and this intrinsic flexibility has been extensively exploited by natural selection. Although TF binding within exons may serve multiple functional roles, our analyses above is agnostic to these roles, which may be complex (*36*).

References and Notes

1. R. Grantham, C. Gautier, M. Gouy, R. Mercier, A. Pavé, *Nucleic Acids Res.* **8**, r49–r62 (1980).
2. T. Ikemura, *J. Mol. Biol.* **151**, 389–409 (1981).
3. R. Grantham, C. Gautier, M. Gouy, M. Jacobzone, R. Mercier, *Nucleic Acids Res.* **9**, r43–r74 (1981).
4. M. Gouy, C. Gautier, *Nucleic Acids Res.* **10**, 7055–7074 (1982).
5. A. Eyre-Walker, M. Bulmer, *Nucleic Acids Res.* **21**, 4599–4603 (1993).
6. D. B. Carlini, W. Stephan, *Genetics* **163**, 239–243 (2003).
7. M. dos Reis, R. Savva, L. Wernisch, *Nucleic Acids Res.* **32**, 5036–5044 (2004).
8. J. L. Parmley, J. V. Chamary, L. D. Hurst, *Mol. Biol. Evol.* **23**, 301–309 (2006).
9. T. Warnecke, C. C. Weber, L. D. Hurst, *Biochem. Soc. Trans.* **37**, 756–761 (2009).
10. W. Gu, T. Zhou, C. O. Wilke, *PLOS Comput. Biol.* **6**, e1000664 (2010).
11. M. F. Lin *et al.*, *Genome Res.* **21**, 1916–1928 (2011).
12. Z. Yang, R. Nielsen, *Mol. Biol. Evol.* **25**, 568–579 (2008).
13. S. Neph *et al.*, *Nature* **489**, 83–90 (2012).
14. S. M. Hyder, Z. Nawaz, C. Chiappetta, K. Yokoyama, G. M. Stancel, *J. Biol. Chem.* **270**, 8506–8513 (1995).
15. G. Lang, W. M. Gombert, H. J. Gould, *Immunology* **114**, 25–36 (2005).
16. D. I. Ritter, Z. Dong, S. Guo, J. H. Chuang, *PLOS ONE* **7**, e35202 (2012).
17. A. H. Khan, A. Lin, D. J. Smith, *PLOS ONE* **7**, e46098 (2012).
18. R. Y. Birnbaum *et al.*, *Genome Res.* **22**, 1059–1068 (2012).
19. Materials and methods are available as supplementary materials on *Science* Online.
20. W.-H. Li, *Molecular Evolution* (Sinauer Associates, Sunderland, MA, 1997).
21. W. Fu *et al.*, *Nature* **493**, 216–220 (2013).
22. C. Coulondre, J. H. Miller, P. J. Farabaugh, W. Gilbert, *Nature* **274**, 775–780 (1978).
23. M. Bulmer, *Nature* **325**, 728–730 (1987).
24. M. Bulmer, *Genetics* **129**, 897–907 (1991).
25. J. Duan *et al.*, *Hum. Mol. Genet.* **12**, 205–216 (2003).
26. J. zur Megede *et al.*, *J. Virol.* **74**, 2628–2635 (2000).
27. J. R. Coleman *et al.*, *Science* **320**, 1784–1787 (2008).
28. R. M. Samstein *et al.*, *Cell* **151**, 153–166 (2012).
29. S. McKnight, R. Tjian, *Cell* **46**, 795–805 (1986).
30. C. Zhang *et al.*, *Nucleic Acids Res.* **34**, 2238–2246 (2006).
31. H. Xi *et al.*, *Genome Res.* **17**, 798–806 (2007).
32. A. Hellman, A. Chess, *Science* **315**, 1141–1143 (2007).
33. D. Zilberman, M. Gehring, R. K. Tran, T. Ballinger, S. Henikoff, *Nat. Genet.* **39**, 61–69 (2007).
34. S. Itzkovitz, U. Alon, *Genome Res.* **17**, 405–412 (2007).
35. S. Itzkovitz, E. Hodis, E. Segal, *Genome Res.* **20**, 1582–1589 (2010).
36. T. R. Mercer *et al.*, *Nat. Genet.*, published online 23 June 2013 (10.1038/ng.2677).

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Supplementary Materials

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Cryptic Variation in Morphological Evolution: HSP90 as a Capacitor for Loss of Eyes in Cavefish

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In the process of morphological evolution, the extent to which cryptic, preexisting variation provides a substrate for natural selection has been controversial. We provide evidence that heat shock protein 90 (HSP90) phenotypically masks standing eye-size variation in surface populations of the cavefish *Astyanax mexicanus*. This variation is exposed by HSP90 inhibition and can be selected for, ultimately yielding a reduced-eye phenotype even in the presence of full HSP90 activity. Raising surface fish under conditions found in caves taxes the HSP90 system, unmasking the same phenotypic variation as does direct inhibition of HSP90. These results suggest that cryptic variation played a role in the evolution of eye loss in cavefish and provide the first evidence for HSP90 as a capacitor for morphological evolution in a natural setting.

A longstanding question in evolutionary biology is the extent to which selection acts on preexisting "standing variation" in a population, as opposed to de novo mutations. Recent studies have indicated that both mechanisms have contributed to morphological evolution (*1*, *2*). Thus, although de novo mutations may exist and contribute to phenotypic evolution, repeated use of standing variation has played an important role in the evolution in these fish. However, these observations also raise a critical question: How is genetic variation maintained in a population if it is not adaptive before new selective conditions?

Waddington proposed that developmental processes are quite robust and produce the same phenotype regardless of minor genotypic variation, a phenomenon he termed "canalization" (*3*). In such conditions, cryptic variation can accumulate and can be maintained without consequence. He further proposed that under certain environmental conditions, this property could be lost ("decanalization"), resulting in expression of the cryptic variation on which selection could act (*4*).

More recently, Lindquist demonstrated that HSP90 (heat shock protein 90) provides a molecular mechanism for buffering genetic variation and releasing it in response to environmental stress (*5*–*10*). The HSP90 chaperone assists in the folding of proteins that are metastable signal transducers, such as kinases, transcription factors, and ubiquitin ligases. HSP90 is normally present at much higher concentrations than needed to maintain these proteins, allowing it to act as a buffer, protecting organisms from phenotypic consequences that would otherwise be caused by genetic variants of these proteins. Because protein folding is so sensitive to environmental stress, changes in the environment can exhaust the chaperone buffer, unmasking vulnerable polymorphisms. And because multiple variants can be unmasked at the same time, this system provides a mechanism to create complex traits in a single step (*11*).

Besides changes in the activities of kinases, phosphatases, transcription factors, and ubiquitin ligases, other distinct mechanisms have been reported by which changes in HSP90 function can lead to changes in phenotype (*5*, *10*, *12*–*16*)

Evidence strongly suggests that this mechanism has operated in microbial populations (*7*, *8*), but its relevance to the evolution of natural populations of higher organisms remains highly controversial. Thus far, examples of HSP90-mediated canalization in multicellular eukaryotes have been limited to lab strains of various model organisms. Moreover, with the exception of some phenotypes in *Arabidopsis*, the phenotypes of HSP90-released canalization in higher organisms are not

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obviously adaptive. Last, it has been unclear how a heat shock or other environmental factor would feature in the normal context of natural selection.

If a long-term environmental stress were to drive the course of phenotypic evolution, it would plausibly arise when species are confronted with a completely foreign set of conditions. Such circumstances are met when organisms, such as the cavefish *Astyanax mexicanus*, are inadvertently introduced into a cave environment.

Cavefish display many phenotypic differences from their surface conspecifics. We chose to focus on the dramatic loss of eyes in the cave morph, a trait that has been shown to be influenced by at least 14 mapped quantitative trait loci. Moreover, genetic evidence suggests that eye loss is very likely to be adaptive (*17*, *18*). Loss of eyes could have had direct adaptive importance—for example, in the energetic cost of maintaining eyes in an environment where they lack utility and/or could have been selected indirectly through the pleiotropic need to expand other sensory systems (supplementary text). It is possible that, to some extent, neutral effects could have also contributed to the process by which eyes were lost in the cave populations of *Astyanax*.

To reduce the capacity of HSP90 chaperone activities during development of the fish (mimicking the effects of environmental stress) (*5*–*7*, *19*, *20*), we used the well-characterized and highly specific inhibitor Radicicol. When developing, *A. mexicanus* were treated with 500 nM Radicicol; this resulted in a strong increase in expression of two marker genes for HSP90 inhibition, *BAG3* and *HSP27*(*HSPB1*), as well as HSP90 itself (Fig. 1A and fig. S1), which is consistent with impairment of chaperone activity. The treated fish displayed an array of different low-penetrance phenotypes in surface, cave, and F2 populations (fig. S2). Similar to previous studies, most of the low-penetrance phenotypes were not obviously adaptive and either not viable or transient. Radicicol was not teratogenic for eye development per se. When an inbred laboratory strain of zebrafish was reared under similar conditions of Radicicol exposure (by using a concentration that elicited a similar transcriptional response of target genes), no difference was observed in eye size or morphology (fig. S3). In contrast, when *A. mexicanus* surface fish were raised in the presence of the drug we observed unusually large variation in eye size in larval fish (Fig. 1B).

We initially characterized the effect of HSP90 inhibition on adult stages in an F2 population derived from a cross between a surface fish and a cavefish, allowing us to examine eye size in the simultaneous presence of surface and cave alleles. Indeed, we observed both larger eyes and smaller eyes in the treated fish, leading to a statistically significant increase in the standard deviation (SD) of the treated fish [+58% compared with dimethyl sulfoxide (DMSO)–treated control groups; two-sided F test, $P = 0.0004$; Bartlett's test, $P = 0.001$; Levene's test, $P = 0.03$] (Fig. 1, C and D). These experiments were conducted in the dark because Radicicol is slightly light sensitive; however, raising *Astyanax* embryos in the presence or absence of light had no effect on eye size (Fig. 1C). Thus, inhibition of HSP90 permits the expression of cryptic variation in eye size present in the *Astyanax* populations.

If the cryptic variation we observed in eye size played a role in the evolution of the reduced eye trait within the caves, one would expect this variation to be present in the modern river population of surface fish (similar to the ancestral form) but to be less prevalent in the cave populations that have undergone selection for this trait. We observed a statistically significant increase in eye size variation (SD, +83% compared with DMSO-treated control fish; two-sided F test: $P = 8.1 \times 10^{-6}$; Bartlett's test, $P < 0.001$, Levene's test, $P < 0.001$) in the parental surface populations, again including both larger and smaller eyes than are ever seen in untreated broods (Fig. 2A). The same result was seen whether measuring the eye itself or the infraorbital bones surrounding the eye socket or "orbit" (SD, +108%; two-sided F test, $P = 3.4 \times 10^{-6}$; Bartlett's test, $P < 0.001$; Levene's test, $P < 0.001$) (Fig. 2B and fig. S4).

A very different result was observed, however, when we conducted parallel experiments on fish from the Tinaja cave population. Cavefish do not possess visible eyes but still retain a cavity in the skull where the eye would be located (fig. S4), so in this case, we focused on the orbit size. We detected no increase in the variation in orbit size in the inhibitor-treated cavefish (SD, –12%; two-sided F test, $P = 0.239$), suggesting that some of the alleles have been selected for in the cavefish population (Fig. 2C). However, we detect a statistically significant decrease in the orbit size in the inhibitor-treated individuals (two-tailed t test, $P = 0.002$) (Fig. 2C). This shows that the alleles that have been selected for in cavefish evolution are alleles that, at least in part, are dependent on and responsive to HSP90. Moreover, the HSP90-dependent alleles remaining in the cave population are specifically those contributing to

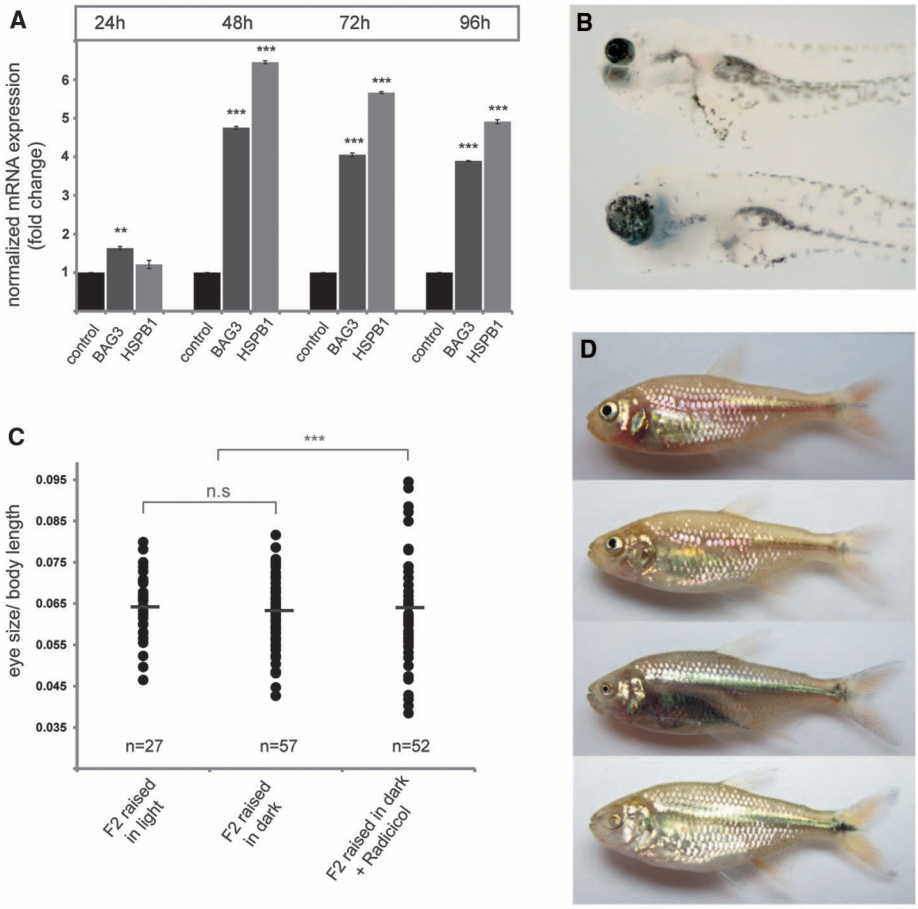

Fig. 1. Reduction of HSP90 levels in *A. mexicanus* using the chemical inhibitor Radicicol. (**A**) Inhibition of HSP90 using 500 nM Radicicol leads to activation of BAG3 and HSPB1 (two-tailed t test, $^{**}P < 0.005$, $^{***}P < 0.0005$). Time scale refers to hours of treatment. (**B**) Variable eye sizes in surface *A. mexicanus* larvae after treatment. (**C**) Quantification of eye size in adult F2 hybrids after larval treatment of Radicicol reveals a significant increase in SD of eye size, whereas average eye size is not affected (two-sided F test: $P = 0.0004$; Bartlett's test, $P = 0.001$; Levene's test, $P = 0.03$). Raising the fish in the dark alone does not affect eye size. Values were corrected for body size by using standard length of the fish. (**D**) Examples of eye size variation in F2 population of hybrid *A. mexicanus*.

reduced eye sockets. These data are consistent with the possibility that HSP90 played a role in the evolution of eye size in Tinaja cavefish.

To test whether the cryptic variation in eye size uncovered by HSP90 inhibition can be genetically assimilated, we treated a population of embryonic surface fish with Radicicol. We then selected for smaller eye size by intercrossing fish from this treated brood whose eye size was smaller than any of the untreated fish raised in parallel. The resultant F2 fish, raised in the absence of the drug, all had eyes and orbit sizes at the lower end of the range of sizes observed in the parental fish and included many individuals with eyes and orbits smaller than any seen in the untreated surface fish and comparable with the smallest of the treated fish (Fig. 3, A and B). Thus, the individuals that develop the smallest eyes in the presence of HSP90 inhibition contain alleles that can contribute to inheritance of small eye size in the absence of treatment.

A critical question, however, is whether a river fish finding itself suddenly trapped in a cave environment would experience a HSP90-related stress response. To characterize abiotic factors that differ between the cave and river environments, we measured pH, oxygen content, temperature, and conductivity in the Tinaja cave and a nearby surface fish habitat in the Sierra de El Abra (table S1). The biggest numerical difference we detected was the much lower conductivity present in the cave water. Further sampling of additional caves revealed conductivities as low as 230 μS (Sabinos cave), compared with the 1300-μS conductivity in adjacent river environments. Low conductivity can elicit a heat shock such as stress response in fish (*21*), making it a good candidate for a cave-specific stress factor.

To investigate, we raised surface fish at the lowest measured conductivity from the Sabinos cave (230 μS). When fish embryos develop under such conditions, they up-regulate HSP90, showing that they are indeed in a state of physiological stress response and, moreover, activate the same heat shock response genes that are up-regulated with HSP90 inhibition by Radicicol (Fig. 4B and fig. S5). Thus, the environment encountered by these fish during their evolutionary transition from surface to cave stresses the protein homeostasis mechanisms of the organism in a manner similar to a specific stress on HSP90 chaperone activities.

Adult river fish placed in low conductivity during larval development displayed statistically significant increases in eye and orbit size variation of 50% (two-sided *F* test, $P = 0.0018$; Bartlett's test, $P = 0.006$; Levene's test, $P = 0.005$) and 58% (two-sided F test, $P = 5.9E\text{-}4$; Bartlett's test, $P = 0.001$; Levene's test, $P = 0.01$), respectively (Fig. 4, C and D). This demonstrates that a cave-specific environmental stress can elicit similar changes in morphological eye development as biochemical inhibition of HSP90.

We detected an increase in variation in eye size in fish treated with Radicicol or in the presence of environmental stresses, representing cryptic variation present in the untreated population. We further demonstrated that in cavefish populations, the alleles that are responsive to HSP90 have undergone selection in the transition from surface to cave forms, leaving only alleles that

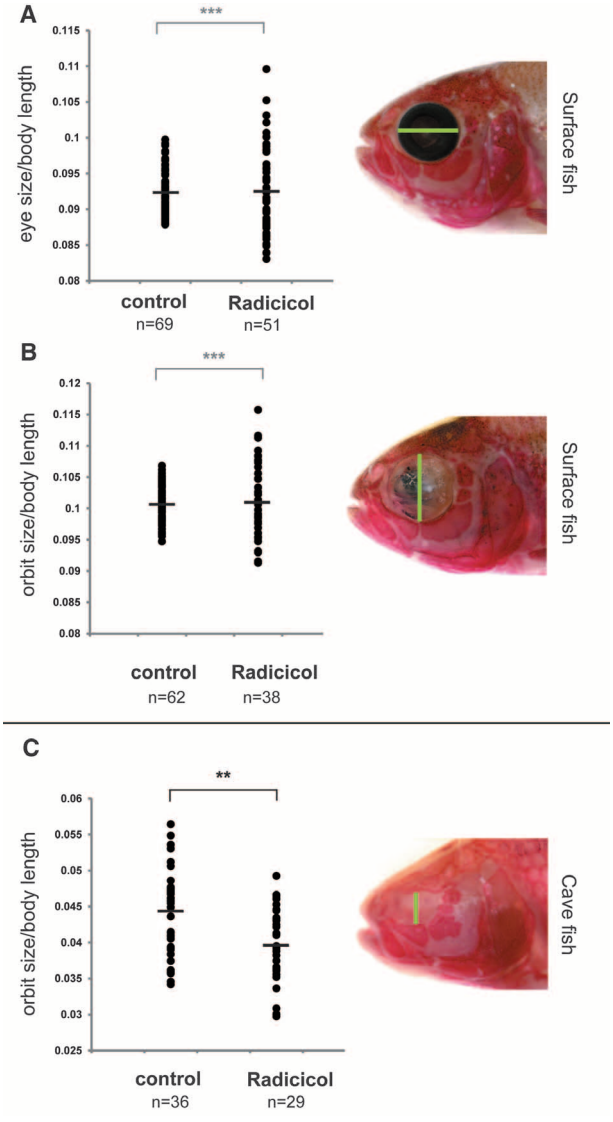

Fig. 2. HSP90 inhibition in natural populations of *A. mexicanus*. (**A** and **B**) HSP90 inhibition in natural populations of surface *A. mexicanus* leads to an increase of variation in (A) eye size and (B) orbit size. Eye size SD, +83%; two-sided *F* test, $P = 8.1 \times 10^{-6}$; Bartlett's test, $P < 0.001$; Levene's test, $P < 0.001$; orbit size SD, +108%; two-sided *F* test: $P = 3.4 \times 10^{-6}$; Bartlett's test, $P < 0.001$; Levene's test, $P < 0.001$. (**C**) Orbit size decreases after Radicicol treatment in Tinaja cave populations (*t* test, $P = 0.02$), while change in variation is observed (SD, –12%; two-sided *F* test, $P = 0.239$). Control is DMSO. Asterisks in (A) and (B) compare SDs, whereas in (C), asterisks compare averages.

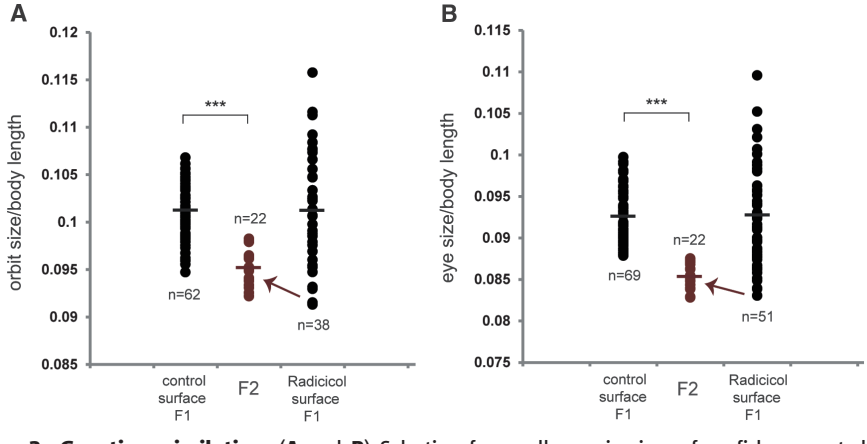

Fig. 3. Genetic assimilation. (**A** and **B**) Selection for small eye size in surface fish generated by Radicicol treatment resulted in offspring with significantly smaller (A) orbit size and (B) eye size in the absence of treatment (two-tailed *t* test, $P = 6.5 \times 10^{-13}$ for orbit size; $P = 7.2 \times 10^{-16}$ for eye size). The resultant range exceeded the range seen in any cross of untreated surface fish.

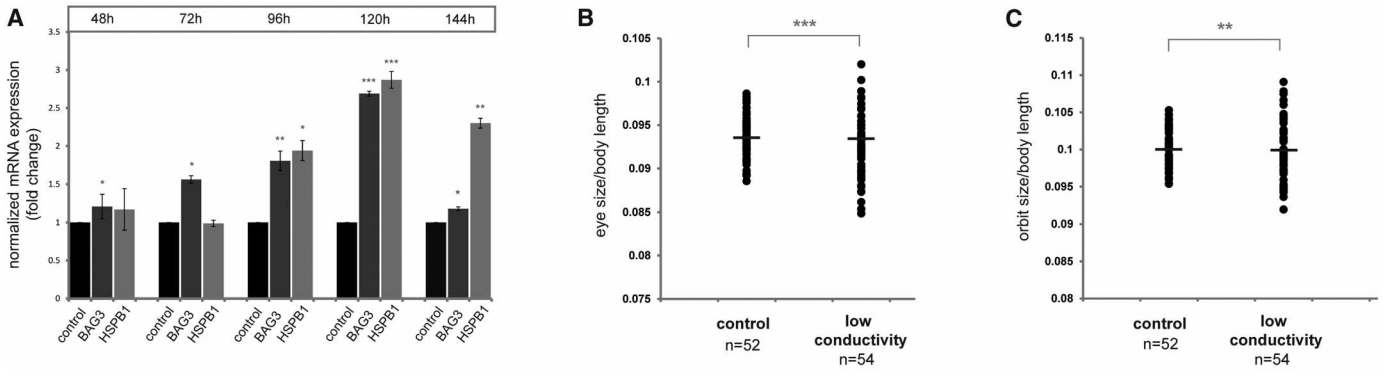

Fig. 4. Low-conductivity conditions in the cave natural habitat have a similar effect to Radicicol treatment on surface populations. (**A**) Quantitative reverse transcription polymerase chain reaction of BAG3 and HSPB1 for surface fish reared under low-conducitivity (230 μS) conditions compared with control conductivity conditions (two-tailed *t* test, $*P < 0.05$; $**P < 0.005$; $***P < 0.0005$). Time scale refers to hours of treatment. (**B** and **C**) Lower-conductivity conditions reveal an increase in variation of (B) orbit size and (C) eye size (eye size SD, +50%; two-sided *F* test, $P = 0.0018$; Bartlett's test, $P = 0.006$; Levene's test, $P = 0.005$; orbit size SD, +58%; two-sided *F* test: $P = 5.9 \times 10^{-4}$; Bartlett's test, $P = 0.001$; Levene's test, $P = 0.01$).

produce a smaller orbit when released from their normal HSP90 interactions. This strongly suggests an involvement of HSP90 in cavefish evolution and provides an actual case in nature for Waddington's classic theory of the role of canalization in evolution. Not all cave-specific traits appear to have relied on HSP90-canalized cryptic variation for their evolution. We examined several other traits and found, for example, that there is no cryptic variation in body size (fig. S6) or in neuromast number (fig. S7) uncovered by HSP90 inhibition in the populations we examined.

It is also reasonable to assume that the change in conductivity is only one factor contributing to the stress response that surface fish might experience after colonizing the caves (such as lower oxygen levels or starvation). However, such environmentally induced stress is likely to have been only transient because the cavefish would have adapted to these new conditions over subsequent generations. Cavefish have higher basal HSP90 levels than those of surface fish (*22*), potentially rendering them more stress-resistant. However, during the transition period when the fish were adapting to the cave conditions, the HSP90-dependent standing variation in eye size we observed in the surface population of *A. mexicanus* would have helped potentiate a rapid response to the cave environment.

Of course, the extreme environment of the cavefish is exceptional in many ways. Yet, environmental challenges are likely to be a driving force for many other adaptations. For example, temperature increases are extremely common in nature, and even simple starvation affects Hsp expression in European Sea Bass (*23*).

References and Notes

1. P. F. Colosimo *et al.*, *Science* **307**, 1928–1933 (2005).
2. F. C. Jones *et al.*, *Nature* **484**, 55–61 (2012).
3. C. H. Waddington, *Nature* **150**, 563–565 (1942).
4. C. H. Waddington, *Evolution* **7**, 118 (1953).
5. S. L. Rutherford, S. Lindquist, *Nature* **396**, 336–342 (1998).
6. C. Queitsch, T. A. Sangster, S. Lindquist, *Nature* **417**, 618–624 (2002).
7. D. F. Jarosz, S. Lindquist, *Science* **330**, 1820–1824 (2010).
8. L. E. Cowen, S. Lindquist, *Science* **309**, 2185–2189 (2005).
9. Y. Xu, M. A. Singer, S. Lindquist, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 109–114 (1999).
10. M. Taipale *et al.*, *Cell* **150**, 987–1001 (2012).
11. D. F. Jarosz, M. Taipale, S. Lindquist, *Annu. Rev. Genet.* **44**, 189–216 (2010).
12. V. Sollars *et al.*, *Nat. Genet.* **33**, 70–74 (2003).
13. D. Mittelman, K. Sykoudis, M. Hersh, Y. Lin, J. H. Wilson, *Cell Stress Chaperones* **15**, 753–759 (2010).
14. G. Chen, W. D. Bradford, C. W. Seidel, R. Li, *Nature* **482**, 246–250 (2012).
15. V. Specchia *et al.*, *Nature* **463**, 662–665 (2010).
16. R. Sawarkar, C. Sievers, R. Paro, *Cell* **149**, 807–818 (2012).
17. M. Protas, M. Conrad, J. B. Gross, C. Tabin, R. Borowsky, *Curr. Biol.* **17**, 452–454 (2007).
18. W. R. Jeffery, *Annu. Rev. Genet.* **43**, 25–47 (2009).
19. T. W. Schulte *et al.*, *Mol. Endocrinol.* **13**, 1435–1448 (1999).
20. P. L. Yeyati, R. M. Bancewicz, J. Maule, V. van Heyningen, *PLoS Genet.* **3**, e43 (2007).
21. C. Y. Choi, K. W. An, *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **149**, 91–100 (2008).
22. T. A. Hooven, Y. Yamamoto, W. R. Jeffery, *Int. J. Dev. Biol.* **48**, 731–738 (2004).
23. E. Antonopoulou *et al.*, *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **165**, 79–88 (2013).

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Supplementary Materials

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Progressive Specification Rather than Intercalation of Segments During Limb Regeneration

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An amputated salamander limb regenerates the correct number of segments. Models explaining limb regeneration were largely distinct from those for limb development, despite the presence of common patterning molecules. Intercalation has been an important concept to explain salamander limb regeneration, but clear evidence supporting or refuting this model was lacking. In the intercalation model, the first blastema cells acquire fingertip identity, creating a gap in positional identity that triggers regeneration of the intervening region from the stump. We used HOXA protein analysis and transplantation assays to show that axolotl limb blastema cells acquire positional identity in a proximal-to-distal sequence. Therefore, intercalation is not the primary mechanism for segment formation during limb regeneration in this animal. Patterning in development and regeneration uses similar mechanisms.

Numerous models to explain proximodistal metazoan limb patterning during regeneration have been proposed. Cell intercalation has become an important concept based on the results of grafting experiments (*1*–*6*). Cell intercalation is a patterning process whereby experimentally