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Repeated removal of developing limb buds permanently reduces appendage size in the highly-regenerative axolotl

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ABSTRACT

Matching appendage size to body size is fundamental to animal function. Generating an appropriately-sized appendage is a robust process executed during development which is also critical for regeneration. When challenged, larger animals are programmed to regenerate larger limbs than smaller animals within a single species. Understanding this process has important implications for regenerative medicine. To approach this complex question, models with altered appendage size:body size ratios are required. We hypothesized that repeatedly challenging axolotls to regrow limb buds would affect their developmental program resulting in altered target morphology. We discovered that after 10 months following this experimental procedure, limbs that developed were permanently miniaturized. This altered target morphology was preserved upon amputation and regeneration. Future experiments using this platform should provide critical information about how target limb size is encoded within limb progenitors.

1. Introduction

From fertilization to cleavage, development, and adulthood a specific and robust developmental program ensures proper animal form. Organs and appendages are grown in a stereotypical and orderly fashion, following a programed timeline that is largely invariant within a given species. Appendage development, for instance, is intimately connected to progenitor cell distribution, growth factors, and patterning mechanisms ((reviewed in Sheeba et al., 2015; Chen and Johnson, 1999; Tabin and Wolpert, 2007; Tickle, 2006), for example, (Riddle et al., 1993; Zhu et al., 2008; Yu and Ornitz, 2008; Kawakami et al., 2001; Cooper et al., 2011)). Over the course of organismal growth, animals calibrate the size of their appendages to the size of their bodies. Once these structures have stopped growing, active molecular processes ensure they are not pathologically overridden; for example, size/growth control is also a key issue in cancer.

Within a species, a relatively constant ratio exists for appendage size versus, for instance, overall body length or height (reviewed in (Lui and Baron, 2011; Gould, 1966)). Permutations of this program have been uncovered in zebrafish by screening for mutants with altered fin:body ratios (Perathoner et al., 2014). This work has uncovered a role for potassium channels in regulating the appendage-body size relationship, but the mechanisms connecting changes in membrane potential to overall appendage size remain murky. Because localized overexpression of potassium channels is sufficient to instigate nearby tissue overgrowth, the control of appendage size may largely be controlled at the local level (Perathoner et al., 2014). The intrinsic ability of the tissue itself to dictate the size of the growing organ was also highlighted in a transplantation experiment with two different sized salamanders where their limb buds were swapped. The limb buds of the larger species produced large limbs on the smaller hosts, and the limb buds of the smaller species produced small limbs on the large hosts (Twitty and Schwind, 1931). This result is consistent with the idea that limb buds are autonomous units programmed with the information necessary to produce the appropriately-sized appendage for the animal that will ultimately grow into an adult. The experiment also suggests that information from the host's body—for example, information about its size—cannot override the pre-defined growth determinant of the graft. However, in these inter-species chimeric experiments communication between the graft and host cells may be

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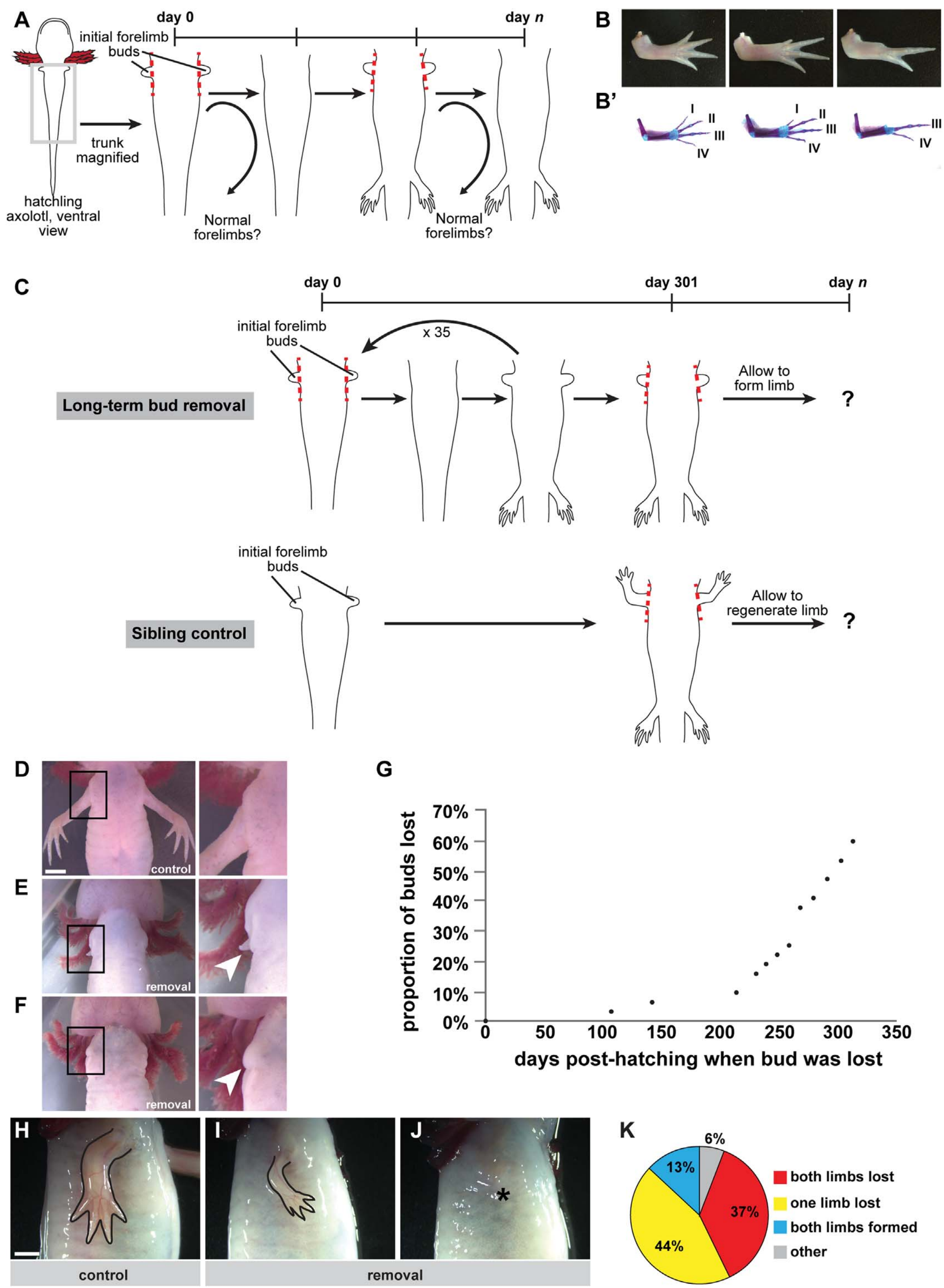

Fig. 1. Experimental design and gross morphological outcomes after repeated bud removal. We performed two primary lines of experimentation: 1) short-term bud removal (i.e. up to 10 removals) with animal drop out and 2) long-term bud removal (i.e. 36 removals) without dropout. (A) Schematic of short-term bud removal experiments. Dashed red lines indicate cutting plane. Curved arrows indicate that animals are dropped out of the experimental protocol at select time points and then allowed to grow a limb. (B) Dorsal view of representative limb morphologies observed when limb formation is delayed. Anterior is at top and posterior is at bottom of the image. (B') Dorsal view of skeletal preparations of limbs shown in (B). Digit identity is noted with Roman letters. (C) Schematic of long-term bud removal experiments. Dashed red lines indicate cutting plane. Control siblings were amputated proximally at the time of the last bud removal. Both control animals and animals that underwent repeated bud removal were allowed to fully form limbs after these final procedures. (D) Representative example of control limbs from a sibling of the exact same age as the experimental animals, ventral view, right forelimb/body junction (area inside black box) magnified at right. (E) Representative example of a reformed bud-like structure (arrowhead in magnified inset) on an experimental animal following 11 days of growth since last removal. (F) Representative example of loss of bud-like structure (arrowhead in magnified inset) following repeated removal, imaged 11 days after last removal. (G) Cumulative distribution plot of loss of bud-like structure as a function of time (n=32 buds/16 animals). (H-J) Representative examples of morphological outcomes when primary limbs are allowed to form at 313 days post-hatching, lateral view of right forelimb. (H) Sibling control amputated at the same time point, 313 days post-hatching, and allowed to regenerate for ~16 weeks (n=26 forelimbs/13 animals). (I) Example outcome in an experimental animal allowed to form a fully patterned/differentiated limb, beginning at 313 days post-hatching (n=12/32 forelimbs). (J) Example outcome in an experimental animal which lost the ability to regrow a limb during the course of the study (n=19/32 forelimbs). Asterisk indicates shoulder joint. (K) Quantification of final outcomes in experimental animals (Both limbs lost: n=6/16 animals; one limb lost: n=7/16 animals; both limbs formed: n=2/16; other: n=1/16). Scale bars are 5 mm; bar in (D) applies to (D-F); bar in (H) applies to (H-J).