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Expression of *cyclin D1*, *cyclin D2*, and *N-myc* in embryos of the direct developing frog *Eleutherodactylus coqui*, with a focus on limbs

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Abstract

Species of frogs that develop directly have removed the tadpole from their ontogeny and form adult structures precociously. To see whether cell cycle regulators could be involved in this altered embryogenesis, we examined the expression of *ccnd1*, *ccnd2*, and *mycn* in embryos of the direct developing frog, *Eleutherodactylus coqui*. Notable differences compared to embryos of *Xenopus laevis*, a species with a tadpole, included prominent expression of *ccnd2* in the midbrain and *ccnd1* in the mandibular neural crest. The former may contribute to the precocious appearance of the adult-type visual system and the latter to the adult-type jaw. Large domains of *ccnd2* and *mycn* presage the early appearance of limb buds, and *ccnd1* and *mycn* are implicated in digit development.

1. Introduction

The tadpole has been removed from the life history at least ten times in the evolution of frogs (Duellman and Trueb, 1986; Hanken, 1999). This direct development from the embryo to the frog, without a feeding larva, couples incomplete or lack of formation of tadpole-specific structures, such as tadpole jaws, gills, and long coiled intestine, with precocious development of frog structures, such as large eyes, frog jaws, and limbs (Elinson and del Pino, 2012). The early formation of frog structures could be due to a larger initial allocation of embryonic cells to these structures or to increased cell division to generate them in the relatively shorter embryonic period. Both of these possibilities appear to contribute to the direct developing embryo.

The best investigated direct developing frog is *Eleutherodactylus coqui* (Callery et al., 2001; Elinson and del Pino, 2012). A casual examination of *E. coqui* early embryos shows a much larger neural plate (Fang and Elinson, 1996; Schlosser, 2003), compared to species with tadpoles. On the other hand, both the retina and the tectum in *E. coqui* have high levels of proliferation compared to species with tadpoles, as indicated by expression of proliferating cell nuclear antigen (PCNA) (Schlosser and Roth, 1997; Schlosser, 2008). Similarly, there are high levels of proliferation in the *E. coqui* early spinal cord (Schlosser, 2003). Both the

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initial large size of the spinal cord and cell division within it contribute to its rapid development.

A second noticeable feature of embryos of *E. coqui* and other direct developing frogs is the early formation and rapid development of the limbs. The initiation and growth of limbs in *E. coqui* look more like that in amniotes, such as birds and mammals, than in frog tadpoles (Elinson, 1990, 2001). In tadpoles, small limb buds form around the time that feeding begins, and they grow slowly until metamorphosis.

In order to compare better the role of cell proliferation in early development between *E. coqui* and *Xenopus laevis*, the model amphibian which has a tadpole, we have examined the expression of three cell cycle regulators in *E. coqui*: *cyclin D1* (*ccnd1*), *cyclin D2* (*ccnd2*), and *N-myc* (*mycn*). *Ccnd1* and *ccnd2* were chosen because their expression patterns are known for *X. laevis* embryos (Vernon and Philpott, 2003), and all three cell cycle regulators are dynamically expressed in chick wing development (Towers et al., 2008; Welten et al., 2011). *Mycn* was chosen because some aspects of its expression are known in *X. laevis* (Vize et al., 1990; Bellmeyer et al., 2003) and because it is expressed strongly in early limb buds of both mouse and chick (Sawai et al., '90; Kato et al., '91; Ota et al., 2007).

2. Results

2.1 Expression of cell cycle regulators in *E. coqui* embryos

We cloned *E. coqui* orthologues of three cell cycle regulators: *cyclin D1* (*ccnd1*) (Genbank JQ700063), *cyclin D2* (*ccnd2*) (Genbank JQ700064), and *N-myc* (*mycn*) (Genbank JQ700062). The parameters of our *E. coqui* clones are presented in Table 1. A dendrogram shows that the *E. coqui* orthologues of *ccnd1* and *ccnd2* fall within their respective groups (Fig. 1).

Expression in embryos from stages TS3 – TS8 was examined by in situ hybridization (Fig. 2). Each of the three genes is expressed in distinct patterns. All of them are expressed in limb buds and developing limbs, and these expression patterns will be considered in later sections. *Ccnd1* is expressed early at TS3 in the neural folds with gaps (Fig. 2A). At TS4, *ccnd1* is expressed in prospective forebrain, at the midbrain-hindbrain and hindbrain- spinal cord boundaries as well as the spinal cord (Fig. 2B, C). There is less expression in midbrain, hindbrain, and anterior spinal cord. Most notable is *ccnd1* expression in the cranial neural crest including the mandibular, hyoid, and branchial streams (Fig. 2B, C). A few days later at TS6–8, a mid-trunk gap in *ccnd1* spinal cord expression appears (Fig. 2E, F). The remaining areas of expression may reflect more active neurogenesis associated with the precocious development of the limbs.

Distinct from *ccnd1*, the most salient feature of *ccnd2* is prominent expression in mid-brain and posterior fore-brain, starting at TS4 (Fig. 2H, I) and continuing through later stages (Fig. 2J–L). At TS4–5, there is expression in the spinal cord including the anterior end (Fig. 2H, J). A mid-trunk gap of spinal cord expression is present at TS6–7 (Fig. 2K), similar to *ccnd1* expression, and spinal cord expression declines by TS8. *Mycn* is expressed strongly throughout the embryos (Fig. 2M–R), with early expression in eye (Fig. 2O), distinguishing it from *ccnd1* and *ccnd2*. Another difference between *mycn* expression and that of *ccnd1* and *ccnd2* is broader expression in the dorsal axis. That broader *mycn* pattern is due to staining of the somites as well as the neural tube (Fig. 2P).

2.2 Comparative overview of *E. coqui* limb development

The early development of the *E. coqui* limb differs from frogs with tadpoles in several significant ways. To highlight these differences, developing limbs of *E. coqui* and *X. laevis*

are aligned in Table 2 according to the major morphological divisions of bud, notch, paddle, and digits. When the *E. coqui* hindlimb bud is first visible (TS4), the diameter at its base is 0.55 mm, while the diameter of an early *X. laevis* hindlimb bud (NF48) is 0.24 mm (Table 2, Fig. 3). Approximating the buds at these stages as hemispheres, the *E. coqui* hindlimb bud is about 12x the volume of the *X. laevis* hindlimb bud. The relatively large size of the *E. coqui* hindlimb bud is emphasized further, since the *E. coqui* TS4 embryo is much smaller than the *X. laevis* NF48 tadpole (Fig. 3).

Development is very rapid in *E. coqui*, taking less than 4 days from the formation of the limb bud (TS4) to a jointed limb with digits (TS8). The comparable period in *X. laevis* is about 18 days (NF48 to NF54). In that interval, the *E. coqui* hindlimb increases in size moderately but much less in absolute terms than that of *X. laevis*. When digits form, the *E. coqui* hindlimb is 1.2 mm long, while the *X. laevis* hindlimb is 2.4 mm long (Table 2). The *X. laevis* limb length increase, however, occurs along with a much greater increase in body size, compared to *E. coqui*. Consequently, hindlimb length, normalized to snout-vent length, remains larger in *E. coqui*, when digits form (Table 2).

2.3 Expression of cell cycle regulators in developing limbs of *X. laevis* and *E. coqui*

The expression of the orthologues of *mycn*, *ccnd1*, and *ccnd2* was examined in limbs of both *X. laevis* and *E. coqui*. In *X. laevis*, small hindlimb buds are visible on either side of the proximal end of the anal canal in tadpoles that have just begun feeding (NF46). All three cell cycle regulators are expressed generally through the hindlimb bud from NF46 to NF53, with stronger expression distally for *mycn* and *ccnd1* (Fig. 4, 5). To ensure that this general expression was not an artifact of in situs on tadpoles, we examined expression of *shh*. Endo et al (1997) found local expression of *shh* along the posterior edge of the developing hindlimb, a pattern we repeated for NF51–52 (not shown).

In *E. coqui*, there is little *ccnd1* expression in the limb forming regions at TS3 (Fig. 2A), but *ccnd1* is expressed in the limb forming regions and early limb buds at TS4 (Figs. 2B, 6). *Ccnd1* is expressed throughout the TS5 limb buds (Fig. 1D, 6), and the expression becomes patterned at TS6 (Fig. 6). *Ccnd1* expression marks the forming digits at TS8 (Fig 2E, 6), and by TS10, *ccnd1* is expressed only in the digits (Fig. 6). Parallel expression changes occur in forelimbs and hindlimbs.

Ccnd2 is initially expressed at TS3 in two broad lateral domains on either side of the posterior neural folds (Fig. 2G) and then in the four limb buds at TS4 and TS5 (Fig 2H, J). As the limbs grow out, there is more *ccnd2* expression proximally and less distally, so that at TS7 and TS8, there is little autopodial expression (Figs. 2K, 7). Unlike *ccnd1*, *ccnd2* is not expressed in the digits (Fig. 7).

Like *ccnd2*, there is a broad posterior, lateral expression of *mycn* at TS3 (Figs. 2M, 8A, 8B), and this expression domain concentrates into the early hindlimb bud at TS4 (Fig. 2N). There is no comparable forelimb expression domain at TS3, but one is present at early TS4. *Mycn* is expressed in both forelimb and hindlimb buds at TS5 and TS6 (Fig. 2Q), making the buds distinct from the surrounding tissue. When the foot paddles appear at TS7, *mycn* expression is more concentrated in several regions of the stylopod and autopod (Fig. 8C). *Mycn* continues to be expressed throughout the limb including the digits as development proceeds (Fig. 2R, 8D). Parallel expression changes occur in forelimbs and hindlimbs.

3. Discussion

3.1 Cell cycle regulators and the derived life history of direct development

Direct development is a derived life history with loss of larval structures and precocious formation of adult ones. The present observations suggest an involvement of cell cycle regulators in this evolution. For example, there are high levels of cell proliferation in the retina and optic tectum of *E. coqui* embryos compared to species with tadpoles (Schlosser and Roth, 1997; Schlosser, 2008). We have detected a high level of *mycn* expression in the early retina and pronounced expression of *ccnd2* in the developing midbrain. While the eye *mycn* expression may not be different from that in *X. laevis* (Vize et al., 1990; Bellmeyer et al., 2003), *X. laevis* *ccnd2* midbrain expression was not prominent (Vernon and Philpott, 2003). Similarly, there are high levels of cell proliferation in the *E. coqui* spinal cord (Schlosser, 2003), and we observed continued high expression levels of *ccnd1* and *mycn* in spinal cord. The *ccnd1* expression in particular may be associated with innervation of the precociously developing legs.

We detected *ccnd1* expression in the streams of cranial neural crest, as delineated by Olsson et al. (2002). Expression in the mandibular stream in *E. coqui* may be significant, since *ccnd1* expression is limited to the hyoid and branchial streams in *X. laevis* (Vernon and Philpott, 2003). A major difference between *E. coqui* and species with tadpoles is the precocious formation of frog-like jaws with Meckel's and palatoquadrate cartilages arising in a mid-metamorphic state (Hanken et al., 1992; Kerney et al., 2010). These cartilages are derived from the mandibular stream (Hanken and Gross, 2005), so *ccnd1* enhancement of proliferation in the mandibular stream may have contributed to the evolution of the derived jaw development in *E. coqui*.

Finally, *E. coqui* *mycn* is expressed throughout embryogenesis. Comparisons to *X. laevis* are presently difficult due to the limited in situ analysis that has been reported (Bellmeyer et al., 2003), but our results raise the possibility of a significant involvement of *mycn* in the rapid, precocious development of *E. coqui*.

It should be noted that the expression of the three cell cycle regulators, reported here, is unrelated to the increased activity of thyroid hormone, associated with metamorphosis. Metamorphic upregulation of thyroid hormone signaling in *E. coqui* begins at TS10 (Jenkins & Hanken 1998; Callery & Elinson 2000; Elinson 2013) and in *X. laevis* at NF58. With the possible exception of *ccnd1* and *mycn* limb expressions at TS10 (Figs. 6, 8), all of our results in both *E. coqui* and *X. laevis* are before metamorphosis.

3.2 Cell cycle regulators and limb development

The early formation of large limb buds in the direct developing frog *E. coqui* contrasts with the delayed formation of small limb buds in the *X. laevis* tadpole. The most striking feature of the expression of cell cycle regulator genes in *E. coqui* is the large expression domains before limb buds are visible. Both *mycn* and *ccnd2* are expressed at TS3 in broad areas where the hindlimb buds will appear, and *mycn* is expressed in very early forelimb buds at TS4. These expression patterns indicate that the initial limb forming fields are much greater in size than those of *Xenopus*, as indicated by histological studies on *X. laevis* hindlimb (Tarin and Sturdee, 1971) and the expression of *Tbx5* in *X. tropicalis* forelimb (Abu-Daya et al., 2010).

Expression of the three cell cycle regulators occurs in the earliest limb buds in both *X. laevis* and *E. coqui*, and this expression continues to the stage of digit formation. There are some differences in limb expression patterns between genes and between *E. coqui* and *X. laevis*, but there does not appear to be heightened expression of cell cycle regulator genes in the

development of the *E. coqui* limbs. Although the limbs remain relatively larger compared to the body in *E. coqui* vs. *X. laevis*, the absolute growth of the limbs is greater in *X. laevis* (Table 1). At the time of digit formation, the length of the *E. coqui* hindlimb is about twice as big as the initial diameter of the limb bud. Conversely, the length of the *X. laevis* hindlimb is about ten times its initial limb bud diameter, and it is twice as long as the *E. coqui* hindlimb when digits form. Of course, the body size of the tadpole has also increased greatly in that developmental interval. Consequently, limbs of tadpoles of all anurans look small relative to body size when compared to other tetrapods, such as birds and mammals and to *E. coqui*.

Among tetrapods, digits form differently between amphibians and amniotes. In amniotes, digits are sculpted from the autopodial palette by apoptosis (Zuzarte-Luis and Hurlé, 2005; Montero and Hurlé, 2010). In several amphibians investigated, apoptosis has no role, and digits form by differential growth (Cameron and Fallon, 1977; Vlaskalin et al., 2004; Franssen et al., 2005). In *E. coqui*, both *ccnd1* and *mycn* are expressed early in prospective digit patterns, and expression continues in the forming digits. These regulators likely are important in anuran digit development.

3.3 Conclusions

Comparative examination of the expression of three cell cycle regulators suggests their involvement in the evolution of direct development. Examples include *ccnd1* expression in the mandibular neural crest, *ccnd2* expression in the midbrain, and large expression domains of *mycn* and *ccnd2* in the prospective limb forming areas. Localized expression of *ccnd1* and *mycn* in autopods may be the basis for the amphibian pattern of digit formation.

4. Experimental Procedures

4.1 Animals and embryos

Adult *Eleutherodactylus coqui* were collected on the Island of Hawaii under Injurious Wildlife Export permits issued by the Department of Land and Natural Resources, Hawaii. Embryos were obtained from natural matings in our reproductive colony and cultured as described previously (Beckham et al., 2003; Singamsetty and Elinson, 2010; Nath et al., 2013). Embryos were staged according to Townsend and Stewart (TS) (1985), from cleaving embryos (TS1) through hatched froglets (TS15). Embryos and tadpoles of *Xenopus laevis* were obtained by in vitro fertilization, according to standard procedures and staged according to Nieuwkoop and Faber (NF) (1994). The Duquesne University Institutional Animal Care and Use Committee (IACUC) approved the protocols for use of *E. coqui* and *X. laevis*.

4.2 Cloning *E. coqui* orthologues of cell cycle regulators

To clone *E. coqui* orthologues of the cell cycle regulators *cyclin D1* (*ccnd1*), *cyclin D2* (*ccnd2*), and *N-myc* (*mycn*), TS4 limb buds were isolated as described previously (Nath et al., 2013). RNA was extracted with Trizol (Invitrogen, Carlsbad, CA); cDNA was synthesized using M-MLV reverse transcriptase, and the cDNA was used as a template for PCR amplification with degenerate primers. Degenerate primers for *ccnd1* and *ccnd2* were designed based on sequences from *X. laevis*, *X. tropicalis*, *Homo sapiens*, and *Gallus gallus*. The sequence of the *ccnd1* forward primer was: 5' TTC AAR TGY GTS CAG AAG GA 3', and the sequence of the *ccnd1* reverse primer was: 5' TGB GCY TGT TTY CGG ATG AT 3'. The sequence of the *ccnd2* forward primer was: 5' AGT GCT CCT ACT TCA ART G 3', and the sequence of the *ccnd2* reverse primer was: 5' GCT CCY ACA CTT CCA GTT G 3'. PCR amplification produced a 415 nt fragment of *ccnd1* and a 509 nt fragment of *ccnd2*. Degenerate primers for *mycn* were designed based on sequences from *X. laevis*, *X.*

tropicalis, *Mus musculus*, and *G.gallus*. The sequence of the *mycn* forward primer was: 5' GGA RGA CAT MTG GAA GAA 3', and the sequence of the *mycn* reverse primer was: 5' TCG CTG TCC TCV GAR TC 3'. A 960 nt fragment was amplified. Complete *ccnd1* and *ccnd2* cDNAs were obtained from an *E. coqui* TS3 embryo library using exact primers in combination with plasmid primers for T7 or T3. A longer *mycn* cDNA was obtained using combinations of exact primers, based on the clone sequence, and degenerate primers.

4.3 In situ hybridization

In situ hybridization was carried out on whole *E. coqui* embryos as described previously (Elinson et al., 2008; Nath et al., 2013). Templates for digoxigenin- labeled sense and antisense probes were *E. coqui* cDNAs for *ccnd1* (0.5 kb), *ccnd2* (1 kb), and *mycn* (0.9 kb), cloned into the TEasy Vector.

Clones to make probes for *X. laevis mycn*, *ccnd1*, *ccnd2*, and *mycn* were obtained from Open Biosystems (Huntsville, AL) and sequenced to confirm their identity prior to use. These cDNAs are cloned into pCMV Sport-6 Vector, so probe templates were prepared using PCR with M13 primers. Digoxigenin-labeled antisense and sense probes were transcribed from the templates with SP6 and T7 RNA polymerases, respectively. A probe template for *X. laevis shh* in pBluescript KS+ was prepared similarly, and antisense and sense probes were transcribed with T3 and T7 RNA polymerases, respectively. Prior to using the probes of the three cell cycle regulatory genes on limb buds, we checked whether they produced patterns on embryos consistent with those in the literature. Our *ccnd1* and *ccnd2* probes replicated the results on NF 22/23 and NF33/34 embryos as reported by Vernon and Philpott (2003), and our *mycn* probe gave results consistent with the whole mount immunohistochemistry of Vize et al. (1990).

For *X. laevis* limb buds, tadpoles were anesthetized, fixed in MEMFA for 4 hr at room temperature, washed in ethanol, and stored at -20°C until use. Prior to carrying out in situ hybridization, tadpoles were individually staged, and pieces containing the limb buds were isolated by cutting NF46 to NF53 tadpoles on either side of the limb bud region. In situ hybridization included a 20 minute treatment with 1 µg/ml proteinase K.

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References

- Abu-Daya A, Nishimoto S, Fairclough L, Mohun TJ, Logan MPO, Zimmerman LB. The secreted integrin ligand *nephronectin* is necessary for forelimb formation in *Xenopus tropicalis*. *Dev Biol.* 2010; 189:246–255.
- Beckham YM, Nath K, Elinson RP. Localization of RNAs in oocytes of *Eleutherodactylus coqui*, a direct developing frog, differs from *Xenopus laevis*. *Evol Dev.* 2003; 5:562–571. [PubMed: 14984038]
- Bellmeyer A, Krase J, Lindgren J, LaBonne C. The protooncogene c-Myc is an essential regulator of neural crest formation in *Xenopus*. *Dev Cell.* 2003; 4:827–839. [PubMed: 12791268]
- Brown DD, Cai L, Das B, Marsh-Armstrong N, Schreiber AM, Juste R. Thyroid hormone controls multiple independent programs required for limb development in *Xenopus laevis* metamorphosis. *Proc Natl Acad Sci USA.* 2005; 102:12455–12458. [PubMed: 16129821]
- Callery EM, Elinson RP. Thyroid hormone-dependent metamorphosis in a direct developing frog. *Proc Natl Acad Sci USA.* 2000; 97:2615–2620. [PubMed: 10706622]

- Callery EM, Fang H, Elinson RP. Frogs without polliwogs: evolution of anuran direct development. *BioEssays*. 2001; 23:233–241. [PubMed: 11223880]
- Cameron J, Fallon JF. The absence of cell death during development of free digits in amphibians. *Dev Biol*. 1977; 55:331–338. [PubMed: 838123]
- Duellman, WE.; Trueb, L. *Biology of Amphibians*. McGraw-Hill Book Co; New York: 1986.
- Elinson RP. Direct development in frogs: Wiping the recapitulationist slate clean. *Sem Dev Biol*. 1990; 1:263–270.
- Elinson RP. Direct development: an alternative way to make a frog. *genesis*. 2001; 29:91–95. [PubMed: 11170349]
- Elinson RP. Metamorphosis in a frog that does not have a tadpole. *Curr Top Dev Biol*. 2013; 103:259–276. [PubMed: 23347522]
- Elinson RP, Fang H. Secondary coverage of the yolk sac by the body wall in the direct developing frog, *Eleutherodactylus coqui*: an unusual process for amphibian embryos. *Dev Genes Evol*. 1998; 208:457–466. [PubMed: 9799426]
- Elinson RP, del Pino EM. Developmental diversity of amphibians. *WIREs Dev Biol*. 2012; 1:345–369.10.1002/wdev.23
- Elinson RP, Walton Z, Nath K. *Raldh* expression in embryos of the direct developing frog *Eleutherodactylus coqui* and the conserved retinoic acid requirement for forelimb initiation. *J Exp Zool (Mol Dev Evol)*. 2008; 310B:588–595.
- Endo T, Yokoyama H, Tamura K, Ide H. Shh expression in developing and regenerating limb buds of *Xenopus laevis*. *Dev Dyn*. 1997; 209:227–232. [PubMed: 9186057]
- Fang H, Elinson RP. Patterns of distal-less gene expression and inductive interactions in the head of the direct developing frog *Eleutherodactylus coqui*. *Dev Biol*. 1996; 179:160–72. [PubMed: 8873761]
- Franssen RA, Marks S, Wake D, Shubin N. Limb chondrogenesis of the seepage salamander, *Desmognathus aeneus* (Amphibia: Plethodontidae). *J Morph*. 2005; 265:87–101. [PubMed: 15880507]
- Hanken, J. Larvae in amphibian development and evolution. In: Hall, BK.; Wake, MH., editors. *The origin and evolution of larval forms*. Academic Press; San Diego: 1999. p. 61-108.
- Hanken J, Gross JB. Evolution of cranial development and the role of neural crest: insights from amphibians. *J Anat*. 2005; 207:437–446. [PubMed: 16313386]
- Hanken J, Klmkowsky MW, Summers CH, Seufert DW, Ingebrigtsen N. Cranial ontogeny in the direct-developing frog, *Eleutherodactylus coqui* (Anura: Leptodactylidae), analyzed using whole-mount immunocytochemistry. *J Morph*. 1992; 211:95–118. [PubMed: 1371162]
- Hanken J, Carl TF, Richardson MK, Olsson L, Schlosser G, Osabutey CK, Klymkowsky MW. Limb development in a “nonmodel” vertebrate, the direct-developing frog *Eleutherodactylus coqui*. *J Exp Zool (Mol Dev Evol)*. 2001; 291:375–388.
- Jennings DH, Hanken J. Mechanistic basis of life history evolution in anuran amphibians: thyroid gland development in the direct-developing frog, *Eleutherodactylus coqui*. *Gen Comp Endo*. 1998; 111:225–232.
- Kato K, Kanamori A, Wakamatsu Y, Sawai S, Kondoh H. Tissue distribution of N-myc expression in the early organogenesis period of the mouse embryo. *Dev Growth Diff*. 1991; 33:29–36.
- Kerney R, Hanken J. Gene expression reveals unique skeletal patterning in the limb of the direct-developing frog, *Eleutherodactylus coqui*. *Evol Dev*. 2008; 10:439–448. [PubMed: 18638321]
- Kerney R, Gross JB, Hanken J. Early cranial patterning in the direct-developing frog *Eleutherodactylus coqui* revealed through gene expression. *Evol Dev*. 2010; 12:373–382. [PubMed: 20618433]
- Martin BL, Harland RM. A novel role for *lhx1* in *Xenopus* hypaxial myogenesis. *Development*. 2006; 133:195–208. [PubMed: 16339190]
- Montero JA, Hurlé JM. Sculpturing digit shape by cell death. *Apoptosis*. 2010; 15:365–375. [PubMed: 20041300]
- Muntz L. Myogenesis in the trunk and leg during development of the tadpole of *Xenopus laevis* (Daudin 1802). *J Embryol Exp Morph*. 1975; 33:757–774. [PubMed: 1176869]

- Nath K, Fisher C, Elinson RP. Expression of a cardiac myosin gene in non-heart tissues of developing frogs. *Dev Genes Evol.* 2013;1007/s00427-012-0421-9
- Nieuwkoop, PD.; Faber, J. Normal Table of *Xenopus laevis* (Daudin). Garland Publishing; New York: 1994.
- Olsson L, Moury JD, Carl TF, Håstad O, Hanken J. Cranial neural crest-cell migration in the direct-developing frog, *Eleutherodactylus coqui*: molecular heterogeneity within and among migratory streams. *Zoology.* 2002; 105:3–13. [PubMed: 16351851]
- Ota S, Zhou ZQ, Keene DR, Knoepfler P, Hurlin PJ. Activities of N-Myc in the developing limb link control of skeletal size with digit separation. *Development.* 2007; 134:1583–1592. [PubMed: 17360777]
- Sabo MC, Nath K, Elinson RP. *Lbx1* expression and frog limb development. *Dev Genes Evol.* 2009; 219:609–612. [PubMed: 20091319]
- Satoh A, Sakamaki K, Ide H, Tamura K. Characteristics of initiation and early events for muscle development in the *Xenopus* limb bud. *Dev Dyn.* 2005; 234:846–857. [PubMed: 16245333]
- Sawai S, Kato K, Wakamatsu Y, Kondoh H. Organization and expression of the chicken N-*myc* gene. *Mol Cell Biol.* 1990; 10:2017–2026. [PubMed: 2183017]
- Schlosser G. Mosaic evolution of neural development in anurans: acceleration of spinal cord development in the direct developing frog *Eleutherodactylus coqui*. *Anat Embryol.* 2003; 206:215–227. [PubMed: 12592573]
- Schlosser G. Development of the retinotectal system in the direct-developing frog, *Eleutherodactylus coqui* in comparison with other anurans. *Front Zool.* 2008; 5:910.1186/1742-9994-5-9
- Schlosser G, Roth G. Development of the retina is altered in the directly developing frog *Eleutherodactylus coqui* (Leptodactylidae). *Neurosci Lett.* 1997; 224:153–156. [PubMed: 9131659]
- Singamsetty S, Elinson RP. Novel regulation of yolk utilization by thyroid hormone in embryos of the direct developing frog *Eleutherodactylus coqui*. *Evol Dev.* 2010; 12:437–448. [PubMed: 20883213]
- Tarin D, Sturdee AP. Early limb development of *Xenopus laevis*. *J Embryol Exp Morph.* 1971; 26:169–179. [PubMed: 5157347]
- Towers M, Mahood R, Yin Y, Tickle C. Integration of growth and specification in chick wing digit-patterning. *Nature.* 2008; 452:882–886. [PubMed: 18354396]
- Townsend DS, Stewart MM. Direct development in *Eleutherodactylus coqui* (Anura: Leptodactylidae); a staging table. *Copeia.* 1985; 1985:423–436.
- Vernon AE, Philpott A. The developmental expression of cell cycle regulators in *Xenopus laevis*. *Gene Exp Patt.* 2003; 3:179–192.
- Vize PD, Vaughan A, Krieg P. Expression of the N-*myc* proto-oncogene during the early development of *Xenopus laevis*. *Development.* 1990; 110:885–896. [PubMed: 2088727]
- Vlaskalin T, Wong CJ, Tsilifidis C. Growth and apoptosis during larval forelimb development and adult forelimb regeneration in the newt (*Notophthalmus viridescens*). *Dev Genes Evol.* 2004; 214:423–431. [PubMed: 15322877]
- Welten M, Pavlovska G, Chen Y, Teruoka Y, Fisher M, Bangs F, Towers M, Tickle C. 3D expression patterns of cell cycle genes in the developing chick wing and comparison with expression patterns of genes implicated in digit specification. *Dev Dyn.* 2011; 240:1278–1288. [PubMed: 21509900]
- Zuzarte-Luis V, Hurlé JM. Programmed cell death in the embryonic vertebrate limb. *Sem Cell Dev Biol.* 2005; 16:261–269.

- *Ccnd1* is expressed in the mandibular neural crest of *E. coqui* but not *X. laevis*, suggesting involvement in the early formation of the adult-type jaw.
- There is prominent expression of *ccnd2* in the midbrain, suggesting involvement in the early formation of the adult-type visual system.
- Expression domains of *ccnd2* and *mycn* in *E. coqui* define early, large limb fields, not seen in *X. laevis*.
- Expression of *ccnd1* and *mycn* in autopods support digit formation in amphibians by differential growth rather than by interdigital apoptosis, characteristic of amniotes.

Figure 1. Dendrogram of *ccnd1* and *ccnd2* genes

The *E. coqui* orthologues cluster with their respective groups.

Figure 2. Expression of *ccnd1*, *ccnd2*, and *mycn* in *E. coqui* embryos

Embryos are oriented with their anterior end at the top of each picture. **(A – F) Expression of *ccnd1*.** **(A)** At TS3, there is *ccnd1* expression along the neural folds. The right side of the prospective midbrain is marked by a white asterisk (*) in this and subsequent pictures. **(B)** At TS 4, there is expression near the midbrain-hindbrain and the hindbrain-spinal cord boundaries, and in the spinal cord. Notable is expression in the streams of cranial neural crest. **(C)** An enlarged, lateral view of the head of a TS4 embryo shows expression in the mandibular stream (m), the hyoid stream (h), and the branchial streams (b) of the cranial neural crest. **(D)** The limb buds are stained prominently at TS5. **(E)** There are distinct patterns of expression in both the limbs and spinal cord at TS8. **(F)** Embryos at TS6 are compared in *in situs* with a control sense (s) and an antisense (as) probe. Little or no stain is

present with the sense probe, and similar results occurred for all stages examined. **(G – L) Expression of *ccnd2*.** **(G)** Embryos at TS3 are compared with an antisense (as) and control sense (s) probe. Little or no stain is present with the sense probe with this and other stages examined. There is broad, diffuse expression of *ccnd2* lateral to the posterior neural folds, and this expression presages formation of hindlimb buds. The damage at the periphery of both embryos is difficult to avoid, since the thin tissues overlying the large yolk mass tear easily. **(H)** There are several sites of expression at TS4, including the midbrain. **(I)** An enlarged view of the head of a TS4 embryo shows the start of midbrain expression. As for the *ccnd1* pictures, the right side of the prospective midbrain is marked by a white asterisk (*) in this and subsequent pictures. **(J)** Midbrain expression intensifies at TS5, along with expression in the spinal cord and limb buds. **(K)** Midbrain expression remains prominent at TS7, an enlargement of which is shown in **(L)**. **(M – R) Expression of *mycn*.** **(M)** At TS3, *mycn* is expressed in the head and neural folds, with broad, diffuse expression lateral to the posterior neural folds as seen also for *ccnd2* **(G)**. **(N)** *Mycn* is expressed throughout the TS4 embryo, including the limb buds. **(O)** This enlarged, lateral view of the head at TS4 shows prominent staining of the eye (e). **(P)** The broad staining of the dorsal axis of the TS4 embryos is due to expression in both the neural tube (n) and the somites (s). To obtain this view, a piece of the dorsal axis was dissected from a stained embryo and splayed out. Two somites are present on either side of the neural tube. *Mycn* is expressed throughout the embryo at TS6 **(Q)** and TS8 **(R)**. As with *ccnd1* and *ccnd2*, there was little or no staining with the *mycn* sense probe (not shown). Live embryos at these stages are ~3.5 – 4 mm in diameter.

Figure 3. Limb buds in *X. laevis* and *E. coqui*

(**A**, **B**) *X. laevis* tadpole (NF48) with small hindlimb bud (white arrow). (**C**, **D**) *E. coqui* embryos (TS4) with large fore- and hindlimb buds. To indicate size differences in limb buds between *X. laevis* and *E. coqui*, (**A**) and (**C**) are at the same magnification, and (**B**) and (**D**) are at the same enlargement.

Figure 4. Expression of *ccnd1* and *ccnd2* in *X. laevis* developing hindlimbs
Numbers indicate NF stages, with *in situs* of *ccnd1* (top) and *ccnd2* (bottom).

Figure 5. Expression of *mycn* in *X. laevis* developing hindlimbs

Numbers indicate NF stages from the initial hindlimb bud (NF46) to the paddle stage (NF53). There was little or no staining with a control sense probe at any stage.

Figure 6. Expression of *ccnd1* in *E. coqui* developing hindlimbs

The panels are labeled with their TS stage. For TS4, 5, 6, and 8, the left hindlimb is seen in dorsal view with anterior at the top (same as Fig. 2). The TS10 panel is a lateral view of the embryo's right side with anterior at the right of the panel.

Figure 7. Expression of *ccnd2* in *E. coqui* developing hindlimbs

The panels are labeled with their TS stage and with hindlimb (HL) or forelimb (FL). For all panels, the right limb is seen in dorsal view with anterior at the top (same as Fig. 2). There is less distal and autopodial expression with development.

Figure 8. Expression of *mycn* in *E. coqui* developing hindlimbs

(A) and (B) As the neural folds meet (TS3), there is *mycn* expression on either side of the posterior end of the forming neural tube. (C) Although there is *mycn* expression throughout the limb (Fig. 2), clearing of this TS7 embryo reveals internal, local patterns of expression in the proximal stylopod and the distal autopod. (D) *Mycn* is expressed throughout the TS10 limb, including the digits.

Table 1

Characteristics of *E. coqui* cell cycle regulator clones.

	mycn	ccnd1	ccnd2
Length (nt)	1223	1128	1056
Length (aa)	357	291	291
Amount ORF	~80% from Start	all	all
nt identity to <i>X. laevis</i>	72%	81%	84%
aa identity to <i>X. laevis</i>	71%	88%	91%

Table 2Morphological comparison of limb development between *E. coqui* and *X. laevis*.

BUD				NOTCH		PADDLE	DIGITS	
<i>Eleutherodactylus coqui</i>								
TS STAGE	4	5		6	7	8		9
Age (days) (22–24C)	4	4.5		5	6.5	7.5		8
Length (mm) [diameter]	[0.55]	0.7		1	1.1	1.2		1.5
Hindlimb/ Snout-vent	-	0.2		0.29	0.3	0.32		0.35
Tissues	muscle precursors ^a					cartilage ^b		muscle ^c
<i>Xenopus laevis</i>								
NF STAGE	48	49	50	51	52	53	54	
Age (days) (22–24C)	7.5	12	15	17	21	24	26	
Length (mm) [diameter]	[0.24]	0.25	0.43	0.8	1.3	1.6	2.4	
Hindlimb/ Snout-vent	-	0.029	0.054	0.082	0.11	0.12	0.15	
Tissues				muscle precursors ^d		muscle ^e cartilage ^f TH required ^g		

^aDetection of *Lbx1* expressing cells in limb (Sabo et al., 2009).^bHanken et al. (2001), Kerney and Hanken (2008).^cElinson and Fang (1998).^dEarliest stage of detection of muscle precursor cells in limb; no experimental results for earlier stages (Satoh et al., 2005). *Lbx1* expressing cells present in limb buds at NF53, but earlier stages not reported (Martin and Harland, 2006).^eDifferentiated muscle (Muntz, 1975; Brown et al., 2005; Satoh et al., 2005).^fNieuwkoop and Faber (1994).^gThyroid hormone (TH) required to develop beyond NF52 (Brown et al., 2005). In contrast, formation of both limb cartilage and muscle in the absence of thyroid hormone synthesis in the *E. coqui* embryo (Callery and Elinson, 2000).