The chilling tail of temperature's influence on thyroid hormone signalling in the post-embryonic developmental response of *Rana catesbeiana* cultured tail fin

By

Emily Koide

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in the Department of Biochemistry and Microbiology

University of Victoria

We acknowledge and respect the lək^{w}əŋən peoples on whose traditional territory the university stands, and the Songhees, Esquimalt and WSÁNEĆ peoples whose historical relationships with the land continue to this day.

Supervisory Committee

The chilling tail of temperature's influence on thyroid hormone signalling in the post-embryonic developmental response of *Rana catesbeiana* cultured tail fin

by

Emily Koide

Supervisory Committee

Dr. Caren C. Helbing
Supervisor, *Department of Biochemistry and Microbiology*

Dr. Chris Nelson
Departmental Member, *Department of Biochemistry and Microbiology*

Dr. Leigh Anne Swayne
Outside Member, *Division of Medical Sciences*

Abstract

Supervisory Committee
Dr. Caren C. Helbing (Department of Biochemistry and Microbiology)
Supervisor
Dr. Chris Nelson (Department of Biochemistry and Microbiology)
Departmental Member
Dr. Leigh Anne Swayne (Division of Medical Sciences)
Outside Member

Thyroid hormone (TH) is a critical signalling molecule for all vertebrate organisms, playing an especially crucial role in postembryonic development. Given its importance, many studies have focused on further elucidating the initial TH signal response and its method of transduction. Although the primary mechanism of TH response is genomic signalling, alternative mechanisms of early TH signal transduction have been relatively poorly studied. The North American bullfrog, *Rana catesbeiana*, is a useful model to study these early responses as tadpole post-embryonic development, or metamorphosis, can be experimentally induced through exposure to TH. The experimental induction of the TH signalling program leads to similar morphological endpoints as seen in natural metamorphosis in the transition of a tadpole to a juvenile froglet, such as regression of the tail. This TH-induced developmental program can also be manipulated through temperature where, as temperatures lower, developmental rate is delayed and at 5°C metamorphosis is completely stalled. Interestingly, when tadpoles exposed to TH at 5°C are introduced to permissive temperatures (24°C), an accelerated developmental program ensues, even when no more endogenous TH signal remains. Previous research has shown that this phenomenon can also be seen on the molecular level where only a select few transcripts have been shown to be responsive to TH at 5°C. However, the characteristic, if not augmented, TH response program is seen on the transcriptomic level when tadpoles are shifted

to 24°C. This indicates that there is a molecular memory where the TH signal is induced in cold temperatures but not executed until more permissive temperatures arise. The extent and regulation of the transcriptomic program involved in this TH-induced molecular memory has yet to be understood. Herein we use the broader probing technique of RNA-seq analysis to identify potential components of the molecular memory. Eighty-one gene transcripts were TH-responsive at 5°C in cultured *R. catesbeiana* tail fin indicating that the molecular memory is more complex than previously thought. A number of these transcripts encoded regulators of transcription. Closer examination of select transcripts including a novel krüppel-like factor family member, *klfX*, at 5°C indicated that not all of the candidate molecular memory transcripts are regulated through active transcription and active translation is not required. When moved into 24°C an accelerated transcriptomic response occurred even when no additional TH is added, suggesting that a priming event occurs by TH exposure at 5°C allowing an accelerated metamorphosis at permissive temperatures. The molecular memory may be used as a means to isolate the initiating TH signalling response and the regulation of this program to allow further elucidation of early TH signalling in post-embryonic development.

Table of Contents

List of Tables

List of Figures

Acknowledgements

Firstly to Caren, I can't thank you enough for all your encouragement, guidance and froggy wisdom throughout my graduate degree. I will always appreciate all that I have learned and how much grown in my time in your lab.

To my supervisory committee, thank you for your valued input, new ideas, continued support and patience with me in my journey.

To my fellow lab members that I will treasure in my heart forever:
Anita, thank you for inspiring me with your genius but also providing Dan approved puns.
Caitlin, thanks for being my first student and guinea pig on my training tactics.
Ethan, the absolute legend who stuck with me for what should have been a month of primer validation but turned into a full year of many, MANY trials, I will be forever in your debt and am grateful for your go getter attitude and encyclopedia brain.
Jacob, you bioinformatics king! Thank you for constantly helping me and answering my silly questions about anything that required touching scripts, also just for your all-around kindness and willingness to help even if it was just to make a baby Groot.
Jess, thank you for being my human google (and by that I mean googling things so I don't have to!), my only friend in the lab for a long time and just generally leading me through life.
Kate, thanks for the moral support and constant encouragement, but not as much thanks for the stairs you made me run.
Kevin and Shireen, thank you for all your training when I had no idea what I was doing and the weird cat sounds that I picked up and will probably come out of my mouth forever now. I am ready for you to come back to Canada now!
Lauren, thank you for being a wise old lady with many teachings while also making sure the lab was never too quiet.
Lorissa, thank you for always being super helpful and providing excellent support and troubleshooting help, even if it came with a pest tax.
Meg, thanks for being a bad egg.
Michael, thank you for being you, for the puns, the stories, and the terrible jokes. You provided love, support and much needed comedic relief.
Sara, even though you abandoned us in the lab you haven't abandoned me in my heart.
Sandy, thank you for teaching us how to sing the most important song of my graduate degree.

To my family, thanks for feeding me.

List of Abbreviations

Abbreviated gene transcripts are italicized and capitalized following the nomenclature as set by http://www.xenbase.org/gene/static/geneNomenclature.jsp for Amphibia and http://www.informatics.jax.org/mgihome/nomen/gene.shtml for Mammalia as demonstrated below:

	Gene Transcript	Protein
Mammalia	*Thra*	Thra
Amphibia	*thra*	Thra

ActD	Actinomycin D
ATP	Adenosine triphosphate
C-fin	Cultured tail fin assay
cDNA	Complimentary DNA
cebp1	CCAAT/enhancer binding protein 1
CHX	Cyclohexamide
CRF	Corticotropin releasing factor
CT	Cycle threshold
Dio1	Type I iodothyronine deiodinase
Dio2	Type II iodothyronine deiodinase
Dio3	Type III iodothyronine deiodinase
DNA	Deoxyribonucleic acid

dNTP	Deoxynucleotide triphosphate
DO	Dissolved Oxygen
EDC	Endocrine disrupting chemical
eef1a	Eukaryotic translation elongation factor 1
ER	Estrogen receptors
FC	Fold change
GO	Gene ontology
HPT	Hypothalamus-pituitary-thyroid
Klf	Krüppel-like factor
klfX	Krüppel-like factor X
MAD	Maximum absolute deviation
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
mRNA	Messenger ribonucleic acid
NuRD	Nucleosome remodeling and deacetylase complex
P66a	Transcriptional repressor p66-alpha
PCA	Principle component analysis
PPAR	Peroxisome proliferator-activated receptor
qPCR	Quantitative polymerase reaction
RAR	Retinoic acid receptor
RIN	RNA Integrity Number
RNA	Ribonucleic acid

RNA-Seq	RNA-sequencing
rpl8	Ribosomal protein L8
rps10	Ribosomal protein S10
rT3	Reverse triiodothyronine
RXR	Retinoid X receptors
six1	Homeobox protein SIX1
six2	Homeobox protein SIX2
T_2	Diiodothyronine
T_3	Triiodothyronine
T_4	Thyroxine
tcp4	RNA polymerase II transcriptional coactivator p15
Tg	Thyroglobulin
TH	Thyroid hormone
thibz	Thyroid hormone-induced basic leucine zipper-containing protein
thra	Thyroid hormone receptor alpha
thrb	Thyroid hormone receptor beta
TR	Thyroid hormone receptor
TRE	Thyroid hormone response elements
TRH	Thyrotrope releasing hormone
TRα	Thyroid hormone receptor alpha
TRβ	Thyroid hormone receptor beta

TSH	Thyroid stimulating hormone
tshb	Thyroid stimulating hormone subunit beta
Tyr	Tyrosine
w/v	Weight/volume

1. Introduction

1.1 Thyroid Hormone

Thyroid hormones are crucial for all vertebrate organisms. Although these signalling molecules (Figure 1) are most infamous to the general population for their regulatory role in adult metabolism, one of their most important roles is in the vertebrate developmental process. The highly regulated release, signalling and metabolism of TH has shown to be most critical during postembryonic development. Also called the perinatal period in many *in utero* developing vertebrates, the postembryonic developmental stage is important for vertebrate growth and organ development. As it was discovered that deficiency of TH during the perinatal period in humans can lead to cretinism, characterized by severe impairment of neurological and physical development, studies on TH have demonstrated its key role in cell proliferation, migration and differentiation of neuronal cells (reviewed by Horn and Heuer, 2010; Pfaff and Joels, 2016), bone development and ossification (reviewed By Bassett and Williams, 2016; Kim and Mohan, 2013), and skeletal muscle development (Lee et al., 2014, Nwoye et al., 1982).

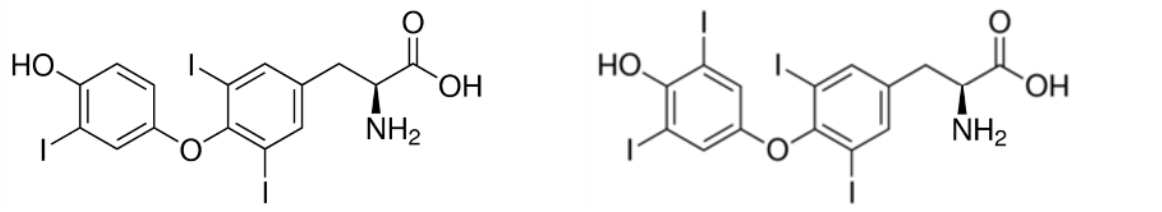


Figure 1: Example Thyroid Hormones.

A more visually dramatic demonstration of TH's role in postembryonic development can be seen in vertebrates that undergo a clear morphological metamorphosis between the larval and juvenile stages. In anurans, TH is an obligatory signal for the life stage transition from a tadpole to a froglet, initiating the necessary programs for apoptosis or reprograming of larval tissue (i.e. the tail and liver, respectively) and *de novo* generation of adult tissues (i.e., the limbs; Gilbert et al., 1996; Shi, 1999). Similarly, in other amphibians such as the axolotl, TH induces gill and tail fin resorption to prepare for the transition from aquatic to terrestrial environments (Brown, 1997; Crowner et al., 2019). Most teleost fish also undergo TH-induced metamorphosis in muscle, blood and skin tissues which is most notably displayed in flatfish metamorphosis transitioning from a symmetric to an asymmetric morphology (reviewed by Campinho, 2019).

1.2 Thyroid Hormone Synthesis, Transport, and Metabolism

Given the important signalling roles of TH it is unsurprising that availability of the hormone is strictly regulated on multiple levels from synthesis to metabolism. The synthesis of TH is under the neuroendocrine control of the hypothalamus-pituitary-thyroid (HPT) axis (Figure 2; Nussey and Whitehead, 2001; Yen, 2001). Signalling for TH production is initiated through environmental signals stimulating thyrotropes in the anterior pituitary to produce thyroid stimulating hormone (TSH). In mammals this stimulation is prompted by hypothalamic release of thyrotropin releasing hormone (TRH; Zoeller et al., 2007). However in amphibians, thyrotropes were discovered to be under the control of corticotropin releasing factor (CRF; Denver, 1993, Denver et al., 1998). Thyrotropes, stimulated by TRH or CRF, regulate the production of TH in the follicles of the thyroid (**Figure 2**).

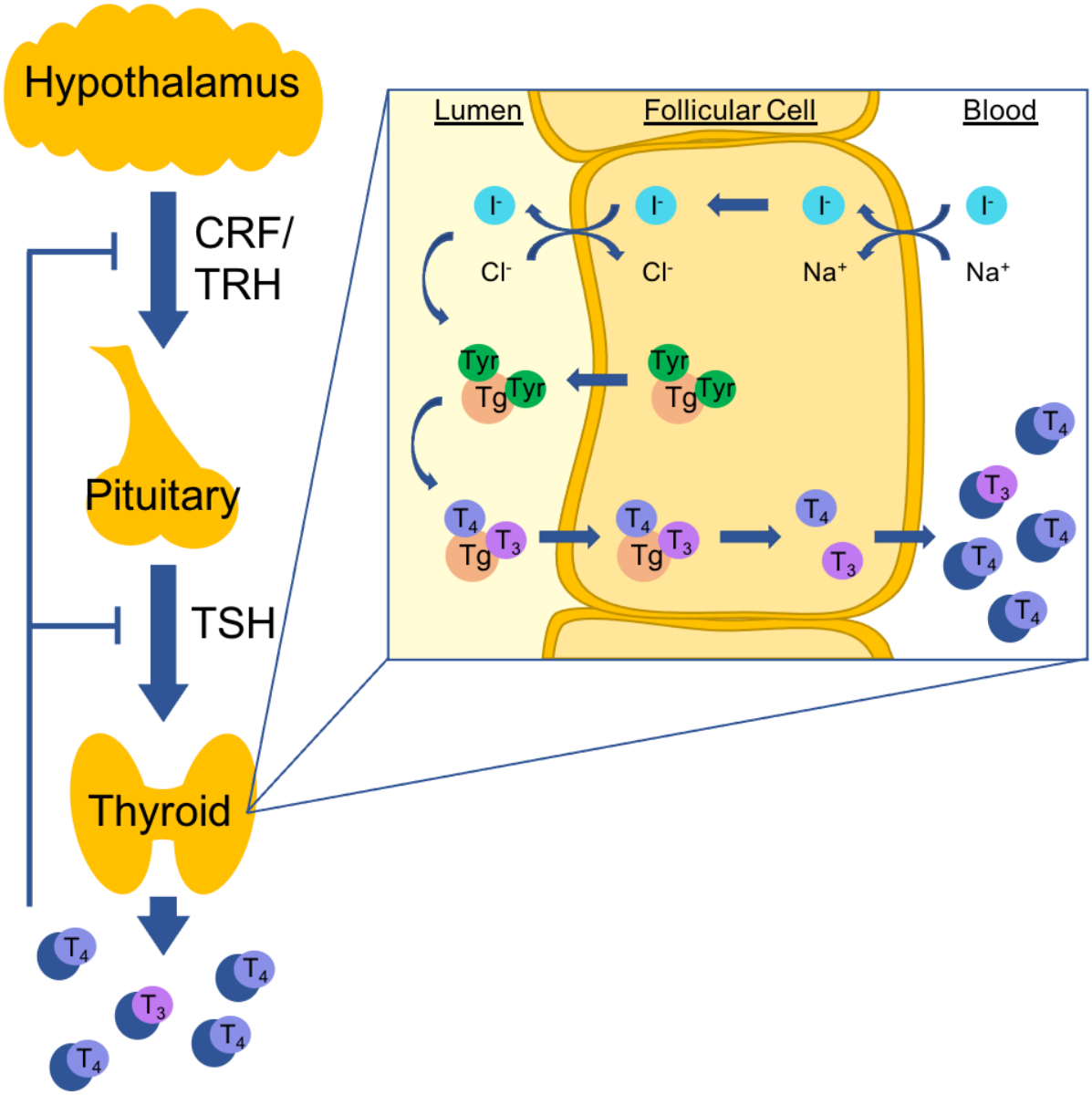


Figure 2: Thyroid Hormone Production.
TH is produced through the hypothalamus-pituitary-thyroid axis. Environmental cues stimulate the hypothalamus to produce mammalian TRH or amphibian CRF, which in turn, signal the pituitary to release TSH. TSH stimulates the production of TH within the follicular cells of the thyroid. Within the follicular cells, iodide is imported into the follicular call through a sodium-iodide symporter and subsequently exported into the follicular lumen along with tyrosine (Tyr) carrying thyroglobulin (Tg). Thyroglobulin acts as a substrate to iodinate the Tyr residues to form T_3 or T_4 which are released into the blood stream through the thyroid follicular cells, with T_4 being released in greater quantities than T_3 and carried to peripheral tissues by carrier proteins. Adapted from Nussey and Whitehead (2001) and Thambirajah et al. (2019).

Within the follicles of the thyroid gland, TH is synthesized by thyroglobulin which acts as the substrate for triiodothyronine (T_3) and thyroxine (T_4) production. Iodide, which is transported into the follicle by a sodium-iodide symporter, reacts with peroxidases and binds to the tyrosine residues of thyroglobulin (Nussey and Whitehead, 2001). This iodination occurs at either the 3 or 3 and 5 positions leading to the production of 3-monoiodotyrosine or 3,5-diiodotyrosine, respectively. Condensation of a 3,5-diiodotyrosine with a neighbouring 3-monoiodotyrosine leads to the production of T_3 whereas condensation with another 3,5-diiodotyrosine leads to the production of T_4 (Dunn and Dunn, 1999; Fort et al., 2007).

T_3 and T_4 are released from thyroglobulin by phagolysosomal hydrolysis and secreted into the bloodstream where they are transported to peripheral tissues with transport proteins such as transthyretin or, in a lesser proportion, as free TH (Gilbert et al., 1996; Larsson et al., 1985; Visser et al., 2011). Although both T_3 and T_4 can be found in the plasma, T_4 is released from the thyroid gland in orders of magnitudes greater than T_3 (Nussey and Whitehead, 2001). Released TH negatively regulates TSH and CRF creating a negative feedback loop on its own production through the HPT axis.

Further regulation of TH occurs on the local level in the peripheral tissues. Once cells take up TH through specific membrane transporters (Visser et al., 2008), deiodinase activity within the cytoplasm maintains TH homeostasis through TH conversion. Type I and II 5'-deiodinase enzymes, Dio1 and Dio2 respectively, convert T_4 into the more biologically active T_3 (Figure 3; (Becker et al., 1997; Luongo et al., 2019). Although T_4 was originally considered solely a pro-hormone and T_3 was the only active TH, it has been shown that T_4 and 3,5-diiodo-

L-thyronine (3,5- T_2) have activation potential as well (Galton and Cohen, 1980; Jackman et al., 2018; Maher et al., 2016; Mendoza et al., 2013). Active THs are metabolized by type III 5-deiodinase activity (Dio3) which converts T_3 and T_4 to the relatively inactive 3,3-L-diiodothyronine (3,3-T_2) or reverse T_3 (rT_3; Becker et al., 1997; Bianco and Kim, 2006). The expression of these deiodinases correspond with the observed competence of the peripheral tissues to respond to TH signal (Cai and Brown, 2004). Metabolism of THs can also occur through sulfation and glucuronidation (van der Spek et al., 2017).

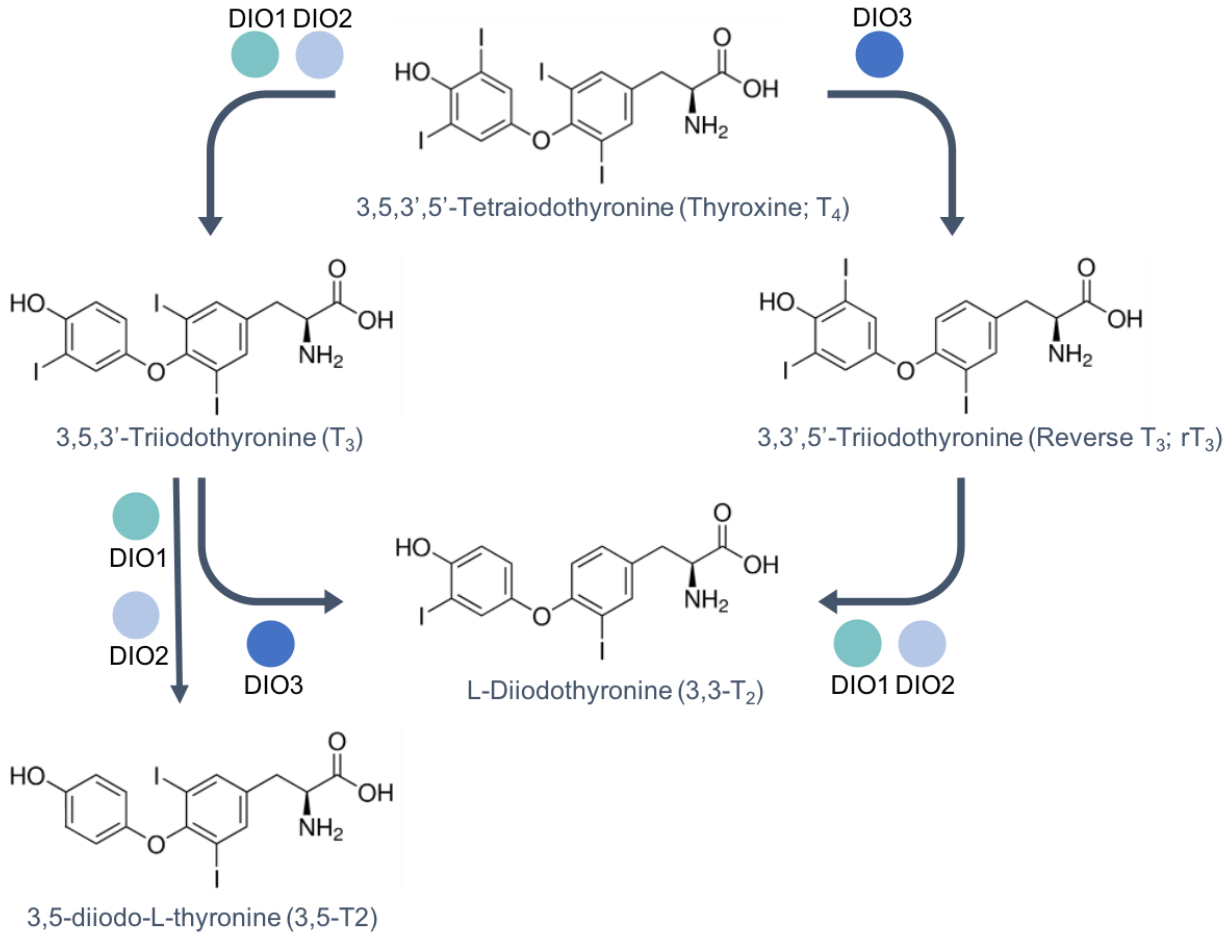


Figure 3: Thyroid Hormone Conversion by Deiodinase Activity.
Type I and II 5'-deiodinase enzymes, Dio1 and Dio2 respectively, convert T_4 into the more biologically active T_3 as well as converting T_3 into the active metabolite 3,3-T_2. Whereas, type III 5-deiodinase, Dio3, converts T_3 and T_4 to the less biologically active to 3,3-T_2 and rT_3, adapted from Köhrle et al. (2020) and Luongo et al. (2019).

1.3 Thyroid Hormone Signalling

TH signals predominantly genomically through the TH receptors (TRs) TRα and TRβ (Gilbert et al., 1996). TRs are in the steroid/thyroid hormone nuclear receptor superfamily along with estrogen receptors (ER) and peroxisome proliferator-activated receptors (PPAR; Evans, 1988; Tsai and O'Malley, 1994). Although TRs can form homodimers, they most commonly form heterodimers with retinoid X receptors (RXR). These heterodimers are constitutively bound to the genome at TH response elements (TREs) located in proximity to the promotor regions of TH response genes (Yen, 2001). In the well described dual function model of TH signalling (Figure 4), TRs negatively regulate TH response gene expression in the absence of TH and positively regulate these genes in the presence of this signalling molecule (Buchholz et al., 2006; Sachs et al., 2000). The TR's ability to regulate gene expression in this manner relies upon co-activators and co-repressors (Cheng et al., 2010; Shi, 2009). Co-activators include acetyl transferases (Paul et al., 2007) and arginine methyl transferases (Matsuda et al., 2009). Whereas, co-repressors interact with histone deacetylases (Ishizuka and Lazar, 2003) and ATP-dependent chromatin remodelers (Xue et al., 1998) indicating that histone modifications in the promotor region may play an important role in transducing the TH signal.

TRα is present in premetamorphic tadpoles allowing for competence to respond to the TH signal (Yaoita and Brown, 1990). Knock out studies of TRα in *Xenopus tropicalis* have confirmed the requirement of this receptor to repress transcription with TH response transcripts increasing in expression upon knock out of this receptor. In the presence of TH, however, these same TH-responsive transcripts were not as responsive to TH as the wild type *X. tropicalis*, also highlighting the role of TRα in competence to respond to TH (Choi et al., 2015; Wen et al.,

2017). Overall, knock out of this receptor lead to an early initiation of metamorphosis but a much slower development. Contrarily to TRα, TRβ is not present until prometamorphosis, rising in response to the TH signal (Yaoita and Brown, 1990). Knock out of TRβ has a similar effect where TH response genes are de-repressed in the absence of TH and their response to TH is muted (Shibata et al., 2020).

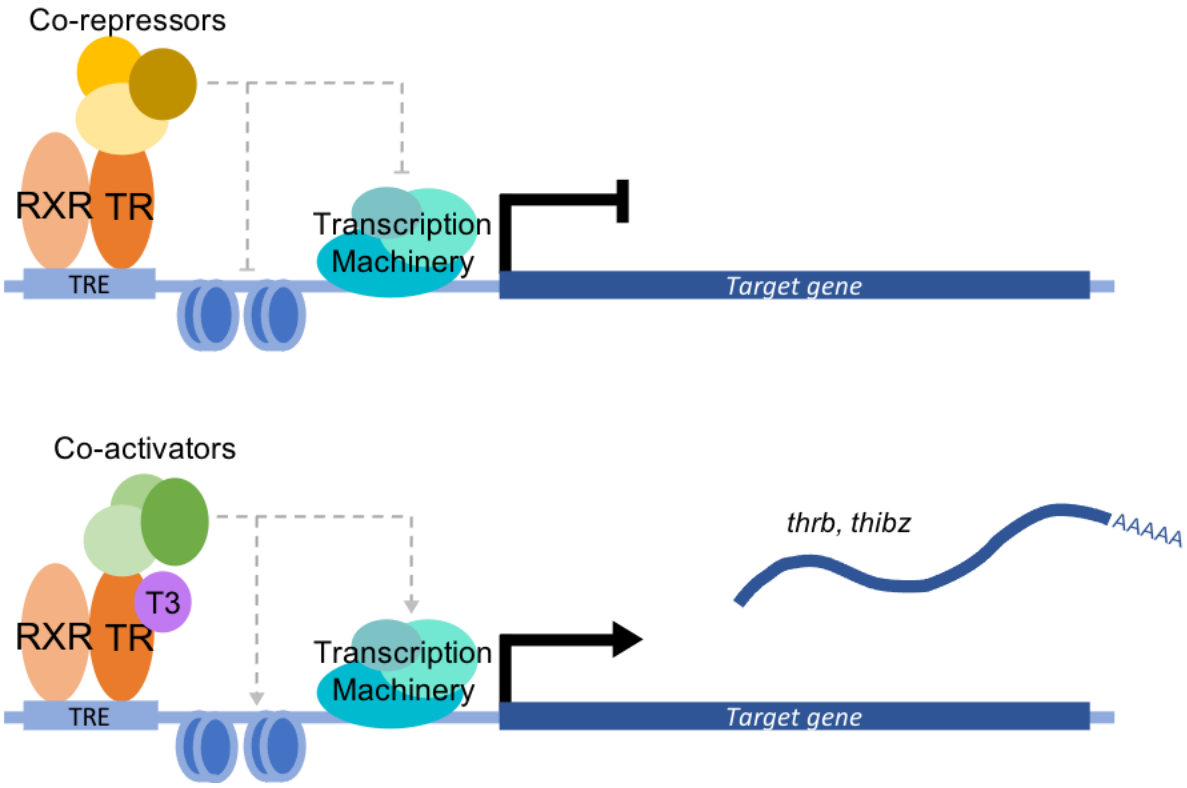


Figure 4: Thyroid Hormone Signalling Dual Function Model.
The Dual Function Model describes TH signalling gene expression as either induced or not induced depending on the presence or absence of TH. When TH is not present, co-repressors interact with the TH receptors (TRs) repressing transcription of the response transcripts. In the presence of TH, however, co-repressors are released and co-activators are recruited leading to the expression of the TH response genes such as the receptor transcripts *TH receptor beta* (*thrb*) and TH-induced basic region leucine zipper-containing transcription factor (*thibz*).

Early studies found that introduction of TH induces a biphasic transcriptomic response with a distinct group of early TH response genes including the TRβ mRNA, *TH receptor beta* (*thrb*), responding to TH signal within 8h followed by a delayed upregulation of secondary transcripts (Wang and Brown, 1993). Although most of the earliest response transcripts were found to continue to be upregulated at later timepoints, a few were transient during early response reducing to untreated levels at later timepoints (Wang and Brown, 1993). This biphasic program includes an induction and an execution phase where during the induction phase the TH signal is received and affects only direct TH response genes. During the subsequent execution phase the initial TH response leads to downstream secondary response of transcripts required for executing the TH signalling program (Das et al., 2009; Hammond et al., 2015; Wang and Brown, 1993). Das et al. (2009) utilized the protein synthesis inhibitor cycloheximide (CHX) to distinguish between transcripts involved in the two phases; hypothesizing that any TH response transcripts not requiring *de novo* protein synthesis would likely be direct targets of TH and therefore may be involved in initiation of the response. Contrarily, any CHX sensitive TH response transcripts must require translation of a direct TH response transcript for their regulation and therefore are more likely to be involved with execution of the signal (Figure 5). Using this method, Das et al. (2009) found that a disproportionate number of genes related to transcriptional regulation and initiation of cell cycle progression are enriched in the induction of the TH signal whereas transcripts related to the later stages of DNA replication and the apoptotic program were sensitive to CHX and likely involved in the execution phase. These secondary response transcripts, although not directly responsive to TH, are critical transcripts in the eventual phenotypic responses to TH signalling. Interestingly, a large proportion of CHX-

resistant transcripts did not overlap with transcripts responsive to only TH treatment. Although this can partially be explained by the ability of CHX to stabilize transcript abundance through alternative pathways, this may also indicate the lower abundance levels of many induction transcripts compared to execution transcripts.

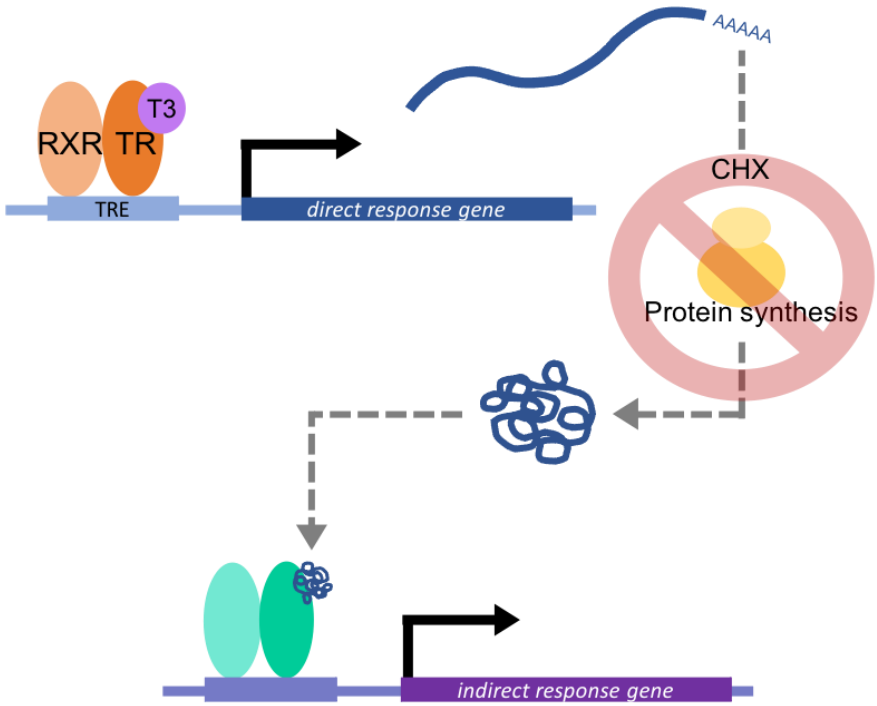


Figure 5: Direct Versus Indirect Thyroid Hormone Response Genes.
One requirement of a direct response gene is that it does not require *de novo* protein synthesis to respond to the TH signal and is therefore resistant to protein synthesis inhibitors such as cycloheximide (CHX). Contrarily, indirect TH response genes may require, for example, the synthesis if a direct response transcription factor making them sensitive to protein synthesis inhibitors.

Countering the dual function model, some transcripts such as the TSH subunit-encoding transcript, *tshb*, are downregulated in the presence of TH. In mice, knockdown of co-activator binding sites have demonstrated that co-activators are required for negative regulation of this transcript by TH (Ortiga-Carvalho et al., 2005). The negative feedback of TH on *tshb*, does not

only occur on the genomic level, but its regulation is also acted on post-transcriptionally. Treatment with TH has shown to lead to *tshb* mRNA destabilization in rat pituitary cells through decreasing the length of the mRNA poly(A) tail (Krane et al., 1991). This decrease in polyadenylation may occur non-genomically as it is not affected by transcription inhibition but does rely on TH interaction with αVβ3 integrin (Bargi-Souza et al., 2018), a membrane protein which contains a surface receptor site that interacts with TH (Bergh et al., 2005; Cody et al., 2007).

As mentioned, TH can also signal non-genomically in conjunction to genomic signalling. TH has been shown to bind to cell surface receptors such as αVβ3 integrin (Bergh et al., 2005; Cody et al., 2007) or extranuclear TRs (Moeller et al., 2006) leading to downstream cellular responses. Non-genomic actions of TH can also indirectly influence active transcription. It has been demonstrated that TH can act through cytosolic TRβ activating the phosphatidylinositol 3-kinase pathway causing the transcription of glucose metabolism genes (Moeller et al., 2006). As well, non-genomic signalling activates mitogen-activated protein kinase pathways leading to the phosphorylation and subsequent de-repression of TRs (Davis et al., 2008, 2000). As noted above, non-genomic TH signalling can also have an effect on post-transcriptional regulation. The synchronous interplay between genomic and non-genomic TH actions highlights the complexities of the signalling mechanisms of this hormone.

1.4 Thyroid hormone and Amphibian Metamorphosis

Studying TH signalling during the postembryonic or perinatal period in typical mammalian model species is problematic due to the need to separate confounding maternal TH as well as

the practical difficulties of *in utero* experimentation. More mature mammalian models require either physical or chemical ablation of the thyroid gland, potentially introducing extrinsic variables to TH signalling pathways. Fortuitously, anurans undergo a free swimming postembryonic developmental period driven by TH where the basic mechanism of TH signalling, as well as many developmental processes that occur in this postembryonic stage, are similar between anurans and mammalians making them ideal models (Sachs and Buchholz, 2017; Tata, 2006). Additionally, further understanding of TH signalling in environmentally relevant anuran models is crucial to improve knowledge on the effect of endocrine disrupting chemicals (EDCs) of TH signalling in sensitive species (Thambirajah et al., 2019).

During the early stages of development, premetamorphic tadpoles do not have a functional thyroid gland and therefore have undetectable levels of circulating TH. As the thyroid gland begins to develop and TH levels begin to rise it induces the transition into prometamorphosis, a developmental stage characterized by synchronized developmental changes that affect virtually every tissue. This metamorphic stage sets up the complex process to prepare for the life stage transition from an aquatic larval tadpole to a terrestrial or semi-terrestrial juvenile froglet, which includes *de novo* synthesis of new tissues such as the limbs, coordinated apoptosis of larval tissues such as the tail as well as complete reprogramming of yet other tissues, such as the liver (Figure 6; Gilbert et al., 1996; White and Nicoll, 1981). The peak endogenous TH levels coincide with the climax of metamorphic changes where larval features are regressing as juvenile features are developing (Figure 6; Gilbert et al., 1996; Leloup and Buscaglia, 1977; Shi, 1999; White and Nicoll, 1981). The utility of athyroid premetamorphic tadpoles in the study of TH signalling is enhanced by the ability to induce postembryonic development experimentally

through TH treatment (Gudernatsch, 1912). Premetamorphic tadpoles can be injected or externally exposed to biologically relevant levels of TH and will undergo metamorphosis in a similar manner as to what is seen during natural metamorphosis. As well, experimentally induced metamorphosis leads to a precocious response of TH response genes allowing the control and timing to study the transcriptomic TH response program. Given the varied fate of each tadpole tissue upon treatment to TH, it is unsurprising that the response of each tissue involves an individual TH response program, however certain transcripts including *thrb* and *thyroid hormone-induced B/Zip* (*thibz*) are involved in the early response to TH signalling irrespective of tissue (Shi and Brown, 1993; Wang and Brown, 1993).

One of the most dramatic displays during anuran metamorphosis is the resorption of the tail. In late metamorphosis the diverse tissues of this organ, including notochord, skeletal muscle, nerve tissue, connective tissue, epidermis and dorsal and ventral tail fins, undergo a synchronized and highly ordered histolysis. Best studied in the tail fin, this histolysis involves coordinated steps starting with breakdown of the outermost epidermal cells which propagate inwards towards the mesenchymal cells. As cells undergo programmed cell death they are either phagocytosed or slough off the animal (Gilbert et al., 1996; Shi, 1999). The tail apoptotic program has shown to be able to occur in an autonomous, tissue specific manner, as isolated tail tips and tail fin biopsies will respond to external exposure of TH, in a similar manner as to what is seen with whole animal exposures (Hinther et al., 2010; Skirrow et al., 2008; Tata, 1966).

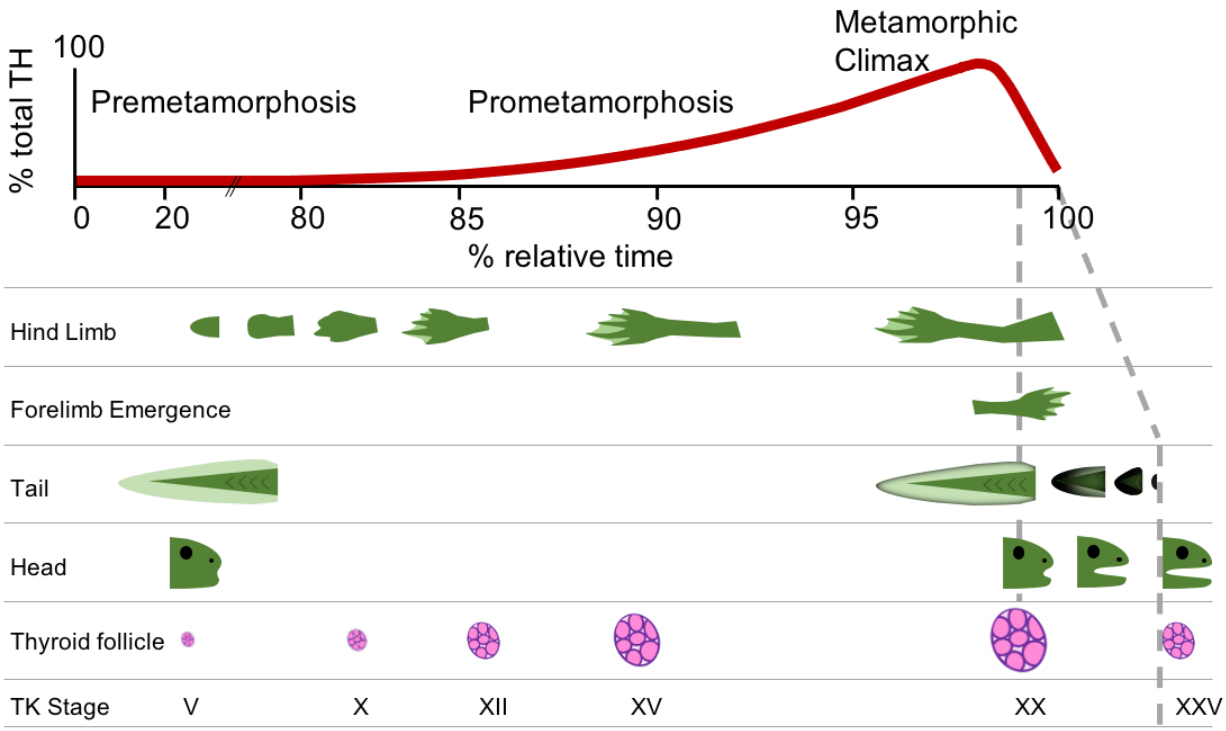


Figure 6: Thyroid Hormone Driven Amphibian Metamorphosis.
Premetamorphic tadpoles have virtually no endogenous thyroid hormone (TH) levels. However, as the thyroid gland develops, and hormone levels rise the tadpole will undergo the metamorphic changes required for the transition from an aquatic larval state to a semi-terrestrial juvenile state including development of limbs, controlled apoptosis of the tail, and head shape reconfiguration. Adapted from Thambirajah et al., 2019.

1.5 Cold Temperature Influence

Throughout phyla, species have evolved to allow temperature to influence their developmental programs. Reptilians such as crocodilians, turtles and some lizards utilize temperature for environmental sex determination (Janzen and Paukstis, 1991), for many plant species temperature cues germination time (Covell et al., 1986; Eh, 1988), and in insects temperature is a main driver for rate of development (Allsopp and Butler, 1987; Ratte, 1985). Temperate latitude living North American Bullfrog, *Rana [Lithobates] catesbeiana* has evolved

to allow temperature to control developmental timing as a strategy to avoid energy demanding postembryonic development during unfavourable conditions (Viparina and Just, 1975).

In the 1960's, it was discovered that *R. catesbeiana* tadpoles exposed to TH underwent slower metamorphosis at temperatures under 25^{o}C, and at 5^{o}C metamorphosis was completely halted (Frieden et al., 1965; Fry, 1972). This observation of low temperatures seemingly blocking TH signalling was not due to an inability of TH to be transported to target tissues as there is no effect of cold temperature on binding to transthyretin transport proteins (Murata and Yamauchi, 2005). Additionally, TH is still able to be taken up within both the cell and nucleus of *R. catesbeiana's* nucleated red blood cells at cold temperatures, although maximum accumulation occurs later at cold temperatures than warm temperatures (Murata and Yamauchi, 2005; Yamamoto et al., 1966).

Given that TH primarily acts genomically, more recent studies have focused on TH's ability to modulate the expression of genes at cold temperatures. TH can still bind TRs (Murata and Yamauchi, 2005), and transcripts that respond to cold temperature stress or lipid pathways continue to be responsive at 5^{o}C, indicating that temperatures are not low enough to halt transcription machinery (Hammond et al., 2015, 2016a; Suzuki et al., 2016). However, TH induction of many well studied TH response transcripts, such as *thrb,* and *deiodinase 3* (*dio3*), is abolished at temperatures around 4-5^{o}C. This corresponds with the epigenetic findings in *R. catesbeiana* liver which showed globally higher levels of the activation mark acetylated H3K9 in permissive temperatures but not cold temperatures (Ishihara et al., 2019) as well as increased transcription of histone acetylation related transcripts at cold temperatures (Ishihara and

Yamauchi, 2019). In permissive temperatures TH exposure leads to increased activation marks di- and tri-methylated H3K36 in the *thrb* promotor region which is not seen in cold temperatures (Mochizuki et al., 2012).

Transcriptomic studies to date have discovered that there are a select few TH response genes capable of responding to the TH signal within 48h at cold temperatures: *thibz* has been found to be responsive in most tissues (Figure 7a) and *CCAAT/enhancer binding protein 1* (*cebp1*) in back skin (Figure 7b; Hammond et al., 2016) as well as additional tissues after longer exposure periods (Hammond et al., 2015). It is yet unknown how regulation on the epigenetic level plays into the control of these genes differently than those TH response genes that do not show abundance changes at cold temperatures. The fact that TH response transcripts that are considered to be direct response genes (*thrb*) do not respond in cold temperatures and yet other direct response genes do (*thibz*; Hammond et al., 2016), indicates that there may be more hierarchical stratification within the induction phase of TH signalling program (Figure 7). This separation in cold temperatures may allow for an experimental method to further elucidate the dynamics of early TH response gene regulation.

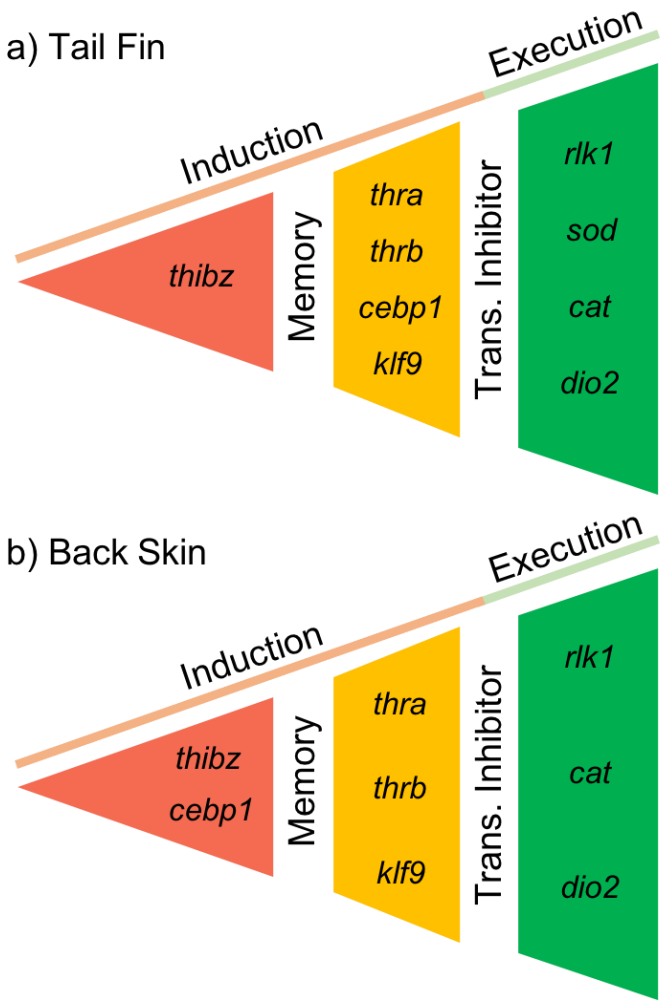


Figure 7: Hierarchy of Early Thyroid Hormone Response Gene Regulation.
Early transcriptomic thyroid hormone response has been delineated into a biphasic response including an induction and execution phase. These phases can be uncoupled through translation (trans) inhibitors as direct TH response genes do not require *de novo* protein synthesis for the induction program. Cold temperature has shown to be able to further stratify the induction program as only certain early transcripts are responsive in cold temperatures, with differences in tissue specific responses. Adapted from Hammond et al., 2016.

Not only do cold temperatures pause the metamorphic program, when *R. catesbeiana* tadpoles are then shifted to more permissive temperatures they undergo a more accelerated metamorphosis (Frieden et al., 1965). This phenomenon occurs even when there is no longer endogenous TH signal (Ashley et al., 1968; Frieden et al., 1965). This indicates that a molecular

memory is formed in cold temperatures where the TH signal is initiated and remembered for when more permissive temperatures occur. Once shifted to warm temperatures, the TH response transcripts that are unreactive in cold temperatures, such as *thrb* have been shown to have similar, if not greater abundance changes in most tissues after 48h (Hammond et al., 2015, 2016a).

Improved technologies for a greater breadth of transcriptomic exploration, as well as resources to be able to functionally assess data, including the recent publication of the *R. catesbeiana* draft genome (Hammond et al., 2017), provide the ability to garner a more in-depth understanding of the mechanisms involved within the establishment of the molecular memory in non-permissive temperatures and the priming for an accelerated metamorphosis when more permissive temperatures occur. As *thibz* is capable to respond to TH in cold temperatures (Hammond et al., 2015, 2016a), as well as a previously determined early response transcript (Das et al., 2009; Wang and Brown, 1993) it insinuates that this transcription factor may be an important regulator of the transcriptomic induction program. Using RNA-seq analysis we show that the response to TH at cold temperatures in cultured tail fin involves a complex transcriptomic response program of which includes transcription factors. The expression dynamics and regulation differs between each transcription factor hinting at the complexities of the transcriptomic response for the establishment of the molecular memory and the priming of the subsequent accelerated response.

1.6 Hypothesis and Objectives

1.6.1 Hypothesis

My overall hypothesis is that the molecular memory observed in the bullfrog tadpole tissues may be a means to further stratify the early response transcripts in the initiation of TH-signalling with the components involved not only remembering the induction signal but also priming for a more accelerated metamorphic program when more permissive temperatures arise.

1.6.2 Objectives

The objectives of this research are to further elucidate components involved in the molecular memory and their response when metamorphosis resumes by:

1) Determining the transcriptomic program involved within the molecular memory at cold temperatures;

2) Examining the regulation of candidate molecular memory factors;

3) Investigating the response dynamics of these candidate factors when permissive temperatures arrive.

2 Methods

Acknowledgement: Ethan Abbott contributed to the validation of primer design for new transcripts: *six1*, *klfX*, *p66a*, and *tcp4.* As well as running qPCR analysis for each experiment on these new primers and probes for all experiments from stored RNA.

2.1 Experimental Animals

Rana catesbeiana tadpoles (Taylor and Krollros stage I-VI (Taylor and Kollros, 1946)) of mixed sexes were collected by WestWind SeaLab Supplies (Victoria, BC, Canada) in freshwater lakes surrounding the Victoria, British Columbia area. Tadpoles were housed in the University of Victoria's Outdoor Aquatics unit in 100 Gallon fibreglass tanks. Tanks contained recirculated dechlorinated municipal water at 15±1°C with pH 6.8 and 96-98% dissolved oxygen (DO). Tadpoles were fed Spirulina flakes (Nutrafin Max, Rolf C. Hagen, Montreal, PQ, Canada) twice a day in the morning and afternoon and outdoor tanks allowed for a natural photoperiod. Periodically during the spring season, tadpoles were treated with 1% Instant Ocean (Dynamic Aqua Supply, Cat No. 15160) to mitigate fungal infections. The care and treatment of animals was in accordance with guidelines established by the Canadian Council on Animal Care and approved by the University of Victoria Animal Care Committee (protocol # 2019-025).

2.2 C-fin Assay

All exposures were done using a cultured tail fin (C-fin) assay (Figure 8). Tadpoles were euthanized by immersion in 0.1% (w/v) tricaine methanesulfonate (Syndel Laboratories, Nanaimo, Canada) prepared in 25 mM $NaHCO_3$ (Fisher Chemical; CAS 144-55-8). All subsequent

steps were performed aseptically in a laminar flow hood. Euthanized tadpoles were rinsed in four successive washes of 100 mL Magnesium-Free Media (pH 7.6) containing 88 mM NaCl (Bio Basic Canada, CAS No. 7647-14-5), 1 mM KCl (Caledon, CAS No. 7447-40-7), 2.4 mM $NaHCO_3$, 7.5mM Tris-HCl (pH 7.6; Bio Basic Canada, CAS No. 1185-53-1), and 0.88 mM $CaCl_2$ (Bio Basic Canada, CAS No. 10043-52-4). Four- or six-mm biopsies were taken from the tail fin tissue using sterile biopsy punches (Miltex; REF 33-34 and REF 33-36, respectively). Biopsies were transferred into the individual wells of polystyrene 24-well tissue culture plates (Becton Dickinson; REF 353947) containing 500 μL 70% Leibovitz's L-15 Medium with GlutaMAX (Gibco, CAT No. 31415029) modified with 10 mM HEPES (Sigma, CAS No. H4034-500), 200 mM GlutaMAX (Gibco; REF 35050-061), and 50 μg/mL penicillin, 50 μg/mL streptomycin and 100 μg/mL neomycin (Gibco; REF 15640-055). Biopsies were acclimated to their experimental temperatures for 24h in either a 5°C or 24°C incubator.

Following acclimation, 500 μL 2X treatment media was added. For 5°C experiments treatment media was brought to 5°C and treatments were carried out on cold blocks to avoid exposing biopsies to warmer temperatures. In contrast for 24°C experiments, treatment media was brought to 24°C and exposures were carried out at room temperature. Biopsies were incubated at their respective temperatures for the timeline of each experiment. All wells were inspected daily for bacterial or fungal growth. In all experiments, no contamination growth was detected. After the exposure length, biopsies were transferred into RNAlater (Life Technologies

Inc., Burlington, ON, Canada), brought to the experimental temperature, and incubated at 4°C for 48h then moved to -20°C for longer term storage.

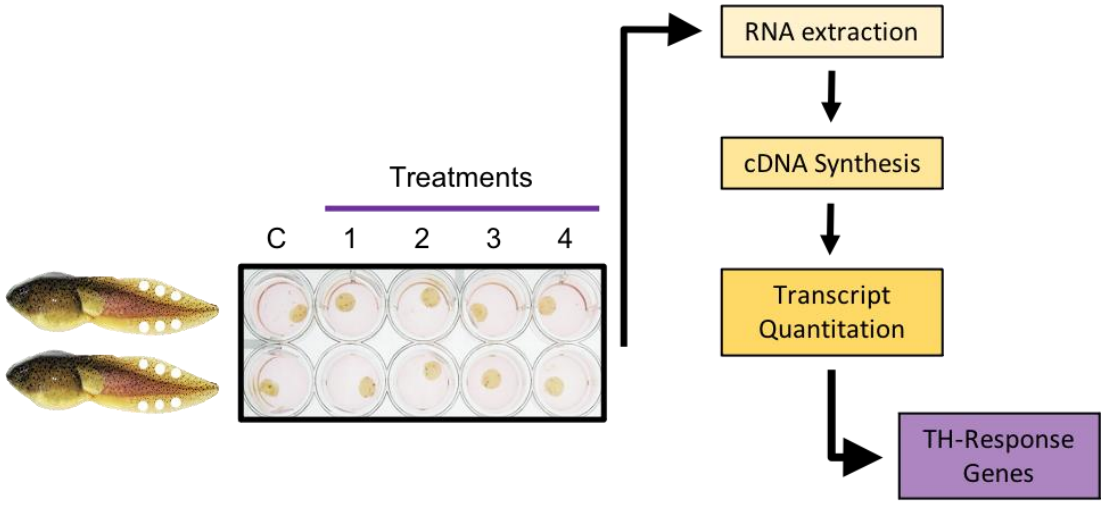


Figure 8: The C-fin Assay.
Biopsies from tadpoles were taken from the dorsal and ventral tail fins and cultured in the different treatment exposures, allowing every tadpole to be exposed to each treatment. To test the transcriptomic response of each C-fin sample, the RNA was extracted and reverse transcribed into cDNA to quantitate transcript abundance. Figure adapted from (Hinther et al., 2010).

2.3 Exposure Design

2.3.1 Assessment of the Influence of Location on the T_3 Response in the C-fin Assay

Biopsies were taken from eight locations on the tail fin and exposed to control of 400 nM NaOH (Fisher Chemical, CAS No. 1310-73-2;) or treatment of 10 nM 3,5,3'-triiodo-L-thyronine sodium salt (T_3; Alfa Aesar, CAS No. 55-06-1) in 400 nM NaOH for eight animals. Control and treatment condition biopsies were evenly divided between the posterior and anterior positions and the dorsal and ventral fins. After a 24h acclimation period, biopsies were exposed to their designated treatment for 48h at 24°C (Figure 9).

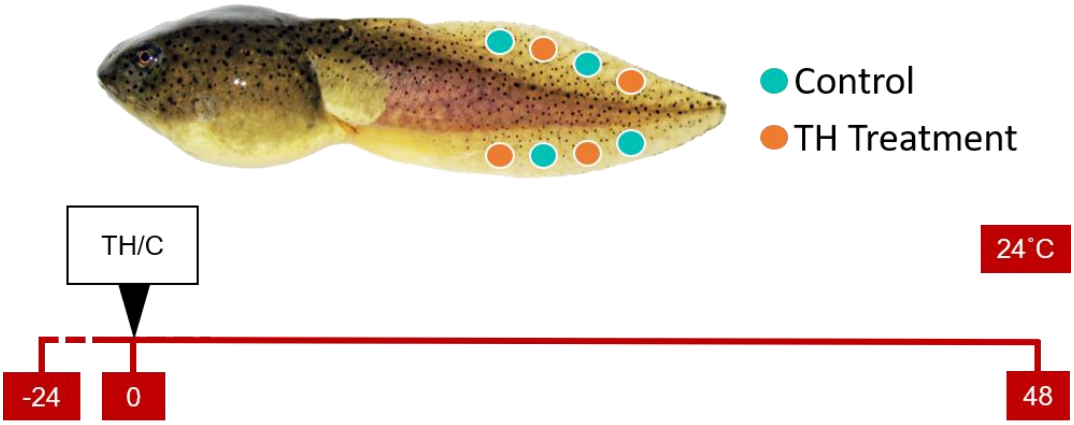


Figure 9: Biopsy Location Experiment.
Biopsies were taken from the tail fin of eight tadpoles and alternately placed in 400 nM NaOH control (C) or 10 nM T_3 in 400 nM NaOH (TH) treatment for 48h at 24°C. Control biopsy locations are highlighted in teal and TH treated biopsy locations are highlighted in orange. Boxes designate the time in hours, with the dashed line showing temperature acclimation time and solid line showing exposure time.

2.3.2 Expression Dynamics of Molecular Memory Transcripts

Eleven biopsies were taken from 12 premetamorphic tadpoles and after a 24h incubation period unexposed biopsies were removed into RNAlater tissue preservative. The remaining biopsies were treated with 10 nM T_3 in 400 nM NaOH for 1.5, 3, 6, 12, 18, 36, 48, 72 or 96h at 5°C (Figure 10).

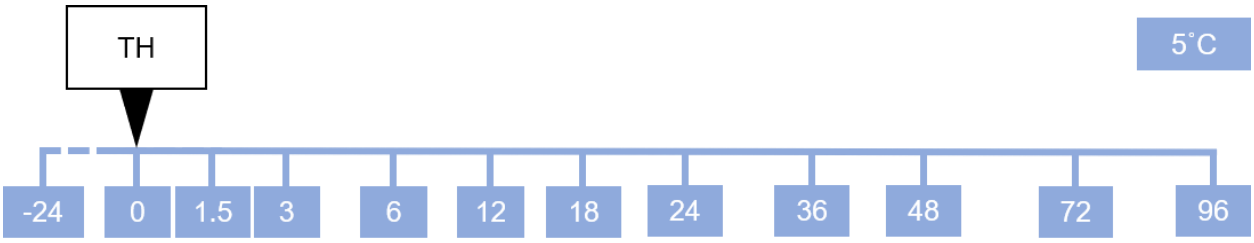


Figure 10: Expression Dynamics Exposure.
Biopsies were treated with 10 nM T_3 in 400 nM NaOH (TH) for a time course from 0 to 96h at 5°C. Each timepoint starting from zero indicates removal of one set of biopsies. See Figure 9 for additional plot details.

2.3.3 Temperature Shift Accelerated Transcriptomic Response

Biopsies from eight of the same animals were acclimated to either 5°C or 24°C for 24h before untreated control biopsies were removed. Those acclimated to 24°C were exposed to T_3 for 1, 2, 4, 8, or 16h (Figure 11a). Biopsies acclimated to 5°C were exposed to T_3 for 48h at 5°C. The 48h exposure period was chosen to ensure establishment of the molecular memory as in permissive temperatures a commitment point is established within 24h to 48h of TH exposure in the tail, after which inhibitors of protein synthesis will not be able to stop progression of the metamorphic program (Gilbert et al., 1996; Wang and Brown, 1993). As well, previous studies have demonstrated that a response to TH can be seen at 5°C within this time period (Hammond et al., 2016a).

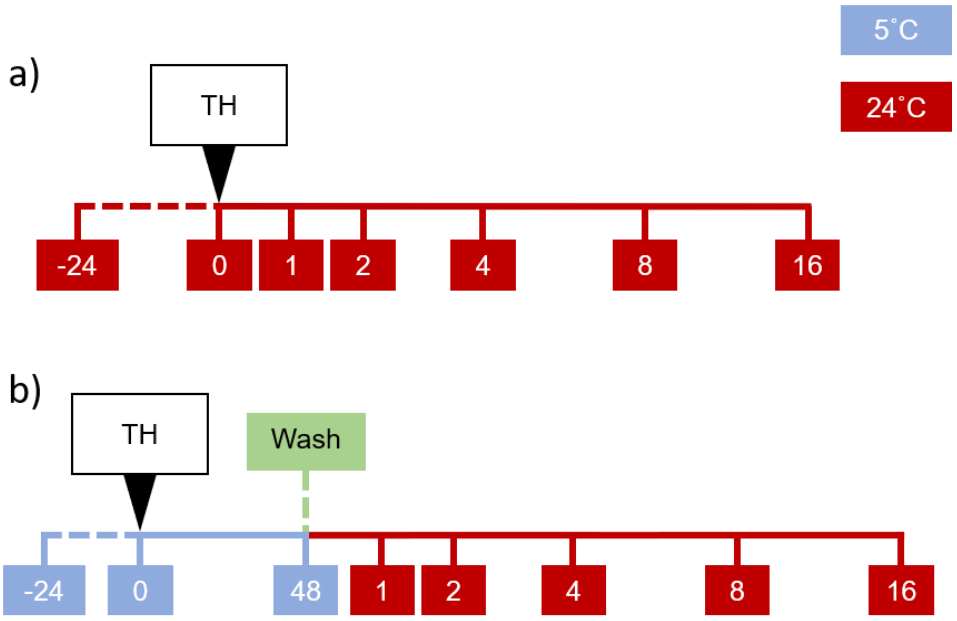


Figure 11: Experimental Designs for Different Temperature Conditions: a) at Permissive Temperature and b) in a Temperature Shift Experiment.
At permissive temperature, biopsies were treated with 10 nM T_3 in 400 nM NaOH (TH) for a time course from 0 to 16h at 24°C. in the temperature shift experiment, biopsies were treated with 10 nM T_3 in 400 nM NaOH for 48h at 5°C, washed and moved into clean media for an identical time course from 0 to 16h at 24°C. Each timepoint starting from zero indicates removal of one set of biopsies. See Figure 9 for additional plot details.

After establishment of the molecular memory at 5°C, one set of biopsies was removed. For the remaining biopsies, the treatment media was removed through rinsing biopsies with three consecutive washes of 70% modified Leibovitz's Media at 24°C. Biopsies were moved into clean, treatment free media at 24°C for the remainder of their exposure period mirroring the 24°C exposure for 1, 2, 4, 8, and 16h (Figure 11b).

2.3.4 Inhibition of Transcription and Translation

Biopsies acclimated at 5°C were exposed to 80 μg/mL of the translation inhibitor cycloheximide (CHX; Sigma, CAS No. 01810) or 20 μg/mL of the transcription inhibitor Actinomycin D (ActD; Sigma, CAS No. A1410) one hour in advance of treatment with NaOH or T_3 to allow sufficient time for the inhibitors to penetrate the tissue before treatment (Figure 12). Subsequently, 400 nM NaOH or 10 nM T_3 in 400 nM NaOH was added to the media and biopsies remained in treatment for 24h at 5°C (Figure 12). Longer exposure times were not used to avoid the cytotoxic effects of the inhibitors.

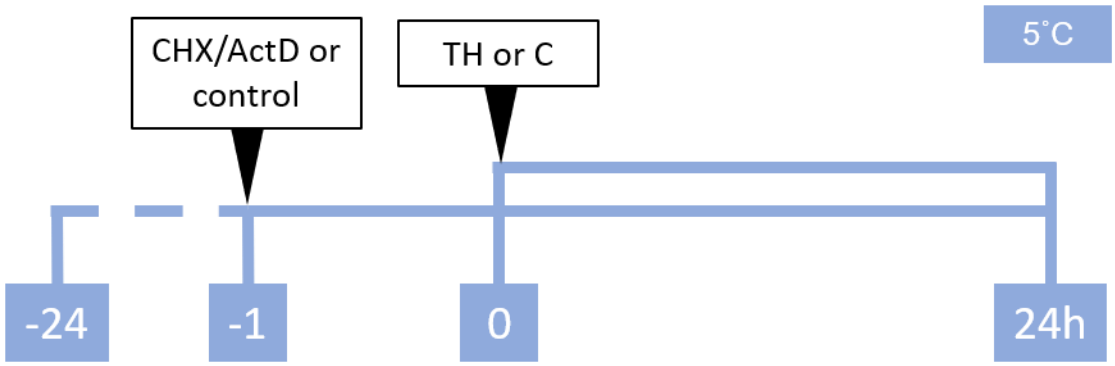


Figure 12: Inhibitor Experiment.
Biopsies were exposed to 80 μg/mL cycloheximide (CHX) or 20 μg/mL actinomycin D (ActD) for 25h starting one hour before being treated with 10 nM T_3 in 400 nM NaOH (TH) or 400 nM NaOH (C) for 24h at 5°C. See Figure 9 for additional plot details.

2.4 Isolation of Total RNA

Biopsies were randomized prior to RNA extraction to reduce experimenter bias. RNA was extracted using the TRIzol reagent as described by the manufacturer (Invitrogen Canada Inc., Burlington, ON, Canada; Cat no. 15596-018). Tissue was mechanically homogenized in 300 µL TRIzol using a Retsch MM301 Mixer Mill (Thermo Fisher Scientific, Ottawa, Canada) with a 1 mm diameter tungsten carbide bead. Homogenization occurred twice for three minutes at 24 Hz with a 180^{o} rotation between mixes. Sixty µL of chloroform was added to the homogenate and centrifuged at 10,000 xg for 10 min to separate the organic and aqueous phases. Twenty µg of glycogen (Invitrogen, CAT No. LT-02241) was added to the aqueous layer of each sample to aid in RNA precipitation with 150 µL isopropanol. RNA was washed with 300 µl 75% ethanol and resuspended in 20 µL UltraPure RNAse Free water (Invitrogen, Ref No. 10977-015). Gel electrophoresis was used with a 2% RNase free agarose gel (Fisher Bioreagents, CAS 9012-36-6) to ensure quality of the RNA and lack of degradation.

2.5 cDNA synthesis

RNA was reverse transcribed to cDNA using an applied high-capacity cDNA reverse transcription kit with RNase inhibitor (CAT No. 4374967, Applied Biosystems Inc.) as per manufacturer instructions. RNA was diluted to allow for 1 µg of RNA per reaction. The reverse transcription reaction was carried out on a MyCycler^{TM} Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A) with a 10 min primer ligation step at 25^{o}C, a 2h DNA polymerization step at 37^{o}C followed by 5 min at 85^{o}C for enzyme deactivation. Synthesized cDNA was diluted 1/20 in and stored at -20^{o}C.

2.6 RNA Quantitation by qPCR

2.6.1 qPCR analysis

Target TH response transcripts were measured using qPCR. Taqman based multiplex reactions were used to detect the TRα transcript (*thra*), *thrb*, and the normalizing transcripts ribosomal protein L8 (*rpl8*), eukaryotic elongation factor 1A (*eef1a*) and ribosomal protein S10 (*rps10*) using a MX3005P qPCR system (Agilent Technologies Canada Inc., Mississauga, ON, Canada) as described in Hammond et al. (2013) and Wojnarowicz et al. (2013). SYBR based reactions were used to detect *thibz* and *cebp1* on a CFX Connect real-time PCR detection system (Bio-Rad Laboratories Inc., Hercules, CA, USA; see Supplementary Table 1 for primer and probe sequences and annealing temperatures). Reactions were carried out as previously described by Veldhoen et al. (2014).

All transcripts were run in quadruplicate and the mean of the cycle threshold (CT) used for further analysis with outliers of greater than 0.5 standard deviation or abnormal melt curves removed. The efficiency and reliability of normalizers, *eef1a*, *rps10* and *rpl8*, was tested for each experiment using Chronbach's alpha test, Normfinder (Andersen et al., 2004) and Bestkeeper Software (Pfaffl et al., 2004). TH response transcripts were normalized to the geomean of the *eef1a*, *rps10* and *rpl8* normalizer transcripts to control for input variation. Relative fold change to control samples was calculated using the $2^{-\Delta\Delta Ct}$ method as described by (Schmittgen and Livak, 2008).

2.6.2 Novel Primer Design

Novel primers were designed for krüppel-like factor X (*klfX*), homeobox protein SIX1 (*six1*), transcriptional repressor p66-alpha (*p66a*) and RNA polymerase II transcriptional coactivator p15 (*tcp4*; see Supplementary Table 1 for primer and probe sequences and annealing temperatures). Where possible, primers were designed to be located within the protein coding region to maximize conserved sequence within the gene, with the exception of *six1* due to the high sequence identity with homeobox protein SIX2 (*six2*). Additionally, primers were designed outside conserved motifs (i.e., DNA or protein binding domains) to avoid areas that may be conserved between highly related genes. Primer Premier 6 (Premier Biosoft, Palo Alto, CA) was used to design primers with an approximately 50% GC content and minimizing the probability of hairpin or self-dimerization. Specificity of primers for the selected transcript was determined through ensuring minimal alignment of designed primers against highly related gene sequences.

A validation process was used to design qPCR run conditions and test for accuracy and efficiency of primer and probe sets in order to ensure primers followed Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE guidelines; Bustin et al., 2009), as described in Veldhoen et al. (2014). The primer validation process included insurance of expected qPCR amplicon through verification of predicted length using gel electrophoresis in a 1.5% agarose gel (Fisher Bioreagents, CAS 9012-36-6) and amplicon sequencing by Eurofins Genomics (Toronto, Ontario). Efficiency of primers was tested through linear amplification performance of a 2-fold serial dilution of cDNA from 1/5 to 1/160. The qPCR reactions of the validated primers and probes for the novel targets used a 15 μL qPCR reaction of 30 mM KCl,

0.01% glycerol, 10 mM Tris, 1 mM Tris–HCl (pH 8.3), 10 mM $MgCl_2$, 0.01% Tween-20, 83.3 nM ROX (Life Technologies), 200 mM dNTPs (Bioline USA Inc., Taunton, MA, USA), 1 unit of Immolase DNA polymerase (Bioline USA Inc.), 5 pmol of each primer, 3 pmol probe, and 2 µL of 1/20 diluted cDNA. All novel targets were run on MX3005P qPCR system (Agilent Technologies Canada Inc., Mississauga, ON, Canada) with *six1*, *p66a* and *tcp4* detected using a qPCR program of one cycle of 9 minutes at 95°C, followed by 45 cycles of 15s at 95°C, 30s at 64°C, and 30s at 72°C. The qPCR program for *klfX* included one cycle of 9 minutes at 95°C, followed by 45 cycles of 15s at 95°C, 30s at 60°C, and 45s at 72°C.

2.6.3 Statistical Analysis

Normality and homogeneity of variances was tested for using the Shapiro Wilk's test and Levene's test, respectively. Common for low sample gene expression data, data was found to not fall under the normality and homogeneity of variances assumptions for a parametric test. As each animal tail fin was biopsied allowing each animal to be exposed to every treatment, repeated-measures statistics were utilized to take into account the decreased biological variance. The non-parametric, repeated measures Friedman test was used to determine significance between treatments with the Wilcoxon signed-rank post hoc test using a p-value ≤ 0.05 to determine significance.

2.7 RNA Quantitation by RNA-seq

2.7.1 RNA Sequencing (RNA-seq) of Molecular Memory Transcripts

C-fin biopsies were exposed to 400 nM NaOH or 10 nM T_3 in 400 nM NaOH for 48h at 5°C (Figure 13). Total RNA was extracted from biopsies using the TRIzol-chloroform method described above. qPCR was used to assure tissue responsiveness to TH at 5°C through insurance that *thibz* responded to TH treatment as expected. A Bioanalyzer 2100 (Agilent, Mississauga, Ontario, Canada) was used for quality assessment of RNA with sequenced samples all having an RNA Integrity Number (RIN) greater than eight. Five samples from each treatment were strand specific sequenced with 75 bp paired-end sequencing using the HiSeq2500 (Illumina, San Diego, California, USA) at the Michael Smith Genome Sciences Centre in Vancouver, BC, as described previously by Jackman et al., 2018.

Figure 13: RNA-seq exposure.
To establish the molecular memory, biopsies were treated with 400 nM NaOH (C) or 10 nM T_3 in 400 nM NaOH (TH) for 48h at 5°C. See Figure 9 for additional plot details.

2.7.2 Bioinformatics

2.7.2.1 Assembly and Annotation

An initial quality control assessment on raw RNA-seq results was done using FastQC to ensure sequence quality and assess presence of adapters, duplication of reads and overrepresentation of

nucleotide bases or k-mers (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), Version 0.11.8). Raw reads were aligned to the *R. catesbeiana* genome version 3 (https://www.bcgsc.ca/downloads/supplementary/bullfrog/; Hammond et al., 2017) using STAR (Dobin et al., 2013) with default parameters. Subsequently, StringTie (Pertea et al., 2015) was used to assemble and quantitate probable transcripts. To minimize low count false positives a cut off rate of 5 or more samples with normalized counts that fell below 0.1 counts per million was used. Differential expression of TH treated versus control samples was calculated using DESeq2 (Love et al., 2014) with the Wald text statistic, controlling for repeated measures through addition of animal to the design formula. Differential fold change was considered significant with a Benjamini-Hochberg multiple testing adjusted p-value ≤ 0.05.

Transcripts were annotated using the highest percent e-value BlastX sequence alignment within the NCBI non-redundant database (Altschul et al., 1990; Bethesda, 1988). In parallel, Trinotate (Bryant et al., 2017) was used to annotate the transcripts using the Uniprot database (The UniProt Consortium et al., 2021) and further to determine Gene Ontology (GO) terms. Where discrepancy existed for four transcripts between the NCBI and Uniprot denoted annotations, the annotation with the higher percent identity was selected.

2.7.2.2 Gene Ontology Analysis

GO analysis was run using two different methods in parallel. Annotations of the significant transcripts were assigned GO terms through the David software (Huang et al., 2009a, 2009b). Ontologies were compared from the second level of biological process and molecular function. Enrichment analysis was performed for these GO terms from the significant transcripts using a modified Fisher's Exact test against the annotation of all the RNA-seq

identified transcripts as described by the developer, with significance being determined at $p \leq 0.05$.

Enrichment analysis for GO terms determined through Trinotate (Bryant et al., 2017) was completed through GO-Seq (Young et al., 2010), again comparing the GO terms defined for the significant transcripts against all the identified transcripts in the C-fin samples. Enrichment analysis through GO-Seq was selected to consider gene length in determining significance at $p \leq 0.05$. Cytoscape (Shannon et al., 2003) was used to visualize the interconnection of significant GO terms that contained greater than two transcripts though data organization using GOGadget (Nota, 2017).

2.7.2.3 Novel Thyroid Hormone Response Transcription Factors

Transcripts for identified transcription factors *six1*, *p66a,* and *tcp4* were verified for their annotation through alignment of the translated protein coding region of the transcript to the amino acid sequence from *Homo sapiens* (NCBI Protein Database Accession Nos.: *p66a* NP_001287875.1, *tcp4* NP_006704.3, *six1* NP_005973.1), *Xenopus tropicalis* (NCBI Protein Database Accession Nos.: *p66a* NP_001016842.1, *tcp4* NP_001004871.1, *six1* NP_001093693.1), *Xenopus laevis* (NCBI Protein Database Accession Nos.: *p66a* NP_001081980.1, *tcp4* NP_001084564.1, *six1* NP_001082027.1), and *Rana temporaria* (NCBI Protein Database Accession Nos.: *p66a* XP_040176515.1, *tcp4* XP_040193950.1, *six1* XP_040188170.1) using ClustalW sequence alignment with MUSCLE sequence aligner (Edgar, 2004). As *klfX* had a highly conserved DNA binding domain, this transcription factor was aligned with several different krüppel-like factor annotations all with a high percent identity in this conserved zinc-finger motif region. To determine the identity of this krüppel-like factor, 17

Homo sapiens krüppel-like factor amino acid sequences were used to extract the highest aligning sequences in the *Rana catesbeiana* genome (NCBI mRNA Database Accession Nos.:*klf1* NM_006563.5*, klf2* NM_016270.4*, klf3* NM_016531.6*, klf4* NM_001314052.2*, klf5* NM_001286818.2*, klf6* NM_001300.6*, klf7* NM_003709.4*, klf8* NM_007250.5*, klf9* NM_001206.4*, klf10* NM_005655.4*, klf11* NM_003597.5*, klf12* NM_007249.5*, klf13* NM_015995.4*, klf14* NM_138693.4*, klf15* NM_014079.4*, klf16* NM_031918.4*, klf17* NM_173484.4). Sequences were verified by Blastp sequence alignment with the NCBI non-redundant database (Bethesda, 1988). The 14 Krüppel-like factors found in *Rana catesbeiana* were phylogenetically compared using Phylip (Felsenstein, 2009) bootstrapping 1000 times and determining the most fit consensus tree using the maximum likelihood Jones-Taylor-Thorton model and the Hidden Markov Model to infer rates of amino acid changes using a gamma distribution of rates. The sequence determined to be the most phylogenetically related was aligned to identify areas of highest identity using ClustalW alignment (MUSCLE sequence aligner; Edgar, 2004).

Analysis of KlfX amino acid sequence features was done using putative motifs identified by NCBI (Altschul et al., 1990; Bethesda, 1988). Predictions of secondary sequence structure, glycosylation and phosphorylation sites were done using ProteinPredict (Altschul et al., 1997) and PROSITE patterns (de Castro et al., 2006; Sigrist et al., 2012).

3 Results

3.1 Effect of Biopsy Location on the Thyroid Hormone Response

Lack of significant abundance changes were observed for the TH response genes *thra*, *thrb*, and *thibz*, suggesting that baseline or response levels of TH response transcripts does not differ locationally along the tail fin (Figure 14). All TH treated locations were significantly upregulated ($p \leq 0.05$) from all control treated biopsy locations for each of the three transcripts.

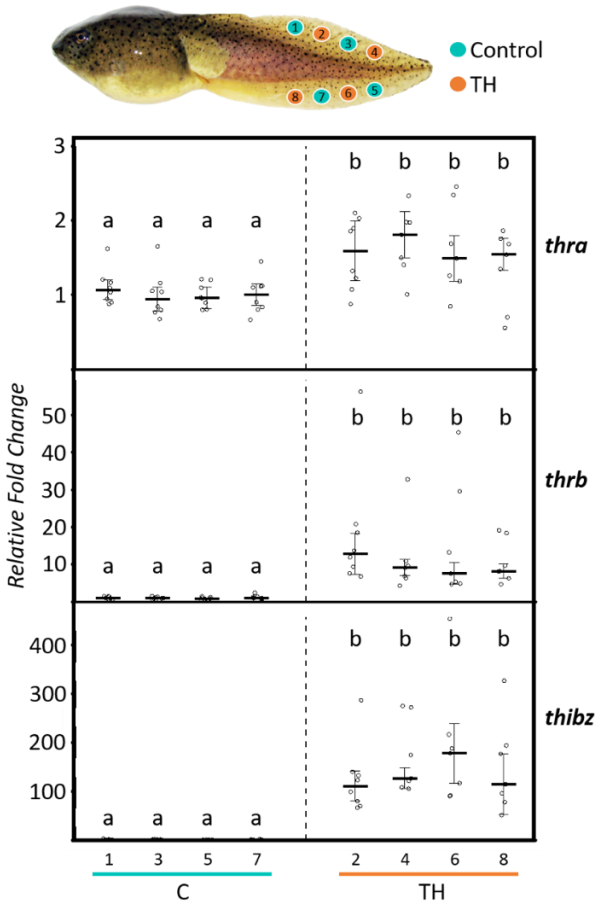


Figure 14: Effect of biopsy location on the *R. catesbeiana* tail fin TH response.
Eight biopsies were taken from the tail fin of eight tadpoles and alternately placed in 400 nM NaOH control (C) or 10 nM T_3 in 400 nM NaOH (TH) for 48h at 24°C. Control biopsy locations are highlighted in teal and TH treated biopsy locations are highlighted in orange on the tadpole tail fin. X-axis number labels correspond to the biopsy location as displayed in the tail fin. qPCR was used to measure the abundance of each transcript. The open circles represent the relative fold change to control samples, with the median designated by the bar and maximum absolute deviation (MAD) designated by the whiskers. Different letters indicate significance from other letters ($p \leq 0.05$).

3.2 Transcript Abundance Regulation Within the Molecular Memory

To identify potential transcripts involved in the molecular memory, tail fin tissue from premetamorphic tadpoles was exposed to 10 nM T_3 in 400 nM NaOH for 48h at 5°C using the C-fin assay followed by RNA sequencing. Each sample had a similar raw read count (Mean = 31,937,896, Standard Deviation = 3,263,646; Supplementary Table 2). Read mapping of the paired reads demonstrated a high degree of mapping with an average of 86.07% (±0.4%) reads aligning to the *R. catesbeiana* genome considering all reads with inaccurate distances between the pairs as unmapped. Final assembled transcripts by Stringtie was a total of 20,332 transcripts found in the C-fin tissue samples. Of these transcripts 67% remained after removal of transcripts with counts below the cut-off of 0.1 counts per million to reduce false positives.

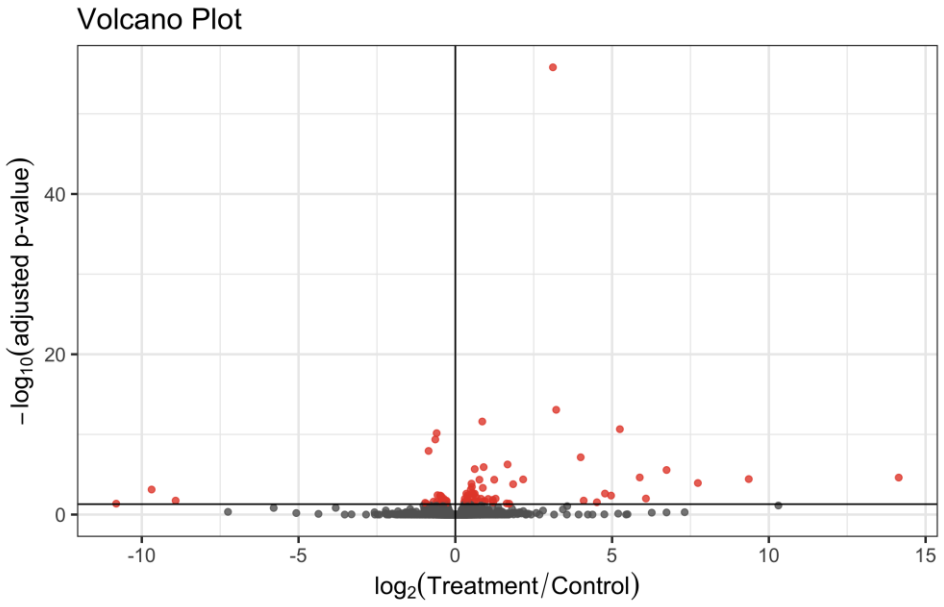


Figure 15: Volcano Plot of Significant Transcripts.
RNA-seq was used to determine significantly altered transcription upon exposure to 10 nM T_3 in 400 nM NaOH compared to 400 nM NaOH control in premetamorphic tadpole *Rana catesbeiana* C-fin (n=5). Red dots indicate the significant transcripts as identified by DESeq2 analysis (p-adj ≤ 0.05). Grey dots indicate transcripts that were not significantly changed. The Logfold response of each transcript can be visualized on the X-axis with the increased transcripts to the right of the zero line and the decreased transcripts to the left.

DESeq2 analysis identified 81 significantly different transcripts upon treatment with TH at 5°C with a Benjamini-Hochberg multiple testing adjusted p-value (p-adj) ≤ 0.05. The greater portion of transcripts regulated by TH at 5°C were upregulated with 53 transcripts increasing in abundance and 28 transcripts decreasing in abundance (Figure 15, Supplementary Table 3).

Despite the majority of significant transcripts having relatively small fold changes (Figure 15), principle component analysis (PCA) of all significantly different transcripts still depicted a separation of control and treatment samples with the first component explaining 39.8% of variation (Figure 16).

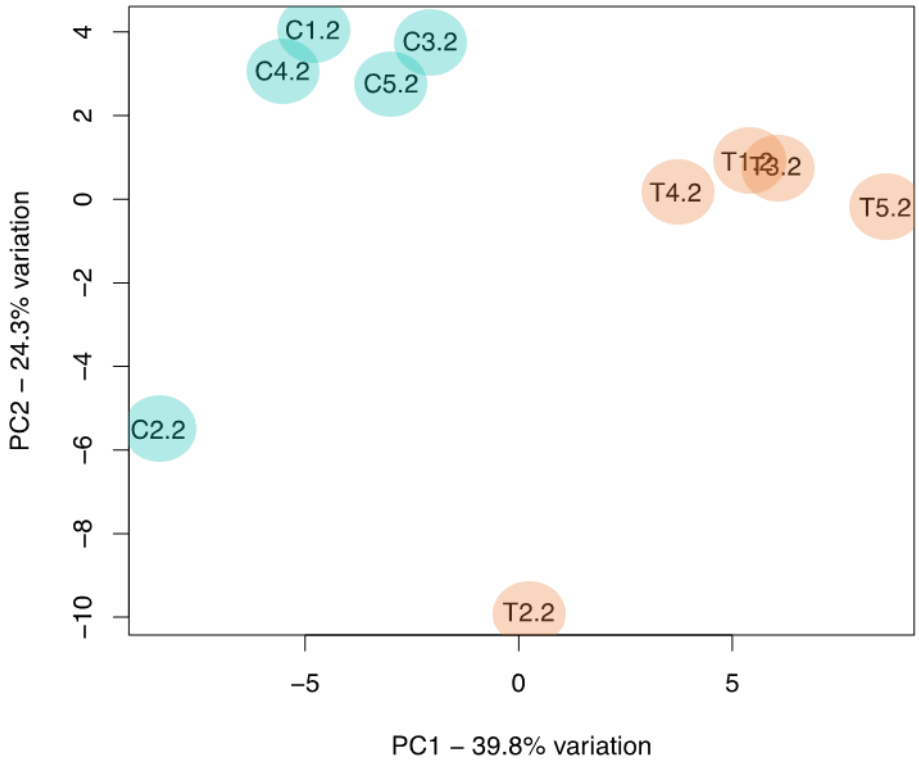


Figure 16: PCA Analysis of all significantly changing transcripts.
Principle component analysis of all significant transcripts identified by DESeq2 (p-adj ≤ 0.05) in response to *Rana catesbeiana* C-fin exposure (n=5) to 10 nM T_3 in 400 nM NaOH treatment (T) or 400 nM NaOH control (C) for 48h at 5°C. Each point represents a different animal with control treated animals highlighted in teal and TH treated animals highlighted in orange.

3.3 Annotation of Significant Transcripts

The NCBI non-redundant and Uniprot databases were able to identify an annotation for 78 out of 81 translated significant transcripts with the average percent identity for the top hit of the identified annotation being 81.5%. When solely functional protein annotations were sought (i.e., not selecting the top annotations consisting of a numerical identifier or hypothetical protein) 41% had greater then 90% identity, 77% had greater than 70% identity and 89% had greater than 50% identity with 4% having no functional annotation (Figure 17).

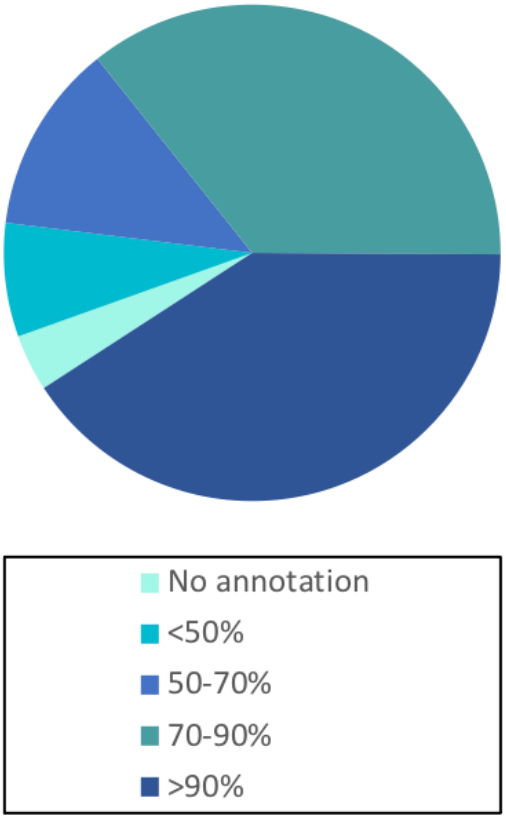


Figure 17: Annotation of significant transcripts.
Count of significant transcripts with the percent identity between the gene used to provide an annotation compared to the significant transcripts being annotated.

3.3.1 Functional Analysis of Molecular Memory Transcripts

GO analysis was used to determine enriched functional categories for which significant transcripts could be grouped to help unveil general biological pathways that may be impacted by TH signalling at cold temperatures. Two parallel methods were used for GO analysis, firstly to identify the functional categories of which the significant transcripts can be categorized (Figure 18) and secondly to explore the relationship between these functional categories (Figure 19). Fifteen percent of annotations were not found in the David database (Huang et al., 2009a, 2009b) and therefore were not included in the GO analysis. Of the remaining 61 transcript annotations that were used, molecular function enriched pathways included those in structural molecule activity, nucleic acid binding, transcription factor activity, and peptidase regulator activity. Nucleic acid binding transcription factor activity had the highest number of transcripts in molecular function. The biological process categories more generally included cellular and biological processes, metabolic process, cell development and adhesion, and immune system response. Given that the number of upregulated transcripts is two-fold greater than downregulated transcripts it is unsurprising that there are more upregulated transcripts in each category. Comparing amongst upregulated transcripts, the majority were grouped into biological or cellular process, or development. The higher number of downregulated transcripts, however, were categorized into metabolic process, multi-cellular organism process and response to stress (Figure 18).

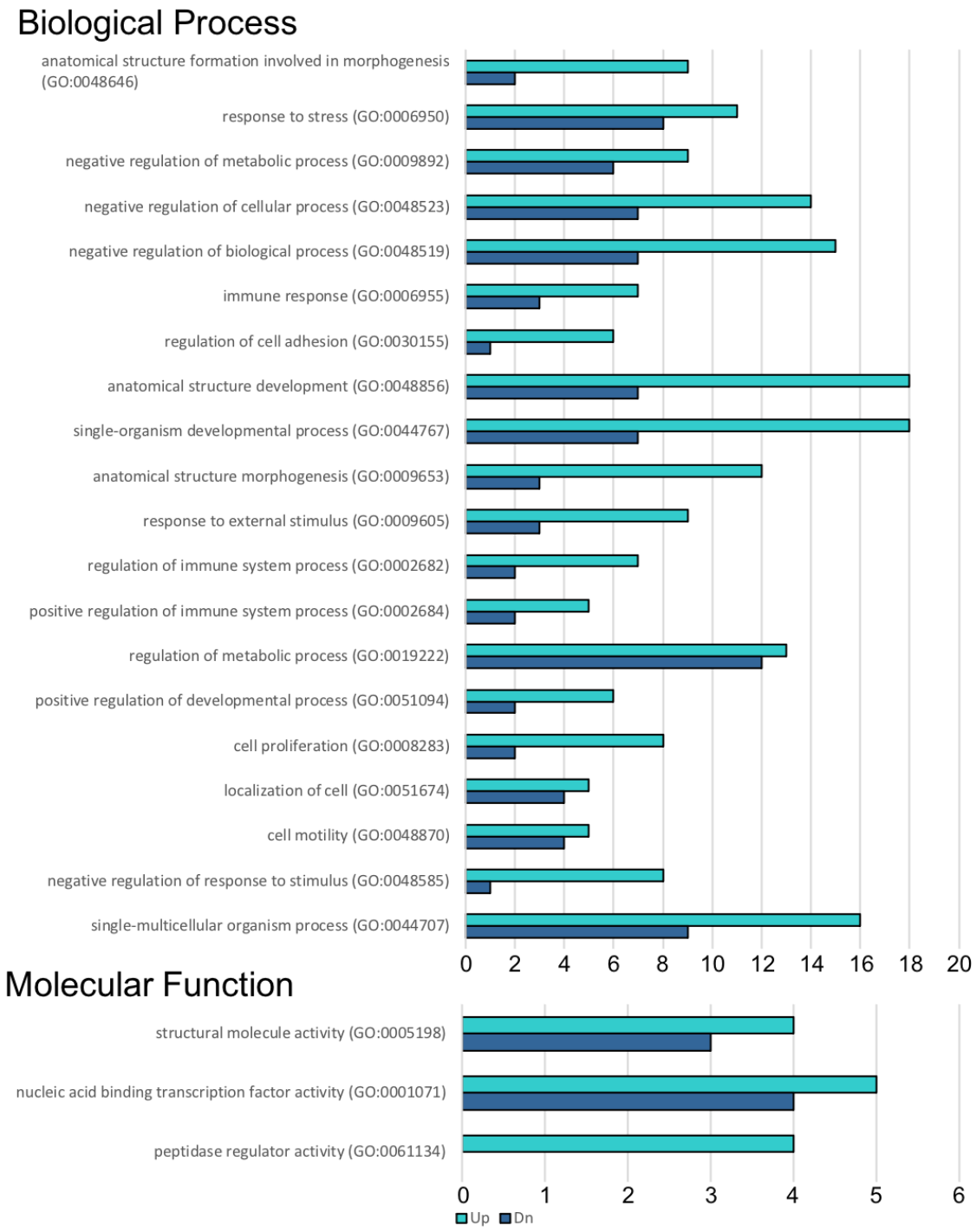


Figure 18: Gene Ontology of Increased and Decreased Transcripts.
Gene ontology analysis was run separately for both significantly increased and significantly decreased transcripts. The X-axis designates the number of increased (aqua) and decreased (navy) transcripts that fell within significantly enriched GO terms ($p \leq 0.05$).

When comparing these enriched terms through a network analysis, these GO categories were tightly interconnected. Seven groupings of these intertwined ontologies could be generally visualized including: biological and metabolic process, DNA binding and transcription factor activity, cell adhesion and differentiation, immune response, enzyme activity, and development (Figure 19). Although a different method of GO designation was used, there was a high amount of overlap between the enriched categories from the two software programs highlighting the robustness of the ontology designations. Similar to the DAVID analysis, the more general categories defined by GO-Seq contained more transcripts, however, had lower significance.

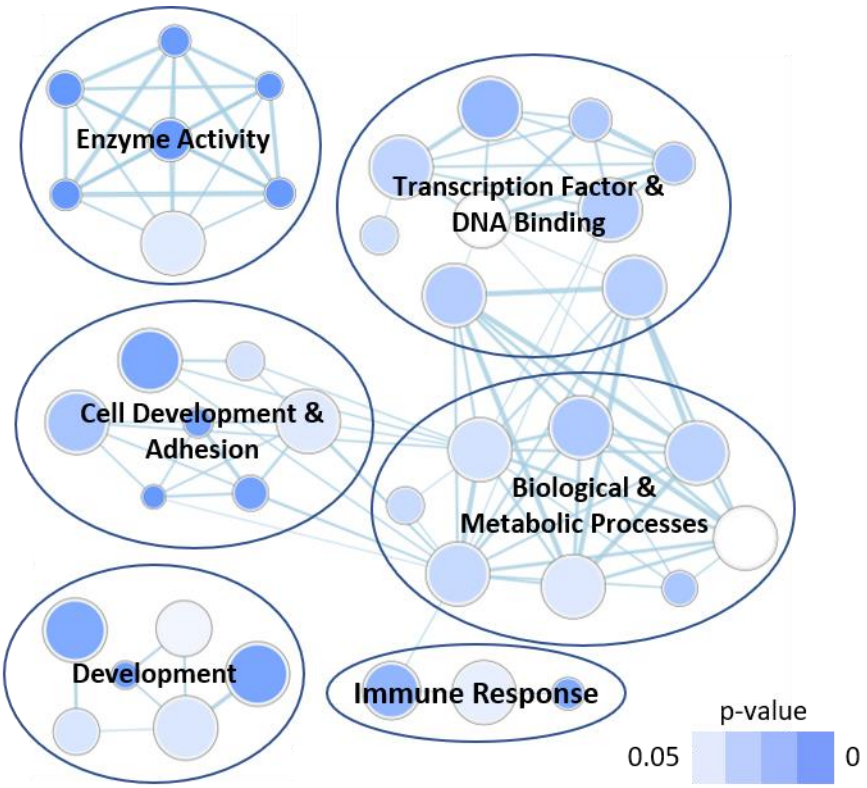


Figure 19: Gene Ontology Enrichment Analysis Network.
Significantly enriched gene ontology categories of significant transcripts in *Rana catesbeiana* C-fin exposed to 10 nM T_3 for 48h at 5°C ($p \leq 0.05$). Node size indicates the number of transcripts found within the category and darker node colour representing lower p-values. Lines represent overlap between transcripts within ontology categories with thicker lines indicating higher numbers of overlapping transcripts.

3.3.2 Transcription Factors associated with the Molecular Memory

There was a total of 14 significant transcription factors identified (Table 1). Half of these transcription factors were upregulated whereas the other half were downregulated. The two greatest increased and decreased transcription factors were *homeobox protein SIX1* (*six1*) and *krüppel-like factor X* (*klfX*), and *transcriptional repressor p66-alpha* (*p66a*) and *RNA polymerase II transcriptional coactivator p15* (*tcp4*), respectively. With the exception of *klfX,* the translated protein sequences of these transcription factors aligned to comparative species genomes including *Homo sapiens*, *Xenopus tropicalis*, *Xenopus laevis,* and *Rana temporaria* improving the likelihood of their designated annotation (Supplementary Figures 5-7).

The KlfX protein sequence was confirmed to be a putative krüppel-like factor as it falls under previously defined sequence specifications of having a three zinc finger motif along a total of 81 amino acids with a canonical aspartic acid residue in the second zinc finger on the 44^{th} residue which stabilizes DNA binding (Presnell et al., 2015). KlfX had the highest alignment identity to the C-terminal end in the conserved DNA-binding zinc finger motif region when compared to the 14 other identified krüppel-like factors in the *R. catesbeiana* genome (Supplementary Figure 8). However, the alignment of KlfX had a low identity on the N-terminal end, the region that interacts with other transcriptional regulators (McConnell and Yang, 2010). Phylogenetically, the unknown KlfX protein sequence was most closely related to Klf5 (Figure 20a). Alignment of KlfX with Klf5 showed that, even with the most closely related krüppel-like factor, the percent identity was only 33.74% with the highest amino acid similarity in the zinc finger domain and a high amount of variation on the N-terminal end (Figure 20b).

Table 1: Significant transcription factors in the molecular memory.
Transcription factors identified as significantly up- or down-regulated by DESeq2 in response to *R. catesbeiana* C-fin exposure to 10 nM T_3 for 48h at 5°C. FC indicates fold change from control condition of 400 nM NaOH with significance determined at a Benjamini-Hochberg adjusted p-value (p-adj) ≤ 0.05.

Transcription factor	FC	p-adj
Homeobox protein SIX1 (six1)	3.6	0.0002
Krüppel-like factor X (klfx)	2.5	0.01
Thyroid hormone-induced B/Zip (thibz)	1.8	0.0005
PR domain zinc finger protein 1	1.4	0.0004
Cysteine/serine-rich nuclear protein	1.4	0.0001
Proto-oncogene c-Fos	1.4	0.04
Zinc finger protein SNAI2	1.3	0.02
High mobility group protein B2	0.75	0.04
Transcription factor SOX-21	0.7	0.02
DNA-binding protein inhibitor ID-4	0.65	0.02
Forkhead box protein Q1	0.65	7E-11
Cyclin L2	0.6	0.02
Transcriptional repressor p66-alpha (p66a)	0.001	0.008
RNA pol II transcriptional coactivator p15 (tcp4)	0.0006	0.04

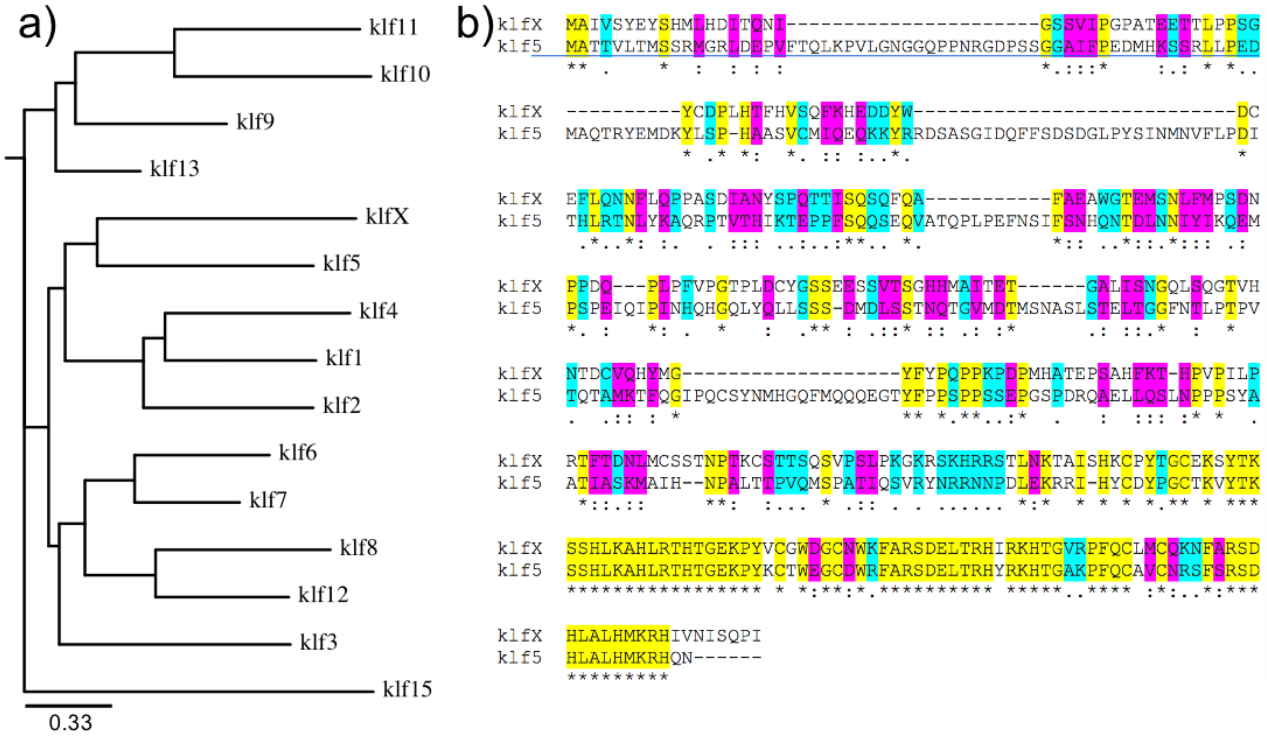


Figure 20: Inferring the relationship of KlfX to previously defined krüppel-like factors through maximum likelihood phylogenetic analysis and sequence alignment.
a) Phylogenetic analysis was used to infer the evolutionary relationship between the krüppel-like factors by protein sequence conservation using the Jones-Taylor-Thorton model. The maximum-likelihood was calculated from the consensus of 1000 bootstrap replicates using the Hidden Markov Model with a gamma distribution to infer rates of amino acid change. b) The protein sequences of KlfX and Klf5 were aligned using ClustalW to highlight identity between these related krüppel-like factors. Yellow or star indicates a conserved residue, pink or ":" indicates a conserved amino acid residue with strongly similar properties, and blue or "." indicates a conserved amino acid residue with weakly similar properties.

Computational analysis of the amino acid sequence features of KlfX show that, similar to other krüppel-like factors (McConnell and Yang, 2010), the putative protein binding sites are on the N-terminal end of the protein whereas the DNA-binding sites (zinc finger domains) are concentrated to the C-terminal end. Based on sequence pattern features the protein is also predicted to be able to be glycosylated and phosphorylated.

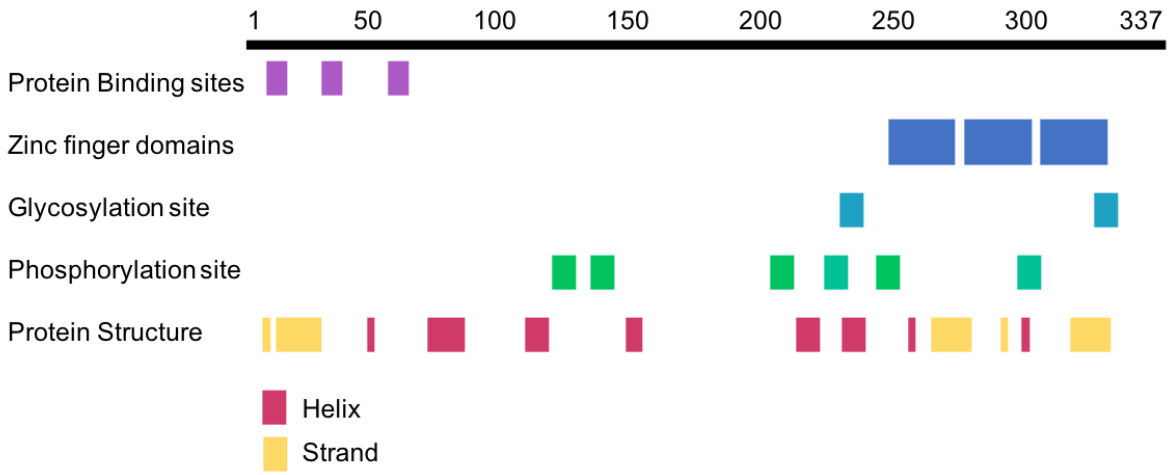


Figure 21: Putative KlfX motifs and structure.
Amino acid sequence-based identification of putative motifs including protein binding sites and zinc finger domains (DNA binding sites). As well as possible glycosylation and phosphorylation sites. Secondary protein structure elements were also predicted based on sequence elements. Analysis of KlfX amino acid sequence features was done using putative motifs identified by NCBI (Altschul et al., 1990; Bethesda, 1988). Predictions of secondary sequence structure, glycosylation and phosphorylation sites were done using ProteinPredict (Altschul et al., 1997) and PROSITE patterns (de Castro et al., 2006; Sigrist et al., 2012).

3.4 Expression Dynamics of Thyroid Hormone Response Genes in Cold Temperatures

C-fin biopsies were treated with TH at 5^{o}C for a time course from 0 to 96h to further elucidate the expression dynamics of TH response genes at cold temperatures. Transcripts that previously were not significantly changed within 48h (Hammond et al., 2016a), including *thra* and *thrb*, had no change in abundance throughout the 96h time course with the exception of *cebp1* (Figure 22). *Cebp1* slightly increased in expression by 1.6-fold over the longer period of exposure of 72h but was insignificant by 96h which could indicate a transient small increase in this transcript's response or some variability within this transcript's regulation at cold temperatures.

For the transcripts that decreased in abundance upon TH exposure*, tcp4* decreased by 0.61 by 24h and continued to decrease to 0.22 by 96h and *p66a* decreased to 0.61 by 48h (Figure 23). Of the significantly increasing transcription factor transcripts tested, *klfX* was upregulated by 12h by 2-fold and continued to increase to a 6.6-fold increase at 72h (Figure 23). *Thibz* was upregulated by 2.5-fold by 18h with a maximal 52-fold change by 72h (Figure 22). *Six1* significantly decreased slightly at 6h by 0.5-fold and increased significantly by 6.1-fold by 24h (Figure 23).

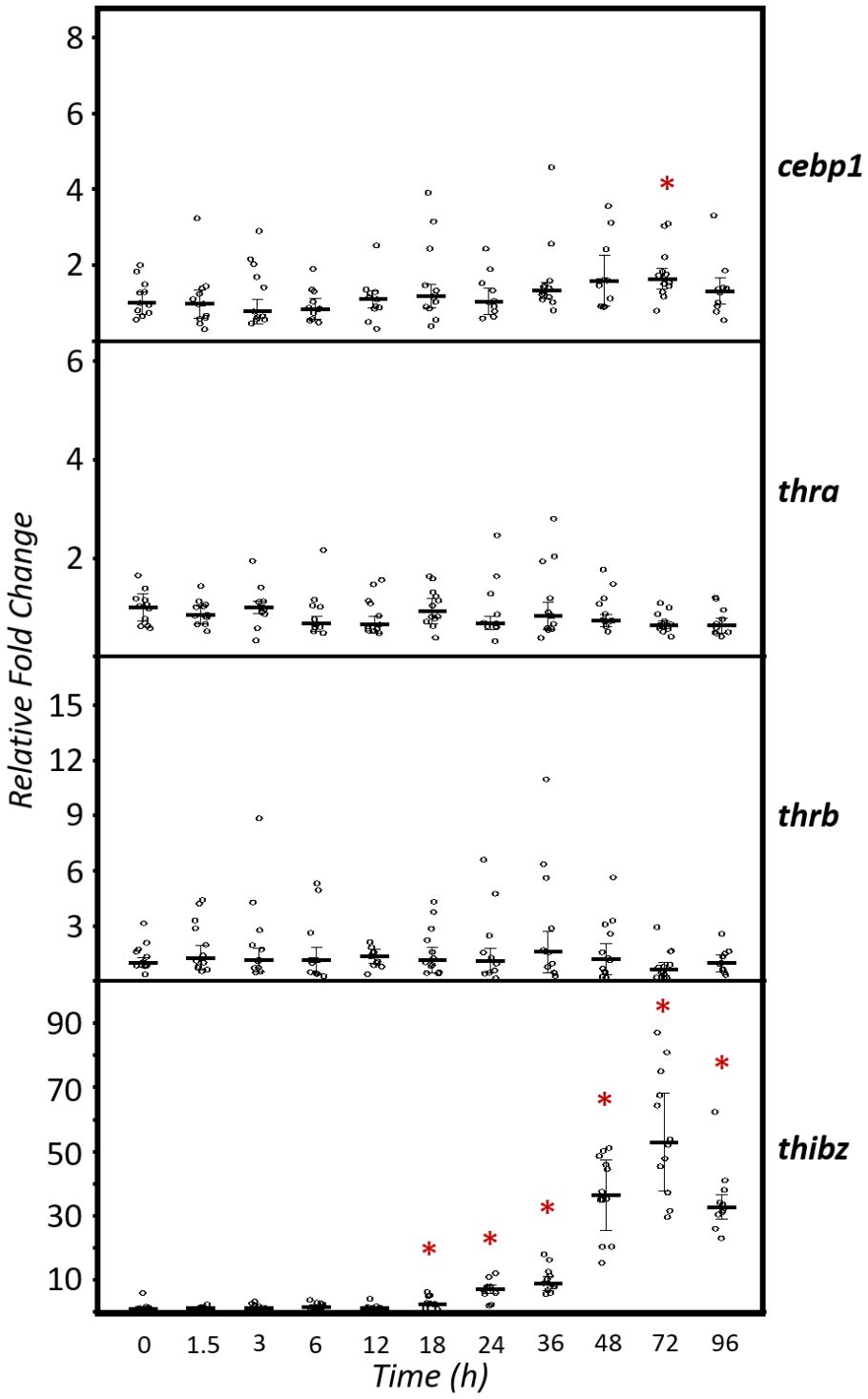


Figure 22: Expression dynamics of TH response genes at 5°C.
Expression of *cebp1*, *thra*, *thrb* and *thibz* in *Rana catesbeiana* tail fin biopsies (n=12) exposed to 10 nM T_3 in 400 nM NaOH for a time course of 0 to 96h at 5°C. * indicates significance in the median relative fold change from the 0h timepoint ($p \leq 0.05$). See Figure 14 for additional plot details.

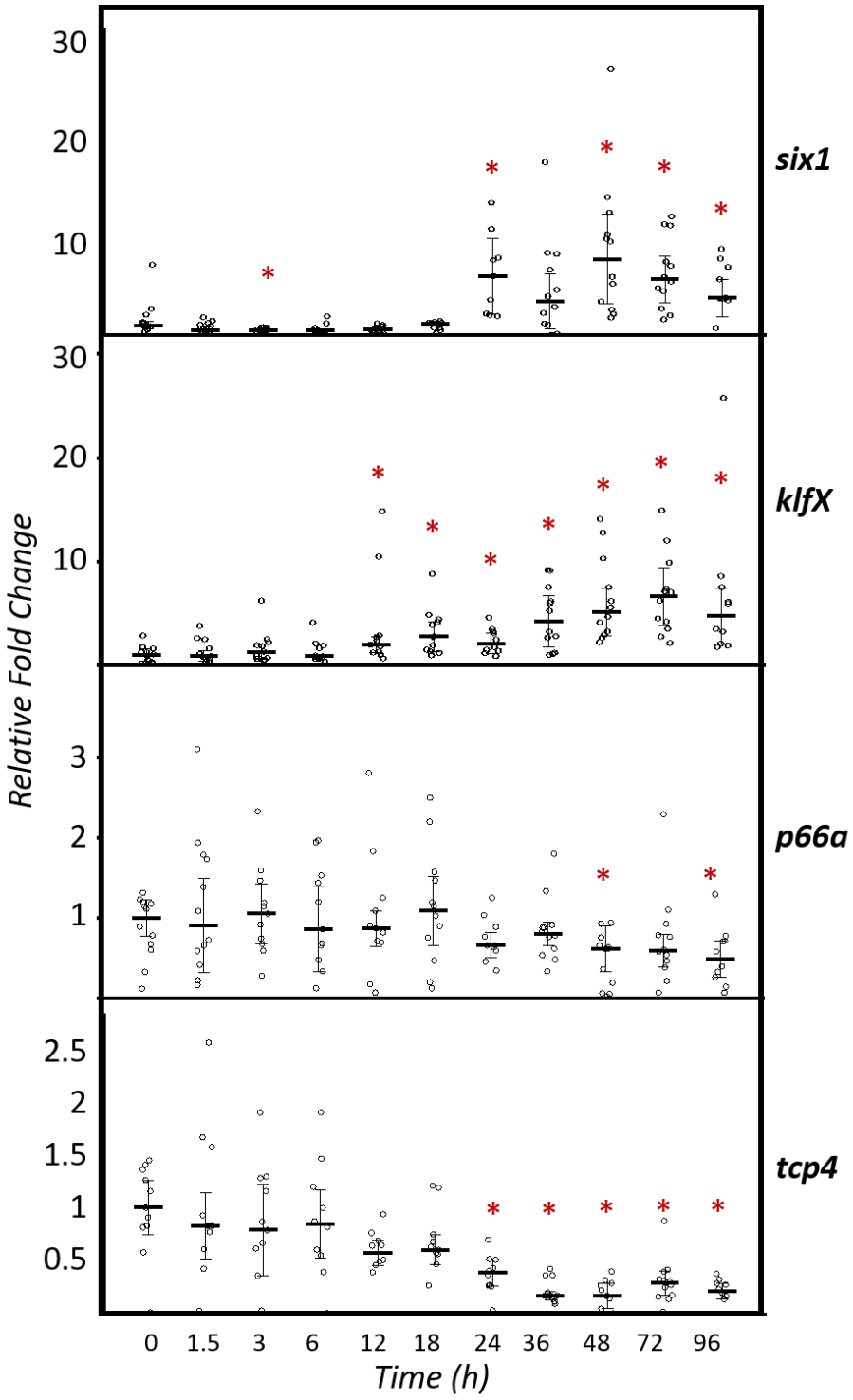


Figure 23: Expression dynamics of TH responsive transcription factors at 5C.
Expression of *six1*, *klfX*, *p66a* and *tcp4* in *Rana catesbeiana* tail fin biopsies (n=12) exposed to 10 nM T_3 in 400 nM NaOH for a time course of 0 to 96h at 5°C. * indicates significance in the median relative fold change from the 0h timepoint ($p \leq 0.05$). See Figure 14 for additional plot details.

3.5 Direct Response Nature of TH Response Genes

Similar to Das et al. (2009) and Wang and Brown (1993), a translation inhibitor was used to query the ability of genes to respond directly to TH. C-fin biopsies were exposed to the translation inhibitor CHX for 1h preceding TH exposure for 24h at 5^{o}C in order to inhibit translation of downstream protein synthesis initiated from TH signalling. CHX had no effect on the ability for *thibz*, *six1*, *klfX*, and *tcp4* to be regulated upon TH exposure, with neither the control nor TH treated conditions decreasing upon exposure to this inhibitor. *P66a* expression was not affected by CHX or TH by 24h (Figure 25) although, due to the shorter exposure period chosen to avoid cytotoxic effects of protein synthesis inhibition, the response of this transcript to TH by this timepoint was not expected. Inhibition of *de novo* protein synthesis slightly, but significantly, increased the expression of *thra* and *thrb* in cold temperatures regardless of TH exposure (Figure 24). Where *thra* increased by 1.1-fold in the control condition and 1.2-fold in the TH treated condition when exposed to ActD. Similarly, *thrb* increased by 1.9-fold in the control condition and 1.4-fold in the TH treated condition. The decrease in abundance of both TRs indicates that there may be active repression of their transcription that occurs in cold temperatures and requires a *de novo* synthesized protein. To ensure tissue responsiveness to CHX, deiodinase genes were queried as transcripts are suppressed by cold temperatures (Mochizuki et al., 2012) and repression of deiodinases depends on active protein synthesis (Chik et al., 2007). Both *dio2* and *dio3* were increased by protein synthesis inhibition in the control condition by 2.1- and 2.6-fold, respectively, indicating a functional CHX exposure, however this effect was eliminated upon exposure to TH (Supplementary Figure 9).

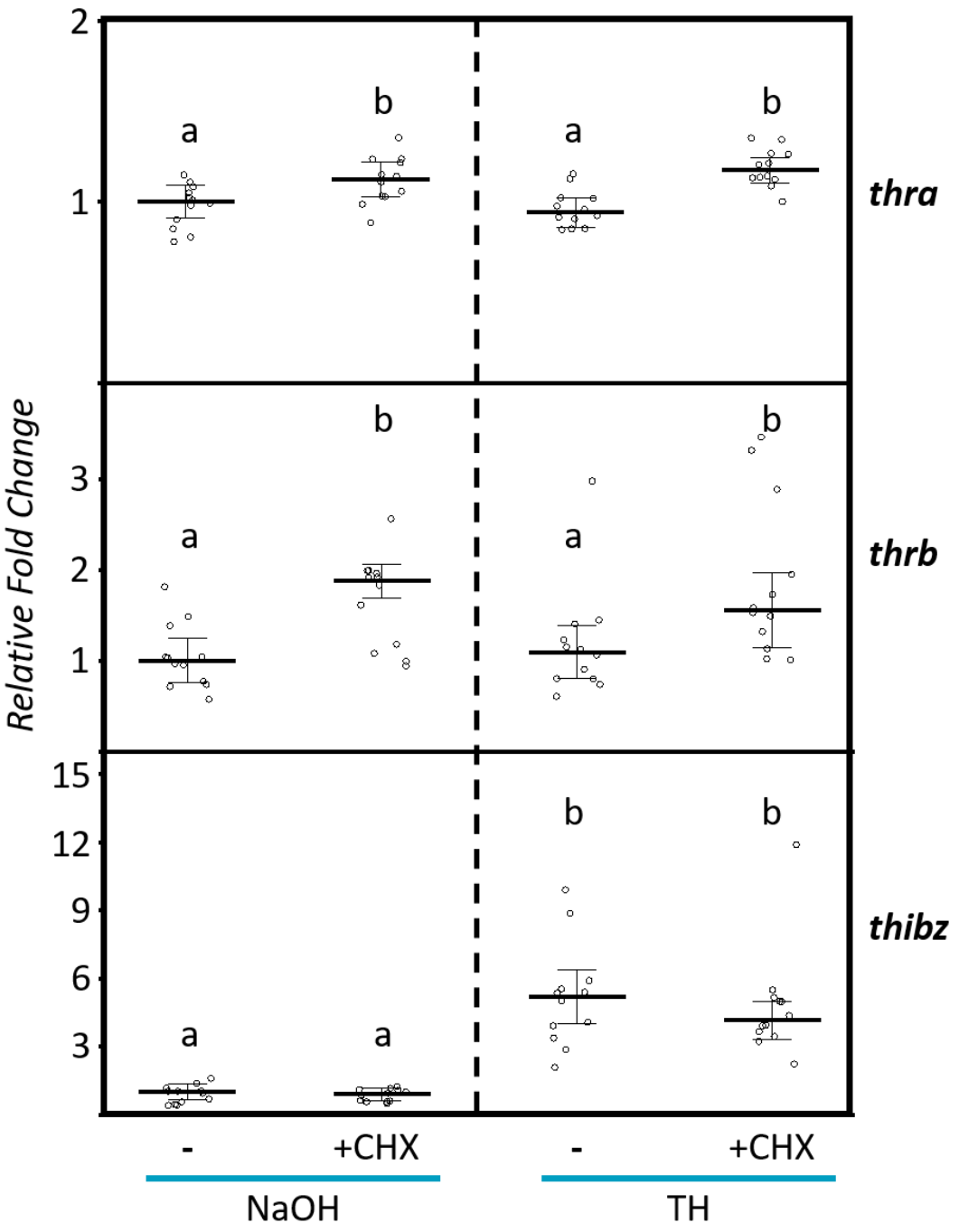


Figure 24 Inhibition of translation on TH response genes.
Median relative fold change in expression of *thra*, *thrb* and *thibz* in *Rana catesbeiana* tail fin biopsies (n=12) exposed to 80 μg/mL CHX, 10 nM T_3 (TH), 80 μg/mL CHX and 10 nM T_3 compared to 400 nM NaOH control (NaOH) for 24h at 5°C. Different letters indicate a significant difference between treatments ($p \leq 0.05$). See Figure 14 for additional plot details.

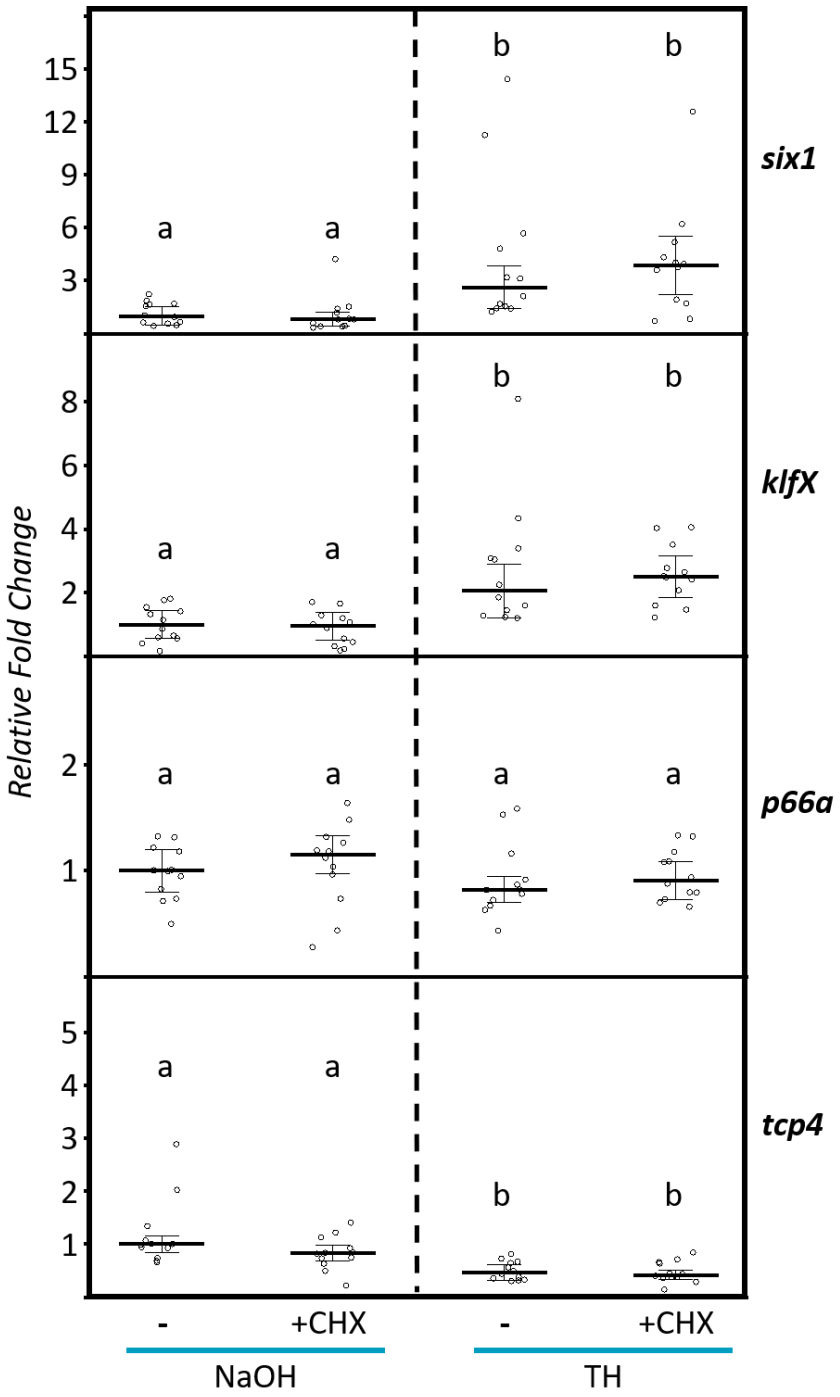


Figure 25: Inhibition of translation on TH response transcription factors.
Median relative fold change in expression of *six1*, *klfX*, *p66a* and *tcp4* in *Rana catesbeiana* tail fin biopsies (n=12) exposed to 80 μg/mL CHX, 10 nM T_3 (TH), 80 μg/mL CHX and 10 nM T_3 compared to 400 nM NaOH control (NaOH) for 24h at 5°C. No transcripts were affected by CHX exposure to the NaOH or T_3 treatment matched control ($p \leq 0.05$). Different letters indicate a significant difference between treatments ($p \leq 0.05$). See Figure 14 for additional plot details.

3.6 Active Transcription Requirements for Molecular Memory Transcripts

To further elucidate the mechanism by which abundance levels of TH response transcripts are altered, biopsies were exposed to the transcription inhibitor ActD. ActD did not have an effect on the TH response of the two TR-encoding transcripts that do not respond to TH at cold temperatures. Although, *thra* decreased by 0.86-fold when exposed solely to the transcription inhibitor, not considering TH exposure (Figure 26).

The upregulated *thibz* (Figure 26) and *klfX* (Figure 27) transcripts, were both responsive to TH within 24h, however for both transcripts, inhibition of active transcription had no impact on their regulation by TH. The only transcription factor-encoding transcript whose regulation was affected was *tcp4* which, as expected, decreased in abundance due to TH exposure by 0.5-fold and further decreased from exposure to ActD both in the control and TH treated condition by 0.5- and 0.6-fold, respectively.

Similar to what was seen in the protein inhibition exposures, *p66a* was unresponsive to either TH or ActD treatment in the 24h exposure (Figure 27). Surprisingly, *six1* was also unresponsive to both treatments (Figure 27). Given that the abundance of this transcript in the control and TH exposed conditions (without exposure to inhibitor) was much lower than the identical exposure conditions in the CHX exposures, the observation of no alteration of regulation is possibly due to a negative response to the acetone solvent. Of note, transcripts used for input normalization, *rpl8*, *rps10*, and *eef1a* also appeared to be affected by the acetone solvent as, for both control and inhibitor treated biopsies, CT values of all three normalizer transcripts was lower. The level of decrease was the same across treatments,

however, allowing this response to be controlled for through comparison to the identically treated control biopsies (Supplementary Figure 10).

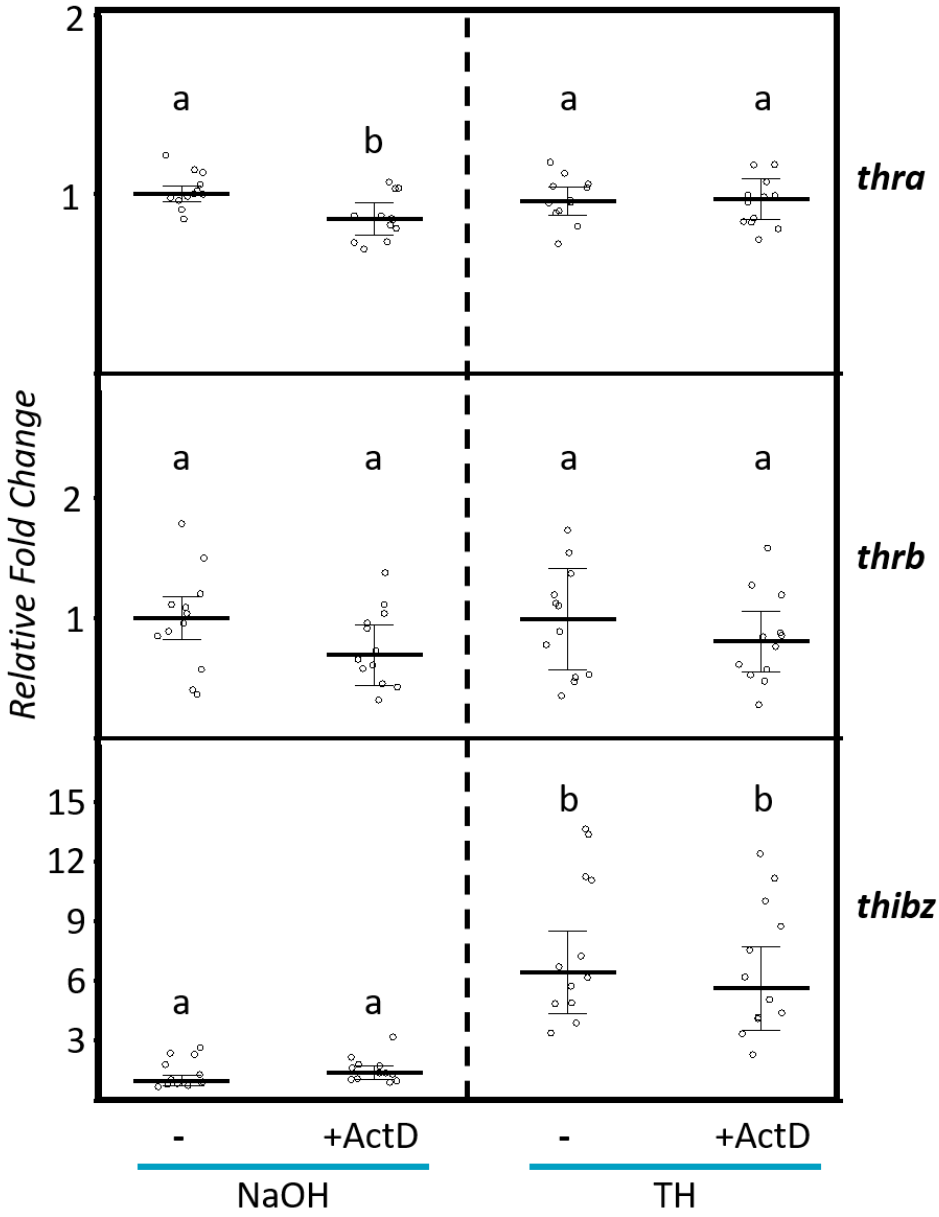


Figure 26: Inhibition of transcription on TH response genes.
Median relative fold change in expression of *thra*, *thrb* and *thibz* in *Rana catesbeiana* tail fin biopsies (n=12) exposed to 20 μg/mL ActD, 10 nM T_3 (TH), 20 μg/mL ActD and 10 nM T_3 compared to 400 nM NaOH control (NaOH) for 24h at 5°C. Different letters indicate a significant difference between treatments ($p \leq 0.05$). See Figure 14 for additional plot details.

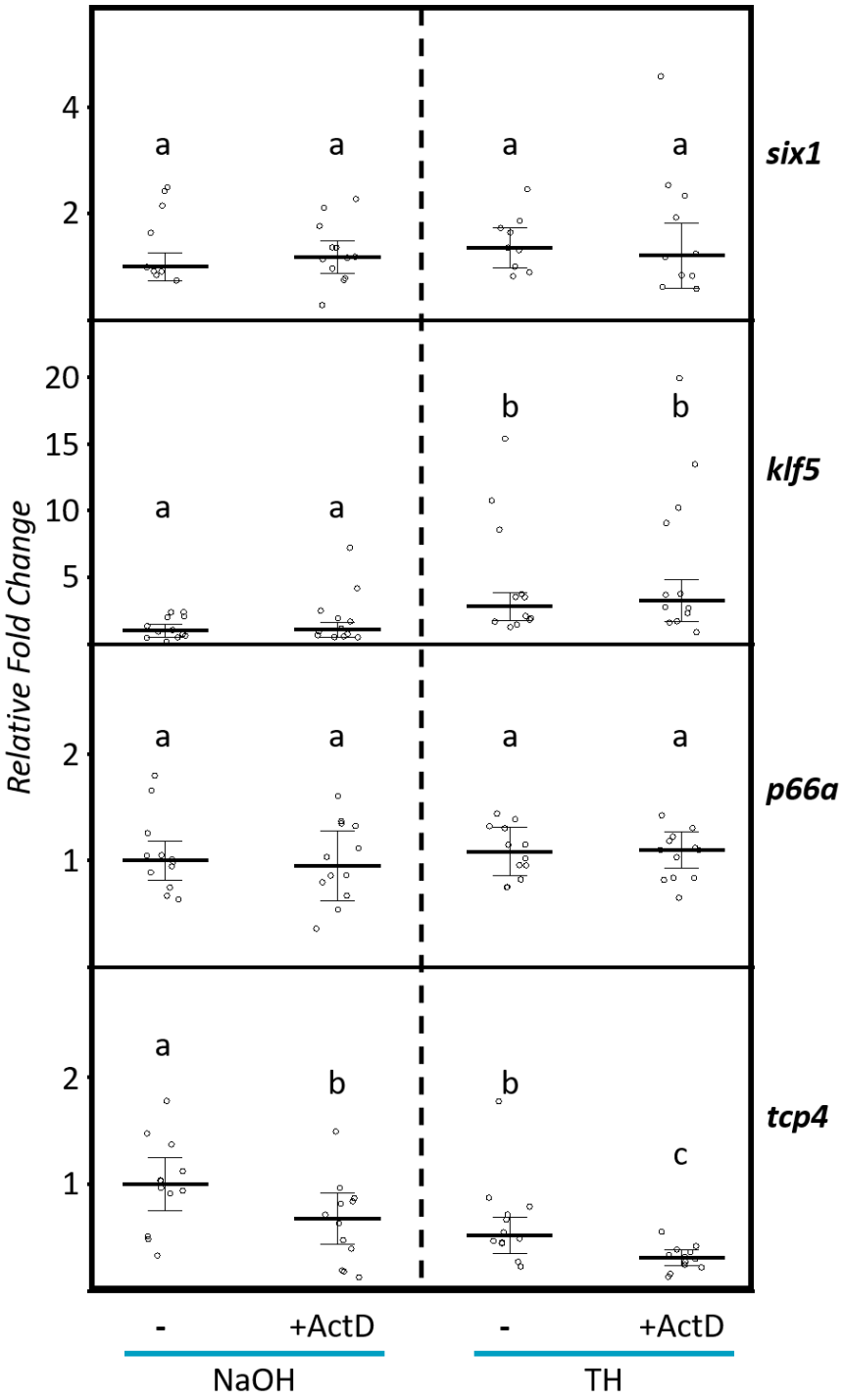


Figure 27: Inhibition of transcription on TH response transcription factors.
Median relative fold change in expression of *six1*, *klfX*, *p66a* and *tcp4* in *Rana catesbeiana* tail fin biopsies (n=12) exposed to 20 μg/mL ActD, 10 nM T_3 (TH), 20 μg/mL ActD and 10 nM T_3 compared to 400 nM NaOH control (NaOH) for 24h at 5°C. Different letters indicate a significant difference between treatments ($p \leq 0.05$). See Figure 14 for additional plot details.

3.7 Acceleration of the TH-Induced Response: Effect of Molecular Memory

To determine whether the molecular memory affects the accelerated response of metamorphosis when more permissive temperatures occur, which has previously been studied on the morphological level (Ashley et al., 1968; Frieden et al., 1965), the regular expression dynamics of early TH response transcripts were queried using a short time course of 0 to 16h at 24^{o}C. This was compared to an identical time course at 24^{o}C after the establishment of the molecular memory through a 48h exposure to TH at 5^{o}C. In permissive temperatures, the canonical TH response transcript *thrb*, which is not responsive to TH within the molecular memory, upregulated by 4.8-fold upon exposure to TH for 16h (Figure 28). When the molecular memory was induced and then biopsies were shifted to 24^{o}C, *thrb* saw a significant increase in abundance at a faster rate than the same exposure without the molecular memory priming, upregulating by 4.5-fold in 8h. *Thrb* also had a significantly higher response to TH after 16h in permissive temperatures after priming with the molecular memory, with about a 24-fold increase. Similarly, *thra* was increased by 1.1-fold in permissive temperature conditions after 8h. When brought into cold temperatures, disregarding TH treatment, *thra* decreased in abundance by 0.5-fold solely due to cold temperature exposure. Although there was an increase of *thra* compared to the untreated condition in cold temperatures, *thra* did not rise to a level significantly greater than the untreated condition in permissive temperatures after 16h of exposure (Figure 28).

The transcription factors *cebp1*, *thibz*, *six1* and *klfX* were upregulated by 48h TH exposure at 5^{o}C (Figure 28 and Figure 29). With the exception of *six1*, these four transcripts were all upregulated in permissive temperatures within the 16h time course with *klfX* having the earliest

response with an 8.7-fold increase at 1h, *thibz* with a 17-fold increase at 4h and *cebp1* with a 3.1-fold increase at 8h. Similar as to what was seen in *thra*, *thibz* transcripts abundance was decreased by 0.3-fold solely due to cold temperature exposure. When exposed to TH for 48h at 5^{o}C, however, *thibz* levels rose 14-fold compared to the permissive temperature control, which is similar to what is seen after 4h of TH treatment in permissive temperatures. Once shifted to permissive temperatures in 4h, *thibz* transcript levels increased by 104-fold which is a similar fold-change as reached by solely permissive temperature exposures by 16h and peaked at 8h at 145-fold greater than the untreated 24^{o}C condition. Similarly, although not as dramatically as *thibz*, *six1* increased by 19-fold from the permissive temperature control after shifting the 5^{o}C exposed biopsies into more permissive temperatures. Both *cebp1* and *klfX* increased by 2- and 4.4-fold, respectively, after 48h exposure at 5^{o}C but when shifted to more permissive temperatures did not significantly increase further.

The two TH responsive transcription factors that decrease in abundance upon TH exposure both had supressed expression after 48h exposure to TH at 5^{o}C by 0.6-fold for *p66a* and 0.3-fold for *tcp4* (Figure 29). For *tcp4*, however, the effect of temperature alone led to the increase in expression of this transcript by 3.1-fold and exposure to TH significantly decreased *tcp4* expression from the untreated 5^{o}C condition but not the untreated 24^{o}C condition. Once shifted to more permissive temperatures the abundance of both transcripts did not change significantly from abundance levels established within the molecular memory. Comparatively, the identical permissive temperature time course without priming in cold temperatures, *tcp4* was downregulated by 0.6-fold by 4h and contrarily *p66a* saw no significant change in abundance throughout the 16h time course.

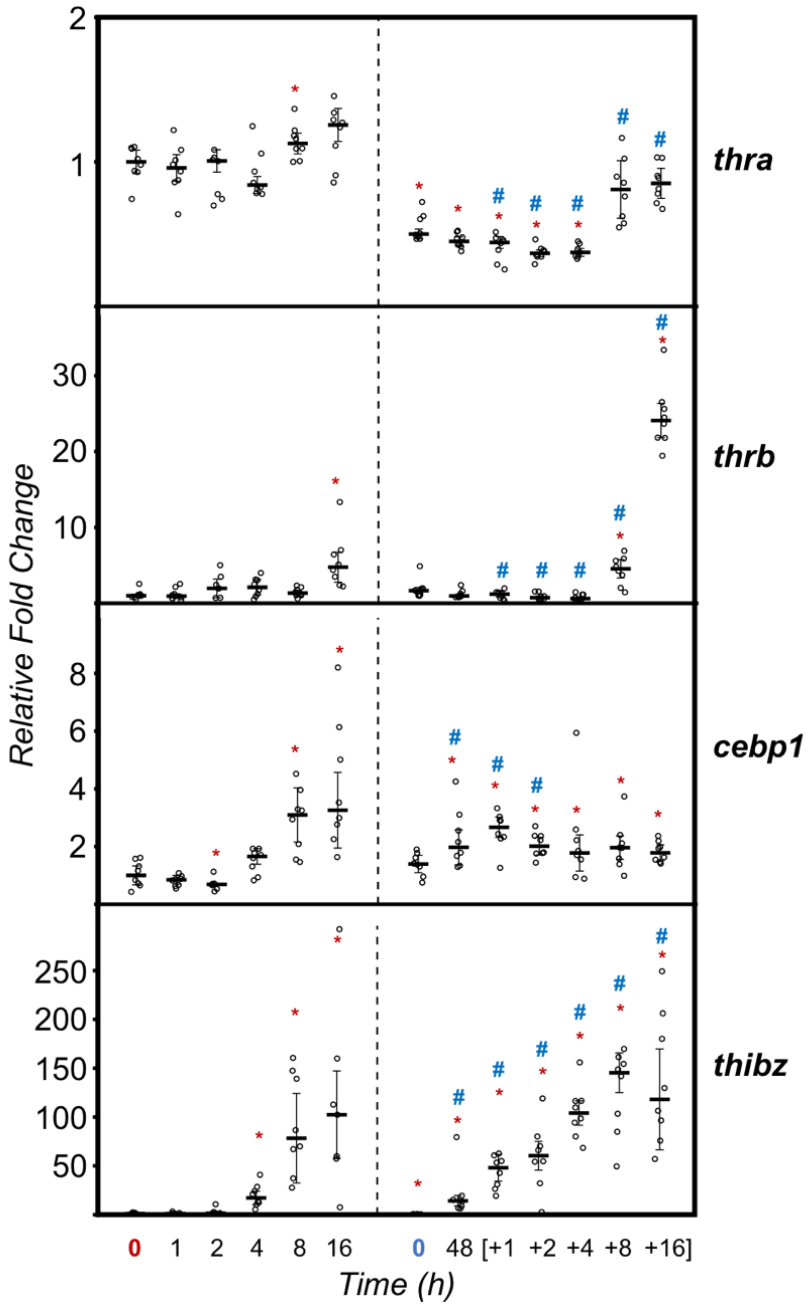


Figure 28: Accelerated response of TH responsive transcripts.
Expression of *thra*, *thrb, cebp1* and *thibz* in *Rana catesbeiana* tail fin biopsies (n=12) exposed to 10 nM T_3 in 400 nM NaOH for a time course of 0 to 16h at 24°C or comparatively, to 10 nM T_3 in 400 nM NaOH for 48h at 5°C then washed to remove exogenous T_3 and shifted to 24°C for a time course of 0 to 16h. * indicates significance in the median relative fold change from the 0h timepoint at 24°C (red zero; $p \leq 0.05$). # indicates significance in the median relative fold change from the 0h timepoint at 5°C (blue zero) for the cold temperature-primed biopsies ($p \leq 0.05$). See Figure 14 for additional plot details.

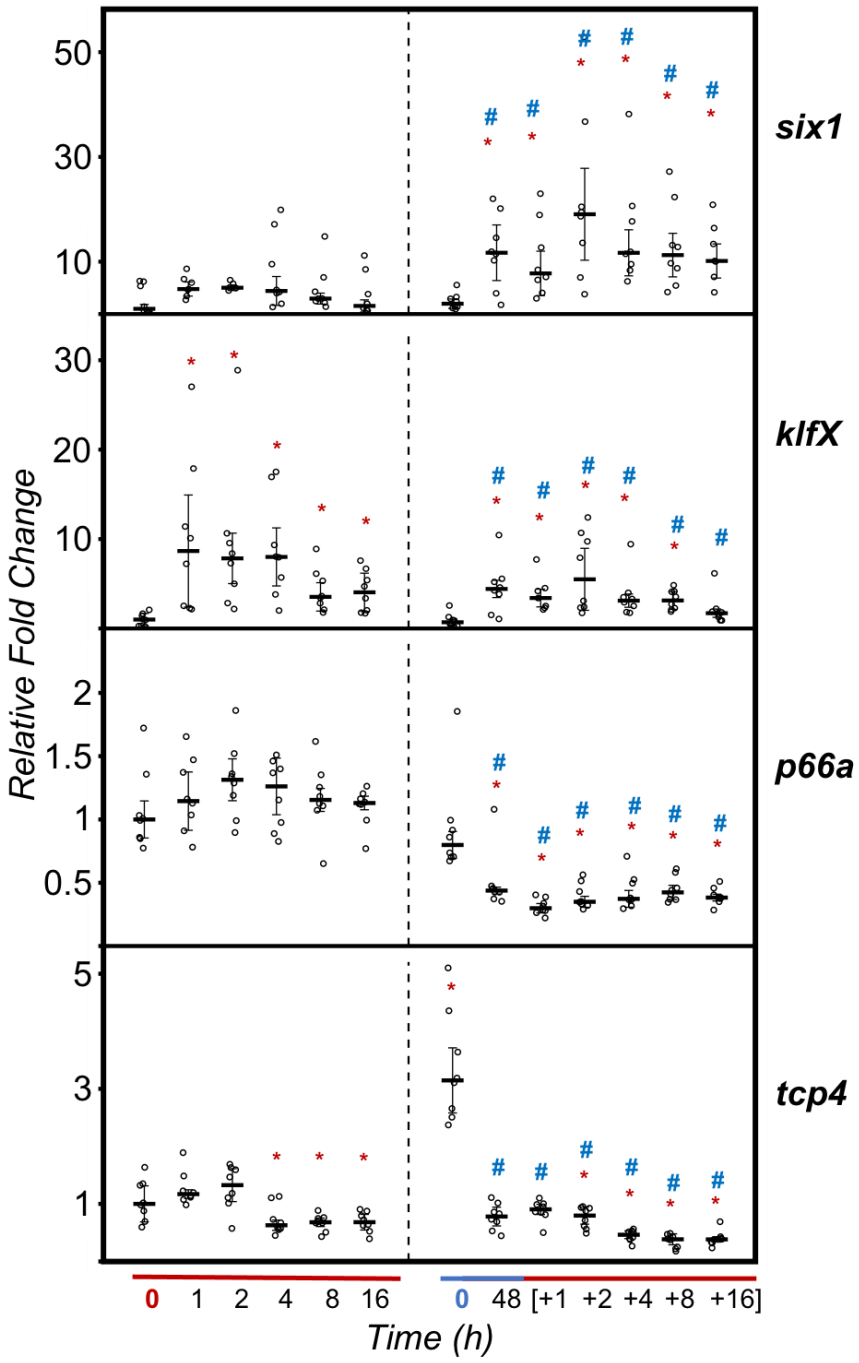


Figure 29: Accelerated response of TH response transcription factors.
Expression of *six1*, *klfX*, *p66a* and *tcp4* in *Rana catesbeiana* tail fin biopsies (n=12) exposed to 10 nM T_3 in 400 nM NaOH for a time course of 0 to 16h at 24°C or comparatively, to 10 nM T_3 in 400 nM NaOH for 48h at 5°C then washed to remove exogenous T_3 signal and shifted to 24°C for a time course of 0 to 16h. * indicates significance in the median relative fold change from the 0h timepoint at 24°C (red zero; $p \leq 0.05$). # indicates significance in the median relative fold change from the 0h timepoint at 5°C (blue zero) for the cold temperature-primed biopsies ($p \leq 0.05$). See Figure 14 for additional plot details.

4 Discussion

4.1 Cold Temperature Transcriptomic Program

Temperate dwelling *Rana catesbeiana* tadpoles have evolved to allow temperature to cue postembryonic development, prompting overwintering tadpoles to metamorphose when a more permissive temperature arrives in spring. Temperature's influence has been well described on the experimental level where at low temperatures, premetamorphic tadpoles will arrest development even when exposed to the initiating hormone, TH (Frieden et al., 1965; Fry, 1972). When then brought to permissive temperatures, cold TH-treated tadpoles will undergo an accelerated metamorphosis even after 80 days when the TH signal has long been metabolized, indicating that a molecular memory is established in cold temperatures where the TH signal is initiated but not executed (Ashley et al., 1968).

As early TH response genes are likely to play a crucial role in transduction of the TH signal to the ensuing complex postembryonic developmental program, there is a need to further understand the regulation of these first initiating responses in TH signalling. Using short time courses in warm temperatures, Wang and Brown (1993) identified in *Xenopus laevis* tadpoles (that are not cold-tolerant) that there appeared to be a two stage program in early TH response, with the earliest transcripts responding within 8h followed by a secondary regulation of transcripts after 15h. The use of protein synthesis inhibitors enabled the uncoupling of this initial group of TH response transcripts by distinguishing between direct response transcripts involved in the induction of the TH response and secondary transcripts involved in the subsequent execution of the TH signalling program (Das et al., 2009; Shi and Brown, 1993;

Wang and Brown, 1993). The transcripts encoding transcription factors *thrb* and *thibz* are within this early direct response category in whole tadpole, hindlimb, head, intestine, and tail, increasing within 2 to 4h after TH induction in permissive temperatures (Kanamori and Brown, 1992; Shi and Brown, 1993). In cold temperatures, however, transcriptomic profiling of *R. catesbeiana* has found that after 48h of TH exposure, *thibz* is competent to respond to TH at cold temperatures and yet *thrb* is not (Hammond et al., 2016, 2015). This discrepancy indicates that the molecular memory may be able to be used as a natural method to further stratify the early response transcripts involved in the induction of the TH response.

To date, the transcription factor *thibz* has been the only transcript found that reliably responds to the TH signal in the tail fin at 5^{o}C. However, given that the tail fin of *Rana catesbeiana* has shown to have almost 5,000 transcripts respond to TH within 48h in permissive temperatures (Partovi, 2018) and in whole *Xenopus laevis* tadpole, 437 TH response genes were identified as putative direct targets of TH through their resistance to protein synthesis inhibitors (Das et al., 2009), it is notable that we found 80 other candidate transcripts potentially involved in the molecular memory.

Many of the most TH-responsive transcripts at 5^{o}C did not overlap those identified at permissive temperatures in tail fin (Partovi, 2018). In fact, a few transcripts showed inverse responses comparing the two temperatures. For example, *Cytokeratin-19* mRNA that encodes an epithelial intermediate filament which has previously been shown in cultured *Xenopus laevis* intestine to be higher in adult progenitor epithelial cells compared to larval cells undergoing apoptosis (Hasebe et al., 2011), is increased by TH at cold temperatures, but decreased by TH at warm temperatures by 48h (Partovi, 2018). The inverse regulation of some transcripts and the

disparity in regulation between 81 differential TH transcripts at cold temperatures and almost 5,000 at permissive temperatures in the *R. catesbeiana* tail fin highlights the different molecular needs for this same tissue which is setting up to undergo different fates at the two temperatures. The TH induced developmental program in cold temperatures is initiating the program just to the point of remembering it for when more suitable conditions arise. Whereas, by 48h of TH signalling in permissive temperatures the metamorphic program has been executed and has passed a commitment point whereby removal of the TH signal or inhibition of protein or mRNA synthesis will no longer stop metamorphosis (Gilbert et al., 1996; Wang and Brown, 1993).

Comparisons between the significant TH-induced functional gene ontologies at 5^{o}C to studies of anurans exposed to TH in permissive temperatures show significant overlap in the more broad cellular, biological and metabolic process categories demonstrating that whether or not the tadpole is fated to execute metamorphosis there is a general tissue response to the TH signal (Das et al., 2009; Helbing et al., 2003; Jackman et al., 2018). Similar to what has previously been found for direct TH-response transcripts in *Xenopus laevis*, cold-temperature TH responsive transcripts were enriched in cell differentiation, cellular development, and transcription factors (Das et al., 2009). In contrast, categories such as apoptosis were not enriched by TH in the tail fin in the cold temperature, suggesting that the molecular memory program leads to a cellular and developmental response similar to the initial response to TH signalling in permissive temperatures, but in cold temperatures TH does not lead to the induction of the metamorphic response program fated for the tail fin organ.

Both the direct response program in *X. laevis* (Das et al., 2009) and the molecular memory program are enriched in transcription factors, consistent with their role to initiate signaling and gene expression program cascades. The two most responsive TH-downregulated transcription factors were *tcp4* and *p66a*. Tcp4 is an activating cofactor involved in the preinitiation complex for polymerase transcription (Conesa and Acker, 2010; Das et al., 2006; Ge and Roeder, 1994). Although *tcp4* was not previously identified as responsive to TH at 4^{o}C in the liver of *Rana catesbeiana*, this transcript did increase in abundance due to cold temperature exposure (Mochizuki et al., 2012). In contrast to Tcp4, P66a acts as a transcription repressor through the nucleosome remodeling and deacetylase (NuRD) complex that interacts with unliganded TRs supressing TH response gene activation (Xue et al., 1998).

Six1, is an upregulated transcript encoding a transcription factor potentially involved in the molecular memory. Six1 is a homeobox protein important to epithelial development (Ikeda et al., 2007; McCoy et al., 2009; Terakawa et al., 2018)and trunk sensory neuron development (Yajima et al., 2014). This transcription factor is involved in regulating the TH axis through the regulation of *tsh* in response to seasonal cues in mouse pituitary (Dardente et al., 2014; Masumoto et al., 2010), although as of yet this has only been demonstrated upon change in photoperiod.

A novel krüppel-like factor, *klfX*, was also upregulated upon exposure to TH at cold temperatures. Although thorough analyses of krüppel-like factor phylogenetics is lacking in anurans, similar to *Xenopus laevis* (Presnell et al., 2015), it was determined that 15 of the 17 *Homo sapiens* krüppel-like factors are found in *Rana catesbeiana*. The involvement of krüppel-like factors in TH signalling pathways has been well studied for *klf9* which is regulated by TH. In

turn, Klf9 is postulated to play a role in the regulation of *thrb* as *Xenopus laevis thrb* has seven potential krüppel-like factor binding regions and Klf9 associates with the *thrb* promoter region in tail and brain (Bagamasbad et al., 2008; Hu et al., 2016). *Klf9* response to TH has been most thoroughly studied in the brain due to its requirement for proper brain development during the post-embryonic development period (Denver and Williamson, 2009; Dugas et al., 2012) but this krüppel-like factor is also regulated by TH in tail fin, intestine and lung (Maher et al., 2016; Veldhoen et al., 2015, 2014) in *Rana catesbeiana* at permissive temperatures. Although *klf9* was not responsive to TH herein or in other shorter term exposures at 5^{o}C (Hammond et al., 2016a), its transcript abundance increases in the brain, back skin, tail fin and lung after long exposure periods at cold temperatures suggesting that this krüppel-like factor may play a longer term role in the maintenance of the TH signal in the molecular memory of overwintering tadpoles (Hammond et al., 2015). Klf2 is also regulated by TRs during lung development in mice (Knoedler and Denver, 2014), but the involvement of the other krüppel-like factor family members in TH signalling has not yet been described. Although Klf5, the most closely related krüppel-like factor to KlfX, has not been directly linked to TH signalling, its involvement in the regulation of related receptors in the nuclear receptor superfamily including estrogen receptor, peroxisome proliferator receptor (PPAR) and retinoic acid receptor (RAR) in cell lines and mammalian models has been demonstrated (Drosatos et al., 2016, p. 5; Guo et al., 2010; Kada et al., 2008).

It is important to note that the understanding of the function of components within the molecular memory is limited to available annotations and bioinformatic resources. For the 81 significant transcripts found to be responsive to TH at 5^{o}C, 77% had a functional annotation

with a percent identity of 70% or greater. Furthermore, annotations and gene ontologies are often model organism-centric, limiting the ability to understand the role of these components in different developmental settings and in different organisms (Armengaud et al., 2014). Further elucidating the function of these components and their role in the molecular memory will be critical in understanding the initiating response of TH signalling.

4.2 Regulation of molecular memory

The effect of cold temperature on TH response genes has previously been hypothesized to involve modulation of the epigenome as epigenetic modifications often provide transcriptional plasticity in response to environmental signals and their involvement in cold temperature response has been well documented (reviewed by Hammond et al., 2016b; McCaw et al., 2020). It has also been well established that histone modifications are important in the transcriptomic response to TH in permissive temperatures (Ishizuka and Lazar, 2003; Matsuda et al., 2009; Paul et al., 2007; Sun et al., 2014; Xue et al., 1998). When comparing global histone changes in the liver of *Rana catesbeiana,* Ishihara et al. (2019) found that winter acclimatized tadpoles had lower levels of the transcriptionally active mark, acetylated H3K9, than summer acclimatized tadpoles. More specific to TH signalling, (Mochizuki et al., 2012) used chromatin immunoprecipitation to demonstrate that upon exposure to TH in permissive temperatures there is an increased association of acetylated H3K9 with the promoter region of *thrb*. However, the increase in this transcription activation mark is not seen in cold temperature TH exposures. To date, epigenetic control of TH response transcripts has only been studied on transcripts that are not differentially regulated at cold temperatures. Further investigation of

the association of active or repressive epigenetic marks is needed to better understand the genomic control of the molecular memory.

Our data suggest that although genomic regulation is likely important in the lack of response of TH-response transcripts including *thrb*, another mechanism of action may play a role in the abundance changes seen in at least some candidate molecular memory transcripts as active transcription was not required for the TH-dependent increase in *thibz* and *klfX* transcripts. One possible mechanism of abundance increases is that TH treatment is leading to transcript stabilization as mRNA abundance is the net of mRNA transcription and decay. The stabilization of mRNA is not a new concept with the structural elements of mRNA impacting its own stability including: the poly (A) tail, the cap structure, the untranslated regions and AU-rich elements (Hollams et al., 2002; Liu et al., 2014; Staton et al., 2000). More recently, the role of post-transcriptional modifications (Boo and Kim, 2020; Zhao et al., 2017), microRNA (Roush and Slack, 2006) and RNA sequestering into RNA granules (Tian et al., 2020) have also demonstrated to be important mechanisms of mRNA stability.

Hormone initiated changes to mRNA stability have been demonstrated with testosterone, estradiol, progesterone, testosterone and cortisol leading to post-transcriptional modification in abundance of response transcripts (Park et al., 1996; Qian et al., 1993; Rydziel et al., 2004). Through modifications of stability of the estrogen receptor mRNA, estradiol is also able to regulate the expression of its own receptor. Estrogen receptor alpha transcript stability is also modulated through being a direct target for microRNA (Pandey and Picard, 2009) as well as through modification of phosphorylation (Keen et al., 2005).

Similar to other hormones, TH has also shown to be able to modulate mRN stability. In rat pituitary, treatment with TH lead to *tshb* destabilization through multiple possible mechanisms including decreasing the length of the mRNA poly(A) tail (Krane et al., 1991). This change in *tshb* stabilization has been postulated to occur non-genomically as it relies on TH interaction with αVβ3 integrin, a membrane protein which has shown to interact with TH (Bargi-Souza et al., 2018). Similar to the regulation of *tshb* (Krane et al., 1991), treatment with the protein synthesis inhibitor CHX did not impact the TH-induced abundance changes of the *thibz* and *klfX* transcripts suggesting that synthesis of *de novo* proteins is not required for the possible increase in stability of these transcripts. TH also plays a role in mRNA stabilization for acetylcholinesterase mRNA, increasing the half-life of this transcript from 4h to no measurable decrease in abundance for over a 24h experimental time course in rat neuroblastoma cell culture (Puymirat et al., 1995). This effect was synergistically increased by inhibition of phosphatases and decreased by kinase inhibitors (Puymirat et al., 1995). This provides another possible method of mRNA stabilization as phosphorylation is known to be important to establish the TH signalling program and metamorphic response (Skirrow et al., 2008; Skirrow and Helbing, 2007). Further study on the impact of TH on the half-life of molecular memory candidate transcripts in cold temperatures is required to elucidate whether the mRNA is being stabilized and if so, whether phosphorylation, modifications to the poly(A) tail or another mechanism is regulating their stabilization and what role non-genomic signalling may play.

The rate at which TH regulates transcript abundance has previously been shown to be much faster through direct genomic regulation compared to modifications in stability (Danzi and Klein, 2005). This difference in timing of regulation perhaps in part explains the observed

lag in abundance change response time of *thibz* and *klfX* in cold temperatures compared to permissive temperatures in *Rana catesbeiana*. This delay in response of transcripts in cold temperatures compared to permissive temperatures might also be explained by the slower accumulation of TH within the nucleus of target tissue cells at cold temperatures. In cold temperatures, TH intake into the nucleus was approximately ½ at one day and reached equilibrium at around three days (Murata and Yamauchi, 2005; Yamamoto et al., 1966). Comparing the rate of TH accumulation to the regulation of *thibz*, *klfX* and *tcp4,* these transcripts were upregulated by 1 to 4h in permissive temperatures whereas they were not responsive until 18, 12 and 24h, respectively, in cold temperatures. These transcription factors reached a maximum response within 48 to 72h, which aligns with the previously identified equilibrium point between cold and warm temperature TH accumulation (Murata and Yamauchi, 2005; Yamamoto et al., 1966). Once in the nucleus, however binding affinity to the TR is unchanged (Murata and Yamauchi, 2005).

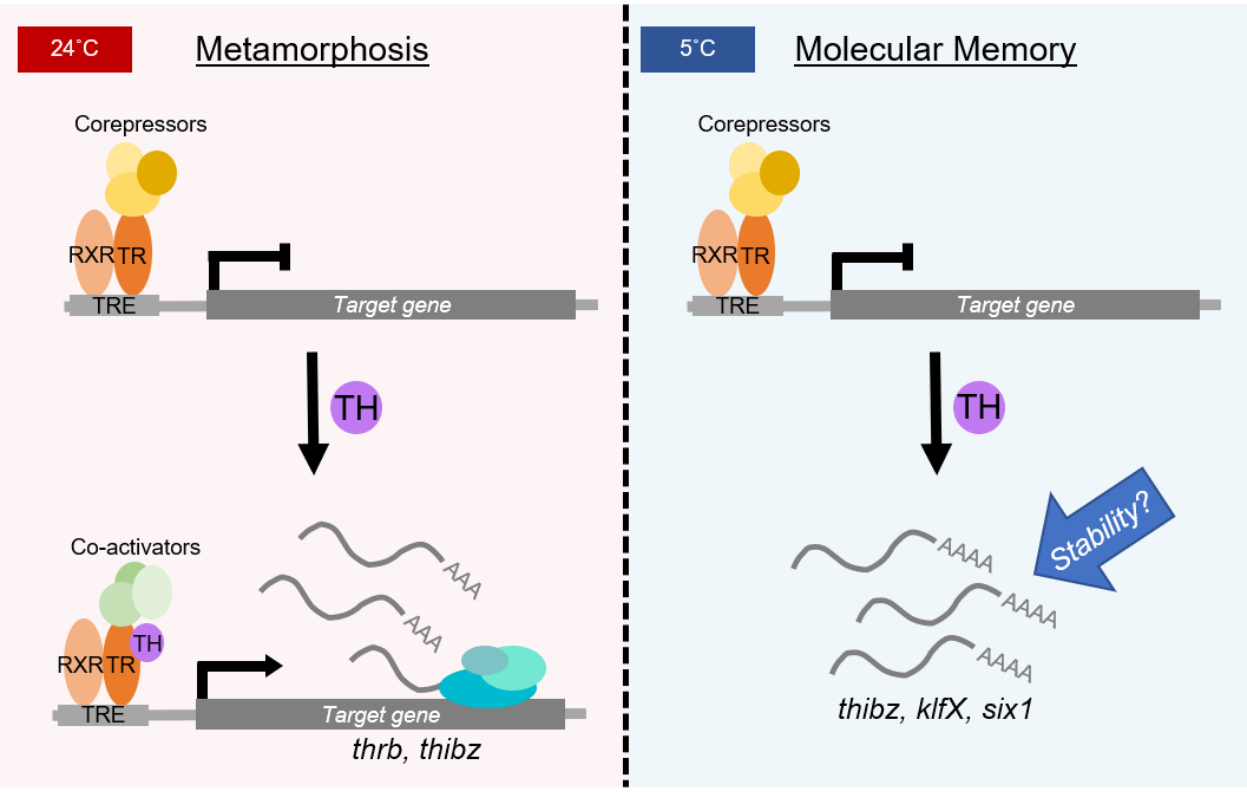


Figure 30: Temperature differentiated regulation mechanisms of TH response transcripts. Overview of differences in TH induced transcript abundance regulation at 24oC and 5oC. In permissive temperatures the primary response is genomic with TH inducing active transcription of TH response genes leading to the downstream metamorphic response. Whereas, in cold temperatures another mechanism is controlling transcript abundance changes, possibly modification to mRNA stability.

That cold temperature leads to differential regulation of select TH-response genes has been well demonstrated (Hammond et al., 2016a, 2015; Ishihara and Yamauchi, 2019; Mochizuki et al., 2012). However, understanding the mechanism of this differential regulation has not yet been described. Given our findings that active transcription may not be required for regulation of some candidate molecular memory transcripts, we propose that temperature may isolate different mechanisms of transcriptomic regulation allowing for a method to tease apart the complex modes of regulation that are encompassed in the TH signalling program (Figure 30). Where in permissive temperatures, multiple modes of gene regulation may be occurring in

synchrony, with the primary mechanism likely being active genomic signalling. In cold temperatures the dominant mode of TH signalling may be through an alternate mechanism such as transcript stability. The mechanism that is occurring to regulate transcription in the molecular memory is enough to induce the TH signal and remember it for when more permissive temperatures arise, yet not enough to execute the metamorphic program.

4.3 Primed Thyroid Hormone Response

The establishment of the molecular memory serves as a retention mechanism of the TH signal for when more permissive conditions occur. Tadpoles exposed to TH in cold temperatures and then shifted to more permissive temperatures resume the metamorphic program even after 80 days when TH has been cleared (Ashley et al., 1968; Frieden et al., 1965). TH exposure at cold temperatures also primes the animal to respond more rapidly when shifted to a warmer temperature without the lag period that is seen in permissive temperature exposures (Frieden et al., 1965).

This phenomenon is not only seen on the phenotypic level but also on the transcriptomic level where transcripts such as *thrb* do not respond in cold temperatures but have an augmented response when shifted to permissive temperatures for 24h (Hammond et al., 2016a). This increased *thrb* is most likely due to a more accelerated program, as *thrb* responded earlier when tadpoles were exposed to TH at cold temperatures to establish the molecular memory and then shifted to permissive temperatures, compared to tadpoles exposed to TH in only permissive temperatures. The transcription factors *thibz* and *tcp4*, although responsive to TH within the molecular memory, also had an augmented response at an accelerated rate

compared to when primed by the establishment of the molecular memory. As with the regulation of TH response transcripts in permissive temperatures, shift from 5^{o}C to 24^{o}C may involve regulation by epigenetic modulations. Ishihara et al., 2019 found that when winter acclimatized *R. catesbeiana* tadpoles, with lower levels of acetylated H3K9 than summer acclimatized tadpoles, were brought into more permissive temperatures for three days the global levels of acetylated H3K9 in the liver rose significantly. The increase in this activation mark may help explain the increase in transcription of the TR transcript, *thrb*, once the C-fin was shifted to permissive temperatures after cold exposure even in the absence of TH, which in turn would act to regulate TH response transcripts on the genomic level.

KlfX responded by 48h at cold temperatures but when shifted to permissive temperatures did not have the same response as seen in permissive temperatures. This transcription factor also had a more transient response in permissive temperature TH signalling and the induction of the TH response as transcript levels began to decrease after 8h. Similarly, the closely related *klf5* has also shown transient expression dynamics in adipogenesis pathways (Cervantes-Camacho et al., 2015). Previous research has predicted the existence of very few of these transient response factors in early TH signalling using subtractive hybridization (Wang and Brown, 1993), however, now with more accurate and in-depth technologies the number and involvement of these more transient early response transcripts can be explored with greater precision.

5 *Conclusions and Future Directions*

TH is a critical signalling molecule in post-embryonic development. Using TH-driven metamorphosis in anurans as a model for TH signalling it has been discovered that TH impacts the developmental program in virtually every tissue, with this one signal leading to complex and diverse downstream response programs. Given the widespread importance of TH, many studies have focused on elucidating the crucial initial response to this hormone to better understand the induction of the extensive TH program. Cold temperature can be used to pause the TH program in the induction phase of the TH signal in *R. catesbeiana* providing a natural method by which to investigate these early responses. Not only that, but this molecular memory also serves to remember this initial TH signal allowing for a more accelerated metamorphosis when permissive temperatures arrive. Herein we expanded the knowledge of what components may be involved in the molecular memory revealing that transcription factors, including a novel krüppel-like factor, may play an important role in the induction of the TH response. Although gene ontology analysis was able to provide a high-level overview of what general functions these molecular memory components might group in, further analysis is needed on the role of these components in establishing the molecular memory and initiating the TH response through knock out studies to better understand the downstream pathways of the important transcripts.

We also shed light on temperature's effect on isolating mechanisms of regulation of the TH transcriptomic response program from the classic active transcription response. In permissive temperatures it has been well established that when TH is not present, corepressors interact with TRs inhibiting transcription. When TH is present however, coactivators are

recruited leading to active transcription of TH response transcripts. It has also been demonstrated that TH has multiple other mechanisms of signal transduction, including non-genomic signalling and transcript stability modulation. Using a transcription inhibitor, it was revealed that the regulation of at least some of these molecular memory transcripts may not need active transcription and therefore TH may be utilizing transcript stability as the primary mode of regulation over the classically studied genomic signalling. The molecular memory may therefore be isolating mechanisms of TH regulation from genomic signalling. It has yet to be determined how the abundance of these transcripts is modulated, if not by active transcription or whether this same mechanism of transcriptomic changes is also occurring at permissive temperatures. The testing of transcript half-life in cold temperatures would allow for further understanding of the changes in stability of these transcripts in cold temperatures. Although yet undetermined, the mechanisms that are used at this cold temperature are sufficient to induce the TH response to the point where the signal will be remembered for when more permissive temperatures arise. The establishment of this molecular memory program is also sufficient to prime for an accelerated metamorphic temperature upon shift to these permissive temperatures.

Given the importance of TH in post-embryonic development it is crucial to have a full picture of its early initiating signalling mechanisms to not only better understand the developmental process, but also to unveil mechanisms by which TH signalling could be disrupted in disease scenarios or in response to endocrine disrupting chemicals.

References

Allsopp, P.G., Butler, D.G., 1987. Estimating day-degrees from daily maximum-minimum temperatures: A comparison of techniques for a soil-dwelling insect. Agric. For. Meteorol. 41, 165–172. https://doi.org/10.1016/0168-1923(87)90076-1

Altschul, S., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic Local Alignment Search Tool. J. Mol. Biol. 215, 403–410.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402. https://doi.org/10.1093/nar/25.17.3389

Andersen, C.L., Jensen, J.L., Ørntoft, T.F., 2004. Normalization of Real-Time Quantitative Reverse Transcription-PCR Data: A Model-Based Variance Estimation Approach to Identify Genes Suited for Normalization, Applied to Bladder and Colon Cancer Data Sets. Cancer Res. 64, 5245–5250. https://doi.org/10.1158/0008-5472.CAN-04-0496

Armengaud, J., Trapp, J., Pible, O., Geffard, O., Chaumot, A., Hartmann, E.M., 2014. Non-model organisms, a species endangered by proteogenomics. J. Proteomics, Special Issue: Proteomics of non-model organisms 105, 5–18. https://doi.org/10.1016/j.jprot.2014.01.007

Ashley, H., Katti, P., Frieden, E., 1968. Urea excretion in the bullfrog tadpole: Effect of temperature, metamorphosis, and thyroid hormones. Dev. Biol. 17, 293–307. https://doi.org/10.1016/0012-1606(68)90066-3

Bagamasbad, P., Howdeshell, K.L., Sachs, L.M., Demeneix, B.A., Denver, R.J., 2008. A Role for Basic Transcription Element-binding Protein 1 (BTEB1) in the Autoinduction of Thyroid Hormone Receptor β. J. Biol. Chem. 283, 2275–2285. https://doi.org/10.1074/jbc.M709306200

Bargi-Souza, P., Goulart-Silva, F., Nunes, M.T., 2018. Posttranscriptional actions of triiodothyronine on Tshb expression in TαT1 cells: New insights into molecular mechanisms of negative feedback. Mol. Cell. Endocrinol. 478, 45–52. https://doi.org/10.1016/j.mce.2018.07.006

Bassett, J.H.D., Williams, G.R., 2016. Role of Thyroid Hormones in Skeletal Development and Bone Maintenance. Endocr. Rev. 37, 135–187. https://doi.org/10.1210/er.2015-1106

Becker, K.B., Stephens, K.C., Davey, J.C., Schneider, M.J., Galton, V.A., 1997. The Type 2 and Type 3 Iodothyronine Deiodinases Play Important Roles in Coordinating Development in Rana catesbeiana Tadpoles 138, 9.

Bergh, J.J., Lin, H.-Y., Lansing, L., Mohamed, S.N., Davis, F.B., Mousa, S., Davis, P.J., 2005. Integrin αVβ3 Contains a Cell Surface Receptor Site for Thyroid Hormone that Is Linked to Activation of Mitogen-Activated Protein Kinase and Induction of Angiogenesis. Endocrinology 146, 2864–2871. https://doi.org/10.1210/en.2005-0102

Bethesda, (MD), 1988. National Library of Medicine (US), National Center for Biotechnology Information; [cited 2019 Aug 08]. Available from: https://www.ncbi.nlm.nih.gov/protein/.

Bianco, A.C., Kim, B.W., 2006. Deiodinases: implications of the local control of thyroid hormone action. J. Clin. Invest. 116, 2571–2579. https://doi.org/10.1172/JCI29812

Boo, S.H., Kim, Y.K., 2020. The emerging role of RNA modifications in the regulation of mRNA stability. Exp. Mol. Med. 52, 400–408. https://doi.org/10.1038/s12276-020-0407-z

Brown, D.D., 1997. The role of thyroid hormone in zebrafish and axolotl development. Proc. Natl. Acad. Sci. 94, 13011–13016. https://doi.org/10.1073/pnas.94.24.13011

Bryant, D.M., Johnson, K., DiTommaso, T., Tickle, T., Couger, M.B., Payzin-Dogru, D., Lee, T.J., Leigh, N.D., Kuo, T.-H., Davis, F.G., Bateman, J., Bryant, S., Guzikowski, A.R., Tsai, S.L., Coyne, S., Ye, W.W., Freeman, R.M., Peshkin, L., Tabin, C.J., Regev, A., Haas, B.J., Whited, J.L., 2017. A Tissue-Mapped Axolotl De Novo Transcriptome Enables Identification of Limb Regeneration Factors. Cell Rep. 18, 762–776. https://doi.org/10.1016/j.celrep.2016.12.063

Buchholz, D.R., Paul, B.D., Fu, L., Shi, Y.-B., 2006. Molecular and developmental analyses of thyroid hormone receptor function in Xenopus laevis, the African clawed frog. Gen. Comp. Endocrinol. 145, 1–19. https://doi.org/10.1016/j.ygcen.2005.07.009

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clin. Chem. 55, 611–622. https://doi.org/10.1373/clinchem.2008.112797

Cai, L., Brown, D.D., 2004. Expression of type II iodothyronine deiodinase marks the time that a tissue responds to thyroid hormone-induced metamorphosis in Xenopus laevis. Dev. Biol. 266, 87–95. https://doi.org/10.1016/j.ydbio.2003.10.005

Campinho, M.A., 2019. Teleost Metamorphosis: The Role of Thyroid Hormone. Front. Endocrinol. 10. https://doi.org/10.3389/fendo.2019.00383

Cervantes-Camacho, C., Beltrán-Langarica, A., Ochoa-Uribe, A.-K., Marsch-Moreno, M., Ayala-Sumuano, J.-T., Velez-delValle, C., Kuri-Harcuch, W., 2015. The transient expression of Klf4 and Klf5 during adipogenesis depends on GSK3β activity. Adipocyte 4, 248–255. https://doi.org/10.1080/21623945.2015.1007823

Cheng, S.-Y., Leonard, J.L., Davis, P.J., 2010. Molecular Aspects of Thyroid Hormone Actions. Endocr. Rev. 31, 139–170. https://doi.org/10.1210/er.2009-0007

Chik, C.L., Wloka, M.T., Price, D.M., Ho, A.K., 2007. The Role of Repressor Proteins in the Adrenergic Induction of Type II Iodothyronine Deiodinase in Rat Pinealocytes. Endocrinology 148, 3523–3531. https://doi.org/10.1210/en.2007-0166

Choi, J., Moskalik, C.L., Ng, A., Matter, S.F., Buchholz, D.R., 2015. Regulation of thyroid hormone-induced development in vivo by thyroid hormone transporters and cytosolic binding proteins. Gen. Comp. Endocrinol. 222, 69–80. https://doi.org/10.1016/j.ygcen.2015.07.006

Cody, V., Davis, P.J., Davis, F.B., 2007. Molecular modeling of the thyroid hormone interactions with αvβ3 integrin. Steroids, FASEB 2006 Summer Research Conference 72, 165–170. https://doi.org/10.1016/j.steroids.2006.11.008

Conesa, C., Acker, J., 2010. Sub1/PC4 a chromatin associated protein with multiple functions in transcription. RNA Biol. 7, 287–290. https://doi.org/10.4161/rna.7.3.11491

Covell, S., Ellis, R.H., Roberts, E.H., Summerfield, R.J., 1986. The Influence of Temperature on Seed Germination Rate in Grain Legumes: I. A COMPARISON OF CHICKPEA, LENTIL, SOYABEAN AND COWPEA AT CONSTANT TEMPERATURES. J. Exp. Bot. 37, 705–715. https://doi.org/10.1093/jxb/37.5.705

Crowner, A., Khatri, S., Blichmann, D., Voss, S.R., 2019. Rediscovering the Axolotl as a Model for Thyroid Hormone Dependent Development. Front. Endocrinol. 10. https://doi.org/10.3389/fendo.2019.00237

Danzi, S., Klein, I., 2005. Posttranscriptional regulation of myosin heavy chain expression in the heart by triiodothyronine. Am. J. Physiol.-Heart Circ. Physiol. 288, H455–H460. https://doi.org/10.1152/ajpheart.00896.2004

Dardente, H., Hazlerigg, D.G., Ebling, F.J., 2014. Thyroid Hormone and Seasonal Rhythmicity. Front. Endocrinol. 5. https://doi.org/10.3389/fendo.2014.00019

Das, B., Heimeier, R.A., Buchholz, D.R., Shi, Y.-B., 2009. Identification of Direct Thyroid Hormone Response Genes Reveals the Earliest Gene Regulation Programs during Frog Metamorphosis. J. Biol. Chem. 284, 34167–34178. https://doi.org/10.1074/jbc.M109.066084

Das, C., Hizume, K., Batta, K., Kumar, B.R.P., Gadad, S.S., Ganguly, S., Lorain, S., Verreault, A., Sadhale, P.P., Takeyasu, K., Kundu, T.K., 2006. Transcriptional Coactivator PC4, a Chromatin-Associated Protein, Induces Chromatin Condensation. Mol. Cell. Biol. 26, 8303–8315. https://doi.org/10.1128/MCB.00887-06

Davis, P.J., Leonard, J.L., Davis, F.B., 2008. Mechanisms of nongenomic actions of thyroid hormone. Front. Neuroendocrinol. 29, 211–218. https://doi.org/10.1016/j.yfrne.2007.09.003
Davis, P.J., Shih, A., Lin, H.-Y., Martino, L.J., Davis, F.B., 2000. Thyroxine Promotes Association of Mitogen-activated Protein Kinase and Nuclear Thyroid Hormone Receptor (TR) and Causes Serine Phosphorylation of TR*. J. Biol. Chem. 275, 38032–38039. https://doi.org/10.1074/jbc.M002560200
de Castro, E., Sigrist, C.J.A., Gattiker, A., Bulliard, V., Langendijk-Genevaux, P.S., Gasteiger, E., Bairoch, A., Hulo, N., 2006. ScanProsite: detection of PROSITE signature matches and ProRule-associated functional and structural residues in proteins. Nucleic Acids Res. 34, W362–W365. https://doi.org/10.1093/nar/gkl124
Denver, R.J., 1993. Acceleration of Anuran Amphibian Metamorphosis by Corticotropin-Releasing Hormone-like Peptides. Gen. Comp. Endocrinol. 91, 38–51. https://doi.org/10.1006/gcen.1993.1102
Denver, R.J., Mirhadi, N., Phillips, M., 1998. Adaptive Plasticity in Amphibian Metamorphosis: Response of Scaphiopus Hammondiitadpoles to Habitat Desiccation. Ecology 79, 1859–1872. https://doi.org/10.1890/0012-9658(1998)079[1859:APIAMR]2.0.CO;2
Denver, R.J., Williamson, K.E., 2009. Identification of a Thyroid Hormone Response Element in the Mouse Krüppel-Like Factor 9 Gene to Explain Its Postnatal Expression in the Brain. Endocrinology 150, 3935–3943. https://doi.org/10.1210/en.2009-0050
Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., Gingeras, T.R., 2013. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29, 15–21. https://doi.org/10.1093/bioinformatics/bts635
Drosatos, K., Pollak, N.M., Pol, C.J., Ntziachristos, P., Willecke, F., Valenti, M.-C., Trent, C.M., Hu, Y., Guo, S., Aifantis, I., Goldberg, I.J., 2016. Cardiac Myocyte KLF5 Regulates PPARr Expression and Cardiac Function. Circ. Res. 118, 241–253. https://doi.org/10.1161/CIRCRESAHA.115.306383
Dugas, J.C., Ibrahim, A., Barres, B.A., 2012. The T3-induced gene KLF9 regulates oligodendrocyte differentiation and myelin regeneration. Mol. Cell. Neurosci. 50, 45–57. https://doi.org/10.1016/j.mcn.2012.03.007
Dunn, J.T., Dunn, A.D., 1999. The importance of thyroglobulin structure for thyroid hormone biosynthesis**We shared with our friend and colleague, the late Gaetano Salvatore, a long–standing interest in thyroglobulin structure, as well as a broader interest in the optimal production of thyroid hormone and its dependence on adequate iodine supply. Over the years, we frequently discussed progress in this field with him and his many co–workers. We now welcome the opportunity to review some of our investigative work in one of his favorite fields, in his honor. Biochimie 81, 505–509. https://doi.org/10.1016/S0300-9084(99)80102-3
Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792–1797. https://doi.org/10.1093/nar/gkh340
Eh, R., 1988. Temperature and seed germination. Symp. Soc. Exp. Biol. 42, 109–132.
eHammond, S.A., eCarew, A., eHelbing, C., 2013. Evaluation of the effects of titanium dioxide nanoparticles on cultured Rana catesbeiana tailfin tissue. Front. Genet. 4. https://doi.org/10.3389/fgene.2013.00251
Evans, R.M., 1988. The Steroid and Thyroid Hormone Receptor Superfamily. Science 240, 889–895.
Felsenstein, J., 2009. PHYLIP (Phylogeny Inference Package) version 3.7a. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle.
Fort, D.J., Degitz, S., Tietge, J., Touart, L.W., 2007. The hypothalamic-pituitary-thyroid (HPT) axis in frogs and its role in frog development and reproduction. Crit. Rev. Toxicol. 37, 117–161.
Frieden, E., Wahlborg, A., Howard, E., 1965. Temperature Control of the Response of Tadpoles to Triiodothyronine. Nature 205, 1173–1176. https://doi.org/10.1038/2051173a0

Fry, A.E., 1972. Effects of temperature on shortening of isolated Rana pipiens tadpole tail tips. J. Exp. Zool. 180, 197–207. https://doi.org/10.1002/jez.1401800207
Galton, V.A., Cohen, J.S., 1980. Action of Thyroid Hormones in Premetamorphic Tadpoles: An Important Role for Thyroxine?*. Endocrinology 107, 1820–1826. https://doi.org/10.1210/endo-107-6-1820
Ge, H., Roeder, R.G., 1994. Purification, cloning, and characterization of a human coactivator, PC4, that mediates transcriptional activation of class II genes. Cell 78, 513–523. https://doi.org/10.1016/0092-8674(94)90428-6
Gilbert, L.I., Tata, J.R., Atkinson, B.G., 1996. Metamorphosis: postembryonic reprogramming of gene expression in amphibian and insect cells. Academic Press., San Diego.
Gudernatsch, J.F., 1912. Feeding Experiments on tadpoles. Arch. Für Entwicklungsmechanik Org. 35, 457–483. https://doi.org/10.1007/BF02277051
Guo, P., Dong, X.-Y., Zhao, K.-W., Sun, X., Li, Q., Dong, J.-T., 2010. Estrogen-induced interaction between KLF5 and estrogen receptor (ER) suppresses the function of ER in ER-positive breast cancer cells. Int. J. Cancer J. Int. Cancer 126, 81–89. https://doi.org/10.1002/ijc.24696
Hammond, S.A., Jackman, K.W., Partovi, S.H., Veldhoen, N., Helbing, C.C., 2016a. Identification of organ-autonomous constituents of the molecular memory conferred by thyroid hormone exposure in cold temperature-arrested metamorphosing Rana (Lithobates) catesbeiana tadpoles. Comp. Biochem. Physiol. Part D Genomics Proteomics 17, 58–65. https://doi.org/10.1016/j.cbd.2016.01.002
Hammond, S.A., Nelson, C.J., Helbing, C.C., 2016b. Environmental influences on the epigenomes of herpetofauna and fish. Biochem. Cell Biol. 94, 95–100. https://doi.org/10.1139/bcb-2015-0111
Hammond, S.A., Veldhoen, N., Helbing, C.C., 2015. Influence of temperature on thyroid hormone signaling and endocrine disruptor action in Rana (Lithobates) catesbeiana tadpoles. Gen. Comp. Endocrinol. 219, 6–15. https://doi.org/10.1016/j.ygcen.2014.12.001
Hammond, S.A., Warren, R.L., Vandervalk, B.P., Kucuk, E., Khan, H., Gibb, E.A., Pandoh, P., Kirk, H., Zhao, Y., Jones, M., Mungall, A.J., Coope, R., Pleasance, S., Moore, R.A., Holt, R.A., Round, J.M., Ohora, S., Walle, B.V., Veldhoen, N., Helbing, C.C., Birol, I., 2017. The North American bullfrog draft genome provides insight into hormonal regulation of long noncoding RNA. Nat. Commun. 8, 1–8. https://doi.org/10.1038/s41467-017-01316-7
Hasebe, T., Buchholz, D.R., Shi, Y.-B., Ishizuya-Oka, A., 2011. Epithelial-Connective Tissue Interactions Induced by Thyroid Hormone Receptor Are Essential for Adult Stem Cell Development in the Xenopus laevis Intestine. STEM CELLS 29, 154–161. https://doi.org/10.1002/stem.560
Helbing, C.C., Werry, K., Crump, D., Domanski, D., Veldhoen, N., Bailey, C.M., 2003. Expression Profiles of Novel Thyroid Hormone-Responsive Genes and Proteins in the Tail of Xenopus laevis Tadpoles Undergoing Precocious Metamorphosis. Mol. Endocrinol. 17, 1395–1409. https://doi.org/10.1210/me.2002-0274
Hinther, A., Domanski, D., Vawda, S., Helbing, C.C., 2010. C-fin: A cultured frog tadpole tail fin biopsy approach for detection of thyroid hormone-disrupting chemicals. Environ. Toxicol. Chem. 29, 380–388. https://doi.org/10.1002/etc.44
Hollams, E.M., Giles, K.M., Thomson, A.M., Leedman, P.J., 2002. MRNA stability and the control of gene expression: implications for human disease. Neurochem. Res. 27, 957–980. https://doi.org/10.1023/a:1020992418511
Horn, S., Heuer, H., 2010. Thyroid hormone action during brain development: More questions than answers. Mol. Cell. Endocrinol. 315, 19–26. https://doi.org/10.1016/j.mce.2009.09.008
Hu, F., Knoedler, J.R., Denver, R.J., 2016. A Mechanism to Enhance Cellular Responsivity to Hormone Action: Krüppel-Like Factor 9 Promotes Thyroid Hormone Receptor-β Autoinduction During Postembryonic Brain Development. Endocrinology 157, 1683–1693. https://doi.org/10.1210/en.2015-1980

Huang, D.W., Sherman, B.T., Lempicki, R.A., 2009a. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4, 44–57. https://doi.org/10.1038/nprot.2008.211

Huang, D.W., Sherman, B.T., Lempicki, R.A., 2009b. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 37, 1–13. https://doi.org/10.1093/nar/gkn923

Ikeda, K., Ookawara, S., Sato, S., Ando, Z., Kageyama, R., Kawakami, K., 2007. Six1 is essential for early neurogenesis in the development of olfactory epithelium. Dev. Biol. 311, 53–68. https://doi.org/10.1016/j.ydbio.2007.08.020

Ishihara, A., Sapon, M.A., Yamauchi, K., 2019. Seasonal acclimatization and thermal acclimation induce global histone epigenetic changes in liver of bullfrog (Lithobates catesbeianus) tadpole. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 230, 39–48. https://doi.org/10.1016/j.cbpa.2018.12.014

Ishihara, A., Yamauchi, K., 2019. Analysis of global and gene-specific acetylation of histones in the liver of American bullfrog (Rana catesbeiana) tadpoles acclimated to low temperature. J. Therm. Biol. 84, 488–495. https://doi.org/10.1016/j.jtherbio.2019.08.002

Ishizuka, T., Lazar, M.A., 2003. The N-CoR/Histone Deacetylase 3 Complex Is Required for Repression by Thyroid Hormone Receptor. Mol. Cell. Biol. 23, 5122–5131. https://doi.org/10.1128/MCB.23.15.5122-5131.2003

Jackman, K.W., Veldhoen, N., Miliano, R.C., Robert, B.J., Li, L., Khojasteh, A., Zheng, X., Zaborniak, T.S.M., van Aggelen, G., Lesperance, M., Parker, W.J., Hall, E.R., Pyle, G.G., Helbing, C.C., 2018. Transcriptomics investigation of thyroid hormone disruption in the olfactory system of the Rana [Lithobates] catesbeiana tadpole. Aquat. Toxicol. 202, 46–56. https://doi.org/10.1016/j.aquatox.2018.06.015

Janzen, F.J., Paukstis, G.L., 1991. Environmental Sex Determination in Reptiles: Ecology, Evolution, and Experimental Design. Q. Rev. Biol. 66, 149–179.

Kada, N., Suzuki, T., Aizawa, K., Munemasa, Y., Matsumura, T., Sawaki, D., Nagai, R., 2008. Acyclic retinoid inhibits functional interaction of transcription factors Krüppel-like factor 5 and retinoic acid receptor-alpha. FEBS Lett. 582, 1755–1760. https://doi.org/10.1016/j.febslet.2008.04.040

Kanamori, A., Brown, D.D., 1992. The regulation of thyroid hormone receptor beta genes by thyroid hormone in Xenopus laevis. J. Biol. Chem. 267, 739–745. https://doi.org/10.1016/S0021-9258(18)48346-9

Keen, J.C., Zhou, Q., Park, B.H., Pettit, C., Mack, K.M., Blair, B., Brenner, K., Davidson, N.E., 2005. Protein Phosphatase 2A Regulates Estrogen Receptor α (ER) Expression through Modulation of ER mRNA Stability *. J. Biol. Chem. 280, 29519–29524. https://doi.org/10.1074/jbc.M505317200

Kim, H.-Y., Mohan, S., 2013. Role and Mechanisms of Actions of Thyroid Hormone on the Skeletal Development. Bone Res. 1, 146–161. https://doi.org/10.4248/BR201302004

Knoedler, J.R., Denver, R.J., 2014. Krüppel-like factors are effectors of nuclear receptor signaling. Gen. Comp. Endocrinol. 203, 49–59. https://doi.org/10.1016/j.ygcen.2014.03.003

Köhrle, J., Lehmphul, I., Pietzner, M., Renko, K., Rijntjes, E., Richards, K., Anselmo, J., Danielsen, M., Jonklaas, J., 2020. 3,5-T2—A Janus-Faced Thyroid Hormone Metabolite Exerts Both Canonical T3-Mimetic Endocrine and Intracrine Hepatic Action. Front. Endocrinol. 10. https://doi.org/10.3389/fendo.2019.00787

Krane, I.M., Spindel, E.R., Chin, W.W., 1991. Thyroid hormone decreases the stability and the poly(A) tract length of rat thyrotropin beta-subunit messenger RNA. Mol. Endocrinol. Baltim. Md 5, 469–475. https://doi.org/10.1210/mend-5-4-469

Larsson, M., Pettersson, T., Carlström, A., 1985. Thyroid hormone binding in serum of 15 vertebrate species: Isolation of thyroxine-binding globulin and prealbumin analogs. Gen. Comp. Endocrinol. 58, 360–375. https://doi.org/10.1016/0016-6480(85)90108-X

Lee, J.-W., Kim, N.-H., Milanesi, A., 2014. Thyroid Hormone Signaling in Muscle Development, Repair and Metabolism. J. Endocrinol. Diabetes Obes. 2, 1046.

Leloup, J., Buscaglia, M., 1977. La triiodothyronine: hormone de la métamorphose des amphibiens. CR Acad Sci. 284, 2261–2263.

Liu, H., Luo, M., Wen, J., 2014. mRNA stability in the nucleus. J. Zhejiang Univ. Sci. B 15, 444–454. https://doi.org/10.1631/jzus.B1400088

Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550. https://doi.org/10.1186/s13059-014-0550-8

Luongo, C., Dentice, M., Salvatore, D., 2019. Deiodinases and their intricate role in thyroid hormone homeostasis. Nat. Rev. Endocrinol. 15, 479–488. https://doi.org/10.1038/s41574-019-0218-2

Maher, S.K., Wojnarowicz, P., Ichu, T.-A., Veldhoen, N., Lu, L., Lesperance, M., Propper, C.R., Helbing, C.C., 2016. Rethinking the biological relationships of the thyroid hormones, l-thyroxine and 3,5,3'-triiodothyronine. Comp. Biochem. Physiol. Part D Genomics Proteomics 18, 44–53. https://doi.org/10.1016/j.cbd.2016.04.002

Masumoto, K., Ukai-Tadenuma, M., Kasukawa, T., Nagano, M., Uno, K.D., Tsujino, K., Horikawa, K., Shigeyoshi, Y., Ueda, H.R., 2010. Acute Induction of Eya3 by Late-Night Light Stimulation Triggers TSHβ Expression in Photoperiodism. Curr. Biol. 20, 2199–2206. https://doi.org/10.1016/j.cub.2010.11.038

Matsuda, H., Paul, B.D., Choi, C.Y., Hasebe, T., Shi, Y.-B., 2009. Novel Functions of Protein Arginine Methyltransferase 1 in Thyroid Hormone Receptor-Mediated Transcription and in the Regulation of Metamorphic Rate in Xenopus laevis. Mol. Cell. Biol. 29, 745–757. https://doi.org/10.1128/MCB.00827-08

McCaw, B.A., Stevenson, T.J., Lancaster, L.T., 2020. Epigenetic Responses to Temperature and Climate. Integr. Comp. Biol. 60, 1469–1480. https://doi.org/10.1093/icb/icaa049

McConnell, B.B., Yang, V.W., 2010. Mammalian Krüppel-Like Factors in Health and Diseases. Physiol. Rev. 90, 1337–1381. https://doi.org/10.1152/physrev.00058.2009

McCoy, E.L., Iwanaga, R., Jedlicka, P., Abbey, N.-S., Chodosh, L.A., Heichman, K.A., Welm, A.L., Ford, H.L., 2009. Six1 expands the mouse mammary epithelial stem/progenitor cell pool and induces mammary tumors that undergo epithelial-mesenchymal transition. J. Clin. Invest. 119, 2663–2677. https://doi.org/10.1172/JCI37691

Mendoza, A., Navarrete-Ramírez, P., Hernández-Puga, G., Villalobos, P., Holzer, G., Renaud, J.P., Laudet, V., Orozco, A., 2013. 3,5-T2 Is an Alternative Ligand for the Thyroid Hormone Receptor β1. Endocrinology 154, 2948–2958. https://doi.org/10.1210/en.2013-1030

Mochizuki, K., Goda, T., Yamauchi, K., 2012. Gene expression profile in the liver of Rana catesbeiana tadpoles exposed to low temperature in the presence of thyroid hormone. Biochem. Biophys. Res. Commun. 420, 845–850. https://doi.org/10.1016/j.bbrc.2012.03.085

Moeller, L.C., Cao, X., Dumitrescu, A.M., Seo, H., Refetoff, S., 2006. Thyroid hormone mediated changes in gene expression can be initiated by cytosolic action of the thyroid hormone receptor β through the phosphatidylinositol 3-kinase pathway. Nucl. Recept. Signal. 4, nrs.04020. https://doi.org/10.1621/nrs.04020

Murata, T., Yamauchi, K., 2005. Low-Temperature Arrest of the Triiodothyronine-Dependent Transcription in *Rana catesbeiana* Red Blood Cells. Endocrinology 146, 256–264. https://doi.org/10.1210/en.2004-1090

Nota, B., 2017. Gogadget: An R Package for Interpretation and Visualization of GO Enrichment Results. Mol. Inform. 36, 1600132. https://doi.org/10.1002/minf.201600132

Nussey, S., Whitehead, S., 2001. The thyroid gland. BIOS Scientific Publishers.
Nwoye, L., Mommaerts, W.F., Simpson, D.R., Seraydarian, K., Marusich, M., 1982. Evidence for a direct action of thyroid hormone in specifying muscle properties. Am. J. Physiol.-Regul. Integr. Comp. Physiol. 242, R401–R408. https://doi.org/10.1152/ajpregu.1982.242.3.R401
Ortiga-Carvalho, T.M., Shibusawa, N., Nikrodhanond, A., Oliveira, K.J., Machado, D.S., Liao, X.-H., Cohen, R.N., Refetoff, S., Wondisford, F.E., 2005. Negative regulation by thyroid hormone receptor requires an intact coactivator-binding surface. J. Clin. Invest. 115, 2517–2523. https://doi.org/10.1172/JCI24109
Pandey, D.P., Picard, D., 2009. miR-22 Inhibits Estrogen Signaling by Directly Targeting the Estrogen Receptor α mRNA. Mol. Cell. Biol. 29, 3783–3790. https://doi.org/10.1128/MCB.01875-08
Park, D., Cheon, M., Kim, C., Kim, K., Ryu, K., 1996. Progesterone together with estradiol promotes luteinizing hormone β-subunit mRNA stability in rat pituitary cells cultured in vitro. Eur. J. Endocrinol. 134, 236–242. https://doi.org/10.1530/eje.0.1340236
Partovi, S.H., 2018. Transcriptomic analysis of thyroid hormone effects on Rana [Lithobates] catesbeiana tadpole tissues with special emphasis on the innate immune system. University of Victoria.
Paul, B.D., Buchholz, D.R., Fu, L., Shi, Y.-B., 2007. SRC-p300 Coactivator Complex Is Required for Thyroid Hormone-induced Amphibian Metamorphosis. J. Biol. Chem. 282, 7472–7481. https://doi.org/10.1074/jbc.M607589200
Pertea, M., Pertea, G.M., Antonescu, C.M., Chang, T.-C., Mendell, J.T., Salzberg, S.L., 2015. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. Nat. Biotechnol. 33, 290–295. https://doi.org/10.1038/nbt.3122
Pfaff, D.W., Joels, M., 2016. Hormones, Brain and Behavior. Elsevier Science & Technology, San Diego, UNITED STATES.
Pfaffl, M.W., Tichopad, A., Prgomet, C., Neuvians, T.P., 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations. Biotechnol. Lett. 26, 509–515. https://doi.org/10.1023/B:BILE.0000019559.84305.47
Presnell, J.S., Schnitzler, C.E., Browne, W.E., 2015. KLF/SP Transcription Factor Family Evolution: Expansion, Diversification, and Innovation in Eukaryotes. Genome Biol. Evol. 7, 2289–2309. https://doi.org/10.1093/gbe/evv141
Puymirat, J., Etongue-Mayer, P., Dussault, J.H., 1995. Thyroid Hormones Stabilize Acetylcholinesterase mRNA in Neuro-2A Cells That Overexpress the β1 Thyroid Receptor (∗). J. Biol. Chem. 270, 30651–30656. https://doi.org/10.1074/jbc.270.51.30651
Qian, K., Franklin, R.B., Costello, L.C., 1993. Testosterone regulates mitochondrial aspartate aminotransferase gene expression and mRNA stability in prostate. J. Steroid Biochem. Mol. Biol. 44, 13–19. https://doi.org/10.1016/0960-0760(93)90146-N
Ratte, H.T., 1985. Temperature and Insect Development, in: Hoffmann, K.H. (Ed.), Environmental Physiology and Biochemistry of Insects. Springer, Berlin, Heidelberg, pp. 33–66. https://doi.org/10.1007/978-3-642-70020-0_2
Roush, S.F., Slack, F.J., 2006. Micromanagement: A Role for MicroRNAs in mRNA Stability. ACS Chem. Biol. 1, 132–134. https://doi.org/10.1021/cb600138j
Rydziel, S., Delany, A.M., Canalis, E., 2004. AU-Rich Elements in the Collagenase 3 mRNA Mediate Stabilization of the Transcript by Cortisol in Osteoblasts *. J. Biol. Chem. 279, 5397–5404. https://doi.org/10.1074/jbc.M311984200
Sachs, L.M., Buchholz, D.R., 2017. Frogs model man: *In vivo* thyroid hormone signaling during development: SACHS and BUCHHOLZ. genesis 55, e23000. https://doi.org/10.1002/dvg.23000

Sachs, L.M., Damjanovski, S., Jones, P.L., Li, Q., Amano, T., Ueda, S., Shi, Y.-B., Ishizuya-Oka, A., 2000. Dual functions of thyroid hormone receptors during Xenopus development. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 126, 199–211. https://doi.org/10.1016/S0305-0491(00)00198-X

Schmittgen TD, Livak KJ, 2008. Analyzing real-time PCR data by the comparative C T method | Nature Protocols. Nat Protocols 3, 1101–1108.

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B., Ideker, T., 2003. Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. Genome Res. 13, 2498–2504. https://doi.org/10.1101/gr.1239303

Shi, Y.-B., 2009. Dual Functions of Thyroid Hormone Receptors in Vertebrate Development: The Roles of Histone-Modifying Cofactor Complexes. Thyroid 19, 987–999. https://doi.org/10.1089/thy.2009.0041

Shi, Y.-B., 1999. Amphibian Metamorphosis: From Morphology to Molecular Biology. Wiley.

Shi, Y.B., Brown, D.D., 1993. The earliest changes in gene expression in tadpole intestine induced by thyroid hormone. J. Biol. Chem. 268, 20312–20317. https://doi.org/10.1016/S0021-9258(20)80730-3

Shibata, Y., Tanizaki, Y., Shi, Y.-B., 2020. Thyroid hormone receptor beta is critical for intestinal remodeling during Xenopus tropicalis metamorphosis. Cell Biosci. 10, 46. https://doi.org/10.1186/s13578-020-00411-5

Sigrist, C.J.A., de Castro, E., Cerutti, L., Cuche, B.A., Hulo, N., Bridge, A., Bougueleret, L., Xenarios, I., 2012. New and continuing developments at PROSITE. Nucleic Acids Res. 41, D344–D347. https://doi.org/10.1093/nar/gks1067

Skirrow, R.C., Helbing, C.C., 2007. Decreased cyclin-dependent kinase activity promotes thyroid hormone-dependent tail regression in Rana catesbeiana. Cell Tissue Res. 328, 281–289. https://doi.org/10.1007/s00441-006-0362-6

Skirrow, R.C., Veldhoen, N., Domanski, D., Helbing, C.C., 2008. Roscovitine inhibits thyroid hormone-induced tail regression of the frog tadpole and reveals a role for cyclin C/Cdk8 in the establishment of the metamorphic gene expression program. Dev. Dyn. 237, 3787–3797. https://doi.org/10.1002/dvdy.21800

Staton, J.M., Thomson, A.M., Leedman, P.J., 2000. Hormonal regulation of mRNA stability and RNA-protein interactions in the pituitary. J. Mol. Endocrinol. 25, 17–34. https://doi.org/10.1677/jme.0.0250017

Sun, G., Fu, L., Shi, Y.-B., 2014. Epigenetic regulation of thyroid hormone-induced adult intestinal stem cell development during anuran metamorphosis. Cell Biosci. 4, 73. https://doi.org/10.1186/2045-3701-4-73

Suzuki, S., Awai, K., Ishihara, A., Yamauchi, K., 2016. Cold temperature blocks thyroid hormone-induced changes in lipid and energy metabolism in the liver of Lithobates catesbeianus tadpoles. Cell Biosci. 6. https://doi.org/10.1186/s13578-016-0087-5

Tata, J.R., 2006. Amphibian metamorphosis as a model for the developmental actions of thyroid hormone. Mol. Cell. Endocrinol. 246, 10–20. https://doi.org/10.1016/j.mce.2005.11.024

Tata, J.R., 1966. Requirement for RNA and protein synthesis for induced regression of the tadpole tail in organ culture. Dev. Biol. 13, 77–94. https://doi.org/10.1016/0012-1606(66)90050-9

Taylor, A.C., Kollros, J.J., 1946. Stages in the normal development of Rana pipiens larvae. Anat. Rec. 94, 7–23. https://doi.org/10.1002/ar.1090940103

Terakawa, J., Serna, V.A., Nair, D., Sato, S., Kawakami, K., Radovick, S., Maire, P., Kurita, T., 2018. SIX1 cooperates with RUNX1 and SMAD4 in cell fate commitment of Müllerian duct epithelium. bioRxiv 427351. https://doi.org/10.1101/427351

Thambirajah, A.A., Koide, E.M., Imbery, J.J., Helbing, C.C., 2019. Contaminant and Environmental Influences on Thyroid Hormone Action in Amphibian Metamorphosis. Front. Endocrinol. 10. https://doi.org/10.3389/fendo.2019.00276

The UniProt Consortium, Bateman, A., Martin, M.-J., Orchard, S., Magrane, M., Agivetova, R., Ahmad, S., Alpi, E., Bowler-Barnett, E.H., Britto, R., Bursteinas, B., Bye-A-Jee, H., Coetzee, R., Cukura, A., Da Silva, A., Denny, P., Dogan, T., Ebenezer, T., Fan, J., Castro, L.G., Garmiri, P., Georghiou, G., Gonzales, L., Hatton-Ellis, E., Hussein, A., Ignatchenko, A., Insana, G., Ishtiaq, R., Jokinen, P., Joshi, V., Jyothi, D., Lock, A., Lopez, R., Luciani, A., Luo, J., Lussi, Y., MacDougall, A., Madeira, F., Mahmoudy, M., Menchi, M., Mishra, A., Moulang, K., Nightingale, A., Oliveira, C.S., Pundir, S., Qi, G., Raj, S., Rice, D., Lopez, M.R., Saidi, R., Sampson, J., Sawford, T., Speretta, E., Turner, E., Tyagi, N., Vasudev, P., Volynkin, V., Warner, K., Watkins, X., Zaru, R., Zellner, H., Bridge, A., Poux, S., Redaschi, N., Aimo, L., Argoud-Puy, G., Auchincloss, A., Axelsen, K., Bansal, P., Baratin, D., Blatter, M.-C., Bolleman, J., Boutet, E., Breuza, L., Casals-Casas, C., de Castro, E., Echioukh, K.C., Coudert, E., Cuche, B., Doche, M., Dornevil, D., Estreicher, A., Famiglietti, M.L., Feuermann, M., Gasteiger, E., Gehant, S., Gerritsen, V., Gos, A., Gruaz-Gumowski, N., Hinz, U., Hulo, C., Hyka-Nouspikel, N., Jungo, F., Keller, G., Kerhornou, A., Lara, V., Le Mercier, P., Lieberherr, D., Lombardot, T., Martin, X., Masson, P., Morgat, A., Neto, T.B., Paesano, S., Pedruzzi, I., Pilbout, S., Pourcel, L., Pozzato, M., Pruess, M., Rivoire, C., Sigrist, C., Sonesson, K., Stutz, A., Sundaram, S., Tognolli, M., Verbregue, L., Wu, C.H., Arighi, C.N., Arminski, L., Chen, C., Chen, Y., Garavelli, J.S., Huang, H., Laiho, K., McGarvey, P., Natale, D.A., Ross, K., Vinayaka, C.R., Wang, Q., Wang, Y., Yeh, L.-S., Zhang, J., Ruch, P., Teodoro, D., 2021. UniProt: the universal protein knowledgebase in 2021. Nucleic Acids Res. 49, D480–D489. https://doi.org/10.1093/nar/gkaa1100

Tian, S., Curnutte, H.A., Trcek, T., 2020. RNA Granules: A View from the RNA Perspective. Molecules 25, 3130. https://doi.org/10.3390/molecules25143130

Tsai, M.-J., O'Malley, B., 1994. Molecular Mechanisms of Action of Steroid/Thyroid Receptor Superfamily Members. Annual Reviews of Biochemistry 63, 451–486.

van der Spek, A.H., Fliers, E., Boelen, A., 2017. The classic pathways of thyroid hormone metabolism. Mol. Cell. Endocrinol., A century of thyroid hormone research - Vol. I: The expanded thyroid hormone network: novel metabolites and modes of action 458, 29–38. https://doi.org/10.1016/j.mce.2017.01.025

Veldhoen, N., Propper, C.R., Helbing, C.C., 2014. Enabling comparative gene expression studies of thyroid hormone action through the development of a flexible real-time quantitative PCR assay for use across multiple anuran indicator and sentinel species. Aquat. Toxicol. 148, 162–173. https://doi.org/10.1016/j.aquatox.2014.01.008

Veldhoen, N., Stevenson, M.R., Helbing, C.C., 2015. Comparison of thyroid hormone-dependent gene responses in vivo and in organ culture of the American bullfrog (Rana (Lithobates) catesbeiana) lung. Comp. Biochem. Physiol. Part D Genomics Proteomics 16, 99–105. https://doi.org/10.1016/j.cbd.2015.09.001

Viparina, S., Just, J.J., 1975. The Life Period, Growth and Differentiation of Rana catesbeiana Larvae Occurring in Nature. Copeia 1975, 103–109. https://doi.org/10.2307/1442411

Visser, W.E., Friesema, E.C.H., Jansen, J., Visser, T.J., 2008. Thyroid hormone transport in and out of cells. Trends Endocrinol. Metab. 19, 50–56. https://doi.org/10.1016/j.tem.2007.11.003

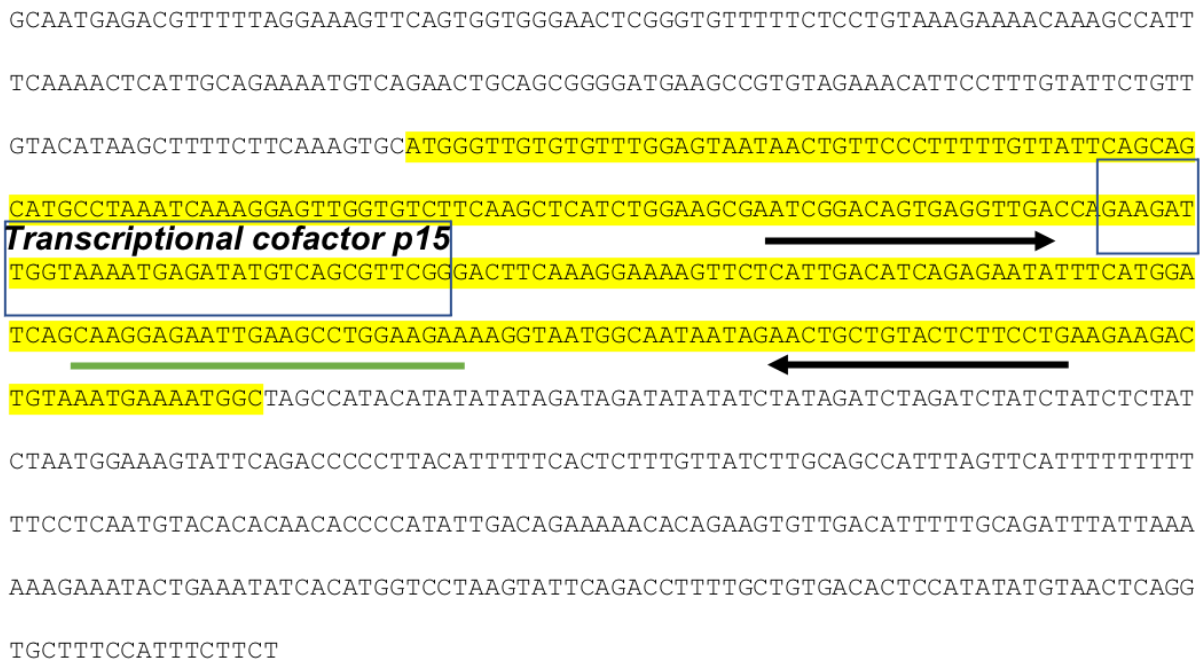
Visser, W.E., Friesema, E.C.H., Visser, T.J., 2011. Minireview: Thyroid Hormone Transporters: The Knowns and the Unknowns. Mol. Endocrinol. 25, 1–14. https://doi.org/10.1210/me.2010-0095

Wang, Z., Brown, D.D., 1993. Thyroid hormone-induced gene expression program for amphibian tail resorption. J. Biol. Chem. 268, 16270–16278. https://doi.org/10.1016/S0021-9258(19)85416-9

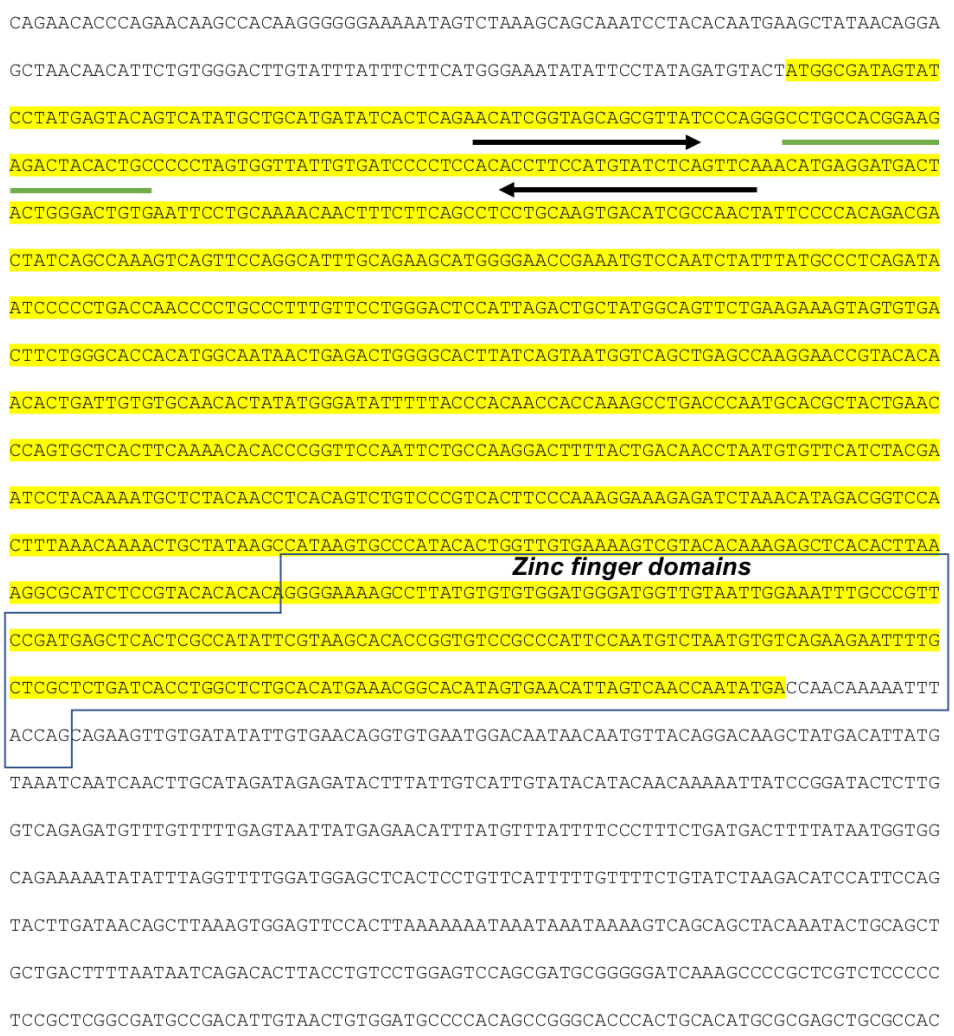
Wen, L., Shibata, Y., Su, D., Fu, L., Luu, N., Shi, Y.-B., 2017. Thyroid Hormone Receptor α Controls Developmental Timing and Regulates the Rate and Coordination of Tissue-Specific

Metamorphosis in Xenopus tropicalis. Endocrinology 158, 1985–1998. https://doi.org/10.1210/en.2016-1953
White, B.A., Nicoll, C.S., 1981. Hormonal Control of Amphibian Metamorphosis, in: Gilbert, L.I., Frieden, E. (Eds.), Metamorphosis: A Problem in Developmental Biology. Springer US, Boston, MA, pp. 363–396. https://doi.org/10.1007/978-1-4613-3246-6_11
Wojnarowicz, P., Ogunlaja, O.O., Xia, C., Parker, W.J., Helbing, C.C., 2013. Impact of Wastewater Treatment Configuration and Seasonal Conditions on Thyroid Hormone Disruption and Stress Effects in Rana catesbeiana Tailfin. Environ. Sci. Technol. 47, 13840–13847. https://doi.org/10.1021/es403767y
Xue, Y., Wong, J., Moreno, G.T., Young, M.K., Côté, J., Wang, W., 1998. NURD, a Novel Complex with Both ATP-Dependent Chromatin-Remodeling and Histone Deacetylase Activities. Mol. Cell 2, 851–861. https://doi.org/10.1016/S1097-2765(00)80299-3
Yajima, H., Suzuki, M., Ochi, H., Ikeda, K., Sato, S., Yamamura, K., Ogino, H., Ueno, N., Kawakami, K., 2014. Six1 is a key regulator of the developmental and evolutionary architecture of sensory neurons in craniates. BMC Biol. 12, 40. https://doi.org/10.1186/1741-7007-12-40
Yamamoto, K., Kanski, D., Frieden, E., 1966. The uptake and excretion of thyroxine, triiodothyronine and iodide in bullfrog tadpoles after immersion or injection at 25° and 6°C. Gen. Comp. Endocrinol. 6, 312–324. https://doi.org/10.1016/S0016-6480(66)80019-9
Yaoita, Y., Brown, D.D., 1990. A correlation of thyroid hormone receptor gene expression with amphibian metamorphosis. Genes Dev. 4, 1917–1924. https://doi.org/10.1101/gad.4.11.1917
Yen, P.M., 2001. Physiological and Molecular Basis of Thyroid Hormone Action. Physiol. Rev. 81, 1097–1142. https://doi.org/10.1152/physrev.2001.81.3.1097
Young, M.D., Wakefield, M.J., Smyth, G.K., Oshlack, A., 2010. Gene ontology analysis for RNA-seq: accounting for selection bias. Genome Biology 14–26.
Zhao, B.S., Roundtree, I.A., He, C., 2017. Post-transcriptional gene regulation by mRNA modifications. Nat. Rev. Mol. Cell Biol. 18, 31–42. https://doi.org/10.1038/nrm.2016.132
Zoeller, R.T., Tan, S.W., Tyl, R.W., 2007. General background on the hypothalamic-pituitary-thyroid (HPT) axis. Crit. Rev. Toxicol. 37, 11–53.

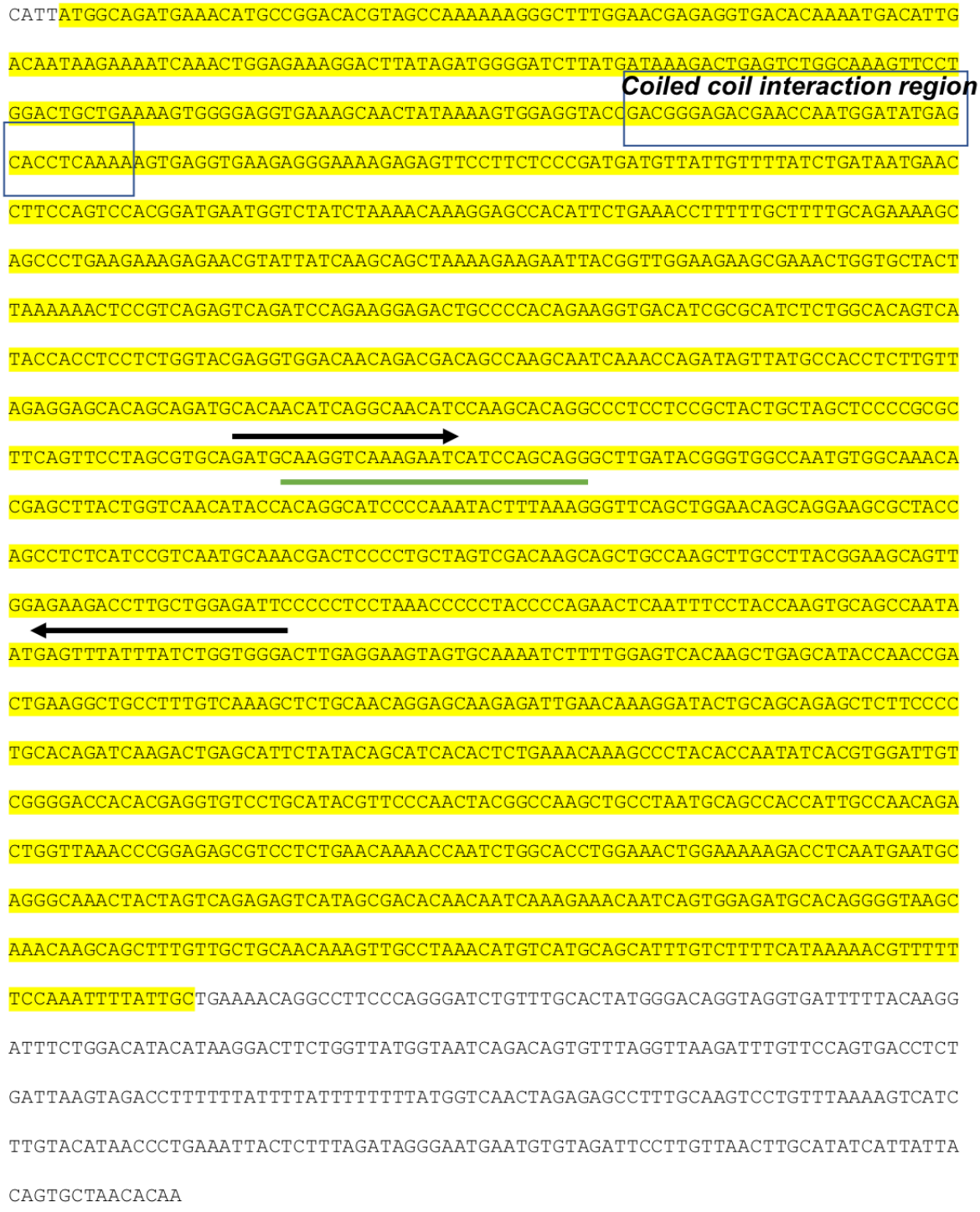
6 Supplementary Figures



Supplementary Figure 1: *tcp4* Primer Design. Primers (black arrows above nucleotide sequence) and probe (green bar above nucleotide sequence) were designed within the protein coding region (yellow highlight) but outside of the transcriptional cofactor p15 highly conserved motif.

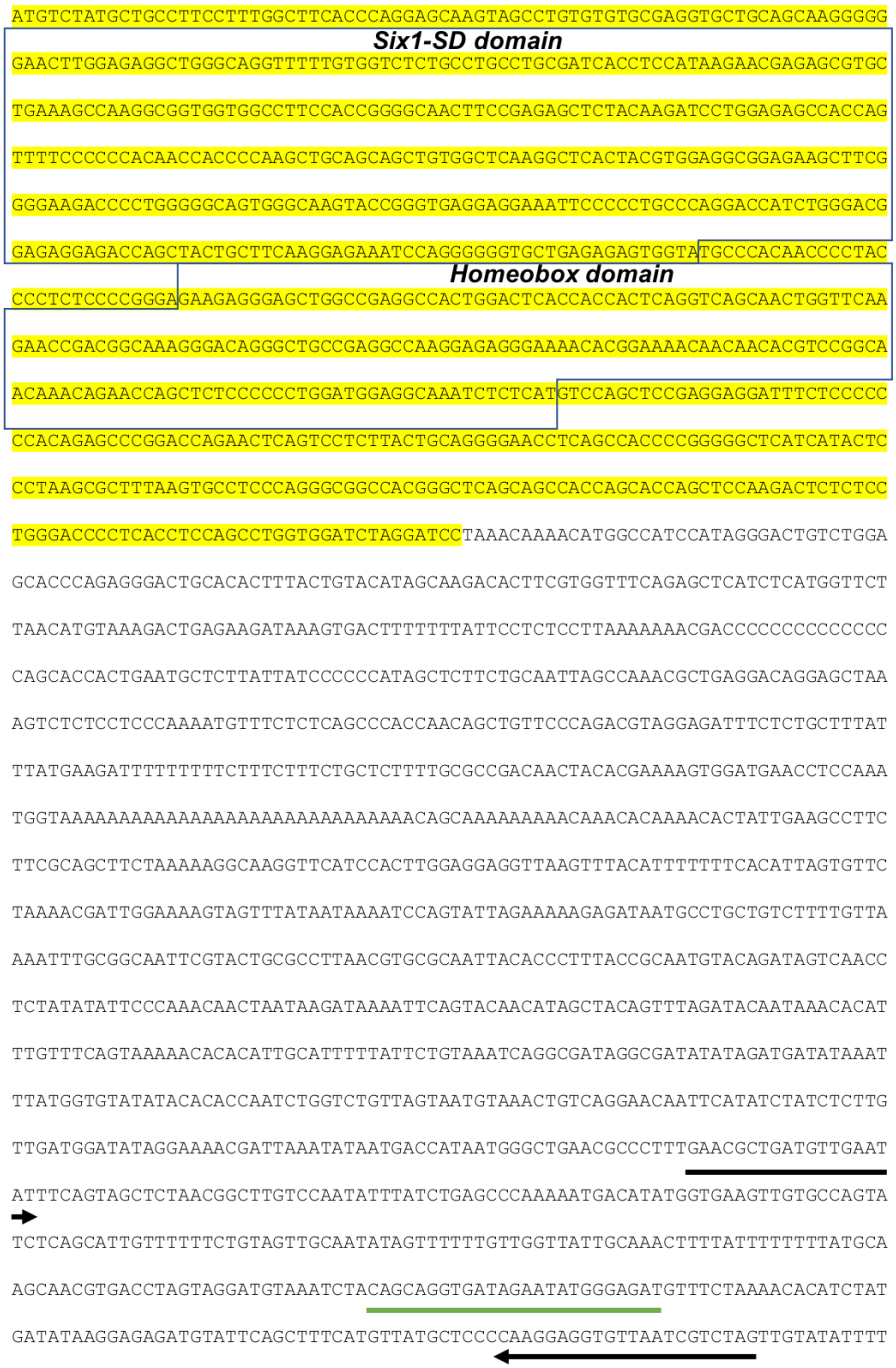


Supplementary Figure 2: *klfX* primer design.
Primers (black arrows above nucleotide sequence) and probe (green bar above nucleotide sequence) were designed within the protein coding region (yellow highlight) but outside of the conserved zing finger domain. Sequence is partial to demonstrate primer location.



Supplementary Figure 3: *p66a* primer design

Primers (black arrows above nucleotide sequence) and probe (green bar above nucleotide sequence) were designed within the protein coding region (yellow highlight) but outside of the conserved coiled coil interaction region. Sequence is partial to demonstrate primer location.



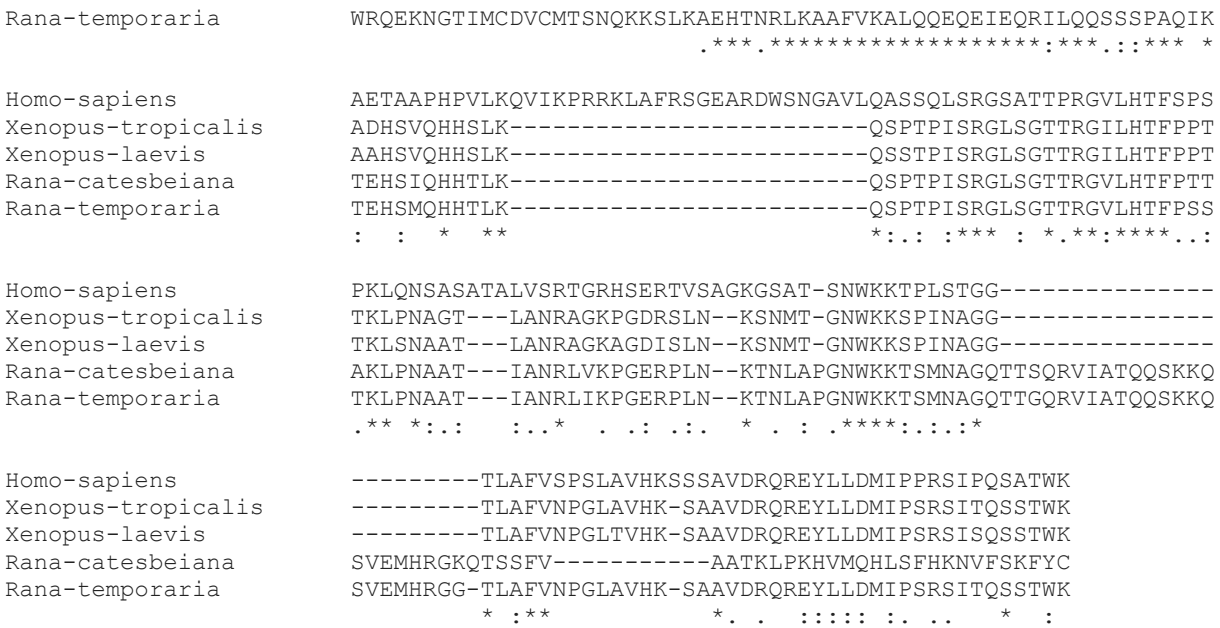
Supplementary Figure 4: *six1* primer design.
Primers (black arrows above nucleotide sequence) and probe (green bar above nucleotide sequence) were designed outside of the protein coding region (yellow highlight) due to high sequence identity with related protein *six2*. Sequence is partial to demonstrate primer location.

```
Homo-sapiens            MSMLPSFGFTQEQVACVCEVLQQGGNLERLGRFLWSLPACDHLHKNESVLKAKAVVAFHR
Rana-catesbeiana        MSMLPSFGFTQEQVACVCEVLQQGGNLERLGRFLWSLPACDHLHKNESVLKAKAVVAFHR
Rana-temporaria         MSMLPSFGFTQEQVACVCEVLQQGGNLERLGRFLWSLPACDHLHKNESVLKAKAVVAFHR
Xenopus-laevis          MSMLPSFGFTQEQVACVCEVLQQGGNLERLGRFLWSLPACDHLHKNESVLKAKAVVAFHR
Xenopus-tropicalis      MSMLPSFGFTQEQVACVCEVLQQGGNLERLGRFLWSLPACDHLHKNESVLKAKAVVAFHR
                        ************************************************************

Homo-sapiens            GNFRELYKILESHQFSPHNHPKLQQLWLKAHYVEAEKLRGRPLGAVGKYRVRRKFPLPRT
Rana-catesbeiana        GNFRELYKILESHQFSPHNHPKLQQLWLKAHYVEAEKLRGRPLGAVGKYRVRRKFPLPRT
Rana-temporaria         GNFRELYKILESHQFSPHNHPKLQQLWLKAHYVEAEKLRGRPLGAVGKYRVRRKFPLPRT
Xenopus-laevis          GNFRELYKILESHQFSPHNHPKLQQLWLKAHYVEAEKLRGRPLGAVGKYRVRRKFPLPRT
Xenopus-tropicalis      GNFRELYKILESHQFSPHNHPKLQQLWLKAHYVEAEKLRGRPLGAVGKYRVRRKFPLPRT
                        ************************************************************

Homo-sapiens            IWDGEETSYCFKEKSRGVLREWYAHNPYPSPREKRELAEATGLTTTQVSNWFKNRRQRDR
Rana-catesbeiana        IWDGEETSYCFKEKSRGVLREWYAHNPYPSPREKRELAEATGLTTTQVSNWFKNRRQRDR
Rana-temporaria         IWDGEETSYCFKEKSRGVLREWYAHNPYPSPREKRELAEATGLTTTQVSNWFKNRRQRDR
Xenopus-laevis          IWDGEETSYCFKEKSRGVLREWYAHNPYPSPREKRELAEATGLTTTQVSNWFKNRRQRDR
Xenopus-tropicalis      IWDGEETSYCFKEKSRGVLREWYAHNPYPSPREKRELAEATGLTTTQVSNWFKNRRQRDR
                        ************************************************************

Homo-sapiens            AAEAKERENTENNNSSSNKQNQLSPLEGGKPLMSSSEEEFSPPQSPDQNSVLLLQGNMGH
Rana-catesbeiana        AAEAKERENTENNNTSGNKQNQLSPLDGGKSLMSSSEEDFSPPQSPDQNSVLLLQGNLSH
Rana-temporaria         AAEAKERENTENNNTSGNKQNQLSPLDGGKSLMSSSDDEFSPPQSPDQNSVLLLQGNLSH
Xenopus-laevis          AAEAKERENTENNNTSTNKQNQLSPLDGGKSLMSSSEEEFSPPQSPDQNSVLLLQGSLTH
Xenopus-tropicalis      AAEAKERENTENNNTSSNKQNQLSPLDGGKSLMSSSEEEFSPPQSPDQNSVLLLQGSLTH
                        **************:* *********:***.*****:::*****************.: *

Homo-sapiens            ARSSNYSLPGLTASQPSHGLQTHQHQLQDSLLGPLTSSLVDLGS
Rana-catesbeiana        PGGSSYSLSALSASQGGHGLSSHQHQLQDSLLGPLTSSLVDLGS
Rana-temporaria         PGGSSYSLSALSASQGGHGLSSHQHQLQDSLLGPLTSSLVDLGS
Xenopus-laevis          PGATSYSLSALSASQGSHGLQGHQHQLQDSLLGPLTSSLVDLGS
Xenopus-tropicalis      PGGSSYSLSALSASQGGHGLQGHQHQLQDSLLGPLTSSLVDLGS
                        . .:.***..*:*** .***. **********************
```

Supplementary Figure 5. Evolutionary conservation of *Rana catesbeiana* Six1 protein sequence.

The protein coding sequence of *Rana catesbeiana* SIX1 was aligned to the SIX1 sequence from *Homo sapiens*, *Rana temporaria*, *Xenopus laevis* and *Xenopus tropicalis* using ClustalW. Star indicates a conserved residue, ":" indicates a conserved amino acid residue with strongly similar properties, and "." indicates a conserved amino acid residue with weakly similar properties.

```
Homo-sapiens            ----------------------------------------MTEEACR-TRSQKRALERD
Xenopus-tropicalis      ----------------------------------------MTDEACR-TRSQKRALERE
Xenopus-laevis          ------------------------------------------------------------
Rana-catesbeiana        ----------------------------------------MADETCR-TRSQKRALERE
Rana-temporaria         MSHIGTHGPSIVGVPSSTLQQLLTDTGAEPAESCDGSGLDNMADEPCRTTRSQKRALERE


Homo-sapiens            PTEDDVESKKIKMERGLLASDLNTDGDMRVTPEPGAGPTQGLLRATEATAMAMGRGEGLV
Xenopus-tropicalis      VTQNDVDTKKIKLEKGML--DIKEDGDLLLKTESSKLP--GLLKSAEVKATI--KLELPA
Xenopus-laevis          ------------------------------------------------------------
Rana-catesbeiana        VTQNDIDNKKIKLEKGLI------DGDLMIKTESGKVP--GLLKSGEVKATI--KVEVPT
Rana-temporaria         VTQNDVDHKKIKLEKGPV------DGDLMIKTESGKVP--GLLKSGEVKATI--KVEVPT


Homo-sapiens            GDGPVDMRTSHSDMKSERRPPSP-DVIVLSDNEQPSSPRVNGLTTVALKETSTEALM--K
Xenopus-tropicalis      GDEPMDMSTSKSDLKREKRVPSP-DVIVLSDNE-PSSPRMNGLTKVPKEPSSETFLLMRK
Xenopus-laevis          ------------------------------------------------------------
Rana-catesbeiana        GDEPMDMSTSKSEVKREKRVPSPDDVIVLSDNE-PSSPRMNGLSKT-KEPHSETFLLLQK
Rana-temporaria         GDEPMDMSTSKSEVKREKRVPSPDDVIVLSDNE-PSSPRMNGLSKT-KEPHSETFLLLQK


Homo-sapiens            SSPEERERMIKQLKEELRLEEAKLVLLKKLRQSQIQKEATAQKPTGSVGSTVTTPPPLVR
Xenopus-tropicalis      SSPEERERMIKQLKEELRLEEAKLVLLKKLRQSQIQKETAPQKVT---------------
Xenopus-laevis          --------MIKQLKEELRLEEAKLVLLKKLRQSQIQKETAPQKVT---------------
Rana-catesbeiana        SSPEERERIIKQLKEELRLEEAKLVLLKKLRQSQIQKETAPQKVT---------------
Rana-temporaria         SSPEERERIIKQLKEELRLEEAKLVLLKKLRQSQIQKETAPQKVT---------------
                               :*****************************::.** *

Homo-sapiens            GTQNIPAGKPSLQTSSARMPGSVIPPPLVRGGQQASSKLGPQASSQVVMPPLVRGA----
Xenopus-tropicalis      ----------------SRISGTVIPPPLVRGGQQAPSK----QSTQIVMPPLVRGA----
Xenopus-laevis          ----------------SRISGTVIPPPLVRGGQHAPSK----QSSQIVMPPLVRGAQPIS
Rana-catesbeiana        ----------------SRISGTVIPPPLVRGGQQTTAK----QSNQIVMPPLVRGA----
Rana-temporaria         ----------------SRISGTVIPPPLVRGGQQ-TAK----QSNQIVMPPLVRGAQPIS
                                      :*:.*:***********: .:*     *.*:*********

Homo-sapiens            ---QQIHSIRQHSSTGPPPLLLAPRASVPSVQIQGQRIIQQGLIRVANVPNTSLLVNIPQ
Xenopus-tropicalis      ----QIHNIRQHPSTGPPPLLLAPRASVPSVQMQGQRIIQQGLIRVANVANTNLLVNIPQ
Xenopus-laevis          VSPQQIHNIRQHPSTGPPPLLMAPRASVPSVQMQGQRIIQQGLIRVANVGNTNLLVNIPQ
Rana-catesbeiana        ---QQMHNIRQHPSTGPPPLLLAPRASVPSVQMQGQRIIQQGLIRVANVANTSLLVNIPQ
Rana-temporaria         VSPQQMHNIRQHPSTGPPPLLLAPRASVPSVQMQGQRIIQQGLIRVANVANTNLLVNIPQ
                            *:*.****.********:**********:*************** **.*******

Homo-sapiens            PTPASLKGTTATSAQANSTPTSVASVVTSAESPASRQAAAKLALRKQLEKTLLEIPPPKP
Xenopus-tropicalis      ASPTSLKGSAGT---ATSISS-----ANSNDSPASRQAAAKLALRKQLEKTLLEIPPPKP
Xenopus-laevis          ASP-SLKGSTGT---ASSLSS-----ANSNDSPASRQAAAKLALRKQLEKTLLEIPPPKP
Rana-catesbeiana        ASPNTLKGSAGTAGSATSLSS-----VNANDSPASRQAAAKLALRKQLEKTLLEIPPPKP
Rana-temporaria         ASQNTLKGSAGTAGSATSLSS-----ANTNDSPASRQAAAKLALRKQLEKTLLEIPPPKP
                        .:  :***::.*   *.* .:     ..: :****************************

Homo-sapiens            PAPEMNFLPSAANNEFIYLVGLEEVVQNLLETQAGRMSAATVLSREPYMCAQCKTDFTCR
Xenopus-tropicalis      PAPELNFLPSAANNEFIYLVGLEEVVQNLLESQGKISQAPS--SHEPYICAQCKTDFTSR
Xenopus-laevis          SSPELNFLPSAANNEFIYLVGLEEVVQNLLESQGKISPASS--SHEPYICAKCKTDFTSR
Rana-catesbeiana        PTPELNFLPSAANNEFIYLVGLEEVVQNLLESQ----------------------------
Rana-temporaria         PTPELNFLPSAANNEFIYLVGLEEVVQNLLESQGKTVPQPP--SHEPYSCAQCKTDFTSR
                        .:**:**************************:*

Homo-sapiens            WREEKSGAIMCENCMTTNQKKALKVEHTSRLKAAFVKALQQEQEIEQRLLQQGTAPAQAK
Xenopus-tropicalis      WRQEKNGTIMCEVCMSSNQKKTLKAEHTNRLKAAFVKALQQEQEIEQRILQQSSSPAQSK
Xenopus-laevis          WRQEKNGTIMCDVCMSSNQKKVLKAEHTTRLKAAFVKALQQEQEIEQRILQQSSSPAQTK
Rana-catesbeiana        ------------------------AEHTNRLKAAFVKALQQEQEIEQRILQQSSSPAQIK
```



Supplementary Figure 6. Evolutionary conservation of *Rana catesbeiana* P66A protein sequence.

The protein coding sequence of *Rana catesbeiana* P66A was aligned to the P66A sequence from *Homo sapiens*, *Rana temporaria*, *Xenopus laevis* and *Xenopus tropicalis* using ClustalW. Star indicates a conserved residue, ":" indicates a conserved amino acid residue with strongly similar properties, and "." indicates a conserved amino acid residue with weakly similar properties.

```
Rana-catesbeiana        MGCVFGVITVPFLLFSSMPKSKELVSSSSSGSESDSEVDQK-------------------
Homo-sapiens            ------------------MPKSKELVSSSSSGSDSDSEVDKKLKRKKQVAP---EKPVKKQ
Rana-temporaria         ------------------MPKSKELVSSSSSGSESDSEVDQKAKRKKPAPPPEKEKPTKKQ
Xenopus-laevis          ------------------MPKSKEILSSSSSGSDSDSEVDQKVKRKKQ-PPPEKEKPAKKQ
Xenopus-tropicalis      ------------------MPKSKEIVSSSSSGSDSDSEVDQKVKRKKQ-PPPEKEKPVKKQ
                                          ******::*******:******:*

Rana-catesbeiana        -------------------------IGKMRYVSVRDFKGKVLIDIREYFMDQQGELKPGR
Homo-sapiens            KTGETSRALSSSKQSSSSRDDNMFQIGKMRYVSVRDFKGKVLIDIREYWMDPEGEMKPGR
Rana-temporaria         KTGGES-SKSSAKQSSKDPEDNMFQIGKMRYVSVRDFKGKVLIDVREYFMDQQGELKPGR
Xenopus-laevis          KTGESSKGGASSRQSS-GPEDNMFQIGKMRYVTVRDFKGKVLIDVREYFMDQAGEMKPGR
Xenopus-tropicalis      KTGESSKGGASSRQSN-APEDNMFQIGKMRYVSVRDFKGKVLIDVREYFMDQAGEMKPGR
                                         *******:***********:***:**  **:****

Rana-catesbeiana        KGNGNNRTAVLFLKKTVNENG------
Homo-sapiens            KGISLNPEQWSQLKEQISDIDDAVRKL
Rana-temporaria         KGISLNPEQWNQLKEQMSDIDNAIRKL
Xenopus-laevis          KGISLNPEQWNQLKEQMSDIDDAIRKL
Xenopus-tropicalis      KGISLNPEQWNQLKEQMSDIDDAIRKL
                        ** . *      **: :.: .
```

Supplementary Figure 7. Evolutionary conservation of *Rana catesbeiana* TCP4 protein sequence.

The protein coding sequence of *Rana catesbeiana* TCP4 was aligned to the TCP4 sequence from *Homo sapiens*, *Rana temporaria*, *Xenopus laevis* and *Xenopus tropicalis* using ClustalW. Star indicates a conserved residue, ":" indicates a conserved amino acid residue with strongly similar properties, and "." indicates a conserved amino acid residue with weakly similar properties.

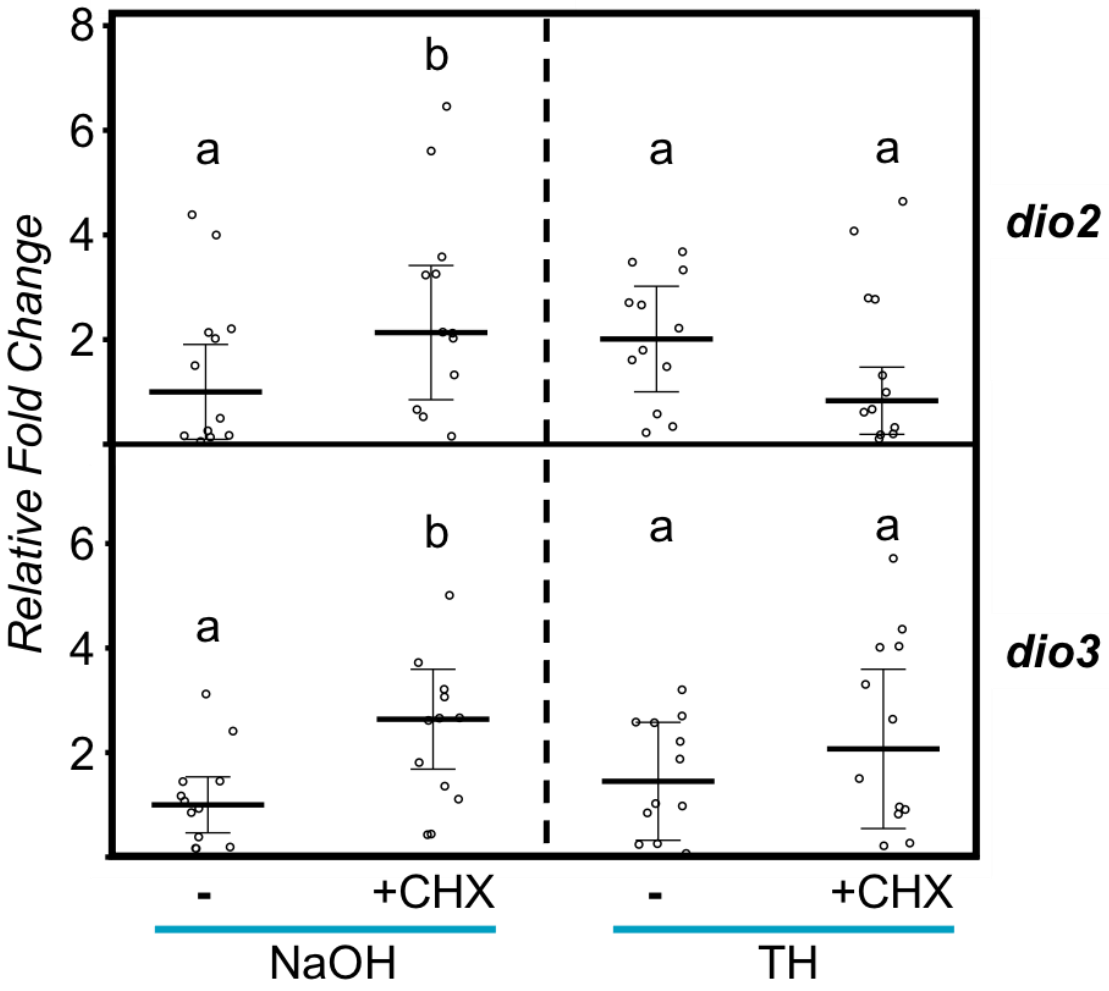

klf15 ----------MVDHLLLRDEC----------FS
klfX ----------MAIVSYEYSHMLHDITQ
klf5 ----------MATTVLTMSSRMGRLDEPVFT
klf6 ----------MDVLPMCS---------IFQ
klf7 ----------MDVLASYS---------IFQ
klf8 ----------MHSETQPAY------IASPIMS
klf12 ----------MLMLDGMPAAR-----VKTELLE
klf11 ----------MINVTTTDRAVDFMDICESILERKRHDSER
klf3 ----------MLMFDPVP-----IKQEAME
klf10 ----------MIGYSTMDRMDVCG-----FQRFGGA
klf4 MALSGTLLPSISTFTAGTPGKDKVLRQASVGNRWREELSLMKRHPALN-----QSRPYDG
klf1 ----------MPCM----------
klf2 ----------MALADTILPSFSTFSNQDKWKAAY
klf9 ----------MTAV----------
klf13 ----------MSSA----------
klf15 TNPSAVGFTTDMLMGG----------
klfX NIGSSVIPGPATE----------
klf5 QLKPVLGNGGQPPNRGDPSSGGAIFPEDMH----------
klf6 ELQIVHDTGYFSALP----------
klf7 ELQIVHDTGYFSAMP----------
klf8 SHCKIGTEASASALLA----------
klf12 TEQVSPKVHSYPDMEAVPLLLN----------
klf11 SSCSTLEQNDFEAVEALVCMSSWGQRSHKGEVLKMRPLTPASDSSDCLLQYESSPSISKD
klf3 PMPMSYQASYLESLKTNN----------
klf10 PWKSTAEKSDVEAVEALMYMSSRWKTEPKSFIDLRPITPASDFSENEDSTITA-------
klf4 LEGETAPFGSRREQEEFNELLDFDFILSNSLIHQESMVATSSSSSSATASSPPPAAPLQD
klf1 ----------
klf2 DSKTTHSSCNMSDVMHNTDPFCSKKEDEDLNNVLEFVLSMGADRNIQTCE----------
klf9 ----------
klf13 ----------
klf15 ----------RVYQMLPSPTS------EDDSDSSSFRSCSSPDSQVLSSSYGSTSSA
klfX ----------ETTLPPSGYCD------PLHTFHVSQFKHE----------
klf5 ----------KSSRLLPEDMA------QTRYEMDKYLSPHAASVCMIQEQKKYRRDS
klf6 ----------SLEEYWQQTCL------ELERYL-QSEPCYVSASEFKFDR-------
klf7 ----------SLEENWQQTCL------ELERYL-QTEPKKMSE----------
klf8 ----------DIKMEPPEQLL------DSDSSLQQSEPVDL----------
klf12 ----------NVKAEPPEDLL------ATDHFQTQTEPVDLSINKARTSPRG-----
klf11 FHSLATLCMTP--PHSPEFTEPCT------TLPPACQVTYSKPVTVMSSSSHCSVSSSNA
klf3 ----------KYSVIYPTPG----------MLHSKLYPCNEAFPAFQMEPV------
klf10 ----------DFTTIPP-FCL------TPPYSPSEFELSHSPSINPVSPSIEKSKPV
klf4 SMSALSSSANCNFTYQLRCPQDTA------GGGSLMYTPRDTTVPTLTFNLADINDVSPS
klf1 ----------ELPPDYGM------KPPMPDRKYTELRVS----------
klf2 ----------DYSLSSPESCTYYQTTPSPGGYSTDHSPPPYTSTSLMTE--------
klf9 ----------AYTDLVAAQC----------
klf13 ----------AFVDHFAAECL----------
klf15 E----------SQDSILDYLLSQTSLSNTSLSWWDKRNQQTMVKEEFFRVP
klfX ----------DDYWDCEFLQNNFL----------QPP----
klf5 ASGIDQFFSDSDGL------PYSINMNVFLPDITHLRTNLYKAQRPTVTHIKTEPPFSQQ
klf6 ----------EEDLWTKFILACEKKDDPDPQ----------
klf7 ----------AFGEDLDCFLNSSIPQGTD----------
klf8 ----------SLHKPKNLIRQSQSPLPSTSVS----------TPPPVIS
klf12 ----------VSSSPLSIATSAASPSNSSSS----------
klf11 SKPFTPAVHSLSSQSCEMS-ERSQPCRAMVTSVIRHTADTSAFHHLPQ-----SPPKAPT
klf3 ----------DLTTNKRNSPPSTG-SSPSPAKYQSPKR-----SSP----
klf10 TSTTPATSANN---------ALSPKQKAQATSVIRHTADAEFCNRRLFT----PKNCDGY
klf4 GGFVAELMRPDLDPVYMQQPQTSLQGKFVLKAAV-DMGDYGQSLSVRKSGSGMDGPMVYT
klf1 ----------NVDIPVTIQVEQMTHLG----------

```
klf2            --------------------LMRSDMDNFCHQNTVQGKFFVSSAFDFSDSIKVEPPMDSY
klf9            --------------------LVSISNRT--RGPE-VRADDE--------------------
klf13           ----------------------VSMSSRAIVHSAGHGG-----------------------


klf15           EFGVDLEE----------TPLFHPTLDEIQEFLEEKMSTDFKAETQHEVRELRNCGHLFS
klfX            --------------------ASDIANYSPQTTISQSQFQAFAEAWGTEMSNLFMPSDNPP
klf5            SEQVATQPLPEFNSIFSNHQNTDLNNIYIKQEMPSPEIQIPINHQHGQLYQLLSSSDMDL
klf6            ------------------LVCIKTEDISDSQNLETTSLNSDISSELSDSSEELSPTTKFT
klf7            ------------------GYARKLDPIVIPVDISTYEKRPSMDILL--------SQDKLL
klf8            LSGLGS------------AIPAVLSPGSILATTQGSSGQRILHVIHTIPSVNLPSKMGGL
klf12           ------------------SSCPVSSPTVITSVPSTASVPTVLTPGPLVASASGVGGQQFL
klf11           AVSSRNCDV---------KHSKLTKETPVQEDLSRSNSPMYLPQTEIPCANAN-TGRQQT
klf3            --ILFMPS----------SSPPMKKLTPSPPAVPHYQMPLAINPLLSAFPRQT-YRNPGL
klf10           NTGVGTQQQSSVVQSTENKVPLPEQVKTETQVLPSFSLANSNHGSVPSVPFQACNGKPNS
klf4            SHQL---P----------HMCQKIKQEPTASCSASCQMDGQVQQSQGQHDFQV-GRVLPS
klf1            ------------------QKIKKERPEQTCMMTPSPTSSDCMGPNLIHMSGQI-GGHAYI
klf2            GPVMGLVP----------QSCSKIKQEGSTACMMAYDQPRLANS----------PQMGGI
klf9            ------------------EPTTLLSPPAATEHLSEHHLRTMAEPLTKEHHDGG-PGEAWK
klf13           ----------------------HRSEPPAPGDPPPEDKAGKENASLFVVAR---------


klf15           EQLKQTPMVVANNMREDTNEESKSFSQEESSSKCSGTLAVEGGIPVILQIQPLEIKQESN
klfX            DQPL--PFVPGTPLDCYGSSEESSVTSGHHMAITETGALISNGQLSQGTVHNTDCVQHYM
klf5            SSTNQ-TGVMDTMSNASLSTELTGGFNTLPTPVTQTAMKTFQGIPQCSYNMHGQFMQQQE
klf6            SDPIG-DMVIKTEP-----------FSSSVTSTPPSSPEQGKESPTHLWN-------AIT
klf7            SETCV-SLQPASSSTECYTSVNQAQLNAVTSLTPPSSPELSR-----HLVKTPQSLTALD
klf8            QTI---PVVVQSLPVVYTTLPTDG-VTAHVTVPLLGADGKTTGYSPTIMVQSLDTEVEDS
klf12           HIIH--PVPPSSPLNLQSSKLSH--VHRIPVVVQSVPVVYTAVRSPGSMNSTIVVPLLED
klf11           AS-VQ-TQEPVKCENEHSNETNP--LVSVPASSSPVLCQMIPVNGQPGVLSAFIKPPSQP
klf3            LPLLQ-PVVVQPVHVQSLMYAPH--LQQQIMVSTVLAEEMESQSSKHIPMTVSDFYENPP
klf10           PDTCVIPMHIPSTPQLVPTP-----VTSNALPQVPVLCQMVPFSSSNSMVTTVVASTPAQ
klf4            RT-NQ-SLTPEELMVRDCHASPQ--TLSHPSLSIPPGYRTSASF-PHFTPDQQLQLPPTQ
klf1            QHQLG---SVEEVQINDCHMIQD--MHCLPIMQMQH-YQLAQPYQPHFNGQGPSQFHGQF
klf2            DTP---PMSPDELMLNECQTQ----LMCHPSLPYSHGYQNTAAFHHPSSIQYQSASQYSL
klf9            DYCTLLTIAKSLLE-----------LNKYRPLPSPDYSDEELSSDSDVTTESGHSYHSAS
klf13           --------------------------ILADLNQQVPGGPTEEKGSPPEI------------


klf15           TSQASPTQFPENIKVAQLLVNIQGQTFALVPQLVQSSNLSSKFVRIAPVPIAAKPMGPGG
klfX            GYFYPQPPK---------PDPMHATEPSAHFKTHPVPILPRTFTDNLMCSSTNPTKCSTT
klf5            GTYFPPSPP------SSEPGSPDRQAELLQSLNPPPS----YAATIASKMAIHNPALTTP
klf6            GELHSPGKV------------RSGCFGKTLEQGSPIS----------------------
klf7            GTVTLKLVA-------------KKASVSSGKATESAS-----------AAITSKCGLSDN
klf8            ESKGDKSHC------YRGHVSLSQNSVKIEPDMCPVE----EISDTDDCIEETSSSFQDS
klf12           GRSHLKAHM--------DPRGLSPRQIKSDSDDDDLPNVTLDSVNETGSTALSIARAVQD
klf11           MCNTIKPILPQTTSLSQQVLMGTPMSPGTVMFVLPQASVTQPSQQCPQTLMTVGNTKLLP
klf3            LLKNIKVEP----GLEQTITEVYPEQMSPGNSTPPKG----MFHEHHPSVIVHPGKRPLP
klf10           TQAVMQPVF----------YMGAQVPKGTVMFVMPQP------VMQNTKTVWASSTRLSP
klf4            LQYQGQSYL----NIYEDPSLNFQLLKQEVMLTPPSS----PL----E--LLTSGCMSEE
klf1            GVYRDPMKV--------------HPAMHGMIVTPPSS----PLVEYYPVM-----NPPED
klf2            FEDSLSMQP----------------SANRGILTPPCS-----------------PLEILD
klf9            SRTDYSPEA--------------------GSTQQPPRP-----------------PSPDTP
klf13           ---------------------------------TPPIQ--------------------PESR


klf15           GAQGQSGHMSGQKFQKNPAAELIKM-HKCSFPGCTKMYTKSSHLKAHLRRHTGEKPFACT
klfX            SQSVPSLPKGKRSKHRRSTLNKTAISHKCPYTGCEKSYTKSSHLKAHLRTHTGEKPYVCG
klf5            VQMSPATIQSVRYNRRNNPDLEKRRIHYCDYPGCTKVYTKSSHLKAHLRTHTGEKPYKCT
klf6            --------------GETSPDGKKRV-HKCLFNGCKKIYTKSSHLKAHQRTHTGEKPYRCS
klf7            EQSGNA--------GEASPENKKRV-HRCQFNGCRKVYTKSSHLKAHQRTHTGEKPYKCS
klf8            TNGSLMQHS-----GTASPDLRKRRVHQCDFEGCNKVYTKSSHLKAHRRIHTGEKPYKCT
klf12           SVSPFSIESTRRQRRSESPDSRRRRIHRCDFEGCNKVYTKSSHLKAHRRTHTGEKPYMCT
```

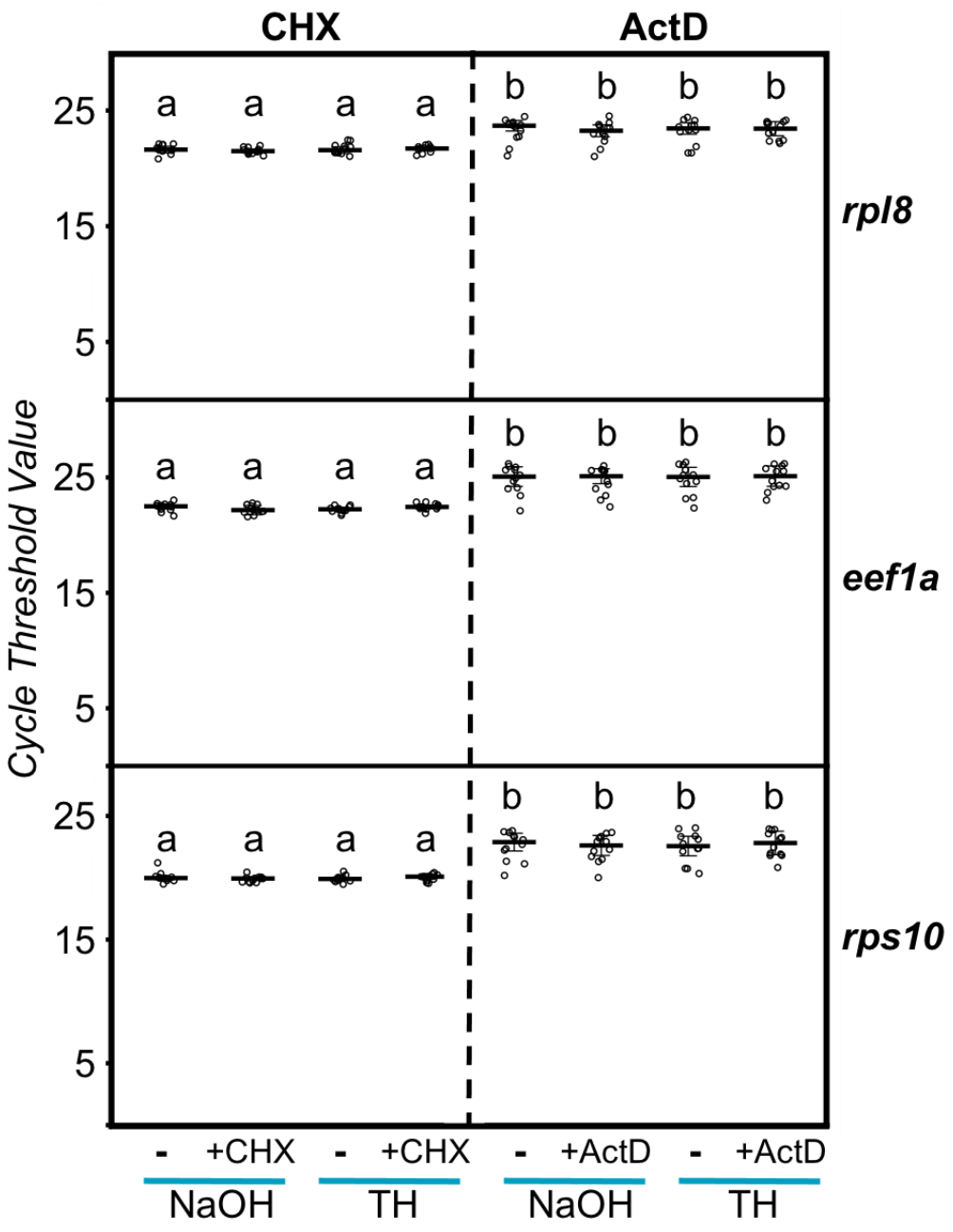
```
klf11           LAPAPVFITSGQTSPPQMDFSRRRN-YVCNFTGCRKTYFKSSHLKAHLRTHTGEKPFSCN
klf3            V-------------ESPETQRKRRI-HRCDYDGCNKVYTKSSHLKAHRRTHTGEKPYQCT
klf10           IAPAPGVSASEPKFPSPMDASRIRS-HICNQPGCGKTYFKSSHLKAHMRTHTGEKPFNCS
klf4            VKPKRG--------RKSWPRKRTAT-HTCEYAGCGKTYTKSSHLKAHLRTHTGEKPYHCD
klf1            CKPKRG--------RRSWARKRTAT-HNCEYPGCGKTYTKSSHLKAHMRTHTGEKPYHCT
klf2            AKPKRG--------RRSWPRKRTAT-HTCSYAGCGKTYTKSSHLKAHLRTHTGEKPYHCN
klf9            SSP-----------PRPAGSTSEKR-HRCPFAGCGKVYGKSSHLKAHYRVHTGERPFPCT
klf13           QRGRRGGK------SRADPESPLKR-HKCPYPGCDKVYGKSSHLKAHLRTHTGEKPFECS
                                         : *   ** * * ******** * ****.*: *

klf15           WPGCGWRFSRSDELSRHRRSHSGVKPYQCAVCEKKFARSDHLSKHIKVHRFPRSSRATRL
klfX            WDGCNWKFARSDELTRHIRKHTGVRPFQCLMCQKNFARSDHLALHMKRHIVNISQPI---
klf5            WEGCDWRFARSDELTRHYRKHTGAKPFQCAVCNRSFSRSDHLALHMKRHQN---------
klf6            WEGCTWRFARSDELTRHFRKHTGAKPFKCTHCDRCFSRSDHLALHMKRHM----------
klf7            WEGCDWRFARSDELTRHYRKHTGAKPFKCNHCDRCFSRSDHLALHMKRHI----------
klf8            WEGCTWKFARSDELTRHFRKHTGIKPFMCSDCDRTFSRSDHLALHRRRHIMM--------
klf12           WEGCTWKFARSDELTRHYRKHTGVKPFKCADCDRSFSRSDHLALHRRRHMLV--------
klf11           WEGCDKKFARSDELSRHRRTHTGEKKFACPLCDRRFMRSDHLTKHARRHMTTKKVPSWQA
klf3            WEGCTWKFARSDELTRHFRKHTGIKPFQCPDCDRSFSRSDHLALHRKRHMLV--------
klf10           WEGCERKFARSDELSRHRRTHTGEKKFACPKCDRRFMRSDHLTKHARRHLSNKKLPTWQM
klf4            WEGCGWKFARSDELTRHYRKHTGHRPFQCQRCDRAFSRSDHLALHMKRHF----------
klf1            WEGCGWKFARSDELTRHFRKHTGHRPFQCHLCERAFSRSDHLALHMKRHM----------
klf2            WEGCGWKFARSDELTRHFRKHTGHRPFQCHLCDRAFSRSDHLALHMKRHM----------
klf9            WPDCLKKFSRSDELTRHYRTHTGEKQFRCPLCEKRFMRSDHLTKHARRHTDFHPSMIKRS
klf13           WEECNKKFARSDELARHYRTHTGEKKFCCPICEKRFMRSDHLTKHARRHANFQPSMLKGR
                *  * ..*:*****:** *.*:* . : *  *:. * *****: * . *

klf15           SN----------------------------
klfX            ----------------------------
klf5            ----------------------------
klf6            ----------------------------
klf7            ----------------------------
klf8            ----------------------------
klf12           ----------------------------
klf11           EVGKLNRITTSEQTRNPGPSLSMLVSMSPPV
klf3            ----------------------------
klf10           EVSRLSDIALPQSSAPVQ-------------
klf4            ----------------------------
klf1            ----------------------------
klf2            ----------------------------
klf9            KRSNSAFI-----------------------
klf13           GGASSRNGSVSDYSRSDASSPAISPASSP--
```

Supplementary Figure 8. Protein sequence alignment of the 14 identified *Rana catesbeiana* KLFs.

The protein coding sequences of *Rana catesbeiana* KLFs, identified through sequence identity with *Homo sapiens* KLF sequences were aligned using ClustalW to query conservation between sequences Star indicates a conserved residue, ":" indicates a conserved amino acid residue with strongly similar properties, and "." indicates a conserved amino acid residue with weakly similar properties.



Supplementary Figure 9. **Inhibition of translation of deiodinase genes.**
Median relative fold change in expression of *dio2* and *dio3* in *Rana catesbeiana* tail fin biopsies (n=12) exposed to 80μg/mL CHX, 10nM T3, 80μg/mL CHX and 10nM T3 compared to 400nM NaOH control for 24h at 5°C. * indicates significance from CHX exposure to the NaOH or T3 treatment matched control ($p \leq 0.05$). Different letters indicate a significant difference between treatments ($p \leq 0.05$). See Figure 14 for additional plot details.



Supplementary Figure 10. **Effects on acetone solvent on transcription of normalizing genes.**

Median qPCR cycle threshold of *rpl8*, *eef1a* and *rps10* in *Rana catesbeiana* tail fin biopsies (n=12) exposures for 24h at 5°C. Biopsies from the same animals were exposed to two identical exposures with two different inhibitors: CHX (NaOH, NaOH and 80 μg/mL CHX, 10nM T3 (TH), 20 μg/mL CHX and 10 nM T3; with the water solvent in each condition) and ActD (NaOH, NaOH and 20 μg/mL ActD, 10 nM T3 (TH), 20 μg/mL ActD and 10 nM T3; with the acetone solvent in each condition). Different letters indicate significant differences than other letters ($p \leq 0.05$). See Figure 14 for additional plot details.

7 Supplementary Tables

Supplementary Table 1. Primer and Probe Information.
Sequences for primers and probes and annealing temperatures used to detect abundance of TH response transcripts, novel molecular memory candidate transcripts and normalizer transcripts using qPCR analysis.

Gene[a]	Primer Sequences		Probe Sequence	Annealing
	Up	Down		Temp (°C)
six1	GAACGCTGATGTTGAATAT	CTAGACGATTAACACCTCCTTG	CAGCAGGTGATAGAATATGGGAGAT	64
klfX	ACATCGGTAGCAGCGTTAT	TGAACTGAGATACATGGAAGGT	CCTGCCACGGAAGAGACTACACTGC	60
p66a	CACAACATCAGGCAACATC	GAATCTCCAGCAAGGTCTTCT	CAAGGTCAAAGAATCATCCAGCAGG	64
tcp4	ATCGGACAGTGAGGTTGA	CAGGAAGAGTACAGCAGTT	CAAGGAGAATTGAAGCCTGGAAGAA	64
thra[b]	TGATAAGGCCACAGGRTACCACTA	CGGGTGATCTTGTCGATRA	ACTATCCAGAAGAACCTGCACCCCTC	64
thrb[b]	CTCATAGAAGAAAACAGAGAAAARAGA	GAAGGCTTCTAAGTCCACTTTTCC	CATGTGGCCACCAATGCACAGG	64
thibz[c]	ASCTCCRCAGAAYCAGCA	TCACGTACCAGGCCAAAA	*NA*	62
cebp1[d]	AAAGTTGAGGAAGAGGGTGA	ACAGTTGCCCATCACTTTGAC	*NA*	60
rpl8[b]	AGGCAGGTCGTGCNTACCA	GGGATGTTCTACAGGATTCATAGC	AAACTGCTGGCCACGTGTCCGT	64
rps10[b]	GCTGCTGGTGTTGGTGART	AGCATGTTGTCACCRTTCC	TACATCAAGAAGATTGGTTACAACCC	60
eef1a[b]	GCYTGGCGTCACTTTTACTG	CACGTCCAAAYCCTCCTCTAA	AAGGCTGAGGCTGGWGCTGGAG	60

[a] Gene name acronyms for *homeobox protein SIX1* (*six1*), *krüppel-like factor X* (*klfX*), *transcriptional repressor p66-alpha* (*p66a*), *RNA polymerase II transcriptional coactivator p15* (*tcp4*), *thyroid hormone receptor α* (*thra*), *thyroid hormone receptor β* (*thrb*), *thyroid hormone induced bZip* (*thibz*), *CCAAT/enhancer binding protein 1* (*cebp1*), *type II iodothyronine deiodinase* (*dio2*), *type III iodothyronine deiodinase* (*dio3*), *ribosomal protein L8* (*rpl8*), *ribosomal protein S10* (*rps10*), *eukaryotic translation elongation factor 1A* (*eef1a*),

[b] As described by Hammond et al. (2013) and Wojnarowicz et al. (2013)

[c] As described by Veldhoen et al. (2014)

[d] As described by Hammond et al.(2015) and Mochizuki et al. (2012)

Supplementary Table 2. RNA-Seq read alignment.
75 bp paired end reads sequenced using RNA-seq from *Rana catesbeiana* C-fin (n=5) identified exposed to 10 nM T3 in 400 nM NaOH (TH) compared to 400 nM NaOH (Control) for 48h at 5^{o}C were mapped to the *Rana catesbeiana* genome version 3 (NCBI Genbank Accession No: LIAG00000000, BioProject PRJNA285814; Hammond et al., 2017).

Treatment	Animal	Input reads*	Mapped reads	% Mapped reads$^{\phi}$
Control	1	34,127,860	29,214,163	85.60
Control	2	34,065,847	29,135,036	85.53
Control	3	31,462,041	27,130,513	86.23
Control	4	26,537,161	22,714,326	85.59
Control	5	32,984,488	28,357,990	85.97
TH	1	29,474,602	25,473,557	86.43
TH	2	30,406,899	26,203,147	86.18
TH	3	30,656,595	26,546,340	86.59
TH	4	38,655,770	33,433,771	86.49
TH	5	31,007,695	26,679,729	86.04
	mean	31,937,896	27,488,857	86.07
	stdev	3,263,646	2,832,335	0.39

* Input reads counts both 75 base paired ends of the paired end reads.

$^{\phi}$% Mapped Reads includes all reads where paired end alignment was not at the specified 250 base pair distance

Supplementary Table 3. Significant Molecular Memory Transcripts.
Log2 fold change of significantly altered transcripts in *Rana catesbeiana* C-fin (n=5) identified by RNA-Seq upon exposure to 10 nM T3 in 400 nM NaOH compared to 400 nM NaOH for 48h at 5°C. Significant transcripts were identified by DESeq2 analysis (p-adj ≤ 0.05) and annotated using the NCBI non-redundant and Uniprot databases.

Transcript	Annotating Species	Log_2 Fold Change	padj
60S ribosomal protein L27a	*Nanorana parkeri*	14.14	0.00002
Zona pellucida-like domain-containing protein 1	*Rana catesbeiana*	9.36	0.00004
Waprin-Phi1-like	*Xenopus tropicalis*	7.74	0.00012
WAP four-disulfide core domain protein 18-like	*Fukomys damarensis*	6.74	$2.76x10^{-6}$
Fibronectin-like	*Xenopus tropicalis*	6.08	0.01006
Fibronectin type III domain-containing protein 7-like	*Xenopus laevis*	5.88	0.00002
Keratin, type I cytoskeletal 19	*Rana catesbeiana*	5.25	$2.21x10^{-11}$
Astacin-like metalloendopeptidase	*Nanorana parkeri*	4.97	0.00428
Integrin beta-1	*Nestor notabilis*	4.77	0.00240
Transmembrane protease serine 9-like	*Nanorana parkeri*	4.52	0.02903
Zona pellucida-like domain-containing protein 1	*Nanorana parkeri*	4.09	0.01803
Olfactomedin-4-like	*Nanorana parkeri*	4.00	$7.11x10^{-8}$
Embryonic protein UVS.2	*Xenopus laevis*	3.22	$2.76x10^{-6}$
CD109 antigen	*Peromyscus bairdii*	3.11	$1.56x10^{-56}$
Immunoglobulin kappa variable 4-1	*Homo sapiens*	2.16	0.00004
Homeobox protein SIX1	*Nanorana parkeri*	1.84	0.00016
Protein shisa-2 homolog	*Nanorana parkeri*	1.72	0.04353
Epidermal differentiation-specific protein	*Cynops pyrrhogaster*	1.67	$5.60x10^{-7}$
Fibronectin type III domain-containing protein 7	*Charadrius vociferus*	1.64	0.04081
krüppel-like factor X	*Xenopus laevis*	1.28	0.01031
Ig heavy chain precursor	*Xenopus laevis*	1.24	0.00004
Erythropoietin receptor	*Nanorana parkeri*	1.21	0.02890
Olfactomedin-4-like	*Nanorana parkeri*	1.19	0.01456
Hydroperoxide isomerase ALOXE3-like	*Nanorana parkeri*	1.05	0.01128
Hemoglobin subunit alpha-3	*Rana catesbeiana*	0.94	0.02063

Pigment epithelium-derived factor	*Nanorana parkeri*	0.90	$1.20x10^{-6}$
Thyroid hormone-induced B/Zip protein	*Rana catesbeiana*	0.88	0.00047
Ig light chain C region	*Rana catesbeiana*	0.86	$2.45x10^{-12}$
No annotation	NA	0.86	0.02528
Leptin	*Nanorana parkeri*	0.82	0.01059
Pigment epithelium-derived factor	*Nanorana parkeri*	0.77	0.00004
C-reactive protein-like	*Nanorana parkeri*	0.67	0.00740
C-reactive protein	*Xenopus Laevis*	0.66	0.02063
Kainate-binding protein	*Rana berlandieri*	0.62	$2.11x10^{-6}$
Chordin-like protein 2	*Pygoscelis adeliae*	0.62	0.00240
No annotation	NA	0.60	0.02421
Stabilin-2	*Nanorana parkeri*	0.58	0.00291
Clustered mitochondria protein homolog	*Nanorana parkeri*	0.54	0.00200
PR domain zinc finger protein 1	*Nanorana parkeri*	0.53	0.00034
40S ribosomal protein S7	*Xenopus tropicalis*	0.52	0.00509
Cysteine/serine-rich nuclear protein 1	*Nanorana parkeri*	0.52	0.00014
Serine/threonine-protein kinase pim-3	*Rana catesbeiana*	0.49	0.00075
Proto-oncogene c-Fos	*Xenopus tropicalis*	0.47	0.03618
PR domain zinc finger protein 1	*Nanorana parkeri*	0.43	0.02421
Tropomyosin beta chain isoform X4	*Myotis brandtii*	0.39	0.00391
Connective tissue growth factor	*Pelophylax nigromaculatus*	0.38	0.00648
Zinc finger protein SNAI2	*Nanorana parkeri*	0.37	0.02421
Plasminogen activator inhibitor 1	*Nanorana parkeri*	0.36	0.04081
Sacsin	*Nanorana parkeri*	0.35	0.04081
Otoraplin	*Rana catesbeiana*	0.35	0.00246
Thread biopolymer filament subunit gamma	*Rana catesbeiana*	0.32	0.02528
Periostin	*Nanorana parkeri*	0.30	0.01060
Dermatopontin	*Nanorana parkeri*	0.28	0.02704
Von Willebrand factor A domain-containing protein 5A-like	*Nanorana parkeri*	-0.28	0.02120
Major vault protein isoform X1	*Nanorana parkeri*	-0.29	0.01823
Non-receptor tyrosine-protein kinase TNK1-like	*Nanorana parkeri*	-0.30	0.04081
Interleukin-17D-like	*Nanorana parkeri*	-0.31	0.02528

Kunitz-type protease inhibitor 2-like isoform X2	*Nanorana parkeri*	-0.34	0.04567
Filamin-B isoform X3	*Xenopus tropicalis*	-0.37	0.01059
Serine/arginine-rich splicing factor 2 isoform X2	*Nanorana parkeri*	-0.37	0.04343
Keratin, type II cytoskeletal	*Rana catesbeiana*	-0.40	0.02528
High mobility group protein B2	*Alligator mississippiensis*	-0.41	0.04045
Otogelin-like protein	*Nanorana parkeri*	-0.41	0.04213
Insulin receptor substrate 2-A-like	*Nanorana parkeri*	-0.42	0.01816
Matrix Gla protein	*Rana catesbeiana*	-0.44	0.00560
Von Willebrand factor A domain-containing protein 5A-like	*Nanorana parkeri*	-0.46	0.03606
Transcription factor SOX-21	*Nanorana parkeri*	-0.47	0.02558
UPF0577 protein KIAA1324 homolog	*Nanorana parkeri*	-0.47	0.04081
60S ribosomal protein L18a	*Rana catesbeiana*	-0.47	0.00438
Cingulin isoform X3	*Nanorana parkeri*	-0.49	0.00437
Putative ferric-chelate reductase 1	*Nanorana parkeri*	-0.57	0.00381
DNA-binding protein inhibitor ID-4	*Xenopus tropicalis*	-0.59	0.02856
Forkhead box protein Q1	*Nanorana parkeri*	-0.60	6.96×10^{-11}
Matrix metalloproteinase-18	*Rana catesbeiana*	-0.64	4.29×10^{-10}
Cyclin L2 L homeolog isoform X2	*Xenopus laevis*	-0.70	0.02421
Thrombospondin-4 precursor	*Xenopus tropicalis*	-0.85	1.15×10^{-8}
Interleukin-8	*Rana catesbeiana*	-0.92	0.04353
No annotation	NA	-0.96	0.03403
Long-chain specific acyl-CoA dehydrogenase	*Nanorana parkeri*	-8.92	0.01816
Transcriptional repressor p66-alpha	*Nanorana parkeri*	-9.69	0.00075
Activated RNA polymerase II transcriptional coactivator p15	*Rana catesbeiana*	-10.82	0.04353