects and WSTF is involved in neural crest cell migration and/or maintenance (Barnett et al., submitted). Previous studies have determined that haploinsufficiency of WSTF contributes the heart development defects in mice similar to that seen in WS patients. Using inexpensive *Xenopus laevis* embryos, which are easy to manipulate, I can further determine mechanisms by which WSTF is involved in early stages of heart development. This study will help us understand more about Williams Syndrome and how loss of WSTF may contribute to the clinical features of WS.

**Project Budget:**

<table>
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<tr>
<th>Item</th>
<th>Cost</th>
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<tr>
<td>Morpholinos</td>
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<td>Plasmid construct of <em>Nkx2-5</em></td>
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<td>Plasmid MidiPrep kit</td>
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<td><em>In vitro</em> transcription kits (Megascript T7™, Megascript Sp6™, Ambion)</td>
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<td>Reagents for microinjection, in situ, and sectioning</td>
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<td><em>Xenopus laevis</em> females, 10 males (20$/frog)</td>
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<td>Micropipette sets and disposables-tips, plates, etc</td>
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<td><strong>Total</strong></td>
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**Budget Justification:**
The reagents listed above reflect the majority of the necessary materials to perform this research. Additional reagents will be made available by the Krebs lab as needed.
References:


Project Timeline:

January-mid February 2012: Do microinjection of WSTF morpholino along with control in Xenopus laevis embryos and collect at desired stages; store embryos in 100% methanol in -20°C as collected.
Mid February-March 2012: Do in situ hybridization using cardiac molecular markers, Cardiac α-actin and Nkx2-5, in selected stages of whole mount embryos.
April 2012: Do sectioning of these whole mount in situ embryos.
Mid-April 2012 – Presentation at the Undergraduate Research and Discovery Symposium
May 15, 2012 – Expenditure deadline
May 30, 2012 – Final written report deadline