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SRC-p300 Coactivator Complex Is Required for Thyroid Hormone-induced Amphibian Metamorphosis*

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Gene activation by the thyroid hormone (T3) receptor (TR) involves the recruitment of specific coactivator complexes to T3-responsive promoters. A large number of coactivators for TR have been isolated and characterized *in vitro*. However, their roles and functions *in vivo* during development have remained largely unknown. We have utilized metamorphosis in *Xenopus laevis* to study the role of these coactivators during post-embryonic development. Metamorphosis is totally dependent on the thyroid hormone, and TR mediates a vast majority, if not all, of the developmental effects of the hormone. We have previously shown that TR recruits the coactivator SRC3 (steroid receptor coactivator-3) and that coactivator recruitment is essential for metamorphosis. To determine whether SRCs are indeed required, we have analyzed the *in vivo* role of the histone acetyltransferase p300/CREB-binding protein (CBP), which was reported to be a component of the SRC·coactivator complexes. Chromatin immunoprecipitation revealed that p300 is recruited to T3-responsive promoters, implicating a role of p300 in TR function. Further, transgenic tadpoles overexpressing a dominant negative form of p300, F-dnp300, containing only the SRC-interacting domain, displayed arrested or delayed metamorphosis. Molecular analyses of the transgenic F-dnp300 animals showed that F-dnp300 was recruited by TR (displacing endogenous p300) and inhibited the expression of T3-responsive genes. Our results thus suggest that p300 and/or its related CBP is an essential component of the TR-signaling pathway *in vivo* and support the notion that p300/CBP and SRC proteins are part of the same coactivator complex *in vivo* during post-embryonic development.

T3 receptor (TR)[3] belongs to the superfamily of ligand-inducible nuclear hormone receptors (1, 2). TR regulates transcription by forming a heterodimer with 9-*cis*-retinoic acid receptor (RXR) and binding to T3 response elements (TREs) on T3-responsive promoters. In the absence of T3, the TR heterodimer represses transcription by recruiting corepressors, whereas in the presence of T3, TRs recruit coactivators to facilitate transcription (3–6). These coactivators may either directly interact with TR or are recruited to the TR activation complex through protein-protein interactions. Coactivators for TR include the steroid receptor coactivator (SRC) or p160 family of proteins (7–15), the mediator-like TRAP/DRIP (thyroid hormone receptor-associated protein/vitamin D receptor-interacting protein) complex (16, 17), the histone acetyltransferases, p300/CREB-binding protein (CBP) (18), and the histone methyltransferases PRMT1 and CARM1 (19, 20).

It is becoming increasingly clear that most nuclear receptor coactivators reside in multiprotein complexes, and gene regulatory circuits can operate through combinatorial cofactor recruitment (21, 22) (3–6,23). Among the most studied coactivators are the steroid receptor coactivators (SRCs). The SRC family comprises three members, SRC1/NCoA-1, SRC2/TIF2/GRIP1, and SRC3/pCIP/ACTR/AIB-1/RAC-3/TRAM-1, which interact directly with the nuclear receptor ligand-binding domain via distinct receptor interaction domains containing L*XX*LL motifs (7–15, 24–26). The SRC family can recruit other coactivators such as histone methyltransferases, PRMT1, and CARM1 (19, 20) or histone acetyltransferases such as p300/CBP (10, 26, 27). A number of studies suggest that the SRC proteins and p300/CBP function in the same activation pathway where p300/CBP is recruited to liganded TR by the SRC proteins (28) (27, 29). p300 and CBP are highly homologous proteins, often referred to as a single entity-p300/CBP. p300 and CBP possess histone acetyltransferase activity (30) and play central roles in diverse cellular processes such as cell cycle control, transformation, differentiation, and apoptosis (31, 32). Although numerous studies have addressed the roles of these coactivators *in vitro*, their utilization by TR and other nuclear receptors in different tissue and cell types *in vivo* is yet to be elucidated. Information on the functional interplay of different coactivators, especially with reference to particular genes *in vivo* during post-embryonic development, remains scarce.

We have utilized metamorphosis in *Xenopus laevis*, the African clawed toad, as a model system to study the role of coactivators in TR function. Anuran metamorphosis exhibits remarkable similarity to post-embryonic development in mammals and is totally dependent on T3 (33–35). The process involves integration of complex spatial and temporal gene regulatory networks that underlie *de novo* morphogenesis, remodeling, and complete regression of some organs, culminating in the transformation of an aquatic herbivorous tadpole to a ter-

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[3] The abbreviations used are: T3, thyroid hormone; TR, T3 receptor; CBP, CREB-binding protein; CREB, cAMP-response element-binding protein; RXR, 9-*cis*-retinoic acid receptor; SRC, steroid receptor coactivator; RT, reverse transcription; GFP, green fluorescent protein; SID, SRC-interacting domain.

restrial carnivorous frog. The metamorphic effects of T3 are essentially all mediated by TR (35–38). The system affords an added advantage in that the process can be induced by adding exogenous T3 to the tadpole-rearing water or blocked by using specific inhibitors of T3 synthesis (34, 39). Moreover, tadpoles are free-living and thus their development is not complicated by maternal influences.

To correlate gene expression and function with metamorphic transformations, we have focused our studies by using intestinal remodeling as a model. The premetamorphic tadpole intestine is a very simple tubular organ made of mostly a single monolayer of larval epithelial cells surrounded by sparse connective tissue and muscles (40). During metamorphosis, essentially all larval epithelial cells die and are eventually replaced by adult epithelial cells developed *de novo*. Concurrently, connective tissue and muscles also develop extensively. Thus, during the early stage of intestinal remodeling (the first few days of T3 treatment of premetamorphic tadpoles or natural metamorphosis), the entire organ behaves largely as a single cell type, the larval epithelial cells, making it possible to carry out molecular analysis of gene regulation mechanisms *in vivo*.

Using this model system, we have shown earlier that the steroid receptor coactivator SRC3 is up-regulated during metamorphosis (41, 42) and is recruited to T3-responsive promoters in a gene- and tissue-dependent manner (43). More importantly, transgenic expression of a dominant negative SRC3 containing only the TR-interacting domain inhibits T3-dependent gene expression and metamorphosis, demonstrating an essential role for coactivator recruitment in this post-embryonic process. Because the dominant negative SRC3 blocks all coactivator binding to liganded TR, it remains possible that coactivators other than SRC family members play the essential role in gene regulation by TR and metamorphosis. To determine whether and how SRCs participate in metamorphosis, we have investigated here the role of SRC-binding protein p300 during metamorphosis. We have shown here that *Xenopus* p300 is recruited to T3-responsive promoters in a T3-dependent manner. Furthermore, using a dominant negative form of p300 that contains only the SRC-binding domain, we have demonstrated an essential role of p300·SRC complex or related complexes in gene regulation by TR and metamorphosis.

EXPERIMENTAL PROCEDURES

Constructs—A dominant negative form of p300, F-dnp300, comprising the SRC interaction domain of *X. laevis* p300 (amino acids 1995–2166) (44), was amplified by reverse transcription (RT)-PCR from total RNA isolated from stage 54 *X. laevis* tadpoles using primers designed to incorporate a FLAG tag and cloned into pCRT7NTTOPO vector (Invitrogen), which has the Xpress and His tags. The clone was verified by sequencing. Next, the p300 fragment was digested with NdeI (filled in with Klenow polymerase) and EcoRI and subcloned into the BglII (filled in with Klenow) and EcoRI sites of the vector pT7Ts under the control of the T7 promoter. This construct has the 5′- and 3′-UTR of the β globin gene and was used for generating mRNA for the oocyte microinjection experiments. For verifying the specificity of the anti-p300 antibody by using *in vitro* translation followed by Western blotting, a construct encoding a p300 fragment containing the peptide TLPQVAVQNLLRALRSP, which was used for immunization, was amplified using RT-PCR from total RNA using the forward and reverse primers 5′-ATGAACCCAATGCCGCCCATAGGA-3′ and 5′-CTAGGAAATAGGGGGCTGTTGTGG-3′, respectively, and cloned into pCRT7TOPO-NT vector (Invitrogen). For transgenesis, the F-dnp300 transgene was placed under the control of the constitutive cytomegalovirus promoter in the vector pCGCG (45), replacing the original green fluorescent protein (GFP) fragment at this location, resulting in the double promoter construct pCF-dnp300CG, which also has the gene for GFP driven by the eye lens-specific γ-crystallin promoter to facilitate the identification of transgenic animals.

Antibodies—The anti-acetylated histone H4 antibody was purchased from Upstate Cell Signaling Solutions. The anti-FLAG M2 antibody was from Sigma. The anti-TR antibodies were described earlier (46, 47). The anti-p300 polyclonal antibodies were generated by coinjecting two peptides derived from *X. laevis* p300, TLPQVAVQNLLRALRSP (multiple antigenic peptide-conjugated) and QPSPHHVSPQTSSPHPGLVGP (keyhole limpet hemocyanin-conjugated) into rabbits (Invitrogen). For the chromatin immunoprecipitation (ChIP) assays performed to study the recruitment of wild type p300 in wild type and transgenic animals, we used affinity-purified polyclonal antibodies generated against the peptides KSEPVELEEKKEEVKTE (amino acids 1033–1049) and KPKRLQEWYKKMLDKSVSER (amino acids 1487–1506) (48, 49).

Animals and Treatment—Wild type tadpoles of the African clawed toad *X. laevis* were obtained from *Xenopus* I, Inc. (Dexter, MI), and developmental stages were determined according to Nieuwkoop and Faber (50). Adult female frogs used for oocyte preparation were obtained from NASCO (Fort Atkinson, WI). Stage 54 premetamorphic tadpoles at a density of 2 tadpoles/liter of dechlorinated water were treated with the indicated amount of T3 for 2–3 days.

Histological Analysis of the Intestine—The intestines of the tadpoles were dissected out and fixed for 2 h at room temperature in 4% paraformaldehyde and 60% phosphate-buffered saline, cryoprotected in 0.5 M sucrose in 60% phosphate-buffered saline, and embedded in O.C.T. compound (TissueTek). The intestines were sectioned in a cryotome at 7.5 μm. Sections were visualized using methyl green pyronin Y (Muto, Tokyo, Japan) (51).

Oocyte Injections and Luciferase Assays—pSP64-TR, pSP64-RXR (46), and T7Ts-FLAG-dnp300 were used to synthesize, *in vitro*, the corresponding mRNAs with the T7 or SP6 *in vitro* transcription kit (mMESSAGE mMACHINE, Ambion). The mRNA (5.75 ng/oocyte) was microinjected into the cytoplasm of 20 *X. laevis* stage-VI oocytes. The reporter plasmid DNA (0.33 ng/oocyte), which contained the T3-dependent *TRβA* promoter driving the expression of the firefly luciferase (52) was injected into the oocyte nucleus together with a control construct that contained the Herpes simplex *tk* promoter driving the expression of the *Renilla* luciferase (Promega, WI) (0.03 ng/oocyte). Following incubation overnight at 18 °C in the absence or presence of 100 nM T3, the oocytes were prepared for luciferase assay using the Dual-Luciferase reporter assay

system (Promega, WI) according to the manufacturer's recommendations.

RNA Isolation and RT-PCR—RNA was isolated using the TRIzol reagent (Invitrogen) according to the manufacturer's recommendations. RT-PCRs were carried out using the Superscript One-Step RT-PCR kit (Invitrogen). The expression of the ribosomal protein L8 (rpl8) was used as an internal control (53). The sequences of the primers used were (5′-3′): CGTGGTGCTCCTCTTGCCAAG and GACGACCAGTACGACGAGCAG for *rpl8* (53), CACTTAGCAACAGGGATCAGC and CTTGTCCCAGTAGCAATCATC for *T3/bZIP* (54), and ATAGTTAATGCGCCCGAGGGTGGA and CTTTTCTATTCTCTCCACGCTAGC for *TRβA* (55). PCR was also done on RNA samples without reverse transcription as a control for genomic DNA contamination (data not shown). 0.5 μg of total RNA was used in a 25-μl reaction and with the following reaction conditions: 42 °C for 30 min for the RT reaction followed by 21–25 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The resulting products were analyzed on an agarose gel stained with ethidium bromide.

Preparation of Tadpole Tissues for Western Blot Analysis—Tadpoles were sacrificed by decapitation on ice. The dissected organs were sliced into small pieces and homogenized in buffer containing 50 mM Tris-HCl, pH 8.0, 1% SDS, 1 mM dithiothreitol and protease inhibitor mixture (Roche Diagnostics). The lysate was centrifuged at 11,000 × *g* for 5 min, and the protein in the supernatant was quantitated by Bradford assay (Bio-Rad). Equal amounts of protein were loaded on an 8–16% Tris-glycine gel (Invitrogen) and transferred onto a polyvinylidene difluoride membrane for Western blot analysis.

Chromatin Immunoprecipitation Assays—ChIP assays using oocytes and tissues from tadpoles was performed as described previously (56). The following antibodies were used in the assay: anti-*Xenopus* TR (46), anti-acetylated histone H4 (Upstate Cell Signaling Solutions, Lake Placid, NY), and anti-*Xenopus* p300. After reverse cross-linking, DNA was purified using a PCR purification kit (Qiagen). Quantitative PCR was carried out with a ChIP DNA sample in duplicate on an ABI 7000 (Applied Biosystems) using promoter-specific primers and FAM (6-carboxyfluorescein)-labeled TaqMan probes (Applied Biosystems) (38). To ensure the validity of the PCR, for each assay, six 2-fold serial dilutions from a large batch of ChIP input DNA, prepared from intestines isolated especially for the purpose of serving as standards, were used for the quantification of the experimental samples. The calculated standard curves ranged in slope from −3.30 to −3.50, where theoretical amplification has a slope of −3.32. Also included was a no-template control where double-distilled water was added instead of sample DNA as a control for PCR product contamination. Results from the experimental samples were within the range of the standard curve (not shown). The primers used for the quantitative PCR were (5′-3′): CCCCTATCCTTGTTCGTCCTC and GCGCTGGGCTGTCCT, for the TRE region of the *TRβA* promoter and GGACGCACTAGGGTTAAGTAAGG and TCTCCCAACCCTACAGAGTTCAA for the TRE region of the *T3/bZIP* promoter. The FAM-labeled probes were (5′-3′) CCTAGGCAGGTCATTTC and ATGAGGCTGAGCATTCA for the *TRβA* and the *T3/bZIP* promoters, respectively.

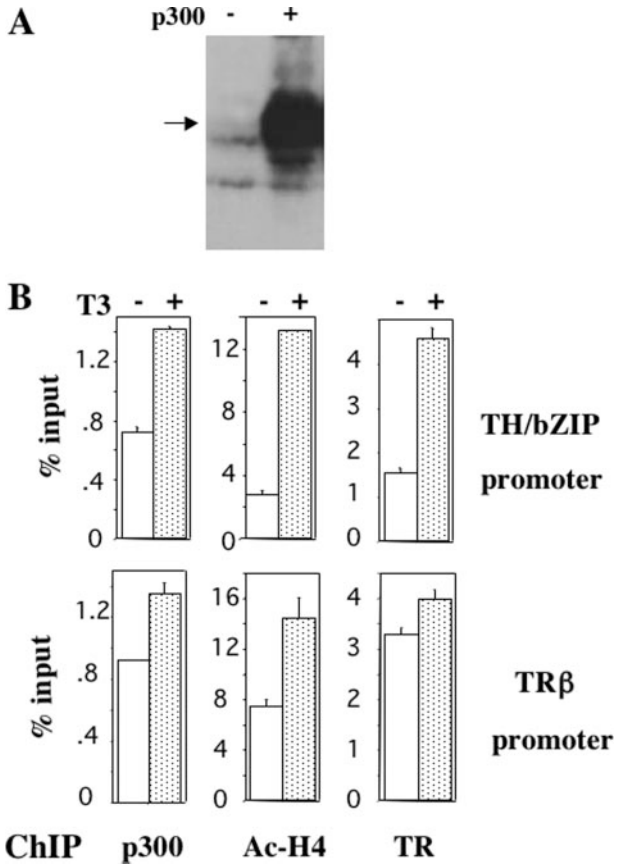

FIGURE 1. **Ligand-dependent recruitment of p300 to the T3-responsive promoters.** *A*, a polyclonal antibody recognizes *X. laevis* p300. *In vitro* coupled transcription/translation was carried out in the absence (−) or presence (+) of the p300 construct harboring the peptide TLPQVAVQNLLRALRSP of *X. laevis* p300, which was used to generate the polyclonal antibody, and the products were subjected to Western blot analysis with the anti-p300 antibody. *B*, T3 treatment leads to the recruitment of p300 to *TRβ* and *T3/bZIP* gene promoters in the tadpole intestine. Premetamorphic tadpoles at stage 54 were treated with 10 nM T3 for 2 days. Chromatin was isolated from the intestine and immunoprecipitated using antibodies recognizing TR, p300, or acetylated histone H4 (*Ac-H4*). The occupancy of the TREs of the two promoters in the ChIP samples was analyzed by real time PCR. Three tadpoles were used for each treatment. The experiments were done independently three times with similar results. In addition, the ChIP assays were also done using an affinity-purified antibody against a different region of p300, yielding similar results (data not shown and Fig. 7*A*).

RESULTS

p300 Is Recruited to Target Promoters in the Tadpole Intestine upon T3 Treatment of Premetamorphic Tadpoles—We have previously shown that p300 is constitutively expressed in whole tadpoles as well as in the intestine and tail throughout metamorphosis (41). To investigate whether p300 is utilized by TR during intestinal remodeling, we generated a polyclonal antibody against two peptides of the *X. laevis* p300 (Fig. 1*A*) and used it for ChIP assay to determine the association of p300 with T3 target genes *in vivo*. For this purpose, we treated premetamorphic tadpoles at stage 54 with 10 nM T3 for 2 days at room temperature, a condition known to induce metamorphosis (34, 39). Intestinal nuclei were isolated and subjected to ChIP assays using antibodies against p300, TR, or acetylated histone H4.

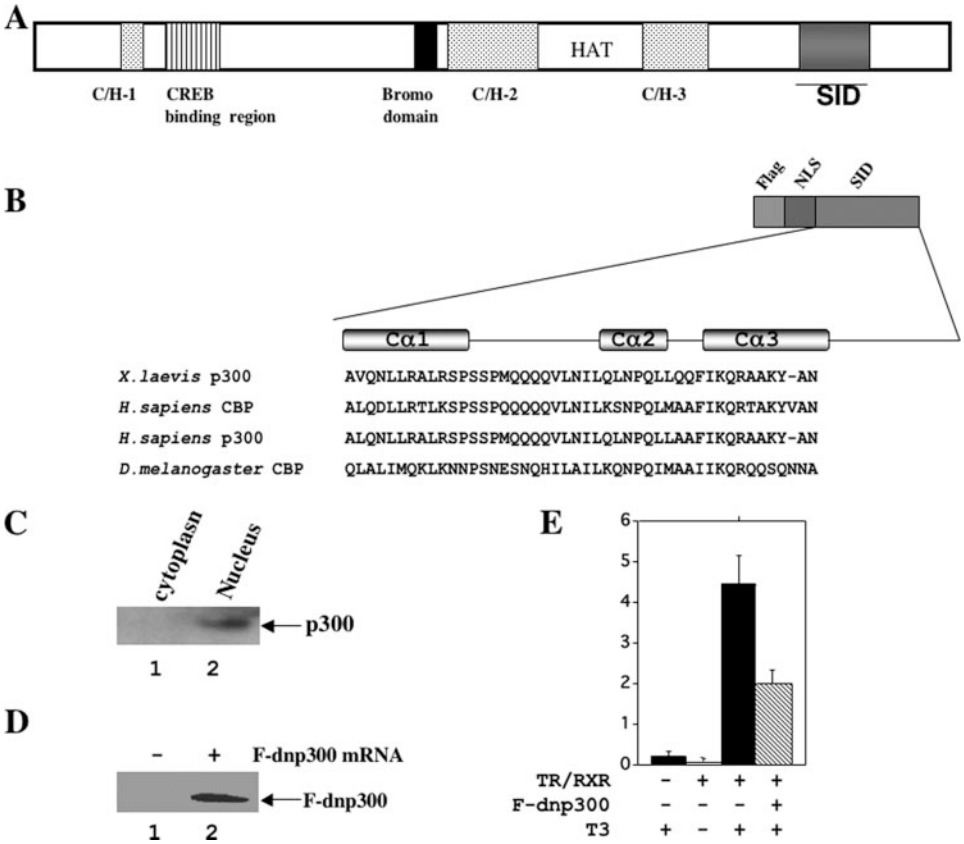

FIGURE 2. **Design and characterization of a FLAG-tagged dominant negative p300 (F-dnp300).** *A*, schematic representation of the full-length p300 illustrating the various domains: cysteine-histidine-rich motifs C/H-1, C/H-2, and C/H-3; the CREB-binding domain; histone acetyltransferase domain (*HAT*); the bromodomain; and the SRC interaction domain (*SID*). *B*, the dominant negative form, F-dnp300, comprising the SRC interaction domain SID fused to an N-terminal peptide containing the FLAG tag and nuclear localization sequences. A sequence alignment of the SID, which comprises three α-helices, Cα1, Cα2, and Cα3, from different species is shown *below* the schematic diagram of F-dnp300, showing that SID is highly conserved in different species. *C*, endogenous p300 is localized in the nucleus in *Xenopus* oocytes. The nuclei from *Xenopus* oocytes were manually dissected out under a light microscope. Extracts were prepared from the nuclei and the rest of the oocytes (*cytoplasm*) followed by Western blotting using a polyclonal anti-p300 antibody. *D*, F-dnp300 can be overexpressed upon injecting mRNA into the cytoplasm of *Xenopus* oocytes. The mRNA (5.75 ng/oocyte) encoding F-dnp300 was injected into the cytoplasm of oocytes and incubated overnight to allow for protein synthesis. Extracts were prepared from oocytes with or without mRNA injection and analyzed by Western blotting using an anti-FLAG antibody. *E*, F-dnp300 inhibits T3-induced transcription of a target promoter *in vivo*. The mRNAs for TR, RXR, and F-dnp300 (5.75 ng/oocyte) were injected into the cytoplasm of oocytes, as indicated. This is followed by the injection of the firefly luciferase reporter vector (0.33 ng/oocyte) (TRE-Luc, under the control of the T3-inducible promoter of *Xenopus TRβA* gene) into the nucleus along with the control *Renilla* luciferase plasmid (0.03 ng/oocyte). After overnight incubation at 18 °C with or without 100 nM T3, oocytes were harvested and assayed for transcription from TRE-Luc. The ratio of firefly luciferase activity from TRE-Luc to that from the control *Renilla* luciferase plasmid was determined for each oocyte, and the average from four oocytes was plotted together with the standard deviation.

The binding of p300 to two T3-responsive promoters, *TRβA* and *T3/bZIP*, was analyzed. As shown in Fig. 1*B*, *Xenopus* p300 was found to be recruited in a ligand-dependent manner to both promoters in the intestine. As a positive control for the ChIP assay, we showed that the levels of histone H4 acetylation, a marker associated with gene activation, was enhanced in T3-treated animals at both promoters (Fig. 1*B*), as seen in our earlier reports (42, 43, 57). Similarly, TR was bound to both promoters with its binding to the *T3/bZIP* promoter increased after T3 treatment, again in agreement with earlier findings (47, 57). These results suggest that p300 plays a role in T3-signaling events during metamorphosis by participating in gene regulation by TR.

Generation of a Dominant Negative Form of p300 to Inhibit T3-induced Transcription—To study the role of p300 in the TR-signaling cascade during development, we reasoned that a dominant negative form of p300 targeting gene regulation by TR should interfere with gene regulation by TR when expressed in metamorphosing tadpoles. We chose this approach, because it is currently impossible to knock out or knock down genes in developing tadpoles. The *Xenopus* p300 is a large protein of 270 kDa and is highly homologous to its counterparts in other species, with multiple domains mediating the interactions with other proteins (Fig. 2*A*). The bromodomain and cysteine/histidine domains designated as C/H1, C/H2, and C/H3 have been implicated in interactions with a large number of proteins (31, 32). The kinase-inducible interaction domain mediates the phosphorylation-dependent binding to the transcription factor CREB (58). The central region of the protein encodes a histone acetyltransferase activity, which can acetylate the lysine residues of histones (30, 59, 60). In addition, p300 also has a structurally distinct activation domain present toward the C terminus of the protein, which functions independently of the histone acetyltransferase activity (61). This region contains the SRC-interacting domain (SID), which contains three α-helices (Cα1, Cα2, and Cα3), and mutations in the helical regions, especially in Cα3, disrupt binding to SRC1 (27, 29, 62). Thus, we designed a dominant negative form of p300 that contained only an SID of p300 to restrict its effects to the SRC pathway (Fig. 2*B*).

The dominant negative form of p300, F-dnp300, was generated as a fusion protein with an N-terminal FLAG tag and a nuclear localization signal (Fig. 2*B*). To verify the effect of the dominant negative on TR-mediated transcription, we utilized the *Xenopus* oocyte system, where gene regulation can be studied in the context of chromatin (46). First, we showed that endogenous p300 was present in the nuclei of *Xenopus* oocytes (Fig. 2*C*, *lane 2*), suggesting that TR may be able to utilize p300 in gene activation in the oocytes. The F-dnp300 protein was expressed in the oocyte by microinjecting *in vitro* synthesized mRNA into the oocyte cytoplasm followed by incubating the

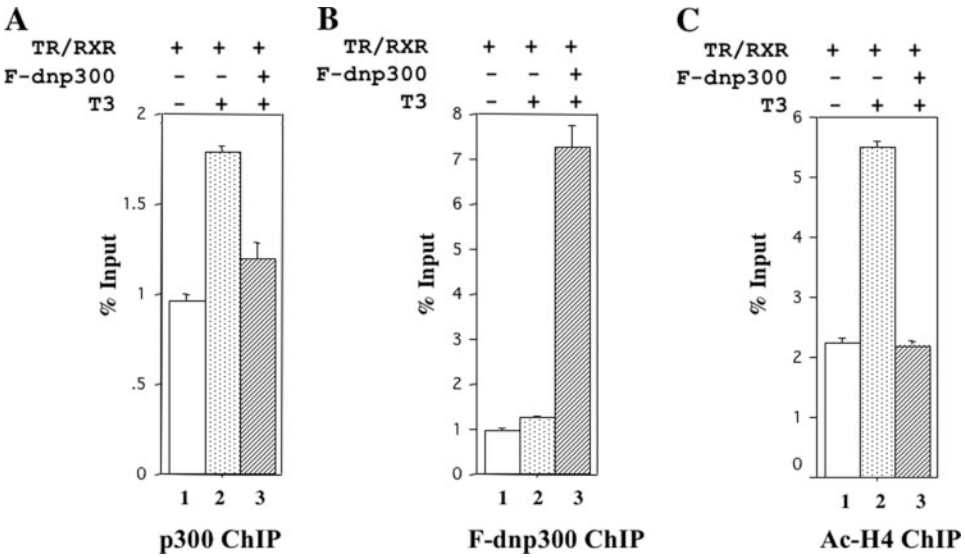

FIGURE 3. **F-dnp300 competes against the endogenous p300 for recruitment to the *TRβA* promoter.** The mRNAs for TR, RXR, and/or F-dnp300 were injected into the cytoplasm of oocytes, and the reporter plasmid TRE_Luc harboring the *TRβA* promoter was injected into the nucleus. After overnight incubation at 18 °C with or without 100 nM T3, ChIP assays were conducted using anti-p300 (*A*), anti-FLAG (for F-dnp300) (*B*), and acetylated histone H4 (*C*) antibodies. The promoter region immunoprecipitated by the antibodies were analyzed by quantitative PCR and shown as the % of input (prior to antibody immunoprecipitation) DNA.

oocytes overnight to permit protein synthesis (Fig. 2*D*). To study the effects of F-dnp300 on gene TR function, a reporter construct, TRE-Luc, harboring the T3-dependent *Xenopus TRβA* promoter driving the firefly luciferase reporter gene (52), was microinjected into the nuclei of *Xenopus* oocytes together with a plasmid harboring the *Renilla* luciferase gene under a T3-independent promoter as an internal control. The mRNAs encoding TR and RXR, with or without F-dnp300 mRNA, were injected into the cytoplasm of the oocytes. Following overnight incubation in the presence or absence of 100 nM T3, oocytes were harvested and assayed for luciferase activity. In the absence of T3, basal activity from TRE-Luc was repressed by TR and RXR, as reported previously (46), whereas in the presence of T3, the promoter was activated (Fig. 2*E*). In the presence F-dnp300 and T3, the transcription from the *TRβA* promoter was markedly diminished (Fig. 2*E*), whereas F-dnp300 alone had no effect on the control *Renilla* luciferase expression or the reporter promoter (data not shown). Thus, F-dnp300 functions as an inhibitor of TR-mediated gene activation by T3 *in vivo*.

To confirm the mechanism of F-dnp300 action, we carried out ChIP assay on the T3-responsive *TRβA* promoter in *Xenopus* oocytes. The oocytes were injected as described above. After an overnight incubation in the presence or absence of 100 nM T3, the oocytes were harvested and processed for ChIP assays. To detect the binding of endogenous p300, we utilized an antibody generated against a peptide of p300 (48, 49), which is absent in the F-dnp300. Our results revealed that endogenous p300 was recruited to the *TRβA* promoter in a T3-dependent manner and that the expression of F-dnp300 inhibited this recruitment (Fig. 3*A*), with concurrent recruitment of F-dnp300 to the promoter (Fig. 3*B*). In addition, F-dnp300 also inhibited the histone acetylation at the promoter (Fig. 3*C*). Thus, F-dnp300 inhibits gene activation by T3 by competing against endogenous p300 for recruitment to the promoter.

Transgenic Tadpoles Expressing F-dnp300 Exhibit Resistance to T3-induced Metamorphosis—To study the effects of interfering p300 function on tadpole development, we next introduced the F-dnp300 into developing tadpoles via the restriction enzyme-mediated integration procedure (63). For this purpose, we placed the F-dnp300 coding sequence under the control of the ubiquitously expressed cytomegalovirus promoter (Fig. 4*A*). The transgenesis construct also harbored the GFP driven by the eye lens-specific γ-crystallin promoter to distinguish the transgenic animals from their wild type siblings by virtue of green fluorescence in the eyes (45) (Fig. 4, *A* and *B*). This facilitated the rearing and subsequent treatments of the wild type and transgenic tadpoles in a single container. The expression of the mutant protein in the transgenic (but not wild type) siblings was confirmed by Western blotting using anti-FLAG antibodies (Fig. 4*C*).

As the transgenic procedure itself can cause developmental defects, only embryos generated from the transgenic procedure that were morphologically normal at stage 20 (neural tube stage, 22 h after fertilization) were used for further analysis. Both wild type and transgenic animals generated from the same transgenic procedure developed apparently normally throughout embryogenesis and up to the end of premetamorphosis (stage 54, ~26 days post-fertilization) (not shown). Thus, although the F-dnp300 in theory can interfere with any cellular processes involving SRC-p300·CBP complexes, under our transgenic conditions, it had little effect on embryogenesis and tadpole growth. This could either be because the level of F-dnp300 was insufficient to cause significant embryonic effects or that the effects were minor and not at the gross morphological level, thus ruling out nonspecific toxic effect of the transgene.

To study the effect of F-dnp300 on metamorphosis, wild type and transgenic siblings at the premetamorphic stage 54 were treated with 5 nM T3, a concentration similar to peak concentrations in the plasma during metamorphosis (64) for 3 days at room temperature. The wild type tadpoles underwent the characteristic T3-induced metamorphosis, most noticeably the regression of the aquatic breathing organs (the gills) leading to an overall reduction in the size of the head (Fig. 4*D*, compare *WT* to *WT + T3*). In addition, the lower jaw protruded out because of the growth and development of the Meckel cartilage, and the anterior end of the olfactory lobe was also closer to the nostrils in these wild type animals treated with T3 (Fig. 4*D*). In contrast, in the T3-treated transgenic animals, all metamorphic changes were drastically inhibited. The gills did not regress significantly, as evident from the large size of the head, and the jaw

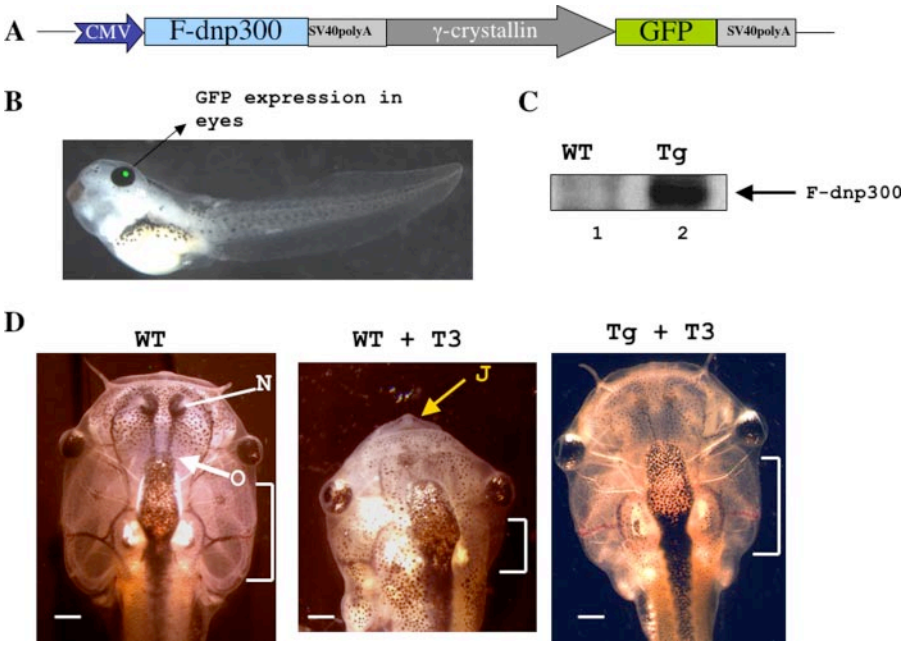

FIGURE 4. **Transgenic overexpressing F-dnp300 inhibits T3-induced metamorphosis.** *A*, schematic representation of the double promoter construct used for generation of transgenic animals. The constitutively active cytomegalovirus promoter drives the expression of the transgene F-dnp300 and is followed by the SV40 polyadenylation signal. The construct also harbors GFP under the control of the eye-specific γ-crystallin promoter, as a marker to identify transgenic animals. *B*, a transgenic *X. laevis* tadpole. The presence of GFP in the eye (*green lens*, *arrow*) indicates the presence of the double promoter construct, which also contained F-dnSRC3 in the tadpole. *C*, F-dnp300 protein is expressed in the transgenic animals. Total protein extract was prepared from a wild type (*WT*) or transgenic (*Tg*) animal and subjected to Western blot analysis with an anti-FLAG antibody. *D*, F-dnp300 inhibits T3-induced metamorphic changes. Wild type and transgenic tadpoles at premetamorphic stage 54 were treated with 5 nM T3 for 3 days in the same container. At the end of the treatment, transgenic tadpoles were identified by the green fluorescence in the eye lens. The wild type tadpoles, which were treated with T3, underwent the typical T3-induced changes, such as resorption of gills (*bracketed*) and development of the Meckel cartilage leading to protrusion of the jaw (marked as *J*). Furthermore, the anterior end of the olfactory lobe (marked as *O* in the wild type animal without T3 treatment) is closer to the nostrils (marked as *N*) in wild type animals treated with T3 as compared with untreated wild type siblings (*D*). These changes were not observed or greatly reduced in F-dnp300 transgenic animals treated with T3. In the absence of T3 treatment, the transgenic and wild type animals were morphologically indistinguishable (data not shown). Each experiment had 3 animals/group, and the experiment was repeated three times with 5 or 10 nM T3 treatment with similar results. Scale bar, 2 mm.

did not undergo remodeling (Fig. 4*D*, compared *bracketed area* in *Tg + T3* to *WT* and *WT + T3*). The morphology of these F-dnp300 transgenic tadpoles treated with T3 was more similar to that of the untreated wild type (Fig. 4*D*) or untreated transgenic animals (data not shown). In the absence of T3 treatment, the transgenic and wild type animals were morphologically indistinguishable (data not shown).

The effect of the transgene on intestinal remodeling was studied through histological analysis of the animals treated with/without T3. The premetamorphic intestine of both wild type (Fig. 5*A*) and transgenic animals (not shown) at stage 54 had a simple tubular structure with a monolayer of columnar epithelial cells lining the lumen, with little connective tissue and musculature. After 3 days of T3 treatment, the intestine in wild type animals underwent dramatic remodeling (Fig. 5*B*), which involves larval epithelial degeneration through apoptosis and adult cell proliferation (65). The intestines of T3-treated transgenic animals displayed a phenotype intermediate between T3-treated wild type and untreated wild type animals (Fig. 5*C*). Thus, the transgenic animals were also impaired in their ability to undergo T3-mediated intestinal remodeling.

The Dominant Negative p300 Inhibits Intestinal Metamorphosis by Competing against Endogenous p300 for Recruitment to Target Genes and Inhibiting Their Expression—To determine whether F-dnp300 blocked metamorphosis by affecting TR-dependent gene regulation in the intestine, we analyzed the expression of three direct, ubiquitous T3-responsive genes, *TRβA*, *T3/bZIP*, and *ST3* (54, 66–68). Premetamorphic wild type and transgenic tadpoles at stage 54 were treated with 5 nM T3 for 3 days, and total RNA isolated from the animals was analyzed by RT-PCR using gene-specific primers. As expected, the three genes analyzed were induced by T3 treatment in wild type tadpoles (Fig. 6, *lane 2*). However, in the transgenic animals, their up-regulation was blocked or inhibited (Fig. 6, *lane 3*), suggesting that the defect in metamorphosis in the transgenic animals is due to the inhibition of T3-responsive genes.

To investigate the mechanism of the observed gene inhibition in transgenic animals, we treated wild type and transgenic premetamorphic tadpoles at stage 54 with T3. Nuclei were isolated from the intestine and subjected to ChIP analysis. Again, to distinguish the endogenous p300 from the transgenic F-dnp300, we utilized the peptide antibody that recognizes the region of p300 not present in F-dnp300 (48, 49). Consistent with the data in Fig. 1, T3 treatment of wild type animals led to a ligand-dependent recruitment of p300 to the TRβA promoter (Fig. 7*A*) and concomitant increase in histone acetylation (Fig. 7*C*). In contrast, in the transgenic animals, the T3-induced recruitment of endogenous p300 to the promoter was reduced (Fig. 7*A*), and the same was true for histone acetylation at the promoter (Fig. 7*C*). As expected, the dominant negative p300 was recruited to the promoter in the transgenic animals in the presence of T3 (Fig. 7*B*). Thus, the mutant protein displaces the endogenous wild type p300 from the promoter, leading to reduced histone acetylation and gene expression.

The Dominant Negative p300 Also Inhibits Natural Metamorphosis—We left some of the transgenic and sibling wild type animals to develop naturally through spontaneous metamorphosis. Although wild type tadpoles underwent complete metamorphosis in 54–62 days (Fig. 8*B*), 3 of the 11 transgenic animals analyzed died around stage 61–62, the climax of metamorphosis when T3 levels peak (64). Seven completed metamorphosis, of which five completed metamorphosis

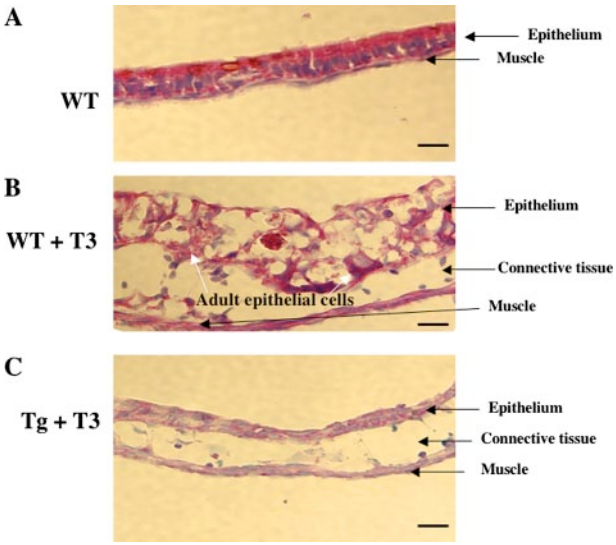

FIGURE 5. **F-dnp300 inhibits T3-induced intestinal remodeling.** Wild type (*WT*) and transgenic (*Tg*) animals were treated with T3 as in Fig. 4. Small intestines were isolated, sectioned using a cryostat, and stained using methyl-green pyronin Y. *A*, the intestine from wild type or transgenic (not shown) animals without T3 treatment had a nice uniform intestinal epithelium strongly stained *red* by the dye because of the abundant cytoplasmic RNA. The connective tissue was thin and lies in between the epithelium and muscles. *B*, the T3-treated WT intestine had increased connective tissue and a degenerating larval epithelium. Some strongly stained cells (*white arrows*) in the epithelium were likely developing adult epithelial cells (65). *C*, the T3-treated Tg intestine had delayed/inhibited remodeling with the morphology in between wild type animals treated with and without T3. *Scale bar*, 25 μm.

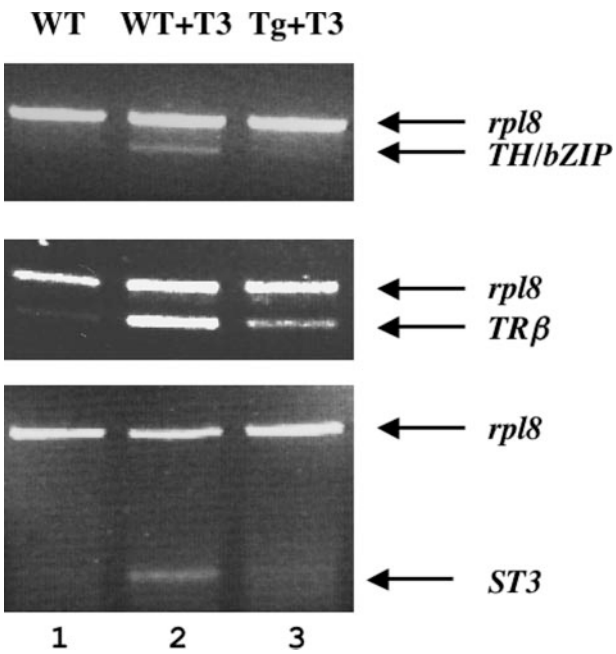

FIGURE 6. **F-dnp300 inhibits T3-induced gene activation.** Wild type (*WT*) and transgenic (*Tg*) animals at premetamorphosis (stages 47–50) were treated with 5 nM T3 for 3 days, and total RNA was isolated and analyzed for the expression of three direct T3-responsive genes, *TRβA*, *T3/bZIP*, and *ST3*.

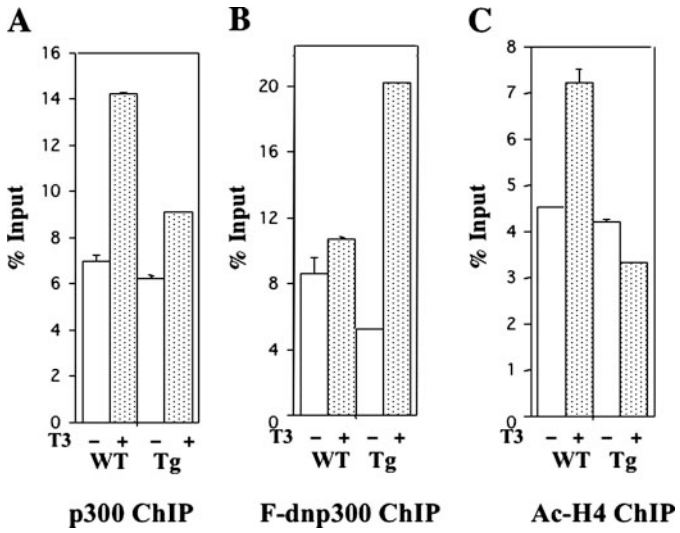

FIGURE 7. **F-dnp300 functions in transgenic animals by competing against endogenous p300 for binding to T3-responsive genes in the intestine.** Wild type (*WT*) and transgenic (*Tg*) animals were treated with 10 nM T3 for 48 h. Chromatin from the intestine was isolated (three tadpoles/treatment with tissues pooled together), and ChIP assays were performed using anti-p300 (*A*), anti-FLAG (for F-dnp300) (*B*), and anti-acetylated histone H4 (Ac-H4) (*C*) antibodies. The TRE region of the *TRβA* promoter was analyzed using quantitative PCR. The wild type animals exhibited a ligand-dependent binding of p300 to the promoter, whereas this recruitment was impaired in the transgenic animals with concurrent recruitment of F-dnp300.

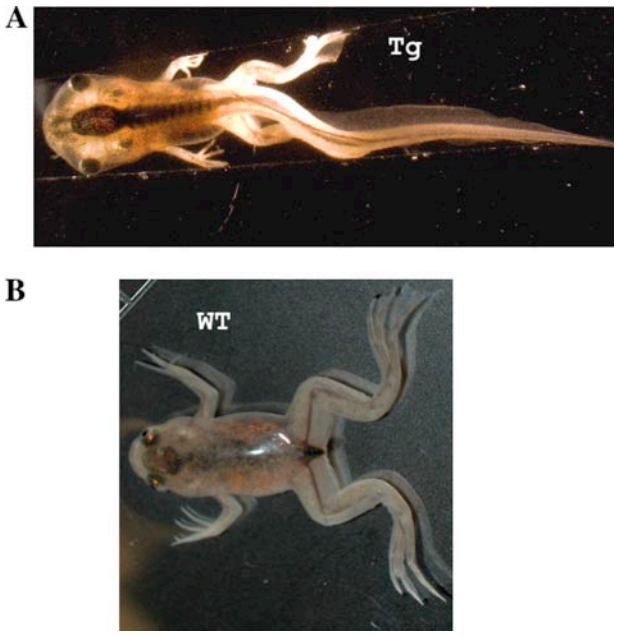

FIGURE 8. **The overexpression of F-dnp300 arrests or delays natural metamorphosis.** The progression of natural metamorphosis of F-dnp300 transgenic animals (*Tg*, *A*) and their wild type siblings (*WT*, *B*) was monitored. Shown in *A* is a tadpole that displays arrested metamorphosis. This tadpole remained at stage 61 for more than a month and then died, possibly due to partial metamorphosis. The wild type animals completed metamorphosis at ~2 months old, with complete gill and tail regression (*B*).

within a similar time frame of wild type animals, one completed on day 73, and the last one on day 83 (*i.e.* 15 and 25 days beyond the normal end of metamorphosis, respectively). Finally, one transgenic animal remained at stage 61 for >1 month and died on day 90 (Fig. 8*A*). The varying degrees of resistance to metamorphosis may reflect different levels of transgene expression, because each transgenic animal might contain a different number of copies of the transgene at different genomic locations. The balance between the levels of endogenous p300 and the mutant protein is expected to determine the severity of any observed effects. Those with a lower dosage of F-dnp300 would

be less affected in their developmental program and undergo metamorphosis without significant defects. In animals expressing higher levels of the transgene, the F-dnp300 would titrate or compete out the endogenous protein, and this would manifest as various dose-dependent defects in metamorphosis, leading to death for those animals that cannot complete metamorphosis.

DISCUSSION

In this study, we have addressed the role of p300/CBP in TR signaling during development. One of the first pieces of evidence for the involvement of p300/CBP in nuclear receptor function was the inhibition of nuclear receptor activity by anti-CBP antibodies in tissue culture cells (69). Subsequently, several *in vitro* studies have shown that p300 is recruited to the nuclear receptor activation complex via its SRC-interacting domain (10, 27–29). However, little was known about the *in vivo* significance of SRC-p300/CBP interaction in nuclear receptor function and animal development/physiology. Although gene knock-out mice lacking various coactivators have been generated, often they have little or relatively mild phenotypes because of cofactor redundancy or embryonic lethal phenotypes. Furthermore, because these cofactors are often involved in multiple transcriptional regulatory pathways, it is difficult to pinpoint the role of a particular cofactor to a nuclear hormone receptor, even when gene knock-out and/or transgenesis result in easily identifiable phenotypes, such as mice deficient in p300 and SRC1–3 (70–73). Here, by using amphibian metamorphosis as a model, we show for the first time *in vivo* that SRC-p300 or related complexes are required for gene regulation by TR and vertebrate development.

TR is a dual function transcription factor that recruits corepressors in the absence of T3 to repress transcription and coactivators in the presence of T3 to activate transcription. A large number of coactivators for TR have been identified and extensively characterized *in vitro*. Coactivators interact with TR in the presence of T3 and activate transcription, either directly or by recruiting accessory cofactors. Coactivators for TR and other nuclear receptors belong to several categories, and cell culture studies have suggested a sequential recruitment of these coactivators (74). Furthermore, cyclical recruitment of coactivators and their receptors has also been observed, where the occupancy on a promoter may be transient, with phases of association and dissociation of the coactivator and receptors (75–78). Thus, it is becoming increasingly clear that gene expression by TR and other nuclear receptors involves not only ligand-induced switches but also spatial and temporal regulation of interacting cofactors.

Essentially all of the existing information on cofactor recruitment has emerged from *in vitro* and cell culture studies, and few studies have explored the significance of these interactions during post-embryonic development in vertebrates, especially with reference to nuclear receptor function, mainly because of the lack of a proper system. Amphibian metamorphosis offers a unique opportunity to study coactivator involvement in receptor function during vertebrate development because of its total dependence on T3 and the tissue- and, possibly, cell-ubiquitous nature of the requirement for T3. Such properties make it possible to correlate developmental phenotypes with molecular pathways in different organs/tissues. Using this system, we have previously shown that the coactivator SRC3 is up-regulated during *X. laevis* metamorphosis and is recruited to T3-dependent promoters (41, 43). Furthermore, overexpression of a dominant negative form of SRC3, comprising only the nuclear receptor-interacting domain, inhibited all aspects of metamorphosis (42). Given the *in vitro* and tissue culture studies showing the importance of p300/CBP in SRC function, our results raise the possibility that p300/CBP is also required for gene regulation by TR and metamorphosis during *X. laevis* development. Indeed, by using similar approaches as the ones we used for the earlier SRC3 studies, we have shown here that, similar to SRC3, p300 is also recruited to TR target promoters in the animal intestine in a T3-dependent manner, similar to that observed in the tail (48). More importantly, transgenic expression of a dominant negative form of p300, which comprises only the SRC-interacting domain, inhibited all metamorphic events induced by T3 that we were able to measure/observe.

Through gene expression analysis, we have shown that the inhibition of metamorphosis was correlated with the inhibition of the expression of T3 response genes. More importantly, our ChIP assay indicated that the molecular basis of this repression was because of the displacement of endogenous wild type p300 from the TR-signaling complex at T3-regulated promoters, accompanied by reduced histone acetylation at these promoters.

It is of interest that the phenotypes and gene expression profiles of transgenic animals expressing F-dnp300 are similar, under our assay conditions, to those of transgenic animals expressing a dominant negative SRC3, containing only the TR-interacting domain (42) or transgenic animals expressing a dominant negative TR (36, 37). These would suggest that F-dnp300 mostly affects TR function through an SRC-dependent pathway during metamorphosis, although in theory, F-dnp300 could affect all processes involving SRC-p300·CBP complexes or other processes in which the SRC-interacting domain of p300 participates, *e.g.* by interacting with and therefore titrating out other molecules capable of binding to this domain. Interpreting our results as a specific effect through TR may be understandable given the fact that TR is the central transcription factor for metamorphosis, both being necessary and sufficient for mediating the effects of T3 during this process (36, 38). On the other hand, transcriptional pathways involving other transcription factors, such as other nuclear hormones or orphan receptors, are also likely inhibited by the transgenic F-dnp300 as well. However, the roles of these other transcription factors in metamorphosis are either unknown or secondary to TR action, even in organs where some may participate, *e.g.* glucocorticoid receptor in the tail (because no ligands for nuclear receptors other than T3 can induce morphological changes in premetamorphic tadpoles) (34). Thus, the unique property of the metamorphosis model made it possible for us (1) to correlate the developmental phenotypes to the gene regula-

tion pathways mediated by TR and (2) to show that coactivator function is required for this process (42). This same property has now allowed us to further demonstrate a specific requirement for the SRC pathway involving SRC-p300·CBP complexes or related complexes in gene activation by liganded TR and metamorphosis. This, to our knowledge, represents the first *in vivo* evidence for an essential, direct role of such complexes in specific gene regulation by nuclear receptors and vertebrate developmental.

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