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Original Article

Multiple stage-dependent roles for histone deacetylases during amphibian embryogenesis: implications for the involvement of extracellular matrix remodeling

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ABSTRACT Histone acetylation has long been implicated in the regulation of gene expression. Recently, a number of histone acetyltransferase and histone deacetylase genes have been identified and cloned. Molecular studies have shown that these enzymes influence transcriptional regulation as components of cofactor complexes that interact with diverse transcription factors. However, relatively little is known about their function during development. Here, we make use of the ability to manipulate *Xenopus laevis* embryos *in vitro* to study the role of histone deacetylases in development. We first demonstrate that the histone deacetylase *Rpd3* and its associated co-repressor *Sin3*A are coordinately expressed during embryogenesis. *Rpd3* and *Sin3*A are known to be part of at least one large corepressor complex, which is involved in transcriptional regulation by many transcription factors, suggesting that deacetylase activity is important for embryogenesis through transcriptional regulation. Indeed, treating developing embryos with a specific histone deacetylase inhibitor, trichostatin A (TSA), leads to embryonic lethality with severe defects in the head and tail regions. Furthermore, the effects of TSA are stage-dependent with the severity of the defects decreasing when treatment is initiated at later stages. On the other hand, a sharp bend (kink) develops in the tail even when TSA treatment begins at tadpole hatching. We provide evidence that this tail defect may be in part due to the TSA-dependent inhibition of the expression of the matrix metalloproteinase gene stromelysin-3, which has been implicated in tail development through extracellular matrix remodeling.

KEYWORDS: *Xenopus laevis, matrix metalloproteinase, histone acetylation, chromatin remodeling, morphogenesis.*

Introduction

The central theme of developmental biology has always been to understand how the fertilization of a single cell, the egg, gives rise to a complex organism. At molecular level, this involves determining how various genes are activated or repressed at various developmental stages to effect cell fate determination and tissue patterning. A key factor in transcriptional regulation is believed to be the state of the local chromatin environment of the gene to be regulated (Wolffe, 1996). Chromatin structure can be influenced by both DNA and histone modifications such as DNA methylation, histone phosphorylation and acetylation as well as histone methylation (Wolffe, 1996; Strahl and Allis, 2000).

It has been suggested that histone acetylation influences gene expression (Pogo *et al.*, 1966; Hebbs *et al.*, 1988; Wolffe, 1996; Wolffe and Pruss, 1996; Grunstein, 1997; Struhl, 1998). Histone acetylation occurs at the lysine residues of the amino-terminal tails of the histones. Each acetyl group neutralizes a positive charge of the histone tails. This reduces the affinity of histones toward DNA (Hong *et al.*, 1993) and thus can alter the nucleosomal conformation and chromatin accessibility, possibly leading to easier access of transcription factors to regulatory regions within chromatin (Norton *et al.*, 1989; Lee *et al.*, 1993; Vettese-Dadey *et al.*, 1996). Consequently, histone acetylation can regulate gene transcription. Further support for a role of histone acetylation in gene regulation has come from recent demonstrations that many diverse transcription factors and cofactors have histone acetylase (or

Abbreviations used in this paper: TSA, trichostatin A; TR, thyroid hormone receptor; *ST3*, stromelysin 3; *Col3*, collagenase 3; *xhh*, *Xenopus* sonic hedgehog.

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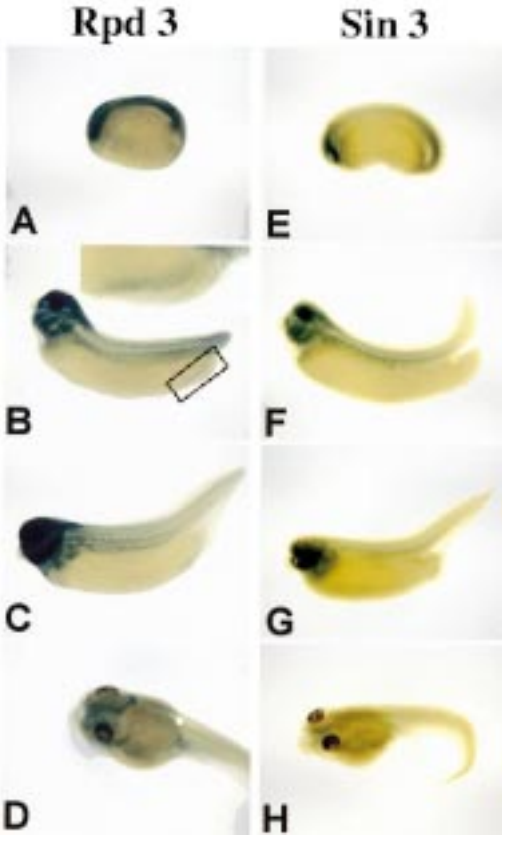

Fig. 1. Coordinated spatial and temporal expression of histone deacetylase *Rpd3* and corepressor *Sin3*. *Digoxigenin labeled Rpd3 and Sin3 antisense mRNA was used as probes in whole mount analysis of their developmental expression in albino Xenopus embryos.* **(A,B,C,D)** *Rpd3 and* **(E,F,G,H)** *Sin3 expression was examined at the end of neuralation (stage 18/21, A,E), tailbud (stage 31/32,B,F), hatching stages (stage 35/36, C,G), and at the onset of feeding (stage 45, D,H). Both Rpd3 and Sin3 were abundantly expressed, particularly in dorsal axial structures and in head and anterior structures. Their expression diminished by tadpole feeding. Their expression profiles overlap extensively. Note that Rpd3 was also expressed in the dorsal region of the embryo in the area of the proctodeal channel (boxed area, which was enlarged in the insert in B), where stromelysin-*

acetyltransferase) activity and that transcriptional corepressors are complexed with histone deacetylases (Wade and Wolffe, 1997; Pazin and Kadonaga, 1997; Struhl, 1998; Ng and Bird, 2000).

One of the best-studied transcriptional co-repressor complexes contains the co-repressor *Sin3* and deacetylase *Rpd3*. *Sin3* can interact with the co-repressors SMRT and N-CoR, which in turn bind to a number of transcription repressors including Mad/Max and unliganded thyroid hormone receptors (TRs) (Heinzel *et al.*, 1997; Nagy *et al.*, 1997; Pazin and Kadonaga, 1997). Thus, specific local changes in histone acetylation through targeting histone deacetylases by transcription factors may serve as a mechanism for specific gene regulation during development. Compared to the extensive biochemical and molecular characterization of histone acetylation and deacetylation, relatively little is known about their developmental functions. Amphibian development is an excellent system with which one can investigate how histone acetylation influences gene expression during development. Oviparous amphibian embryogenesis can be easily manipulated *in vitro* without any concerns of maternal effects as occurs with mammalian development, and amphibian embryos are easily accessible for molecular analysis.

Here we make use of *Xenopus laevis* development to investigate the developmental roles of histone deacetylases. We first determined the spatial and temporal expression of histone deacetylase *Rpd3* and corepressor *Sin3*, which interacts with *Rpd3*. Using a specific chemical inhibitor of histone deacetylases, trichostatin A (TSA), we directly demonstrate the requirement for histone deacetylases in *Xenopus* embryogenesis. We further identify a potential downstream mediator of this effect, the matrix metalloproteinase stromelysin-3, in a specific developmental morphogenetic process.

Results

Rpd3 and Sin3 are co-localized during Xenopus embryogenesis

To gain insight into the possible role that histone acetylation may play in the regulation of developmental events, we focused on the involvement of histone deacetylases due to the availability chemical inhibitors of these enzymes for possible embryonic studies. One histone deacetylase, *Rpd3*, and its likely interacting partner in the co-

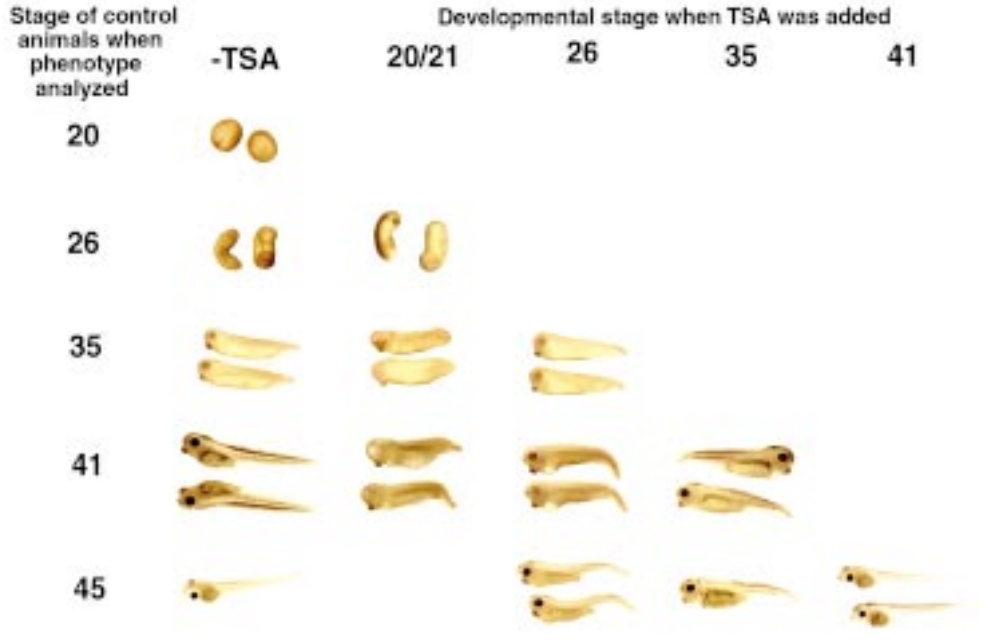

Fig. 2. Blocking histone deacetylases leads to stage-dependent developmental defects. *TSA was added to embryo or tadpole-rearing water at a concentration of 100 nM at various developmental stages as indicated and phenotypic effects were subsequently assayed when control animals reached indicated stages. All embryos within a treatment group developed essentially identical phenotypes (also see Table 1). In general all treatment groups were delayed in their development, and treatment of embryos up to and including stage 26 resulted in developmental arrest and death when control embryos reached feeding stage. All treatments, except when started at stage 41 or later, resulted in kinked tails. Earlier treatments (starting prior to stage 26) also resulted in embryos with eye and head defects, ventral swelling, and impaired swimming ability.*

Fig. 3. TSA treatment prior to and at stage 20 results in severe head and eye defects. *Embryos treated with 100 nM TSA beginning at stage 18 displayed severe head and tail defects when examined when control sibling reached stages 35* **(A and B)** *and 41* **(C)**. *The two sides of the same four embryos were pictured in A and B to demonstrate the presence of only one pigmented structure, presumable the rudiment of the eye, on only one side of the embryo (A, arrow) but not on the other (B). Note also the irregularly shaped swollen head, protruding cement gland, swollen tail tip, and the lack of a prominent tail fin. By stage 41 (C), the swelling in the head is more pronounced and in addition to the single pigmented area present in earlier embryos, often an additional pigmented structure develops on the same side (arrowhead).*

repressor complexes, *Sin3*, have been cloned in *Xenopus laevis* (Wong *et al.*, 1998; Vermaak, *et al.*, 1999). Thus, we first performed whole mount *in situ* hybridization for both *Rpd3* and *Sin3* to determine their spatial and temporal expression profiles during embryogenesis (Fig. 1). Consistent with the idea that *Rpd3* and *Sin3* function together in a deacetylation complex, the expression profiles of both genes largely overlapped throughout embryogenesis. Both genes were found to be abundantly expressed in head and axial structures of embryos from early neurula to tailbud stages (Fig. 1 A,B for *Rpd3*, and Fig. 1 E,F for *Sin3*, respectively). Expression in anterior and axial structures was within the area of the embryo where most differentiation was occurring (as opposed to the medial and ventral endoderm where there were relatively few differentiating tissues at these stages). By hatching stage, when the differentiation of many axial structures was near completion, the levels of both *Rpd3* and *Sin3* in the dorsal axis decreased (Fig. 1 C,G). Similarly, as feeding begins and the tadpole entered a growth rather than differentiation stage of development, expression of both *Rpd3* and *Sin3* further decreased (Fig. 1 D,H). Thus, histone deacetylase activity is likely to be playing an important role in regulating genes necessary for tissue differentiation and morphogenesis, especially head and axial development.

Inhibition of histone deacetylase activity causes stage-dependent developmental abnormalities

To investigate the function of histone deacetylases in embryogenesis, we took advantage of the fact that one can inhibit this family of enzymes with chemical inhibitors. In particular, the drug trichostatin A (TSA), has been shown to specifically inhibit histone deacetylases both *in vitro* and *in vivo* (Yoshida *et al.*, 1990, 1995; Finnin *et al.*, 1999). Addition of TSA to embryo rearing water led to global and stage-dependent defects in embryos and tadpoles (Fig. 2). Notably most or all embryos within a treatment group developed identical phenotypes (Table 1). All embryos were delayed in their development. In addition, when treatment began prior and up to stage 35, developmental arrest occurred shortly and thereafter the embryos died (Table 1). Treatment with TSA at very early, pre-gastrulation

TABLE 1

PHENOTYPES OF EMBRYOS TREATED WITH TSA AT VARIOUS DEVELOPMENTAL STAGES

Developmental stage of control embryos	TSA added stage 20/21	TSA added stage 26	TSA added stage 35	TSA added stage 41
26	50/50 largely normal			
35	49/50 abnormal head and eye development; cement gland structure and position; kinked, short tail	50/50 eye and cement gland abnormal; swollen thorax; kinked, short tail		
41	49/50 delayed development; single pigmented area (eye?); cement gland structure and position abnormal; swollen head, thorax, ventral mesoderm; kinked, short tail	48/50 delayed development; eyes abnormal; cement gland structure and position abnormal; swollen head, thorax, ventral mesoderm; kinked, short tail	40/45 delayed development; slight swollen head; swollen thorax; kinked short tail 5/45 normal except for kinked, short tail	
45	41/50 development arrest – death within 2 days	47/50 delayed development; head structures better; swollen cranium and thorax; kinked, short tail	34/45 delayed development; swollen thorax; kinked short tail 10/45 normal except for kinked, short tail	37/40 normal
47		41/50 development arrest – death within 3-4 days	25/45 development arrest – death within 3-4 days 11/45 normal	30/40 normal

The numbers shown are of embryos with a particular phenotype over the total number of embryos treated with TSA. Unaccounted for numbers represent embryos that died by the assay stage.

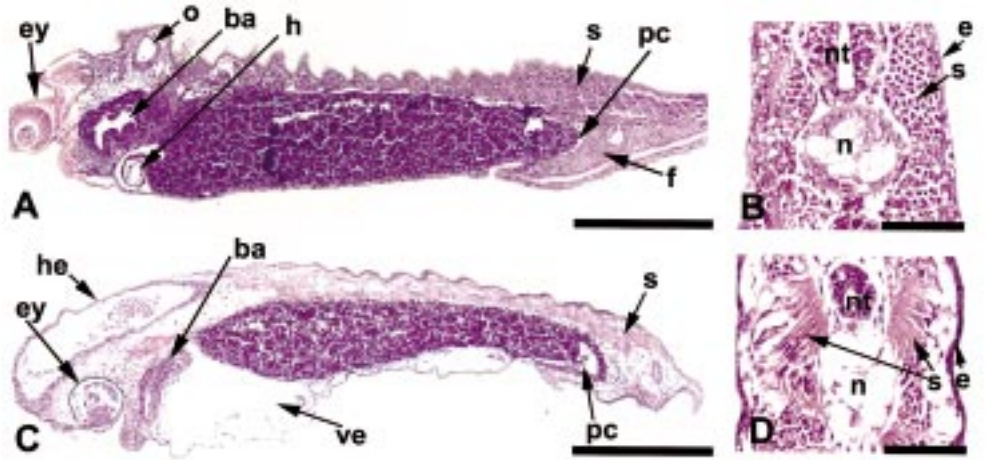

Fig. 4. TSA treatment causes numerous tissue defects in embryos. *Embryos treated without (control) or with TSA beginning at stage 20 were fixed when control embryos reached stage 35 and sectioned for histological examination. (A,C) Longitudinal and (B,D) cross sections were examined of control (A,B,) and TSA treated (C,D) embryos.* **(A,B)** *Control embryos revealed well defined structures such as eye (ey), branchial arches (ba), otic vesicle (o), heart (h), well organized somites (s), tail fin (f), and narrow proctodeal channel (pc).* **(C)** *Mid trunk cross sections revealed a well-organized neural tube (nt), notochord (n) epidermis (e) and somites (s).* **(D,E)** *TSA treatment resulted in swelling in the head (he) and thorax (ve), a poorly organized eye (ey), poorly organized somites (s), and a wide and irregularly shaped tail and proctodeal channel (pc). In cross section (D) the neural tube (nt) and the notochord (n) were seen to be irregular, the epidermis appears thickened (e), and the somites (s) were disorganized. Bar represents 1 mm in A and C and 0.4 mm in B and D.*

stages led to severe developmental defects, including improper induction/formation of the axis, head and mesoderm (data not shown, Almouzni *et al.*, 1994). All treatments initiated between stage 20 and stage 35 resulted in kinked tails, eye and head defects, and ventral swelling and edema. Eye and head defects were most severe in embryos treated at very early stages (stage 20, Table 1 and Fig. 2) and included the presence of only one pigmented structure (Fig. 3), presumable remnants of the eye, on only one side of the embryo (3A) but not on the other (3B). They also had deformed and swollen heads, protruding cement glands, and swollen tail tips (Fig. 3). When control siblings reached stage 41, the swelling in the heads of the treated embryos was more pronounced, and a second pigmented spot developed on the same side of the embryo (Fig. 3C). In general, these TSA-treated embryos were also characterized by ventral swellings, a ventral arching of the entire body axis, lack of a prominent tail fin, and poor or no heart development.

When TSA treatment began at stage 26-35, the embryos developed largely normal heads, eyes, and axes, but had delayed overall development. All embryos treated with TSA developed kinked tails. Most appeared to be arrested at stage 34-39 and died about 24 h after control embryos reached feeding stage (stage 45) or shortly after. Compared to TSA treatment beginning at stage 26, when TSA treatment began at stage 35, the severity of the tail bending was less and decreased further as the animal developed to about stage 45 (Fig. 2 and Table 1).

When treatment began at stage 41, the animals developed normally up to stage 47, without any noticeable morphological differences compared to control animals (Fig. 2 and Table 1), although they too died within 4 days of treatment (Table 1). On the other hand, TSA treatment of postfeeding stage (stage 45) tadpoles, e.g., at stage 55, for up to a few weeks had no observable effects on the animals. Thus, histone deacetylases are important for embryogenesis and organogenesis but not essential for tadpole growth.

TSA treatment causes numerous tissue defects in embryos

To examine the tissue defects underlying the gross morphological abnormalities, embryos were treated with TSA at stage 20 and fixed for histological analysis when control embryos reached hatching (stage 35). In contrast to control embryos (Fig. 4 A,B) numerous internally defects were found as a result of TSA treatment (Fig. 4 C,D). Obvious defects included edemas and swellings in the head, tail and ventral region (Fig. 4C) and the poor organization or differentiation of a number of tissues including the eyes, somites, and heart, branchial arches, tail fin. The swelling in the head and tail appeared to be due to a swelling within the anterior and posterior neural tube. The proctodeal opening and channel (Fig. 4 A,C) were also abnormal in the TSA treated embryos and appeared to, in part, result in the ventral bending of the tail seen at this stage. Major axial structures such as the notochord, neural tube and somites were present in TSA treated embryos, but their organization and morphology was abnormal (Fig. 4 B vs. D).

Tissue specific downregulation of matrix metalloproteinase gene stromelysin-3 by TSA is associated with the formation of a sharp bend (kink) in the tail

TSA is a highly specific drug against histone deacetylases (Yoshida *et al.*, 1990, 1995). Thus, we reasoned that the morphological defects caused by TSA were likely due to specific changes in developmental gene expression. The lack of known TSA regulated genes in development prompted us to take a candidate gene analysis approach. Given the major defects in the head and axial structures, we focused on genes that are expressed in these regions and are likely involved in morphogenesis. We previously showed that the matrix metalloproteinase genes collagenase 3 (*Col3*) and stromelysin-3 (*ST3*) and the morphogen sonic hedgehog (*xhh*) were expressed in these regions during embryogenesis (Damjanovski *et al.*, 2000 and unpublished observation). As these genes are likely involved in the development of these structures, we analyzed their expression by whole mount *in situ* hybridization on control and TSA treated embryos at stages 35 (Fig. 5A-C) and 41 (Fig. 5 D-F) (referring to the stages of control sibling animals). *ST3* was found to be strongly expressed in the head and in the dorsal/proctodeal endoderm region (Fig. 5A, arrow, top animal) of the untreated embryo while *Col3* mainly in the axial structures (Fig. 5C, top animal). The morphogen *xhh* was also found to be expressed in the head and the around the notochord (Fig. 5B, top animal). Following treatment with TSA the expression of *ST3* was greatly downregulated in the dorsal/proctodeal endoderm region of the tail (Fig. 5A, arrow, bottom animal), although

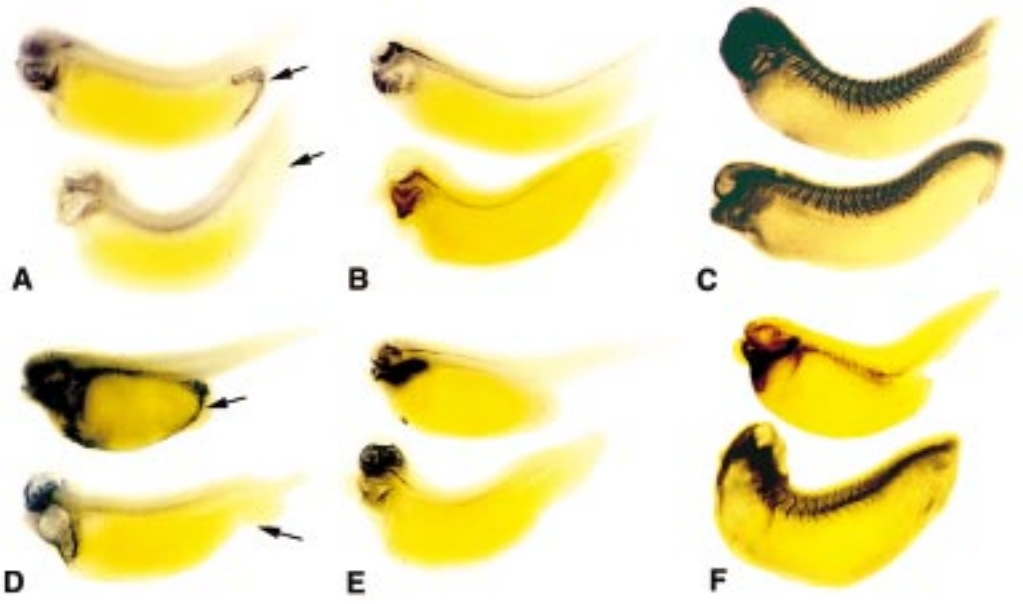

Fig. 5. Stromelysin-3 (*ST3*) expression, but not hedgehog (*xhh*) or collagenase-3 (*Col3*) expression is altered by TSA. *Albino embryos without (control, top of each panel) or with (bottom of each panel) 100 nM TSA treatment from stage 20/21 were fixed when control embryos reached stage 33-35 (A,B,C), or stage 41 (D,E,F). ST3* **(A, D)**, *xhh* **(B, E)** *and Col3* **(C, F)** *mRNA expression was examined by whole mount in situ hybridization on fixed animals. ST3 was largely expressed in the head and the dorsal/proctodeal endoderm region (A, D single arrow) of control animals. Following treatment with TSA, the expression of ST3 was largely unchanged with the exception of the reduction in dorsal/ proctodeal endoderm region (A, D arrow). Despite the TSA induced changes in phenotype, the expression patterns of xhh and Col3 were essentially unchanged (compared the top to bottom animal in B, C, E, F). Note that the kink in the tail of TSA-treated embryos was not very obvious compared to that in Fig. 2. This is due to the whole mount in situ procedure. The protease digestions, high temperatures, and dehydration process caused all embryos to be dorsally curved, thereby diminishing the kinked tail phenotype associated with TSA.*

it remained essentially unchanged in the rest of the embryo (Fig. 5A, bottom animal). On the other hand, despite the drastic embryonic deformation caused by TSA, the expression of *xhh* (Fig. 5B, bottom animal) and *Col3* (Fig. 5C, bottom animal) was largely unaffected. This tissue specific downregulation of *ST3* by TSA persisted up to stage 41 (Fig. 5 D, E, F for *ST3*, *xhh*, *Col3*, respectively), after which *ST3* expression was repressed during normal development (Patterton *et al*., 1995).

To confirm the inhibition of *ST3* expression in embryonic tail by TSA treatment as seen by whole mount *in situ* , semi-quantitative RT-PCR was then performed to compare the levels of *ST3* expression in stage 35 total embryos or tail region from control embryos and embryos treated with TSA from stage 20 (Fig. 6). Overall *ST3* mRNA levels in the total embryo was not affected by TSA, however, there was a dramatic decrease in *ST3* expression in the tail, in complete agreement with the findings of the whole mount *in situ* hybridization. Analysis of a control gene, the ribosomal protein L8 or rpl8, showed that an equal amount of total RNA was present in the control and TSA treated samples. Together, these results showed that the downregulation of *ST3* expression in the tail region prior to stage 41 was temporally associated with TSA-induced bending in the tail. After stage 41, *ST3* was downregulated in control tadpoles (Patterton *et al*., 1995), suggesting that it was no longer required for normal development. Consistently, TSA treatment at or after stage 41 did not cause tail bending (Fig. 2, Table 1, and data not shown).

Discussion

Embryogenesis involves a complex regulation of gene expression in a tissue and developmental stage-dependent manner. To accomplish this, diverse families of transcriptional regulatory proteins are employed to activate or repress gene transcription in various regions/tissues of the embryos. The organization of eucaryotic genome into complex yet poorly understood chromosome argues that the nature of the chromatin will undoubtedly be an important factor in influencing the interactions of these regulators with their target genes. It has been well established that during development in many species, the structural proteins within the chromosome are known to change in both composition and chemical modification (Poccia, 1986; Wolffe, 1991). As the structure backbone of the chromatin, histones play a critical role in developmental gene regulation. Histone acetylation is known to change during *Xenopus* embryogenesis (Dimitrov *et al*., 1993). Such changes are likely determined by the action of histone

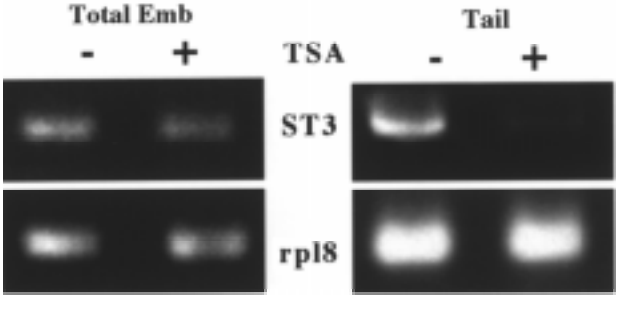

Fig. 6. RT-PCR analysis confirms the reduction in *ST3* expression in embryonic tails by TSA treatment. *Embryos were treated without (control, -) or with (+) TSA beginning at stage 20. When control embryos reached stage 35, RNA was isolated from total embryos or just the tail region (the posterior 25% of the embryo). The RNA was used for semi-quantitative comparison of the levels of expression of ST3 and the control gene encoding the ribosomal protein L8, rpl8, whose expression did not vary due to TSA treatment in the whole embryo or the tail section. ST3 expression in the total embryo was not affected by TSA but there was a dramatic decrease in ST3 expression in the tail.*

acetyltransferases and histone deacetylases. However, the developmental functions of the changes in histone acetylation are unclear. We have provided here two complementary pieces of evidence to support stage and tissue-dependent roles for histone deacetylases during vertebrate development. First, we have shown a correlation of the developmental expression profiles of histone deacetylase *Rpd3* and its associated transcriptional corepressor *Sin3* with development. Second, we have directly demonstrated a requirement for histone deacetylases during embryogenesis with inhibitor studies.

The temporal and spatial expression of *Rpd3* and *Sin3* correlates well with the observed defects by TSA treatment. For example, both *Rpd3* and *Sin3* are highly expressed in the anterior end or the head of the embryo at early stages. The addition of TSA to rearing water of early embryos has a profound effect on development in nearly 100% of the embryos. TSA treatment before gastrulation results in severe developmental abnormalities during which few head and axis differentiation events occur (data not shown). Addition of TSA at stage 20 results in deformations, which include prominent head swelling and ventral tail bending. On the other hand, such embryos and those treated with TSA at stage 26 do develop extensively, albeit at a slower rate. Their development is, however, arrested at the tailbud stages. These animals never appear to go past a stage which appears to be around stage 34-39 (as based on tail elongation) even after control sibling reach stage 47. After stage 41, the expression of both *Rpd3* and *Sin3* decreases. Correspondingly, the animals can develop further in the presence of TSA, although all eventually die as well.

It should be pointed out that TSA may influence the function of proteins other than *Rpd3* to affect embryogenesis. TSA is a highly specific inhibitor for histone deacetylases. Thus, its effects on embryonic development are likely through affecting histone deacetylases. On the other hand, multiple histone deacetylases are known to exist in vertebrates (Ng and Bird, 2000), it is quite possible that TSA inhibition of deacetylases other than *Rpd3* also contributes to the observed effects. Studies of these other histone deacetylases, once they are cloned in frogs, should help to clarify their developmental roles.

Detailed analysis of the TSA-induced defects revealed that different regions/tissues of the embryo have different developmental requirements for histone deacetylases. For example, TSA head and eye development requires histone deacetylase activity during early period of development (up to stage 26), while tail development appears to require the action of histone deacetylases up to stage 35. It is unclear how TSA treatment leads to such defects. It may alter early induction processes, such as the induction of lens differentiation by neural tissue on ectoderm (Zygar *et al.,* 1998, Streit and Stern 1999). Molecularly, TSA is likely to regulate the expression of genes responsible for morphogenetic and remodeling events. It is very likely that one or more gene regulation steps downstream of histone deacetylases are involved to produce the observed morphological defects. Our analysis has provided evidence that one candidate gene important for tail development encodes the matrix metalloproteinase stromelysin-3.

Our previous studies have identified several candidate genes that are important for development during early tailbud stages. These include the matrix metalloproteinase genes stromelyin-3 (*ST3*) and collagenase–3 (*Col3*), and the morphogen sonic hedgehog (*xhh*) (Stolow and Shi, 1995; Damjanovski *et al*., 2000, and unpublished observation). Whole mount *in situ* analyses of the expression of these genes suggested that they are unlikely to be involved in TSA-induced embryonic defects with the exception of tail development. *ST3* but not *Col3* or *xhh* is expressed in the dorsal/proctodeal endoderm region of the tail, adjacent to the region where the kink forms upon TSA treatment. The downregulation of *ST3* expression by TSA is thus correlated both spatially and temporally with the formation of the kinked tail. Further support for a role of premature downregulation of *ST3* in TSA-induced tail kinking comes from the fact that TSA does not cause tail bending when added to embryo rearing water at or after stage 41, when *ST3* is already downregulated even in control embryos (Patterton *et al*., 1995). As a matrix metalloproteinase, *ST3* presumably participate in tail development by remodeling the extracellular matrix.

The exact mechanism by which TSA affects embryogenesis and *ST3* gene expression is unclear. TSA is likely to influence *ST3* expression through multiple gene regulation steps, possibly involving the upregulation of a transcriptional repressor of *ST3* gene. Several lines of evidence point to the involvement of histone deacetylases. First, TSA is a highly specific inhibitor of histone deacetylases (Yoshida *et al*., 1990, 1995). Second, at least one histone deacetylase, *Rpd3*, and its associated transcriptional corepressor *Sin3* are highly expressed in regions where TSA has the greatest effects. Third, the downregulation of *ST3* by TSA also occurs in a region where *Rpd3* is expressed. The fact that *ST3* is not affected in other parts of embryo indicates that the regulation of gene expression by TSA is also tissue-dependent, consistent with the observation that TSA causes developmental abnormalities in a tissue and stage-dependent manner.

The embryonic abnormalities that result from TSA treatment are difficult to interpret due to complex developmental interactions among different organs/tissues. The histological analysis of the TSA treated embryos shows poor notochord and neural development. These structures are themselves responsible for the proper induction and differentiation and patterning of other tissues, such as the neural crest, somites, etc. The future challenge is to identify the early events and the corresponding genes that are responsible for mediating the effects of TSA. In this regard, the possibility of manipulating the amphibian embryo means that this model will continue to be valuable for not only investigating the role of histone acetylation/deacetylation in regulating developmental gene expression, but also dissecting out the functions of genes that are important for vertebrate development and the corresponding mechanisms.

Materials and Methods

In vitro *fertilization of Xenopus eggs and Trichostatin-A treatment*

Xenopus laevis females were primed and ovulated eggs fertilized by standard methods (Puzianoska-Kuznicka *et al*., 1997). The jelly coat was removed by washing the fertilized eggs with 3% cysteine, pH 8.0, followed by 4 - 6 washes with 0.1X MMR. Healthy looking embryos were collected just after beginning of the first division and maintained in 0.1X MMR. *Xenopus* embryos, were staged according to Nieuwkoop and Faber (1956). Trichostatin-A (TSA) (WAKO RLP9340), at a final concentration of 100 nM, a concentration sufficient to inhibit histone deacetylases in *Xenopus* and starfish embryos (Ikegami *et al*., 1993 ; Almouzni *et al*., 1994), was added to and maintained in embryonic and tadpole rearing water at various developmental stages (stages 20, 26, 35 and 41). The rearing water and TSA were changed daily. Phenotypic effects were subsequently assayed at (untreated control) sibling stages 26, 35, 41, 45, and 47.

Whole mount* in situ *hybridization

The protocol used was essentially that of Harland (1991). Briefly, albino *Xenopus* embryos were fixed with paraformaldehyde, rinsed with PBS and treated with proteinase K. Following refixation with paraformaldehyde, embryos were acetylated with acidic anhydride and prehybridized in hybridization solution. Full length clone containing *Xenopus Rpd3* (Wong *et al*., 1998), *Sin3*A (Vermaak, *et al*., 1999), *ST3* (Patterton *et al*., 1995), *Col3* (Brown *et al*., 1996), or hedgehog (Stolow and Shi 1995) cDNA in pBluescript ks(-) vector (Stratagene) was linearized with appropriate restriction enzymes. The DNA was transcribed with T3 or T7 RNA polymerase using a Roche Genius 4 kit to generate digoxigenin labeled antisense and control, sense RNA probes. Hybridization was carried out at 60ºC overnight followed by high stringency washes with 0.2% SSC, 0.1% CHAPS at 60ºC (Damjanovski *et al*., 2000). An alkaline phosphatase-conjugated digoxigenin antibody was used for colorimetric reaction using NBT and BCIP. Embryos were then post-fixed in Bouin's, dehydrated with methanol and cleared with benzyl benzoate and benzyl alcohol for photography.

Histological analysis

Embryos were fixed for 2 h with 4% paraformaldehyde in 1X PBS. The embryos were dehydrated through an ethanol series and xylene and then embedded into paraplast wax (Sigma). Following sectioning at 7 µm thickness, samples were left on a 42°C slide warmer overnight. They were then de-waxed in xylene and rehydrated through an ethanol series into water. The samples were stained 5 minutes in each Mayer's hemotoxylin (Sigma) followed by Eosin Y (Sigma). They were dehydrated again with ethanol and xylene and finally mounted with Permount for photography.

RNA extraction and RT PCR

RNA from the whole embryos, or the 25% most posterior section (the tail) was extracted using RNAzol –B (Teltest Inc.) according to the manufacturer's instructions. Following resuspension in DEPC treated water and UV quantitation, reverse transcription was preformed using 2g total RNA. Briefly, RNA and a specific primer (the reverse primer, see below) for stromelysin-3 (*ST3*, Patterton *et al*., 1995) and another one for the control gene, the ribosomal protein L8 or rpl8 (Shi and Liang, 1994) were annealed in 10 µl at 65°C for 5 min followed by cooling down to room temperature. A mixture (10 µl) contained 5x first strand buffer (4 µl, Gibco-BRL), DTT (2 µl, 0.1 M, Gibco-BRL), dNTP mix (1 µl, 25 mM each, Pharmacia), RNAsin (0.1 µl, 10 U/µl, Gibco-BRL) and reverse transcriptase SuperScript™II (0.5 µl, 200 U/µl, Gibco-BRL) was added to the annealed RNA and primer solution. The mixture was incubated for 1 h at 42°C. Two µl of the resulting cDNA solution were used for PCR in 50 µl with 10x Ex Taq buffer (5 µl, Takara, Intergene), dNTP mix (8 µl, 2.5 mM each, Takara, Intergene), four primers (reverse and forward primers for *ST3* and rpl8, 2 µl of 2 µM each, Gibco-BRL) and Ex Taq polymerase (0.5 µl, 5 U/µl, Takara, Intergene). PCR was performed for 30 cycles consisting of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. PCR using RNA without reverse transcription were performed was done as a control for genomic DNA contamination (data not shown). PCR products (10 µl) were electrophoresed on 2% agarose gels and stained with ethidium bromide. The reverse and forward primers are : for *ST3*, 5' GTTCATCCTGGAAAGCAG 3' (1503 to 1482) and 5' CCTGATGCATGCAAAACT 3' (1038 to 1055, Patterton *et al*., 1995) ; for rpl8, 5' GACGACCAGTACGACGA 3' (749 to 732) and 5' AAAGAGAAACTGCTGGC 3' (586 to 602, Shi and Liang, 1994), respectively.

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