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REVIEW

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Frogs model man: *In vivo* thyroid hormone signaling during development

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Abstract

Thyroid hormone (TH) signaling comprises TH transport across cell membranes, metabolism by deiodinases, and molecular mechanisms of gene regulation. Proper TH signaling is essential for normal perinatal development, most notably for neurogenesis and fetal growth. Knowledge of perinatal TH endocrinology needs improvement to provide better treatments for premature infants and endocrine diseases during gestation and to counteract effects of endocrine disrupting chemicals. Studies in amphibians have provided major insights to understand *in vivo* mechanisms of TH signaling. The frog model boasts dramatic TH-dependent changes directly observable in free-living tadpoles with precise and easy experimental control of the TH response at developmental stages comparable to fetal stages in mammals. The hormones, their receptors, molecular mechanisms, and developmental roles of TH signaling are conserved to a high degree in humans and amphibians, such that with respect to developmental TH signaling "frogs are just little people that hop." The frog model is exceptionally illustrative of fundamental molecular mechanisms of *in vivo* TH action involving TH receptors, transcriptional cofactors, and chromatin remodeling. This review highlights the current need, recent successes, and future prospects using amphibians as a model to elucidate molecular mechanisms and functional roles of TH signaling during post-embryonic development.

KEYWORDS

amphibian, Xenopus, metamorphosis, perinatal endocrinology, gene regulation, molecular mechanism

1 | THYROID HORMONE SIGNALING DURING HUMAN PERINATAL DEVELOPMENT

Lack of thyroid hormone (TH) during perinatal development causes debilitating mental deficits and short stature known as cretinism (Delange, 2005), but even subtle reductions in TH signaling can cause significantly lower IQ (Biondi & Cooper, 2008). Mutations in components of TH signaling, such as TH receptors, plasma membrane TH transporters, deiodinases, and cytosolic TH binding proteins cause various forms of hypo- and hyperthyroidism (Abe et al., 2003; Hernandez, Martinez, Fiering, Galton, & St. Germain, 2006; Refetoff, 2005; Visser, W. E., Friesema, & Visser, T. J., 2010). Endocrine disrupting chemicals that interfere with any of these TH signaling components can also compromise short-term and long-term health and fitness (Gore et al., 2015).

The critical dependence on appropriate TH signaling for proper development of the brain and other organs motivates efforts to look for treatment options during pregnancy with jeopardized TH signaling, such as in Hashimoto's thyroiditis, Grave's disease, premature birth, and endocrine disruption. Unfortunately, many mechanisms pertinent to perinatal endocrinology are not well understood, and studies are needed to reduce known and unknown treatment consequences for fetal growth and development (Forhead & Fowden, 2014). The fact that the rate of preterm delivery with incomplete TH-dependent organ maturation is increasing despite extensive efforts to stop it and the increasing prevalence of endocrine disrupting chemicals points to the importance of broadening the net of basic research to better diagnose, treat, and/or prevent medical issues that may affect perinatal development (Goldenberg, Culhane, Iams, & Romero, 2008; Wang, Chen, W., Chen, C., 2014).

2 | THE VALUE OF *XENOPUS* TO STUDY TH SIGNALING DURING DEVELOPMENT

Understanding the critical, complex, and experimentally intractable period of perinatal development in humans requires studies in model organisms. Model organisms provide simplified systems that are accessible and easily manipulated and can reveal the basic operating principles and disease etiologies associated with TH signaling that are nearly the same across vertebrates. Mice are the most commonly used model to study human health and disease because of their small size, ease of breeding in lab, and evolutionary closeness to humans. However, studies to elucidate developmental mechanisms of TH signaling are constrained in mammalian systems by the difficulty of observing relatively subtle or cryptic TH-dependent changes and of obtaining samples from fetuses *in utero*. An additional difficulty is that fetal tissues are constantly exposed to maternal hormones through the placenta (Forhead & Fowden, 2014), such that manipulation of fetal endocrine signaling to examine receptor function in plus or minus hormonal states is difficult to achieve without potentially introducing artifacts from altered maternal endocrine physiology. Furthermore, as with all model systems, none can fully recapitulate the human situation. For example, mutations in the TH transporter MCT8 in humans cause the crippling disorder, Allan-Herndon-Dudley syndrome (Friesema et al., 2004), whereas equivalent mutations in mice are nearly asymptomatic (Heuer & Visser, 2013).

For elucidating the molecular mechanisms of TH signaling during development, amphibians have intrinsic experimental advantages that make them the model of choice for several reasons (Figs. 1, 2). First, the dramatic TH-dependent molecular and morphological changes that occur during metamorphosis are unrivaled among terrestrial vertebrates (Dent, 1968). Second, tadpoles are large and accessible throughout their development, including TH-dependent stages, comparable to perinatal stages in humans (Buchholz, 2015). Third, signaling via TH and their receptors is necessary and sufficient to initiate nearly all developmental events during metamorphosis (Das et al., 2010; Dodd & Dodd, 1976). Fourth, plasma levels of TH undetectable by radioimmunoassay occur naturally during the frog larval period prior to metamorphosis (Leloup & Buscaglia, 1977), indicating that virtually all TH receptors *in vivo* are in the unliganded condition. Fifth, endogenous TH levels increase to a peak at metamorphic climax (Leloup & Buscaglia, 1977), such that simple exogenous addition of TH to the rearing water during premetamorphosis enables precise temporal control of TH receptors to the liganded state that can mimic natural metamorphosis (Dodd & Dodd, 1976). Sixth, mechanisms of TH signaling in gene regulation and development are highly conserved between frogs and humans (see below), such that fundamental processes can be worked out in frogs and then applied to specific situations in mice and humans. Seventh, amphibians produce large numbers of free-living eggs and embryos that are easy to culture without specialized media or temperature requirements making tadpole studies fast, easy, and cheap with respect to comparable stages in mice (i.e., perinatal stages). Thus, because frogs are easy to breed and maintain in the laboratory, are the closest relatives to humans with easily accessible embryos, and have all the modern tools of a genetic model system, such as a sequenced genome (Hellsten et al., 2010; Session et al., 2016), an ORFeome (Grant et al., 2015), and established methods for gene knockout (Tandon, Conlon, Furlow, & Horb, 2016) and transgenesis (Buchholz, 2012; Ishibashi, Love, & Amaya, 2012), amphibian development is a particularly compelling model system from experimental and fiscal perspectives for use in elucidating TH signaling applicable to human perinatal development.

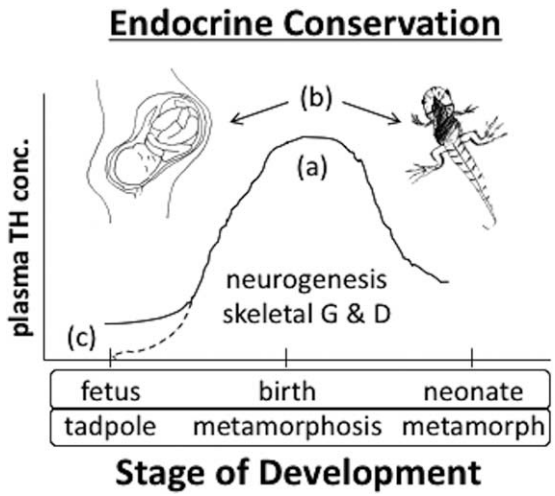

FIGURE 1 Conservation in endocrine mechanisms of TH signaling during development in mammals and amphibians. (a) During the aquatic to terrestrial transitions at birth and metamorphosis, humans and frogs each have a peak in plasma TH level, which most notably regulates neurogenesis and skeletal growth and development (G & D). (b) TH affects these processes in frogs to a higher degree than in mammals in order to accomplish the dramatic changes in diet (herbivorous to carnivorous) and locomotion (swimming to jumping). (c) A notable difference between groups is that TH is virtually absent in tadpole plasma (dashed line) until after limb bud development, whereas human and rodent fetuses are exposed to maternal TH starting at neural tube closure (Forhead and Fowden 2014), such that a distinct, experimentally advantageous switch from an unliganded to liganded condition of the TH receptors occurs naturally only during frog development.

3 | CONSERVATION IN TH SIGNALING BETWEEN FROGS AND MAN

Many developmental events are regulated by TH in common in humans and frogs, including central and peripheral nervous systems (Kollros, 1981; Patel, Landers, Li, Mortimer, & Richard, 2011; Préau, Le Blay, Saint Paul, Morvan-Dubois, & Demeneix, 2016; Thompson & Cline, 2016), musculoskeletal growth and development (Dodd & Dodd, 1976; Van Vliet, 2005), and interaction with glucocorticoids to promote terminal organ maturation (Buchholz, 2015; Fowden & Forhead, 2013). Underlying these conserved TH-dependent developmental events are conserved molecular components (Fig. 3) (Buchholz, Paul, Fu, & Shi, 2006; Furlow & Neff, 2006). As in mammals, amphibians have two types of TH receptors (TRα and TRβ) (Helbing, Gergely, & Atkinson, 1992; Schneider, Davey, & Galton, 1993; Wang, Matsuda, & Shi, 2008;

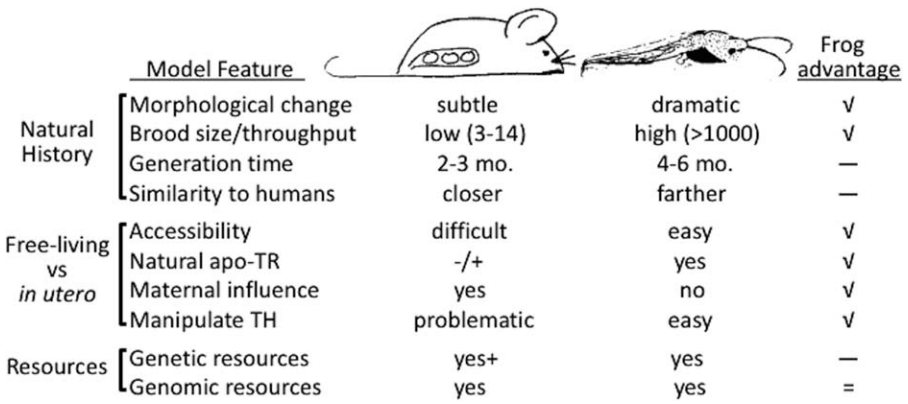

FIGURE 2 Comparison of mouse and frog models for study of *in vivo* TH signaling during development. Several unique aspects in frogs provide advantages for serving as a model to elucidate molecular mechanisms of TH signaling during development in vertebrates including humans. The key natural history assets for frogs are their exaggerated TH-dependent development, large clutch size, and free-living embryos/tadpoles giving rise to (1) ease of observation, tissue accessibility, and hormone manipulation, (2) lack of influence of maternal endocrine system on fetal development, and (3) natural development with unliganded TRs. The shorter generation time, historical advantage of mice for genome manipulation, and evolutionary closeness to humans are powerful aspects of the mouse model for TH signaling studies. However, with advances in gene disruption technology and sequencing of *X. tropicalis* and *X. laevis* genomes, the disparity in genomics tools between frogs and mice is closing.

Yaoita, Shi, & Brown, 1990) with similar alternative splicing of multiple mRNA isoforms and high amino acid sequence similarity (Yaoita et al., 1990). In addition, mammals and frogs share similar TR heterodimer partners (retinoic acid receptors alpha, beta, gamma), TR-associated co-repressors and co-activators, and canonical TH response elements in enhancer or promoter regions of TH target genes (Furlow & Neff, 2006). Furthermore, all known vertebrate TRs commonly bind genomic DNA sequences consisting of two direct repeats of a consensus hexameric AGGTCA sequence separated by four nucleotides (Das, Heimeier, Buchholz, & Shi, 2009). Prior to TR binding, TH enters cells in mammals and frogs via conserved TH transporters (Lat1, MCT8, MCT10, OATP1c1 (Choi, Moskalik, Ng, Matter, & Buchholz, 2015; Connors, Korte, Anderson, & Degitz, 2010; Ritchie et al., 2003)), followed by deiodinase-mediated metabolism (deiodinases I, II, III, (Brown, 2005; Kuiper et al., 2006)) and possibly binding cytoplasmic proteins (CRYM, PKM2, and others (Choi, Moskalik, et al., 2015; Shi, Liang, Parkison, & Cheng, 1994; Yamauchi & Tata, 2001)). Thus, amphibian TH signaling behaves as in humans to regulate many developmental events in common via comparable mechanisms with similar cytoplasmic TH signaling components and nuclear receptors (Wong & Shi, 1995).

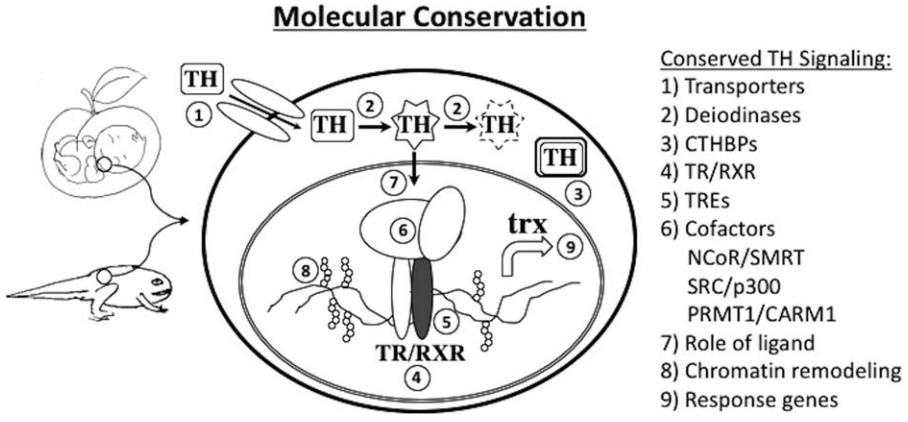

FIGURE 3 Conservation in molecular mechanisms of TH signaling in mammals and amphibians. Processes from TH transport into the cell to altered gene expression share homologous proteins and mechanisms in humans and frogs. (1) TH transporters, such as LAT1 and MCT8, enable TH entry into cells where (2) deiodinase type I, II, and III function to remove iodine atoms from TH to activate or deactivate it. Before entry into the nucleus, (3) cytoplasmic TH binding proteins (CTHBPs), e.g., mu-crystallin, modulate cytoplasmic occupancy. In the cell nucleus, (4) TH receptor (TR) heterodimerizes with retinoid-X-receptor (RXR) and binds to DNA at (5) TH response elements (TREs), where either (6) co-repressors, e.g., NCoR or SMRT, or co-activators, e.g., SRC, p300, PRMT1, CARM1, are recruited depending on (7) the absence or presence of TH. The cofactors alter (8) the state of chromatin ultimately leading to (9) induced expression of TH response genes, e.g., klf9, TRβ, in mammals and frogs.

4 | RECENT ADVANCES ELUCIDATED FIRST IN FROGS

4.1 | Role of unliganded TH receptor in post-embryonic development

The action of TH receptors (TRs) on gene regulation depend on TH, such that TRs actively repress genes in the absence of TH and induce those same genes in the presence of TH (Cheng, Leonard, & Davis, 2010). Such ligand-dependent gene regulation was derived from *in-vitro* cell culture studies, but the dual function model for how TRs might act during development was first proposed in frogs to account for TR action during critical periods of tissue differentiation when TRs transition from unliganded to liganded states (Buchholz et al., 2006; Sachs et al., 2000; Shi, 2009; Yaoita & Brown, 1990). Numerous studies in frogs and mice established that TR-mediated gene induction in response to TH is required for tissue differentiation, but only recently were two groups, working in frogs, able to address the role of unliganded TRα during development (Choi, Suzuki, et al., 2015; Wen & Shi, 2015). Using TALENs, they showed that TRα knockout led to earlier initiation of metamorphosis and acceleration of development caused by higher TH response gene expression from lack of TRα-mediated repression. Equivalent knockout models were previously made in mice (Flamant & Samarut, 2003), but effects of unliganded TRs have not been unequivocally demonstrated (Bernal & Morte, 2013), revealing the importance of the frog model to detect activities of TR during development. The production of a knockout of TRβ is highly expected to define the relative contribution of each TR isoform.

4.2 | Identification of TH direct response genes in developing tissues

The effects of TH on development are mediated mainly through transcriptional regulation of TH response genes (Buchholz, Tomita, Fu, Paul, & Shi, 2004). Identification of such direct response genes is thus of critical importance in understanding TH action during development. However, only a limited number of direct T3 response genes are known in different model systems, thus hampering our understanding of how TH regulates development *in vivo*. The first global analysis to identify such genes in development was conducted in frogs using cycloheximide-treated tadpoles to block translation thereby blocking secondary transcriptional regulation by TH-induced transcription factors (Das et al., 2009). They identified 188 up-regulated and 249 down-regulated genes by TH in the absence of new protein synthesis in whole animals, and gene ontology analysis showed that the direct up-regulated genes are enriched in categories important for transcriptional regulation and protein degradation-dependent signaling processes but not DNA replication. These findings thus revealed the pathways induced by TH at the earliest step of TH-dependent development.

Another experimental approach is to use chromatin immunoprecipitation (ChIP) to identify direct response genes (Grøntved et al., 2015). With ChIP-Seq technology, high throughput sequencing of purified DNA fragments enriched in TR binding sites followed by location of sequencing reads on the genome sequence supply genome-wide TR binding profiles. However, ChIP-seq suffers from a major limitation, as it provides no evidence of the functional connection between the TR binding sites and the target genes (only nearby locations can be inferred). Furthermore, it is now clear that transcription factors can act over large distances through DNA looping with their target promoter. To take into account these concerns, chromatin interaction analysis by paired-end tags sequencing (ChIA-PET) was carried out to map genome-wide TR binding sites and to link them with their target promoter by resolving long-range interaction between enhancer and promoter (Buisine et al., 2015). ChIA-PET requires high-resolution genome (assembly and annotation) that is achieved in *Xenopus tropicalis* (Buisine et al., 2015; Hellsten et al., 2010) in order to provide ChIP-seq and chromosome conformation capture analyses simultaneously. Preliminary data show that, indeed, TR can act over large genomic distances and that identification of TR direct target genes is optimized (Buisine et al., 2015). This study is the first to benefit from such technology using chromatin material isolated directly from animal tissues (Buisine et al., 2015). Analyses of the TR ChIA-PET data are ongoing. This will represent a big step in understanding TH and TR action in a way not yet addressed in mammals.

4.3 | Recruitment of cofactors to TH-response genes in vivo

Numerous cofactors (corepressors and coactivators) have been found to interact with TR using various biochemical approaches *in vitro* (Glass & Rosenfeld, 2000). Genetic studies and the first use of ChIP from living tissue was performed in frogs to elucidate cofactor recruitment in TH/TR action *in vivo* during development (Sachs & Shi, 2000). Nuclear receptor corepressor (NCoR) and histone acetylase 3 (HDAC3) are major corepressor components recruited to TREs in the absence of TH during early development and released following TH treatment. Experimental confirmation of the importance of NCoR in TH-mediated gene repression *in vivo* was first obtained in frogs using overexpression of dominant negative NCoR by *in vivo* gene transfer into tadpole tail muscle (Sachs et al., 2002) and later by transgenesis (Sato, Buchholz, Paul, & Shi, 2007), which led to the loss of repression by unliganded TR and increased tadpole development rate. Like corepressors, coactivators, namely steroid receptor coactivator (SRC/p160), histone acetyltransferase (p300), coactivator associated arginine methyltransferase 1 (CARM1), and protein arginine methyltransferase 1 (PRMT1), were first shown to be recruited in a TH-dependent manner to TH-response genes in frogs *in vivo* during development (Matsuda, Paul, Choi, Hasebe, & Shi, 2007; Matsuda, Paul, Choi, & Shi, 2009; Paul, Buchholz, Fu, & Shi, 2005; Paul, Fu, Buchholz, & Shi, 2005; Paul, Buchholz, Fu, & Shi, 2007). Recruitment of SRC3 in a gene and tissue dependent manner to TREs demonstrates the value of *in vivo* studies (Havis, Sachs, & Demeneix, 2003; Paul, Buchholz, et al., 2005; Paul, Fu, et al., 2005). Transgenic overexpression of dominant negative SRC3 and dominant negative p300, which compete for recruitment of endogenous coactivators, prevented TH-dependent gene regulation and thereby caused

delayed or arrested metamorphosis (Paul, Buchholz, et al., 2005; Paul, Fu, et al., 2005; Paul et al., 2007).

4.4 | TH-dependent chromatin modifications in vivo

Chromatin modifications (covalent changes to DNA or histones) include DNA methylation, post-translational histone modifications, and altered chromatin structure, including histone composition and nucleosome displacement or removal. All of these aspects of chromatin modification were analyzed in the context of TH-dependent gene regulation in amphibians (Grimaldi, Buisine, Miller, Shi, & Sachs, 2013). Antibodies for studying chromatin modifications are against antigens highly conserved among vertebrates, which when coupled with advantages of metamorphosis make the frog system a superior model for elucidating mechanisms of TH-dependent chromatin remodeling *in vivo*. Several studies have shown that liganded TR induces chromatin remodeling *in vivo*. First, analysis in the reconstituted frog oocyte system suggests that TR is able to recognize a TRE within chromatin, TR makes use of chromatin assembly process to silence transcription efficiently, and TR directs the disruption of TRE chromatin structure in response to TH (Li, Imhof, Collingwood, Urnov, & Wolffe, 1999; Wong, Li, Levi, Shi, & Wolffe, 1997; Wong, Shi, & Wolffe, 1997; Wong, Shi, & Wolffe, 1995; Wong, Liang, Sachs, & Shi, 1998). The chromatin disruption at the TRE corresponds to the loss of 2 to 3 nucleosomes, a process that has been observed during metamorphosis (Matsuura, Fujimoto, Fu, & Shi, 2012). BRG1 and BAF57, recruited by liganded TR (Heimeier, Hsia, & Shi, 2008), are good candidates to remove the nucleosomes at the target genes. In the oocyte-reconstituted system, p300 recruitment by liganded TR modifies histones to initiate the recruitment of BRG1 (Huang, Li, Sachs, Cole, & Wong, 2003). Before metamorphosis, histones H3 and H4 are deacetylated around TRE loci in the absence of TH and are later acetylated when TH level rises (Sachs & Shi, 2000). Many studies *in vivo* in frogs have provided more detail than the analyses done in mammals and have highlighted (1) the importance of histone acetylation, i.e., a correlation with the levels of gene expression, TR binding, and RNA polymerase II recruitment (Bilesimo et al., 2011; Matsuura et al., 2012), (2) treatment with HDAC inhibitor (Sachs & Shi, 2000; Sachs, Amano, & Shi, 2001; Sachs, Amano, Rouse, & Shi, 2001), (3) specific lysine acetylation in agreement with cofactor recruitment with HAT or HDAC activity (Havis et al., 2006), (4) transgenic overexpression of dominant positive TR (Buchholz et al., 2004) or dominant negative TR (Buchholz, Hsia, Fu, & Shi, 2003), and (5) transgenic overexpression of a dominant negative SRC3 (Paul, Buchholz, et al., 2005; Paul, Fu, et al., 2005).

Histone methylation is a more complex type of modification, which can correlate with transcriptional silencing or activation (Kouzarides, 2007). ChIP assays were carried out with antibodies against different histone modifications in several tissues (brain, intestine, and tail fin) in premetamorphic tadpoles and during TH-induced or natural metamorphosis. First, the repressive marks, H3K9 dimethylation and trimethylation, are not involved in TR-mediated TH response gene regulation (Bilesimo et al., 2011; Matsuura et al., 2012). Second, Me3H3K27, another repressive mark, showed a tissue-specific deposition in premetamorphic tadpoles inversely correlated with the basal low level of TH-response gene expression measured in the absence of TH (Bilesimo et al., 2011). Following TH treatment, Me3H3K27 levels at TREs decreased pointing to a role of Polycomb and Trithorax for regulating TH-response genes (Bilesimo et al., 2011). Next, the level of Me3H3K79 and Me2H3R17, two active marks, increased upon TH treatment (Matsuura et al., 2012), suggesting the recruitment of Dot1L to methylate H3K79 and correlating with CARM1 recruitment for H3R17 (Matsuda, Paul, Choi, & Shi, 2007). Dot1L knockdown with TALENs revealed its important role together with Me3H3K79 leading to retarded growth and lethality prior to metamorphosis (Wen, Fu, Guo, Chen, & Shi, 2015). Finally, H3K4 methylation marks are more ambiguous because they can correlate with either activation or repression and have gene- and tissue-specific variations in the context of TH-dependent regulation of transcription in premetamorphic tadpoles following TH treatment (Bilesimo et al., 2011). Histone methylation is under intense investigation to clarify their *in vivo* roles in gene regulation, with frog studies pushing the field, showing that H3K4 methylation levels correlated with TR binding to TRE (Bilesimo et al., 2011), an observation in accordance with its association with mammalian enhancer or promoter regions (He et al., 2010; Heintzman et al., 2009) and the possible co-occurrence of the active Me3H3K4 mark with the repressive Me3H3K27 mark (Bilesimo et al., 2011).

4.5 | Tool for screening TH-active compounds

The extreme sensitivity and responsivity of tadpoles to TH signaling provides a great platform for assaying chemicals that affect TH signaling. At the organismal level, the thyroid system represents an important target of endocrine disruption (Boas, Feldt-Rasmussen, & Main, 2012; Brucker-Davis, 1998), thereby prompting development of the *Xenopus* metamorphosis assay for screening TH-disrupting chemicals (Opitz et al., 2005). A high-throughput, whole-tadpole method using transgenic animals that express green fluorescent protein upon exposure to TH-disrupting chemicals was also developed (Castillo et al., 2013; Fini et al., 2007). At the receptor level, the two TR isoforms, α and β, have distinct tissue-specific expression patterns (Cheng et al., 2010), such that use of TR antagonists and TR isoform-selective agonists may enable targeting specific tissues and avoid affecting off-target tissue-specific side-effects. Tadpoles were the *in vivo* model of choice to assess chemicals to modify TR function, including NH3 (TR antagonist (Lim, Nguyen, Yang, Scanlan, & Furlow, 2002)), CO23 (TRα-selective agonist (Ocasio & Scanlan, 2006)), and GC1 (TRβ selective agonist (Furlow et al., 2004)). The compounds GC1 and CO23 were then used to show that in tadpole brain development, TRα and not TRβ is responsible for neural progenitor proliferation (Denver, Hu, Scanlan, & Furlow, 2009). In a further use of frog metamorphosis to reveal new insights, numerous microarray studies were used to identify targets of endocrine disruptors (Kulkarni & Buchholz, 2013). For example, the known xenoestrogen bisphenol A (BPA) was shown to antagonize the regulation of most TH-response genes, thereby suggesting that BPA predominantly affected TH-signaling pathways during development (Heimeier, Das, Buchholz, & Shi, 2009). More importantly, this result provided

molecular evidence for the likely deleterious effects of BPA on human development and the importance of studying endocrine disruption in a developmental context *in vivo*.

5 | FUTURE POTENTIAL TO ELUCIDATE *IN VIVO* TH SIGNALING MECHANISMS USING FROGS

Future potential benefits regarding the preceding topics were covered in the sections above. Beyond those topics, TH signaling is such a dominant feature of frog post-embryonic development that many aspects of development related to or dependent on TH signaling are also powerfully studied using the frog system. Such TH-dependent processes where fundamental discoveries are being made include neurogenesis (Denver et al., 2009; Préau et al., 2016; Thompson & Cline, 2016), origin of adult intestinal stem cells (Ishizuya-Oka & Shi, 2005; Ishizuya-Oka et al., 2009), early eye development (Havis et al., 2006; Bronchain et al, 2016), spinal cord regeneration (Bhumika & Darras, 2013; Gibbs, Chittur, & Szaro, 2011; Lee-Liu et al., 2014), and interaction between TH and stress hormones at global and local scales (Bagamasbad et al., 2015; Kulkarni & Buchholz, 2012).

Of special note, because it has the potential to impact studies on not only TH signaling but also gene regulation more generally, is the use of the frog model in network analysis to integrate transcriptome analyses, transcription factor binding, and epigenetic analyses to dramatically change how fundamental questions in biology are addressed. Such ambitious studies depend on the exploitation of the sequenced *Xenopus tropicalis* genome (Hellsten et al., 2010) and *Xenopus leavis* genome (Session et al., 2016) and the rise of high-throughput sequencing technologies and their applications to RNA (RNA-Seq). The execution of biological processes induced by TH (or any other bioregulatory molecule) requires the interaction and regulation of thousands of molecules. Systematic approaches to study large numbers of genes have revealed complex molecular networks as well as novel insights in understanding basic mechanisms controlling normal biological processes and pathologies (Zhu, Gerstein, & Snyder, 2007). Collection of large-scale data sets has begun and is being assembled into a network format whose topological structure contains significant biological properties (Sachs unpubl.). The integration of all interactions/modifications along with their dynamics will reveal a more complete description of how complex biological processes occur and can be controlled. These data based on work in frogs will allow more precise modeling human disease and to test more accurately corrective actions.

6 | CONCLUSIONS

Starting with the discovery that thyroid extracts fed to tadpoles induces metamorphosis (Gudernatsch, 1912), a century of discoveries stemmed from metamorphosis research, highlighted by hypothalamic and pituitary control of TH secretion (Allen, 1938; Dodd & Dodd, 1976), TH induction of mRNA and protein synthesis pointing to a nuclear action for TH receptors (Tata, 1965, 1966), TH induction of biochemical changes during development (Frieden & Just, 1970), and identification of TH-response gene regulation cascades (Brown et al., 1995, 1996; Brown et al., 2005; Buckbinder & Brown, 1992; Shi & Brown, 1990; Wang & Brown, 1993). Since these discoveries, the genome sequence, genetic methods (*in vivo* gene transfer, transgenesis, gene knockout) have been added to the intrinsic benefits of frog biology (Buchholz, 2012; Grant et al., 2015; Hellsten et al., 2010; Ishibashi et al., 2012; Session et al., 2016; Tandon et al., 2016). Elucidating the complexity of TH signaling at molecular and organismal levels will continue to require exemplary animal models, such as frog development, with its inherent biological advantages and continued development of experimental and bioinformatics tools. Potential application of fundamental discoveries in the treatment of disease and effects of endocrine disrupting chemicals can also be tested using the *in vivo* frog model. The frog system has been and will be a full-service model at the forefront in the study of TH-dependent development. Another century of discovery awaits as we utilize the amazingly dramatic frog metamorphosis model to study *in vivo* mechanisms of TH signaling during development.

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