Mechanisms of Self-organization in Planarian Regeneration

by

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Submitted to the Department of Brain and Cognitive Sciences
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY IN NEUROSCIENCE

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

June 2019

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ABSTRACT

There is an unbreakable link between shape and function. In biology, the architecture of cells, tissues and organisms, that have evolved adapting to the world around them, translate into specific functional outcomes. Self-organization is an adaptive, non-linear and dynamic process, where diverse ordered patterns emerge from an initially disordered and noisy state through local interactions between the elements of a system. This can lead to the fascinating biological diversity and functional complexity in such systems. Unwavering storms on the surface of Jupiter, patterns on the wing of a butterfly, a regenerating planarian eye, development of a neuronal circuit in the human brain can all be studied systematically using the conceptual tools derived from the field of self-organization.

Here, I sought to address a central, but understudied, problem in animal regeneration: How do regenerative progenitors organize into complex replacement structures in the context of adult anatomy? I used the planarians as a system for studying regenerative progenitors and focused on eye regeneration to elucidate the mechanisms. I found that self-organization has a major role in determining the behavior of regenerative progenitors. This work revealed three properties that govern regenerative progenitor behavior, and these three properties in concert explain many previously mysterious aspects of how regeneration works: (i) self-organization, (ii) an extrinsic migratory target for progenitors, and (iii) a broad progenitor specification zone that allows progenitors to be targeted into self-organizing systems even if they are transiently in incorrect locations during the process of regeneration. These components yield a model with broad explanatory and predictive power. As an example, we were able to generate wild-type animals with 3, 4, or 5 eyes instead of 2 by simple manipulations of the system using the model developed. Remarkably, the extra eyes were stably maintained throughout the life of the animal, resulting in wild-type animals with an alternative and stable anatomical state. This model prominently incorporates self-organizing principles, which have been little explored in regeneration. The new conceptual model with broad explanatory power allowed us to address some of the fundamental previous mysteries of regeneration.

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ACKNOWLEDGEMENTS

In life, out of a googol of options one chooses a single one at a time, generating a path that is seemingly laid out by a series of choices. There is also the possibilities, leading to all the actions and the reactions that happen in and outside of the choice universe that affect one’s path. Our ability to predict outcomes of these choices or calculate the results of all of the possibilities is limited. Yet, one can be happy with outcomes, mainly when they go beyond what was initially imagined to be possible. When people describe their journey in life they often talk or write about great turning points, achievements such as graduations, promotions, a new job, a move to a different continent, marriage... Although it is all a continuum, we favor specific points in time and mark life with them. To me life is in the unique moments in any ordinary day. Some are noticed by one only because s/he was paying attention. Science is also just like this, it is in those rare moments where sometimes you are the only one privileged enough to witness how the universe works. Those moments, when enough attention is paid and care is given, turn into knowledge that places us more firmly in space and time. Those moments, for me, define the true joy of living life, and they mean much more when the path that contains them is shared.

Being in the Reddien Lab has been a profound journey of personal growth and scientific training. At times I thought, one can spend lifetimes working on these diverse set of puzzles in this place. This is mainly a result of careful and gentle attention and dedication given by Peter to nurture this environment. I am eternally grateful and privileged to have had Peter as a mentor. Peter is singular in the way he guides you by being the example, encourages you while reminding you all the other myriad dimensions of life, teaches you subtleties that will lead to deep insights. His unique intellect, deep wisdom, limitless enthusiasm and kindness, and unconditional friendship have been inexpressibly valuable for me. The many hours we spent exploring, thinking, discussing many dimensions of science and life are among my most cherished memories. Together we transformed a part of the universe from unknown to known.

Being a part of the Reddien Lab was also a wonderful experience thanks to my lab mates. Sam LoCascio, was the best (brain) bay mate one could ever ask for. He has been a great source of motivation and many fun memories, while being a great collaborator with his optimistic energy, gumption and his selfless dedication to his work. Kate Malecek, Lucila Scimone and Zack Kneckt were also collaborators of mine on other projects that shaped my time in the lab. Kate reaffirmed my belief in persistence and dedication while we undertook one of the most challenging projects of all time. Lucila showed me what can be possible with true hard work, strong ethics and a passion for doing science. Zack, although he arrived only within the last year, he has been greatly fun and engaging to collaborate with. I am grateful for the community of graduate students, research assistants and post-docs in the lab. The limitations of this section may not allow me to truly reflect my thoughts and feelings on my wonderful lab mates: Aneesha Tewari, Chris Fincher, Catherine McQuestion, Kwadwo Owusu-Boaitey, Lauren Cote, Dayan Jack Li, Conor McMann, Isaac Oderberg, Amelie Raz, Travis Rogers, Sarah Stern, Ashley Bonneau, Tom Cooke, Josien van Wolswinkel and Jen Cloutier have been constant sources of joy and support. I am also thankful to Nick Polizzi for his enduring friendship and kindness.

I have had the great fortune of having Martha Constantine-Paton, Myriam Heiman, Chip Quinn and Troy Littleton as my committee members. They have been a source of insight, support and guidance over the course of my training. I had the most wonderful time teaching with Martha and learned so much from her. Her exceptional insights and life energy has been truly inspiring. Myriam has always been boundlessly kind and enthusiastically supportive from day one and onwards. I have been
exceptionally privileged and grateful to have her as a committee member, for she has been a constant source of gentle guidance and valuable insights. Chip shared his singular humor and deep knowledge with me over the years and made this journey even more memorable. Troy’s devotion to the graduate student experience in the MCN Program allowed me to have a truly extraordinary graduate training and social experience. His key advise at multiple critical turning points has been invaluable for me.

Genes in Space carried me to new heights beyond this atmosphere. Ezequiel Alvarez-Saavedra, Sebastian Kraves, Emily Gleason and Scott Copeland have become a second scientific family and allowed me to be a mentor to a wonderful group of mentees over four years, with whom we sent pioneering molecular biology experiments to the International Space Station, contributing to humanity’s journey among the stars.

I am forever indebted to Ray W. Guillery for generously sharing his wit and knowledge with me and enabling me to carry my scientific journey to a place beyond my dreams. It was a distinct privilege being his last mentee and his friend.

I am endlessly grateful to Chris A. Walsh for inviting me to his lab to start my scientific tenure in the US. Chris has been a source of great support and a source of wisdom for me over the years always being there for me with his kind and gentle guidance. The wonderful experience in his lab before my PhD training has taught me so much and made all the subsequent steps possible.

My mentors and teachers have brought me to this point in life. Sultan Kandemir taught me one is never alone in life and held my hand as I grew up through education. Burçin Kantarci and Sevil Erkutay taught me English in a way that now allows me to complete a PhD. Over the years of my training, Türker Kılıç and Kenneth L. Moya have been a great sources of advice, fun and support. My undergraduate advisor Muhittin Arslanyolu has always been a generous with his support and advice. Early in my training, he spent long days and nights in the lab with me, teaching me all aspects of molecular biology research, and taught me how to ask the right questions.

My dear friends Jenny Yang, David Ferrero, Gilad Evrony, Benjamin Hills, Abdul Mohsen Al-Husseini, Tarek Madany, Kevin and Nalini Broadbelt, Emily and Brian Wilson, Ozan Toraman, Elif Murat Dalkara, Yusuf Iseri, Erdem Karaman, Umut Sahin, Korhan Kodaman, Korhan Çelikkaya, Timucin Avsar, Nurçin Küşçükoğlu; my wonderful BCS classmates; talented bandmates Zak Swartz and David Pincus, and Space Science-mate Guy Bushkin have been constant sources of intellectual and emotional support and joy over the years.

I am thankful for my father for he lit the fire of curiosity in me and my mother for lovingly helping me preserve it. None of this would be possible without the unconditional support and love of all my family.

My wife Elif is my source of love and happiness. She makes every day fascinating and beautiful, turning ordinary moments into unique and extraordinary ones. She inspires me with her energy, with her every thought and action, and with her sense of humor, making this voyage through space and time truly wonderful. I am profoundly grateful for her presence in my life, and for our journey together.

Lastly, I am thankful for the MIT Presidential Fellows Program, HHMI International Student Research Fellowship and the Jerome and Florence Brill Graduate Fellowship for their support, allowing me to carry on my research with comfort and peace of mind.

To the memory of my loving grandparents Bahriye and Fehmi Atabay; and Ray W. Guillery.
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Chapter 1:

Introduction
SELF-ORGANIZATION OF FORM AND FUNCTION IN BIOLOGY

“How can the events in space and time which take place within the spatial boundary of a living organism be accounted for by physics and chemistry?” Erwin Schrödinger asked this question in 1943, examining the set of special properties that differentiate a living system from a collection of non-living matter (Schrödinger 1944). Biology as a distinctive field, attempts to logically explain what it means to be a living system; what life is and how living systems are organized. Earlier, in his attempts to characterize the elusive properties of living organisms, Immanuel Kant questioned what an organism is, setting Biology as a separate and distinctive field. In his ‘Critique of Judgment’, Kant defined an organism as a “self-organized” being, referring to its organized complexity (Kant 1790).

Formation of tissue and organs during development is a greatly complex process that involves diverse local interactions at the molecular and cellular levels (Nakano et al. 2012). These interactions guide the mechanisms that integrate biological subunits, such as cells, into coherently functioning modules or systems, such as cell circuits or tissues respectively. Self-organization is the emergence of structure and function out of local interactions between the basic components of an initially disordered state. Self-organization occurs at many different levels of complexity involving mathematical, physical, chemical, biological, mechatronic and cognitive systems. Prevalent instances of self-organization comprise thunderstorms, chemical oscillators, crystallization, animal swarming, neural circuits, and mass migration. In self-organizing systems, lower level components of the system produce the global architecture or pattern without referring to an inclusive “blueprint” of the ultimate structure. In this way, the main pattern of the system is...
not externally influenced, but is shaped through intrinsic properties of the components (Bonabeau et al. 1999).

Living organisms are thermodynamically open systems. They are in a state of perpetual flux, constantly exchanging matter and energy with their surroundings. It is this state of flux and the resistance to preserve form or function that defines the dynamic stability of a self-organizing system. Living organisms are also described by their complex organization that is a consequence of myriad molecular interactions with a high degree of non-linearity. In such systems, the variables that are intrinsic to the components interact with each other to build the system, while responding extrinsic states. The combination of these features allows complex organized states displaying emergent features (Walleczek 2000). Stability and maintenance of a self-organizing structure also depends on the interaction between the micro- and macro-level processes (Walleczek 2000). Accordingly, emergence can be observed at a high level of complexity in a self-organizing system (Figure 1).

Self-organizing systems are non-linear, adaptive and dynamic. Studies of non-linearity in the fields of mathematics and physics in the 1970s led to a variation in the description of self-organization (Keller 2007). The term, from this viewpoint, was defined as “the emergence of dissipative structures that are low in entropy and far from equilibrium” by Ilya Prigogine (Nicolis and Prigogine 1977). The distinction in the meaning of self-organization lies in the modularity and organized complexity of living systems rather than an emergence of complexity, observed in ocean currents or storms or the Belousov - Zhabotinsky reaction (Figure 1B) as examples. The difference here is between disorganized and organized complexity (Keller 2007). In other words, complex
orderly states that have stability, function, and a purpose, selected through external pressures, separate self-organization of living organisms from an inanimate self-organizing system with emergent complexity.

Self-organization in biological systems has been studied in terms of emergent formation of tissue architecture and function, pattern, rhythmic/oscillatory and collective cellular or behavioral states.

**Self-organization in complex collective behavior**

Formation of behavioral patterns in nature does not always stringently follow the same set of rules, which lead the components of the system to produce an overall pattern without referring to a global ultimate structure. In particular instances, as in migratory animal behavior, individuals within the group can adjust their overall behavior through feedback from the emergent global self-organized state of the group. This behavior of the system may lead the group to move in a specific direction (Couzin 2003).

Pattern formation in biological systems has been extensively studied at different levels of complexity ranging from the collective behavior of unicellular organisms to social dynamics in primate societies (Gerhart and Kirschner 1997, Camazine 2001, Couzin 2003). Such processes have been explored in the context of group migration and sorting among cells during embryonic development (Xiong et al. 2013) as well as in the social texture of emergent insect behaviors, as observed in ant colonies or bee nests (Theraulaz and Bonabeau 1995, Franks and Deneubourg 1997, Deneubourg et al. 2002). This patterned behavioral activity in the context of collective animal behavior (e.g., fish schools, bird flocks or army ant colonies) may emerge through the
interactions between the individuals within the system reacting to the information they derive from neighboring activities (Couzin 2003). Such behaviors ultimately produce a cohesive emergent pattern that react and adapt to changing external states (e.g., wind direction, a predator or availability of food). For example, a single engineered mutation in the gene orco that normally allows ants to integrate pheromone signals, which are released by other ants in the colony, is enough to set the individual ants with the mutation behaviorally apart from the colony leading the mutants to become social outcasts (Trible et al. 2017).

The behavior of fish schools can produce diverse emergent states that have been explored through mathematical modeling, in silico reconstruction, and observation-based studies. Accordingly, these different dynamically-stable collective behavioral states were categorized as swarming, milling and polarized swimming (Tunstrøm et al. 2013) (Figure 2). Slow and more individually guided swimming behavior lead to swarms, which produce relatively dense and disordered states. Interestingly, a change in the velocity of swimming can lead to a transition between two different states, between locally-disordered and locally-ordered, producing either milling or polarized group dynamics. Stability of these emergent states is linked with the number of individuals in the fish school and transitions between these states are induced by both external and internal factors (Tunstrøm et al. 2013). Similarly, in migrant birds, such as storks that use air thermals to ease their migratory behavior, it was observed that a small number of individuals (leaders) separate from the group and navigate towards searching for thermals and larger group (followers) are guided by their flight path, ultimately producing a global migratory pattern (Flack et al. 2018). Here, the emergence of the spatiotemporal pattern has two components, the leaders exhibiting more irregular
flight patterns that guide the global behavior of the whole flock, and the followers falling into a more stable emergent state (Flack et al. 2018).

In primate societies, contrary to popular assumption, group dynamics are found to be more dominant rather than leaders guiding the collective behavior. Although primate societies exhibit a more hierarchical and socially structured form in comparison to schooling animals such as fish, a study using GPS to track movements of wild Kenyan olive baboon troops revealed that an emergent shared group behavior guides decision-making process rather than the troop leaders. This process is led by simple rules involving following the majority, when a conflict between two decisions arises (Strandburg-Peshkin et al. 2015).

In humans, many social aspects have been linked to emergent social patterns such as migration behavior (Schweitzer 1997), social decision-making process, contagion and conflict resolution (Dyer et al. 2009). More specifically, it was shown that in small human groups, both group size and the presence of uninformed individuals can influence the decision between two contrasting directional options and the probability of the group splitting. Spatial location of informed individuals within groups is also shown to affect the pace and precision of human group movements. These tendencies detected in small human groups can also be applied to large crowds. Consequently, it was also revealed that only a small minority of informed individuals is usually required to guide a larger uninformed crowd (Dyer et al. 2009), possibly impacting many layers of social dynamics such as political, architectural and linguistic evolution as well as mass migration upon nucleation of driving decisions from an initially small number of individuals.
**Self-organization of rhythmic or oscillatory behavior**

Higher order behaviors of some social biological systems such as bees, ants and fireflies often emerge through self-organizing processes and can lead to rhythmic or oscillatory states, such as those observed in the synchronized flashing behavior of fireflies that are native to South East Asia (Buck 1966). The elusive synchronous flashing of a population of male fireflies in Thailand was first reported over two centuries ago, yet, the true purpose of it has not been fully resolved yet. Purpose in behavior is also a distinctive feature of living systems and is a result of organized complexity. In the case of rhythmic firefly flashing behavior, each firefly acts as an oscillator adjusting its individual rhythm based on the flashing patterns of other fireflies within proximity. A unified rhythm emerges as a result of coupled oscillatory behavior (Mirollo 1990).

Another form of rhythmic or oscillatory behavior, collective cell migration or signaling, has been studied in both multicellular and unicellular organisms. Collective cell migration or communication is prevalent in unicellular organisms (Woods et al. 2014, Mavridou et al. 2018, Hashimura et al. 2019) and metazoans, guiding processes such as morphogenesis, regeneration and immune responses (Johnston 1966, Sadaghiani and Thiebaud 1987, Banchereau and Steinman 1998, Xavier Trepat 2009, Currie et al. 2016). This process in multicellular organisms has mainly been explored by molecular tagging, tracing and live imaging approaches (Xiong et al. 2013, Currie et al. 2016). The slime mold, *Dictyostelium discoideum*, has also been extensively used to study collective cellular behavior in the form of cell migration because of its simple cell-cell interactions mainly through diffusible signals (Bonner 1971). *Dictyostelium* cells develop as unicellular organisms at their vegetative phase, yet they transition from this unicellular state to a multicellular state through aggregation, if they are starved. During this aggregation process,
starved cells characteristically migrate towards the center to shape a multicellular mass. This synchronized and rhythmic cellular migration is guided by the self-organization of cyclic-adenosine monophosphate (cAMP) gradients and also by chemotaxis towards extracellular cAMP (Hashimura et al. 2019). Extracellular cAMP leads to activation of adenylyl cyclase (AC), an enzyme with crucial signaling and regulatory roles in cells. cAMP is produced by AC in response to extracellular cAMP and secreted to stimulate nearby cells to also generate cAMP. Concurrently, this transitory buildup of extracellular cAMP triggers actin polymerization that results in cellular migration. During this process, cAMP signals travel as waves, named the cAMP relay, and lead to spiraling waves of cytosolic cAMP oscillations and possibly migration towards the center forming a multicellular aggregate (Hashimura et al. 2019) (Figure 2B).

At a different level of complexity, coordinated electrical activity of neuronal ensembles also produce oscillatory behavior that lead to different cognitive states (Buzsáki 2006). In this case, a collection of neurons in a network with antagonistic activity patterns can synchronize in a way that produces stable oscillatory states, the “rhythms of the brain” (Buzsáki 2006). An essential example to this is the sharp-wave ripples that are produced by sub-areas of the hippocampus, a region in the brain that is linked with encoding and accession of memories (Foster and Wilson 2006). This self-organized neural population behavior in the hippocampus, the generation of sharp-wave ripples, is thought to represent transiently stored memories, which are then “transferred” to the neocortex for long-term consolidation.

Remarkably, the neuronal ensembles that produce oscillations also organize cell assembly patterns (Buzsáki 2006). This phenomenon is possibly guided by the self-organizing features of patterned
neuronal activity. Firing activity of a neuron at a given time is controlled by only two sources: An input external to the system and self-organized rhythmic activity (Buzsáki 2006). Such self-organized states can be examined by real-time observation of the activities of many individual neurons in various brain regions, yet, any self-organized state can emerge because of an external stimulus or from internal activity, making it difficult to characterize the “nucleating” influencers. Neuronal ensembles are produced as transitory states of harmonic discharges by reciprocal self-organizing neuronal interactions and may be a foundation of cognitive activity. Recent evidence also indicate that these neuronal networks produce connectome-specific harmonic waves through excitatory-inhibitory interactions within the brain (Atasoy et al. 2016).

The central nervous system in vertebrates and in invertebrates is typically packed with diverse types of excitatory and inhibitory neurons exhibiting different properties ranging from synaptic diverse connections, activity patterns and molecular and cellular signatures. How these neurons arrange themselves into patterned, functional areas has been extensively studied primarily focusing on the formation of cortical columns, patterned neuronal clusters located mainly in the visual and somatosensory cortices (Maruoka et al. 2017). More recently, it has been shown that layer 5 subcerebral excitatory neurons in the mouse cortex are structurally and functionally organized into a hexagonal lattice with a systematic topography (Maruoka et al. 2017). Furthermore, another class of (layer 5) cortical projection neurons also form microcolumns and show synchronized activity within these microcolumns, revealed by calcium imaging. The neurons within a microcircuit are electrically coupled through gap junctions. These neurons are shown to be clonally unrelated, coming from different neural progenitor lineages and they generate the electrical coupling at the
end of the first postnatal week (Maruoka et al. 2017). This structural and functional organization is another example for a complex outcome generated by local and simple coupling interactions.

**Self-organization in synthetic biology**

Development of functional tissue architectures involves self-organization. During this process, cells divide, migrate, differentiate, interact with each other and spatially organize themselves ultimately forming the tissue they become a part of. Although, cells can achieve these feats mainly through intrinsic cell-cell interactions guided by external signaling cues, 2- or 3-dimensional *in vitro* tissue structures composed similar cell types sampled from similar temporal states are not as spatially reproducible and as functional as their *in vivo* counterparts. To better understand how *in vitro* tissue formation can recapitulate the *in vivo*, there have been attempts to use synthetic genetic programming of sequential molecular events leading to formation of cell circuits or multicellular assemblies. These studies showed that simple gene circuits guiding cell-cell interactions, such as synthetic Notch receptor system (SynNotch), allows the formation of complex layered multicellular assemblies with predictable shapes formed by layers of different cells and regenerative response upon disruption (Toda et al. 2018). SynNotch receptors comprise the core regulatory domain signaling receptor Notch, linked to a chimeric extracellular recognition domain and a chimeric intracellular transcriptional domain (Toda et al. 2018). Upon recognition of a similar ligand on a neighboring cell, the synNotch receptor undergoes cleavage and releases the intracellular transcriptional domain to translocate to the cell nucleus and initiate expression of custom target genes. These properties yield a range of design options simply by permitting molecular variations in cell-cell interaction parameters, ultimately leading to the formation of different 3-dimensional tissue structures (Figure 2C). These experiments demonstrate how simple
rules can produce complex outcomes allowing the study of the self-organization of multicellular structures in a more predictable manner.

Alternatively, generation of synthetic embryo-like structures that can lead to the formation of different cell and tissue types has also been attempted (Sozen et al. 2018). This study demonstrated that three different stem cell types comprise the ability to self-organize into gastrulating embryo-like bodies, recapitulating most of the early cellular and tissue-level events of the gastrulating mammalian embryo in vitro. Remarkably, it was revealed that embryonic stem cells, trophoblast stem cells and extra-embryonic stem cells, when co-cultured, have the capacity to spontaneously self-organize and self-sort into embryo-like structures that undergo anterior-posterior patterning and morphogenesis leading to the formation of endoderm and mesoderm layers (Sozen et al. 2018). The capacity of these cells to self-organize and initiate a program that leads to many the key events indicate that self-organization might be a dominant strategy used by stem cells and their progenitors to orchestrate myriad subsequent developmental programs guiding in vivo tissue formation, growth and maturation.

Self-organization in development and regeneration

Self-organization has been invoked to explain key aspects of embryonic development (Turing 1952, Nicolis and Prigogine 1977, Stocum and Melton 1977, Halder et al. 1995, Tsiairis and Aulehla 2016, Corson et al. 2017, Harrison et al. 2017, Shyer et al. 2017) and regeneration (Stocum and Melton 1977) and the in vitro formation of complex structures, such as the optic cup (Eiraku et al. 2011, Sasai 2013) and formation of organoids, three-dimensional organ-like structures that form through aggregation of stem cells (Lancaster et al. 2013) (Figure 2D). These processes are
mostly linked with nucleation, growth, maturation and replacement of structures that will give rise to tissues and organs, and ultimately acquire a specific form that will give rise to function and a behavior.

Embryonic development requires molecular and cellular orchestration of various processes to achieve induction or nucleation of different tissues and organs in space and time. These processes involve context-dependent and dynamic cell fate choices (Bedzhov and Zernicka-Goetz 2014). To elucidate this, historically, a systematic and reductionist approach involved conceptually dissecting complex systems into their components, with an goal to explain the whole by elucidating the features of these parts. Although, this approach is greatly effective for uncovering key mechanisms underlying certain biological processes, it does not always explain how the whole, organized state, emerges. The challenge is mostly because of the fact that sufficiently complex systems display emergent properties that cannot be predicted by only studying the parts that make them (Kauffman 1993).

In self-organizing biological systems, activation of intrinsic programs such as specific cell fate decisions, might depend on receiving the “correct” external signals. After that point in time, the intrinsic properties, which will lead to proper local interactions and the emergence of structure and function potentially become active. In 2011, Yosiki Sasai and his group demonstrated that the in vitro formation of a three-dimensional optic cup and retina depends on coordinated migration and orchestrated cellular interactions that ultimately build the structure. Accordingly, formation of a three-dimensional optic cup from mouse embryonic stem cell (ECS) aggregates was achieved using a multipurpose floating culture in serum-free and growth-factor-reduced medium (Eiraku et
It was noted in a following study that the optic cup formation, in this system, was observed to be driven by an intrinsic, self-organizing program that spontaneously manifested once the external conditions such as media, culture ingredients and mechanical substrates were provided (Nakano et al. 2012).

Studies of morphogenesis in embryoid bodies, three-dimensional aggregation of pluripotent stem cells, in mice led to a view that programmed cell death or apoptosis shapes the epiblast, the outermost layer of the embryo that later gives rise to the mesoderm and ectoderm layers. Conversely, it was demonstrated that basal membrane-stimulated integrin signaling, not apoptosis, directs induction of polarization of epiblast cells. Furthermore, it was shown that this basal membrane signaling can be replaced in vitro by a set of extracellular matrix (ECM) proteins that initiate this process. These experiments indicate a model by which ECM can activate a self-organizing program for morphogenesis in developing embryoid bodies (Bedzhov and Zernicka-Goetz 2014).

Patterns in developing tissues and organs can also result from feedback interaction between secreted proteins. In ‘A Chemical Basis for Morphogenesis’, Turing provides a mechanism that involves long-range signal inhibition and short-range cellular activation, which can lead to emergence of specific patterns, such as the formation of stripe patterns on mammalian coats (Mallarino et al. 2016), that arise from initially uniform states (Turing 1952). Another example where diffusion driven Turing system models have been applied is for the formation of fingers in vertebrates, involving a feedback system between Wnt, Bmp and Sox9 signaling pathways, leading
to digit formation through participation of Hox and Fgf gene expression (Raspopovic et al. 2014, Cooper 2015) (Figure 3A).

Distinct domains of cells in developing tissues can result from morphogen gradients that provide inductive signals specifying different cell fates at various signal intensities. This process is dependent on the interpretation of the morphogen gradient into discrete and stable gene expression domains at specific positions (Li et al. 2018). This introduces a challenge since the cells in developing tissues are often in a state of movement or migration, which may affect how they integrate the morphogen signals because of varying degrees of exposure. Yet, a specific pattern emerges as an end result of development. A careful labeling and imaging-based analysis of the specification patterns and movement trajectories of neural progenitors in the developing zebrafish neural tube showed that specified progenitors at different stages of cell fate commitments appear spatially mixed during noisy Sonic Hedgehog signaling across the developing tissue. Cell sorting processes can re-arrange the cells into sharply bordered domains, revealing that active cell re-arrangements can correct imprecision of spatiotemporal patterning caused by noisy inductive signals (Xiong et al. 2013). This also is another example that indicated how the emergence of countless varieties of spatiotemporal patterns during development depends on both positional cues and communication between cells responding to signals, often through molecular or mechanical feedback (Shyer et al. 2017).

An example from the chick development includes dynamics of formation of microvilli in the developing gut, which depends on activity of signaling pathways such as BMP, Sonic Hedgehog (Shh) and Wnt, and mechanical forces such as epithelial folding, leading to an increase in signal
concentration, stimulating the growth (Walton et al. 2016). Similarly, formation of follicle patterns in the developing chick skin relies on aggregation through contractility-driven stretch of the skin tissue. In parallel, this dermal cell aggregation induces the mechanosensitive activation of β-catenin protein, a protein involved in regulation of cell-cell adhesion (in this context), in neighboring epidermal cells and initiates the follicle gene expression program. In this emergent self-organizing process, morphological and molecular programs simultaneously respond, while the progenitor cells diversify their ultimate fates also leading to a highly organized structure and pattern formation (Shyer et al. 2017).

Formation of neural maps through spatial organization of neuronal connectivity within the developing brain in vertebrates is another aspect of development that has been widely explored (Constantine-Paton and Law 1982, Pons et al. 1991, Kaas 1997, Wedeen et al. 2012, Weigand et al. 2017). Existence of radially arranged orientation preference maps (OPM) and striped patterns of ocular dominance columns (ODC) revealing locally competing binocular inputs in primate, ungulate and carnivore visual cortices are not observed in all species of mammals. Such complex map structures was thought to be a result of clade- and neuron-specific connectivity patterns in the developing cortex, yet OCDs can also be achieved in the frog tectum through a transplantation of an eye primordium into the forebrain region developing frog (Rana pipiens) embryo demonstrating an existence of a common connectivity rule linked with neurodevelopmental plasticity and the number neuronal inputs to a developing brain area (Constantine-Paton and Law 1978, Constantine-Paton and Law 1982) (Figure 3B). A computational modeling study focused on the formation of spatial neural maps and also supported a view that an increase in neuronal number alone, without changing the selectivity of the connections, can lead to a universal switch guiding the formation
of OPM and OCD-like maps. Additionally, these authors postulated that the number of connections neuron make in a field contributes to formation of different map structures (Figure 3C) (Weigand et al. 2017). This model also may also help explain the structural differences between rodents, exhibiting a salt-and-pepper arrangement of binocular inputs in their visual cortex, and carnivores, which contain OPMs and ODCs in their visual cortex.

Similarly, map-like organization of the grid cells in the entorhinal cortex of mammals both structurally and functionally exhibit self-organizing patterns (Hafting et al. 2005, Gu et al. 2018). Grid cells represent the external topographical space internally. The geometrical organization of grid cell firing properties suggests that there is a relationship between the topography of theses neural circuits and their function (Gu et al. 2018). Following this line of questioning, it was elucidated that there is considerable anatomical micro-organization among the grid cells in the medial entorhinal cortex, forming a modular two-dimensional lattice, where the spacing of the grid cells matches their spatial tuning properties (Gu et al. 2018). Firing fields of grid cells exhibit a repetitive triangular structure across the tissue in rodents (Hafting et al. 2005). Strikingly, in bats, because of the ability of flight, head direction cells are organized across a three-dimensional structural and functional gradient in the presubiculum area of the brain. These cells have azimuth tuning properties, allowing a transition between two-dimensional to three-dimensional, toroidal, representation of coordinate space (Finkelstein et al. 2015). These examples showing strong relationships between anatomical and functional correlations, which might also indicate that self-organizing attractor dynamics provide the guiding principles of the formation of such systems.
The process of regeneration also leads to formation of specific tissues, organs and even large regions of body-axes - similar to what occurs in embryonic development. Yet, by contrast, regeneration does not start from a specific origin point such as a fertilized oocyte. The majority of the animals that can regenerate, initiate this process through formation of an undifferentiated mass of cells at the wound site called the blastema. The spatiotemporal aspects of cell movements and cell differentiation within the blastema remain poorly understood. Observations in regeneration axolotl (a neotenic salamander with high regenerative capacity) digits revealed that diverse progenitor cell populations, which give rise to all the differentiated cells in the regenerating tissue are choreographed spatiotemporally, some migrating and some proliferating only, eventually leading to a complete regeneration of the missing tissue (Currie et al. 2016).

Self-organization is a widely prevalent phenomenon in biology leading to an emergence of myriad shapes and functions in space and time. Here, I propose that self-organizing processes contribute to diverse aspects of regeneration, ultimately renewing and maintaining form and function. I explore this question using the planarian *Schmidtea mediterranea* as an animal model, mainly because of its remarkable regenerative ability and unique biology, which ultimately allow them to attain this capacity. In the following section, I describe the mechanisms orchestrating patterning and morphogenesis as well as the distinctive biology of planarian regeneration.
MORPHOGENESIS AND PLANARIAN REGENERATION

Regeneration is one of the most enigmatic phenomena in biology. Regeneration encompasses the replacement of missing cells, tissues, organs or entire body parts. Capacity for regeneration is widespread, yet variable in the animal kingdom. Elegant studies showed that members of arthropods, ctenophores, hemichordates, annelids, acoels, platyhelminthes, and chordates exhibit diverse regenerative features (Sánchez Alvarado and Tsonis 2006, Reddien 2011, Tanaka and Reddien 2011, Srivastava et al. 2014, Reddien 2018). Capacity of human for regeneration is relatively limited, yet we still are able to regenerate or replace various tissues and organ systems such as blood, skin, liver and gut epithelium (Baddour et al. 2012).

How do the remaining tissues, following injury, “know” what is missing upon an injury or loss; and how is restoration of size, shape, tissue proportions, and overall function re-established in regeneration? Although the capacity to regenerate varies, some of the molecular and cellular strategies used in the process of replacement of tissues and organs are conserved.

Mechanisms of tissue patterning and morphogenesis

How cells temporally and spatially organize and generate patterns, ultimately forming an organism with diverse functions is a fascinating and central question of biology. To form the organism, different cell types lead to formation of distinct tissue types with specific functions connecting at a systems level, maintaining a cohesive and harmonious set of interactions that are constantly regulated. A better understanding of these processes at different levels of complexities ranging
from molecular to systems levels, is crucial to elucidate how morphogenesis and regeneration occur.

Tissue formation depends on physical cell-cell interactions through cell surface proteins and the activity of molecular signaling pathways. Cell-cell interactions and cell-matrix interactions are generally guided by adhesion molecules. There are four major groups of cell adhesion molecules (CAMs): Cadherins, Integrins, the immunoglobulin (Ig) super family of cell adhesion molecules (IgCAMs) and Selectins (Lodish 2000). In addition to cell adhesion junctions, gap-junctions mediated by connexins, and tight-junctions mediated by occludin and claudins, guide the formation of tissue architecture and regulate tissue-specific functions. Cadherins are Ca2+ dependent proteins have crucial roles guiding tissue formation during development. They hold cells together by homophilic interactions between them, and for these interactions to occur the cytoplasmic domain for the cadherin is linked to the cytoskeletal elements by anchoring proteins called catenins. A large subgroup of the cadherin superfamily, protocadherins; as well as DSCAMs and Dpr-DIPs (members of the IgCAMs) are predominantly expressed in the developing nervous system (Zipursky and Sanes 2010, Chen and Maniatis 2013, Carrillo et al. 2015). These proteins regulate specific assembly of neuronal circuits. The assembly process is guided through combinatorial expression of protein isoforms and interactions between interacting partners. As a result, cells acquire unique identification tags building an interaction code dictated by geometric complementarity within the binding interface, providing unique cellular tags, and allowing molecular distinctions nearly at the level of the single cells (Zipursky and Sanes 2010, Chen and Maniatis 2013). These molecular tags regulate self-recognition and avoidance, controlling processes such as circuit wiring and cellular tiling across a region (Chen et al. 2017). Roger
Sperry’s optic nerve regeneration experiments and John Langley’s spinal nerve regeneration experiments, as carefully reviewed in Zipursky and Sanes, 2010, also indicate that these circuit-specific molecular wiring mechanisms have central roles during regeneration as well.

Molecular signaling pathways comprise extracellular secreted ligands that bind to a receptor, triggering intracellular signal-transduction processes that alter protein interactions and phosphorylation states, usually leading to gene expression changes. Most of the signaling pathways are evolutionarily conserved and utilized by countless species guiding similar processes of development and regeneration. Moreover, these conserved signaling pathways, such as the Wnt, Transforming Growth Factor-beta (TGF-beta), Fibroblast Growth Factor (FGF), Epidermal Growth Factor (EGF), Notch-Delta, Eprins, Semaphorins, Ntrins, Retinoic Acid (RA) and Sonic hedgehog (Shh), guide the process of developmental patterning (Wolpert 2015), as well as organization in regenerating systems (Sánchez Alvarado and Tsonis 2006, Petersen and Reddien 2009a). Spatial and temporal control and regulation of these pathways can lead to activation of distinct cellular types or states, ultimately guiding formation of organismal scale pattern (Petersen and Reddien 2009a). This type of molecular control is achieved by morphogens, molecules that interact directly with cells and activate different molecular and cellular states based on their local concentration in development. Alan Turing’s reaction-diffusion model (Figure 4A), as mentioned in a previous section, invokes morphogens that guide alternating fluctuations generating rhythmic or oscillatory patterns of cell states in a developing area (Turing 1952). Another classical patterning model for morphogenesis is the “French Flag Model”, which posits that formation multiple different cell states across a field would be possible with a diffusing gradient of a morphogen from its source (Wolpert 2015) (Figure 4B). Even though there exists an inherent
molecular noise during the development of an embryo (because of cell migration and division affecting timing of exposure to the morphogenic signals), the cells are able to sort to form precise boundaries (Xiong et al. 2013), which indicates that progenitors can act to fine-tune imprecision in spatial patterning (Figure 4C). Hence, formation of precise boundaries is possibly a result of both cell-cell interactions, regulating precision and refinement, and also morphogen gradients, regulating coarse to fine patterning.

A famous example of early pattern formation is observed and well described in the developing Drosophila embryo (Driever and Nusslein-Volhard 1988, Driever and Nusslein-Volhard 1989, Driever et al. 1989). Regionalization along the anterior-posterior axis in this system is mainly regulated by transcription factor gradients generated by translation of regionally restricted maternal mRNAs and the diffusion of subsequently encoded proteins across the cytoplasm of the syncytial blastoderm of the embryo. Here, the mRNA of the transcription factor Bicoid is shuttled along the microtubules to the anterior terminal of the embryo and in combination with its protein distribution, it initiates a sequence of precise patterning, ultimately resulting in segmentation of the embryo (Frigerio et al. 1986). This process also involves the biased expression and diffusion of hunchback, nanos and caudal mRNAs and encoded proteins (Rivera-Pomar and Jackle 1996, Wolpert 2015). Later, gap genes, such as giant, huckebein, hunchback, knirps, Krüppel and tailless; and pair-rule genes and segment polarity genes such as even-skipped and engrailed, ultimately fine-tune the patterning process more locally (Rivera-Pomar and Jackle 1996, Wolpert 2015) (Figure 4D).
Another set of key experiments defined the “organizer” concept. An organizer is a part of the developing embryo, usually comprised of a relatively small number of cells, that influences the fates of neighboring cell populations. This concept was revealed through experiments in newt embryos, where the dorsal blastopore lip was transplanted and fused onto the ventral side of another newt embryo and induced the formation of an ectopic neural tube (Spemann 1923). Recipient newt embryos formed another axis developing into a ventrally conjoined embryo.

The recipient embryos developed into two newts conjoined on their ventral side. Subsequently, work of numerous labs revealed various mechanisms of genetic interactions involving the secretion of Bone Morphogenetic Protein-4 (BMP-4) from the ventral side of the embryo across the organizer region. The organizer prevents BMP-4 from binding to the adjacent ectoderm through the secretion of the proteins, Chordin and Noggin. These proteins then bind to BMP-4 near the organizer, preventing BMP-4 from interacting with receptor proteins expressed in the ectoderm. This sequence of events leads the ectodermal cells near the organizer to assume central nervous system cell fates (Smith and Harland 1992, Zimmerman et al. 1996, De Robertis 2006). The Spemann-Mangold organizer has been shown to have analogues in both birds (Hensen 1876, Boettger et al. 2001), called the Hensen’s node; and fish (Oppenheimer 1936), named the Embryonic Shield, that guide patterning during embryonic development. These key experimental instances and concepts provide crucial insights into the significance of establishment of positional information in metazoan development and regeneration.

Regulated cell death is another crucial mechanism of many developmental and regenerative programs. Cell death is used by metazoans for both development and morphogenesis, mainly to
control cell number, or eliminate damaged or mutated cells, as well as help construct tissue or organ shape. The process of cell death is essential for development and it generally implicates the production of excess cells and removal of those that are redundant (Vaux and Korsmeyer 1999). In metazoan evolution, molecular regulation of cell death through signaling mechanisms became an essential method that emerged as cell diversification and specialization occurred. Most of the cell death that occur in development is facilitated by caspases, a protein family of cysteine proteases (Alnemri 1997). An example is the case of the gene CED-3, when mutated in *C. elegans*, the programmed 131 total somatic cells deaths that normally take place during development does not occur (Ellis and Horvitz 1986). Moreover, cell engulfment process promotes programmed cell death and mutations in genes that the regulate engulfment process permit the survival of a population of cells that are normally eliminated during development (Reddien et al. 2001, Reddien and Horvitz 2004). Inhibition of cell death can alter the normal course of development and regeneration and disrupt patterning and morphogenesis (Vaux and Korsmeyer 1999).

*Introduction to planarian regeneration, patterning and scaling*

Planarians are freshwater flatworms that belong to the phylum Platyhelminthes and are found throughout the world. Their regenerative abilities have been documented for over two centuries (Dalyell 1814, Johnson 1825, Morgan 1898). Planarians, one the masters of regeneration, can regenerate from an unlimited variety of injuries (Morgan 1898, Reddien and Sánchez Alvarado 2004, Reddien 2018).

Planarians are bilaterally symmetric and possess a complex anatomy including a brain, two symmetrically positioned eyes, a complex musculature, a diffuse and multi-branched intestinal
system, kidney-like protonephridia, and an epidermis, all comprised of myriad different cell types (Fincher et al. 2018) (Figure 5A). The nervous system is comprised of a centralized two-lobed cephalic ganglia linked to two ventral nerve cords (Figure 5B). It includes a wide diversity of neuronal types that are found in different phyla as well. Eyes are located on the dorsal side of the animal and comprise pigmented optic cups and photoreceptor neurons that project their axons ipsilaterally and contralaterally to the brain, which is located ventral to the eyes (Figure 5B). Ingestion of food and excretion of waste occurs through a muscular tube, called the pharynx, which is connected to the multi-branched intestinal system (Figure 5C). The epidermis surrounds the animal and is heavily ciliated ventrally, which together with muscle contractions enables movement. This complex anatomy and the precise proportions of the cell types found throughout the body are remarkably restored within days to weeks following any type injury damaging or removing body parts (Morgan 1898, Reddien and Sánchez Alvarado 2004, LoCascio et al. 2017, Reddien 2018) (Figure 5D).

Planarians continually renew all cells in their bodies via continuous cellular turnover. This involves the only dividing somatic cell population in the adult organism, called neoblasts, marked by smedwi-1 and -2 expression (Reddien et al. 2005). Neoblasts include pluripotent stem cells (Wagner et al. 2011). Pluripotent neoblasts generate diverse specialized neoblast classes that differentiate into particular 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 and the brain (Lapan and Reddien 2011, Lapan and Reddien 2012) (Figure 5E). Regeneration in planarians, with tissues and organs being restored to their correct
proportions, can also occur in the absence of nutrients, without growth (Morgan 1901). This process engages growth and de-growth states of existing tissues (Baguñà 1981).

Neoblasts are located throughout the parenchyma of the adult animal and are mostly excluded from the pharynx and the head tip (Newmark and Sánchez Alvarado 2000, Reddien et al. 2005). Neoblasts are small (5-8µm), round cells that constitute about 10-15% of all cells in the animal (Reddien et al. 2005). They uniquely express the PIWI-family encoding *smedwi-1* gene, along with an array of genes that are previously implicated in maintenance of germ cell state and pluripotency in stem cells (Reddien et al. 2005, Resch et al. 2012, Wagner et al. 2012, van Wolfswinkel et al. 2014). Neoblasts are highly proliferative cells. This feature is revealed by continuous BrdU administration, which labels the entire neoblast population within 24 hours (Newmark and Sánchez Alvarado 2000). Because they are the only diving somatic cell population in the animal, neoblasts can be selectively ablated by irradiation (Dubois 1949, Baguñà 1989, Reddien et al. 2005). This results in the death of the organism due to the loss of homeostatic tissue maintenance as well as regenerative capacity. Neoblasts from an unirradiated donor can rescue homeostatic tissue turnover and the regenerative capacity, when transplanted into irradiated recipients (Baguñà 1989). Transplantation of a single neoblast also can result in reconstitution of the whole neoblast population, and restore the regenerative capacity as well (Wagner et al. 2011, Zeng et al. 2018). The pluripotent neoblasts that can restore the regenerative ability by reconstituting the whole animal are named clonogenic neoblasts (cNeoblasts). Remarkably, transplantation of a single from an unirradiated asexual host into an irradiated sexual recipient permitted a single nucleotide (SNP) polymorphism analysis that indicated that the sexual genotype was entirely replaced by the asexual genotype within the time frame of two months (Wagner et al. 2011). Recent work identified
defined Tetraspanin+ neoblasts as being capable of rescuing irradiated hosts (Zeng et al. 2018) possibly implicating this population as cNeoblasts.

A large fraction of neoblasts express certain transcription factors that mark specific differentiated cell lineages, indicating that an extensive amount of heterogeneity exists within this cell population. These lineage-specific transcription factors regulate specification of neoblasts into various progenitor cell states, leading into generation of distinct cell types (Lapan and Reddien 2011, Scimone et al. 2011, Lapan and Reddien 2012, Cowles et al. 2013, Currie and Pearson 2013, März et al. 2013, Adler et al. 2014, Scimone et al. 2014, van Wolfswinkel et al. 2014, Fincher et al. 2018). Inhibition of many of these transcription factors using RNA interference (RNAi) results in loss of that distinct cell lineage; therefore these RNAi animals can not maintain through renewal or regenerate the associated differentiated cell type (Scimone et al. 2011, Lapan and Reddien 2012, Adler et al. 2014, Scimone et al. 2014). The case of eye progenitors is one of the best-elucidated examples of lineage-specific progenitors in planarians (Lapan and Reddien 2011, Lapan and Reddien 2012). Eye progenitors express the transcription factor ovo and give rise to the entire eye lineage. ovo is required for the homeostatic maintenance and regeneration of the eyes. New eye progenitors, marked by smedwi-1 and ovo expression, are specified broadly posteriorly to the existing eyes. They migrate anteriorly and incorporate into the existing eyes during homeostatic maintenance, or nucleate de novo eyes during regeneration. As they migrate, they progressively up-regulate that are genes distinct to the individual eye cell type, which can be photoreceptor neurons or pigmented optic cup cells (Lapan and Reddien 2011). Elements of this process, from broad specification of progenitors to progenitor migration and subsequent cell differentiation are crucial in understanding how homeostatic tissue maintenance and regeneration is achieved.
Specialized neoblasts, which constitutes tissue-specific progenitors are generally specified in a spatially coarse manner in the animal. In other words, this pattern of specification is visualized to be much broader than their target differentiated tissues (Lapan and Reddien 2011, Adler et al. 2014, LoCascio et al. 2017). These coarsely specified tissue-specific progenitors constantly incorporate into their target tissues to replace the cells that die during tissue turnover. How tissue-specific regeneration is achieved via specialized neoblasts has been a fascinating puzzle. A possible explanation for tissue-specific regeneration would engage feedback-based system (Adler and Sánchez Alvarado 2015), positing that tissue-specific signals inhibit its own specialized neoblast production. More recent work on characterizing how tissue-specific regeneration can be achieved through focusing on eye regeneration is directly linked to this process. It demonstrates that regeneration specificity is achieved, for this tissue type, without detection of eye absence.

When the animal is decapitated, ovo+ eye progenitors in the trunk fragment are amplified along with all the other specialized progenitors. This leads to formation of a new head with new eyes rapidly. Strikingly, selective resection of the eye(s) does not affect the number of ovo+ eye progenitors, yet the eye(s) regenerate (LoCascio et al. 2017) (Figure 6A). Moreover, in animals with only one regenerating eye upon unilateral eye resection, it was observed that incorporation of progenitors occurs at the same homeostatic rate in both eyes. During this process the regenerating eye will grow and the uninjured one will preserve its size. The size for the eye is mainly established by both incorporation of new progenitors and also cell death. This study demonstrated that when the rate of new progenitor cell incorporation into a regenerating eye is constant, the rate of cell death should be lower in the regenerating eye (in terms of cell death events per eye) than the uninjured one (LoCascio et al. 2017). This elegant model, named “target-blind progenitor
specification” might have wide explanatory power across different tissue types (Tewari et al. 2018), as well as across diverse regenerative species (Figure 6B). A study focusing on axolotl central nervous system regeneration upon unilateral lesioning of the dorsal pallium also noted increased production of neurons in uninjured regions in the brain (Amamoto et al. 2016).

Planarian regeneration is initiated by an injury prompting extension of the epidermis and subsequent muscle contraction to help seal the wound in planarians (Morita and Best 1965). Upon an injury, a generic wound response occurs within 1-6 hours after injury. This response induces broad neoblast proliferation, local cell death and activation of gene expression for approximately 200 genes (Baguñà 1976, Pellettieri et al. 2010, Wenemoser and Reddien 2010, Wenemoser et al. 2012, Wurtzel et al. 2015). Amputations leading to tissue loss, and injuries that do not result with missing tissues, both are sufficient to induce this generic wound response. Separately, significant tissue loss is linked with another set of responses named “the missing tissue response”. The missing tissue response drives a sustained second mitotic peak near the wound site, a global increase in cell death, and continued activity of multiple wound induced genes greater than 24 hours post-injury (Pellettieri et al. 2010, Wenemoser and Reddien 2010, Wenemoser et al. 2012). Around 48 hours post-injury, a second wave of mitoses occur close to the wound site (Wenemoser and Reddien 2010, van Wolfswinkel et al. 2014) (Figure 7A). At 72 hours post-injury, gene expression signatures for cellular differentiation can be detected. After this point on, morphallaxis, re-establishment of the correct tissue proportions and shape, occurs via global cell death, homeostatic turnover and constant re-arrangement of existing tissues (Pellettieri et al. 2010, Forsthoefel et al. 2011).
follistatin, a gene that encodes an inhibitor of Activin signaling, is required for the missing tissue response and is required for regeneration specifically at anterior-facing wounds has been studied in detail (Gaviño et al. 2013, Roberts-Galbraith and Newmark 2013, Tewari et al. 2018). Interestingly, inhibition of follistatin was shown to have no effect on the generic wound response despite being required for the missing tissue response (Gaviño et al. 2013). Moreover, inhibition of follistatin in uninjured animals does not affect homeostatic maintenance of existing tissues, indicating that follistatin could be required specifically for the increased proliferative response that occurs in response to injuries associated with tissue absence (Gaviño et al. 2013). A recent study focused on elucidating epigenetic control of regeneration in Hofstenia miamia, a regenerative member of a sister lineage to all bilaterians, including planarians, revealed that follistatin is directly regulated by the early growth response protein (Egr), which also activates the expression of many wound-induced genes (Gehrke et al. 2019). The case of follistatin provides a powerful tool to study the cellular and molecular regenerative responses that are required for regeneration.

As described above, planarians are able to regenerate upon minor injuries, such as eye resection, as an emergent property of homeostatic tissue turnover without increased neoblast proliferation (LoCascio et al. 2017). Major injuries further challenge the system through the loss of positional information or distinct progenitor populations. Although the missing tissue response (comprised of increased proliferation of neoblast proliferation, global cell death and sustained wound induced gene expression) might intuitively seem to be necessary for coping with a major regenerative challenge, it is elegantly demonstrated that regenerative responses guiding the patterning of the AP axis in planarians can be achieved by Wnt signaling induced by generic wound response along with progenitor production that maintains homeostatic tissue turnover (Tewari et al. 2018). (Figure
7B). Following wound-induced responses that occur within 24 to 48 hours, post-injury anterior-posterior patterning starts to be re-established in regenerating tissue fragments. For example, the posterior expression gradient of \textit{wntP-2} in an amputated tail fragment rescales its anterior expression such that expression is more restricted towards the posterior end of the piece (Petersen and Reddien 2009, Gurley et al. 2010). Concurrently, the anterior region of this tail fragment initiates expression of normally anteriorly expressed patterning genes, re-establishing a miniature version of the original planarian “gene expression map”, which provides the necessary positional information for guiding regeneration. Continuous maintenance of positional information in planarians guides homeostatic tissue maintenance and regeneration (Witchley et al. 2013).

Inhibition of \(\beta\)-\textit{catenin-1} by RNAi results in two-headed animals upon amputation, with both heads facing opposite directions. Strikingly, during homeostatic turnover under \(\beta\)-\textit{catenin-1} RNAi, many ectopic heads appear around the rim of the animal (Iglesias et al. 2008, Petersen and Reddien 2008, Gurley et al. 2010). Accordingly, Wnt signaling is implicated in the anterior versus posterior decision-making process at transverse wound faces. Comparably, inhibition of Wnt signaling components (Adell et al. 2009, Petersen and Reddien 2009b, Almuedo-Castillo et al. 2011, Owen et al. 2015, Reuter et al. 2015) can produce the same phenotype. The planarian genome contains two \(\beta\)-\textit{catenin} homologs and two truncated homologs, however, only \(\beta\)-catenin-1 was observed to participate in canonical Wnt signaling (Chai et al. 2010, Su et al. 2017). Remarkably, different planarian species that are incapable of head regeneration, can restore this ability upon inhibition of \(\beta\)-\textit{catenin} will restore head regeneration (Liu et al. 2013, Sikes and Newmark 2013, Umesono et al. 2013). Inversely, up-regulation of Wnt signaling by APC or \textit{notum} RNAi can result in the regeneration of tails instead of heads at both anterior- and posterior-facing wound planes (Gurley
et al. 2008, Petersen and Reddien 2011). *notum*, a secreted inhibitor of Wnt pathway through de-palmitoylation of Wnt proteins (Petersen and Reddien 2011, Kakugawa et al. 2015, Zhang et al. 2015), is normally expressed at the anterior tip of the animal. *notum* expression after injury is activated preferentially at anterior-facing wounds and is required for anterior fate establishment through deacetylation of Wnts to generate a low-Wnt environment, which is thought to drive anterior cell fates (Petersen and Reddien 2011). Conversely, at posterior-facing wounds, a high-Wnt environment establishes posterior tissue fates (Petersen and Reddien 2009).

While Wnt signaling establishes anterior-posterior axis, the planarian dorsal-ventral (DV) axis is formed and maintained by the conserved Bmp signaling. This was revealed by the phenotypes that occurred upon *bmp-4*, or *smad-1, smad-4* and *tolloid* RNAi, which disrupted the formation of DV axis (Molina et al. 2007, Orii and Watanabe 2007, Reddien et al. 2007). The medial-lateral (ML) axis is shown to be regulated by a *slit-wnt-5* circuit, with laterally expressed *wnt-5*, inhibiting medially expressed *slit*. RNAi of *slit* causes a collapse of the midline, and conversely, RNAi of *wnt-5* expands the midline tissues (Cebrià et al. 2007, Adell et al. 2009, Gurley et al. 2010, Oderberg et al. 2017). Further reports on patterning phenotypes (that arise upon inhibition of other constitutively expressed patterning genes) showed duplications in the trunk region through *ndl-3, wntP-2* and *ptk7* RNAi (Lander and Petersen 2016, Scimone et al. 2016), as well as posterior expansion of the central nervous system through *nou darake, fz5/8-4* and *wntA* RNAi (Cebrià et al. 2002, Kobayashi et al. 2007, Adell et al. 2009, Scimone et al. 2016). Genes that exhibit a continuous and regional expression profile in planarians that are linked with a patterning phenotype upon RNAi, or a patterning pathway contributing to establishing and maintaining the anatomical pattern in planarians, are named the position-control genes (PCGs) (Witchley et al. 39)
Expression domains for PCGs also indicate that they take part in the patterning of the animal (Figure 8), further establishing the presence of a dynamic and homeostatically maintained adult positional information guiding regeneration and tissue turnover (Reddien 2011). Strikingly, PCG expression occurs in muscle cells (Witchley et al. 2013).

In planarians, body-wall muscle includes longitudinal, circular, and diagonal fibers (Cebrià 2016). It was found that, myoD and nkx1 are required for the homeostatic maintenance and regeneration of longitudinal and circular muscle fibers, respectively (Scimone et al. 2017). Upon RNAi of both genes, significant loss of body-wall muscle fibers occurs leading to a sharp decrease of PCG expression, which result in patterning phenotypes and ultimately death (Scimone et al. 2017). Collectively, these results show that establishment and the dynamic nature of the positional information in planarians is central to form and maintain pattern during homeostatic tissue turnover, tissue and organismal-scale size regulation and regeneration.

Planarian size regulation during homeostasis is a fascinating biological problem. Previous studies indicated that global neoblast production rate is affected by feeding, starvation, and overall animal size (Baguñà 1976, Baguñà 1981) and size is globally allometrically regulated (Oviedo et al. 2003). Upon major tissue loss, during early phases of regeneration, resulting pieces that lack the pharynx and the brain cannot eat until missing anatomy has been is restored. The blastema forms only some of the missing tissues, and the rest of the anatomy is re-shaped to restore the correct proportions through morphallaxis. In the resulting regenerating animal, initially, some tissues can be overabundant and these overabundant tissues alter their size relative to the ultimate final size of

During homeostasis, proportions are always preserved, even when the animal is fed and growing, or its starved and shrinking. Presumably, during this process cell numbers are adjusted to increase or decrease while preserving functionality of all the organ systems. This is particularly interesting in the case of nervous system: In the starved animal, neuron numbers in the brain decrease, yet it can still drive the normal plethora of behavioral repertoire. This might indicate a functional redundancy or a currently undetectable loss of functional precision in the system. Conversely, in a growing animal, new neurons are added at a faster rate than the steady-state dynamics preserving brain size, resulting in the growth of the brain while brain proportions and functionality is maintained. In any case, preserving organ shape and function during growth and de-growth states possibly requires molecular, cellular and systems-level strategies and adaptations. Uncovering the biological rules for integrating new cells or eliminating the old ones in a set system, without disrupting functionality, could be a fascinating research direction with the advancement of new molecular tools in this system.

The remarkable regenerative feats, simple body plan yet complex anatomy, and existence of uniquely dynamic cellular and systems-level regulatory programs in planarians make them an outstanding testbed for the investigation of the possible roles of self-organization during regeneration.
CONCLUSION

Self-organizing dynamics are employed in the formation and integration of tissues and systems during development and regeneration in diverse living systems. From the transition to multicellularity, to the formation of modular organs, to functional neural strategies expanding cognitive capacity, formation of self-organizing attractor dynamics can be observed. Although this phenomenon has been characterized mainly in the fields of chemistry, physics and mathematics, as well as in developmental biology and neuroscience, its roles in organism-wide regeneration and homeostatic maintenance has not been deeply explored. Planarians are masters of regeneration. They can regenerate from infinite amount of injuries, ultimately restoring their anatomical features and functionality. Planarians are in a state of constant flux at an organismal scale because of the continuous incorporation of new cells forming all of the tissues in the system, and the continuous cell death that occurs as tissues turnover. This dynamic homeostatic system constantly remodels the organism, in pursuit of preserving shape and function. The work presented in this thesis explores the questions of how regeneration and homeostatic tissue maintenance work at cellular and systems-level scales. It seeks to elucidate fundamental rules guiding specific aspects of these homeostatic and regenerative processes.

I will describe results in Chapter 2 that show that self-organization and progenitor targeting are being used as a strategy to maintain form and function in homeostasis and regeneration in planarians, using the planarian eye and the central the nervous system as a test beds.
Figure 1. Self-organization is prevalent in nature.

A) As opposed to self-assembly, self-organization is a continuous process that is adaptive, responsive and regenerative. In self-organizing systems, multiple local interactions between the elements of a system constantly interact forming stable attractors, leading to emergence of a robust pattern and/or function.

B) Examples of self-organization in chemical, physical, and biological systems: Belousov-Zhabotinsky reaction (left) (Credit: Michael C. Rogers and Stephen Morris, Experimental Nonlinear Physics, University of Toronto. Used with permission); Stable hexagonal cloud pattern at north pole of Saturn captured by the Cassini Spacecraft (Credit: NASA/JPL) (mid-left); Flocking starling birds (Credit: Walter Baxter, cc-by-sa/2.0) (mid-right); Transcriptional and morphological self-organization of the attached early human embryo, OCT4 (green), GATA6 (red), GATA3 (blue) (modified from Deglincerti et al. 2016).
Figure 2. Self-organization can guide complex collective behavioral dynamics, rhythmic and oscillatory cellular behaviors; development and regeneration of *in vitro* tissue structures

A) Characteristic swimming configurations exhibited by the golden shiner fish school: swarm state, polarized state and milling state (Tunstrøm et al. 2013).

B) cAMP signaling dynamics through starvation stages of *Dictyostelium discoideum* cells, visualized through cAMP indicator, Flamindo2 (Hashimura et al. 2019; CC BY 4.0).

C) A variety of *in vitro* self-organizing multicellular structures that are designed using synNotch system (Toda et al. 2018).

**Figure 3. Formation of digits, ocular dominance columns and other cortical maps.**

A) Molecular interactions between Bmp, Sox9, and Wnt and their modifiers Hox and Fgf, resulting in a Turing network that drives digit specification. Illustration of a mouse forelimb digits indicated (orange) (Raspopovic et al. 2014, Cooper 2015).

B) Formation of ocular dominance columns in three-eyed frogs. Distribution of eye-specific autoradiographic signal in the optic tectum of a 3-month post-metamorphic three-eyed frog after injection of 10μCi of [3H] prolinein to the vitreous body of one of the original eyes (Constantine-Paton and Law 1978, Constantine-Paton and Law 1982).

C) Model showing emergence of different topographic maps upon increasing interconnectivity between simulated neurons. Accordingly, 1D connectivity leads to layer formation, 2D interconnectivity to a topographic map, and ocular dominance column-like arrangement emerges when 4×4 grids are interconnected (Weigand et al. 2017).
A. Reaction-Diffusion model

1. Concentration diffusion of inhibitor
   inhibition of activator

   Biological space

2. Concentration

   Biological space

B. French Flag Model

Concentration

CT1

CT2

Concentration

Biological space

C. Dynamics of a noisy morphogen gradient and cell sorting

Concentration

CT1

CT2

Concentration

Sorting (local cellular re-arrangements)

Biological space

D. Patterning of the Drosophila embryo

bicoid

nanos

A

P

gaint

Krüppel

gaint

even-skipped

wingless

Time
Figure 4. Formation of pattern in developing (and regenerating) tissues

A) Turing’s Reaction-Diffusion Model guiding pattern formation in developing tissues and organs. Examples include digit formation and establishment of coat patterns in mammals (Turing 1952).

B) Wolpert’s Positional Information Model leading the formation of a “French Flag”. Here, a pattern can result upon a graded distribution of a morphogen, and cells across this gradient make different fate choices based on their distance from the source of the morphogen. Formation of the “French Flag” is mainly guided by two critical concentration thresholds in this model, yet there can be many specific concentration gradients forming numerous boundaries across the developing tissue (illustration adapted from Green and Sharpe 2015; Wolpert 2015).

C) Cellular sorting can fine-tune aberrations in newly patterning tissue caused by noisy morphogen gradients (Xiong et al. 2013).

D) Patterned gene expression and protein distribution at different stages of the developing Drosophila embryo. Biased or polarized initial distribution of these proteins lead to formation of distinct boundaries and segmentation through inhibitory or activating interactions among each other (Driever and Nusslein-Volhard 1988, Driever and Nusslein-Volhard 1989, Rivera-Pomar and Jackle 1996, Wolpert 2015).
Figure 5. Anatomy and regeneration of the planarian *Schmidtea mediterranea*

A) Live image of an adult *Schmidtea mediterranea* (dorsal view), indicating eyes that are formed by photoreceptor neurons that project ipsi- and contralaterally to the brain, and the complex two-lobed brain anatomy revealed by whole-mount fluorescence in situ hybridization (FISH) of choline acetyltransferase (*ChAT*). Different sections of the z-axis are pseudo-colored, displaying ventral nerve cords (purple), cephalic ganglia (blue), dorsal photoreceptor neurons (red), and peripheral head rim neurons (orange).

B) FISH using different RNA probes (*smedwi-1, madt, collagen, opsin, ChAT*) to reveal cell types marking indicated tissue-types (on a DAPI background).

C) Pharynx, the feeding tube, of the planarian, is innervated by neurons and mostly made of muscle cells (Image courtesy of Carolyn E. Adler; Adler et al. 2014).

D) Planarians can regenerate complex anatomy with the correct proportions upon an infinite different types of injuries anatomy after diverse injuries. Restoration of proportions restoration occurs without net organismal growth and the resulting are smaller than the original intact animal. Regenerated animals can feed and grow after regeneration.

E) Neoblasts give rise to diverse specialized progenitors that can be marked by the enduring SMEDWI-1 (blue) protein in the cells, while the cells are at their early phase of differentiation. Here, SMEDWI-1/*ChAT*(orange) double-positive cells (boxed and zoomed) indicate newly differentiating neurons in the brain.
A. Intact | Decapitation | Eye resection

B. "Target Blind" Regeneration

Homeostasis  
Eye resection
Regeneration  
Homeostasis

incorporation = cell death  
incorporation only
incorporation > cell death  
incorporation = cell death

- photoreceptor neuron
- optic cup cell
- ovo+ eye progenitor
- dying cell

Graph: ovo+ eye progenitors per animal

No injury  
Decapitation  
Eye resection
Figure 6. “Target blind” model of regeneration showing that eye absence is not required for eye progenitor amplification

A) FISH with *ovo*+ RNA probe, 3 days after surgery, showing homeostatic levels of *ovo*+ progenitor specification upon eye resection, but an increase in *ovo*+ progenitors after decapitation. Eyes regenerate in both cases (LoCascio et al. 2017).

B) “Target blind” regeneration model showing tissue-specific eye regeneration does not involve a regulation of stem cells triggered by the presence or absence of the eye itself. This model offers a simple, elegant and dynamic platform for homeostatic tissue maintenance and regeneration, which does not comprise complex feedback mechanisms to achieve tissue-specific regeneration (adapted from LoCascio et al. 2017).
Figure 7. Wound induced gene expression response in regeneration

A) Comparison between minor and major injuries in terms of regenerative responses indicated that major injuries activate and sustain a second peak of wound response program termed the “Missing Tissue Response”, that involves, neoblast proliferation, a global increase in cell death and sustained wound induced gene expression (Pellettieri et al. 2010, Wenemoser and Reddien 2010, Wenemoser et al. 2012).

B) *follistatin* inhibition using RNA interference was elegantly used to study the requirements for successful regeneration in terms of wound induced response programs. The results of this study indicate that Missing Tissue Response accelerate regeneration, but it is dispensable for regeneration itself (Tewari et al. 2018).
Figure 8. Positional Control Gene expression guide regeneration

A) A map of PCG gene expression domains on the AP axis (Witchley et al. 2013, Scimone et al. 2016; illustration (left) is adapted from Reddien 2018), and examples of three different PCG expression domains revealed by whole-mount FISH using wntP-2, sFRP-2 and ndl-4 RNA probes.
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Chapter 2:

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

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Published as:

SUMMARY

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 the 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 used 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.
INTRODUCTION

The capacity to regenerate is widespread and variable throughout the animal kingdom. There is much current interest in understanding how regenerative progenitors reform missing organs and tissues. Complex tissue architectures can be formed through an adaptive, nonlinear, and dynamic process called self-organization, described by the emergence of orderly structure through local interactions between the basic elements of a system (E. Bonabeau 1999, S. Camazine 2003). In self-organizing systems, ordered patterns emerge from an initially disordered and noisy environment. Self-organization has been invoked in embryonic development (Turing 1952, G. Nicolis 1977, Halder et al. 1995, Tsiairis and Aulehla 2016, Corson et al. 2017, Harrison et al. 2017, Shyer et al. 2017), regeneration (Stocum and Melton 1977, Stuckemann et al. 2017), and the in vitro formation of complex structures such as the optic cup (Eiraku et al. 2011) and organoids (Sato et al. 2009, Lancaster et al. 2013). 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 (Wagner et al. 2011) 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 (Lapan and Reddien 2011, Lapan and Reddien 2012, Adler et al. 2014).
RESULTS

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 composed of photoreceptor neurons, which project axons ipsilaterally and contralaterally to the brain, and pigmented optic cup cells (Fig. 1A) (Lapan and Reddien 2012). 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 (Fig. 1, A and B, and figs. S1A and S2A) (Lapan and Reddien 2011, Lapan and Reddien 2012, LoCascio et al. 2017). ovo+ progenitors are formed coarsely in the dorsal prepharyngeal region and migrate anteriorly, where they can incorporate into existing eyes or nucleate eyes de novo (fig. S1, A to D) (Lapan and Reddien 2012).

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 (Morgan 1898). Morphallaxis involves new cell production, cell death (Pellettieri et al. 2010), and the remodeling...
of differentiated tissues (Forsthoevel et al. 2011). During morphallaxis, existing organs and major tissues are retained but gradually change their proportions and relative positions (Fig. 1, C and 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 (over 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 (Witchley et al. 2013, Scimone et al. 2016). Inhibition of multiple PCGs (such as *bmp4*, *wntP-2*, and *ndl-3*) by means of RNA interference (RNAi) causes patterning phenotypes, such as ectopic heads, pharynges, or eyes (Witchley et al. 2013, Hill and Petersen 2015, Lander and Petersen 2016, Scimone et al. 2016). After amputation, PCG expression domains are rapidly reestablished in muscle (in a matter of 2 to 3 days). For example, within 48 hours after decapitation, a head fragment initiates posterior PCG expression at the wound, and anterior PCG expression domains shift toward the head tip. Initial PCG expression domain regeneration does not require neoblasts (Witchley et al. 2013) 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 and fig. S3, A to 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 and fig. S3, A to E). Eyes slowly shrink and move anteriorly (morphallaxis) to ultimately align anatomy and PCG expression domains (Fig. 1, C and D). Regardless of whether PCGs directly control this process, their expression changes indicate that rapid positional information shifting during regeneration leads to its discordance with anatomy (Fig. 1E and fig. S3, A to E). Because progenitors continuously target the eye during tissue turnover (Lapan and Reddien 2012), 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 or tissue formation as the “target zone” (TZ). We assessed targeting decisions of eye progenitors by amputating heads and then unilaterally resecting one eye after 3 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 (LoCascio et al. 2017). 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, \textit{de novo} eye nucleation occurred at more anterior locations than the position of the remaining eye, generating asymmetric animals (Fig. 2A and fig. S4, A and 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 and fig. S4).

We predicted that if positional coordinates were shifted in different directions, then 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 day later. Accordingly, regenerating eyes shifted posteriorly (Fig. 2C). These data indicate that rapidly changing positional information after injury can define the eye progenitor TZ.

\textit{Eyes act as self-organizing centers and can trap migratory eye progenitors}

In the experiments above, why did eye progenitors not 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 and fig. S5A), preventing them from reaching more anterior positions. In self-organizing biological systems, molecular coupling strategies lead to maintained, functional anatomical architectures (Keller 2009). Self-organizing systems act as attractors in such cases, leading to the stability of shape and function. To test whether 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, then 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 after amputations). As predicted, animals formed a third eye, located anteriorly and on the same side (left) as the uninjured eye (Fig. 3, A and B, and fig. S5D). All three eyes were targeted by eye progenitors (Fig. 3, C to E, and 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 and fig. S5, G and 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. S8, A to C; and movies S1 to S4).
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 continuously after amputation; eye position would depend on its time of nucleation after amputation. To test this possibility, we decapitated animals and unilaterally resected right eyes on subsequent days (days 1 to 6). De novo eye nucleation occurred at progressively more anterior locations depending on the time of eye resection after decapitation (Fig. 4A). This indicates that eye TZ rescaling is a continuous process after 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 along 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 after 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 ectopic left eyes (Fig. 4, B and 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. 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 through 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. S7, A and B). These findings suggest that 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* (Cebria et al. 2007). The slit medial expression domain is restricted by laterally expressed *wnt5* (Gurley et al. 2010). *wnt5* and *slit* RNAi can affect ML eye formation (Oderberg et al. 2017). After *wnt5* RNAi, serial eye nucleation was observed, progressing laterally as *slit* expression boundaries expanded (Fig. 5, A and 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. S10, A to C). These results implicate a *slit-wnt5* circuit (acting directly or indirectly) as a ML TZ determinant.

*notum* encodes a broadly conserved Wnt inhibitor (Petersen and Reddien 2011) expressed near the anterior brain and in the anterior pole and is involved in anterior tissue patterning (Petersen and Reddien 2011, Hill and Petersen 2015). *notum*(RNAi) animals develop a set of much more anterior and medial eyes under homeostatic conditions (Hill and Petersen 2015). We postulated that this anatomical pattern can be explained by 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 those in 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. 3, C to 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 mispositioned 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 or tissue. The TAZ includes the TZ, but when larger than the TZ, it allows targeting of self-organizing centers in incorrect (non-TZ) locations. TZ and TAZ concepts make testable predictions. We first asked whether eyes would be regenerated in three-eyed animals through 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 after removal (Sakai et al. 2000).

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. 6, C to F, and fig. S11, B to F). Transplanted eyes in the anterior were maintained, whereas eyes transplanted into tails shrunk and ultimately disappeared (Fig. 6, E and F, and fig. S11, B and D). Transplanted eyes sent projections toward 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 used RNAi phenotypes to study the TAZ concept. nou darake(RNAi) animals generate ectopic posterior eyes (Cebria et al. 2002). After removing these animals from RNAi conditions, all eyes remained. We performed unilateral eye resections 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 \textit{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 \textit{wnt5}(RNAi) animals removed from RNAi conditions showed that both medial and lateral eyes were targeted by progenitors (fig. S12, B and C). Similarly, \textit{slit}(RNAi) animals off RNAi also maintained medial eyes (fig. S12D). Last, \textit{notum}(RNAi) animals showed anterior eye regeneration after 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) by 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 TZ (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 TAZ, 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 after injury. The coarse progenitor specification zone is essential, allowing progenitors to maintain organs or 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 TAZ 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 that define TZs at the appropriate position for final anatomy. If TZs were not discretely defined, anatomy duplications would also emerge (fig. S13B). This model identifies a set of rules that are used for stabilizing the system and its coherent functional properties. The existence of adaptive TZs and TAZs 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 that guide coherent
tissue formation and maintenance in dynamic and noisy biological processes such as regeneration.
MATERIALS AND METHODS

Surgical Procedures and Eye transplants

Animals were placed on moist filter paper on a cold block in order to limit movement, and a microsurgery blade was used to remove desired tissues. For eye transplants donor animals were lethally irradiated to prevent any carry over eye progenitor cell. After anesthesia using 0.2% Chloreton in planarian H$_2$O (1,1,1-Tricloro-2-methyl-2-propanol) for two minutes, a thin slit cut was made to desired locations of the recipient animals and a small hole was generated by gently moving the surgical blade up and down within the slit cut. Recipient animals were washed in Holtfreter’s Solution (Guedelhoefer and Sanchez Alvarado 2012) for 2 minutes and rested in 0.2% Chloreton for 2 minutes, briefly washed in Holtfreter’s Solution and transferred on the cold block to introduce the excised eyes. Eye resections were performed with a dissecting microscope by trimming the pigmented tissue around the eyes with a surgical blade, leaving only the white area and the visible optic cup of the eyes. The pigmented ventral side of this tissue was also trimmed away before transplantation. The eyes were gently pushed inside the previously generated holes in the recipient animals. Transplanted animals were immobilized using Type IV, 5% ultra-low melting agarose (Sigma) on top of Whatman$^\text{TM}$ (GE Healthcare, Life Sciences) filter paper. Solidified gel was covered using Rasta Royale ultra-thin rolling paper soaked in Holtfreter’s Solution. A separate eye transplantation procedure was also performed, using eyes isolated following a collagenase-based eye dissociation protocol developed previously (Lapan and Reddien 2012). For this procedure, isolated eyes (fig. S11) were delivered inside a cut slit in the recipients using a glass capillary via mouth pipetting. The immobilization strategy using 5% ultra-low melting agarose was not used for these animals, instead two layers of rolling paper
were placed on top of the animals following soaking with Holtfreter’s Solution. Transplanted animals were kept in 10°C overnight and recovered by cutting the gel around and also on top of the animal. Animals were placed in planarian H2O and kept at 22°C for recovery.

**Fluorescence In Situ Hybridization (FISH)**

Animals were sacrificed in 5% NAC in 1X PBS and fixed in 4% formaldehyde in PBSTx (0.1% Triton X-100 in 1X PBS). The samples were stored in methanol at -20°C until use. All animals were bleached in 1X SSC solution containing 5% deionized formamide and 1.2% hydrogen peroxide for 2 hours, exposed to bright light. Animals were treated with 2 mg/ml Proteinase-K in PBSTx containing 0.1% SDS and hybridized with RNA probes diluted 1:800 in a solution of 50% formamide, 5X SSC, 1 mg/ml yeast RNA, 1% Tween-20, and 5% dextran sulfate at 56°C, overnight. Animals were blocked for 1-2 hours prior to labeling overnight at 4°C with anti-DIG-POD (1:1500, Roche), anti-FITC-POD (1:2000, Roche), or anti-DNP-HRP (1:100, Perkin-Elmer) in blocking solutions of PBSTx containing 5% heat inactivated horse serum and 5% 10X casein solution (Sigma) for anti-DIG-POD, 10% western blocking reagent for anti-FITC-POD, and 5% horse serum and 5% western blocking reagent (Roche) for anti-DNP-HRP. For tyramide development, animals were placed for 10 minutes in borate buffer (0.1M boric acid, 2M NaCl, pH 8.5), followed by 10 minutes in borate buffer containing rhodamine (1:1000) or fluorescein (1:1500) tyramide and 0.0003% hydrogen peroxide. Before antibody labeling for a second probe, peroxidase inactivation was performed in 1% sodium azide overnight at 4°C. Animals were stained in a solution of 1 mg/ml DAPI (Sigma) prior to mounting on slides. FISH protocol was adapted from previous work (Pearson et al. 2009, King and Newmark 2013). GenBank accession numbers for sequences used for all FISH probes are provided in Table S1.
**RNAi**

dsRNA was synthesized by in vitro transcription (Promega) from PCR-generated templates with flanking T7 promoters, ethanol precipitated, resuspended in water and annealed, and diluted in liver for delivery by feeding (Petersen and Reddien 2008, Rouhana et al. 2013). RNA was quantified using a Nanodrop (Thermo Fisher Scientific) and determined to be at least 5 mg/ml. *wnt5, slit, notum,* and *Caenorhabditis elegans unc-22* (control) (Benian et al. 1989) dsRNA were fed (mixed with blended liver) every 3 days for a total of 12 times. Animals were collected at different time points (indicated in figures) for FISH and analysis. GenBank accession numbers and sequences used for all dsRNA preparations are provided is in Table S1.

**Immunohistochemistry**

Prior to immunostaining, FISH was performed as described above. After FISH steps, peroxidase inactivation was performed in 1% sodium azide overnight at 4°C. Animals were washed in PBSTx (0.1% Triton X-100 in 1X PBS) and placed in blocking solution (10% horse serum in PBSTx) for 1-2 hours. After PBSTx washes and blocking, samples were labeled with either a mouse anti-ARRESTIN (1:5000) (kindly provided by Kiyokazu Agata) or a rabbit anti-SMEDWI-1 antibody (Scimone et al. 2010) (1:1000) in block overnight at 4°C. Samples were then developed with fluorescein tyramide in borate buffer containing 0.0003% hydrogen peroxide and or fluorescein (1:1500) tyramide, and stained with DAPI prior to mounting.

**Irradiation**

Animals were irradiated using a dual Gammacell-40 Cesium-137 source to deliver 6000 rads.
**Behavior**

A layout for the behavior arena was generated using Adobe Illustrator CC software (Fig. 3K). The arena was generated using an iPad as a surface displaying the generated arena continuously. A rectangular plate containing planarian H$_2$O was placed on top the iPad and the arena was covered with a box to eliminate any directional light from the test environment. An iPhone was placed on top the box to record videos of behaving animals under different test conditions. 8 animals per testing group were placed in the middle of the arena within the boundaries of 5th and the 6th bands in the arena at the beginning of each trial. Positions of each animal at the end of each minute were recorded for a total of 5 minutes. For analysis positions of each animal at the end of each minute was averaged and compared for each time point for every group. Sham surgeries outside of eye areas were applied to test if the surgery itself can affect behavior in animals with intact eyes. Both eyes were resected in the negative control group and finally original eyes were resected leaving only the ectopic eye on day 1 of testing in the ectopic eye group. Ectopic eyes were later resected to test the loss of negative phototaxis behavior on day 2 after initial surgeries.

**Image Acquisition, Measurements and Quantification**

Live images were acquired using a Zeiss Discovery V8 stereomicroscope with an AxioCam HRC camera. Fluorescence image acquisition was performed using a Zeiss LSM 700 confocal microscope. ImageJ software (Fiji), AxioVision and ZEN digital imaging software (Zeiss) was used for processing and quantification of all images. ovo+ eye progenitor quantification was performed on maximum intensity projections (MIPs) of optical sections using blind manual counting. ovo+, ops+in, SMEDWI-1+/opsin+ and ARRESTIN+ cells were quantified blind in
files with randomized numerical names by examining all fluorescence channels in pre-defined regions of animals as shown in figures. Measurements of anterior and posterior eyes were made using AxioVision software using actual distance units, marking the most anterior point of the de novo nucleated eye and non-resected eyes and measuring the absolute distance between these points (fig. S4A). The same strategy was used for posteriorly nucleated eyes, marking the most posterior points of the two eyes and measuring the absolute distance between these two points (fig. S4A). Absolute medial distance between the two eyes was measured in full sagittal amputation groups reading out the medial shift in de novo nucleated eyes.

**Quantification and Statistical Analysis**

All statistical analyses were performed in GraphPad Prism software. Statistical tests, significance, data points, error bars, and other information relevant to figures are described and explained in corresponding legends.
ACKNOWLEDGMENTS

We thank all Reddien lab members for valuable discussions and I. Oderberg for \textit{wnt5} RNAi discussions. Funding: We acknowledge NIH (R01GM080639) support. K.D.A. is supported by a Howard Hughes Medical Institute (HHMI) International Student Research Fellowship and the Massachusetts Institute of Technology (MIT) Presidential Fellowship Program. S.A.L. was supported by a National Defense Science and Engineering Graduate Fellowship. P.W.R. is a HHMI Investigator and an associate member of the Broad Institute of Harvard and MIT. 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 are available in the manuscript or the supplementary materials.
Figure 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 morphallaxed 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 fluorescence in situ hybridization (FISH)] rescales before anatomy changes occur. White arrowheads indicate brain posterior. (F) Positional information pattern regenerates faster than the anatomical changes. Experimental strategy to reveal a predicted target zone (nucleation target) shift in a decapitated and day 3 eye-resected head fragment. Scale bars, 200 µm.
Figure 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+ (LoCascio, Lapan et al., 2017)] target both non-resected eyes and regenerating eyes at different positions in the same animal (white 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. In (A), (C), and (D), red arrowheads indicate regenerating eyes. Student’s t test, **P \leq 0.01, ****P \leq 0.0001. Scale bars, 200 \mu m.
Figure 3. Generation of an alternative stable neuroanatomical state.

(A and B) Decapitation and parasagittal amputation in large sexual animals plus day 3 unilateral eye resection results in three-eyed animals. Red arrowhead indicates regenerating eye. (C) Map of ovo+ cells from 20 three-eyed animals (day 16 after surgery). (D and E) SMEDWI-1+/opsin+ newly differentiated cells (white arrowheads) were detected in all three eyes (n = 144 eyes examined). (F) Arrestin immunohistochemistry and opsin FISH. Ectopic eyes integrate into visual circuitry. White arrowheads indicate axonal projections. (G) Decapitation and left parasagittal amputation on three-eyed animals resulted in five-eyed animals. (H) Behavior analyses: Misplaced eyes drive negative phototaxis. CA, control arena; TA, test arena. Statistical significance: one-sample t-test comparing each column mean with a hypothetical value of 6 corresponding to chance (n = 8 animals per cohort); Bonferroni correction was applied. Scale bars, (A), (D), (E), and (G), 200 μm; (B), (C), and (G), 100 μm. ER, eye resection. Statistical significance: one-way analysis of variance (ANOVA): *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001. NS, not significant.
Figure 4. Eyes nucleate at predictable positions during dynamic positional information shifting.

(A) Unilateral eye resection time course (days 1 to 6 after decapitation) reveal a continuously shifting TZ. (B and C) Unilateral eye resections after decapitation and parasagittal amputation. In (A) and (B), red arrows and arrowheads indicate regenerating eyes. Statistical significance: one-way ANOVA: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001. NS, not significant. Scale bars, 200 µm.
Figure 5. *slit, wnt5, and notum are involved in establishing a TZ.*

(A) *wnt5, slit,* and control RNAi in uninjured animals (more than eight double-stranded RNA feedings, more than 4 weeks). FISH: *slit* expression expansion and reduction after *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 after notum RNAi led to anteriorly shifted eye nucleation without decapitation, implicating notum in AP TZ regulation. Red arrowheads indicate regenerating eyes. Asterisks indicate ratio of animals with outcome as shown. Scale bars, 200 μm.
Figure 6. Ectopic eyes are maintained in a broad TAZ.

(A) Long-term feeding of wild-type three-eyed animals. All eyes are stably maintained. (B) Selective eye resections reveal a TAZ where ectopic eyes can be maintained but not regenerated (white arrowheads). (C) Eye transplantation strategy into or outside of the TAZ. Donor animals were irradiated so as to lack progenitors. (D) Transplanted eyes sent projections into the visual circuit. (E) Transplanted eyes (red arrowheads) were maintained in the TAZ, but not outside of it (tail). (F) Only TAZ-transplanted eyes incorporated progenitors (SMEDWI-1+/opsin+ cells, analyzed 12 to 16 days after transplantation) and SMEDWI-1+/opsin+ cell quantification (***P ≤ 0.01). White arrowhead indicates SMEDWI-1+/opsin+ cell. (G) ndk(RNAi) animals, off RNAi, with unilateral eye resection. Only one eye regenerated (in TZ); non-resected eyes remained. Red arrowheads in (B) and (G) indicate resected and regenerating eyes. (H) Eye progenitor specification zone by mapping ovo+ cells in 13 animals and generalized model for progenitors integrating self-organization, a TZ, and a TAZ. Red arrowheads indicate newly specified migratory progenitors and an example for system-level behavior predicted by the model in response to a regenerative challenge. Scale bars, 200 µm.
Figure. S1

(A) ovo+ eye progenitors nucleating *opsin*+ photoreceptors are shown on Day 1, 2, and 3 after decapitation in newly regenerating heads, (B) and on Day 0, 1, 2 and 3 after unilateral eye resection in newly regenerating head. (C) Quantification of ovo+ and opsin+ cells at the time of eye nucleation. (D) Anterioposterior and dorsoventral distribution of ovo+ cells are shown. Student’s t-test: ***p 0.001. Scale bars, 200µm.
Fig. S2. (A) Measurements for eye positioning in different sizes of Schmidtea mediterranea. An ideal worm is illustrated with predicted coordinates of the eyes based on animal length.
Figure S2.

(A) Measurements for eye positioning in different sizes of *Schmidtea mediterranea*. An ideal worm is illustrated with predicted coordinates of the eyes based on animal length.
Figure S3.

(A-C) Regionally expressed genes reset their spatial expression patterns during morphallaxis. (D) Quantification of anterioposterior distance between landmarks in the brain (eye and posterior boundary of \textit{cintillo}+ neurons) and a rescaling PCG (\textit{ndl-2}) upon decapitation in headpieces. (E) A rescaling PCG (\textit{ndl-2}) versus eyes upon anterior tip cut on day 0 and 4; green arrow indicates debris/background signal; white arrows show anterior expression boundaries. Statistical significance was assessed by one-way ANOVA: ****p 0.0001; ns, not significant.
Figure S4.

(A) Measurement strategy for asymmetrically nucleated eyes in head fragments is shown. (B) Illustration depicting asymmetric eye nucleation following decapitation and unilateral eye resection in both eyes. (C) SMEDWI-1+/opsin+ photoreceptor neuron counts and their localization (mapped and overlaid) in each eye following decapitation and unilateral eye resection. (D) Distribution of ovo+ progenitors upon decapitation in day 0 and day 4 in headpieces; newly specified cells (ovo+/smedwi-1+) are circled. Scale bars, 200µm. (ns, not significant).
Figure S5.

(A) Illustration showing the suggested local attractor nature of existing eyes. (B) Illustration showing how a ML targeting shift of eye progenitors, such as could occur following a parasagittal amputation, could lead to escape of progenitors from the attractive nature of an existing eye, enabling movement to a more anterior position. (C) Full parasagittal amputation and eye resection (on the same side) on day 3 resulted in medially nucleating eyes, further confirming that eye nucleation can be directed at predicted locations and directions upon surgery (*p 0.05). (D) Generation of 3- and 4-eyed animals after decapitation and lateral sagittal cut without eye resection. (E) Strategy for measuring the distribution of ovo+ progenitors in 20 3-eyed animals on day 16 (in Fig 3C) (F) Eye nucleation sequence following decapitation-parasagittal amputation groups followed by a day 3 unilateral eye resection. (G) Day 19 pictures for decapitated and left or right parasagittally cut 3-eyed animals. (H) Decapitation and right parasagittal amputations on 3-eyed animals resulted in 4-eyed animals. Student’s t-test: *p 0.05. Scale bars, 200µm.
1. Surgery

2. Unilateral eye resection (day 5); reset complete for theoretically correct eye position

3. Eye nucleation

4. Morphallaxis
Figure S6.

(A) Illustration showing prediction for *de novo* eye nucleation coordinates upon decapitation-parasagittal amputation and right eye resection.
Figure S7.

(A) Asymmetric nucleation of day 3 unilaterally resected eyes upon decapitation relative to existing brain anatomy. (B) Asymmetric nucleation of eyes, nucleation of a third eye because of the newly established coordinates in smaller head fragment, and unilaterally asymmetric regeneration of the brain after decapitation and day 3 sagittal amputation. White dashed lines indicate distance between eyes, and red dashed lines indicate distance between right and left cephalic ganglia in both experimental conditions. Scale bars, 200µm.
Figure S8.

(A) Negative phototaxis behavior is recovered in animals with bilateral eye resection after day 5. (B) Negative phototaxis behavior is maintained in decapitated head fragments on day 1 after decapitation. (C) Negative phototaxis behavior present in animal with only a misplaced eye is lost after resection of the eye. (Statistical significance was assessed by one-sample t-test comparing each column’s mean with the hypothetical value of 6, defining chance, in all groups).
Figure S9.

(A) Illustrations depicting formation of ectopic lateral and medial eyes because of the expansion and loss of a slit expression zone under \textit{wnt5} and \textit{slit} RNAi, respectively. Also depicted are mediolateral shifts in the position of de novo regenerating eyes upon unilateral eye resections (before the appearance of lateral eyes) under \textit{wnt5} and \textit{slit} RNAi.
Figure S10.

(A-C) Distribution of ovo+ progenitors in control, wnt5, and slit RNAi animals; newly specified cells (ovo+/smedwi-I+) are circled. Scale bars, 200μm.
Figure S11.

(A) opsins+/SMEDWI-1+ cells shown in old eyes that are outside of TZ in late-stage 3-eyed animals. (B) Examples of eye transplantation into the targetable zone and the tail, which reveal that eyes that are transplanted into tails shrink and disappear within a month. (C) Characterization of enzymatically purified and surgically trimmed eyes by FISH using a muscle marker, collagen; a brain marker, Choline acetyltransferase (ChAT); and a photoreceptor neuron marker, opsins. Scale bars, 100µm. (D) Transplants using enzymatically-purified eyes show only eyes transplanted in TAZ perdured, yet at a lower frequency. (E) Examples of eye transplants into different positions along the AP axis of the animals. Eye transplants into the targetable zone were maintained. Boundaries of the targetable zone roughly coincide with the ovo+ progenitor specification zone. Eyes transplanted into the tail were maintained upon fissioning of the animals in tail fragments because of regenerative rescaling of positional information (N=8). (F) Eyes transplanted into tails sent axonal projections anterior and posteriorly. Scale bars, 200µm.
wnt5 RNAi
Before eye resection
Day 1 after eye resection
Day 18 after eye resection

B: wnt5 RNAi (off RNAi - 3 months)
opsin / SMEDWI-1

C: Number of opsin+/SMEDWI + cells
* 0 2 4 6

D: off RNAi - 3 months
slit RNAi

E: Long-term notum RNAi

17/22

10/10
Figure S12.

(A) Unilateral eye resection after long-term wnt5 RNAi (12 RNAi feedings) showing laterally shifted TZ based on nucleation of the most lateral eye (day 18 post eye resection). (B-C) SMEDWI-1+/opsin+ cells and quantification in off wnt5 RNAi animals (off RNAi 3 months) showing all eyes are targeted by eye progenitors. (D) Maintenance of the medial eye 3 months after slit RNAi (off RNAi 3 months) indicating eyes at (or near) the midline can be targetable. (E) Long-term notum RNAi animals develop anterior sets of eyes indicating a sliding Target Zone (TZ). Extra rows of eye are maintained, indicating they are targetable. One-way ANOVA: *p 0.05. Scale bars, 200µm.
Figure S13.

(A) Model illustrating key components (B-C) Simulated predictions of the model illustrating behaviors of a system with a weak self-organizing center and a larger Target Zone (TZ).
**Supplementary Table 1.** GenBank accession numbers for genes that were used for FISH probes and dsRNA:

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REFERENCES


Chapter 3:

Discussion
EMERGENCE OF STABLE PATTERNS IN DEVELOPMENT AND REGENERATION

During regeneration, planarians face multiple systems-level challenges to solve. Upon a major injury, positional information starts resetting faster than the anatomical changes take place. This can produce a discordance between the new positional information and the existing anatomy. Formation of dynamic and adaptive attractor centers in self-organizing systems allows them to solve some of these challenges. When progenitors encounter their differentiated discrete target tissue, which can behave as an attractor, they can be incorporated into that existing structure, even during dynamic positional resetting. This process ensures that progenitors will continue targeting their self-organizing differentiated tissue structures, even if they are in the “wrong” position with respect to the resetting positional information. This process ensures continuation of progenitor targeting towards the remaining anatomical structures during regeneration. It also prevents iteration of existing structures when the system is faced with such regenerative challenges: Remaining modular tissue structures prevent progenitors from reaching to their new Target Zones (TZ), thus stopping a new nucleation of the same tissue/organ, ultimately blocking formation of iterative structures that might interfere with normal function. In this way, many of the regenerating animal pieces can also remain functional by preserving spatial interactions among cells and tissues throughout the regeneration process.

Broad and coarse specification of progenitors is a critical and fundamental feature of successful regeneration. This feat enables progenitors to target and maintain existing anatomy as positional coordinates change. A Targetable Zone (TAZ) also indefinitely maintains ectopic organs, which can form upon a series of specific major regenerative challenges, but these ectopic organs that are
in incorrect locations with respect to their actual TZ are not regenerated upon their resection. Although maintenance dynamics of an ectopic organ might appear complex, from this viewpoint, it is essentially guided by simple rules in planarians.

Homeostatic size regulation in planarians is another striking aspect of their biology, where self-organization and broad progenitor specification are crucially involved. In a growth state, increased neoblast proliferation give rise to increased number of progenitors targeting their differentiated tissues and leading to proportional growth of the animal, while preserving overall shape. In a degrowth state, the rate of neoblast proliferation decreases below the rate of the cell death that occurs in the system, which results in shrinkage of the animals, while preserving shape and proportions. Self-organization when combined with extrinsic progenitor targeting cues and a broad progenitor specification zone is sufficient to explain and predict the systems-level homeostatic and regenerative behaviors in planarians. Below, I focus on the molecular aspects of self-organizing interactions, and I consider the general implications of this model in other biological contexts in general. These concepts may have applications in: (i) explaining development and regeneration of different anatomical configurations in different species; (ii) diverse phenotypes encountered upon blocking a specific gene function affecting patterning and morphogenesis in planarians; (iii) systems-level reconfiguration of existing structures such as during metamorphosis in insects and amphibians, as well as the functional aspects of different tissue systems, such as neural dynamics in the developing, regenerating or re-configuring nervous systems; and (iv) clinical settings targeted to regenerative strategies.
Molecular correlates of the self-organizing attractive cues

Exploration and identification of the intrinsic, cellular and molecular, properties of “attractive” self-organizing interactions is essential. Such self-organizing molecular cues or the particular combination of factors needed for a given tissue are likely tissue-specific and possibly conserved across species. Cell-cell adhesion molecules, transmembrane proteins like integrins, ephrins, cadherins and other CAMs, and/or locally secreted factors hold promise to play roles in this process. A genome-scale molecular analysis targeting the genes involved in these processes can reveal likely targets for initial testing. Specifically, along with rates of incorporation and cell death in the planarian eyes, these factors could also play roles in size and shape maintenance of this tissue. Presumably, similar scenarios can also play out for every other discrete/modular tissue type in this organism.

A migrating progenitor initially follows long-range migratory cues that are either permissive or repulsive. We have shown that the genes *notum*, *slit* and *wnt-5* are involved in long-range targeting of the *ovo+* eye progenitors. Existence of an ectopic eye along the migratory course can lead to incorporation of the eye progenitor, yet we have observed that this effect is more local, indicating a local boundary for the “attractiveness” of the eye. This effect suggests that a local secreted factor might be involved in rendering the eye as an attractor. Possibly, cell adhesion molecules help cellular sorting within the eye tissue and contribute to the overall shape of the eye, along with regulating the attraction dynamics. Another possibility is that CAMs guide the attractor state through direct physical contact, through a Velcro-like interaction between the newly incorporating eye progenitors and the existing differentiated cells in the eyes. Clearly, the molecular players that are responsible for the self-organization for the eye are present in the differentiated eye tissue but
not in the migratory eye progenitors as we have not observed clustering interactions among them along their migratory path.

An understanding for how progenitors nucleate eyes, or other discrete organs such as the brain, *de novo*, is also crucial for understanding self-organization and the rules for successful regeneration in general. External progenitor targeting cues along with intrinsic self-organizing interactions might be involved in this process. Equally, elucidating the behaviors of existing discrete organ systems after a regenerative challenge (during morphallaxis), such as the slow anterior movement of the eyes in a cut headpiece, is another fascinating conceptual and experimental direction.

**Diverse anatomical configurations in different planarian species could be achieved through simple biological rules**

Captivatingly, there is a wide diversity of eye, brain, pharynx and overall shape configurations across different planarian species (Figure 1A) (Kenk 1972). In this section, I explore this diversity and posit that the same rules in this thesis with minor variations could be involved in generating such anatomical differences. *Polycelis remota*, a New England planarian species, have dozens of smaller eyes distributed along the anterior rim of the animal (Figure 1B) (Smith 1988). *Phagocata woodworthi* is multipharyngeal with all of the pharynges positioned in the same pharyngeal cavity (Hyman 1945). What leads to these variations in such anatomical configurations?

In *Schmidtea mediterranea* TZ for the eyes is patterned as two symmetrical points in the head region, at the anterior edge of the brain but located dorsally. Upon resection of eyes, *de novo* eyes form in these two spots. Upon head amputation, *de novo* eyes form in these exact positions in the
blastema. In the amputated head piece, the existing eyes very slowly move to their new TZ, dictated by the reset positional information. Because of the self-organizing nature of the eyes, a new set of anterior eyes is not observed. How do the dozens of eyes in *Polycelis remota* position themselves and how does this configuration respond to regenerative challenges? Our model predicts that, when two variables are re-adjusted such configuration can arise: A wider TZ, spanning the rim of the animal and weak or no self-organization of the eye tissue (Figure 1C):

Upon a regenerative challenge in *Polycelis remota*, a transverse cut, the number of the eyes initially increase and later decrease in the head piece, as the length of the piece increases and width decreases (Figure 1D, E). Our model predicts that if there is weak or no self-organization of the eyes in this species, they would not be able to attract incoming eye progenitors that would normally result in growth of the eyes, and more eyes would nucleate as TZ boundaries change due to positional reset. Some eyes would be maintained because they fall in the TAZ. Because of the changing positional coordinates, new TZ at the rim would also rescale, that would ultimately result in a decrease of the number of eyes that fall out of the TZ boundaries. After positional resetting, the configuration will reach homeostasis ultimately stabilizing the number of eyes (Figure 1E).

Similarly, if self-organization is weaker but not absent for the pharynx in *Phagocata woodworthi*, the system might nucleate multiple pharynges in the same pharyngeal cavity due to increased local noise in nucleation and growth of this organ. An expansion of the TZ could in principle also result in two separate pharynges as observed in other planarian species (Kenk 1972). This effect can also be recapitulated by RNAi of *wntP-2* (Scimone et al. 2016), *ndl-3* and *ptk7* (Lander and Petersen 2016). The rules that pattern such complex configurations are presumably globally integrated at the systems-level rather than solely regulating local dynamics that lead to different form and functions.
Predictions of the model regarding different RNAi phenotypes can produce biological insights

Genetic control of planarian regeneration has been elegantly studied using RNA interference for over a decade. Interrupting gene function yielded numerous phenotypes that include patterning defects, which occur during homeostasis or regeneration (Reddien, 2018). Some of these patterning phenotypes involve ectopic formation or expansion of different tissues/organs. Analyzing a phenotype when first encountered can be a challenge since this system is significantly dynamic and multiple variables can be responsible for certain phenotypes. Prior knowledge regarding gene function is often helpful gaining biological insights, yet analyzing morphological changes requires a more integrated approach. Establishment of TZs, TAZs and self-organization guiding discrete organ formation gives rise to coherent maintenance and regeneration in this system. For instance, ectopic eyes can form upon inhibition of *notum* (Petersen and Reddien 2011), *ndk* (Cebria et al. 2002), *wnt-5* (Adell et al. 2009, Gurley et al. 2010, Oderberg et al. 2017, Atabay et al. 2018), *slit* (Cebria et al. 2007, Gurley et al. 2010, Oderberg et al. 2017, Atabay et al. 2018), *wntA* (Kobayashi et al. 2007, Scimone et al. 2016), *fzd 5/8-4* (Scimone et al. 2016) and *β-catenin* RNAi (Petersen and Reddien 2008), yet the configuration of these ectopic eyes are significantly different from each other under these RNAi conditions (Figure 2A). These differences can be evaluated in terms of the changes that occur in TZ, TAZ and self-organizing dynamics. Accordingly, a gene that is responsible for establishing anterior-posterior or medial-lateral aspects of the TZ, will lead to an anterior-posterior or medial-lateral shift of the *de novo* eyes when inhibited, upon eye resection. A gene that causes shape changes in the TZ when inhibited may lead to a more stochastic nucleation of the eyes affecting symmetry. A gene that changes the boundaries of the TAZ upon inhibition will affect which ectopic eyes can be maintained under RNAi. Finally,
a gene that regulates self-organization of the eye will lead to significant shape changes of the eye structures and loss of attraction of the eye when inhibited. A simple head amputation could cause formation of anteriorly spread eye cells under this condition. These rules can also be applied to any discrete/modular organ, where the differentiated tissue can act as an attractor for its broadly specified and targeted progenitors. Moreover, various aspects of developmental and regenerative dynamics in other species may be regulated by these rules. Concepts from this model may be applicable to similar cellular processes resulting in discrete pattern formation such as establishment of localized neuronal circuits or layers, migratory and aggregation behavior of the neural crest, and initiation of the specialized tissue formation in the early embryo. In these examples, migratory progenitors are generally broadly specified, targeted towards specific locations, and are organized into modular units.

*Metamorphosis requires partial preservation and reconfiguration of the existing nervous system*

The process of metamorphosis, a change from an immature form to a mature form through two or more distinct stages, enables insects to exist in multiple specific forms and consequently efficiently perform diverse functions essential for survival and reproduction (Truman 1990, Levine et al. 1995). This process necessitates a significant re-organization and cell death, regulated through neural development, within the nervous system. Although certain individual neurons are eliminated and changed through metamorphosis, many of the essential features of neural architecture are common between the larval and the adult *Drosophila* (Truman 1990). This aspect of metamorphosis is also mostly conserved across other insects (Levine et al. 1995). Remarkably, specific larval neurons that are retained can re-organize their dendritic morphology and synaptic connections during this transition (Technau and Heisenberg 1982, Levine et al. 1995). Similar to
planarian growth state, metamorphosis is not just the substitution and re-arrangement of the larval neurons by the adult ones, but also the gradual integration of new neurons or neuronal circuits into an existing neural circuitry. Through a self-organization viewpoint, the insect larva exhibits homeostatic regulation during metamorphosis to stabilize and maintain the preserved aspects of this neural architecture. Similar to regeneration, the metamorphosis process does not construct a new nervous system from scratch, and it works with the one that exists. Differently, metamorphosis transforms the existing nervous system into a fairly different shape that will yield a novel behavioral repertoire. Tracking of individual cells and cell lineages allowed to map which parts of the nervous system is preserved through metamorphosis (Harris et al. 2015). Although the involvement of the larval neuroendocrine system as a global regulator is shown to have roles in this process, the complete picture has not been fully elucidated. Remarkably, as another set of evidence for the preservation of certain larval aspects of the nervous system, studies focused in memory showed that behaviors such as conditioned odor avoidance that was produced in larvae was still observable in the adult Drosophila (Tully et al. 1994). In Drosophila, amnesiac and dunce mutants, that are unable to form memory, did not exhibit learning or the retention of the memory in adults (Tully et al. 1994). Similar experimental results were also observed in other insects (Ray 1999, Blackiston et al. 2008). Retention of memory through metamorphosis is a striking possible clue to an existence of a functional homeostat that is maintained through new incorporation and re-arrangement processes that occur in the nervous system.

Are there any new TZs established during metamorphosis? How do the anatomical boundaries of TAZs change in this process? How do self-organizing interactions regulate the massive re-arrangements in the metamorphosing neural circuitry? Are the activity patterns of the retained
larval neuronal ensembles somehow linked to them being preserved? It would be fascinating to study these questions using the concepts of that are introduced in Chapter 2. Moreover, how these rules apply in the metamorphosis processes of ascidians, echinoderms and amphibians would also be attractive research directions.

**Possible implications of the model in clinical studies focusing on regenerative applications**

Humans can regenerate certain tissue types such as skin, liver, blood and intestine, yet other organ systems such as the nervous system regeneration is particularly limited. It is possible that the conceptual arguments of the model introduced in this thesis can aid guided/induced human regeneration for non-regenerative tissue types. Investigations targeting re-introduction of external migratory cues along with re-programmed specialized migratory progenitors upon injury and simply allowing self-organization of the differentiating cell lineages to form the exact missing tissue rather than a similitude tissue patch could prove crucial on this path. The concepts of TZ and TAZ could help guide the process of reintroduction of progenitors in the injured system. It would be interesting to assess if self-organizing interactions would emerge when the “correct” external conditions, that initially existed during development, are present.
CONCLUSION

Self-organization has a major role in regeneration, determining the behavior of migratory regenerative progenitors. This work revealed three properties that govern regenerative progenitor behavior: (i) self-organization, (ii) an extrinsic migratory target for progenitors, and (iii) a broad progenitor specification zone that allows progenitors to be targeted into self-organizing systems even if they are transiently in incorrect locations during the process of regeneration. These components yield a model with broad explanatory and predictive power. This model can help elucidate diverse processes in development and regeneration across biology.
Figure 1. Planarian species exhibit diverse anatomical configurations

A) Various planarian species are aligned: 1) Schmidtea mediterranea, 2) Schmidtea polychroa, 3) Planaria torva, 4) Dendrocoleum lacteum, 5) Polycelis tenuis, 6) Polycelis felina, 7) Polycelis nigra (Image source for 1-5, Rozanski et al. 2015).

B) Polycelis remota, a New England species, has dozens of eyes positioned across the anterior rim of the animal.

C) Hypothetical Target Zone (TZ) shapes in the Polycelis remota and Schmidtea mediterranea. Theoretically, aside from an expanded TZ, Polycelis eyes also do not exhibit coalescence and growth because of weak or no self-organization among eye cells.

D) Regeneration of Polycelis remota anterior piece upon a transverse cut is shown. Eye configurations exhibit changes during the regeneration process.

E) Positions of the eyes are mapped in relation to an early time point, day 8, post-surgery. A sharp increase in eye number during regeneration was followed by a decrease and stabilization of eye number after complete morphallaxis.
Figure 2. Different RNAi phenotypes generate varied ectopic eye configurations

A) Homeostatic RNAi phenotypes affecting eye configuration in various ways are shown.
REFERENCES


