

# Homology and Evolutionary Novelty in the Deployment of Extracellular Matrix Molecules During Pigment Pattern Formation in the Salamanders *Taricha torosa* and *T. rivularis* (Salamandridae)

DAVID M. PARICHY\*

Section of Evolution and Ecology and Center for Population Biology,  
University of California at Davis, Davis, California 95616

**ABSTRACT** Salamander larvae exhibit a diverse array of pigment patterns shortly after hatching. Previous studies have identified roles for the extracellular matrix and lateral line sensory system in promoting the development of a phylogenetically common pattern of horizontal melanophore stripes. In contrast, salamanders in the genus *Taricha* exhibit evolutionarily derived pigment patterns and pattern-forming mechanisms. *Taricha torosa* larvae exhibit compact melanophore stripes that develop via redundant, lateral line-independent mechanisms, whereas *T. rivularis* larvae lack stripes and instead have melanophores uniformly distributed over the flank. In this study, I test roles for candidate patterning molecules of the extracellular matrix in promoting the development of species-specific pigment patterns in *Taricha*. I show that tenascin deposition is negatively correlated with melanophore distributions both intraspecifically and interspecifically: this matrix molecule is present where melanophores do not localize in *T. torosa* and is absent from these same regions where melanophores are abundant in *T. rivularis*. Embryological manipulations further indicate that transient expression of tenascin in a prospective interstripe region of *T. torosa* reflects a phylogenetically conserved effect of lateral line development. Finally, anti-laminin immunoreactivity is negatively correlated with melanophore distributions in *T. torosa*, and this species exhibits a general retardation of extracellular matrix development that may allow persistent, evolutionarily novel melanophore motility in this species. Together these findings identify tenascin and laminin, or molecules co-regulated with these matrix components, as candidates for promoting early larval pigment pattern development in *Taricha*. *J. Exp. Zool. (Mol. Dev. Evol.)* 291:13–24, 2001. © 2001 Wiley-Liss, Inc.

Neural crest cells contribute to a wide range of derivatives in vertebrates, including the skull, teeth, peripheral nervous system, and externally visible pigment patterns (Gans and Northcutt, '83; Reedy et al., '98; Groves and Bronner-Fraser, '99; Hall, '99). A deeper understanding of vertebrate evolution thus requires a knowledge of how phylogenetic changes in the patterning of neural crest cells and their derivatives has been achieved. One approach to answering this question is to examine developmental mechanisms underlying traits that differ between closely related species. Because of their diversity and accessibility to experimental manipulation, pigment patterns of salamanders provide an opportunity to investigate the cellular and molecular bases for evolutionary changes in an ecologically important neural crest-derivative (Parichy, '96a).

The genus *Taricha* (family Salamandridae) includes three species with markedly different larval pigment patterns (Twitty and Bodenstern, '39; Twitty, '42, '45; Fig. 1). At hatching stages, *Taricha granulosa* exhibit a horizontal stripe pattern comprising neural crest-derived melanophores along the dorsal myotomes and covering the dorsal yolk mass, and a melanophore-free region over the lateral myotomes. *Taricha torosa* also have horizontal stripes, but these stripes are more compact than in *T. granulosa*, and melanophores are not present either over the yolk or in the fin. Finally,

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\*Correspondence to: David M. Parichy, Institute for Cellular and Molecular Biology, University of Texas at Austin, Austin, TX 78712. E-mail: dparichy@mail.utexas.edu

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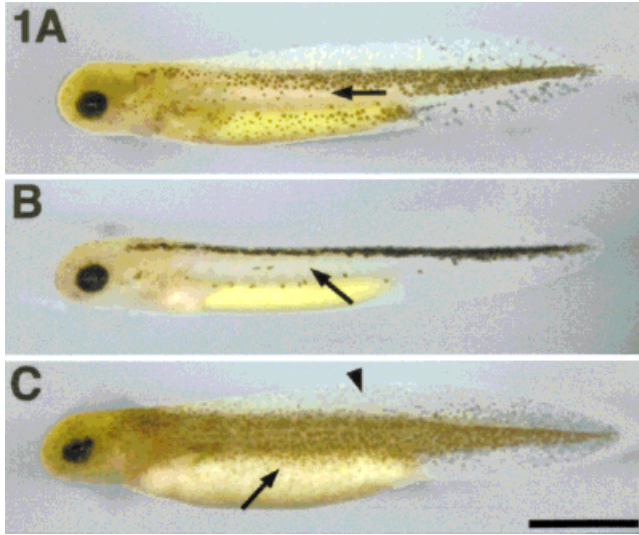


Fig. 1. *Taricha* larvae exhibit diverse early larval pigment patterns. (A) *T. granulosa* exhibit a pattern of horizontal stripes that depends on interactions between melanophores and the lateral line sensory system (see text; Parichy, '96a). Arrow indicates the level of the midbody lateral line. (B) *T. torosa* exhibit horizontal stripes in which melanophores are more densely packed along the dorsal apex of the myotome. In contrast to *T. granulosa* and other species, a melanophore-free "interstripe region" (arrow) develops independently of interactions between melanophores and the lateral lines. *T. torosa* also lack melanophores in the dorsal fin and over the yolk, though a ventral melanophore stripe comprising a few melanophores develops at the junction of the myotome and yolk mass. (C) *T. rivularis* lack stripes, and in contrast to *T. torosa*, melanophores are present both within the dorsal fin (arrowhead) and over the yolk (arrow). Scale bars: 1.5 mm (A); 2 mm (B,C).

*Taricha rivularis* lack stripes: melanophores are distributed nearly uniformly over the myotomes, and are present both over the yolk and in the fin. Phylogenetic and developmental analyses indicate that horizontal stripes similar to those of *T. granulosa* are ancestral, whereas the more organized stripes in *T. torosa*, and the absence of stripes in *T. rivularis* each are evolutionarily derived (Fig. 2). Correlated with the presence and absence of stripes are the breeding habits of these species. Whereas *T. granulosa* and *T. torosa* each breed in ponds or slow moving parts of streams, *T. rivularis* breed in rapidly moving mountain brooks (Twitty, '42; Bishop, '62). *Taricha rivularis* also exhibit other traits characteristic of stream-dwelling salamander larvae, including a smaller dorsal fin, absence of balancers, and development from a large egg. Although similar stream-type morphologies have arisen independently in other species (Duel-

man and Trueb, '86), the mechanistic bases for these transformations remain unknown.

Previous studies have focused on roles for the extracellular matrix and lateral line sensory system in determining melanophore distributions. These analyses suggest a model for how melanophores are organized into stripes in most species and identify mechanisms that may have changed during the evolutionary elaboration of stripes in *T. torosa*, and the evolutionary loss of stripes in *T. rivularis*. In all taxa studied to date, stripes form principally due to factors that influence melanophore movements (rather than the pattern of differentiation), and in the three species that have been examined in greatest detail, these melanophore movements correlate with the expression of molecules within the extracellular matrix (reviewed in Epperlein et al., '96; Parichy, '96c). For example, tenascin is a non-adhesive substratum for melanophores, melanocytes, and other neural crest-derived cells (Mackie et al., '88; Chiquet-Ehrismann, '88, '95; Le Poole et al., '97; Parichy, unpublished data), and in *Triturus alpestris* and *Ambystoma tigrinum tigrinum* this matrix molecule is deposited in the middle of the flank as melanophores evacuate this area, and is also abundant in the fin where melanophores are not found (Epperlein and Löfberg, '90; Parichy, '96c). Similarly, chondroitin sulfate proteoglycans are unfavorable for neural crest cell and melanophore adhesion, and these molecules increase in abundance in the prospective interstripe region in *Tr. alpestris* and are present in the fin of *T. torosa* (Tucker and Erickson, '84, '86b; Epperlein and Löfberg, '90; Perris and Johansson, '90; Perris et al., '96). Moreover, hyaluronan inhibits the differentiation of melanophores, is non-adhesive for neural crest cells, and is present in the fin of *T. torosa* (Tucker and Erickson, '84, '86b). Finally, fibronectin is an adhesive substratum for melanophores and other neural crest-derived cells (Boucalt et al., '84; Rogers et al., '90a) and in *T. torosa*, fibronectin is reported to be more abundant in prospective stripe regions (Tucker and Erickson, '86a,b). Thus, candidate patterning molecules have been identified for excluding melanophores from the middle of the flank and the fin (tenascin, hyaluronan, chondroitin sulfate proteoglycans) or for arresting melanophores at sites of stripe formation (fibronectin).

Additional studies have identified cellular interactions required for stripe development. In most salamanders, the lateral line sensory system is essential for normal stripe formation, as

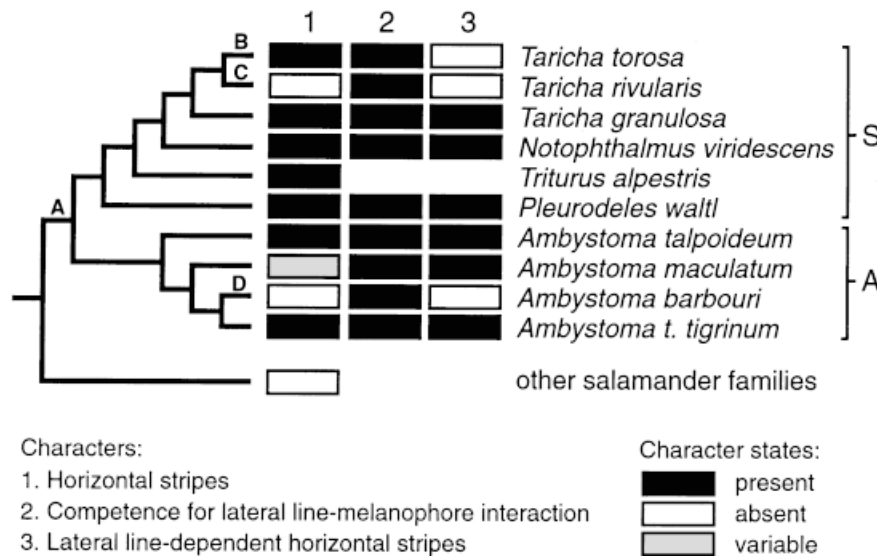


Fig. 2. Phylogenetic relationships and pattern-forming mechanisms of salamanders in families Ambystomatidae and Salamandridae. Shown is an abbreviated phylogeny including ten species in which early larval pigment pattern development has been studied (Ambystomatidae and Salamandridae together comprise ~85 species). Character states are as follows: black = present; white = absent; gray = variable. Character 1 is an early larval pigment pattern comprising horizontal stripes. Such stripes are common in both ambystomatids and salamandrids, though not in other salamander families, and are inferred to be ancestral for Ambystomatidae (A) and Salamandridae (S). In contrast, stripes have been lost independently in *T. rivularis* and *A. barbouri*. Character 2 is competence of the lateral lines to influence melanophore distributions as evidenced by experimental manipulations: the lateral lines have the potential to influence melanophore localization in all species examined, and lateral line-dependent pattern-forming mechanisms are inferred to have been present

in the common ancestor of Ambystomatidae and Salamandridae (A in cladogram; Parichy, '96a). Character 3 is a lateral line-dependence of the early larval pigment pattern. Although the potential for melanophore–lateral line interactions is present in all species tested, the magnitude of these effects differ among taxa. In *T. torosa*, the lateral lines are not essential for stripe development, whereas in *T. rivularis* and *A. barbouri*, the lateral lines have only subtle effects on melanophore distributions (as a striped pattern never develops normally). Taxa in which lateral line-dependent patterns are absent thus are inferred to exhibit evolutionarily derived patterning mechanisms (B, C, D in cladogram) that promote either stripes (*T. torosa*) or uniform patterns (*T. rivularis*, *A. barbouri*). Potential effects of the lateral lines on melanophore distributions have not been tested in *Tr. alpestris* or salamanders of other families. For phylogenetic relationships see: Shaffer et al., '91; Shaffer, '93; Larson and Dimmick, '93; Titus and Larson, '95; Jones et al, '93.

prevention of lateral line development also perturbs the striped pattern (Parichy, '96b; Fig. 2). The lateral lines arise from cranial ectodermal placodes that generate primordia that migrate caudally along the inner epidermis. As the primordia migrate, melanophores in their path recede dorsally and ventrally to establish a striped pattern, and during later stages, the lateral line nerve can act as a barrier to prevent melanophores from returning to the middle of the flank. The appearance of tenascin in the middle of the flank is correlated with lateral line development in *Tr. alpestris* (Figs. 21–24 in Epperlein and Löfberg, '90) and *A. t. tigrinum*, and prevention of lateral line development reduces the local abundance of tenascin in the latter species (Parichy, '96c). Thus, lateral line-dependent tenascin deposition may

contribute to excluding melanophores from the prospective interstripe region.

In contrast to most species, prevention of lateral line development does not perturb the striped pattern of *T. torosa* (Fig. 2). Rather, embryological grafting experiments suggest that novel and redundant, lateral line-independent mechanisms have evolved in this species, whereas the ancestral lateral line-dependent mechanisms have been retained in a latent form (Parichy, '96b). Nevertheless, the derived stripe-forming mechanisms in *T. torosa*, and the derived mechanisms underlying stripe loss in *T. rivularis*, have yet to be identified.

In this study, I test potential roles for extracellular matrix molecules in the evolutionary modification, and elimination, of stripes in *Taricha*. I show that most previously identified candidate

molecules are not expressed in a manner consistent with roles in determining species-specific pigment patterns. In contrast, the distribution of tenascin is negatively correlated with the distribution of melanophores: in *T. torosa*, tenascin is present in regions where melanophores are not found; in *T. rivularis*, tenascin is absent from these same regions, where melanophores are abundant. I then show that the cellular basis for tenascin deposition supports a model in which ancestral lateral line-dependent stripe-forming mechanisms have been retained in *T. torosa*, and I exclude tenascin as a candidate molecule contributing to these derived mechanisms. Finally, I show that another candidate, laminin, also is negatively correlated with melanophore distributions in *T. torosa*. Together, these findings provide insights into roles for the extracellular matrix in stripe development in *T. torosa*, and how changes in the expression of extracellular matrix molecules or their receptors may have contributed to stripe loss in *T. rivularis*.

## MATERIALS AND METHODS

### *Embryos, culture conditions, and staging*

*T. torosa* and *T. rivularis* embryos were collected in the field during the breeding season (February–April) from populations in Napa or Sonoma counties, California. As *T. granulosa* eggs are laid individually and could not be collected in large numbers, *A. t. tigrinum* and *Pleurodeles waltl* embryos were examined as representative taxa exhibiting lateral line-dependent stripes (Fig. 2; Parichy, '96b). *Ambystoma t. tigrinum* were obtained from the Charles Sullivan Co., Inc. (Nashville, TN), and *P. waltl* were a gift from D. Glahn. Embryos were maintained in plastic dishes containing 20% HEPES buffered Steinberg's solution (HSS; plus 37.5 U/ml penicillin, 75  $\mu$ /ml streptomycin; Asashima et al., '89; 6–16°C, 12L:12D). Staging followed Twitty and Bodenstern ('62).

### *Immunohistochemistry and histochemistry*

Antibody staining and lectin immunohistochemistry were used to examine the distribution of extracellular matrix molecules during pigment pattern formation. To maximize the retention of labile extracellular matrix components, embryos were fixed by freeze substitution (Robards and Sleytr, '85; Parichy, '96c). Embryos were cut transversely then frozen in isopentane over liquid N<sub>2</sub>, at which time they were transferred to methanol chilled over liquid N<sub>2</sub>, and placed at –80°C. Embryos were thawed over several days, and were

then cleared in xylene, embedded in Paraplast X-tra, and sectioned at 12  $\mu$ m. For each embryo, 10 sections from the anterior, middle, and posterior trunk were mounted on slides, and for each extracellular matrix molecule multiple embryos at a range of stages for each species were stained simultaneously. Slides were deparaffinized in xylene, rehydrated through ethanol, rinsed in phosphate-buffered saline (PBS), and blocked in 0.1% bovine serum albumin/1% Carnation fat-free powdered milk/PBS. Sections were incubated with primary antibodies (18 hr, 4°C), washed in PBS, and then incubated in the appropriate fluorescent secondary antibody (Cappel; 1.5 hr, room temperature). Sections were then washed and coverslipped with antifade mounting medium. For each extracellular matrix component, sections were photographed with Kodak Tri-X film using identical exposures and comparisons were made only within rounds of staining. For each molecule, at least 10 embryos were examined of each stage and species.

Monoclonal antibodies were used against chondroitin-4 and -6 sulfate (CS-56; Avnur and Geiger, '84; Sigma) and chondroitinase-treated chondroitin sulfate proteoglycans ( $\Delta$ Di-6S; Couchman et al., '84; ICN); for the latter, sections were pretreated with 0.2 U/ml chondroitinase ABC (Calbiochem) for 1 hr at 37°C; extensive digestion abolished staining with CS-56. A polyclonal antiserum against *Xenopus laevis* tenascin (Riou et al., '91) was provided by J.F. Riou, and monoclonal antibody M1 against chicken tenascin-C (Chiquet and Fambrough, '84) was provided by R.P. Tucker; both antibodies yielded identical staining patterns. Specificity of anti-tenascin antibodies was confirmed by immunoblot (data not shown). A polyclonal antiserum against *Ambystoma mexicanum* fibronectin was provided by T. Darribère, and polyclonal antisera against mouse laminin, type I collagen, and type IV collagen were provided by H.K. Kleinman. A polyclonal antiserum against bovine vitronectin was obtained from Chemicon, and monoclonal antibody II-II6B3 against chicken type II collagen was obtained from the developmental studies hybridoma bank. B. Caterson provided monoclonal antibodies 2D3 and 8C2 against keratan sulfate proteoglycans, and S. Hoffman provided a polyclonal antiserum against chicken phosphacan. With the exception of CS-56 and  $\Delta$ Di-6S, enzymatic predigestion of tissues with collagenase, chondroitinase, or hyaluronidase prior to antibody incubation did not result in qualitatively different staining patterns. The distribution of hyaluronan was examined using a biotinylated fragment of hyaluronan

binding protein (supplied by R. Stern) and FITC-conjugated Neutralite avidin (Molecular Probes). Finally, peanut agglutinin (PNA) was used to assess the distribution of PNA-binding carbohydrates as described (Parichy, '96c).

### ***Embryological manipulations***

Microsurgical procedures followed Parichy ('96b,c). Embryos were rinsed in sterile 20% HSS, passed through several changes of 100% HSS (plus 75 IU/ml penicillin, 75 µg/ml streptomycin) and manipulations were performed in sterile agar-lined dishes containing 100% HSS. Operations were performed with tungsten needles, and grafted tissues were held briefly in place using fragments of glass coverslips. To prevent lateral line development, lateral line placode ectoderm was removed at stages 24–33 and replaced with belly epidermis from a donor embryo. To ablate trunk neural crest, neural folds containing prospective neural crest cells were removed bilaterally at stages 15–16.

## **RESULTS**

### ***Distributions of most candidate patterning molecules do not correlate with species-specific pigment patterns***

I used immunohistochemistry and histochemistry to test potential roles for extracellular matrix molecules in determining species-specific pigment patterns in *Taricha*. With the exceptions of tenascin and laminin (below), no correlations were found between the distributions of melanophores and candidate patterning molecules, including chondroitin sulfate proteoglycans, collagens types I, II, and IV, fibronectin, hyaluronan, keratan sulfate proteoglycans, and molecules that bind peanut agglutinin (Fig. 3; for reviews see: Erickson and Perris, '93; Parichy, '96a; Henderson and Copp, '97; Reedy et al., '98). Most of these candidates are thus unlikely to have roles in the evolutionary elaboration or loss of stripes in *Taricha*.

### ***Distribution of tenascin correlates with pigment pattern differences between *T. torosa* and *T. rivularis****

In *T. torosa* during early stages of neural crest cell migration (stages ≤ 33), anti-tenascin immunoreactivity was not detectable beneath the epidermis but was present medially between the neural tube or notochord and the somites, as in *A. t. tigrinum*, *A. mexicanum*, and *X. laevis* (Mackie et al., '88; Parichy, '96c). Subsequently, tenascin staining appeared in the subepidermal

basement membrane of the fin and over the yolk, and appeared transiently in the middle of the flank as stripes formed (stages 35–37; Fig. 4A–D). During later development when stripes become increasingly compact (stages > 37), subepidermal tenascin staining was not detectable over the myotomes (Fig. 4A), but was maintained in the fin and over the yolk, in contrast to a wider distribution of this matrix molecule within the subepidermal basement membrane of *A. t. tigrinum* (Fig. 4H) and *P. waltil* (data not shown).

In *T. rivularis*, tenascin staining was never observed in the subepidermal basement membrane of the fin or over the yolk (Fig. 4E–G), though very weak staining appeared transiently in the middle of the flank between stages 35–37 (data not shown); this staining correlates with a region of slightly diminished melanophore density in the immediate vicinity of the developing midbody lateral line (though this difference in melanophore density is not marked enough to result in a distinctive melanophore-free region or horizontal stripe pattern, presumably due to population pressure exerted by the much greater total number of melanophores in this species; see: Parichy, '96b). The different pattern of tenascin staining in *T. torosa* and *T. rivularis* is unlikely to reflect differential cross-reactivity of antibodies as medial tenascin staining, between the notochord and somites, was equally intense between species. Thus, the distribution of tenascin is negatively correlated with the distributions of melanophores: in *T. torosa* tenascin is present in regions that lack melanophores; in *T. rivularis* tenascin is absent from these same regions, where melanophores are found.

### ***Lateral line-dependent and fin-dependent tenascin deposition***

When the lateral lines are ablated in *A. t. tigrinum*, levels of tenascin are reduced in the middle of the flank and stripes do not form, consistent with a model in which lateral line-dependent tenascin deposition serves as a cue for melanophore localization during stripe development. In contrast, novel lateral line-independent cues were inferred to promote the development of stripes in *T. torosa* (Parichy, '96b,c). Together, these findings suggest two alternative hypotheses for the cellular bases of tenascin deposition in the prospective interstripe region in *T. torosa*: (i) tenascin expression could reflect an ancestral lateral line effect on the extracellular matrix; or (ii) tenascin expression could be independent of the

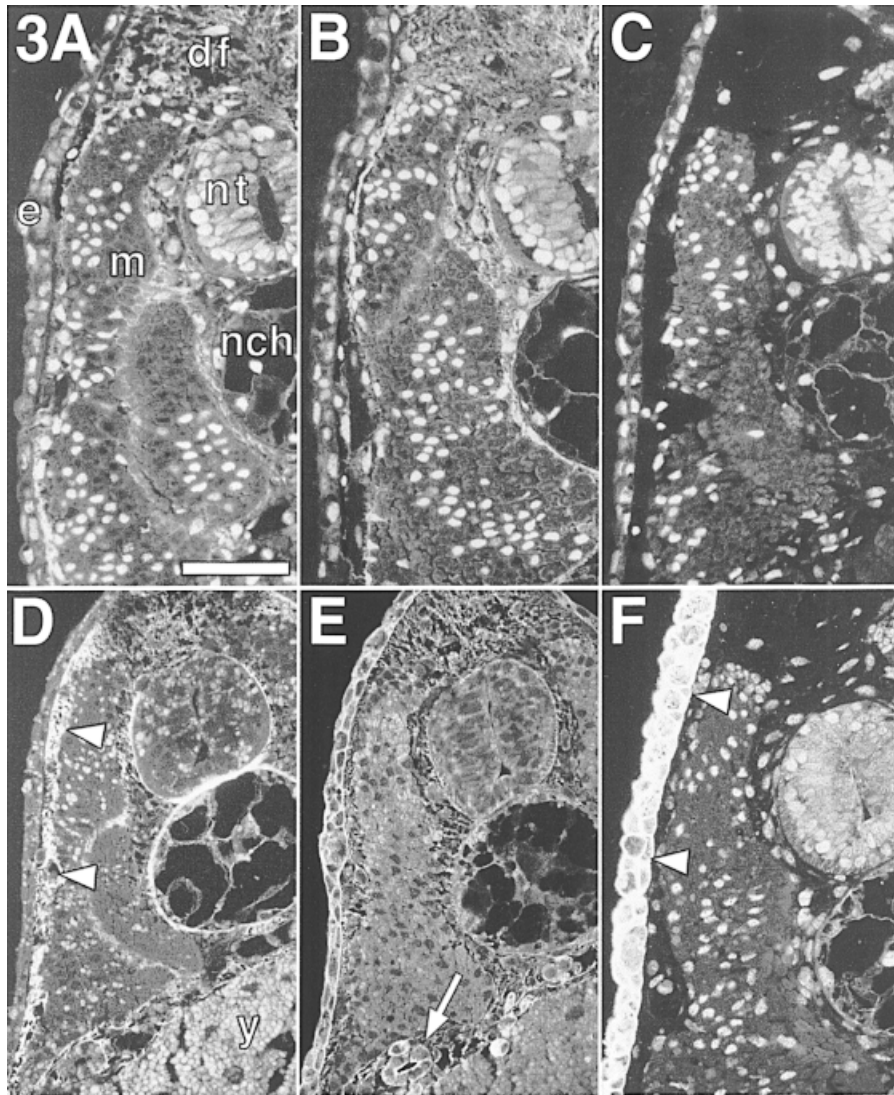


Fig. 3. Distributions of most candidate patterning molecules of the extracellular matrix do not correlate with melanophore distributions within or between species. All embryos shown here are stage 37. (A–C) Staining for hyaluronan in *T. torosa* and *T. rivularis*. (A) In *T. torosa*, hyaluronan is present within the dorsal fin (df) matrix and between the epidermis (e) and myotome (m). Nuclei are stained non-specifically by the avidin used for detecting the biotinylated hyaluronan binding protein fragment used as a probe; nt, neural tube; nch, notochord. (B) In *T. rivularis*, a similar distribution of hyaluronan is observed. (C) In a control section, hyaluronidase digestion abolishes staining in extracellular matrices. (D) Chondroitin sulfate proteoglycans are widely

distributed in both *T. torosa* and *T. rivularis*. In the *T. torosa* embryo shown here, chondroitin sulfate proteoglycans (recognized by mAb CS-56) are present beneath the epidermis (arrowheads) in regions with and without melanophore stripes; y, yolk. (E) Fibronectin is widely distributed in interstitial extracellular matrices and basement membranes in both *T. torosa* and *T. rivularis*. Shown is a *T. torosa* embryo. Arrow indicates the pronephric duct, which is somewhat more intensely stained than surrounding tissues as previously reported (Tucker and Erickson, '86a). (F) Type IV collagen is abundant within the epidermis (arrowheads), and this staining is indistinguishable between species; shown is *T. rivularis*. Scale bar, 100  $\mu$ m.

lateral lines, suggesting an evolutionary decoupling of tenascin regulation and lateral line development (coincident with the decoupling of stripe formation and lateral line development). To distinguish between these hypotheses, I examined the distribution of tenascin in *T. torosa* embryos

in which I ablated the lateral lines. Figure 5 shows that although tenascin is abundant in the presence of lateral lines, tenascin staining is not detectable when lateral line development is prevented. Thus, tenascin deposition in the middle of the flank depends on the lateral lines, demonstrating a phy-

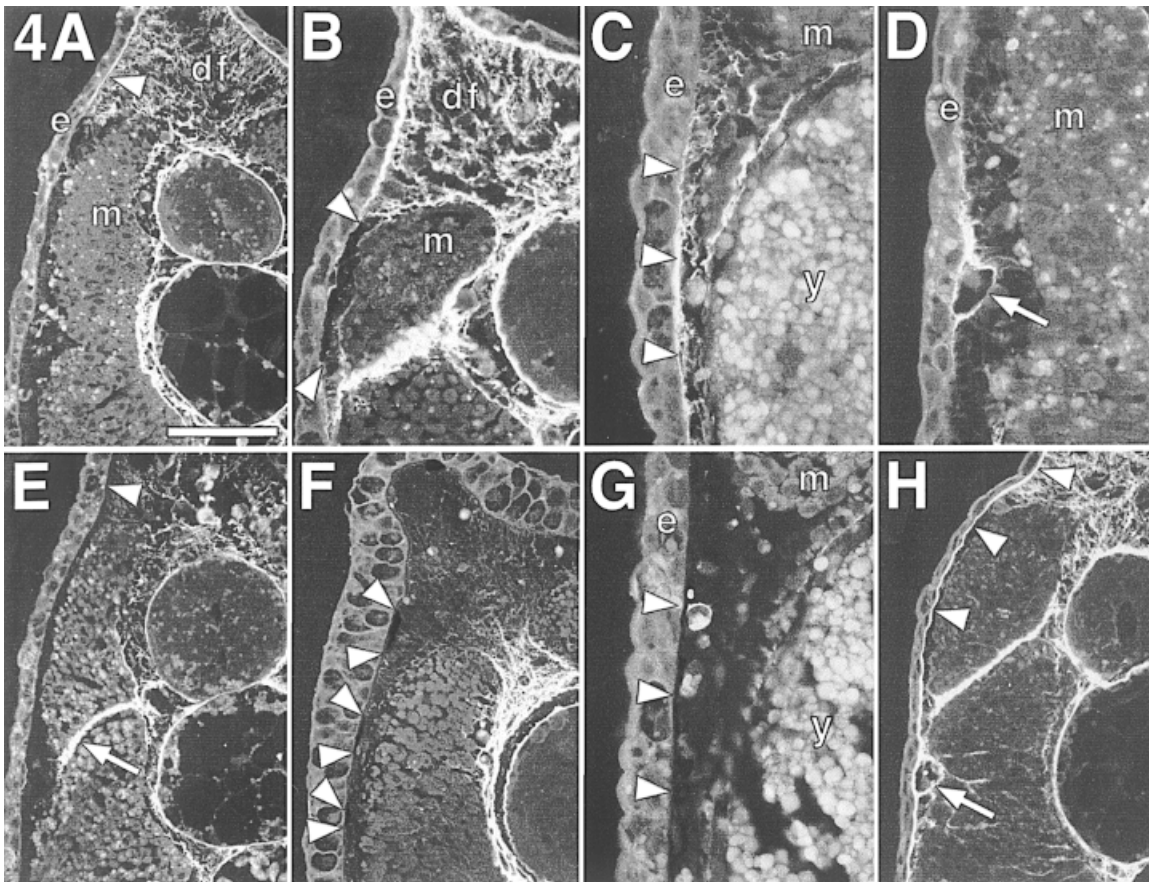


Fig. 4. Tenascin staining differs between *T. torosa* (A–D) and *T. rivularis* (E–G) and correlates with differences in melanophore distributions between species. (A) In *T. torosa* (stage 38), anti-tenascin immunoreactivity is intense within the interstitial matrix of the dorsal fin (df), lining the subepidermal basement membrane of the dorsal fin (arrowhead), within the vertical myosepta (not shown), and medially within the embryo; e, epidermis; m, myotome. (B) Higher magnification view of dorsal fin tenascin staining in a stage 39 *T. torosa* embryo. Arrowheads indicate melanophores in the dorsal stripe. (C) Tenascin staining is abundant in the subepidermal basement membrane over the yolk (arrowheads), where melanophores are not found; y, yolk. (D) Detail showing transient tenascin staining in the middle of the flank (prospective interstripe region) in the vicinity of the lateral line nerve (arrow). Shown is a *T. torosa* embryo at stage 37. (E) In *T. rivularis* (stage 38), anti-tenascin immunoreactivity is limited to medial regions of the embryo and vertical myosepta

(arrow), and is not found in the interstitial matrix of the fin or the subepidermal basement membrane of the fin (arrowhead). (F) Higher magnification view of dorsal fin and epidermis in a *T. rivularis* larva ~2 weeks after hatching (> stage 39), showing the continued absence of anti-tenascin immunoreactivity in this species. Arrowheads indicate melanophores, which are abundant in this region. (G) Detail showing the absence of tenascin staining in the subepidermal basement membrane overlying the yolk in *T. rivularis* (arrowheads), where melanophores are often found. (H) Tenascin staining in *A. t. tigrinum*. In contrast to *Taricha*, *A. t. tigrinum* at a comparable stage exhibit tenascin staining lining the subepidermal basement membrane throughout the embryo (arrowheads), though this staining is more intense in the vicinity of the lateral line nerve (arrow; also see: Parichy, '96c). Scale bars: 100  $\mu\text{m}$  (A,E,H); 50  $\mu\text{m}$  (B,F); 40  $\mu\text{m}$  (C,G); 45  $\mu\text{m}$  (D).

logenetically conserved effect of the lateral line sensory system on the extracellular matrix. Since stripes form in *T. torosa* when lateral line development is prevented, these findings also demonstrate that tenascin deposition in the middle of the flank is not essential for stripe development in this species, though it may be essential in other species with lateral line-dependent stripes (Fig. 2). Finally, examination of serially sectioned *T.*

*torosa* embryos revealed that the few melanophores that localize in the middle of the flank in this species typically contact the lateral line nerve within an outpocketing of the subepidermal basement (e.g., Fig. 4D), and appear to avoid the surrounding tenascin-immunoreactive extracellular matrix (data not shown).

The negative correlation between tenascin staining and melanophore distributions in the fin raises

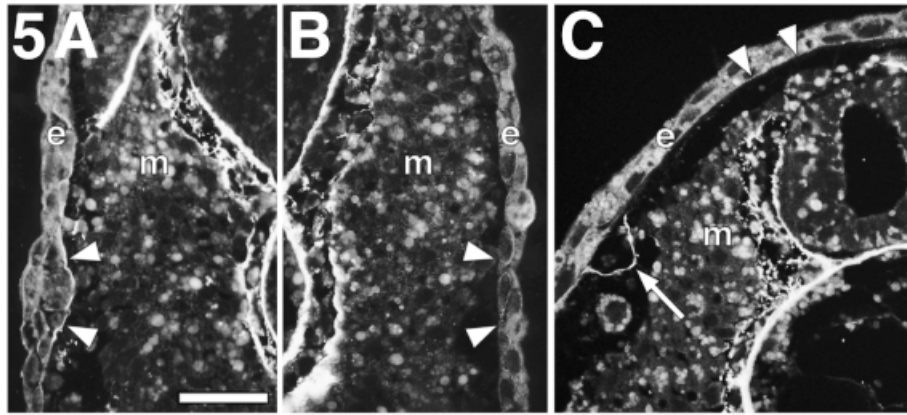


Fig. 5. Tenascin deposition depends on lateral line and fin development. (A) Detail of the unoperated side of a stage-36 *T. torosa* embryo, showing tenascin staining in the vicinity of the lateral line primordium (arrowheads); e, epidermis; m, myotome. (B) On the opposite side of the same embryo, on which lateral line development has been prevented,

tenascin staining in the middle of the flank is absent (arrowheads). (C) Ablation of the neural folds and prevention of fin outgrowth eliminates dorsal tenascin staining in *T. torosa* (e.g., compare region indicated by arrowheads to Fig. 4A). In contrast, lateral line-associated tenascin deposition is unaffected (arrow). Scale bars: 80  $\mu\text{m}$  (A,B); 100  $\mu\text{m}$  (C).

the possibility that tenascin may contribute to excluding melanophores from this region in *T. torosa* (as well as in *A. t. tigrinum*, *P. waltil*, and *Tr. alpestris*), whereas an absence of tenascin may permit melanophores to colonize the fin in *T. rivularis*. Since this difference in tenascin staining also correlates with a reduced fin in the stream-breeding *T. rivularis*, and tenascin deposition is associated with mechanical stress in tissues undergoing developmental remodeling (Chiquet, '99), I asked whether fin outgrowth is ancestrally required for tenascin deposition. To test this possibility, I prevented fin development by ablating the neural folds and prospective neural crest in *T. torosa* (Twitty and Bodenstern, '41). These embryos exhibited no tenascin immunoreactivity beneath the dorsal epidermis (Fig. 5C), indicating that fin epidermis, neural crest cells, or both are required for tenascin deposition in this region.

#### ***Laminin expression correlates with nonstripe regions in T. torosa***

Patterns of laminin staining were indistinguishable between *T. torosa* and *T. rivularis*. Anti-laminin immunoreactivity was most pronounced in the developing myotomes (Fig. 6A), and the onset of expression correlated with a shift in the adhesive affinity of melanophores from the somites to the overlying epidermis (Tucker and Erickson, '86a). Nevertheless, groups of cells at the dorsal and ventral apices of the myotomes remained unstained, and these regions coincide with sites of stripe formation in *T. torosa* (Fig. 6B,D,E). Mel-

anophores comprising both dorsal and ventral stripes frequently contacted unstained myotomal cells, but did not contact stained cells. Moreover, photographic series and serial sectioning indicate that melanophores initially localizing along the pronephric duct to form the ventral stripe are internalized as the myotomes overgrow the pronephric duct (as reported previously: Tucker and Erickson, '86a), but subsequently these melanophores return to the epidermis in the vicinity of laminin-negative cells along the ventral myotomes (data not shown). In contrast to dorsal tenascin deposition, the failure of dorsal myotomal cells to stain for laminin does not depend on interactions with neural crest or dorsal epidermis, as neural fold-ablated embryos continued to exhibit laminin-negative, presumptive myotomal cells in dorsal regions of the embryo (Fig. 6F). Similar staining patterns were observed with antibodies raised against bovine vitronectin and chicken phosphacan (data not shown). Thus, a subpopulation of myotomal cell fails to express laminin and the position of these cells correlates with the position of melanophore stripes in *T. torosa*.

#### **DISCUSSION**

The results of this study identify correlations between extracellular matrix molecules and melanophore distributions in salamanders of the genus *Taricha*. These findings provide insights into potential roles for the extracellular matrix during stripe development, as well as evolutionary conservatism and novelty in stripe-forming mecha-



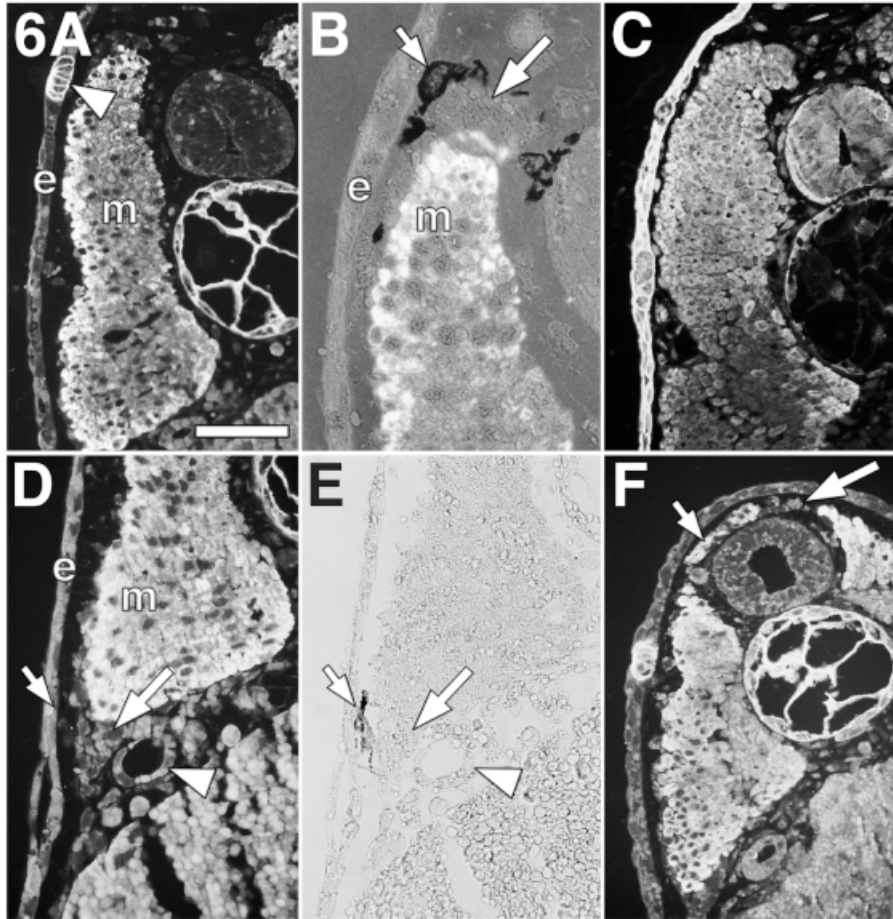


Fig. 6. Anti-laminin immunoreactivity correlates with the interstripe region in *T. torosa*. (A) In a stage-39 *T. torosa* embryo, anti-laminin immunoreactivity is prominent surrounding myotubes within the myotomes (m), and surrounds cells of lateral line neuromasts (arrowhead); e, epidermis. (B) Double exposure showing a melanophore (small arrow) in contact with dorsal myotomal cells that do not stain with anti-laminin antibody (large arrow). (C) In *A. t. tigrinum*, anti-laminin immunoreactivity is intense both in the epidermis and myotomes, and distinctive populations of unstained dorsal and ventral myotomal cells are not observed. (D) Ventral detail of *T. torosa*

at stage 37, showing cells at the ventral margin of the myotome that do not stain with anti-laminin antibody (large arrow); arrowhead, pronephric duct. (E) Brightfield view of the image in D, showing a melanophore (small arrow) in contact with laminin-negative cells of the ventral myotome (large arrow). (F) Ablation of the neural folds results in the myotomes extending dorsally over the neural tube (Twitty and Bodenstern, '41) and interspersed cells that are stained (small arrow) and unstained (large arrow) with anti-laminin antibody. Scale bars: 100  $\mu$ m (A,C,F); 50  $\mu$ m (B); 65  $\mu$ m (D,E).

nisms. These data also suggest potential cellular and molecular mechanisms for the evolutionary loss of stripes in *T. rivularis*.

#### ***Extracellular matrix in stripe development and evolution***

A variety of extracellular matrix molecules have been suggested to have roles in neural crest migration and pigment pattern development in salamanders (reviewed in: Epperlein et al., '96; Parichy, '96a). In this study, tenascin immunoreactivity was negatively correlated with melanophore distributions in *T. torosa*. Since tenascin is

a non-adhesive substratum for melanocytes and other neural crest-derived cell types (Mackie et al., '88; Le Poole et al., '97), its abundance in the subepidermal basement membrane of the fin and over the yolk suggests that this molecule, or a co-regulated molecule, may contribute to excluding melanophores from these regions. In *Tr. alpestris* and *A. t. tigrinum*, tenascin in the middle of the flank may produce an unfavorable environment for melanophore localization, causing these cells to move dorsally and ventrally to more adhesive regions to form stripes (Epperlein and Löffberg, '90; Parichy, '96c). In *T. torosa*, tenascin appears

transiently in the middle of the flank, but this deposition depends on lateral line development. These findings thus support a model in which *T. torosa* retains lateral line effects on the extracellular matrix that contribute ancestrally to stripe development, and demonstrate that tenascin is not itself the derived lateral line-independent stripe-forming cue inferred to be present in this species (Parichy, '96b).

Previous studies suggest several potential candidates for novel lateral line-independent stripe-forming cues in *T. torosa*. Although tenascin does not serve such a role, the distribution of anti laminin immunoreactivity is consistent with lateral line-independent effect of this matrix component on melanophore distributions. During pigment pattern development in *T. torosa*, melanophores initially adhere to the somites but as stripes form these cells change their adhesive affinity and instead associate principally with the epidermis (Tucker and Erickson, '86a). This change in preference occurs as the myotomes develop, and when laminin staining first becomes evident around individual myotubes. Subsequently, melanophores remain associated with the myotomes only at their dorsal and ventral apices where laminin staining is absent. Although laminin is a permissive substratum for neural crest cell migration (Perris et al., '89; Rogers et al., '90b, several studies suggest that some laminin isoforms can be poor substrates for neurite outgrowth and melanophore adhesion (Calof and Lander, '91; Fukuzawa et al., '92; Calof et al., '94; Brandenberger and Chiquet, '95). These findings raise the possibility that maturation of basal laminae in the developing myotomes generates an unfavorable environment for melanophores and thereby contributes to the dorsal and ventral localization of these cells. Or, laminin-negative cells may represent a distinct subpopulation of myotomal cells that produces a chemotactic or haptotactic cue attractive for melanophores (e.g., Adachi et al., '92; Wehrle-Haller and Weston, '95).

Finally, this study and a previous analysis (Parichy, '96c) reveal global increases in the abundance of tenascin, laminin, and several other extracellular matrix molecules during terminal stages of pigment pattern development in *A. t. tigrinum* and *P. waltl*, and this general elaboration of the matrix is hypothesized to stabilize melanophore positions once a striped pattern has formed (also see: Erickson and Perris, '93). In contrast, neither laminin nor tenascin staining increased in the subepidermal basement membrane

during corresponding stages in *Taricha*. Since *T. torosa* melanophores remain motile longer than *A. t. tigrinum* melanophores (Parichy, '96b), the retardation of matrix development in *Taricha* may allow *T. torosa* melanophores to search out more specific, evolutionarily derived cues for stripe formation.

#### *Developmental bases for stripe loss*

The evolutionary loss of stripes has occurred independently in several salamander taxa, and this pigment pattern alteration is associated with a shift to a stream habitat (Henry and Twitty, '40; Duellman and Trueb, '86; Parichy, '96a; Storer et al., '99). An intriguing finding of this study is that tenascin immunoreactivity is not present either beneath the fin or over the yolk in *T. rivularis*, both regions in which melanophores are abundant in this species, but not *T. torosa*. These findings are consistent with a role for tenascin in determining the distribution of melanophores, and identify tenascin or a co-regulated molecule as a candidate for contributing to the loss of stripes in *T. rivularis*. The dependence of tenascin deposition on fin development further raises the possibility that the evolutionarily novel localization of *T. rivularis* melanophores in the fin may reflect a correlated response to selection on fin morphology in a stream habitat, as opposed to direct selection on the pigment pattern. Nevertheless, a variety of factors are likely to contribute to stripe loss. For example, embryological grafting experiments implicate differences autonomous to the neural crest in determining whether or not stripes form (Twitty, '45). Moreover, *in vitro* analyses suggest that *T. rivularis* melanophores cease differentiating at an earlier stage than *T. torosa* melanophores, proliferate more actively, and fail to develop intercellular contacts present among late stage *T. torosa* melanophores (Twitty, '45; Youngs, '57). Conceivably, such autonomous differences could also include the expression of receptors for laminin or co-regulated molecules, such that *T. torosa* melanophores are competent to respond to cues present in both *T. torosa* and *T. rivularis* extracellular environments, whereas *T. rivularis* melanophores lack this capability. It will be interesting to identify additional molecular changes associated with stripe loss in *T. rivularis*, and whether similar (parallel) or different (convergent) mechanisms are responsible for stripe loss in other phylogenetic lineages.

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