Published in final edited form as:

Science. 2018 April 27; 360(6387): 404–409. doi:10.1126/science.aap8179.

Self-organization and progenitor targeting generate stable patterns in planarian regeneration

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Abstract

During animal regeneration cells must organize into discrete and functional systems. We show that self-organization, along with patterning cues, govern progenitor behavior in planarian regeneration. Surgical paradigms allowed manipulation of planarian eye regeneration in predictable locations and numbers, generating alternative stable neuroanatomical states for wild-type animals with multiple functional ectopic eyes. We utilized animals with multiple ectopic eyes and eye transplantation to demonstrate that broad progenitor specification, combined with self-organization, allows anatomy maintenance during regeneration. We propose a model for regenerative progenitors involving (i) migratory targeting cues, (ii) self-organization into existing or regenerating eyes, and (iii) a broad zone, associated with coarse progenitor specification, in which eyes can be targeted by progenitors. These three properties help explain how tissues can be organized during regeneration.

The capacity to regenerate is widespread and variable throughout the animal kingdom. There is much current interest in understanding how regenerative progenitors re-form missing organs and tissues. Complex tissue architectures can be formed through an adaptive, non-linear, and dynamic process called self-organization, described by the emergence of orderly structure through local interactions between the basic elements of a system (1, 2). In self-organizing systems, ordered patterns emerge from an initially disordered and noisy environment. Selforganization has been invoked in embryonic development (3–9), regeneration (10, 11), and in the *in vitro* formation of complex structures such as the optic

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Author Contributions: K.D.A. and P.W.R. conceived of, designed, and interpreted experiments, performed all experiments, acquired and analyzed data, and wrote the manuscript. S.A.L. and K.D.A. designed and performed behavior assays. T.D.H. developed a gel encasement protocol that was later used in eye transplant procedures.

Competing interests: The authors have no competing interests.

Data and materials availability: All data is available in the manuscript or the supplementary material.

cup (12) and organoids (13, 14). To explore the role of self-organization in regeneration, we investigated the ability of planarians to completely regenerate missing organs.

Planarians are flatworms with a remarkable ability to regenerate complex tissues, such as their centralized nervous system. Planarians also continually renew all cells in their bodies via cellular turnover, involving dividing cells called neoblasts, which include pluripotent stem cells (15) and diverse specialized neoblasts that are progenitors for different cell types. Specialized neoblasts can be specified in broad spatial domains and produce migratory progenitors that converge and differentiate at defined target locations, such as the eyes (Fig 1A) and brain (16–18).

Transient discordance between anatomy and positional information in regeneration

As a target tissue for studying self-organization in regeneration, we selected planarian eyes. Planarians have two eyes comprised of photoreceptor neurons, which project axons ipsilaterally and contralaterally to the brain, and pigmented optic cup cells (17) (Fig. 1A). Because the eyes are visible and dispensable for animal viability, they are amenable to extensive manipulation for regeneration studies. Eyes are located at two predictable bilaterally symmetric positions relative to the existing body size and are regenerated and maintained during tissue turnover from *ovo*⁺ eye-specific progenitor cells (16, 17, 19) (Fig. 1A, B; fig. S1A; fig. S2A). *ovo*⁺ progenitors are formed coarsely in the dorsal pre-pharyngeal region and migrate anteriorly where they can incorporate into existing eyes or nucleate eyes *de novo* (17) (fig. S1A–D).

Understanding how progenitors are targeted to a specific location and organize into new tissues is central to understanding regeneration. We realized that progenitors can face conflicting targeting choices during regeneration, and that understanding how this dilemma is resolved could explain key regeneration principles. Synthesis of two pieces of information reveals the existence of these conflicting choices. First, restoration of full anatomy and proportions in planarian regeneration involves both blastema formation (new tissue growth at wounds) and substantial changes (called morphallaxis) to the remaining body fragment itself (20). Morphallaxis involves new cell production, cell death (21), and the remodeling of differentiated tissues (22). During morphallaxis, existing organs and major tissues are retained, but gradually change their proportions and relative positions (Fig. 1C, D). Whereas new tissue formation in a blastema occurs within days, morphallaxis can take up to several weeks (depending on degree of proportion resolution needed and nutrient status). For example, after decapitation, the eyes and brain of a head fragment gradually (weeks) shrink and move anteriorly, while continuously maintaining their form and function.

Second, regenerating correct planarian anatomy depends upon regionally and constitutively expressed genes called position control genes (PCGs), which are proposed to act in muscle to establish adult positional coordinates (23, 24). Inhibition of multiple PCGs (such as *bmp4*, *wntP-2*, and *ndl-3*) by RNA interference (RNAi) causes patterning phenotypes, such as ectopic heads, pharynges, or eyes (23–26). After amputation, PCG expression domains are rapidly re-established in muscle (in a matter of 2–3 days). For example, within 48 hours

following decapitation, a head fragment initiates posterior PCG expression at the wound and anterior PCG expression domains shift towards the head tip. Initial PCG expression domain regeneration does not require neoblasts (23) and precedes new differentiated tissue generation.

PCG expression domain regeneration upon amputation proceeds faster - by days-to-weeks - than do changes to the scale and position of existing differentiated anatomy during morphallaxis. Therefore, there is a substantial period of mismatch between PCG expression patterns and pattern of underlying anatomy in planarian regeneration (Fig. 1E, fig. S3A–E). For example, for several days during regeneration of head fragments, eyes and brain are mispositioned with respect to PCG expression domains, but are located in the correct position with respect to remaining differentiated tissues (Fig. 1E, fig. S3A–E). Eyes slowly shrink and move anteriorly (morphallaxis) to ultimately align anatomy and PCG expression domains (Fig. 1, C, D). Regardless of whether PCGs directly control this process, their expression changes indicate rapid positional information shifting during regeneration leads to its discordance with anatomy (Fig. 1E, fig. S3A–E). Because progenitors continuously target the eye during tissue turnover (17), we presume that eye progenitors are incorporated into existing eyes during morphallaxis. These factors together suggest the targeting choice dilemma that regenerative progenitors must resolve: should the cells target their "correct" anatomical location or their "correct" position with respect to positional coordinates during this period of positional information - anatomy discordance in regeneration?

Dynamic positional coordinates guide regenerative progenitor targeting

We refer to the position where progenitors nucleate in *de novo* organ/tissue formation as the "target zone" (TZ). We assessed targeting decisions of eye progenitors by amputating heads and then unilaterally resecting one eye after three days (Fig. 1F). Prior work demonstrated that eye resection does not cause eye progenitor amplification, with eye regeneration occurring as an emergent property of constant progenitor production and progenitor-target equilibrium dynamics (19). Therefore, we anticipated that eye progenitors would form new eye cells at equal rates on both sides of these head fragments. Does the regenerating eye nucleate at the original, correct anatomical location or does it form at the new, correct position with respect to shifting positional coordinates (Fig. 1F)? As is predicted by a model in which positional information guides progenitors and shifts early in regeneration, *de novo* eye nucleation occurred at more anterior locations than the position of the remaining eye - generating asymmetric animals (Fig. 2A, fig. S4A, B). The original and regenerating eyes were both targeted by progenitors, which originated posterior to the eyes, demonstrating that eye progenitors targeted two different locations in these head fragments (Fig. 2B, fig. S4).

We predicted that if positional coordinates were shifted in different directions, different asymmetric eye configurations would emerge. We tested this prediction by removing the anterior head tip, which shifts positional information posteriorly (fig. S3C), and unilaterally resecting eyes 3 days later. Accordingly, regenerating eyes shifted posteriorly (Fig. 2C). These data indicate that rapidly changing positional information following injury can define the eye progenitor TZ.

Eyes act as self-organizing centers and can trap migratory eye progenitors

In the experiments above, why didn't eye progenitors go to their correct position with respect to positional information on the side with the remaining eye? We postulate that existing eyes act as stable attractors and "trap" migrating progenitors (Fig. 1A, fig. S5A), preventing them from reaching more anterior positions. In self-organizing biological systems, molecular coupling strategies lead to maintained, functional anatomical architectures (27). Self-organizing systems act as attractors in such cases, leading to the stability of shape and function. To test if existing eyes behave as attractors, we decapitated animals and, after a delay, partially resected eyes. Even a small amount of remaining eye tissue was sufficient to prevent incoming eye progenitors from reaching their new TZ, resulting in eye regeneration at the original eye location (Fig. 2D).

We hypothesized that if eyes act as attractors and have limited or local attractive boundaries, moving the migration path of incoming eye progenitors outside of such a boundary could cause *de novo* nucleation of an ectopic third eye (fig. S5B). Parasagittal amputation, lateral to an eye, combined with unilateral eye resection, led to medially shifted eye regeneration (fig. S5C). This raised the possibility that combining decapitation with parasagittal amputation in large animals would allow progenitors to medially evade the attractive boundary of an eye, like light escaping an event horizon. We tested this possibility (also resecting right eyes 3 days post-amputations). As predicted, animals formed a third eye, located anteriorly and on the same side (left) as the uninjured eye (Fig. 3A, B, fig. S5D). All three eyes were targeted by eye progenitors (Fig. 3C–E, fig. S5E) and third eyes extended projections into the visual circuitry (Fig. 3F). In this injury context, eye progenitors arrived at two different locations on the same side - the original eye and a more anterior location.

We also decapitated and concurrently inflicted parasagittal amputation on three-eyed animals (from Fig. 3A), generating four and five-eyed animals, with all eyes integrated into visual circuitry (Fig. 3G, fig. S5G, H). We tested misplaced eye function by examining three-eyed animals (from Fig. 3A) - misplaced eyes were alone sufficient to drive negative phototaxis in a light-graded arena (Fig. 3H, fig S8A–C, movies S1–4).

Eye progenitors can nucleate in a stable position during dynamic positional information shifting

In the three-eyed animals generated above, we noticed that the third eye (the anterior left eye Fig. 3A) was always anterior to the regenerated right eye. This third eye also regenerated later than the right eye did (fig. S5F); we hypothesized that this delay might explain the relative anterior-posterior (AP) eye positions. Specifically, the TZ might have shifted further anteriorly, after right-eye nucleation by the time the third (medial-left) eye nucleated. This could occur if the uninjured left eye trapped progenitors past the new right eye nucleation time point (fig. S5F). This interpretation predicts that the TZ location moves gradually and continually following amputation - eye position would depend on its time of nucleation post-amputation. To test this possibility, we decapitated animals and unilaterally resected right eyes on subsequent days (day 1–6). *de novo* eye nucleation occurred at progressively more anterior locations depending on the time of eye resection post-decapitation (Fig. 4A). This

indicates that eye TZ rescaling is a continuous process following injury and that eye regeneration occurs wherever the shifting TZ is located when eye progenitors first nucleate. We further propose that nucleated eyes became fixed in location, growing and incorporating progenitors even when the TZ continued to move away from this position. This self-organizing process would ensure that progenitors do not form a long trail of differentiated cells deposited along a moving target front, but as discrete, organized structures, even if the structure forms at a position that is ultimately incorrect.

Consistent with this model, shifting injury timing yielded predictable eye positioning. We resected right eyes on days 1, 3, or 5 post-decapitation and left-side parasagittal amputation. As predicted, the later (day 5) right-eye resection group displayed roughly equal positioning of the regenerating right and the ectopic left eyes (Fig. 4B, C). We also observed formation of two eyes on the right side in the day 1 right-eye resection group, leading to four-eyed animals with ectopic, anteriorly shifted left and right eyes (Fig. 4B). The ectopic right eye was always less anterior than the ectopic left eye. This four-eye configuration can be explained with the conceptual model described above: eye progenitors read their TZ at any particular time-point during positional coordinate shifting. After day 1 resection, a new right eye nucleates close to its original location. Note that because a head fragment is much smaller than the original animal, the theoretically correct eye positions should be more medial on both sides (fig. S6A). Because of coordinate rescaling, migrating progenitors ultimately medially escape the attractive nature of both the left (uninjured) and the newly forming right eye. Because the first-formed right eye is small, progenitors escape its influence before progenitors on the left can escape the influence of the non-resected eye. Therefore, the second-formed right eye will nucleate less anteriorly than the ectopic left eye.

Understanding eye progenitor targeting dynamics allowed even further predictable anatomy changes by simple implementation of injury type and timing. Decapitation and eye resection did not lead to anterior shifting of the brain. By contrast, decapitation combined with sagittal amputation 3 days later caused both the brain and the eye to form more anteriorly on the regenerated side. These animals also generated an anterior third eye on the uninjured side (fig. S7A, B). These findings suggest similar self-organizing principles are at play for the brain as well as the eye, and that the position of the eye and the brain can be decoupled.

Molecular nature of the target zone

We next explored the molecular attributes of the TZ with PCG RNAi. Medial-lateral (ML) planarian patterning involves *slit* (28). The *slit* medial expression domain is restricted by laterally expressed *wnt5* (29). *wnt5* and *slit* RNAi can impact ML eye formation (30). Following *wnt5* RNAi, serial eye nucleation was observed, progressing laterally as *slit* expression boundaries expanded (Fig. 5A, B). Unilateral eye resection in *wnt5* RNAi animals, before ectopic eye appearance, resulted in the eye regenerating laterally, indicating that existing eyes can locally influence ectopic lateral eye formation (Fig. 5B). Eye progenitors normally move from posterior to anterior. We therefore posit that, unlike the case of AP TZ movement (such as in wild-type head fragments), ML TZ movement allows new, serial eye nucleation without existing eyes "shielding" the new TZ (fig. S9A). Additionally, *wnt5* and *slit* RNAi affected both the ML migration and specification pattern

of eye progenitors (fig. S10A–C). These results implicate a *slit-wnt5* circuit (acting directly or indirectly) as an ML TZ determinant.

notum encodes a broadly conserved Wnt inhibitor (31) expressed near the anterior brain and in the anterior pole, and is involved in anterior tissue patterning (25, 31). *notum(RNAi)* animals develop a set of much more anterior and medial eyes under homeostatic conditions (25). We postulated that this anatomical pattern can be explained using the model described above for experiments in wild-type animals. Specifically, we postulate that *notum* RNAi leads to progressive anterior TZ movement, but eyes do not appear anteriorly initially because of the attractive influence of remaining eyes. Ultimately, with sufficient TZ anterior movement, arcing medially, eye progenitors could escape existing eyes and nucleate new eyes (similar to Fig. 3A). As predicted by this model, unilateral *notum(RNAi)* eye resection, before ectopic anterior eye appearance, resulted in anteriorly shifted eye regeneration. A third eye, anterior to the intact eye also later formed in these animals as predicted by the model (Fig. 5C).

A broad targetable zone enables maintenance of anatomy in incorrect locations

We next examined what happens to the extra eyes formed by the surgical manipulations described above. We fed three-eyed wild-type animals (as in Fig. 3A), allowing them to grow and undergo long-term tissue turnover. All eyes remained (Fig. 6A). Therefore, these wild-type animals now stably maintained an alternative and functional anatomical state. All three eyes incorporated new progenitors (early-stage: Fig. 3C–E; late-stage: fig. S11A). Because eyes were maintained in their incorrect anatomical positions, we hypothesized that there exists a “targetable zone” (TAZ), where a mis-positioned eye can be maintained because of its self-organizing nature. We define the TAZ as the region where regenerative progenitors are capable of going to maintain or regenerate an organ/tissue. The TAZ includes the TZ, but when larger than the TZ it allows targeting to self-organizing centers in incorrect (non-TZ) locations. Target zone and targetable zone concepts make testable predictions. We first asked if eyes would be regenerated in three-eyed animals by selective eye resection. The original left eye in these animals never reached its correct position (the TZ) during morphallaxis, presumably because the second, more anterior left eye occupied this position. Despite being an original, normal eye, we hypothesized that this posterior eye should not regenerate upon resection, because in its absence as an attractor progenitors should go to the TZ. Indeed, resected posterior eyes did not regenerate, whereas resected anterior eyes (in the TZ) did (Fig. 6B). Supernumerary eyes can rarely appear spontaneously during errors in asexual planarian reproduction, and consistent with the above data, these supernumerary eyes do not regenerate following removal (32).

We postulated that the region where eye progenitors are specified approximates the TAZ. Indeed, mapping *ovo*⁺ eye progenitors from many uninjured animals showed that the eye progenitor specification zone is regional (in the anterior), but coarse spatially (from eyes to near the centrally located pharynx) - much broader than the location of the eye itself (Fig. 6H). We propose that this broad eye progenitor specification zone explains the TAZ: an eye

in this region would have access to eye progenitors and through its self-organizing properties could maintain itself, allowing alternative anatomical states to be indefinitely maintained.

To further test the TAZ concept, we developed eye transplantation strategies (Fig. 6C–F, fig. S11B–F). Transplanted eyes in the anterior were maintained, whereas eyes transplanted into tails shrank and ultimately disappeared (Fig. 6E, F, fig. S11B, D). Transplanted eyes sent projections towards their targets in the brain (Fig. 6D). Transplanted eyes also had neighboring cell types (fig. S11C); understanding how eye cells directly or through neighboring cells can attract eye progenitors in the TAZ will be an interesting future direction. After asexual fissioning, transplanted eyes in the posterior were maintained, presumably because positional information resetting placed them in a new TAZ (fig. S11E).

We also utilized RNAi phenotypes to study the TAZ concept. *nou darake(RNAi)* animals generate ectopic posterior eyes (33). After removing these animals from RNAi conditions, all eyes remained. Unilateral eye resections were performed after removal from RNAi. On the eye-resected side, only one eye regenerated, occurring at the wild-type eye location (Fig. 6G). On the contralateral side, all ectopic eyes were maintained. Similarly, unilateral eye resections were performed in *wnt5(RNAi)* animals, in this case under long-term RNAi (fig. S12A). As predicted by TAZ/TZ concepts, only the lateral-most eye regenerated (a lateral-shifted TZ) whereas all eyes were maintained on the non-resected side. SMEDWI-1 labeling of *wnt5(RNAi)* animals removed from RNAi conditions showed both medial and lateral eyes were targeted by progenitors (fig. S12B, C). Similarly, *slit(RNAi)* animals off RNAi also maintained medial eyes (fig. S12D). Finally, *notum(RNAi)* animals showed anterior eye regeneration following eye resection (Fig 5C), yet multiple eye sets were maintained during long-term RNAi (fig. S12E). These findings demonstrate continuous progenitor targeting to eyes in a variety of locations (the TAZ) using RNAi phenotypes.

Discussion

We propose a model based on findings described above for the properties governing the behavior of migratory, mesenchymal eye progenitors in regeneration (Fig. 6H). The model involves three key components: (i) an extrinsic target zone (different than the anatomical structure itself) that guides regenerative progenitor migration; (ii) self-organization, in which progenitors can be stably incorporated into existing anatomy, even if this is at the “wrong” location; and (iii) a targetable zone, involving a much broader progenitor specification zone than the target location for those progenitors. These three properties yield a systems level process that can explain how new tissues and organs are formed and maintained in noisy and continuously dynamic conditions, such as changing positional coordinates following injury. The coarse progenitor specification zone is essential, allowing progenitors to maintain organs/tissues that remain entirely or partially present after injury, as opposed to duplicating them in new locations. For example, if progenitor specification occurred very locally, near the TZ, reiterated structures could be deposited as this location shifted during positional rescaling (Fig. S13B). Without the targetable zone and self-organization properties, anatomy would get scrambled as progenitors targeted shifting positional coordinates after injury (Fig. S13B). Replacement of completely missing structures is guided by externally acting positional cues defining target zones at the appropriate position for final anatomy. If target

zones were not discretely defined, anatomy duplications would also emerge (Fig. S13B). This model identifies a set of rules that are employed for stabilizing the system and its coherent functional properties. The existence of adaptive target and targetable zones could be a broadly used strategy by diverse developmental and regenerative processes, to form and maintain complex self-organizing modules in appropriate relative positions. These findings explain principles guiding coherent tissue formation and maintenance in dynamic and noisy biological processes such as regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank all Reddien Lab members for valuable discussions and I. Oderberg for *wnt5* RNAi discussions. Funding: We acknowledge NIH (R01GM080639) support. K.D.A. is supported by a HHMI International Student Research Fellowship and the MIT Presidential Fellowship Program. S.A.L. was supported by an NDSEG Fellowship. P.W.R. is an HHMI Investigator and an associate member of the Broad Institute of Harvard and MIT.

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Fig. 1. Discordance between positional coordinates and anatomy during regeneration

(A) Eye progenitors emerge from neoblasts in a broad specification zone and migrate anteriorly to form eyes. (B) Eye nucleation after unilateral eye resection. (C) An amputated head fragment morphallaxes slowly, with eyes moving anteriorly, matching final animal proportions over time (30 days shown). (D) Eye positioning occurs relative to existing body size. Head fragments slowly morphallax to resolve proportions. (E) Positional control gene expression in head fragments (*ndl-2* and *wntP-2* FISH) rescales before anatomy changes occur (white arrowheads: brain posterior). (F) Positional information pattern regenerates faster than do anatomical changes. Experimental strategy to reveal a predicted target zone (nucleation target) shift in a decapitated and day 3 eye-resected head fragment.

Fig. 2. Planarian eyes act as attractors and re-nucleate at predictable positions

(A) Resected eyes on day 3 after decapitation nucleate more anteriorly. (B) Eye progenitors (SMEDWI-1+ (19)) target both non-resected eyes and regenerating eyes at different positions in the same animal (arrowheads). (C) Resected eyes on day 3 after a head-tip cut nucleate more posteriorly. (D) Partially resected eyes prevent anterior eye nucleation. ER, eye resection. Red arrowheads: regenerating eyes. (F, H and I) Student's t-test: ** $p < 0.01$, *** $p < 0.0001$). Bars, 200 μm .

Fig. 3. Generation of an alternative stable neuroanatomical state

(A, B) Decapitation and parasagittal amputation in large sexual animals plus day 3 unilateral eye resection results in 3-eyed animals. (C) Map of *ovo*⁺ cells from 20 3-eyed animals (day 16 post-surgery). (D, E) SMEDWI-1⁺/*opsin*⁺ newly differentiated cells were detected in all three eyes (n=144 examined). (F) Arrestin immunohistochemistry and *opsin* FISH: ectopic eyes integrate into visual circuitry. (G) Decapitation and right or left parasagittal amputations on 3-eyed animals resulted in 4 or 5-eyed animals. (H) Behavior analyses: misplaced eyes drive negative phototaxis (CA; control arena). Statistical significance: one-sample t-test comparing each column mean with a hypothetical value of 6 corresponding to chance (n=8/cohort); Bonferroni correction was applied. Bars: A, D, E and G, 200μm; B, C and G, 100μm. ER, eye resection. Red arrowheads: regenerating eyes. Statistical significance: one-way ANOVA: *p 0.05, **p 0.01, ***p 0.001, ****p 0.0001; NS, not significant.

Fig. 4. Eyes nucleate at predictable positions during dynamic positional information shifting
(A) Unilateral eye resection time course (days 1–6 post-decapitation) reveal a continuously shifting TZ. **(B, C)** Unilateral eye resections following decapitation and parasagittal amputation. Red arrowheads: regenerating eyes. Statistical significance: one-way ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; NS, not significant.

Fig. 5. *slit*, *wnt5*, and *notum* are involved in establishing a target zone (TZ)

(A) *wnt5*, *slit*, and control RNAi in uninjured animals (> 8 dsRNA-feedings). FISH: *slit* expression expansion and reduction following *wnt5* and *slit* RNAi, respectively. (B) Unilateral eye resections after *wnt5*, *slit*, and control RNAi reveal the local attractive nature of eyes, preventing *de novo* eye nucleation and a mediolateral TZ shift. (C) Unilateral eye resection following *notum* RNAi led to anteriorly shifted eye nucleation without decapitation implicating *notum* in AP TZ regulation. Red arrowheads: regenerating eyes. Asterisks: ratio of animals with outcome as shown. Bars, 200 μ m.

Fig 6. Ectopic eyes are maintained in a broad targetable zone (TAZ)

(A) Long-term feeding of wild-type 3-eyed animals: all eyes are stably maintained. (B) Selective eye resections reveal a targetable zone (TAZ) where ectopic eyes can be maintained but not regenerated (white arrows). (C) Eye transplantation strategy into or outside of the TAZ. Donor animals were irradiated to lack progenitors. (D) Transplanted eyes sent projections into the visual circuit. (E) Transplanted eyes (arrows) were maintained in the targetable zone, but not outside of it (tail). (F) Only TAZ-transplanted eyes incorporated progenitors (SMEDWI-1⁺/opsin⁺ cells, analyzed 12–16d post-transplantation) and SMEDWI-1⁺/opsin⁺ cell quantification (**p < 0.01). (H) *ndk(RNAi)* animals, off RNAi, with unilateral eye resection. Only one eye regenerated (in TZ); non-resected eyes remained. (H) Eye progenitor specification zone by mapping *ovo*⁺ cells in 13 animals and generalized model for progenitors integrating self-organization, a target zone, and a targetable zone. Red arrowheads: newly specified migratory progenitors and an example for system-level behavior predicted by the model in response to a regenerative challenge. Red arrowheads (except in E): resected and regenerating eyes. Bars, 200µm.