Expression of novel ING variants is regulated by thyroid hormone in the *Xenopus laevis* tadpole

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Running title: Frog ING variants are TH responsive

Keywords: tumor suppressor; ING1; alternative splicing; amphibian metamorphosis; ING2; thyroid hormone
Summary

The candidate tumor suppressor gene, *ING1*, encodes several protein isoforms as a result of alternative splicing that may possess agonistic and antagonistic roles in the control of cell proliferation and apoptosis. Recently a related gene, *ING2*, was isolated in human whose expression is increased in adenocarcinomas. Little is known about the cellular function and regulation of these ING family members, but the fact that ING proteins contain a PHD finger suggests that these proteins may modulate transcription factor-mediated pathways. In order to elucidate how ING may interact in different tissues to modulate function, we used amphibian metamorphosis as a model system in which a single stimulus, thyroid hormone (TH), initiates tissue-specific proliferation, differentiation and apoptosis. We have isolated the first *Xenopus laevis* ING2 and demonstrate that transcript levels increase in response to TH treatment. We provide evidence for the existence of splice variants that are differentially expressed in tissues with different TH-induced fates. Western blots using an antibody directed against the highly conserved C-terminal end of ING proteins reveal a tissue-specific pattern of ING isoform expression in adult *Xenopus* tissues. Analyses of premetamorphic tadpole tissues show a TH-induced accumulation of ING proteins in tail, whereas the levels in the leg are not affected. This TH-induced accumulation is also observed in serum-free tail organ cultures and is prevented by inhibitors of tail apoptosis. Therefore, this work presents the first link between ING expression and a hormonally-regulated nuclear transcription factor-mediated apoptotic response opening the possibility that ING family members may be involved in transducing the signal initiated by TH that determines cell fate.
**Introduction**

The *ING1* (inhibitor of growth 1) gene was first isolated by PCR-mediated subtractive hybridization for the enrichment of transcripts found in non-tumorigenic breast epithelial cells followed by a novel in vivo positive selection procedure for growth inhibitors (1). *ING1* is implicated in the control of several key cellular processes (for review, see (2)) including cellular proliferation (1,3,4), apoptosis (5-7), senescence (3), and drug resistance (8). *ING1* transcript levels are depressed (1,9-13) and the *ING1* gene is a target for loss of heterozygosity or rearrangement (1,3,13-16) in a variety of cancer cells suggesting that *ING1* functions as a tumor suppressor. At least four *ING1* transcripts are ubiquitously expressed in adult and fetal tissues with varying levels; products of alternative splicing of a variable first exon and a common second exon (4,9,17,18). Several known protein products are encoded by these transcripts; however, no systematic analysis of protein expression in different normal tissues has yet been reported.

Recently, two distinct *ING1* homologs, one encoding a 33 kDa protein and the other a 5 kDa protein, have been identified in humans and mice, but much less is known about them compared to *ING1* (9,19,20)(Cal et al., unpublished; Genbank Accession # AJ006851). The former gene has been referred to as *INGL* and *ING2*, whereas the latter has been referred to as *ING2* but is distinct from the former. We will refer to the former gene as *ING2* and the latter as *ING4* to avoid confusion. A more distantly related ING3 mRNA encoding a putative 47 kDa protein has also been reported in mouse (Zenklusen and Green, unpublished; Genbank Accession #AY007790). *ING2* maps to a different chromosome than *ING1*. Its putative protein product is predicted to also be 33 kDa in size and has 54% sequence identity to p33ING1b (19,21). *ING4* encodes a putative protein of 5 kDa representing the C-terminal end of *ING1* sequences with 69% amino acid identity to p33ING1b.
Both \textit{ING2} and \textit{ING4} transcripts are ubiquitously found in fetal and adult human tissues. The gene structure of \textit{ING2} appears to be similar to \textit{ING1} with 2 exons (Nagashima \textit{et al}, unpublished), but no splice variants have been described. \textit{ING2}, like p33\textsuperscript{ING1b}, can regulate the expression of genes involved in apoptosis such as \textit{p21} and \textit{bax} (20). \textit{ING4} transcript levels are decreased in breast and melanocyte cancer cell lines (9) and \textit{ING2} transcripts are elevated in adenocarcinomas compared to adjacent normal tissue (19) suggesting that each ING family member is independently regulated and has its own unique effects.

\textit{ING} proteins belong to a family of plant homeodomain (PHD) finger-containing proteins that include transcription factors and proteins that regulate chromatin structure (22). Although the mechanisms of action for \textit{ING} proteins have yet to be fully elucidated, there is evidence that \textit{ING} proteins may affect the activity of p53 (6,20,23), histone acetyl transferases (HATs) (24,25), and histone deacetylases (HDACs) (25,26). The combination of splice variants, multiple potential protein products and at least three related genes allows for considerable possibilities for \textit{ING} to modulate cellular effects as both agonists and antagonists. Indeed, recent reports suggest that p33\textsuperscript{ING1b} and p24\textsuperscript{ING1c} are functional antagonists with respect to modulation of p53 (4) and HDAC activity (26) and that p33\textsuperscript{ING1a} and p47\textsuperscript{ING1} have opposite effects on HAT activity (25).

In order to elucidate how \textit{ING} modulates cellular outcome in different tissues, we turned to amphibian metamorphosis as a model system in which a single stimulus, thyroid hormone (TH), initiates tissue-specific proliferation, differentiation and apoptosis. The metamorphosis of the tadpole to a frog is absolutely dependent upon a substantial increase of endogenous levels of 3,5,3’-triiodothyronine (T\textsubscript{3}) from undetectable levels in the plasma (27,28). The other TH, thyroxine (T\textsubscript{4}), also increases, but it is the predominant form transported to target tissues where it is converted to the more active T\textsubscript{3} form. Virtually every tissue in the tadpole is a target of TH and these changes
can be precociously induced by exogenous TH administration in vivo and in culture (29-35). TH functions to selectively activate tissue-specific genetic programs by regulating gene transcription via specific nuclear receptors (TRs) (29,35-42). TRs have important roles as repressors and activators of gene transcription during Xenopus development (for review see (43)). In premetamorphic tadpoles, TRs function as repressors of TH-inducible genes in the absence of appreciable levels of TH thereby preventing precocious metamorphosis. When endogenous TH levels rise, they act as activators of these genes thereby initiating metamorphosis. Thus, the presence or absence of ligand plays a critical role in gene regulation. However, what still remains enigmatic is how TRs can promote the development of multiple cell fates such as proliferation, reprogramming and apoptosis during metamorphosis. Several factors that modulate TR activity have been described and include HATs/HDACs and p53 (44-56) and it is postulated that tissue-specific factors may modulate the TH-induced outcome (for review see (43)).

Herein, we describe the isolation, cloning and initial characterization of the first frog ING2 gene (xING2 for Xenopus ING2) and provide evidence suggesting that xING2 is subject to alternative splicing. We demonstrate that transcript levels differentially increase in response to T3 treatment in tissues with different metamorphic fates. Western blots using an antibody directed against the highly conserved C-terminal end of ING proteins reveal a complex pattern of expression in adult Xenopus tissues. While premetamorphic tadpole tissues show T3-induced accumulation of ING proteins in the tail, the levels in the leg are not affected. This T3-induced accumulation is also observed in serum-free tail organ cultures and is abrogated by inhibitors of tail apoptosis. Therefore, this work presents the first link between ING expression and a hormonally-regulated nuclear transcription factor-mediated response. Since ING proteins appear to associate with chromatin (7,25), we speculate that ING family members may be involved with the
modulation of thyroid hormone receptor activity and may be a link to tissue-specificity of the TH-induced response.

**Experimental Procedures**

**Isolation of RNA, generation of cDNA libraries and 5′/3′ RACE of ING fragments**

Total RNA was isolated from various tissues using TRIZOL (Life Technologies, Burlington, ON) following the manufacturer’s instructions. cDNA libraries were made from poly A+ mRNA isolated from *Xenopus* tadpole brain and tail using the Marathon cDNA amplification kit for 5′ and 3′ RACE (Clontech, Palo Alto, CA).

The initial RACE reactions used the AP1 primer supplied with the kit (5′-CCATCCTAATACGACTCACTATAGGGC) paired with either the H2 gene specific primer (5′-CATAGGAGACCTGGTTGCACAGACAGT) from the human sequence that spans the beginning of the PHD finger domain for 5′ RACE or the reverse and complement, H1 (5′-ACTGTCTGTGCAACCAGGTCTCCTATG), for 3′ RACE. Primer design was accomplished using Primer Premier software (Premier Biosoft International, Palo Alto, CA). Touchdown PCR was performed for all the RACE-PCR reactions (5 cycles at 94°C for 30 seconds, 72°C for 3-6 minutes; 5 cycles at 94°C for 30 seconds and 70°C for 3-6 minutes and; 25 cycles at 94°C for 20 seconds and 68°C for 3-6 minutes) as per the manufacturer’s instructions (Clontech).

Different primers were used for the second round of amplification. For 3′ RACE, the frog primer (5′-CGTGTTCGTATCGGTATTGCT3′) and AP1 adaptor-specific primer were used at 20 pmol per reaction on the *Xenopus* brain and tail cDNA libraries. The PCR cycling conditions were 5 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 62°C and 2 min at 72°C, plus 10
min at 72°C. For 5’ RACE, the frog primer (5’GGTACTCCTCCACATAGCTCACCAGTT3’) and AP1 adaptor-specific primer were used as above except 10 pmol per reaction were used.

The PCR reactions were run on 1% agarose gels and transferred to Nytran Plus membrane (Mandel Scientific Co. Ltd., Mississauga, ON) using a VacuGene XL vacuum blotting apparatus (Amhersham Pharmacia Biotech, Baie d’Urfe, PQ). The transfer was carried out as described in Protocol No. 1 supplied by the manufacturer except that the membrane was UV crosslinked in a UV Stratalinker 2400 (Stratagene, La Jolla, CA) immediately after transfer to immobilize the DNA. The membranes were hybridized overnight with a human ING1 cDNA exon 2 fragment and bands were detected with CDP-Star reagent using AlkPhos Direct Kit reagents (Amersham Pharmacia Biotech).

Those PCR reactions found to be positive for frog ING sequences through Southern blotting were used directly in cloning or, in the case of 3’ RACE reactions, were first purified with a Qiaquick PCR Purification Kit (Qiagen, Mississauga, ON). The TOPO TA Cloning Kit Dual Promoter System with PCR II-TOPO vector and TOP10 cells (Invitrogen, Groningen, Netherlands) was used for ligation and bacterial transformation as suggested by the supplier using 2 µL of PCR products for the reaction. The potentially positive clones were picked for creation of liquid stocks in LB broth containing 100 µg/mL ampicillin (Sigma-Aldrich, Oakville, ON) and subsequently spotted onto nitrocellulose Trans Blot Transfer Medium (Bio-Rad Laboratories, Mississauga, ON). These membranes were placed on LB plates with 100 µg/mL ampicillin and grown overnight at 37°C. The bacteria were then lysed on the membrane by placement on filter paper with 0.5M NaOH for 5 min, 1M Tris-HCl pH 8.0 for 5 min, and 0.5M Tris pH 8.0/1.25M NaCl 5 min. The DNA was UV cross-linked to the nitrocellulose as described previously and then washed with 2X SSC/2% SDS followed by 2X SSC. The membranes were hybridized with human ING1 cDNA.
probe as described above but for only 1 h prior to the detection with CDP-Star reagent. Positive clones were used to inoculate 5 mL cultures of LB broth with 100 µg/mL ampicillin which were grown overnight at 37°C with shaking. Plasmids were harvested with a Qiaprep Spin Miniprep kit (Qiagen) and subsequently digested with EcoRI. A 1% agarose gel was used to separate the inserts from plasmid vector and the products were Southern blotted overnight as described previously. Positive clones were then sequenced. The DNA and derived amino acid (aa) sequences were aligned using Clustal W version 1.8 software (57).

Northern Blot Analyses

Analyses of RNA transcripts was done according to the method described in (58) using the frog ING2 ORF fragment or a human 300 bp ING1 PCR fragment from exon 2. This latter fragment has limited sequence identity to frog ING2 and under the hybridization conditions used, does not cross-hybridize with ING2 transcripts.

RT-PCR Analyses

To determine the relative expression levels of xING2 transcripts, RT-PCR analyses were performed. The primers spanning the putative exon 1/2 boundary (XB5/XB8) and within the putative exon 2 (XB9/XB10) are indicated in Figure 1 and yield amplicons of 635 and 253 bp, respectively. All reactions were determined to be in the linear range of amplification and normalized to L8 ribosomal protein transcript whose expression is not affected by T3 treatment (59). For amplification of control L8 ribosomal protein transcript, the sense primer (5’CAGGGGACAGAGAAAAGGTG3’) and antisense primer (5’ACGACGAGCAGCAATAAGAC3’) were used to generate a 700 bp amplicon. The levels of
TRα and TRβ transcripts were determined by amplifying 480 and 342 bp amplicons, respectively. For TRα, the sense primer (5’ CACTACCGCTGTATCCTTG3’) and antisense primer (5’ GGTTGATTATCTTGGTA3’) were used (60). For TRβ, the sense primer (5’ CCAGTGCCAAGAATGCTG3’) and antisense primer (5’ GTAAACTGGCTGAAGGCT3’) were used (60). All primers were used at 20 pmol in a typical 50 µl reaction containing 1.5 U Taq DNA polymerase (Ambersham Pharmacia), 10 nmol dNTPs (Life Technologies) and 1.5 mM MgCl2. The PCR reaction was: 7 min at 94°C, 35 cycles of 60 s at 94°C, 60 s at 55°C (for TRα, TRβ, and L8) or 54°C (for xING2 primers), and 1 min at 72°C. A final 10 min extension at 72°C was done. The L8 reaction was the same except only 30 cycles were used. The amplified products were separated on 2% agarose gels and visualized by ethidium bromide staining.

Protein Isolation and Western Immunoblotting Analyses

Tissue samples were obtained fresh and homogenized immediately in 3 ml/g tissue of 50 mM HEPES, 150 mM NaCl, 0.1% Tween 20, 2.5 mM EGTA, 1 mM EDTA, 100 mM PMSF, 20U/ml aprotinin, 10 mM β-glycerophosphate, 0.1 mM Na3VO4, 1 mM NaF and 1 mM DTT and processed as described in (58). Protein samples were resolved by electrophoresis through 5-15% gradient SDS-polyacrylamide (PAGE) gels and transferred to nitrocellulose membrane as was previously described (58).

Western blot analyses were performed using a rabbit polyclonal antibody generated using a human GST-ING1 C-terminal end fusion protein (1) using methods described previously (58) with minor modifications. Briefly, blocking was performed overnight at 4°C in PBS containing 5% skim milk, 2% fetal bovine serum (Life Technologies) and 0.1% v/v Tween-20 in PBS pH 7.2. The polyclonal antibody was used at a 1:10,000 dilution in blocking buffer. The antibody incubation
was carried out at room temperature for 1 h. Membranes were washed extensively in PBS with 0.1% v/v Tween-20 for 10 minutes and incubated with goat anti-rabbit IgG polyclonal antibody conjugated to horseradish peroxidase (Calbiochem, La Jolla, CA). Peroxidase activity was detected by using an ECL kit according to the manufacturer’s instructions (Amhersham Pharmacia Biotech). Binding specificity was determined by comparison of unblocked antibody with antibody incubated with GST only or blocked with GST-p33\(^{\text{ING1b}}\). As a positive control, we have confirmed that this antibody can recognize bacterially-expressed xING2 protein (data not shown).

**Thyroid hormone treatment of *Xenopus* tadpoles**

Premetamorphic (NF stage 50-54; (61)) *Xenopus laevis* tadpoles were immersed in dechlorinated tap water at 25°C to which 3,5,3’-triiodothyronine (\(T_3\); Sigma-Aldrich) was added at the indicated concentrations dissolved in dimethyl sulfoxide (DMSO; ACP Chemicals Inc., St. Leonard, PQ). Control animals had an equal volume of DMSO added to their water. The tadpoles were sacrificed at the indicated times after treatment for the isolation of RNA or protein as described above.

**Organ culture of tadpole tails**

Tadpoles were anaesthetized in 0.1% MS222 (Syndel Laboratories, Vancouver, BC) and quickly immersed (5 s each) in a series of sterile water, 70% ethanol and two more beakers of sterile water. The tails were severed under aseptic conditions with a sterile scalpel and placed into culture dishes containing culture medium. The culture medium consisted of a 55% dilution of 1X alpha MEM (ICN Pharmaceuticals, Costa Mesa, CA) pH 7.2 supplemented with 14.5 mM NaCl, 1.1 mM Na\(_2\)HPO\(_4\), 1.1 mM NaH\(_2\)PO\(_4\), 2 mM L-glutamine, 1 mM L-methionine, 25 mM HEPES, 10 \(\mu\)g/ml fungizone and 50 \(\mu\)g/ml gentamycin sulfate. The cultures were incubated at 25°C under air and the medium was changed daily. The tails were allowed to recover overnight before the addition of 100
nM T3 with or without 2 mM EGTA, pH 8.0 (Sigma-Aldrich) that has previously been shown to inhibit tail regression (62).

**Results**

*Isolation of a novel Xenopus laevis xING2 gene*

Using a gene-specific primer from the conserved region 5’ to the region encoding the PHD finger of human ING1, we isolated multiple clones from Xenopus laevis tadpole brain and tail that bear closest sequence homology to ING2 identified in human (19,20) (Cal et al, unpublished; Genbank Accession # AJ006851) and mouse (Nagashima et al, unpublished; Genbank Accession # AF078834). Alignment of xING2 and human ING2 cDNA sequences (Figure 1) shows 71% sequence identity within the ORF. The xING2 ORF is 831 nt long; 6 nt shorter than that for human ING2, and is predicted to encode a 32 kDa protein. The presumed 5’ UTRs in both xING2 and human ING2 , unlike ING1, do not contain an in-frame stop codon leaving open the possibility that this transcript may encode a longer protein. However, the presumed 5’ and 3’ UTRs display a lower degree of sequence conservation suggesting that a longer ORF is unlikely to exist. Like at least two splicing isoforms of the ING1 gene, the human ING2 gene contains 2 exons (Nagashima et al, unpublished; Genbank Accession # AF062747, AF062748). Since the exon boundary spans a completely conserved codon, it is possible that the Xenopus gene structure may be similar to human (Figure 1). Using the XB5/XB8 primer set (Figure 1) and PCR amplification and sequencing of genomic DNA, we have confirmed that this splice site is conserved in Xenopus (data not shown).

Comparison of the 277 aa xp32ING2 predicted protein product with previously reported human and mouse sequences reveals that xp32ING2 is 68% identical to human p33ING2, 67% identical to murine p33ING2, 31% identical to human p47ING1a, 54% identical to human p33ING1b,
58% identical to murine p3ING1b and 62% identical to human p5ING4 (Figure 2). The C-terminal PHD finger domain is completely conserved between ING1 and ING2 proteins and the 90 aa region spanning the PHD finger also displays a high degree of conservation.

Previous work on human tissues indicated that ING2 is ubiquitously expressed as two major transcripts of 1.3 and 1.5 kb with the highest expression in the testis (19). Northern blot analyses of adult Xenopus tissues show a similar trend. xING2 is expressed in all tissues examined with testis having the highest expression levels followed by brain and skin in similar amounts then muscle and liver showing very low levels. A 1.3 kb transcript is found in all tissues with an additional 1.0 kb band found only in testis (Figure 3A). Finding multiple bands in the Northern blot suggests that, at least in the testis, multiple splice variants or transcripts from genes highly related to ING2 are present. These results were obtained using the entire open reading frame of xING2.

Since ING1 is subject to alternative splicing and since the gene structure of ING2 is similar to ING1, we wanted to test whether different splice variants for xING2 exist as well. To test this, we used differential RT-PCR analyses using two primer sets. One primer set (XB5/XB8; Figure 1) specifically amplifies the transcript we have reported in Figure 1. The resultant amplicon is referred to as xING2(1/2) in Figure 3B. The other primer set (XB9/XB10) amplifies a region in the conserved 3' end of the ORF that should be common to all ING2 variants (assuming that splicing occurs in a manner similar to ING1) and is referred to as xING2(2) in Figure 3B. Neither primer set amplifies ING1 sequences (data not shown). If no splice variants are present, then one would expect that relative levels of amplicons generated using the two primer sets would be equal. The RT-PCR results are consistent for the existence of xING2 splice variants in brain, testis and skin (Figures 3B and C).
Expression of ING proteins in adult *Xenopus* tissues

Much of our current knowledge about ING is based upon data of transcript levels and on overexpression studies. Very little is known about the normal expression levels of ING protein and there is some evidence that transcript levels do not necessarily reflect cellular protein levels in breast cancer cells (12). Using a polyclonal antibody directed against the conserved C-terminus of ING1, we have performed Western blots on total protein homogenates from several adult tissues (Figure 4A). Since this region of ING is highly conserved between ING types, it is expected that all ING-like proteins are detected with these antibodies. ING protein is detected in all tissues which correlates with previous data from mouse *in situ* analyses and PCR analyses of transcripts (4,17,19). However, the expression profile in each tissue varies (Figure 4B). All tissues have a 130 and 90 kDa band that varies in quantity with the eye and lung having the highest levels. Testis has the greatest number of bands with major bands at 90 and 37 kDa followed by brain with a major band at 37 kDa. These two tissues share many of the same bands, an observation consistent with general expression patterns found with other proteins. Liver and skin have very prominent bands at 50 and 47 kDa respectively. At this point, we cannot distinguish between different ING gene products. Generation of more specific ING1 and ING2 antibodies is required and is currently underway.

* lng2 expression is elevated in *Xenopus* tadpole tissues during T3-induced and spontaneous metamorphosis

In order to better define the roles that ING plays in a wide range of critical cellular processes, we utilized the versatile TH-induced metamorphosis of the frog tadpole as our developmental system in which cell proliferation, apoptosis and remodeling occur simultaneously in response to a single
stimulus. We first investigated the effect of T3 on the levels of ING2 transcripts in the brain, tail and leg of premetamorphic tadpoles as examples of organs that undergo remodeling, apoptosis and proliferation/differentiation, respectively. Northern blot analyses show that ING2 transcripts are present at low levels in control tadpoles (Figure 5A; 0 h T3) and immersion of the tadpoles in 10^{-7}M T3 results in an increase in the level of the 1.3 kb transcript in all tissues with leg showing the greatest amount of induction followed by brain and tail (Figure 5A).

Closer inspection of the expression profiles of xING2 transcripts reveal that the relative induction of xING2 expression by T3 is reproduced with the putative exon 2 primer set (compare Figure 5A with the 0 and 24 h xING2(2) RT-PCR amplified products in Figures 5B-D). Moreover, maximal levels of induction occur at 48 h in tail and at 6 h in leg (Figures 5B and C). TRα and TRβ transcript levels showed increases as was previously reported (33). In the leg, the xING2(2) induction pattern closely resembles that of T3-induced TRα expression in these tissues where a biphasic response is observed at 6 h and 24 h with maximal levels at 6 h, rather than that observed for TRβ (single peak at 24 h; Figure 5C). In the tail, the xING2(2) induction pattern is similar to both TRα and TRβ with an initial modest increase at 2 h reaching maximal levels at 48 h (Figure 5B). In the brain, xING2(2) amplicon levels increase 24 h after T3 treatment and TRα levels decrease slightly. TRβ levels show a marked increase in this tissue (Figure 5D).

RT-PCR analyses using primers spanning the exon 1/2 splice site show a different expression pattern compared to the exon 2 primer set. T3-induced accumulation is delayed until 48 h in the tail, whereas induction can be detected as early as 2 h in the leg with biphasic peaks at 12 and 48 h (Figure 5C). xING2(1/2) expression levels decrease at 24 h in the brain.

We also examined the expression profiles in tail and leg during spontaneous metamorphosis (Figures 5E and F). Overall xING2 expression follows the TRα and TRβ expression patterns in tail
similar to that observed in the T3 induction experiments (xING2(2) in Figure 5E). xING2 expression gradually increases from low levels at NF stage 58 to maximal levels at NF stage 63, whereas TRα levels are maximal at NF stage 60 and decline by NF stage 63, and TRβ levels reach maximal levels at NF stage 60 and remain high (Figure 5E). The leg does not show any induction of overall xING2 expression similar to the TRα expression pattern (Figure 5F). TRβ levels peak at NF stage 58 and decrease thereafter. These observed patterns of TR expression concur with those previously reported (33). These data also show that the relative levels of both TRα and xING2 are much lower in the leg compared to the tail during spontaneous metamorphosis.

RT-PCR analyses using primers spanning the exon 1/2 splice site show that this presumed splice variant of xING2 increases to maximal levels at NF stage 62 in the leg in contrast to the overall pattern of xING2 expression (compare xING2(1/2) to xING2(2); Figure 5F). At this stage, the relative amount is approximately twice that found at maximal levels in the tail at NF stage 62. In the tail, this presumed splice xING2 variant exhibits a slight delay in increased expression levels (maximal at NF stage 62; xING2(1/2) in Figure 5E) compared to overall xING2 expression; a result that is reminiscent of the T3-induction experiments (Figure 5A).

Specific ING protein levels increase upon T3-induced apoptosis of the tail in vivo and in culture

Given that ING2 transcript levels are T3-responsive, we next asked whether ING protein levels are altered upon induction of T3-dependent metamorphosis. Western blot analyses of leg and tail protein homogenates using an antibody directed against the human ING1 C-terminal end show that T3 treatment does not alter the expression levels of ING proteins in the leg (Figure 6A). However, the tail shows increased amounts of 90, 70 and 37 kDa bands by 6 h and of 130 and 60 kDa bands by 24 h (Figure 6A). These bands also increase dramatically during spontaneous
metamorphosis with the 60 kDa band increasing substantially at NF stage 55, the 37 kDa band increasing at NF stage 60 and the other bands increasing by stage 62 (Figure 6B).

Since ING1 levels in the tadpole appear to be related to apoptosis, we examined the possible relationship of T3-induced apoptosis in the tail and ING protein levels using serum-free organ culture. Administration of T3 to the tail cultures results in the induction of apoptosis within days ((34) and data not shown) and this induction can be blocked by coadministration of a variety of inhibitors including cycloheximide/anisomycin, EGTA and the H7 PKC inhibitor (38,62). Tails were maintained in organ culture for the indicated times in the presence of T3 with or without 2 mM EGTA and total protein homogenates were analysed by Western blot. Incubation with T3 alone results in the pronounced accumulation of 90-130 kDa ING proteins by 48 h with modest accumulation of 60 and 37 kDa bands (Figure 6C). When T3-induced tail regression is inhibited, the 90-130 and 60 kDa bands do not increase, whereas the 37 kDa band remains unaffected. Similar results are observed using cycloheximide/anisomycin and H7 inhibitors (data not shown).

Given that the 1.3 kb transcript detected in the tail (Figure 5) is too short to produce the 90-130 or 60 kDa bands, we suspect that these bands are more likely to represent ING1 gene products or unidentified splice variants of xING2. Northern blot analyses using a heterologous ING1 probe shows that a 3.9 kb transcript is identified that is increased upon T3 treatment in tail tissue providing support for this idea (Figure 6D). Together, these data show that accumulation of ING proteins correlates with the ability of the tail to undergo TH-induced apoptosis and provide the first evidence that ING is subject to hormonal control.
**Discussion**

ING proteins have been implicated in the control of cell proliferation and apoptosis (1,3-7). In this study, we have investigated the expression patterns of ING proteins in a developmental model system in which a single stimulus (TH) can induce both outcomes. We have isolated the first *Xenopus laevis ING2* homolog that has a high degree of identity with human and murine counterparts and we demonstrate for the first time that ING expression is hormone responsive. Moreover, *xING2* was found to be an early response gene along with *TRα* and *TRβ* (38,63) placing it in a potentially important role for the control of cell fate during TH-dependent metamorphosis. The ING2 gene is conserved between frogs and humans and we provide the first evidence of differential regulation of presumed splice variants of this gene. Given the high degree of conservation of gene structure between *ING1* and *ING2* (4,9,17,18)(our work and Nagashima *et al*, unpublished), and given that several *ING1* splice variants have already been identified (4,9,17,18), it is highly probable that alternative splicing contributes to the tissue-specific regulation of *ING2*. We also present the first systematic analysis of ING protein expression in adult and tadpole tissues using an antibody that is capable of recognizing both ING1 and ING2 proteins. We demonstrate that there is a great deal of similarity in expression pattern between brain and testis tissues and that there are distinct tissue-specific isoforms. We were unable to determine which protein bands correspond to ING1 *versus* ING2 proteins and are currently producing isoform-specific antibodies to address this question.

ING1 plays an important role in apoptosis (4-7,20). We have shown that ING protein expression is elevated in response to TH-induced tail regression and that the timing of this event corresponds to the point of commitment for cells to complete a TH-induced program (38). This study has provided evidence that the 90-130 kDa ING proteins may be important in regulating
hormone-induced apoptosis. The tail and brain, two tissues that undergo extensive apoptosis, show a TH-dependent increase in these proteins whereas the leg, whose main response is proliferation and growth, does not. In addition, induction of these proteins is inhibited by a variety of agents that inhibit TH-induced apoptosis in the tail. Recently, Szelei et al identified a PHD finger-containing protein transcript that was associated with estrogen-induced apoptosis in E8CASS breast cancer cells suggesting a role for proteins containing this domain in hormone-dependent programmed cell death pathways (64).

It is unclear whether p53 plays an important role in tail apoptosis, however a p53 target protein, Bax, is required (65). Recently p33ING1b and p33ING2 have been shown to induce bax and p21 expression in established cancer cells (20). In contrast, Shinoura and colleagues observed that Bax and Fas expression were not upregulated by coinduction of p33ING1b and p53, (although most mitochondria examined showed morphological damage in glioma cells (6)), suggesting that apoptosis can be induced via distinct pathways in different cell types.

Clearly defining the relationship between ING proteins and chromatin-associated components promises to give us important insights into the relationship between the control of apoptosis and chromatin structure. Presumably, the variable N-termini of the different isoforms confer unique abilities to interact with cellular components. Since our data strongly suggest that ING2 may also have splice variants, the possibility of more complex interactions among ING1 and ING2 isoforms to execute cellular effects is highly likely. It is not known if ING2 interacts with the same proteins as ING1, but both proteins can regulate gene expression by a currently unknown mechanism (20).

It is clear that cellular context is an important determinant of the TH-regulated response (33), but the mechanism is still poorly understood. TRs bind predominantly as heterodimers with
RXR to accessible stimulatory TREs in the absence of T₃ (66-70). This receptor complex recruits transcriptional co-repressors such as N-CoR, Sin3 and mRPD3 that associate and form a functionally active histone deacetylase (44-50). Histones are deacetylated resulting in repression of transcription. In the presence of T₃, the co-repressors are released and acetyltransferases (p300/CBP, P/CAF, TAFII250) are recruited (46,51,52). Histone acetylation then permits transcription. TR action is further modulated by interaction with many other proteins including auxiliary proteins (TRAPs) and the tumour suppressor p53 (53-56,71-73). Since p53, histone acetylases and deacetylases have been reported to interact with ING1 proteins (23-26), it is reasonable to speculate that ING protein isoforms may modulate TR activity through affecting HAT/HDAC activity to produce tissue-specific outcomes during tadpole metamorphosis.

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**Acknowledgements**

C.H. gratefully acknowledges the Alberta Cancer Board for a Postdoctoral Fellowship for the initial part of the work and the Natural Sciences and Engineering Council for a University Faculty Award and operating grant. This work was also supported by grants to K.R. from the National Cancer Institute of Canada and to R.N.J. from the Alberta Cancer Board.
Figure Legends

Figure 1. The nucleotide sequence of a novel ING2 transcript from *Xenopus laevis* aligned with human ING2. The entire cDNA sequences of xING2 (*Xenopus*) and human ING2 (human; Genbank Accession # AF053537, AJ006851, AB012853; Cal *et al.*, unpublished; (19,20)) were aligned using Clustal W alignment software (57). The putative start and stop codons are in bold and underlined. Identical nucleotides are indicated by an asterisk and the exon 1/exon 2 boundary determined in the xING2 and human ING2 gene is indicated (Nagashima *et al.*, unpublished; Genbank Accession # AF062747, AF062748). The position of the primers used for RT-PCR are indicated in gray with the names and direction indicated above. The xING2 sequence has been deposited in Genbank under Accession # AY014017.

Figure 2. Multiple sequence alignment of the derived aa sequence of xING2 with the aa sequences of ING2 (19,20), ING1 (4,9,13,17,18), and ING4 (9) from human, mouse (m) and *Xenopus* (x). Gaps that were inserted for optimal alignment are indicated by a dash. Identical amino acids are shaded. The putative translation start site of truncated variants are indicated in bold and underlined. The PHD finger domain core amino acids are indicated by the "%" symbol. The most commonly reported variants for p47ING1a and p33ING1b are shown with the unique N-terminus of p47ING1a shown beginning at aa 111. The exon 1/2 boundaries for human (Nagashima *et al.*, unpublished) and *Xenopus* ING2 and murine and human ING1 (4,9) are indicated by a question mark and an asterisk, respectively. The alignment was done using Clustal W alignment software (57). The Genbank Accession numbers for each of the sequences used are AB012853, NM_02353, AF181849, AF181850, AF078834, NM_011919, AF17775352 and AF149724.
Figure 3. Expression levels of xING2 transcripts in adult Xenopus tissues. A, Northern blot of total RNA isolated from adult brain, muscle, testis, liver, and skin (upper panel). Analyses were performed as described in the “Materials and Methods” using the entire ORF of xING2. The sizes of the detected bands are indicated in kilobases (kb) to the right as determined by comigration of a standard RNA ladder (Life Technologies). Relative RNA loading is indicated by the intensity of the 28S rRNA bands as visualized by ethidium bromide staining of the gel (lower panel). B, RT-PCR analyses of adult tissues using primers spanning the putative exon 1/2 boundary (xING2(1/2)) or within the putative exon 2 (xING2(2)). L8 ribosomal protein transcript, known to remain constant between tissues (59), is shown below and was used to normalize the xING2 amplification products. C, Graph comparing the fold differences in normalized xING2(1/2) (hatched bars) and xING2(2) (solid bars) transcript levels relative to the liver.

Figure 4. Western blot analyses of adult Xenopus tissues. A, Total protein homogenates were prepared and separated by size by SDS-PAGE as described in the “Materials and Methods”. The separated proteins were transferred to nitrocellulose membrane and probed with antibody that is specific for the common region of ING. The relative sizes of specific bands were determined by comparison with comigrating standard protein markers and are indicated to the right in kiloDaltons. Non-specific bands are indicated by an asterisk. Lane assignments are as follows: brain (B), muscle (M), testis (T), liver (Li), eye (E), heart (H), spleen (Sp), intestine (I), skin (S), and lung (Lu). B, Summary of specific bands detected in each tissue. The most prominent bands are indicated in bold.
Figure 5. Induction of ING expression in response to 100 nM triiodothyronine (T₃) treatment and during spontaneous metamorphosis in *Xenopus laevis* tadpole tissues. A, Northern blot analyses of total RNA isolated from the brain, tail and leg of premetamorphic tadpoles immersed in T₃ for the indicated times probed with the entire *xING2* coding region (upper panel). Only one band of 1.3 kb is detected in all samples. Relative RNA loading is indicated by the intensity of the 28S rRNA bands as visualized by ethidium bromide staining of the gel (lower panel). RT-PCR analyses of tadpole tail (B and E), leg (C and F) and brain (D) tissues using primers spanning the putative exon 1/2 boundary (*xING2*(1/2)) and within the putative exon 2 (*xING2*(2)). Expression of TRα and TRβ transcripts for each sample is also shown. L8 ribosomal protein transcript, known not to vary between tissues and in response to T₃ (59), was used to normalize each sample. Panels B-D show the relative expression levels during T₃ induced metamorphosis and Panels E and F show the response during spontaneous metamorphosis. Tadpole stages according to Nieuwkoop and Faber (NF; (61)) are indicated.

Figure 6. Western blot analyses of ING-specific proteins on A, *Xenopus* tadpole leg and tail tissues from total protein homogenates that have been isolated at the indicated times after immersion of premetamorphic NF stage 50-54 tadpoles in 10⁻⁷ M T₃, and, B, tail homogenates that were isolated at various stages during spontaneous development. Total protein homogenates were prepared and separated by size by SDS-PAGE as described in the “Materials and Methods”. The separated proteins were transferred to nitrocellulose membrane and probed with antibody that is specific for the common region of ING. C, Western blot analyses of total proteins isolated from cultured premetamorphic tails. Tails were cultured in serum-free medium using the method described in (74). Tails were treated with 100 nM T₃ in the presence or absence of 2 mM EGTA that inhibits
tail regression (62). Similar results were obtained by inhibiting tail regression with
cycloheximide/anisomycin (38) and the protein kinase C inhibitor, H7 (62) (data not shown). Total
protein homogenates were isolated at the indicated times and Western blotted as above. The
relative sizes of specific bands were determined by comparison with comigrating standard protein
markers and are indicated in kiloDaltons. D, Northern blot analyses of total RNA isolated from the
tail of premetamorphic tadpoles immersed in T₃ for the indicated times probed with a human 300
bp ING1 PCR fragment from exon 2 (upper panel). Only one band of 3.9 kb is detected. Relative
RNA loading is indicated by the intensity of the 28S rRNA bands as visualized by ethidium
bromide staining of the gel (lower panel).
Wagner et al Figure 1
Wagner et al. Figure 3

A

Brain Muscle Testis Liver Skin

1.3 kb
1 kb

26S rRNA

B

Brain Muscle Testis Liver Skin

xING2 (1/2)
xING2 (2)
L8

C

Expression level relative to liver

0 1 2 3 4 5

brain muscle testis liver skin
Wagner et al Figure 4

A

B

| Tissue    | ING antibody-reactive polypeptides (kDa) |
|-----------|------------------------------------------|
| brain     | 130, 90, 70, 55, 47, 37, 35, 33, 24      |
| muscle    | 130, 90, 70, 60, 47                      |
| testis    | 130, 120, 90, 70, 65, 55, 50, 37, 35, 24, 16 |
| liver     | 130, 90, 50, 16                          |
| eye       | 130, 90, 40                              |
| heart     | 130, 90, 35                              |
| spleen    | 130, 110, 90, 37                         |
| intestine | 130, 110, 90, 50, 37, 31                 |
| skin      | 130, 90, 50, 47, 40, 37, 33, 26           |
| lung      | 130, 90, 40, 31                          |
Wagner et al. Figure 5

A

|          | Brain | Tail | Leg |
|----------|-------|------|-----|
| Hours T₃ | 0     | 24   | 0   |

1.3 kb

28S rRNA

B

| Hours T₃ |
|----------|
| 0        |
| 0.5      |
| 2        |
| 6        |
| 12       |
| 24       |
| 48       |

C

| Hours T₃ |
|----------|
| 0        |
| 0.5      |
| 2        |
| 6        |
| 12       |
| 24       |
| 48       |

xING2 (1/2)
xING2 (2)
TRα
TRβ
L8

D

| Hours T₃ |
|----------|
| 0        |
| 24       |

E

| NF Stage |
|----------|
| 58       |
| 60       |
| 62       |
| 63       |

F

| NF Stage |
|----------|
| 58       |
| 60       |
| 62       |
| 63       |

xING2 (1/2)
xING2 (2)
TRα
TRβ
L8
Wagner et al Figure 6

A

| Leg | 0 | 2 | 6 | 12 | 24 | 48 | hT₃ |
|-----|---|---|---|----|----|----|-----|
|     |   |   |   |    |    |    | 130 |
|     |   |   |   |    |    |    | 90  |
|     |   |   |   |    |    |    | 70  |
|     |   |   |   |    |    |    | 60  |
|     |   |   |   |    |    |    | 50  |
|     |   |   |   |    |    |    | 37  |

| Tail | 0 | 2 | 6 | 12 | 24 | 48 | hT₃ |
|------|---|---|---|----|----|----|-----|
|      |   |   |   |    |    |    | 130 |
|      |   |   |   |    |    |    | 90  |
|      |   |   |   |    |    |    | 70  |
|      |   |   |   |    |    |    | 60  |
|      |   |   |   |    |    |    | 50  |
|      |   |   |   |    |    |    | 37  |

B

| NF Stage | 41 | 48 | 55 | 60 | 62 | hT₃ |
|----------|----|----|----|----|----|-----|
|          |    |    |    |    |    | 130 |
|          |    |    |    |    |    | 90  |
|          |    |    |    |    |    | 70  |
|          |    |    |    |    |    | 60  |
|          |    |    |    |    |    | 50  |
|          |    |    |    |    |    | 37  |

C

| EGTA | 0 | 24 | 48 | hT₃ |
|------|---|----|----|-----|
|      |   |    |    | 130 |
|      |   |    |    | 90  |
|      |   |    |    | 70  |
|      |   |    |    | 60  |
|      |   |    |    | 37  |

D

| 0 | 12 | 48 | hT₃ |
|---|----|----|-----|
|   |    |    | 3.9 kb |
|   |    |    | 28 S rRNA |
Expression of novel ING variants is regulated by thyroid hormone in the Xenopus laevis tadpole
Mary J. Wagner, Marketa Gogela-Spehar, Rachel C. Skirrow, Randal N. Johnston, Karl Riabowol and Caren C. Helbing

J. Biol. Chem. published online October 12, 2001

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