Nerve-mediated amputation-induced stem cell activation primes distant appendages for future regeneration events in axolotl

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Summary

Animals exhibit extreme diversity in regenerative ability. This likely reflects different, lineage-specific selective pressures in their evolutionary histories, but how specific molecular features of regenerative programs help solve species-specific challenges has not been examined in detail. Here we discover that, in the highly-regenerative axolotl salamander, a conserved, body-wide stem cell activation response triggered in response to limb removal primes undisturbed limbs for regeneration upon subsequent amputation. This response should be particularly useful to salamanders, which frequently lose limbs in response to cannibalism. We further demonstrate the body-wide response requires both peripheral nervous system input at these distant sites and mTOR signaling. We defined gene expression changes within the nerves and nearby tissues, harboring responsive stem cells, leading to identification of candidate genetic pathways influencing distant stem cell activation following amputation. Functional experimentation confirmed a requirement for adrenergic signaling in amputation-induced activation of distant stem cells. These findings reveal a direct link between systemic cellular activation responses to local tissue damage and overall regenerative ability. Similar systemic activation responses to tissue removal have been observed in animals with widely differing regenerative abilities (e.g., planaria to mice), suggesting that it is the responses downstream of these signals, likely sculpted by differing evolutionary pressures, that ultimately distinguish regenerators from non-regenerators.

Keywords: regeneration, stem cells, axolotl, limb, systemic responses, peripheral nervous system

Main

Loss of a body part provokes cellular and molecular changes at the injury site and systemically [1]. Most research in vertebrate models has focused on local responses, within the remaining “stump” tissues, as cells within these tissues contribute directly to the construction of the new body part. Yet, understanding the mechanism and consequence of systemic responses is critical as they may contribute to regeneration and because comparing them across species may reveal insights related to the evolution of regeneration [2]. The axolotl, a highly-regenerative salamander for which molecular genetic tools now exist [3], provides an extraordinary model for studying regeneration events due to its ability to regenerate complex tissues and organs such as limbs, hearts, and parts of the nervous system. Following amputation in axolotl, a structure essential for regeneration called the blastema forms at the amputation site [4]. The blastema is largely composed of a dense population of highly-proliferative progenitor cells that ultimately undergo differentiation, repatterning, and growth to replace the lost structure. Our current understanding of limb regeneration has focused primarily on local events at the injury site. However, the discovery of a body-wide response to amputation in axolotls [5], in which many distant stem cells are activated to re-enter the cell cycle, raises the possibility that systemic activation may be a component of the regeneration process on which local growth responses are elaborated. Indeed, the activation of stem cells distant from the site of injury is evolutionarily conserved amongst many other species, both super-regenerators (such as planarians) and those with more limited natural regenerative prowess (such as mice) [1, 6-8]. The extensive conservation of this distant-activation phenomenon suggests it is an evolutionarily ancient response and raises the question of whether it is used differently in species with differing regenerative potentials, and, if so, how this relates to specific evolutionary pressures these species face.

Here, we investigate the consequence and mechanism of systemic stem cell activation following amputation in axolotls. We identify potential selective advantages of systemic activation and discover a distant stem cell-priming program driving rapid activation of progenitors and faster proliferation of blastema cells in animals recently challenged with contralateral amputations. Our results highlight mTOR signaling and innervation as critical mediators of systemic activation, with a vital role in the regenerative process. We also use transcriptomics to identify additional candidate signaling pathways controlling systemic activation and implicate adrenergic signaling as a key regulator of systemic activation. Our work reveals the impact evolutionarily conserved systemic cues play in the regenerative process with implications for our broader understanding of the evolution of regenerative competency.

Systemically-activated cells are primed for future regeneration events

We recently reported systemic cell cycle re-entry, defined to be “activation,” following limb amputation in axolotl, and identified muscle satellite stem cells to constitute a sizeable fraction of these systemically-active cells [5]. We now demonstrate that among the other cells activated in this system are epidermal stem cells, as defined by their basal position in the tissue and expression of Keratin 10 (K10) (Extended Data Fig. 1). We first reported that systemically-activated cells undergo at least one round of cell division, as evidenced by their expression of phosphorylated Histone H3 [5]. We performed a similar single-pulse EdU experiment, but with a long chase (six weeks). This experiment revealed that systemically-activated cells form small clones, typically 2-4 cells in size (Extended Data Fig. 2), indicating that the response is limited to one or two cell divisions.

To determine if systemic cell activation confers a selective advantage by enhancing the regenerative ability of axolotls, we compared regeneration rates of limbs of systemically-activated animals to those of controls (Fig. 1a). Systemically-activated animals, which received contralateral amputations 7 days prior, exhibited faster blastema growth (blastema area) compared to control animals only receiving a single set of amputations (Fig. 1b, c). The limbs of systemically-activated animals differentiate faster than those of controls, entering the palette stage faster, with outlines of the future digits appearing sooner than in controls, and internal cartilage nodules clearly visible (Fig. 1b, d-e) [9]. Systemic activation of cells in limbs contralateral to an amputation primes them for future regeneration events, enabling the activated limbs to initially coalesce a blastema population of progenitors and then also differentiate them more quickly, ultimately leading to faster regeneration.

mTOR signaling is required for systemic activation

The upregulation of mTOR signaling during regeneration and wound healing is conserved across many species such as planaria, zebrafish, mice, and axolotl [5, 10-13]. In planaria, mTORC1 is necessary for proliferation of neoblast stem cells to promote regeneration [11], and a specific subset of neoblasts characterized as “slow-cycling” during homeostasis relies on mTOR signaling to mount a regenerative response [14]. Furthermore, experimental inhibition of mTOR signaling can counteract hyperproliferation of neoblasts in response to amputation caused by RNAi against *smg1*, a PI3-Kinase-related kinase [10]. Zebrafish caudal fin regeneration also requires mTOR signaling for progenitor cell proliferation, although activation of this pathway in tissues or cells distant to amputation has not been examined in detail [12]. In mice, which repair discrete tissues such as muscle but cannot regenerate full appendages, local tissue injury has been demonstrated to lead to systemic changes as distant stem cells are provoked to enter an “alert” phase of the cell cycle characterized by increased nuclear size and genome replication [8]. The “alert” state sensitizes distant stem cells to respond more quickly to similar injuries in the future [8]. The conserved activation of mTOR across regenerative models led us to explore mTOR signaling as a requirement for both local and systemic stem cell activation during limb regeneration in axolotl. We sought to answer this question by inhibiting mTORC1 signaling via rapamycin. Rapamycin-treated animals produced smaller blastemas compared to controls (Extended Data Fig. 3). Treatment with rapamycin wholly blocked cell-cycle activation of stem cells systemically in contralateral limbs (Fig. 2a-b). These findings indicate that mTOR signaling is required for stem cell activation at both the local injury site, as well as at distant locations.

Overlap of cells engaging in mTOR signaling and those actively proliferating in systemically activated limbs is scarce [5], implying that non-cell-autonomous effects may control mTOR mediated activation of stem cells at locations distant to the site of injury. In an attempt to identify the conduit transmitting the essential non-cell-autonomous mTOR signal throughout the axolotl body, we examined mTOR activation elsewhere in systemically-activated animals. We analyzed the brains of systemically-activated axolotls, and found robust overlap between expression of phosphorylated s6 Kinase (pS6), a marker for mTOR activation, and NeuN, a marker post-mitotic neurons [15], within the ipsilateral anterior telencephalon, suggesting significant mTOR activation in this region of the brain (Extended Data Fig. 4), and prompting us to explore the nervous system more generally. We examined mTOR activation in potential neuronal processes upstream and downstream of the brain, with relation to limb, such as local peripheral nervous system ganglia. We found that the dorsal root ganglia, which contain the cell bodies of peripheral sensory nerves, are positive for pS6 signal in homeostasis, and that signal intensity

increases specifically in the DRG serving the amputated limb (Extended Data Fig. 5). These data imply peripheral nerves may be important for the mediating systemic responses.

Nerves drive systemic activation

Nerve input in the stumps of amputated limbs is an essential requirement for limb regeneration in axolotl and newt [16, 17]. It is also a critical local input in other regenerative contexts in many species, such as heart regeneration in zebrafish and neonatal mice [18]. Recently, the nervous system has been shown to mobilize several stem cell niches in mammalian systems [19, 20]. The upregulation of mTOR signaling in both the central and peripheral nervous systems of systemically activated animals led us to hypothesize that nerves may be the potential agent communicating systemic cues and signals across the body of the axolotl post-amputation (Extended Data Fig. 4 and 5). To test this hypothesis, we transected nerve bundles innervating limbs contralateral to amputations and assayed for successful systemic activation in denervated and sham-operated states (Fig. 3a). Nerve transections performed in intact animals did not induce systemic cell cycle re-entry (Fig. 3b-c). Denervation of limbs contralateral to amputations completely impeded the activation of cells in those appendages demonstrating limb innervation is a requirement in appendages for systemic activation (Fig. 3b-c),

Using cell and tissue morphology and location, we classified responding tissues in sham and denervated contralateral limbs to examine the relationship between innervation and cell-type-specific activation. Tissues were classified into three categories: epidermis, skeletal elements, and other internal tissues. Skeletal and internal soft tissues in sham amputated animals displayed a significant increase in EdU+ nuclei compared to intact controls (Fig. 3d-f). Denervated limbs in both intact and amputated states did not exhibit an increase in EdU+ nuclei compared to unamputated controls (Fig. 3d-f). Our results suggest that nerve-mediated systemic activation of distant progenitors is not tissue type specific, indicating peripheral nervous system input is likely required for systemic activation of all limb tissue types in axolotl.

Transcriptomic analyses reveal pathways mediating systemic activation

We next sought to identify candidate factors regulating nerve-mediated systemic cell activation. We performed bulk-tissue RNA sequencing on peripheral nerves, excluding cell bodies, and surrounding tissues in the contralateral limbs of denervated and sham-operated regenerating animals (Fig. 4a). We uncovered 273 differentially-expressed transcripts between intact and transected nerves, and 82 differentially-expressed transcripts between activated and unactivated surrounding tissue samples (Supplementary File 1). Our RNA-seq analysis uncovered many genes with known roles in regeneration and cell proliferation, both in axolotl and other regeneration models. Among the transcripts upregulated in transected nerve ends, we identified hepatocyte growth factor (Hgf), a systemic factor shown to stimulate quiescent distant tissue stem cells [8, 21] and transcripts encoding kazal-type serine protease inhibitor 2 (Kazald2) and von Willebrand factor D and EGF domains (VWDE), both of which are blastema cell markers required for axolotl limb regeneration [22, 23]. In surrounding tissue samples of systemically activated animals, we discovered transcripts encoding PCNA (proliferating cell nuclear antigen) to be more highly expressed compared to denervated limbs in which systemic activation was blocked. Conversely, we found downregulation of transcripts encoding serine/threonine-protein kinase Lats1. Inhibition of Lats1, a negative regulator of YAP1 in the Hippo signaling pathway, promotes proliferation in murine cardiomyocytes and also cells in the inner ear [24].

We then directed our attention to candidate pathways controlling distant cell activation which have not been extensively studied in the context of limb regeneration. We prioritized investigating transcripts of genes known to encode proteins in signaling pathways, with established chemical inhibitors, for further functional study. Among these candidates was α -2A adrenergic receptor (Adra2a), with transcripts 7.4-fold upregulated in transected nerve ends compared to controls. Adra2a activates hepatic stellate cells in mouse hepatocellular carcinoma models [25] and its silencing has been shown to increase cell proliferation in cervical cancer cell lines through inhibition of the PI3K/AKT/mTOR pathway [26]. We explored the potential role of this gene in systemic cell cycle re-entry by inhibiting the function of its encoded protein with yohimbine, a well-studied Adra2a antagonist [27-29]. In animals treated with yohimbine, limbs contralateral to amputation had a significantly lower fraction of EdU+ cells compared to vehicle-treated controls (Fig. 4b-c). Thus, we conclude that adrenergic signaling is required for distant cell activation in response to amputation, and we propose this signal originates in the nerve.

Other candidate pathways we have tested include insulin and Wnt signaling. Our RNA-seq dataset revealed that the expression of canonical Wnt signaling pathway transcription factor lymphoid enhancer binding factor 1 (Lef1) is 5.9-fold increased in severed nerve ends. Intriguingly, Lef1 has recently been shown to prime adult skin to enhance regeneration outcomes in mice [30] and is expressed in the wound epidermis of regenerating zebrafish fins [31]. While Lef1 itself has not been directly studied in the context of axolotl limb regeneration, Wnt signaling has been shown to regulate limb regeneration in axolotl and *Xenopus* and fin regeneration in zebrafish [32]. Surprisingly, the inhibition of Wnt signaling by the tankyrase inhibitor IWR-1 did not block systemic cell cycle re-entry (Extended Data Fig. 6a-b), suggesting that the Wnt requirement may act locally, at the amputation site, in axolotls. We also found transcripts encoding insulin-like growth factor 1 receptor (Igf1r) to be 19.2-fold upregulated in tissues surrounding the nerve in denervated samples compared to sham-operated controls. Igf1r is required for axonal regeneration of retinal ganglion cells from rats, and has been implicated in progenitor activation mechanisms in several regeneration studies [33-35]. Inhibition of Igf1r by an ATP-competitive inhibitor, NVP-ADW742 [35], blocked distant cell activation in limbs contralateral to amputation, indicating that Igf-1 signaling is also required for this process (Extended Data Fig. 6a-b). Taken together, our data suggest that nerve-mediated Adra2a signaling and Igf-1 signaling in the responding cells mediate systemic cell activation in response to amputation injury in axolotl.

Discussion

Limb blastemas form at the site of amputation in salamanders, which has led the field to focus on events occurring at the injury site. Early localized irradiation studies demonstrated that only cells within the remaining limb stump are required to divide for regeneration [36]. While this lends support to a model wherein only cells at the amputation site give rise to the blastema, understanding how cells interpret injury signals in response to amputation is crucial to understanding regeneration. The traditional view postulates that progenitor cells in the injured limb are activated upon amputation, migrate to the tip of the stump, and proliferate to form the blastema. However, our earlier work demonstrating that injury signals activate distant tissue progenitors [5] supports a model where cell activation and blastema cell commitment are two separate events, and predicts that some activated cells do not become blastema cells. This possibility led us to ask whether there could be an advantage to having this system-wide activation that is later refined to the injury site. Here, we show that global activation primes distant progenitors to respond faster in the event of an additional injury (Fig. 4d). This

“jump start” could serve as a selective advantage in salamanders, many species of which lay eggs in high concentrations in small vernal pools [37], leading to dense aggregates of siblings and other conspecifics. Salamanders developing in environments with limited space and resources are cannibalistic [38-41], often resulting in amputation injuries to more than one limb in juveniles. Becoming poised for a future injury upon losing a limb would allow the animal to quickly respond to subsequent loss of another limb, and to produce new functional limbs, which is essential for their survival.

Our work provides mechanistic insights into how injury signals are transmitted to distant locations. The nervous system has recently been implicated in mammalian systems in stem cell mobilization [19, 20] and our work supports the idea that nerves can directly communicate with resident progenitor cells to initiate cell cycle re-entry. Studies of other regeneration models have also implicated circulation as a means to transduce injury signals [8, 13, 21]. While our work does not exclude the possibility that factors circulating in the blood may also transmit injury signals, here we report a role of nerves. Future work will help uncover whether similar signals are also carried through the blood in axolotls. Our findings should prompt a re-evaluation of the role of peripheral nerves in the regenerative process *per se*, including within the amputated limb itself, because it indicates a role for nerves in the primary decision cells make to re-enter the cell cycle rather than simply serving to maintain proliferation of cells that have independently made this choice.

Regeneration of complex body parts is often explained as an all-or-nothing phenomenon for a given species. Yet, evidence that partial responses can occur, such as adult frogs regenerating cartilaginous spikes missing many internal tissues and lacking appropriate patterning [42, 43], and lizards regenerating tails lacking dorsal-ventral polarity and neuronal cell bodies [44-47], challenges this view. Our data regarding systemic cellular activation in axolotl argues this feature may be a component of the regenerative process and that it has a potentially selectable consequence of enhancing future regenerative responses of distant tissues. Combined with previous reports of systemic activation following injury in mice, we argue that a paradigm worth considering is that both species typically classified as non-regenerators and as regenerators engage in a similar initial post-amputation response that activates prospective progenitor cells throughout the body. This systemic response may therefore be ancestral, present in mammals as a vestigial component of a broader regeneration program. In this scenario, perhaps the key critical difference between the two types of animals is their ability to harness this cellular activation at the site of injury, coalescing activated cells into a blastema, and sustaining proliferation there long enough to spur regeneration. These issues have direct implications for development of regenerative medicine approaches because a pool of proliferative progenitors is an essential pre-requisite of coaxing a limb to regenerate without resorting to cellular transplantation. We predict future therapies may harness endogenous stem cells that have been naturally and successfully primed for appendage regeneration but require molecular instruction to transition to a blastema-like state and, once instructed, these cells may organize a response more akin to salamander limb regeneration.

Methods

Animal Experimentation

All animal experimentation was approved by and conducted in accordance with Harvard University's Institutional Animal Care and Use Committee. Leucistic axolotls were used for all animal experimentation except RNA sequencing and maintained as previously described [22]. For RNA sequencing, *βIII tub:GAP43-EGFP* transgenic animals were used [48]. Animals in all experiments were both age and size matched. Animals were anesthetized in 0.1% (w/v) tricaine prior to all procedures involving amputation, denervation, or injection. After all surgical procedures, axolotls were allowed to recover overnight in 0.5% (w/v) sulfamerazine. All limb amputations were performed at the mid-stylopod and the bone was then trimmed back from the amputation plane. For forelimb denervations the brachial plexus nerves were transected according to [49] and for hindlimb denervations the sciatic nerves were transected according to [50]. Sham operations as controls for denervations were performed by anesthetizing animals and cutting the skin open with sterile scissors, locating the nerve bundles with closed sterile forceps, but not cutting the nerves.

Drug treatments and EdU incorporation

Rapamycin was dissolved into a 25 mg/ml stock solution in ethanol. Rapamycin treatments were delivered via both water treatments at a concentration of 1 μM for 16 days with water changed daily, and through daily intraperitoneal injections at a concentration of 5 mg/kg/day for 16 days, both starting two days before amputation. All intraperitoneal injections were administered at a volume of 20 μL/g in a vehicle solution comprised of APBS with 5% Tween80, 5% PEG-400. All stock solutions were frozen at -80 °C in 1-mL aliquots. Yohimbine (Y3125, Sigma-Aldrich) was dissolved into a 10 mg/mL stock solution with sterilized water. Yohimbine was administered daily at a dose of 1mg/kg for 14 days via intraperitoneal injection. A stock solution of IWR-1 (I0161, Sigma-Aldrich) was created by dissolving 5mg/mL in DMSO. IWR-1 was delivered daily at a dose of 10mg/kg/day for 9 days via intraperitoneal injection. A stock solution of NVP-ADW742 (5247, Tocris) was created by dissolving 2mg/mL in DMSO. NVP-ADW742 was injected daily at a dose of 10mg/kg/day for 14 days.

5-ethynyl-2'-deoxyuridine (EdU) was purchased from ThermoFisher and stock solutions were created by dissolving the powder in dimethyl sulfoxide as instructed by the manufacturer. Following anesthetization, axolotls were administered 20 μL/g of 400 μM EdU in 0.7X PBS via intraperitoneal injection 18 hours before tissue harvest.

Skeletal and tissue preparations

For tissue sections, post-collection, tissue samples were fixed in 4% paraformaldehyde in DEPC-treated PBS overnight at 4°C. Tissues were then cryopreserved in a sucrose gradient following a wash in DEPC-PBS and incubated in 30% sucrose/DEPC-PBS at 4°C overnight. Tissues were then embedded and frozen on dry ice in Tissue-Tek O.C.T. Compound (Sakura) and stored at -80°C. All blocks were sectioned at 16 μm thickness using a Leica CM 1950 cryotome and sections were stored at -80°C until use.

For skeletal preparations, post-collection, limbs incubated overnight in 95% ethanol at room temperature and then incubated in 100% acetone under the same conditions. Skeletal elements were visualized with alcian blue and alizarin red S stains followed by a clearing of non-skeletal tissue with potassium hydroxide.

Immunofluorescence

Sections were rehydrated with PBS and then permeabilized with 0.5% Triton-X-100 PBS. Post-permeabilization, slides were rinsed twice with PBS and EdU visualization was performed via sulfo-cyanine 3 azide (Lumiprobe). Post-EdU visualization slides were washed twice with PBS. Staining and labeling with any additional antibodies was conducted post-EdU labeling. Primary antibodies used were rabbit anti-phospho-S6 ribosomal protein (Ser235/236, 1:200; Cell Signaling 4858), mouse anti-NeuN (1:200; Abcam ab104224), and rabbit anti-keratin 10 (BioLegend 905401). Secondary antibodies used were Cy3-goat anti-mouse (1:250, Jackson ImmunoResearch 115-165-146), Alexa Fluor 488-Goat Anti-Rabbit (1:250, Jackson ImmunoResearch, 111-545-003) and Cy3-goat anti-rabbit (1:250, Jackson ImmunoResearch 111-165-003).

Sections stained with all antibodies were blocked with 10% serum from the host species of the secondary antibody in 0.1% Triton-X-100 in PBS. Primary and secondary antibodies were diluted in 2% BSA, 0.1% Triton-X-100 in PBS. Sections labeled with anti-pS6 and NeuN underwent an antigen retrieval step after permeabilization, in which slides were boiled in 0.1 M sodium citrate (pH 6.0) and then rinsed once in PBS, before EdU was visualized, followed by blocking and primary antibody incubation. All slides were incubated with DAPI (Sigma D9542) before cover slipping.

Western blot

Samples were harvested and blinded, protein extracted and western blots performed as in [22]. Primary antibodies used were anti-pS6 (1:2000, Cell Signaling Technology 4858), anti-S6 (1:1000, Cell Signaling Technology 2317S), and α -tubulin (1:2000, Sigma-Aldrich T9026); secondary antibodies used were goat anti-rabbit HRP (1:5000, Jackson ImmunoResearch 111-035-003) and goat anti-mouse HRP (1:5000, Bio-Rad 1721019).

Imaging and quantification

Imaging was performed on a Zeiss Axio Scan.Z1 automated slide scanner, Leica M165 FC equipped with Leica DFC310 FX camera, and a Nikon Eclipse Ni microscope with a DS-Ri2 camera. Quantification of all images was conducted blindly. For imaging of intact contralateral limbs, representative sections displaying all tissue types (bone and cartilage, soft internal tissues, and epidermis) were selected. For the tissue type analysis of EdU+ nuclei, tissues were classified as epidermis, “skeletal elements” (bone, cartilage), and “soft tissues” (muscle, joint, tendon, ligaments, dermis, vasculature, and nerves) based on nuclear morphology and location as previously described (Whited et al, 2013). All cell counts were conducted utilizing CellProfiler [51]. Measurements of blastema area were performed using Fiji [52].

Statistical analysis

Statistical analysis of blastema differentiation was conducted using a Fisher’s exact test where $p < 0.05$ was considered to be significant. For statistical analysis of the number of digit outlines, a Chi-square test comparing no digits, 2 digits and 3+ digits distribution between activated and control groups was utilized in which $p \leq 0.05$ significant. Statistical analysis of the blastema area and length was performed using an unpaired, two-tailed Welch’s t-test, with Holm-Šidák correction for multiple hypothesis testing, and an adjusted p-value < 0.05 was considered significant.

For the quantification of %EdU+ nuclei in the limbs of animals in the denervation, long-chase EdU, and inhibitor experiments the average of both the forelimb and hindlimb were taken and valued as a single data point. Multiple unpaired, two-tailed Welch’s t-tests were used to analyze effect of mTOR inhibition

and denervation, with Bonferroni-Dunn correction for multiple hypothesis testing. For the remaining experiments, a Welch's ANOVA was used to compare more than 2 groups, or an unpaired two-tailed Welch's t-test, used to compare exactly two groups, were conducted assuming unequal variance. The ANOVA was conducted under the parameters of post-hoc pairwise t-tests with Dunnett T3 correction for multiple hypothesis testing. For all statistical analyses an adjusted-p-value < 0.05 was considered significant.

RNA isolation, sequencing, and computational analysis

βIII tub:GAP43-EGFP transgenic animals were used for RNA sequencing experiments to allow for easily identifiable collections of tissues [48]. RNA was extracted from tissue samples with Trizol (15596018, Ambion) and chloroform (288306, Sigma Aldrich). RNA was then isolated from tissue samples using a Zymo Clean Up and Concentrator kit (R1013). RNA samples were processed at the Bauer Core Facility at Harvard University using the Illumina NextSeq 500. Paired End reads were demultiplexed with bcl2fastq2 v2.2.

The reads were trimmed for quality and adapters with Trimmomatic v0.39 [53] with the following parameters: LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36. An index of the previously published axolotl transcriptome [22] was created using Kallisto v0.45.1 [54]. Sequence based bias correction was performed using Kallisto with 200 bootstraps across 20 threads. The transcript-level abundances from Kallisto were summarized and imported into R as a matrix of counts using tximport v1.0.3 [55].

Differentially expressed genes were determined by estimating fold change and dispersion using DESeq2 [56]. Genes were considered significant if they had a logarithmic fold change greater than 1.5 and adjusted p value of 5%. These significant genes were visualized using pheatmap v1.0.12 [57]. Pathway analysis was performed in R using pathfinder [58].

Data availability

The sequencing data associated with this study have been deposited in the Gene Expression Omnibus (GEO) with the accession code GSE192477.

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Author Contributions

J.L.W., D.P.-D and S.E.W. conceived of the project. D.P.-D, S.E.W., B.E., S.H., J.L., J.V.M, and J.L.W. designed the experiments; D.P.-D, S.E.W., and J.L.W. wrote the paper; S.J.B. performed computational analyses; L.C. performed statistical analyses; D. P.-D., S.E.W., B.E., S.H., J.V.M., A.W., J.L., S.M and H.S. performed sample preparation, experiments, and experimental data analyses. All authors contributed to manuscript editing.

Competing Interests

J.L.W. is a co-founder of Matice Biosciences. Other authors declare no conflicts of interest.

Figures

Figure 1. Systemically-activated cells are primed for future regeneration events.

a, Experimental design. We compared regeneration rates of systemically-activated and naïve, control limbs. Pink dots, activated, EdU positive nuclei. **b**, Representative images of regenerating systemically-activated or control limbs. **c-e**, Quantification of blastema area at 14 days post-amputation (**c**), differentiating limbs (**d**), and number of digit outlines (**e**) at 25 days post-amputation (n=15 animals for each condition in blastema area measurement, analyzed by two-tailed Welch's t-test, n = 30 limbs for each condition in differentiation and digit outline quantification, analyzed by Fisher's exact test for differentiation and Chi-square test for digit outlines). All data shown as mean $\pm$ SD. Scale bars, 2 mm. dpa, days post-amputation. EdU, 5-ethynyl-2'-deoxyuridine.

Figure 2

Figure 2. mTOR signaling is required for systemic activation. **a**, pS6 and EdU staining of blastema, intact (homeostatic) and contralateral limb tissue sections from animals treated with saline, vehicle, or rapamycin at 14 dpa. **b**, Quantification of EdU positive nuclei at 14 dpa. (n = 12 limbs for intact saline, intact vehicle and intact rapamycin groups, n = 10 limbs for amputated saline and amputated vehicle, and n = 8 limbs for amputated rapamycin groups, analyzed by unpaired two-tailed Welch's t-tests with Bonferroni-Dunn correction). All data shown as mean $\pm$ SD. Scale bar, 500 μ m. rapa, rapamycin. Int, intact. contra, contralateral. dpa, days post-amputation. pS6, phospho-S6 ribosomal protein; EdU, 5-ethynyl-2'-deoxyuridine.

Figure 3

Figure 3. Nerves are required for systemic activation. **a**, Experimental design. Nerve bundles innervating limbs contralateral to unilateral amputations were transected or sham-operated, and animals were assayed for systemic activation. Animals receiving unilateral denervations or sham-operations with no amputations were used as controls. **b**, EdU and DAPI staining of intact and contralateral limb tissue sections of denervated and sham-operated animals in the intact state or at 14 days post amputation. **c-f**, Quantification of percent EdU positive nuclei in denervated and sham-operated limbs (**c**), epidermis (**d**), skeletal elements (**e**), and soft internal tissues (**f**) at 14 dpa. (n = 11 animals for sham, n = 10 animals for denervated, n = 13 animals for sham amputated and denervated amputated, analyzed by unpaired two-tailed Welch's t-tests with Bonferroni-Dunn correction). All data shown as mean $\pm$ SD. Scale bar, 500 μ m. den, denervated. int, intact. contra, contralateral. EdU, 5-ethynyl-2'-deoxyuridine.

a

b

c

d

Figure 4

Figure 4. Transcriptome analyses reveal pathways regulating systemic activation. Nerves servicing limbs contralateral to unilateral amputations were transected or sham-operated. Nerve ends and the immediate tissue surrounding them were collected 14 days post amputation. **a**, Differential gene expression analysis identifies transcripts that are enriched in nerve ends (left) and responding tissues (right). **b**, EdU and DAPI staining of limbs contralateral to unilateral amputations treated with yohimbine or vehicle control, harvested at 7 days post amputation. **c**, Quantification of percent EdU positive nuclei in contralateral limbs in **(b)** ($n = 15$ animals for each group, analyzed by unpaired two-tailed Welch's t -test). All data shown as mean $\pm$ SD. Scale bar, 500 μ m. EdU, 5-ethynyl-2'-deoxyuridine. **d**, Model summarizing the main findings of the study. Limbs activated by a prior contralateral amputation regenerate faster than naïve, control limbs. This effect is mediated by nerves, mTOR signaling, and adrenergic signaling.

Extended Data Figure 1

Extended Data Figure 1. A subset of activated cells are epidermal stem cells. Keratin 10, EdU and DAPI staining of limbs contralateral to amputation harvested 14 days post amputation, 18 hours after EdU administration. Data shown as mean $\pm$ SD. Scale bars, 200 μ m. K10, keratin 10. EdU, 5-ethynyl-2'-deoxyuridine.

Extended Data Figure 2

Extended Data Figure 2. Activated cells in distant limbs form clones. **a**, EdU and DAPI staining of intact limbs (left) and limbs contralateral to amputation (right) harvested 6 weeks after EdU administration. **b**, Quantification of percent EdU positive nuclei of intact and contralateral limbs. (n = 15 animals for intact and n = 12 animals for contralateral group, analyzed by unpaired two-tailed Welch's t-test). Data shown as mean $\pm$ SD. Scale bar, 500 μ m. EdU, 5-ethynyl-2'-deoxyuridine.

Extended Data Figure 3

Extended Data Figure 3. Rapamycin-treated animals produce smaller blastemas.

a, Regenerating limbs at 7 dpa treated with rapamycin or vehicle control. Arrowheads mark the amputation plane. **b**, Blastema growth curves of rapamycin or vehicle treated animals. (n = 10 animals per group, analyzed by unpaired two-tailed Welch's t-test with Holm-Šídák correction). **c**, Representative skeletal preparations of regenerated limbs with Alcian blue and Alizarin red at 10 weeks post amputation. Scale bars, 0.5 mm.

Extended Data Figure 4

Extended Data Figure 4. mTOR signaling is upregulated in the brains of animals with regenerating limbs. pS6 and NeuN staining of anterior telencephalon sections of animals with intact (left) or regenerating (right) limbs harvested at 7 days post amputation. Scale bar, 200 μ m. pS6, phospho-S6 ribosomal protein. NeuN, neuronal nuclear protein.

Extended Data Figure 5

Extended Data Figure 5. mTOR signaling is differentially regulated in the dorsal root ganglia of sensory nerves innervating the regenerating limb. **a**, pS6 and NeuN staining of dorsal root ganglia servicing intact, regenerating, and contralateral limbs at 7 days post amputation. **b**, Western blot of pS6 and total S6 protein levels in dorsal root ganglia servicing regenerating and contralateral limbs harvested at 3 days post amputation. Alpha-tubulin serves as loading control. Lanes with same numbers correspond to dorsal root ganglia harvested from the same animal. Scale bar, 200 μ m. pS6, phospho-S6 ribosomal protein. NeuN, neuronal nuclear protein.

a

b

Extended Data Figure 6

Extended Data Figure 6. Test of additional candidate pathways regulating systemic activation. **a**, EdU and DAPI staining of limbs contralateral to unilateral amputations treated with tankyrase inhibitor IWR-1, Igf1r inhibitor NVP-ADW742 or vehicle control, harvested at 7 days post amputation. **b**, Quantification of percent EdU positive nuclei of contralateral limbs in **(a)**. (n = 11 animals for vehicle control, n = 14 animals for IWR-1 and NVP-ADW742, analyzed by Welch's ANOVA with Dunnett's T3 correction, W = 4.883). All data shown as mean $\pm$ SD. Scale bar, 500 μ m. EdU, 5-ethynyl-2'-deoxyuridine.