INVESTIGATING THE ROLES OF NEUROPEPTIDE SIGNALING AND HETEROTRIMERIC G PROTEINS IN PLANARIAN REGENERATION

by

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(Under the Direction of Rachel Roberts-Galbraith)

ABSTRACT

Regeneration is the regrowth of damaged tissues and structures, which encompasses a complex process of cell activation and coordination. Freshwater flatworms called planarians are incredibly regeneration-competent, with the ability to regenerate virtually all body parts. Therefore, understanding the fundamental cellular mechanisms that promote planarian regeneration can unveil more universal themes required for this heightened level of healing in animals. While several essential planarian traits and genetic responses for regeneration are identified, less is known about the mechanisms that drive and coordinate these processes after injury. In this dissertation, I take a dual approach – investigating both upstream signaling ligands and downstream signal transducers – to define potential signaling pathways necessary for successful regeneration in the planarian species *Schmidtea mediterranea*.

Regarding the upstream signals, by targeting a key neuropeptide processing enzyme-encoding gene, *prohormone convertase 2* (*pc2*), I show that putative neuropeptide signaling plays many important roles including promoting proper animal behavior, robust regeneration, and tissue maintenance. Additionally, I uncovered a novel role for planarian *pc2* in stem cell differentiation,

which I propose is the primary phenotype resulting in defects to regeneration and tissue maintenance.

Regarding the downstream signal transducers, I explored potential roles for G protein-coupled receptor pathways in planarian regeneration via the canonical downstream heterotrimeric G proteins. I identified and phylogenetically classified all the heterotrimeric G protein subunit genes in *S. mediterranea*, then performed a functional characterization for these subunits. I found that the planarian heterotrimeric G protein subunits showed diverse roles including promoting proper behaviors, survival, and regeneration. I further show that two subunits critical for regeneration, *Gαq1* and *Gβ1-4a*, facilitate reestablishment of key polarity domains, which is a primary outcome contributing to regeneration in these animals.

My results support the hypothesis that neuropeptide- and GPCR-related signaling pathways play crucial roles in stem cell regulation and planarian regeneration. This work provides a foundation for future investigation into these important gene families as well as specific candidate pathways to continue exploring. Further delineating these pathways will provide valuable knowledge to how an animal regulates pluripotent stem cells *in vivo* and coordinates cellular responses to achieve complex tissue regeneration.

INDEX WORDS: Planarian, Regeneration, Stem cells, Neuropeptides, Prohormone convertase, PC2, Heterotrimeric G proteins, G protein-coupled receptors, GPCR, Signaling, Neurobiology, Polarity, Behavior, *Schmidtea*

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DEDICATION

I dedicate this dissertation to all the wonderful people whose support got me through graduate school (during a pandemic). To my partner, Chris, who was my rock as I went through the growing pains of becoming an independent researcher. To My PI, Dr. Rachel Roberts-Galbraith, who consistently amazed me with her intelligence, perseverance, and compassion. To my lab mates, the Worm Squad, whose friendship made every day in the lab something to look forward to. I cannot begin to describe the impact you had on my life. Thank you!

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Regeneration is the process of regrowing lost tissues and body parts. We see examples of regeneration when interacting with the world. It is seen when a child grabs a lizard by the tail and the lizard sheds the structure, regenerating it after escaping the immediate danger. It is seen when a gardener propagates a plant through cutting. Imagery of regeneration is also intertwined into cultures and history, such as the Hydra monster of Greek mythology (Augustyn, 2022). Along with the vast references to regeneration in culture, the first known documented description of animal regeneration came from Aristotle around 350 BCE (Aristotle et al., 1965). A wave of scientific investigation searching for animals with regenerative abilities followed in the eighteenth century and documented newly discovered examples: polyps of the Cnidarian genus *Hydra* regenerate after transection (Trembley, 1744), annelid worms can be cut into pieces and regenerate new worms (Bonnet, 1745), arthropods regenerate lost appendages (de Réaumur, 1712), and even vertebrate animals such as salamanders have regenerative ability (Spallanzani, 1768). After these first official documentations, through observation of the natural world, the vast range of regenerative abilities became clear. Regeneration is widespread, though not uniformly, throughout many branches of life. We've come to find that animals lie on a regeneration-competency spectrum.

At the less-regenerative end of the spectrum are animals that can only heal from superficial injuries to specific tissues after they reach adulthood. Poor regeneration is seen in many mammalian and bird species (Bely & Nyberg, 2010; Carlson, 2007). In these examples, if a wound

exceeds the level of severity that these wound-healing mechanisms can handle, scars form (Gurtner et al., 2008). Scars can be painful and limit the function of such tissues (Aarabi et al., 2007; Shirakami et al., 2020). In the middle of the regeneration range, some animals have specialized regeneration limited to specific structures, typically those which may be lost due to their lifestyle (e.g., reproductive competition). Examples include the antlers of deer or the large claw of fiddler crabs (Backwell et al., 2000; Goss, 1983; D. Wang et al., 2019; Yamaguchi, 1973). Regeneration-competent animals show the ability to regrow multiple body parts and tissue types, establishing scarless, fully functional regeneration. Organisms that achieve this level of regeneration include axolotls and sea stars (Bölük et al., 2022; Byrne, 2020; McCusker et al., 2015). Finally, among the most incredible examples of regeneration-competent animals are those that regenerate all tissues of their body. Some extreme examples of whole-body regeneration are *Hydra* and sea sponges, which can even be dissociated into single cells and reaggregate to reform the animal anew (Ereskovsky et al., 2021; Kirillova et al., 2018).

Humans reside in the limited end of regeneration competencies, as evidenced by the devastating outcomes that can occur after injury and disease (Centers for Disease Control and Prevention, 2022b; Haagsma et al., 2022; World Health Organization, 2021). Healing and regenerative ability in people are restricted to certain tissues, typically ones with associated multipotent stem cell populations, with few exceptions (e.g., the liver) (Barker et al., 2010; Ozaki, 2020). For example, superficial wounds to the skin can heal through the combined activity of multiple stem cell populations, including those of the hair follicle and epithelium (Barker et al., 2010; Ito et al., 2005; Levy et al., 2007). However, scarring results once the wound is deep enough, penetrating the dermis layer of the skin (Dunkin et al., 2007). Importantly, people do not heal or regenerate well from injuries to complex body structures such as limbs or the central nervous

system, leading to severe issues after injury (Centers for Disease Control and Prevention, 2022a; Hanger Clinic, 2022). Because humans cannot regenerate well, observation of animals with robust regeneration raises pressing questions. How does an animal achieve complex tissue regeneration in nature? Furthermore, can we improve regenerative processes in ourselves?

In an attempt to answer these questions, many scientists – including myself – study highly regeneration-competent animals. The rationale is that understanding the fundamentals about how these animals achieve complex tissue regeneration might inform how to improve regeneration of our bodies. Insights can range from general themes, such as the formation of a specialized wound epidermis in vertebrates learned from salamander species (Gawriluk et al., 2016; Globus et al., 1980; Thornton, 1957) to specific molecular mechanisms typically associated with successful regeneration. For the purpose of my dissertation work, I decided to explore mechanisms of regeneration in the planarian flatworm model.

Planarians are a model for robust regeneration

Planarians are free-living, soft-bodied, acoelomate flatworms of the phylum Platyhelminthes (Ivankovic et al., 2019; Sluys & Riutort, 2018). The planarian flatworm is an animal model known for its incredible ability to regenerate complex tissues. The first surviving mention of planarian regeneration originated from a Chinese text written in 860 AD, which stated that terrestrial planarians could "easily separate into several pieces" (T'uan, 860). In fact, planarians regenerate all tissues so robustly that a study reported they can regenerate an entire functional animal from a piece that was 1/279th the original volume of the intact worm (Morgan, 1898, 1901)! Future estimates were more conservative (e.g., the remaining piece requires at least 10,000 cells

(Montgomery & Coward, 1974)), but the regenerative capacity of planarian flatworms is still a captivating feature.

However, not all planarian species show equal regeneration-competence (Brøndsted, 1955; Vila-Farré & C. Rink, 2018). Therefore, much of the regeneration research focuses on a handful of species known for their capability for whole-body regeneration: *Schmidtea mediterranea*, *Dugesia japonica*, and *Girardia/Dugesia tigrina* (Newmark & Alvarado, 2002; Salo et al., 2009). The specific planarian species favored by investigators also greatly depends on the questions being asked and the availability of tools. For example, *S. mediterranea* has a fully sequenced diploid genome, which makes genetic studies more straightforward (Grohme et al., 2018; Robb et al., 2015). In contrast, *D. japonica* harbors a mixoploid genome, but is a better model than *S. mediterranea* for toxicology studies (Ireland et al., 2020; Orii et al., 1999). Regardless of the specific species used, planarian research has shown a great resurgence since the development of more sophisticated molecular tools, such as in situ hybridization, RNA interference, and single-cell transcriptomics (Fincher et al., 2018; Newmark & Alvarado, 2002; Plass et al., 2018; Rouhana et al., 2013).

Below I have provided a summary of the biology of freshwater planarians, with a focus on the most common species used in regeneration research. For a review on the complexity of lifestyles and regeneration competencies among planarians, I encourage readers to refer to (Sluys & Riutort, 2018; Vila-Farré & C. Rink, 2018).

External features

Freshwater planarians can range in size from a few millimeters to a few centimeters, and they grow or degrow in response to availability of nutrients (Baguñá & Romero, 1981; C. M. Miller &

Newmark, 2012; Sluys & Riutort, 2018). These animals possess a bilateral, left-right symmetrical body plan containing anterior-posterior, dorsal-ventral, and medial-lateral axes of polarity (Figure 1.1A-B) (Hyman, 1951; Reddien et al., 2007; Sureda-Gomez & Adell, 2019). The primary axes of polarity are controlled by largely conserved developmental signaling pathways, which I will detail below. The coloration of freshwater planarians is typically a shade of brown and black (or unpigmented) due to sub-epidermal pigment cells (Sluys & Riutort, 2018). Additionally, pigmentation is often darker on the dorsal side and paler on the ventral side.

The planarian body is covered by a monolayered epidermis that connects to a basement membrane at the cells' basal surfaces, with the nuclei oriented in a uniform layer toward the basal side of the cells (Hay & Coward, 1975; Rompolas et al., 2010; Skaer, 1965). The post-mitotic cell types within the epidermis include variably ciliated cuboidal epithelial cells and mucus-secreting, rhabdite-containing cells (Pedersen, 1976). The epithelial cells on the dorsal side of the animal are largely nonciliated, except for cells of the dorsal ciliated stripes (Farnesi & Tei, 1980; K. G. Ross et al., 2018). In contrast, the epithelial cells on the ventral side of the animal are multiciliated (Figure 1.1B) (Rompolas et al., 2010). The epidermis has many functions supporting the planarian**.** It provides a barrier for the body, playing a key role in tissue protection as well as prevention of infection (Gao et al., 2017). The epidermis is also the location for exchange of gases necessary to support life, such as the absorption of oxygen and release of carbon dioxide (Quijada-Rodriguez et al., 2017). Finally, ciliated epidermal cells on the ventral surface along with secreted mucus support the gliding locomotion typical of these animals (Figure 1.1B) (Martin, 1978; Rompolas et al., 2010).

In addition to the epidermis, other organs and features are visible externally on planarians. A pair of eyespots, important for light detection, reside at the dorsal and anterior region of the

body (Figure 1.1A) (Carpenter et al., 1974; Krugelis MacRae, 1964). A feeding organ, called a pharynx, is situated in a pouch at the middle of the body (Figure 1.1A) and protrudes from a hole (referred to as a mouth) at the ventral face of the body during feeding (Kreshchenko, 2009). I describe these structures in more detail while discussing their associated organ systems below.

Internal features

The internal anatomy of planarians is surprisingly complex, with multiple distinct organ systems and numerous cell types. The planarian digestive system is comprised of the aforementioned pharynx, which connects to a blind, tri-branched intestine (giving these worms the triclad distinction) (Figure 1.2A) (Hyman, 1951; Sluys & Riutort, 2018). The intestine is tubular and composed of a single layer of columnar epithelium with a highly extensive branching system (Forsthoefel et al., 2011; Ishii, 1965; Willier et al., 1925). The intestinal epithelium contains cell types that support its function, and the degree of cellular and regional diversity within these cells has recently gained attention (Forsthoefel et al., 2020). The broad cell type categories within the intestinal epithelium include absorptive phagocytes that engulf ingested food, secretory goblet cells that secrete digestive enzymes, and basal cells that are thought to contribute to metabolism (Bowen et al., 1974; Fincher et al., 2018; Forsthoefel et al., 2020; Garcia-Corrales & Gamo, 1988; Ishii, 1965; Willier et al., 1925). The intestine is also surrounded by enteric muscles, which promote processes such as waste elimination and peristalsis (Cebrià, 2016; Kobayashi et al., 1998; Orii et al., 2002).

The pharynx itself shows a high degree of complexity and is composed of multiple cell types. The pharynx includes a musculature with multiple layers, excretory cells, and its own pharyngeal nerve network (Bueno et al., 1997; Ishii, 1965; MacRae, 1963). The pharynx also has

its own axis of polarity, with many genes associated with the Wingless/Integrated (Wnt) signaling pathway showing localized expression at either the distal or proximal end of the pharynx (Gurley et al., 2010). Amazingly, when the pharynx is removed from the rest of the body, this separated organ still displays attraction to and consumption of food, suggesting that aspects of feeding behavior are orchestrated by the pharynx itself (Miyamoto et al., 2020).

A second major organ is the body musculature. The planarian body contains multiple layers of muscle fibers in the body wall, which support the shape of the body and aid in coordination of animal movements (Figure 1.2B) (Kobayashi et al., 1998; Morita, 1965). These fibers show a defined organization of orientation from the most exterior of the animal to the deepest tissues (Cebrià et al., 1997; Scimone et al., 2017). Most superficially, the muscle fibers are oriented circularly, enwrapping the body transversely (Scimone et al., 2017). The second layer of body wall muscle fibers runs longitudinally from anterior to posterior (Scimone et al., 2017). The third layer of muscle fibers runs diagonally to the other layers, forming a crisscross pattern (Scimone et al., 2017). The fourth layer contains a second set of longitudinal muscle fibers (Scimone et al., 2017). The fifth and deepest layer of muscle fibers extends perpendicular to the other layers through the parenchyma along the dorsoventral axis (Scimone et al., 2017).

One of the most surprising examples of organization in planarians is their nervous system, which includes distinct central and peripheral nervous systems (K. G. Ross et al., 2017). The central nervous system is composed of 1) a bi-lobed brain/cephalic ganglia connected largely by an anterior commissure and 2) two ventral nerve cords that span the length of the body and connect through transverse commissures (Figure 1.2C) (Cebria et al., 2002; Okamoto et al., 2005; K. G. Ross et al., 2017). The brain extends lateral projections, called brain branches, to the periphery of the planarian head (Figure 1.2C) (MacRae, 1967). Brain branches are proposed sensory structures,

specifically thought to contribute to chemosensation (Okamoto et al., 2005; Pigon et al., 1974; Roberts-Galbraith et al., 2016). The aforementioned light-detecting eyespots are composed of a pigment cup and cluster of photoreceptors, which have axons that project to the visual center of the brain (Carpenter et al., 1974; Lapan & Reddien, 2012). The peripheral nervous system consists of three nerve plexuses: the subepidermal plexus located between the epidermis and body wall musculature; the submuscular plexus internal to the body wall musculature; and the gastrodermal plexus surrounding the intestine (Baguñà & Ballester, 1978; K. G. Ross et al., 2015). Furthermore, the nervous system contains >50 distinct neural cell types as well as a recently discovered glial cell population (Chandra et al., 2023; Fincher et al., 2018; Plass et al., 2018; Roberts-Galbraith et al., 2016; I. E. Wang et al., 2016).

In addition to the digestive system, musculature, and nervous system, planarians harbor additional tissue types and organ systems. The water balance of planarians is maintained through an excretory system of protonephridia (Ishii, 1980a, 1980b; McKanna, 1968). The space between individual organs is filled by a matrix of connective tissues called the parenchyma (Cote et al., 2019; Pedersen, 1961; Roberts-Galbraith et al., 2016). Finally, while I use an asexual planarian strain in my research, there are also sexual planarians that serve as models for reproductive development and regeneration (Collins et al., 2010; Newmark & Alvarado, 2002). Sexual planarians are cross-fertilizing hermaphrodites, wherein individual animals contain both male (e.g., testes and seminal vesicles) and female (e.g., ovaries and oviducts) tissues (Chong et al., 2011; Hyman, 1951).

Planarians can regenerate all of their tissues

Considering the internal complexity of planarians that I just discussed, their regenerative potential becomes even more astonishing. Planarians are among the most regeneration-competent bilaterians, showing the incredible ability to re-establish the same body plan after an almost infinite number of injury scenarios (Figure 1.3A-D) (Ivankovic et al., 2019; Morgan, 1898; Randolph, 1897; Reddien & Alvarado, 2004). After an injury resulting in tissue loss, a mass of differentiating cells (called a blastema) forms at the injury site (Figure 1.3B-D) (Baguñà, 1976; Newmark & Sánchez Alvarado, 2000; Wenemoser & Reddien, 2010). This structure results from stem cell activation in response to the injury, wherein stem cells migrate to the injury site and proliferate to send differentiating progeny into the blastema (Eisenhoffer et al., 2008; Guedelhoefer & Sánchez Alvarado, 2012; Wenemoser & Reddien, 2010). Once the blastema is established and missing structures are forming, the rest of the body undergoes remodeling, which contributes to the restoration of correct anatomical proportions (Figure 1.3C-D) (reviewed in Pellettieri, 2019).

One of the most striking examples of planarian regeneration is their ability to regenerate the adult central nervous system, including the brain, *de novo* (Ross et al., 2017 for review). After amputation, markers for new neurons are detectable in the regenerating blastema within 24-48 hours, and neurons begin to organize into localized patterns within two days (Cebria et al., 2002). Physical connections of these neurons, such as the anterior commissure connecting the two lobes of the brain, are detectable within three days (Cebria et al., 2002; Umesono et al., 1997). Proper brain morphology is restored between three to five days after head amputation (K. G. Ross et al., 2017). Finally, key brain function resumes as early as one week after head amputation, though it may take longer to reestablish full complex brain morphology (such as glial cells) and function resembling intact animals (Chandra et al., 2023; Inoue et al., 2004, 2014).

Multiple required characteristics are defined for planarian regeneration

With existing molecular approaches available in planarian research, the community has described three fundamental traits underlying planarian regeneration. For the purpose of this work, I am focusing on regeneration in the context of large tissue loss and not covering superficial wound healing. Additionally, the list I provide here is not exhaustive. Other key processes also occur, such as organized cell death to aid in reestablishing body proportions (Pellettieri, 2019; Pellettieri et al., 2010). Here, I provide a brief review on the characteristics most relevant to my current work.

Planarian Trait 1: Wound Response

When significant injury occurs, some of the first steps that follow include covering the wound site and alerting the remaining tissue to the damage. In planarians, the wound surface is covered by the remaining cells from the dorsal and ventral epidermis (Hori, 1989; Morita & Best, 1974). These epithelial cells loosen contacts with the basement membrane, adopt a more flattened morphology, and migrate over the wound (Hori, 1989; Morita & Best, 1974; Pedersen, 1976). The process of epithelial coverage of wounds is aided by muscle contraction, which brings the sides of the wound closer together and reduces the surface area of the injury (Hori, 1989; Morita & Best, 1974; Pedersen, 1976). In addition to providing physical protection to the wound, the new direct connection between the epidermal cells and the parenchyma is thought to also trigger signaling events to elicit the molecular wound response (Chandebois, 1980; Scimone et al., 2022).

The molecular wound response in planarians constitutes a program of four different waves of gene expression after significant wounding. The first category of genes, called W1, reaches peak upregulation at three hours after injury and includes many conserved "immediate early genes", such as transcription and signaling factors (e.g. *jun-1* and *fos-1*) (Lamph et al., 1988; Müller et al.,

1984; Wenemoser et al., 2012). This response is translation-independent (Wenemoser et al., 2012). The second and third categories, called W2 and W3, consist of known patterning factors and reaches peak upregulation at six hours after injury (Wenemoser et al., 2012). The main difference between these categories is that W2 genes are typically expressed subepidermally at the wound site, whereas W3 genes are expressed in the epidermis in regions excluding the wound (Wenemoser et al., 2012). Surprisingly, these factors are generically upregulated regardless of the missing tissue type, supporting the hypothesis that the early response to injury is context neutral (Petersen & Reddien, 2009a; Wenemoser et al., 2012). Finally, a category of genes called W4 is upregulated in the population of stem cells, and includes examples required for proper production of differentiated progeny (Wenemoser et al., 2012).

Planarian Trait 2: Dynamic Polarity

Once the remaining tissue is alerted to the injury, the proper body plan needs to be reestablished. The identity of tissues within the body plan is largely influenced by polarity signaling genes, and the patterns of expression of polarity genes dynamically change in response to injury (Gurley et al., 2010; Reddien, 2018; Witchley et al., 2013). One of the most well understood polarity axes in planarians is the anterior-posterior (AP) axis. Key features of the animal body plan organized by the AP axis include the head (with a brain and two eyespots) at the anterior of the animal, the pharynx localized at the center of the body, and the tail at the posterior. The AP axis is established and maintained primarily through Wnt signaling (Adell et al., 2009; Gurley et al., 2008; Iglesias et al., 2008; Petersen & Reddien, 2008, 2009a, 2009b, 2011). In intact animals, Wnt signaling factors are expressed largely in the posterior of the animal and promote tail identity (Petersen & Reddien, 2008). In contrast, genes that encode inhibitors of Wnt signaling and promoters of

anterior identity (e.g., *notum* and *secreted Frizzled-related protein* [*sFRP-1*]) are expressed at the anterior of the animal and specifically in a polarity structure called the anterior pole (Gurley et al., 2010; Petersen & Reddien, 2008, 2011). Additional inhibitors that target separate, but intertwined, posterior-promoting pathways are also expressed at the anterior pole and contribute to the establishment and stabilization of the anterior domain (e.g., *follistatin*, which inhibits posterior-promoting Activin signaling) (Gaviño et al., 2013; Roberts-Galbraith & Newmark, 2013; Tewari et al., 2018).

After amputation along the AP axis, *notum* is upregulated primarily at anterior-facing wounds and *wnt1* is upregulated regardless of the wound direction (Petersen & Reddien, 2009a, 2011). Due to the high levels of *notum* at anterior-facing wounds, anterior wounds take on anterior identity (eventually forming an anterior pole) (Petersen & Reddien, 2011). In contrast, the high Wnt environment of posterior wounds, unchecked by *notum*, promotes posterior identity and subsequent posterior pole formation (Petersen & Reddien, 2011). Polarized upregulation of *notum* was recently determined to rely on Activin signaling, suggesting that there is still much to learn about the relationships between polarity signaling pathways (Cloutier et al., 2021).

A second polarity axis is the dorsal-ventral (DV) axis. Key features of the body organized by the DV axis include the ciliated ventral epidermis and the ventral nerve cords of the central nervous system. The DV axis is controlled largely by Bone Morphogenetic Protein (BMP) signaling; the presence of BMP signaling promotes dorsal identity whereas inhibition of BMP signaling promotes ventral identity (Molina et al., 2007, 2011; Orii & Watanabe, 2007; Reddien et al., 2007). In intact animals, *bmp4* expression is high along the dorsal side of the body, whereas *anti-dorsalizing morphogenetic protein* (*admp*), an inhibitor of BMP signaling, is expressed at the

ventral midline and at the DV boundary of the body margin. (Gaviño & Reddien, 2011; Molina et al., 2007, 2011; Orii & Watanabe, 2007; Reddien et al., 2007).

A third defined polarity axis is medial-lateral (ML) axis. The ML axis promotes the proper size and bilateral patterning of structures of the body, including many of the structures already mentioned (the central nervous system, the pharynx, the eyespots, etc.). The ML axis is controlled predominantly by the reciprocal, antagonistic activity of *slit* and *wnt5* (Gurley et al., 2010). In intact animals, *slit* is expressed at the midline of the body and inhibits lateral identity (Cebrià et al., 2007; Gurley et al., 2010). In contrast, *wnt5* is expressed on the ventral side of the dorsal/ventral boundary and just lateral to the ventral nerve cords and inhibits medial identity (Gurley et al., 2010). BMP signaling has also shown roles in regulating the ML axis (Reddien et al., 2007).

Although the field has defined major signaling pathways controlling the establishment and maintenance of the primary axes, evidence indicates a degree of inter-connectivity between these pathways (Clark & Petersen, 2023; Cloutier et al., 2021; Roberts-Galbraith & Newmark, 2013; Scimone et al., 2022; Tewari et al., 2018). Therefore, the body plan is maintained through the interplay of signals along each axis. Additionally, many genes are expressed in a locally restricted manner along the body plan, but the precise ways these genes influence tissue identity remains to be determined (Fincher et al., 2018; Scimone et al., 2016; Witchley et al., 2013). Through the combined activity of polarity signaling throughout the animal, planarians establish their body plan and dynamically regulate proper body proportions after injury.

Planarian Trait 3: Pluripotent Stem Cells

Planarians contain a heterogenous population of stem cells that is activated to generate required cell types and new growth material. The planarian stem cell population accounts for ~20% of the

total cells in the body (Hayashi et al., 2006; Reddien, Oviedo, et al., 2005; Wagner et al., 2011). Planarian stem cells are the only dividing cells of the animal and display a characteristic morphology including a small, round shape (~5–10 μm in diameter), a high nucleus-to-cytoplasm ratio, and the presence of cell-specific organelles called chromatoid bodies (Baguñá & Romero, 1981; Coward, 1974; Morita & Best, 1984; Newmark & Sánchez Alvarado, 2000; Rouhana et al., 2014). Planarian stem cells are localized within the parenchyma (Hyman, 1951; Pedersen, 1961).

Planarian stem cells display characteristic proliferative responses to injury, which can be summarized as two peaks of cell division. The first proliferative peak happens around six hours after injury, is body-wide, and occurs regardless of injury type (Baguñà, 1976; Saló & Baguñà, 1984; Wenemoser & Reddien, 2010). The second proliferative peak happens around two days after injury, with more specific localization to the wound site, and only occurs after injuries that result in significant tissue loss (Baguñà, 1976; Saló & Baguñà, 1984; Wenemoser & Reddien, 2010).

Strikingly, some stem cells display functional pluripotency in adult planarians (Baguna et al., 1989; Wagner et al., 2011). The fascinating existence of adult pluripotent stem cells in planarians was validated by studies showing that a single transplanted stem cell from a non-irradiated doner could rescue tissue turnover and regeneration in a lethally irradiated host (Wagner et al., 2011; A. Zeng et al., 2018). The stem cells that could repopulate irradiated animals were termed clonogenic neoblasts (Wagner et al., 2011; A. Zeng et al., 2018).

However, while the functional pluripotency of individual planarian stem cells has been established, defining the molecular signatures of pluripotent cells remains an active area of research. A prominent model describing the nature of potency in planarian stem cells is called the "Specialized Neoblast Model" – neoblast is a term historically used to refer to the stem cells in planarian literature (Baguñà, 2012; Reddien, 2013). The Specialized Neoblast Model was largely

derived from work indicating that the planarian stem cell population is heterogenous, containing pluripotent stem cells and mitotic progenitors destined for specific fates (Fincher et al., 2018; Plass et al., 2018; Scimone et al., 2011; van Wolfswinkel et al., 2014; Wagner et al., 2011; A. Zeng et al., 2018). Intriguingly, recent work provides evidence suggesting that even stem cells displaying lineage-specific markers can retain potency and produce less specialized progeny through asymmetric division (Raz et al., 2021). The newly appreciated plasticity of planarian stem cells could suggest that pluripotency is not restricted to a rare or unique stem cell class (Raz et al., 2021).

While a molecular wound response, dynamic polarity, and pluripotent stem cells play demonstrated roles in successful planarian regeneration, there remain many questions about how regeneration is orchestrated. We understand wound response gene expression, but it is unclear what factors initiate the molecular wound response and how waves of gene expression are timed or translated into robust regeneration. We do not know what factors instruct stem cells to promote progeny fate and/or whether polarity signaling directs particular cell types to be created from stem cells. A more thorough understanding of the signaling environment and downstream mechanisms that drive and coordinate the regenerative response in planarians could unlock potential themes underlying animal regeneration, such as the cellular source of pro-regenerative instructions to the rest of the body.

Is neural tissue a pro-regenerative signaling source?

When thinking of likely candidates for cell types involved in orchestrating regeneration, I used results in other animals to inform my search. Nervous tissue provides key signals to promote regeneration in many branches of the animal kingdom (Farkas & Monaghan, 2017; A. Kumar & Brockes, 2012). A role for nerves in tissue growth and repair was first discovered in the context of

the salamander limb, with the broad takeaway of the work being that innervation is required for limb regeneration (A. Kumar et al., 2007, 2011; Todd, 1823). This theme is also seen in arm regeneration in sea stars (Huet, 1975), fin regeneration of teleost fish (Geraudie & Singer, 1977), and digit tip as well as heart regeneration in mice (Mahmoud et al., 2015; Rinkevich et al., 2014).

Although neurons display pro-regenerative roles in many animals, there are some examples where neurons are not required for a regenerative response, which challenges the necessity of nerves. In *Hydra vulgaris*, neurogenesis is required for regeneration in wild-type animals (Miljkovic-Licina et al., 2007), but a slower mechanism for regeneration compensates after the elimination of nerves (Sugiyama & Fujisawa, 1978). Evidence indicates that the compensatory mechanism results from epithelial expression of genes typically expressed in wild-type neurons (Hornberger & Hassel, 1997). A second example, in *Xenopus laevis*, shows that the role of nerves can also depend on the developmental stage of the animal. Limb regeneration in early *Xenopus* larvae is nerve-independent, but denervation eliminates limb regeneration in later larval stages (Cannata et al., 2001). As a last intriguing example, the salamander limb (while having a definitive requirement for nerves in the wild-type) does not require nerves to regenerate when a limb develops without innervation (Yntema, 1959).

Studies in highly regenerative animals from diverse clades of life could show whether pro-regenerative roles for neural tissue are widespread and/or conserved. Additionally, a broader understanding of neural roles in regeneration could indicate whether there are intrinsic mechanisms we could activate in humans to improve our regenerative response. The body of work investigating how nerves impact animal regeneration was a great motivation for my thesis research. I found it fascinating that nerves could be utilized in such a variety of ways. I also marveled at how the planarian model provided a unique opportunity to study the relationship

between neural tissue and regeneration in an animal that robustly regenerates their entire nervous system! Because I hoped to contribute to our understanding of nerve roles in regeneration, in my thesis I sought to identify the mechanisms through which neural tissue might promote regeneration in planarians.

Neurons secrete neuropeptide signals which typically activate GPCRs

To understand how neural tissue might support regeneration, I considered the key biology of neurons. Neurons use multiple mechanisms to communicate with other cells. Once a neuron is activated, an action potential is generated through successive membrane depolarizations, which travels along the neuron (Levitan & Kaczmarek, 2015). Upon the action potential reaching the axon terminals of the neuron, it can trigger the release of small-molecule chemical signals (e.g., serotonin, acetylcholine, and dopamine) into the synaptic cleft (Levitan & Kaczmarek, 2015). Receptors on the postsynaptic neuron then receive the secreted neurotransmitters, and remaining unbound small-molecule transmitters are quickly removed from the synaptic cleft through reuptake mechanisms (Levitan & Kaczmarek, 2015). Neurotransmitters typically travel short distances within the synaptic cleft once secreted (van den Pol, 2012). However, there is an additional signal type that is less understood and shows functional diversity as well as variable traveling distances: neuropeptide signaling.

Neuropeptides are short peptide signals that are typically produced from neural or neuroendocrine tissue (Fricker, 2012). Neuropeptides originate as larger precursor proteins, which are referred to as prohormones (also sometimes called proproteins, propeptides, or proneuropeptides in the literature) (Figure 1.4A) (Fricker, 2012). A prohormone contains a signal peptide at the N-terminus of the polypeptide, which targets the prohormone to the secretory

pathway in the cell (J. Coleman, 1985). Once in the secretory pathway, the prohormone is highly processed and modified by a series of enzymes (Figure 1.4A-C) (Fricker, 2012). The first step that occurs in neuropeptide processing is removal of the signal peptide by the enzyme Signal Peptidase (Dalbey & von Heijne, 1992). Then, the prohormone is cleaved into individual neuropeptides largely through a family of proteases called the prohormone/proprotein convertases (Figure 1.4B-C) (Rouillé et al., 1995).

One of the earliest eukaryotic neuropeptide cleavage enzymes discovered was Prohormone/Proprotein Convertase 2 (PC2) (Smeekens & Steiner, 1990). PC2 is a subtilisin/kexin type serine protease that recognizes dibasic amino acids within prohormones (Figure 1.4B) (Steiner, 1998). PC2 cleavage sites reside at the carboxyl side of each dibasic pair (Figure 1.4B) (Yoon et al., 2022). PC2 protein itself undergoes processing before becoming a mature enzyme and assuming its biological function of neuropeptide processing (Benjannet et al., 1992; Mbikay et al., 2001). The molecular activity of PC2 requires a specific environment (including calcium ions and an optimum pH), and thus PC2 is most active in dense core secretory vesicles (N. Seidah et al., 2008).

Including PC2, there are 9 proteases in the mammalian prohormone/proprotein convertase family (Garten, 2018). The individual prohormone convertases display substrate preference and specificity *in vivo* (N. Seidah et al., 2013). Additionally, depending on the combinations of proteases expressed within specific cell types, different neuropeptide products can be made from the same prohormone polypeptide. The Pro-opiomelanocortin (POMC) prohormone provides a powerful example of the different neuropeptide products that can result from different processing enzymes (Harno et al., 2018). In the human pituitary, adrenocorticotropic hormone (ACTH) is one of the products from the POMC prohormone, because the main protease expressed in the cells of

the pituitary is Prohormone Convertase 1/3 (PC1/3) (Harno et al., 2018). However, in other tissue types that also express PC2 in addition to PC1/3 (e.g., the hypothalamus), ACTH undergoes further processing, resulting in α-melanocyte stimulating hormone (Harno et al., 2018). The ability to generate different neuropeptide products in a cell-specific manner allows an additional layer of complexity to neuropeptide regulation and function.

Once neuropeptides are cleaved via protease activity, additional enzymes further modify the neuropeptide intermediates to increase their stability and bioactivity (Figure 1.4C). Carboxypeptidases remove the remaining basic residues at the C-terminus of the neuropeptides after prohormone convertase cleavage (Fricker, 1988). Peptidylglycine α-amidating monooxygenase (PAM) then converts the C-terminus to an amide, which greatly increases the neuropeptide's bioactivity (Eipper et al., 1992; Eipper & Mains, 1988). Interestingly, while mature neuropeptides can be released along with small-molecule transmitters at axon terminals, neuropeptides can also be released from other regions of the neuron such as the dendrites and cell body (Huang & Neher, 1996; Ludwig & Leng, 2006; Morris & Pow, 1991; van den Pol, 2012). When released along with small-molecule neurotransmitters, neuropeptides can show modulatory activity – increasing or decreasing the activity of the cotransmitter – though a neuromodulatory role for neuropeptides is not established in planarians (M. E. Adams & O'Shea, 1983; Glantz et al., 2000). However, due to their high stability and various release sites, neuropeptides can also act at variable distances and act directly as signaling molecules (Herkenham, 1987; Jan & Jan, 1982; Nässel, 2009; Nässel & Zandawala, 2022).

Neuropeptides commonly signal via G protein-coupled receptors (GPCRs) (Frooninckx et al., 2012; Hewes & Taghert, 2001; Mirabeau & Joly, 2013). GPCRs are one of the largest and most highly conserved families of cell surface receptors, with GPCRs utilized in organisms

ranging from diverse metazoans to unicellular organisms (Baig & Ahmad, 2017; Caers et al., 2012; Frooninckx et al., 2012; Krishnan et al., 2012). GPCRs are characterized by a conserved seven-transmembrane domain composed of alpha-helices, which form a hydrophobic core wherein many ligand binding sites localize (Hilger et al., 2018; K. L. Pierce et al., 2002). The amino-terminus of the receptor resides extracellularly, and the carboxy-terminus of the receptor terminates within the cell (K. L. Pierce et al., 2002). GPCRs also have extracellular domains between transmembrane helices that can influence signal perception and are specific to the receptor subfamily and associated ligands (Lagerström & Schiöth, 2008).

The classification of GPCRs has been complicated, with multiple classification systems proposed (Alexander et al., 2021; Kolakowski, 1994; Munk et al., 2016; Schiöth & Fredriksson, 2005; Scholz et al., 2019). One of the first widely accepted classification systems was based on sequence homology (Kolakowski, 1994). The sequence homology-driven classification system resulted in grouping GPCRs into six classes: Class A (rhodopsin-like), Class B (secretin receptor family), Class C (metabotropic glutamate), Class D (fungal mating pheromone receptors), Class E (cyclic AMP receptors) and Class F (frizzled/smoothened) (Alexander et al., 2021; Kolakowski, 1994). However, the sequence homology classification resulted in issues, including the inability to group some receptors into any of these classes (Alexander et al., 2021; Kolakowski, 1994; Schiöth & Fredriksson, 2005). An alternative classification based on phylogenetic analysis is now broadly used and solves many of the pitfalls associated with earlier classifications (Alexander et al., 2021; Schiöth & Fredriksson, 2005). The phylogenetic classification system is called GRAFS, which is an acronym of the five main families established by the GRAFS system: Glutamate, Rhodopsin, Adhesion, Frizzled, and Secretin (Schiöth & Fredriksson, 2005). For simplicity, I follow the GRAFS system when referring to receptor types throughout this work. In multiple

animals, most characterized GPCRs that receive neuropeptides are in the Rhodopsin or Secretin families (Bauknecht & Jékely, 2015; Caers et al., 2012; Foster et al., 2019; Frooninckx et al., 2012; Tran et al., 2019).

Signals perceived by GPCRs are canonically propagated through a mechanism mediated by a heterotrimeric G protein complex (Hilger et al., 2018). A heterotrimeric G protein complex is composed of three distinct components called an α, β and γ subunit (in the text I refer to these subunits as Gα, Gβ and Gγ, for clarity) (Figure 1.5A) (Oldham & Hamm, 2008). These proteins get the name "G protein", because the Gα subunit associates with guanine nucleotides: guanosine diphosphate (GDP) in the resting state and guanosine triphosphate (GTP) once activated (Figure 1.5A-B) (Oldham & Hamm, 2008). Additionally, while in resting state, all three subunits bind together and may be associated with a receptor (Figure 1.5A) (Hilger et al., 2018). Once the receptor is activated, the receptor acts as a Guanine nucleotide exchange factor (GEF), causing the Gα subunit to exchange GDP for GTP and the heterotrimer dissociates into the activated Gα subunit and Gβ/γ subcomplex (Figure 1.5B-C) (Hilger et al., 2018). Each subunit is key for the function and stability of the heterotrimeric G protein complex, but the Gα subunit typically enables receptor specificity and has been the focus of most research (Figure 1.5B) (Heydorn et al., 2004; Smrcka, 2008).

Because the downstream functions of Gα subunits are more well understood than the emerging roles for Gβ/γ subunits, GPCR pathways are typically defined by the type of Gα subunit that is activated. Gα class proteins are divided into four families according to functional and structural homology: Gs, Gi/o, Gq/11, and G12/13 (Syrovatkina et al., 2016). Each Gα class family is best known for specific characterized downstream outcomes (Syrovatkina et al., 2016). Gs stimulates adenylyl cyclase, which produces cyclic adenosine monophosphate (cAMP) as a second

messenger and leads to the activation of protein kinase A (PKA) (Cassel & Pfeuffer, 1978; Gill & Meren, 1978; E. M. Ross & Gilman, 1977). PKA then phosphorylates a variety of proteins to cause cellular responses (e.g., inhibiting glycogen production by phosphorylating glycogen synthase) (Sassone-Corsi, 2012). Gi/o, in contrast, inhibits adenylyl cyclase (AC) and reduces intracellular cAMP (Bokoch et al., 1983; Codina et al., 1983; Neer et al., 1984). Gq/11 activates phospholipase C (PLC), which cleaves phosphatidylinositol 4,5-bisphosphate to produce the second messengers inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) (Strathmann & Simon, 1990; Taylor et al., 1990). IP3 induces the release of Ca^{2+} from the endoplasmic reticulum and DAG activates Protein Kinase C (Kamato et al., 2015). Gα12/13 can activate RhoGEF proteins, which influence Rho GTPase activity and therefore cytoskeletal dynamics (Seasholtz et al., 1999).

Interestingly, the Gβ/γ subcomplex also plays diverse downstream roles, but researchers gained appreciation for the essential roles played by Gβ/γ subcomplex later in history compared to the Gα subunit (Figure 1.5C) (Smrcka, 2008). The Gβ/γ subcomplex can activate ion channels; the acetylcholine-regulated inwardly-rectifying K^+ channel was the first characterized ion channel to be directly bound and activated by Gβ/γ (Smrcka, 2008; Wickman et al., 1994). The Gβ/γ subcomplex also can interact with several effectors, many of which were initially thought to be activated uniquely by Gα subunits (e.g., PLC and AC) (Boyer et al., 1992; Smrcka, 2008; Tang & Gilman, 1991). The many downstream effects of individual G protein subunits allow for a high level of signaling regulation, with different combinations of heterotrimeric G protein subunits activated by a variety of GPCRs eliciting diverse cellular outcomes.

Neuropeptide and GPCR signaling pathways show an incredible diversity in function throughout the animal kingdom

Neuropeptide functions

Neuropeptides themselves have incredible functional diversity. Neuropeptide signals are highly involved in regulation of animal behaviors in multiple species, from swimming depth and settlement of the annelid *Platynereis dumerilii* larva (Conzelmann et al., 2011, 2013) to reproductive behaviors in molluscs (Morishita et al., 2010). Strikingly, work with *Drosophila* indicates that neuropeptides regulate most aspects of daily life, such as behavior (e.g., feeding, locomotion, and aggression), physiology (e.g., development, ecdysis, and stress response), and maintenance of homeostasis (e.g., water and ion homeostasis) (Nässel & Zandawala, 2019).

Importantly, neuropeptides are emerging as key signals that directly or indirectly regulate stem cells. Much of the current work exploring the relationships between neuropeptides and stem cell populations comes from models such as rodents and *Drosophila*. Neuropeptide F (NPF) in *Drosophila* induces proliferation of germline stem cells through activation of a receptor on the ovaries (Ameku et al., 2018). The glia-sourced insulin-like peptide (ILP) neuropeptide in *Drosophila* directly activates neuroblasts in response to availability of nutrients (Chell & Brand, 2010; Sousa-Nunes et al., 2011). Neuropeptides play important roles in rodent neurogenic niches and regulation of neural stem cells. For examples, neuropeptide Y (NPY) stimulates proliferation in stem cell populations such as basal cells within the olfactory epithelium (Hansel, Eipper, et al., 2001a, 2001b) and neural stem cells within the dentate gyrus in the hippocampus (Cheung et al., 2012; Decressac et al., 2011; Howell et al., 2003, 2005). An additional neuropeptide, pituitary adenylate-cyclase-activating polypeptide (PACAP), also promotes olfactory neurogenesis by inducing basal cell proliferation as well as promoting survival of the differentiating neurons

(Hansel, May, et al., 2001). These results show that there is much to learn about the regulatory effects of neuropeptides on stem cell populations *in vivo*. Research in more diverse models could uncover shared themes and indicate the level of conservation for these roles.

While neuropeptides play diverse roles within animal models, PC2, one of the key processors of neuropeptides, is surprisingly not essential. PC2 null mouse embryos are viable with reduced growth and chronic hypoglycemia (Furuta et al., 1997, 2001). The *Drosophila* PC2-encoding gene, a*montillado*, is required for multiple stages of embryonic development and lifecycle (Rayburn et al., 2003, 2009; Siekhaus & Fuller, 1999). However, studies targeting *amontillado* in adult flies, once again, describe a more restricted role in maintenance of sugar homeostasis (Rhea et al., 2010). The C. *elegans kpc-2* (otherwise known as *egl-3*) mutant presents neuromuscular dysfunction and defects in egg laying behavior (Kass et al., 2001; Trent et al., 1983). However, the eggs produced can result in viable progeny, indicating that germ cell development is functional in *kpc-2* mutants (Trent et al., 1983). PC2 may be less essential than downstream neuropeptides due to functional redundancy with other prohormone convertases in these animals (e.g., there are 9 prohormone convertase family proteases in mice) (Garten, 2018; N. Seidah et al., 2013).

After reading up on neuropeptide research, I was impressed and fascinated by the diverse roles neuropeptides play in animals and the multitude of mechanisms through which neuropeptides can act! I then considered GPCRs, the most common receptors of neuropeptides. What animal roles do GPCRs play? Furthermore, are there verified examples of GPCRs mediating neuropeptides in the examples provided above?

GPCR functions

GPCRs are one of the most functionally diverse family of receptors. Mutations to GPCRs lead to diseases such as Alzheimer's disease, Parkinson's disease, and schizophrenia (Borroto-Escuela et al., 2017; Sushma & Mondal, 2019). This, along with their extracellular accessibility, makes GPCRs some of the most sought-after targets for therapeutics, with GPCRs accounting for ~30% of current pharmaceuticals (Hauser et al., 2017). Abundant research in vitro has described the molecular structures and mechanisms of action for GPCRs, however *in vivo* work is necessary to understand how they function within the animal context (Bradley et al., 2014).

GPCRs and heterotrimeric G proteins play key roles in stem cell regulation, wound response, and regeneration of animal tissues in diverse species. I provide examples of these roles below, but for a more comprehensive list of GPCR roles, refer to (Choi et al., 2015; Doze & Perez, 2013). The GPCR *CXCR4* and Gs class subunits regulate the homing function of hematopoietic stem cells to the bone marrow microenvironment in mice (G. B. Adams et al., 2009; Ma et al., 1999; Petit et al., 2002). The zebrafish GPCR *agtrl1b* is required for proper early myocardial progenitor formation and cell movements to develop the heart (Scott et al., 2007; Zeng et al., 2007). In *Drosophila*, the GPCR *Methuselah-like 10* mediates signaling after epithelial injuries and triggers repair mechanisms (O'Connor et al., 2021). Wound signaling is also mediated by GPCRs in rodents and *Caenorhabditis elegans* (Kiseleva et al., 2014; Ziegler et al., 2009; Zugasti et al., 2014). Melanopsin (a light-sensitive GPCR) and coupled Gq/11 signaling promotes axon regeneration after optic nerve crush in mice (S. Li, Yang, et al., 2016). Similarly, the GPCRs *srg-36* and *srg-37* along with downstream Gq *egl-30* (Brundage et al., 1996) activity promote adult axon regeneration in *C. elegans* (Sakai et al., 2021; Shimizu et al., 2022). Furthermore, Go class subunits are inhibitory to axon regeneration in *C. elegans* primarily due to inhibition of Gq

signaling (Shimizu & Hisamoto, 2020). Regeneration of multiple other tissues and organs is promoted by specific G protein subunits in mice, including liver and muscle (Lu et al., 2016; Minetti et al., 2014).

GPCRs are also characterized as the downstream receptors that mediate some of the aforementioned neuropeptide roles in stem cell regulation. In *Drosophila*, the proliferative effect of the neuropeptide NPF on germline stem cells is mediated by the GCPR NPFR (Ameku et al., 2018). Similarly, in rodents, the proliferative effect of the neuropeptide NPY on basal cells in the Olfactory Epithelium is mediated by the GPCR Y1R (Doyle et al., 2008; Hansel, Eipper, et al., 2001a). These findings indicate key functions for neuropeptide- and GPCR-related signaling pathways in regulating aspects of healing and regeneration – including initial wound response and stem cell regulation – in multiple animal species.

Do neuropeptide- and GPCR-related signaling mechanisms regulate robust planarian regeneration?

Planarians provide an incredible system to explore mechanisms that coordinate cells throughout the animal during whole-body regeneration. However, a remaining gap in knowledge is how regenerative processes are initiated, coordinated, and integrated to promote a functional regenerative response. Due to the broad roles of neuropeptides and GPCRs described in other animals, I reasoned that neuropeptide and/or GPCR signaling pathways offer likely candidates for coordinating regenerative processes in planarians. What is currently known about neuropeptides, GPCRs, and heterotrimeric G proteins in planarians? Does any of the current literature suggest that these pathways are important in regeneration? Below, I provide a review of the current state of knowledge of planarian neuropeptides, GPCRs, and heterotrimeric G proteins.

Planarian neuropeptides

The first evidence of neuropeptide signaling in planarians came from the characterization of one of the primary processing enzymes in neuropeptide biosynthesis, *prohormone convertase 2* (*pc2*) (Cummins et al., 2009; Fuller et al., 1988; R. Miller et al., 2003; Steiner, 1998). Planarian *pc2* is commonly used as a broad neural marker, due to its enriched expression in many cells of the central nervous system (CNS) (Agata et al., 1998; Collins et al., 2010; Gurley et al., 2008; Roberts-Galbraith et al., 2016). In fact, the exact structure of the planarian CNS was first described in the species *Dugesia japonica* using the expression pattern of *pc2* and localization of PC2 protein (Agata et al., 1998; Okamoto et al., 2005). The expression pattern of a functional domain in an additional neuropeptide processor (peptidylglycine α-amidating monooxygenase - PAM) was also determined to be enriched in the central nervous system of *D. japonica* through in situ hybridization (Asada et al., 2005). However, other neuropeptide processors are not characterized in planarians.

After *pc2* is knocked down by RNA interference (RNAi), the animals present a characteristic phenotype of paralysis and locked ventral curling (Nogi et al., 2009; Reddien, Bermange, et al., 2005; Stevenson & Beane, 2010). However, recent work suggests additional roles for *pc2* and potential downstream signals. In *D. japonica*, *pc2(RNAi)* animals lose the pharyngeal extension behavior required for feeding (Shimoyama et al., 2016). Additionally, during a broad screen for genes important for regeneration, stem cell function, and tissue maintenance in *S. mediterranea*, *pc2* was implicated as a regulator of tissue maintenance and potentially regeneration (Reddien, Bermange, et al., 2005). Of note, the role for *pc2* in planarian regeneration is not completely clear, with work in *D. japonica* reporting no effect on regeneration in *pc2(RNAi)* animals (Nogi et al., 2009). *pc2* also regulates key processes in *S. mediterranea* sexual

development, with *pc2(RNAi)* animals displaying regressed testes due to improper germ cell differentiation and maintenance (Collins et al., 2010). Results implicating *pc2* in regulation of multiple aspects of planarian biology suggest that there are many exciting downstream neuropeptide pathways to explore.

The current defined neuropeptide complement in *S. mediterranea* includes products from 52 prohormone genes (Collins et al., 2010; McVeigh et al., 2009; Ong et al., 2016). These planarian prohormone genes encode neuropeptides belonging to diverse families including Neuropeptide Y, Insulin/Insulin-like Growth Factor, RFamide, and Pedal Peptide (Collins et al., 2010). Expression for most (~85%) planarian prohormone genes is highly enriched in the central nervous system (Collins et al., 2010). Many prohormones in the nervous system show remarkable regional diversity and neural subtype specificity, increasing the field's understanding of the complexity of the planarian brain (Collins et al., 2010; Ong et al., 2016; Shimoyama et al., 2016).

Limited functions are described for individual planarian neuropeptide prohormone genes. Five neuropeptide prohormones in *D. japonica* (*DjNpM24*, *DjNpP02*, *DjNp19*, *DjNp42* and *DjNp47*) were identified as mediators of pharynx extension (Shimoyama et al., 2016). Application of synthetic NPF peptides sourced from the tapeworm species, *Moniezia expansa*, produced pro-regenerative and pro-mitotic effects in the planarian species *Dugesia tigrina* (also referred to as *Girardia tigrina*) (Kreshchenko et al., 2008). In *S. mediterranea*, an insulin-like peptide (*ilp-1*) regulates homeostatic animal growth, wherein targeting this peptide leads to degrowth in planarians regardless of food intake/availability (C. M. Miller & Newmark, 2012). *ilp-1* also regulates germline stem cells, promoting spermatogenesis (C. M. Miller & Newmark, 2012). Another prohormone involved in sexual regulation is the CNS-expressed neuropeptide-encoding gene *npy-8*, which supports development and maintenance of reproductive tissues (Collins et al.,

2010; Saberi et al., 2016). Neuropeptides also have defined roles in key behaviors, indicating potential roles in regeneration or proper neural function. For example, two neuropeptide prohormone-encoders in *D. japonica*, *1020HH* and *eye53*, promote negative phototaxis after regeneration, despite no observed effects on the morphology of the visual system (Inoue et al., 2004).

Planarian GPCRs and heterotrimeric G proteins

Looking downstream, the genome of the planarian species *S. mediterranea* contains genes that encode a repertoire of 566 currently identified GPCRs (Saberi et al., 2016; Zamanian et al., 2011). Of these predicted receptors, 91% belong to five conserved GPCR families: rhodopsin; glutamate; adhesion; frizzled; and secretin (Fredriksson et al., 2003; Saberi et al., 2016; Schiöth & Fredriksson, 2005). However, 9% of planarian GPCRs do not have known homologs (Saberi et al., 2016). Previous work demonstrated expression of R-opsin GPCRs in planarian photoreceptor neurons (Lapan & Reddien, 2012; Sánchez Alvarado & Newmark, 1999). Saberi and colleagues further investigated the expression patterns for 54 of the 566 GPCR genes. Of the GPCR-encoding genes that they detected through in situ hybridization, the expression patterns were in diverse tissue types including the brain, sexual structures, and the intestine (Saberi et al., 2016).

The vast number of GPCR genes adds difficulty in assessing the functions of this family of receptors. However, diverse roles have been characterized for a small percentage (~3%) of the *S. mediterranea* GPCR-encoding genes. Two receptor-encoding genes of the atypical GPCR Frizzled family (*fzd1* and *fzd4-1*) play roles in promoting posterior identity by mediating Wnt signals during regeneration (Pascual-Carreras et al., 2021; Scimone et al., 2016; Stückemann et al., 2017). The serotonin-receiving GPCR-encoding genes, *smed-ser39* and *smed-ser85*, play roles

in planarian gliding locomotion (Agbedanu, 2012; Zamanian, 2011). Subsequent work in *S. mediterranea*, as well as studies with other planarian species, provides additional support of this role for homologs of *smed-ser85* and serotonin signaling in modulation of planarian locomotion and regenerative polarity (Chan et al., 2015, 2016; Currie & Pearson, 2013; Farrell et al., 2008; März et al., 2013; Zamanian et al., 2012). The NPY GPCR-encoding gene, *npyr-1*, functions with ligand *npy8* in supporting sexual maturity (Collins et al., 2010; Saberi et al., 2016). An additional orphan GPCR gene, *ophis*, also promotes sexual maturity by supporting germline stem cell differentiation and testes regeneration (Saberi et al., 2016). Finally, five Opsin family GPCR-encoding genes (*rhodopsin1*, *rhodopsin2*, *rhodopsin10*, *melanopsin2*, and *melanopsin3*) impact eye regeneration and function (Lozano, 2015).

Perhaps even less understood in planarian literature is the diversity and function of the downstream signal transducers of GPCRs, the heterotrimeric G proteins. Thus far, there are only five G protein subunit genes identified in *S. mediterranea*. One Gα class subunit (*gpas*) is homologous to a gene first identified in *D. japonica* (called *Dj-1791hh*) (Cebria et al., 2002; Iglesias et al., 2011). *gpas* is used as a molecular marker and is highly expressed in the planarian brain branches and pharynx (Cebria et al., 2002; Iglesias et al., 2011). The other four G protein subunit genes identified in *S. mediterranea* include 2 additional Gα class subunits (*gna-q* and *gna-o*), a Gβ class subunit (*gnb*), and a Gγ class subunit (*gnc*) (Lapan & Reddien, 2012). These four G protein subunits are highly expressed in the photoreceptor neurons and were uncovered during a transcriptome analysis of the planarian eye (Lapan & Reddien, 2012). Work in *D. japonica* also used a Gβ class subunit (*Dj-1997hh*) as a neural marker to characterize cultured cells from the head-abundant cell fraction by fluorescence-activated cell sorting (Asami et al., 2002). Importantly, there has not been a comprehensive investigation of planarian heterotrimeric G

proteins, so we do not know how many G protein subunits planarians possess nor the diversity of tissue types that may express heterotrimeric G proteins.

The functional significance of planarian heterotrimeric G proteins also remains unclear, and specific roles for individual G protein subunit-encoding genes have not been evaluated. However, pharmacological analyses validated the coupling of Gαs class subunits to homologs of *S7.1R*, the serotonin GPCR responsible for regulation of gliding in *D. japonica* (Chan et al., 2015, 2016; K. Nishimura et al., 2009; Zamanian et al., 2012). Because much of the current research regarding GPCR-related pathways focuses on the receptor first then addresses G protein-coupling (typically through pharmacological, *ex vivo* assays), there is a lot to learn about how heterotrimeric G proteins operate *in vivo*. Additionally, because of the plethora of GPCR-encoding genes, assessing function for GPCR pathways at the receptor level provides challenges such as potential functional redundancy and difficulty in screening. Therefore, new strategies to identify candidate GPCRs via heterotrimeric G proteins can accelerate the progress of GPCR research in the planarian field.

Thesis Objectives

Assessing the state of neuropeptide and GPCR research in the planarian field, it became clear that there are many open questions. How might PC2 and downstream neuropeptides promote tissue maintenance and regeneration? Are there additional roles for PC2 and neuropeptides not yet discovered? How many heterotrimeric G protein subunit-encoding genes do planarians harbor and how conserved are planarian G protein subunits? Are planarian G protein subunits functionally redundant, or will individual subunits play significant roles in the animal? Where in the planarian body are the G protein subunits expressed? Can we connect phenotypes of G protein subunits to

an associated GPCR(s)? In the following chapters, I hope to contribute to the progress of this field and address some of these key questions.

Neuropeptides can be functionally pleiotropic, work in additive and redundant ways, and can travel variable distances due to their stability (making target location uncertain). Through a thorough understanding of broader roles for putative neuropeptide signaling, I hope to inform future investigations into individual peptide mediators. Therefore, in Chapter 2, I provide a detailed description of the *pc2(RNAi)* phenotype (Figure 1.6). I find that *pc2* is necessary to maintain various aspects of planarian biology, including tissue turnover and behavior. Whole-body regeneration is also significantly decreased in *pc2(RNAi)* animals, with a correlated strong reduction in planarian stem cells and progeny quickly after RNAi. My results describe many exciting animal roles to explore in neuropeptide research, including stem cell regulation.

One approach to further understanding cellular mechanisms behind neuropeptide signaling in regeneration is to learn the cellular targets of these signals. Therefore, identifying the receptors responsible for mediating the signals would provide more resolution. However, the most common neuropeptide receptors – GPCRs – are the largest receptor family in planarians, with a vast list of over 500 predicted GPCR-encoding genes. Additionally, little is known about how the downstream heterotrimeric G proteins function in planarians. In Chapter 3, I identify, classify, and functionally characterize the complete planarian heterotrimeric G protein repertoire (Figure 1.6). My work with G protein subunits can suggest the ways in which GPCR-related signaling operates in planarians and shows that heterotrimeric G proteins indeed regulate regeneration. Additionally, I show that there is possibility for a G protein subunit-first approach to work backwards and identify candidate GPCRs.

Taken together, in the following chapters I investigate whether neuropeptide- and heterotrimeric G protein-related signaling mechanisms promote robust regeneration in planarians. My results support the hypothesis that these pathways are highly involved in regeneration and display roles connected to currently defined planarian regenerative traits such as reestablishment of key body polarity patterns after amputation and regulation of stem cells. Identifying the neuropeptide signals and GPCRs that mediate the phenotypes presented in this work will help elucidate the specific molecular mechanisms driving regeneration in planarians and potentially contribute to the broader field of neuropeptide and GPCR research.

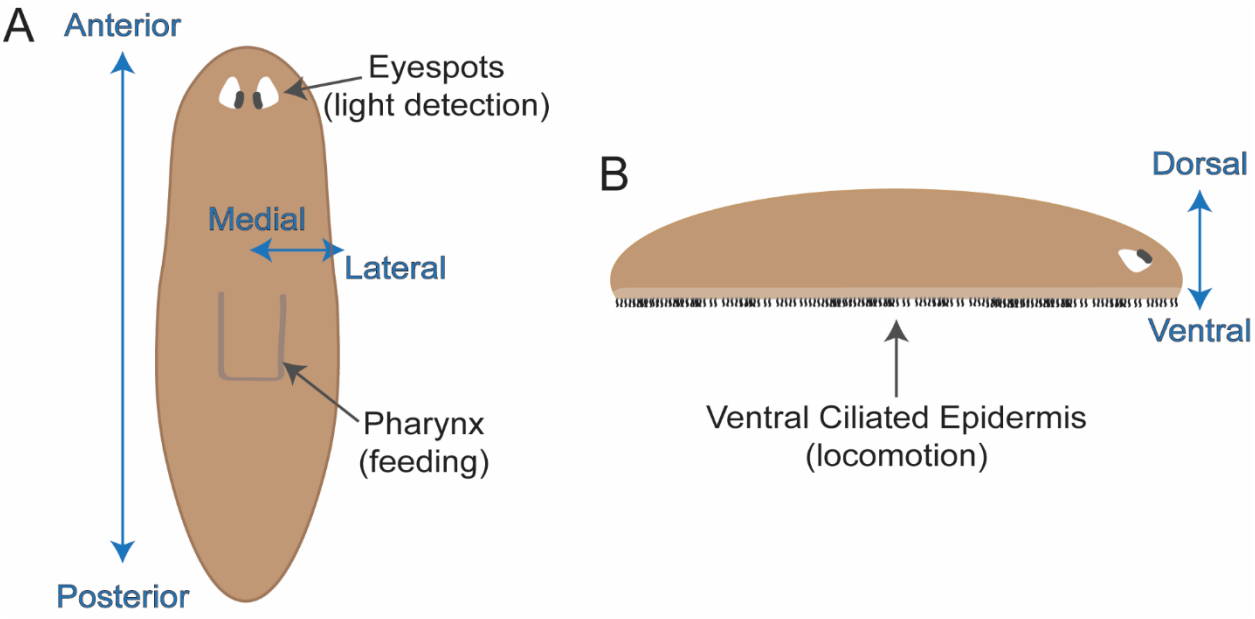


Figure 1.1 Planarian external features and primary axes of polarity. Graphic depictions of *S. mediterranea* from the **(A)** dorsal and **(B)** lateral view. The primary axes of polarity for the body plan are labeled blue. External features of the body are labeled in black. The main function associated with each feature is indicated in parentheses.

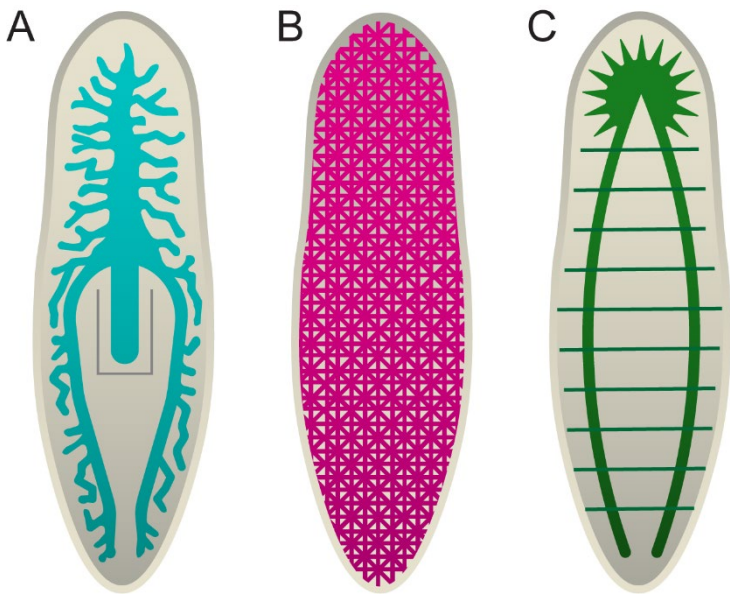


Figure 1.2 Examples of planarian internal complexity. Simplified graphics of **(A)** the digestive system (Forsthoefel et al., 2011; Ishii, 1965; MacRae, 1963; Willier et al., 1925), **(B)** the body wall musculature (Cebrià et al., 1997; Kobayashi et al., 1998; Morita, 1965; Scimone et al., 2017), and **(C)** the central nervous system (Cebria et al., 2002; MacRae, 1967; Okamoto et al., 2005; K. G. Ross et al., 2017), all of which were discussed in this introduction. For more information about these structures and additional organ systems, refer to (Roberts-Galbraith & Newmark, 2015).

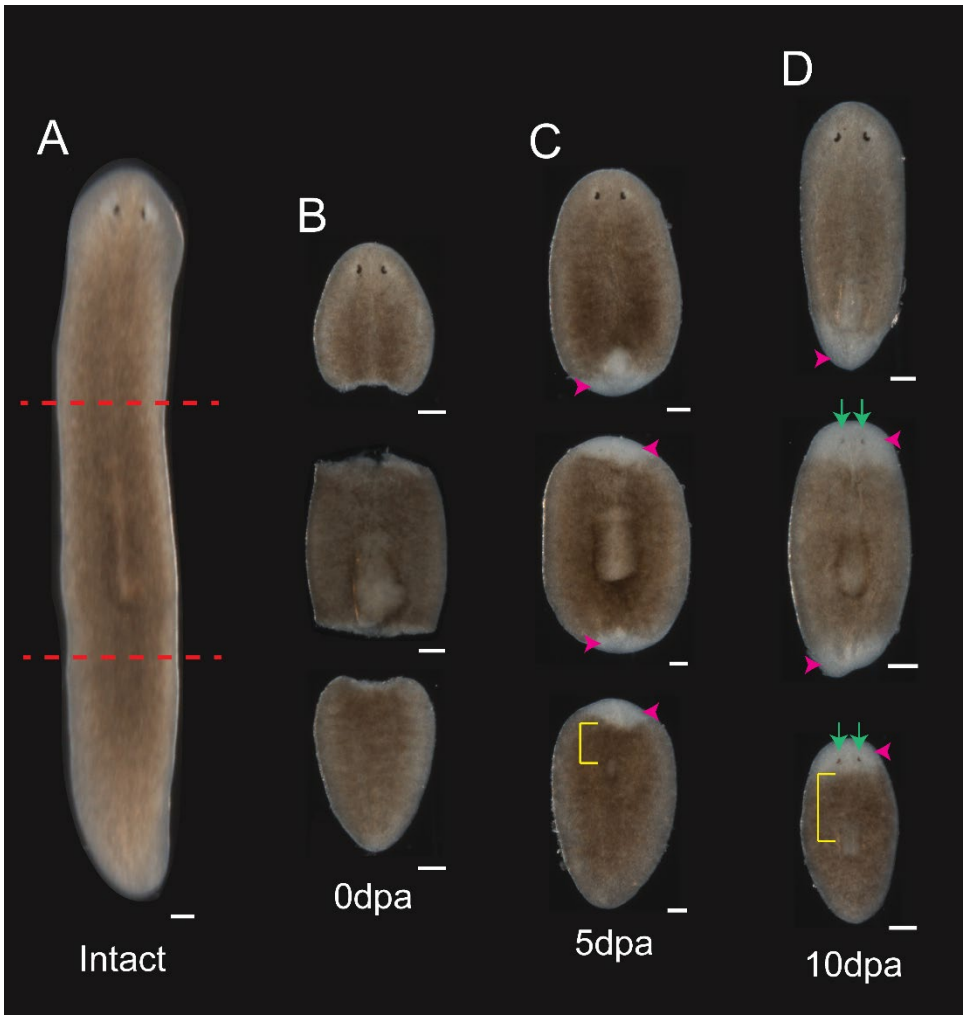


Figure 1.3 Planarians regenerate all missing structures within two weeks. (A) Live image of intact *S. mediterranea*, with the amputation sites indicated in red, dashed lines. Images of fixed regenerating animals at **(B)** 0 days after amputation, **(C)** 5 days after amputation, and **(D)** 10 days after amputation. The anterior is towards the top of the page in all images. The magenta arrowheads indicate the regenerating blastemas. The green arrows indicate newly regenerated eyespots. The yellow brackets show the distance between the amputation site and regenerating pharynx. The lower placement of the pharynx at 10dpa is indicative of re-establishing body proportions. dpa = days post amputation. Scale bars = 200 μm.

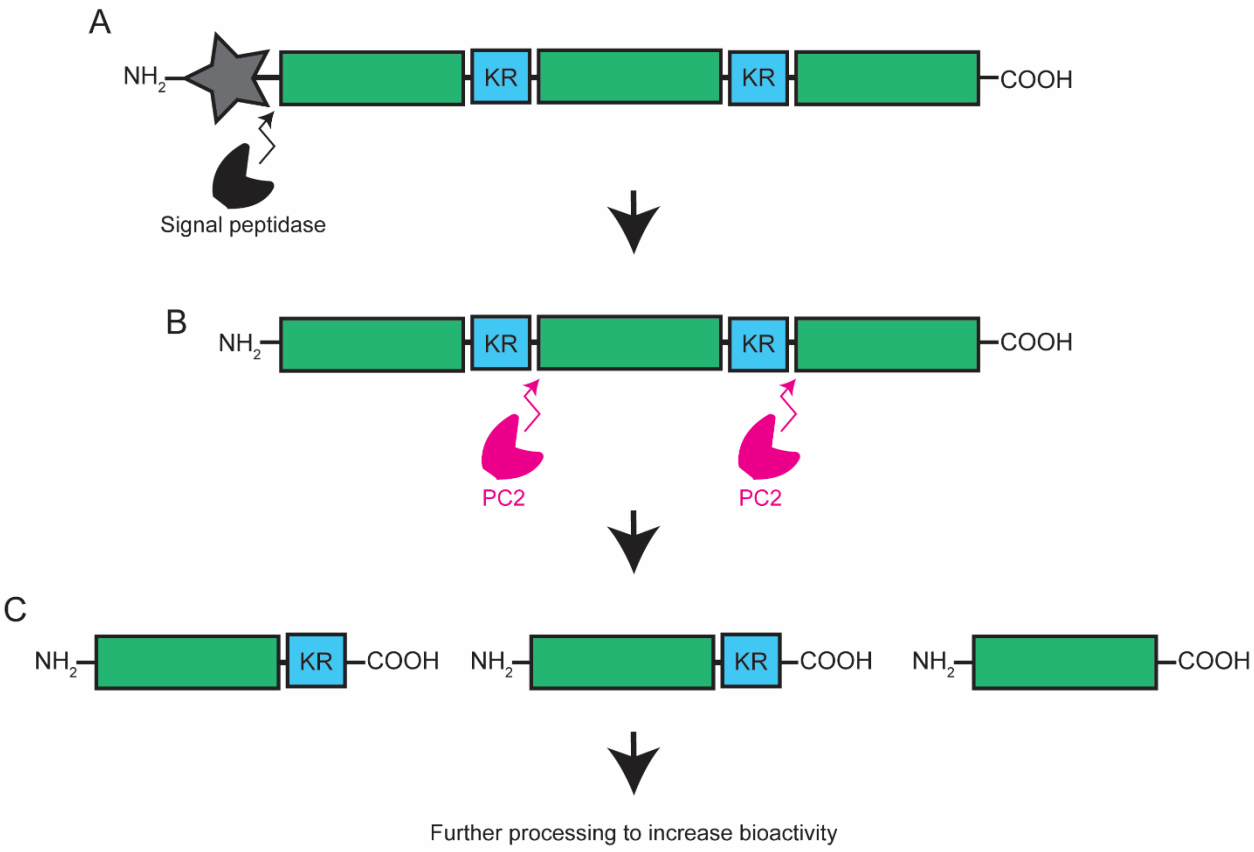


Figure 1.4 Neuropeptides are processed by multiple enzymes from a precursor polypeptide. **(A)** Graphic of the simplified structure of a generic neuropeptide prohormone, including key features such as a signal peptide (indicated as a gray star) and dibasic amino acid residues between the individual neuropeptide modules (indicated as the blue boxes with "KR" inside), which are recognized by Convertase cleavage enzymes. KR refers to the most common basic amino acids in prohormone dibasic pairs: K = Lysine and R = Arginine. One of the first steps in neuropeptide processing, the removal of the signal peptide by Signal peptidase, is also displayed. **(B)** Graphic indicating the step of neuropeptide processing executed by Prohormone Convertase 2 (PC2). PC2 cleaves at the carboxyl end of the dibasic pairs, releasing individual neuropeptides from the precursor polypeptide. **(C)** Graphic of the resulting products after PC2 cleavage. The intermediate products then undergo further processing to increase peptide stability and bioactivity.

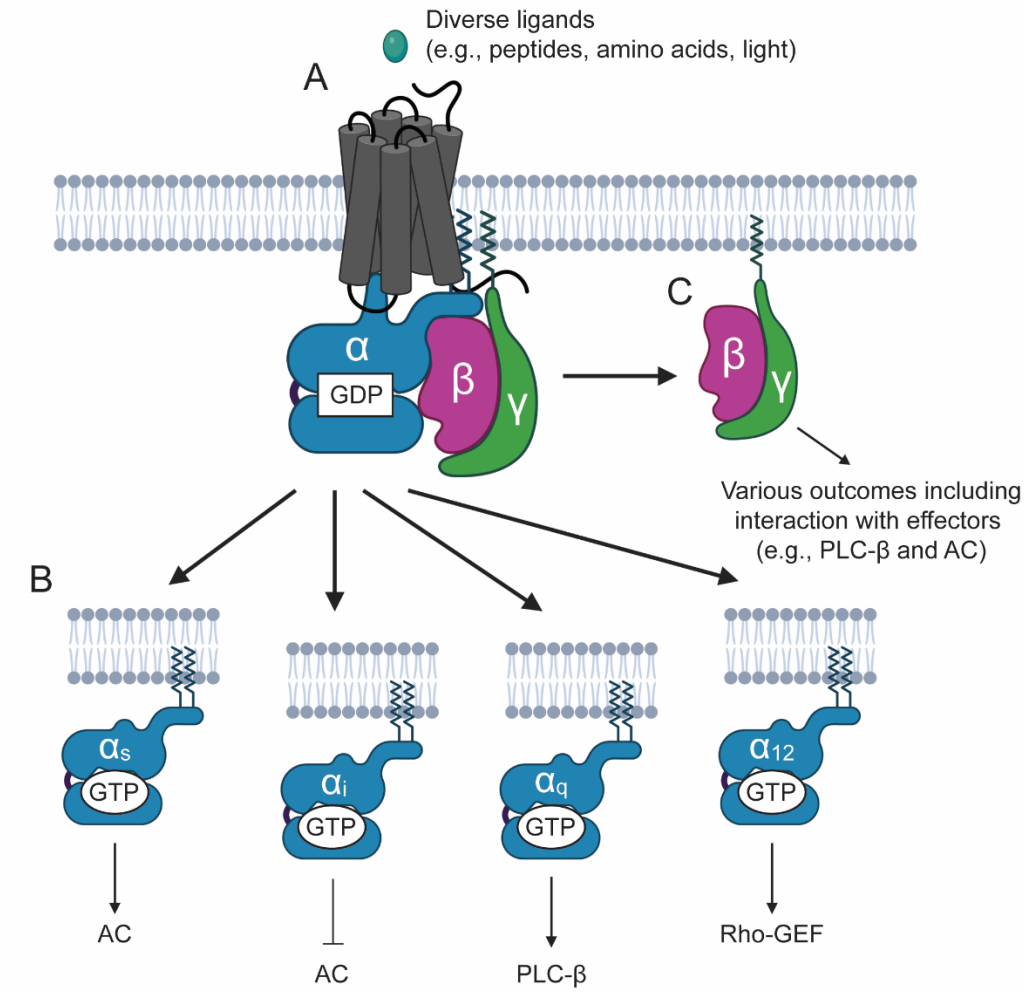


Figure 1.5 G protein-coupled receptors canonically signal through a heterotrimeric G protein complex. (A) Simplified graphic of the structure of an inactivated GPCR and associated heterotrimeric G protein complex, with the Gα subunit bound to GDP. The diversity of activating ligands is indicated above the receptor. **(B)** Graphics showing activated Gα subunits bound to GTP and dissociated from the Gβ/γ subcomplex. The activated Gα subunits are categorized into families based on downstream functional outcomes. Pointed arrows indicate that the Gα subunit activates the effector protein, and the blunt-ended line indicates an inhibitory effect. **(C)** Graphic of the dissociated Gβ/γ subcomplex, which also performs key downstream roles, such as activating and/or inhibiting effectors. AC = Adenylyl cyclase. PLC-β = Phospholipase C β. Rho-GEF = Rho Guanine nucleotide Exchange Factor. The graphics in this figure were created using BioRender.com.

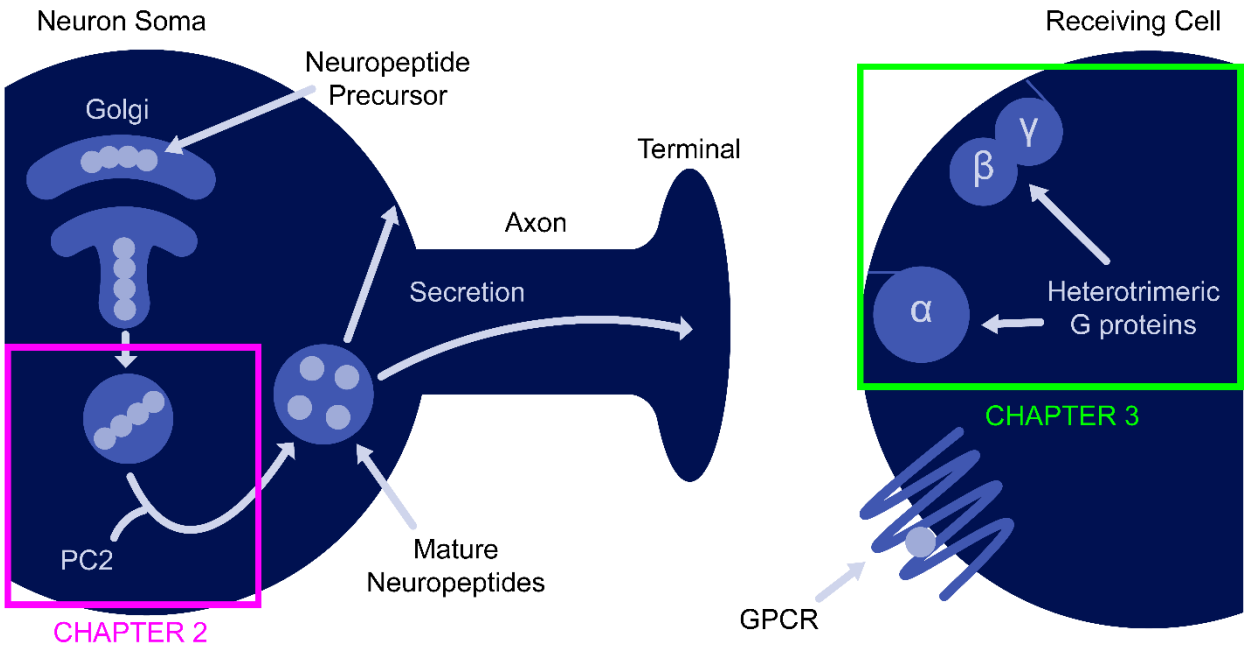


Figure 1.6 My thesis takes a two-sided approach to investigate signaling in regeneration. Simplified graphic of a typical neuropeptide signaling pathway - with neuropeptide production in a source neuron, a GPCR as the receptor on the receiving cell, and a downstream heterotrimeric G protein complex propagating the signal. The outline of my thesis objectives is overlain on the graphic, showing the two-sided, dual approach I took in investigating the relevance of these pathways in planarian regeneration.

CHAPTER 2

A NOVEL ROLE FOR PLANARIAN *PROHORMONE CONVERTASE 2* IN STEM CELL MAINTENANCE AND FUNCTION [1]

[1] Jenkins, J.E., Villalobos, A.E., and Roberts-Galbraith, R.H. 2023. To be submitted to *Developmental Biology*

Abstract

Stem cells display a cell state which can make more stem cells and produce differentiating progeny. The regulation of stem cells *in vivo* is paramount to preserve the integrity of tissues and is presumably a complex process requiring input from the surrounding environment. Freshwater flatworms called planarians are animals that contain a population of pluripotent adult stem cells and provide a powerful model to understand how stem cells are regulated *in vivo*. In this work, we aimed to further understand the required signaling environment that regulates this highly potent planarian stem cell population and drives regeneration by investigating neuropeptide signaling. To assess broad functions for neuropeptides, we targeted a neural-expressed gene encoding a subtilisin-like protease required for neuropeptide biosynthesis, called *prohormone convertase 2* (*pc2*). Here, we validate and quantify roles for *pc2* in planarian behavior, whole body regeneration, and animal survival. We also provide some of the first evidence for a mechanism behind the phenotypes regarding regeneration and survival, showing that the maintenance of stem cells and differentiating progeny is significantly affected by *pc2* knockdown. Our work lays an important foundation to support future investigations into roles for neuropeptide signaling in planarians. This work also could contribute an exciting narrative to explore, which suggests a conserved role for neuropeptide signaling in regulation of stem cells *in vivo*.

Introduction

Stem cells are defined by their ability to self-renew and give rise to new cells of one or more lineages (Becker et al., 1963; Till & McCulloch, 1961; Weissman, 2000). Stem cells provide the building material for tissues during development, homeostatic tissue turnover, and regeneration after injury. Additionally, misregulation of stem cells can result in pathologies such as cancer, which makes understanding how these cells operate vital (Cairns, 1975; G. B. Pierce, 1974; Potter, 1978). From work thus far, some broader themes are understood. To regulate stem cells within an animal, surrounding cells and tissues form a "niche" to signal and provide physical support (Barker et al., 2007; Lemischka, 1997; Ritsma et al., 2014; Schofield, 1978; Sun et al., 2014; Takeda et al., 2011)). While we have made progress in understanding the niche environment in specific tissues, there is still much to learn about cell signaling mechanisms behind stem cell regulation *in vivo*, particularly for pluripotent stem cell populations in highly regenerative organisms.

Neuropeptides recently emerged as a key family of stem cell regulatory signals (Geloso et al., 2015; Park et al., 2015; Peng et al., 2017; Ulum et al., 2020). Neuropeptides are short peptide signals (typically ~3-40 amino acids) sourced from neural or neuroendocrine tissues, which are defined by their biosynthesis and manner of release (van den Pol, 2012). Neuropeptides undergo extensive processing throughout the secretory pathway, requiring activity from multiple enzymes (Strand, 1999). Once bioactive, neuropeptides can either be secreted at a synapse, where they typically act as neuromodulators, or in an endocrine fashion, where they travel variable distances due to their stability (van den Pol, 2012). Neuropeptides have been shown to directly and/or indirectly target multipotent stem cells to regulate their activity in multiple contexts. For examples, NPY peptides in rodents directly target neural stem cells and promote neurogenesis in the dentate gyrus of the brain (Cheung et al., 2012; Decressac et al., 2011; Howell et al., 2005), and Insulin-

like peptides in *Drosophila* stimulate neuroblast activation in response to nutrient availability (Chell & Brand, 2010; Sousa-Nunes et al., 2011). Although extensive stem cell research has investigated regulation of multipotent stem cells *in vivo* and pluripotent stem cell activity *in vitro*, there remains much to learn about how pluripotent stem cells might act within an *in vivo* context. Of particular interest, we must learn how these stem cells can be regulated to elicit a functional, robust regenerative response without triggering tumorigenesis.

Freshwater flatworms called planarians are an excellent model with which to study cell signaling regulation of a dynamic, pluripotent stem cell population *in vivo*. Planarians contain adult stem cells with functional pluripotency, wherein a single stem cell has the potential to make progeny contributing to all adult tissues (Raz et al., 2021; Wagner et al., 2011; A. Zeng et al., 2018). Planarians also regenerate robustly and can regrow an entire animal from even small fragments of the original worm (Baguna et al., 1989; Elliott & Sánchez Alvarado, 2013; Morgan, 1898). After injury resulting in significant tissue loss, stem cells undergo defined events to regenerate the missing structures, including organized patterns of cell division (Wenemoser & Reddien, 2010), migration to the wounding site (Guedelhoefer & Sánchez Alvarado, 2012), and production of differentiating progeny (Bohr et al., 2021; Scimone, Kravarik, et al., 2014; van Wolfswinkel et al., 2014). Because planarians undergo continuous tissue turnover and regenerate all tissues, these animals provide a unique opportunity to investigate pluripotent stem cell regulation during homeostatic cell replacement and regeneration.

Planarians also utilize a repertoire of neuropeptide signals, including highly conserved peptides (Collins et al., 2010; C. M. Miller & Newmark, 2012; Ong et al., 2016; Shimoyama et al., 2016). Our understanding of the relationship between neuropeptide signaling and planarian stem cell biology is developing, with much of the current work focusing on the germline in the sexual

strain of *S. mediterranea*. A key processing enzyme in neuropeptide biosynthesis, *prohormone convertase 2* (*pc2*) promotes germ cell differentiation and sexual maturation/maintenance (Collins et al., 2010). A specific neuropeptide – from prohormone *npy-8* – and its receptor *npyr-1* were identified as specific regulators of sexual development (Collins et al., 2010; Saberi et al., 2016). Insulin-like peptide (*ilp-1*) and its cognate receptor (*inr-1*) also promote spermatogenesis from germline stem cells (C. M. Miller & Newmark, 2012). In asexual *S. mediterranea*, the *ilp-1*/*inr-1* pathway also regulates homeostatic growth via nutritional cues (C. M. Miller & Newmark, 2012). Finally, RNAi of *pc2* implicates neuropeptide signaling in tissue maintenance and regeneration (Reddien, Bermange, et al., 2005). While these results display a connection between neuropeptide signaling and planarian physiology, the potential ways by which *pc2* and downstream neuropeptides promote tissue maintenance and regeneration through the somatic stem cell population remain to be explored.

To understand whether *pc2* affects somatic stem cells in homeostasis and regeneration, we targeted *pc2* expression to broadly downregulate neuropeptide pathways. We define with temporal detail the key functional outcomes of *pc2* in homeostasis, showing that it supports planarian viability, feeding, and movement. Additionally, we find that *pc2* promotes whole body regeneration, with effects on the stem cell population. Further, *pc2* strongly affects epidermal and eye progenitors, suggesting a key role for putative neuropeptide signaling in stem cell differentiation and/or maintenance. This work details the impacts of *pc2* on planarian physiology and somatic stem cells in asexual *S. mediterranea*. Our results provide a foundation for informed investigation into specific neuropeptide pathways regulating planarian stem cell maintenance and function.

Results

pc2 supports planarian viability and behavior

To understand the complex, multifaceted ways by which *pc2* (and putative neuropeptide signaling) supports planarian physiology, we performed a detailed behavior analysis of planarians after *pc2* knockdown. *pc2(RNAi)* animals lose gross movement and present ventral curling, which paralyzes the animals (Nogi et al., 2009; Reddien, Bermange, et al., 2005; Stevenson & Beane, 2010). We repeated RNAi to document how quickly *pc2* knockdown triggers loss of motility. Additionally, we sought to uncover other potential functions for PC2-regulated signals.

We first optimized our method of RNAi and found that 2-3 feedings three days apart results in robust knockdown. Using this paradigm, we started our behavior analysis (Fig 2.1A). By day 6, the locomotion displayed by *pc2(RNAi)* animals was strongly reduced, manifesting first as loss of forward movement and a relaxed body morphology (Sup Movie S2.1). With continued absence of *pc2* expression, by day 11, the characteristic ventral curling body posture arose (Fig 2.1C; Sup Movie S2.1). This confirms that *pc2* supports planarian movements and demonstrates that loss of *pc2* function causes locomotion and posture defects within 6 days.

In addition to paralysis, we also noted dysfunction in the digestive system. By the second RNAi feeding, we documented visible food retention, which is evident due to green dye added to the food (Fig 2.1G). To quantify this phenotype, we took images of individual animals and blinded the data by assigning labels at random, then a colleague categorized the images in a binary score (either “green” or “not green”). When quantified, 72.7% of *pc2(RNAi)* animals had detectable green coloring within their intestine four days after RNAi feeding, compared to only 45.5% of controls (Fig 2.1H). While the difference between control and *pc2(RNAi)* animals was not significant through Chi-square analysis (Fig 2.1H), the binary categorization did not represent the

higher intensity of food retention seen in *pc2(RNAi)* animals (representative images seen in Fig 2.1G). While this phenotype needs further exploration, our results suggest that *pc2(RNAi)* animals may show higher rates of food retention.

Additionally, in the planarian species *Dugesia japonica*, extension behavior of the feeding organ (called the pharynx (Ishii, 1965; MacRae, 1963; Wulzen, 1917)) requires *pc2* and five identified neuropeptide prohormones (Shimoyama et al., 2016). Surprisingly, in our analysis using *S. mediterranea*, we saw complete pharynx ejection and detachment in *pc2(RNAi)* animals (Fig 2.1D). The animals began detaching their pharynges on day 11, and by day 26, at least 50% of *pc2(RNAi)* animals had lost their pharynges (this behavior was more difficult to document in curled animals). These results indicate that *pc2* supports maintenance and attachment of the planarian feeding organ.

Finally, it was previously noted that *pc2* knockdown causes lysis (Reddien, Bermange, et al., 2005). We sought to determine how quickly planarians perish without *pc2* function. During our analysis, we first detected visible issues with the epidermis in *pc2(RNAi)* animals on day 11. This manifested as unevenness of epidermal pigment patterning (Fig 2.1E). Next, by day 18 epidermal lesions were detected (Fig 2.1E). These lesions did not appear to have anatomical specificity and instead appeared along the bodies of the animals. Lesions continued over time until *pc2(RNAi)* animals began to die due to lysis (Fig 2.1E). To quantify lethality, we generated a survival curve. We showed that whereas all control animals survived, *pc2(RNAi)* animals began to die at day 25 of RNAi, then by day 43, 80% of *pc2(RNAi)* animals had died due to lysis (Fig 2.1F). These results demonstrate the need for *pc2* function in supporting planarian viability.

Taken together, our results build on prior knowledge and show that *pc2* function supports multiple aspects of planarian biology. The accumulation of multiple phenotypes clearly displays the necessity for *pc2* and related pathways in planarian survival.

pc2 promotes planarian regeneration

We next sought to investigate the degree to which *pc2* is involved in the robust tissue regeneration displayed by planarians. While it was documented that *pc2* knockdown leads to reduced head blastema size (Reddien, Bermange, et al., 2005), we aimed to quantify this role and detail the tissue types affected to inform future studies into downstream PC2-related signals. We first investigated *pc2* in the context of brain regeneration, because brain size is a highly robust way of detecting regeneration defects (Roberts-Galbraith et al., 2016) (Fig 2.2B). We found that targeting *pc2* lead to a 50% decrease in regenerated brain size (Fig 2.2A; 2.2C). This demonstrates that brain regeneration is significantly stunted in the absence of *pc2*.

Because we noted defects in planarian viability in our behavior analysis, we wanted to determine whether *pc2* had additional roles in regeneration or if regeneration defects indirectly resulted from other phenotypes such as loss of tissue integrity. To differentiate between primary regeneration defects and regeneration defects as secondary outcome due to lysis, we measured head regeneration at a timepoint before epidermal defects are detectable (day 13 of RNAi) and compared these measurements to regeneration at a later timepoint when the animals are showing lesions and are less healthy overall (day 23 of RNAi). We observed a 28.65% decrease in head blastema size at early timepoints in *pc2(RNAi)* animals (Fig 2.2D-E). At later timepoints, *pc2(RNAi)* animals showed a 49.9% reduction in head blastema size (Fig 2.2F-G). These results

indicate that *pc2* is required for robust regeneration, but that the illness (also resulting from *pc2* downregulation) might exacerbate this phenotype.

Finally, we questioned whether defects in regeneration were specific to the head or whether *pc2* function supports whole-body regeneration, regardless of the missing tissue type. To investigate whether *pc2* supports tail regeneration, we measured tail blastemas after regeneration at the early and late timepoints. We saw a 43.68% decrease in tail blastema size at the early timepoint (Fig 2.2D-E) and a 54.89% decrease at the late timepoint (Fig 2.2F-G) in *pc2(RNAi)* animals compared to controls. This was an equally robust decrease in the context of tail regeneration that we saw for head regeneration. Taken together, our results suggest that *pc2* supports regeneration of multiple tissues in planarians, and that the regenerative defect increases in severity as these animals show other phenotypes (e.g., lysis).

pc2 supports maintenance of the planarian stem cell population

After observing the lysis phenotypes and defective regenerative responses in *pc2(RNAi)* animals, we hypothesized that these phenotypes could result from improper maintenance of the stem cell population. To first determine whether intact *pc2(RNAi)* animals lose their stem cells over time, we performed in situ hybridization (ISH) with *Smedwi-1* (a marker for planarian stem cells (Reddien, Oviedo, et al., 2005)). To our surprise, we saw a consistent decrease in *Smedwi-1*$^{+}$ cells as early as day 9 of RNAi, before the animals showed lesions or lysis (Fig 2.3B). Furthermore, while there appeared to be further loss of *Smediw-1*$^{+}$ cells in the head over time, the body-wide reduced population persisted (though there was a range of phenotype severity), even with robust RNAi of *pc2* (Fig 2.3B; Sup Fig S2.1). Intriguingly, we could not detect a significant reduction of *Smedwi-1* in intact animals at day 13 through RT-qPCR (Fig 2.3D). Additionally, we noted that in

the event of lesions or pharynx ejection, we saw a local enrichment of *Smedwi-1*$^{+}$ cells (Fig 2.3B; Sup Fig S2.1). Together, our results indicate that the stem cell population quickly decreases after loss of *pc2*, but that some stem cells remain and might respond to injury even as the animals enter lysis.

We then investigated stem cell persistence during regeneration. Because we saw highly significant regeneration phenotypes in the context of the later timepoint (Fig 2.2F-G), we investigated whether stem cells were lost at this timepoint in *pc2(RNAi)* animals. We saw that *Smedwi-1*$^{+}$ cells were markedly reduced in *pc2(RNAi)* head regenerates, with the most obvious reduction seen in the tail area (Fig 2.3C; Sup Fig S2.1). With a quantitative approach, we also detected a significant reduction of *Smedwi-1* through RT-qPCR at day 13 of RNAi, 5 days after head amputation (Fig 2.3D). Surprisingly, in our ISH results, stem cells appear enriched at the amputation site in both control and *pc2(RNAi)* animals (Fig 2.3C). Additionally, as seen in our intact timeline, when the animals had lesions, we saw a broader enrichment of *Smedwi-1* near all wounds (Fig 2.3C; Sup Fig S2.1). These results suggest that the reduced stem cell population in *pc2(RNAi)* animals superficially responds to injury.

Taken together, our results indicate that loss of *pc2*, and potential loss of neuropeptide signaling, results in a reduced stem cell population that detects injury but cannot functionally restore damaged or lost tissues.

pc2 promotes maintenance of multiple progenitor populations

Because the stem cells in *pc2(RNAi)* animals appear to respond to injury but cannot restore tissues, we reasoned that additional dysfunction in stem cell behaviors could prevent the remaining stem cells from replacing the necessary cell types. To investigate whether the remaining stem cells were

able to produce differentiating progeny, we examined defined progenitor markers in *pc2(RNAi)* animals. We first observed epidermal progenitors, because the epidermal lineage is one of the most well characterized in planarians and there are defined markers expressed only in distinct progenitor populations (not the mature differentiated cells) (Eisenhoffer et al., 2008; Tu et al., 2015). We saw a strong reduction in staining for both an earlier epidermal progenitor marker (*Prog-1*) and later progenitor marker (*AGAT-1*) (Eisenhoffer et al., 2008; Tu et al., 2015) after *pc2(RNAi*), which was confirmed through RT-qPCR (Fig 2.4A-D). Intact *pc2(RNAi)* animals displayed comparable, strong reductions in *Prog-1*$^{+}$ cells at both early (day 13 of RNAi) and late (day 23 of RNAi) timepoints (Fig 2.4A). In regenerating animals, *Prog-1*$^{+}$ cells were also reduced; however, stronger phenotypes were observed at the late timepoint (Fig 2.4B). These results indicate that *pc2* expression supports production and/or maintenance of differentiating epidermal lineage progenitors.

We next investigated whether progenitor depletion was specific to the epidermal lineage, or whether multiple progenitor populations required *pc2* function for their differentiation and/or maintenance in planarians. To distinguish between these two options, we observed expression of a transcription factor-encoding gene required for formation of the planarian eye, *ovo* (Lapan & Reddien, 2012). *ovo* is expressed in all eye cells, including eye progenitors that appear in visible trails posterior to the eye (Lapan & Reddien, 2012). In intact animals, the homeostatic progenitor trail is reduced quickly after *pc2* knockdown then further lost over time, with almost no detectable *ovo*$^{+}$ trail progenitors by day 23 (Fig 2.4E). During regeneration in control animals, *ovo*$^{+}$ cells are detected in the regenerating eyes and in progenitor trails with higher density compared to the trails seen in intact animals (Fig 2.4F) (Lapan & Reddien, 2012). In some regenerating *pc2(RNAi)* animals, *ovo*$^{+}$ cells form a medial cluster at day 13 of RNAi (Fig 2.4F). Defects are even more

prevalent by day 23, with a range of phenotypes including staining that resembles the expected patterns of earlier stages of regeneration (Fig 2.4F) (Lapan & Reddien, 2012). These results suggest that multiple lineage progenitor populations are greatly reduced in the absence of *pc2* function.

In summary, our results support a role for *pc2* and related downstream signals in promoting multiple aspects of planarian biology, including regulation of the dynamic stem cell population in homeostasis and regeneration.

Discussion

Planarians contain a dynamic, highly regulated population of stems cells that must continuously support homeostatic tissue turnover and respond appropriately to wounding. In this work, we take a step toward discovering new potential signaling pathways that regulate the behavior of stem cells by characterizing the phenotype resulting from downregulation of a gene encoding a key processor for neuropeptide biosynthesis, *pc2*. We first confirmed that PC2 function supports multiple aspects of planarian viability, including movement, feeding, and maintenance of tissue integrity (Fig 2.5). We then demonstrated a role for *pc2* in whole-body and brain regeneration. We further brought new clarity to tissue integrity and regeneration defects by showing that they are correlated with a reduction to the stem cell population, with multiple cell lineage progenitor pools also appearing depleted (Fig 2.5). Our findings support the notion that *pc2* and putative neuropeptide signaling support multiple key processes in planarian biology, including a potentially conserved role in regulating stem cells.

Identification of the molecular substrates for PC2 in planarians would resolve the multifaceted pc2(RNAi) phenotype

One of the critical next steps for understanding the cellular and molecular mechanisms behind the pleiotropic phenotype after *pc2(RNAi)* would be identification of the specific PC2 substrates responsible for each role. The most common and conserved molecular targets for PC2 are neuropeptide precursors/prohormones (Cummins et al., 2009; Husson et al., 2007; Luxmi et al., 2018; R. Miller et al., 2003; Steiner, 1998). Neuropeptides display incredible functional diversity in animals, with a range of roles in behavior, physiological homeostasis, development, reproduction, and stem cell biology (Collins et al., 2010; Conzelmann et al., 2011; C. Li, 2008; Malva et al., 2012; Morishita et al., 2010; Nässel & Zandawala, 2019; Shakya & Lindberg, 2021). Therefore, future work should identify the specific PC2-depleted signals responsible for the many phenotypes in *pc2(RNAi)* planarians.

While the work presented here does not identify specific signals responsible for the phenotypes of *pc2(RNAi)*, evidence in the planarian field supports a conserved function for planarian PC2 in neuropeptide biosynthesis. Collins III and colleagues generated peptidomic evidence of planarian PC2 processing a subset of the predicted neuropeptide prohormones in *S. mediterranea* (Collins et al., 2010). These same researchers also documented defects in sexual development and maintenance in *pc2(RNAi)* animals and further characterized a neuropeptide prohormone gene (*npy8*) as a specific mediator of this phenotype (Collins et al., 2010; Saberi et al., 2016). Additionally, in another planarian species, *Dugesia japonica*, Shimoyama and colleagues documented pharyngeal extension phenotypes in *pc2(RNAi)* animals and subsequently identified five downstream neuropeptide prohormone genes responsible for the regulation of this behavior (Shimoyama et al., 2016). These findings further support the notion that the phenotypes

documented in this work after *pc2(RNAi)* likely result from loss of neuropeptide signals. The vast roles of neuropeptides in the animal kingdom, along with the diverse tissue types affected in *pc2(RNAi)* animals, suggests that there are many new exciting signaling pathways to explore in planarians.

We completed an initial screen of the 52 previously identified prohormone genes (Collins et al., 2010; Ong et al., 2016). We detected mild regeneration phenotypes after perturbation of 7 neuropeptide prohormones, *spp-15*, *spp-16*, *grh-1*, *spp-18*, *npp-8*, *spp-14*, and *eye53-1* (Sup Fig S2.2). However, a more thorough investigation using a more aggressive knockdown regimen could improve detection of subtle neuropeptide roles and strengthen these results. Additionally, peptide signals sometimes work additively and/or redundantly, such as that seen in the insulin-like peptide regulation of growth in *Drosophila* (Grönke et al., 2010; H. Zhang et al., 2009). In that case, targeting multiple neuropeptides in tandem may be required to achieve the regeneration phenotype severity seen with *pc2(RNAi)*. Finally, through the combined efforts of the planarian community, more complete genome and transcriptome sequences have become available since the first neuropeptide prohormone characterization (Grohme et al., 2018; O. Nishimura et al., 2015; Robb et al., 2015). Therefore, additional prohormone genes may still remain to be identified. As proof of principle, Ong and colleagues found an additional prohormone (*spp-20*) with peptidomic methods (Ong et al., 2016). The potential redundancy of neuropeptide signals, along with improved resources available in planarian research, suggests a that a concerted effort to further explore candidate neuropeptides in logical and aggressive ways may uncover the key signals mediating the phenotypes in *pc2(RNAi)* planarians.

From published work, we can make informed choices about candidate neuropeptides to investigate as potential signals downstream of PC2. Considering planarian literature, *pc2(RNAi)*

animals cannot maintain growth, which is a demonstrated role for neural-produced insulin signaling (*ilp-1*/*inr-1*) (C. M. Miller & Newmark, 2012). Therefore, *ilp-1* may be a candidate for the observed growth function. Another strategy could use the published expression patters (Collins et al., 2010) to suggest neuropeptide-encoding candidates for specific phenotypes in *pc2(RNAi*) animals. Using the pharynx ejection and detachment phenotype documented in this work as an example, there are multiple prohormone genes expressed in or around the pharynx (e.g., *npp-1*, *spp-1*, and *spp-8*), making these prohormone genes potential candidates as mediators for pharyngeal phenotypes (Collins et al., 2010). Additionally, recent work characterized proteins upregulated during brain regeneration and detected peptides from many prohormones (particularly those derived from Secreted peptide prohormones, Pedal peptide prohormones, and NPY prohormones) (Ong et al., 2016). Many of these prohormone-encoding genes were hits in our initial brain regeneration screen (Sup Fig S2.2). Therefore, thorough investigation into these prohormone candidates may uncover functional roles in regeneration.

Work outside of planarian literature may also suggest logical candidate approaches in the identification of neuropeptide mediators of *pc2* function. For example, NPY-related peptides act as mitogenic and neurogenic signals in animals ranging from mammals to arthropods (Ameku et al., 2018; Hansel, Eipper, et al., 2001a, 2001b; Lee et al., 2003; Malva et al., 2012). Currently, there are 11 predicted planarian NPY prohormone genes, with a sexual-specific NPY prohormone gene (*npy8*) influencing sexual development (Collins et al., 2010). The roles for NPYs in other animals, along with the demonstrated bioactivity of *npy8*, makes planarian NPYs intriguing candidates for the stem cell and regeneration phenotypes resulting from *pc2* knockdown. Once candidate neuropeptides are found, this could open the door to delineating the cellular mechanism.

Characterization of the cellular targets will clarify the mechanisms through which PC2-related signals support planarian biology

The cellular targets of PC2-processed signals are another important consideration when defining the cellular mechanisms through which *pc2* influences planarian biology. Regarding the phenotypes related to stem cells and regeneration, while there is a clear effect on stem cells after *pc2(RNAi)*, one of the remaining questions is *how* PC2 function impacts stem cells. The signals processed by PC2 may directly target the stem cell population (as seen with neural stem cells in rodent brain (Decressac et al., 2009; Howell et al., 2005)) and/or PC2-processed signals might affect other cell types which then indirectly influence stem cell behavior (as that in *Drosophila*, where the peptide signal Bursicon targets gut muscles to regulate intestinal stem cells (Scopelliti et al., 2014)). Future work should aim to identify the downstream signals (stated above) and potential receptors of these signals to enable the characterization of these cellular targets.

A strategic family of receptors to investigate is the G protein-coupled receptor (GPCR) family. GPCRs 1) are commonly neuropeptide receptors and 2) have been characterized as the downstream mediators of neuropeptide effects on stem cells in other animals (Ameku et al., 2018; Doyle et al., 2008; Frooninckx et al., 2012; Hansel, Eipper, et al., 2001a; Hewes & Taghert, 2001; Mirabeau & Joly, 2013). Planarians have a defined set of GPCR-encoding genes including ~50 predicted peptide receptors (Saberi et al., 2016; Zamanian et al., 2011). Investigation into the planarian peptide receptors could uncover GPCRs downstream of *pc2* for roles related to stem cells and regeneration. Additionally, we recently characterized the canonical signal transducers of GPCRs, called heterotrimeric G proteins (Jenkins & Roberts-Galbraith, 2023). Many planarian heterotrimeric G protein subunits displayed roles related to regeneration, and we showed that working with the G protein subunits can accelerate identification of candidate GPCRs (Jenkins &

Roberts-Galbraith, 2023). If the GPCRs and/or heterotrimeric G Proteins that mediate the functions described here for *pc2* are discovered, the expression of these downstream components would help clarify the tissue types through which neuropeptides act. Furthermore, if the specific receptors are uncovered, the homology of the GPCR(s) could inform investigation into the neuropeptide prohormones (e.g., if the GPCR is an NPY-predicted receptor, the NPY prohormones would be important to further investigate). Therefore, looking downstream at the potential receptors and signal transducers could both suggest candidate ligands and indicate the potential tissues targeted by neuropeptide signals.

Considering the potential tissue types that receive neuropeptides, one tissue which logically could be the conduit between planarian neuropeptides and stem cells is muscle. Planarian muscle cells provide much key information to the stem cell population, ranging from polarity cues to modifications to the extracellular matrix (Cote et al., 2019; Witchley et al., 2013). The *pc2(RNAi)* phenotype includes strong effects on contractile muscle function, particularly the locked ventral curling posture of these animals. It is important to understand whether the malfunction of muscle tissue is 1) connected with the phenotypes observed with stem cells and/or 2) a result of defects to the muscle cells themselves or from improper crosstalk with neurons.

Neurons do influence muscle tissues directly and/or indirectly through neuropeptides in multiple animals (M. E. Adams & O'Shea, 1983; S. Li, Koziol-White, et al., 2016; v. Euler & Gaddum, 1931). Additionally, neuropeptides act as neuromodulators in other organisms, so the effect on muscle may result from altered response to other key small-molecule neurotransmitters (reviewed in (Merighi et al., 2011)). If the effects on stem cells do not result from direct targeting of neuropeptides and instead manifest as a secondary effect of neuropeptides targeting muscle to induce a proper response, understanding this relationship could still be an important missing link

to the instructive signaling environment muscle provides to stem cells in homeostasis and regeneration. Therefore, key future work would also attempt to clarify this relationship and/or decouple the effects seen on regeneration and stem cells from the effects seen on muscle.

The planarian pc2 phenotype suggests a potential conserved role for neuropeptides in stem cell regulation

Peptide signaling is considered an ancient, conserved mechanism of cell signaling, with peptide signals and associated receptors detected in single-celled organisms like *Saccharomyces cerevisiae* (Fuller et al., 1988; Michaelis & Barrowman, 2012) and non-bilaterian metazoans like Cnidarians (Anctil & Grimmelikhuijzen, 1989; Fujisawa & Hayakawa, 2012; Grimmelikhuijzen & Graff, 1986; Takahashi et al., 1997). Additionally, emerging evidence implicates neuropeptides in regulation of multipotent stem cell populations (Agasse et al., 2008; Álvaro et al., 2008; Amcheslavsky et al., 2014; Ameku et al., 2018; Chell & Brand, 2010; Decressac et al., 2009; Hansel, Eipper, et al., 2001a; Howell et al., 2005; Park et al., 2015, 2016; Thiriet et al., 2011). However, it is unclear whether these mechanisms of stem cell regulation are widespread throughout the animal kingdom and whether the connections represent a conserved/ancestral function of neuropeptides. Furthermore, due to the limitations of commonly used models in animal research, potential roles for neuropeptide signaling in regulation of highly potent and/or pluripotent stem cell populations *in vivo* are not well explored. In this work, we investigated potential roles for peptide signals in the planarian flatworm, *S. mediterranea*, which harbors a population of adult pluripotent stem cells and robustly regenerates all tissues. Our results show strong effects on planarian somatic stem cells resulting from targeting the gene encoding a key enzyme in neuropeptide biosynthesis, *pc2*. Further exploration of the role for planarian *pc2* and downstream

signals could be expanded to explore neuropeptide regulation of adult pluripotent stem cells among diverse animal species.

To define the ways by which *pc2* and downstream signals influence planarian stem cell biology and regeneration, future work should delineate the downstream cellular mechanisms responsible for the animal-wide roles of *pc2* (as stated above) as well as the exact dysfunctions displayed by stem cells in *pc2(RNAi)* animals. Because the reduced stem cell population still seems to mount a superficial response to wounding without achieving functional regeneration (or homeostatic tissue turnover), we believe that additional stem cell behaviors are dysfunctional in these animals. Looking to other work, PC2 activity, neuropeptides, and related peptide hormones directly and/or indirectly support multiple multipotent stem cell populations by influencing behaviors including proliferation, exit from quiescence, survival, and differentiation (Amcheslavsky et al., 2014; Ameku et al., 2018; Czarnecka et al., 2019; Foronda et al., 2014; Gueron et al., 1997; O'Brien et al., 2011; Park et al., 2015, 2016; Scopelliti et al., 2014). Our results indicate a reduction in the stem cell population, which could result from lower rates of proliferation (Fig 2.3). However, with the current data, we cannot say whether stem cell proliferation is affected by *pc2(RNAi)*. Therefore, future work should determine whether stem cells are dividing at the expected rate during homeostasis and regeneration.

Perhaps more compelling, our results also show that stem cell progeny are highly reduced, with two lineages investigated (Fig 2.4). Furthermore, we quantitatively looked at epidermal progenitors and found that the earlier marker (*Prog-1*) was more significantly reduced than the later marker (*AGAT-1*) at the early timepoint (Fig 2.4). This could indicate a top-down effect, with the stem cells failing to specify epidermal fate, depleting the progenitor numbers over time. Considering the model that the stem cells are not being driven to differentiate, this could also

contribute to why we see modest reductions in *Smedwi-1* expression, because early stem cell progeny still express low levels of *Smedwi-1* (Molinaro & Pearson, 2016; A. Zeng et al., 2018). Therefore, the reduction of whole mount *Smedwi-1* staining may reflect the depletion of these early progeny cells. However, we have yet to explore how *pc2* influences stem cell differentiation of additional progenitor lineages (e.g., muscle, pharyngeal, and intestinal). Detailed investigation into differential effects on multiple progenitor lineages and further investigation into defined stem cell behaviors could inform the model for how the *pc2* phenotype manifests, clarifying how downstream signaling regulates planarian stem cells.

In summary, identifying the specific neuropeptide pathways that mediate the phenotypes of *pc2(RNAi)* are likely to uncover novel mechanisms and tissue sources of signaling required for the behaviors of pluripotent stem cells in planarians. This knowledge would contribute to the field's understanding of *in vivo* stem cell regulation in homeostasis and regeneration.

Materials and Methods

Animal Maintenance: Asexual planarians of the species *S. mediterranea* (CIW4 (Alvarado et al., 2002)) were maintained at 18°C in 1X Montjuïc salts (1.6 mmol/L NaCl, 1 mmol/L $CaCl_2$, 1 mmol/L $MgSO_4$, 0.1 mmol/L $MgCl_2$, 0.1 mmol/L KCl and 1.2 mmol/L $NaHCO_3$ prepared in ELGA PURELAB [ELGA LabWater, Woodridge, IL] ultrapure water) (Cebrià & Newmark, 2005). Either weekly or biweekly, animals were fed with locally sourced beef liver puree (White Oak Pastures, Bluffton, GA). For experiments, animals ranging from 1-5 mm were separated from stocks, starved and treated with 1:1000 Gentamicin sulfate (50 mg/mL stock [Gemini Bio, West

Sacramento, CA]) to reduce microbes in the water at least one week prior to starting the experiment.

RNA interference (RNAi) experiments: dsRNA was transcribed in vitro from the pJC53.2 vector (Collins et al., 2010) containing a 300–1000 bp fragment of each gene of interest as described (Collins et al., 2010; Rouhana et al., 2013). dsRNA concentrations were estimated from band intensity after gel electrophoresis of 1:10 dilutions. For experiments, 10-12 animals were transferred to 60 mm Petri dishes. RNAi was performed through dsRNA feeding (Rouhana et al., 2013), with the standard paradigm involving dsRNA feedings once every three days for 2-3 total feedings (after which the animals became paralyzed and unable to eat). For each feeding, 3µg dsRNA was mixed into ~30µL beef liver paste diluted 4:1 liver:salts. 1 µL green food dye was added to the mixture to color the intestines of animals that consumed the food. dsRNA targeting *green fluorescent protein* (*GFP)* was fed to negative control animals. After each feeding and periodically through experiments, the animals were washed and put into fresh dishes then the water was treated with 1:1000 gentamicin sulfate (50 mg/mL stock [Gemini Bio, West Sacramento, CA]). Images and videos of live animals were captured using either an iphone SE or a Zeiss Axiocam 506 Color camera mounted on a Zeiss Axio ZoomV.16 microscope with ZEN 2.3 (blue edition) software (ZEISS Microscopy, Jena, Germany). Videos were post-processed using iMovie software (Apple Inc., Cupertino, California).

In situ hybridization (ISH): Single-stranded, antisense riboprobes matching genes of interest were transcribed in the presence of a nucleotide derivative marked with Digoxygenin (Dig-11-UTP) (Sigma-Aldrich, St. Louis, MO) using standard molecular methods (Collins et al., 2010). ISH was performed as a modified version of the protocol previously described (King & Newmark,

2013). Instead of 7.5% N-Acetyl Cysteine (NAC), 10% NAC solution was used as a reducing agent and anti-mucolytic. Additionally, we substituted 2 µg/mL Proteinase K solution instead of 5 µg/mL to prevent animal degradation. Hybridized riboprobes were detected with anti-digoxigenin antibodies (1:2000 dilution) with Fab fragments (Sigma Aldrich, St. Louis, MO), which were conjugated with an alkaline phosphatase for subsequent development. Post-antibody washes used TNTx (0.1 M Tris pH 7.5, 0.15 M NaCl, and 0.3% Triton X-100) and were as follows: one 5-minute wash, one 10-minute wash, and six 20-minute washes. Colorimetric ISH staining development utilized nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3'-indolyphosphate (BCIP) (both Sigma-Aldrich, St. Louis, MO) in an alkaline buffer (100mM Tris pH 9.5, 100mM NaCl, 50 mM $MgCl_2$, 0.1% Tween-20, brought up in 10% Polyvinyl alcohol) to promote phosphatase activity. Animals were cleared and mounted in 80% glycerol. Stained animals were imaged using a Zeiss Axiocam 506 Color camera mounted on a Zeiss Axio Zoom. V16 microscope running ZEN 2.3 (blue edition) software (ZEISS Microscopy, Jena, Germany).

Image Quantification: To quantify brain regeneration images, we generated brain/body ratios as previously described (Roberts-Galbraith et al., 2016). Triplicate brain and body area measurements were completed manually with FIJI (Fiji is just ImageJ) software (Schindelin et al., 2012). In regeneration assays measuring blastema size, the same method was used to generate blastema/body ratios. Resulting data were graphed and statistically analyzed in Prism - GraphPad Version 9.0 software (GraphPad Software, San Diego, CA). Specific tests employed are included in the corresponding figure legends.

Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR): To quantify transcript abundance, we extracted RNA from control and *pc2(RNAi)* animals with Trizol Reagent

(Thermo Fisher Scientific, Waltham, MA) as described (Liu & Rink, 2018). We degraded any remaining DNA in the samples with RQ1 RNase-free DNase (Promega Corporation, Madison, WI) at 37°C for a 15 min incubation. The purified RNA was used to synthesize cDNA with an iScript kit (Bio-Rad, Hercules, CA). Reactions for RT-qPCR used SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA) in a QuantStudio® 3 real-time PCR system (Applied Biosystems, Foster City, CA). Primers targeting sequences ~100 bp were generated with Primer3 (Rozen & Skaletsky, n.d.), and *pc2* primers matched a region not included in the construct used for dsRNA. Amount of the control gene *β tubulin* (Collins et al., 2010) was used to normalize transcript abundance for genes of interest in RT-qPCR reactions. Triplicate biological replicates (12 animals each) were used for experiments, then RT-qPCR reactions for each biological replicate were also done in technical triplicate. Data were statistically analyzed and visualized using Prism - GraphPad Version 7.0 software (GraphPad Software, San Diego, CA). Specific tests employed are in the corresponding figure legends.

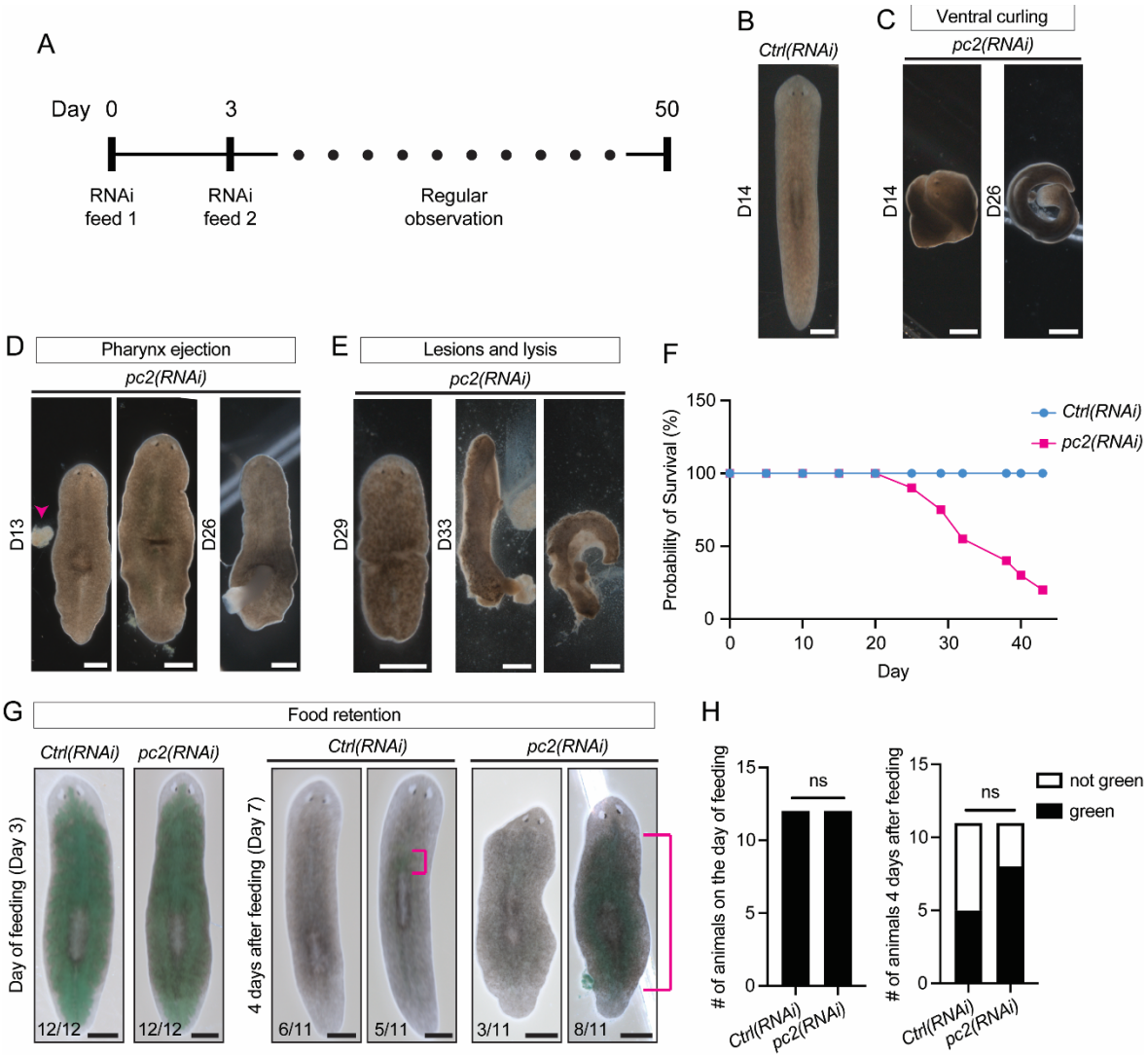


Figure 2.1 *pc2(RNAi)* produces a highly pleiotropic homeostatic phenotype, including reduced survival. (A) Schematic of the RNAi paradigm used for behavior investigation. **(B)** Image of a reference negative control animal. *pc2(RNAi)* animals displaying **(C)** the characteristic ventral curling posture, **(D)** ejection and detachment of the feeding organ (pharynx), and **(E)** tissue integrity issues including lesions and lysis. **(F)** Survival curve showing the relative percentage of alive animals throughout the course of RNAi. **(G)** Representative live images of control and *pc2(RNAi)* animals immediately after RNAi feeding and 4 days later. (**H**) Binary quantification of the proportion of animals per RNAi condition that had detectable green coloring in their intestine (categorized as "green") vs. animals with no detectable green in their intestines (categorized as "not green"). Data were statistically analyzed with Chi-square analysis. Magenta arrowhead indicates a detached pharynx in (D). Magenta brackets indicate regions of the intestine with detectable green in (G). Scale bars = 500 µm.

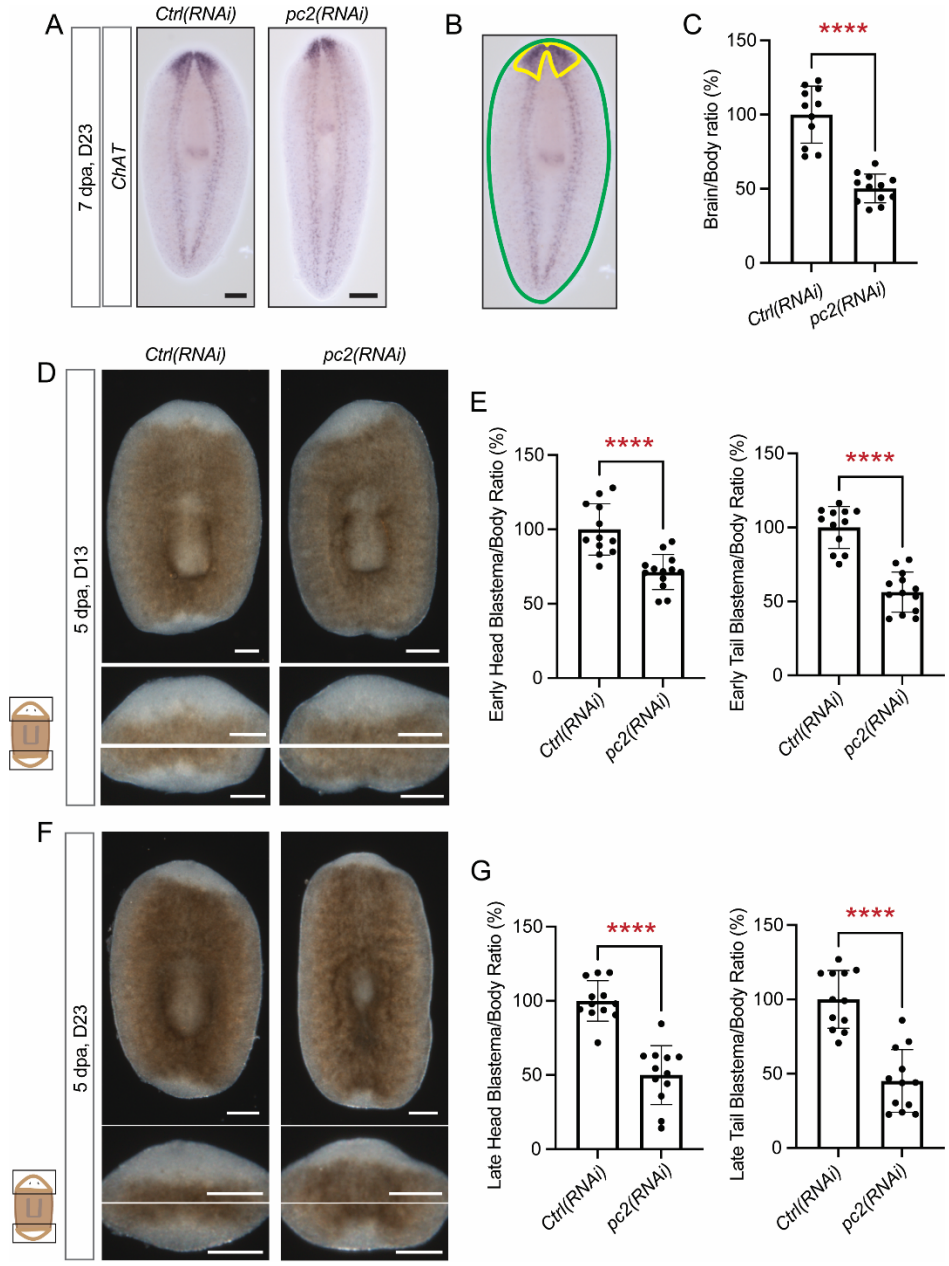


Figure 2.2 *pc2(RNAi)* significantly reduces whole-body regeneration. (A) Representative images of *ChAT* ISH (a marker for cholinergic neurons (K. Nishimura et al., 2010)), visualizing the central nervous system after 7 days of head regeneration. **(B)** Diagram showing the method of quantification for brain regeneration images. The brain (yellow) and body (green) are traced in triplicate to generate average brain/body ratios, as described previously (Roberts-Galbraith et al., 2016). **(C)** Bar graph displaying quantified brain/body ratios, with each individual ratio normalized to the average of the control ratios. Representative images of animals at **(D)** Day 13 and **(F)** Day 23, after 5 days of head and tail regeneration. Zoomed insets of the head and tail blastemas are included below the whole animal images. Bar graphs displaying head and tail blastema/body ratios at **(E)** Day 13 and **(G)** Day 23. Bar Graphs are displayed as mean with standard deviation. Data were statistically analyzed with unpaired t-test. **** = p value ≤ 0.0001. Scale bars = 200 µm.

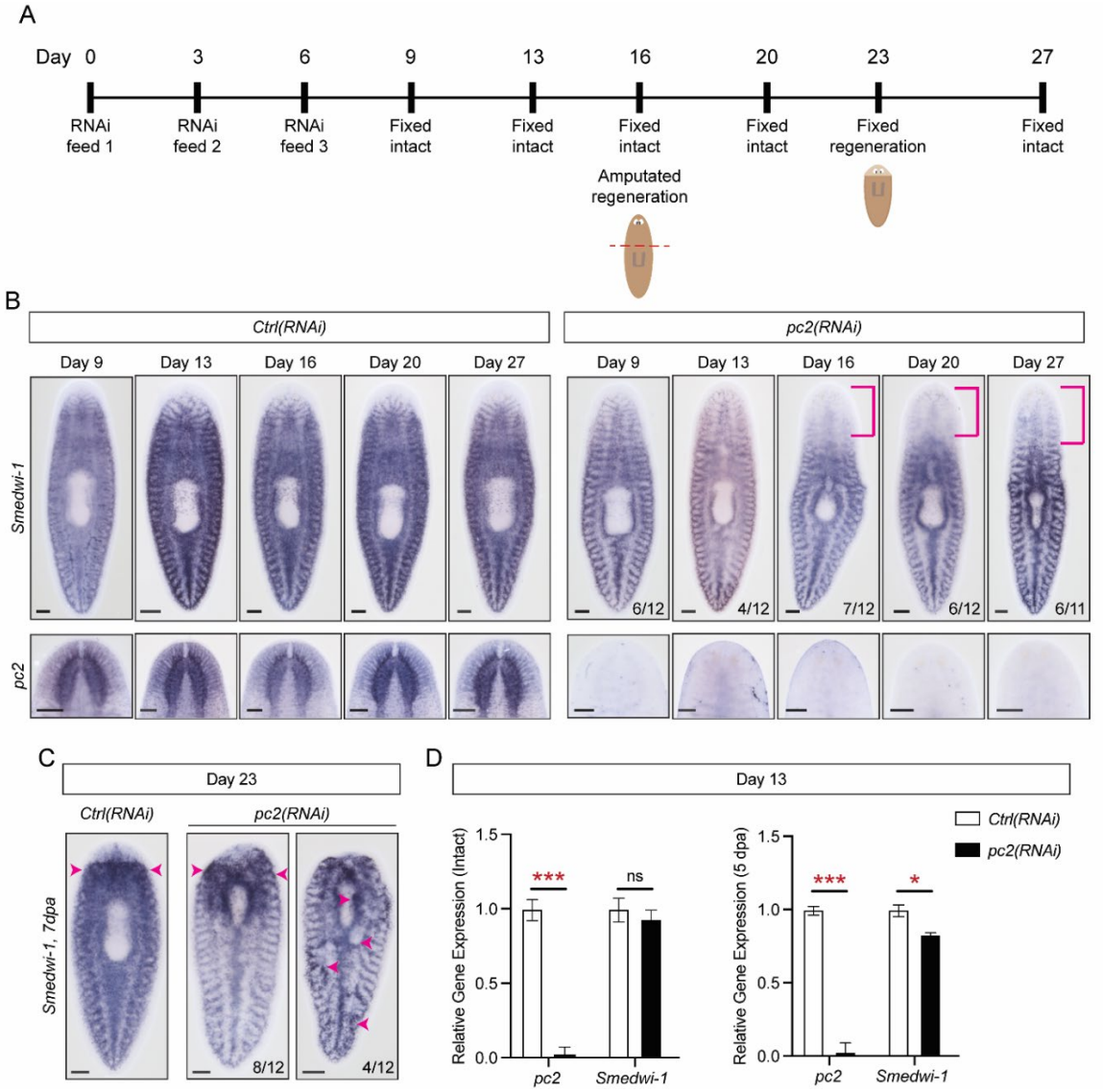


Figure 2.3 The *Smedwi-1*$^{+}$ stem cell population moderately decreases, but still superficially responds to injury, after *pc2(RNAi)*. **(A)** Schematic showing the RNAi paradigm to investigate the total stem cell population after knockdown of *pc2*. For intact stem cell maintenance, animals were fixed at Day 9, 13, 16, 20, and 27 (animals had begun lysing at the last fixation). To observe the stem cells in regeneration, heads were amputated on Day 16, then the trunks were fixed after one week of head regeneration. Representative images of *Smedwi-1* (Reddien, Oviedo, et al., 2005) ISH in **(B)** intact and **(C)** regenerating control and *pc2(RNAi)* animals at the specified timepoints of RNAi. Associated *pc2* ISH images are displayed below the *Smedwi-1* images to represent strength of *pc2* knockdown at each intact timepoint. **(D)** Relative expression levels of *pc2* and *Smedwi-1* in intact and 5 dpa animals, measured by RT-qPCR. Error bars represent SEM. Differences in sample means were statistically analyzed with unpaired t-tests. * = p value ≤ 0.05. *** = p value ≤ 0.0005. Magenta brackets indicate specifically reduced expression in the head region in (B). Magenta arrowheads indicate enriched *Smedwi-1* at wound sites in (C). More expanded examples of the data are found in Supplemental Figure S2.1. dpa = days post head amputation. Scale bars = 200 μm.

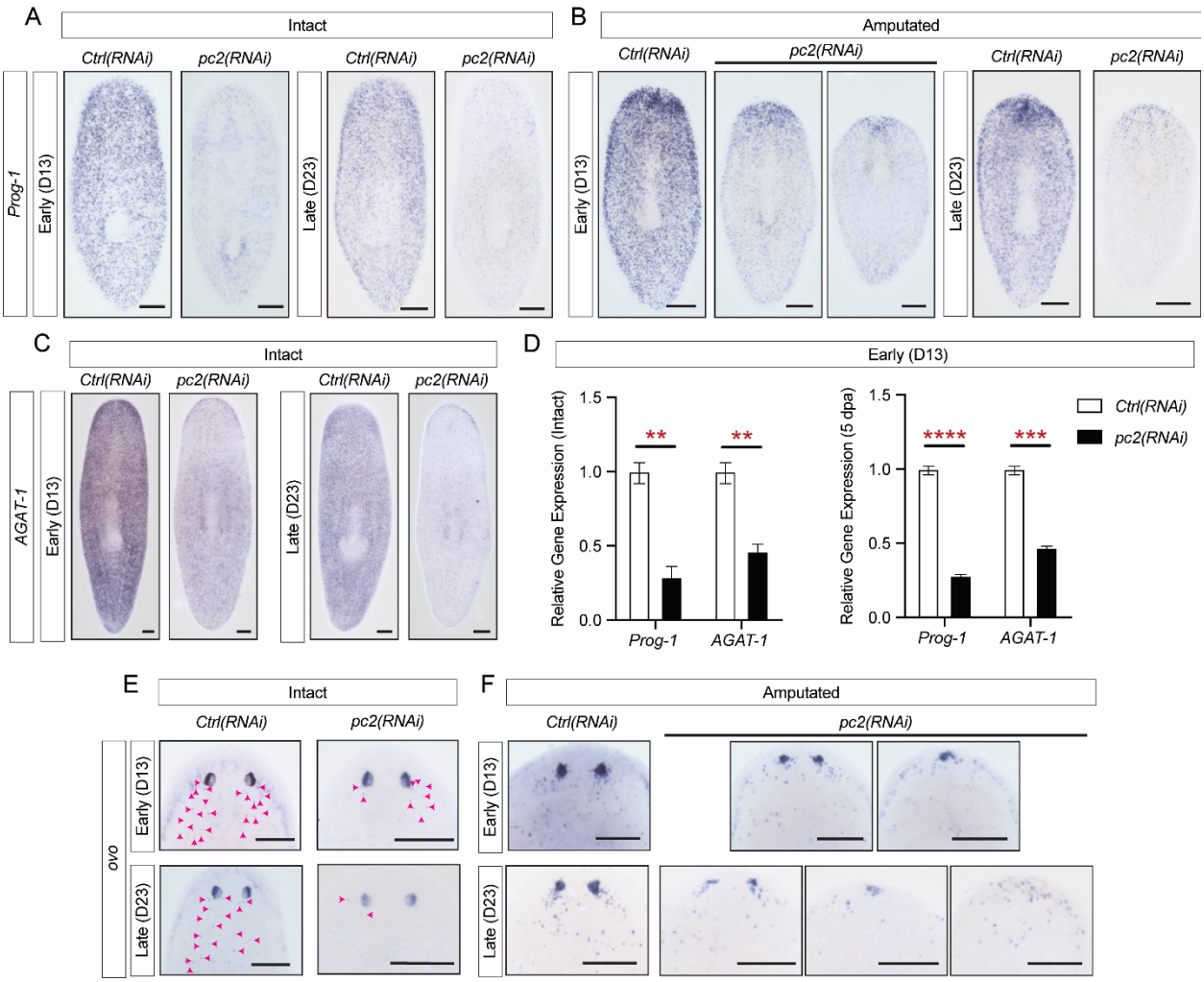


Figure 2.4 *pc2(RNAi)* reduces epidermal and eye progenitors. Representative images of ISH revealing markers expressed in epidermal progenitors (*Prog-1* and *AGAT-1* (Eisenhoffer et al., 2008; Tu et al., 2015)) in **(A and C)** intact animals and **(B)** animals 5 days post head amputation. **(D)** Relative expression levels of *Prog-1* and *AGAT-1* in intact and 5 dpa animals, measured by RT-qPCR using the same cDNAs as in Figure 2.3. Error bars represent SEM. Differences in sample means were statistically analyzed with unpaired t-tests. ** = p value ≤ 0.005. *** = p value ≤ 0.0005. **** = p value ≤ 0.0001. Representative images of ISH revealing an eye lineage marker (*ovo* (Lapan & Reddien, 2012)) in **(E)** intact animals and **(F)** animals 5 days post head amputation. Magenta arrowheads indicate *ovo*$^{+}$ trail progenitors. Scale bars = 200 µm.

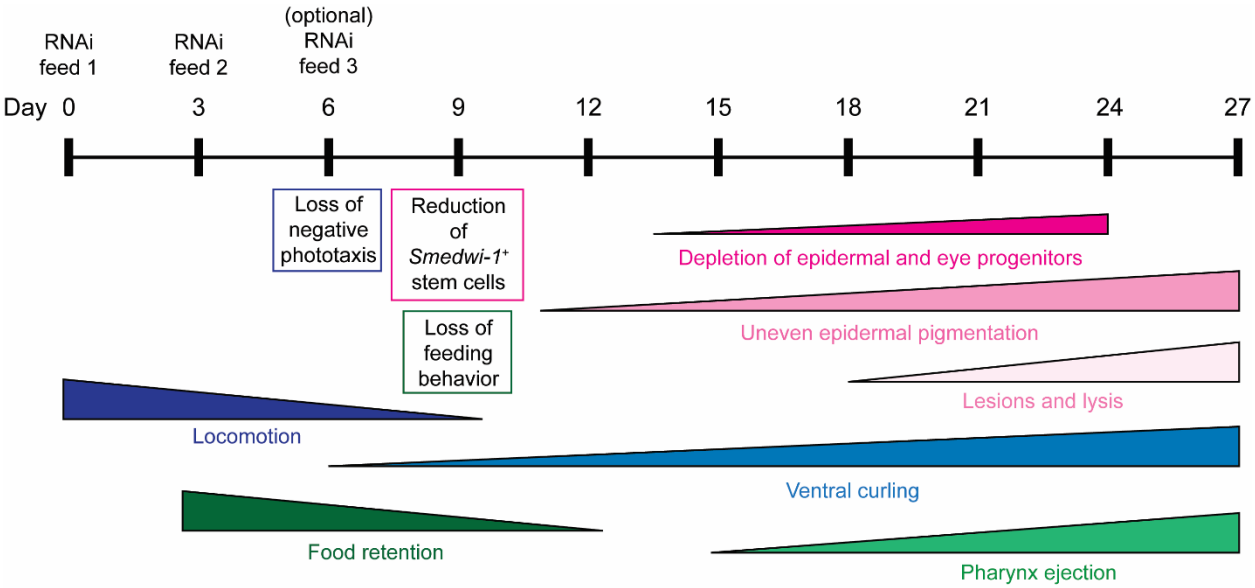
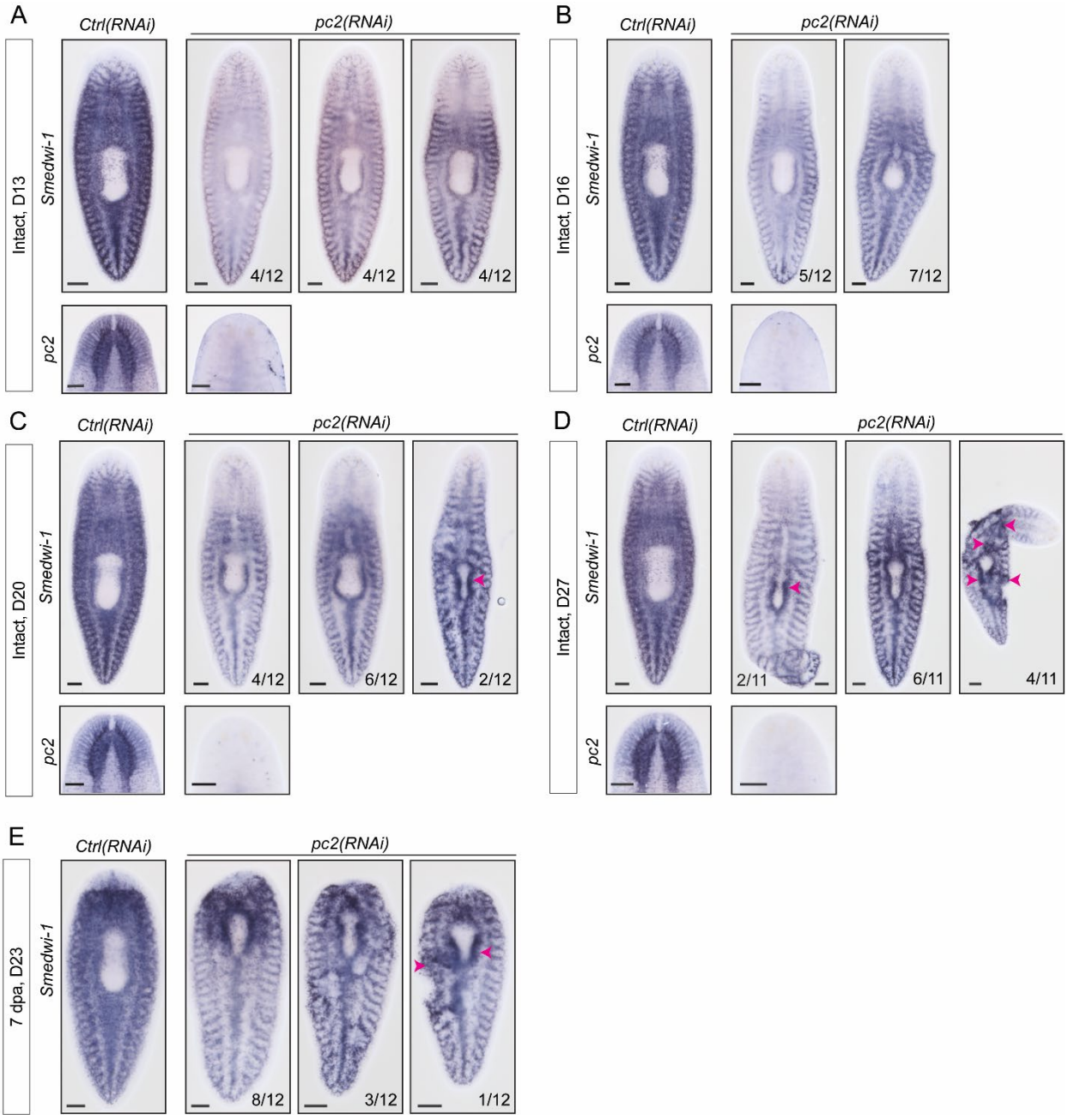
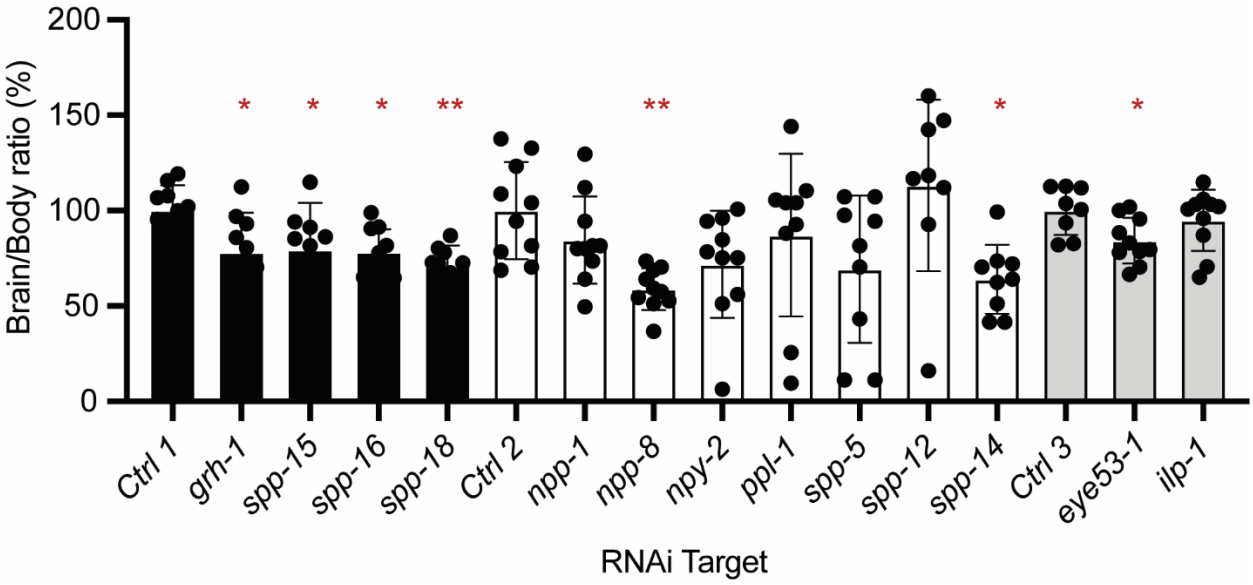


Figure 2.5 Estimated timeline of phenotype development after *pc2* knockdown. Temporal summary of sightings for each phenotype resulting after reduction of *pc2* function. Phenotype descriptions are color coded depending on broader function classification (blue for movement, green for feeding, and pink for stem cell regulation). Phenotypes that changed in penetrance over time are indicated with a horizontal bar of increasing/decreasing width. These datapoints were described from an individual experiment, and we note that the exact timing of phenotype manifestation can vary per experiment depending on the size of the animals and strength of knockdown. We also note that we saw equally robust knockdown strength when the third RNAi feeding was removed.



Supplemental Figure S2.1 Expanded *Smedwi-1* ISH timecourse. Representative images of ISH against *Smedwi-1* (Reddien, Oviedo, et al., 2005) in intact animals at Day **(A)** 13 **(B)** 16 **(C)** 20 and **(D)** 27 of *pc2* knockdown. Associated *pc2* ISH images are displayed below the *Smedwi-1* images to represent strength of *pc2* RNAi at each intact timepoint. **(E)** Representative images of ISH against *Smedwi-1* in regenerating animals, 7 days after head amputation at Day 23 of *pc2* knockdown. Magenta arrowheads are placed on images not included in Main Figure 2.3 and indicate regions of increased *Smedwi-1* staining at regions of wounding from pharynx ejection or epidermal lesions. Scale bars = 200 µm.



Supplemental Figure S2.2 Planarian neuropeptide prohormones show subtle roles in regeneration. Bar graph of quantified brain/body ratios (using the same quantification method in Main Figure 2.2) after RNAi of neuropeptide prohormones. Animals of RNAi treatments with a perceived reduction in regeneration were quantified, other *neuropeptide(RNAi)*s were not quantified. Bars are color coded to match samples and negative controls from the same experiment. Results are displayed as individual data points, with mean and standard deviation included. Data of the black and gray bar samples were statistically analyzed using one-way ANOVA, and white bar samples were analyzed using Brown-Forsythe and Welch ANOVA. * = p value ≤ 0.05. ** = p value ≤ 0.005.

Supplemental Movie S2.1 *pc2* knockdown quickly causes paralysis. 20 second video showing locomotion effects at 0, 6, 13, and 20 days of RNAi against *pc2*. Video playback is set to 20X speed.

CHAPTER 3

HETEROTRIMERIC G PROTEINS REGULATE PLANARIAN REGENERATION AND BEHAVIOR [1]

[1] Jenkins, J.E. and Roberts-Galbraith, R.H. 2023. *Genetics*. 223(4), iyad019
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Abstract

G protein-coupled receptors play broad roles in development and stem cell biology, but few roles for G protein-coupled receptor signaling in complex tissue regeneration have been uncovered. Planarian flatworms robustly regenerate all tissues and provide a model with which to explore potential functions for G protein-coupled receptor signaling in somatic regeneration and pluripotent stem cell biology. As a first step toward exploring G protein-coupled receptor function in planarians, we investigated downstream signal transducers that work with G protein-coupled receptors, called heterotrimeric G proteins. Here, we characterized the complete heterotrimeric G protein complement in *Schmidtea mediterranea* for the first time and found that seven heterotrimeric G protein subunits promote regeneration. We further characterized two subunits critical for regeneration, *Gαq1* and *Gβ1-4a*, finding that they promote the late phase of anterior polarity re-establishment, likely through anterior pole-produced Follistatin. Incidentally, we also found that five G protein subunits modulate planarian behavior. We further identified a putative serotonin receptor, *gcr052*, that we propose works with *Gαs2* and *Gβx2* in planarian locomotion, demonstrating the utility of our strategy for identifying relevant G protein-coupled receptors. Our work provides foundational insight into roles of heterotrimeric G proteins in planarian biology and serves as a useful springboard towards broadening our understanding of G protein-coupled receptor signaling in adult tissue regeneration.

Introduction

G protein-coupled receptors (GPCRs) represent one of the largest, most highly conserved, and functionally diverse families of cell surface receptors (Anantharaman et al., 2011; Krishnan et al., 2012; Langenhan et al., 2015). GPCRs also comprise ~30% of drug targets, due to their broad involvement in cell signaling (Garland, 2013; Hopkins & Groom, 2002; Wise et al., 2002). GPCRs possess structures that include seven transmembrane domains, extracellular domains for signal perception, and intracellular domains for interaction with signal transducers (Figure 3.1A) (Lagerström & Schiöth, 2008; K. L. Pierce et al., 2002). Canonically, activation of a GPCR initiates dissociation of a heterotrimeric G protein complex (Figure 3.1A) into an α subunit and a β/γ subcomplex, both of which impact cellular function (Oldham & Hamm, 2008; Smrcka, 2008).

Importantly, GPCR signaling regulates wound response throughout the animal kingdom in organisms that include nematodes, fruit flies, and mammals (Choi et al., 2015; Doze & Perez, 2013; Guo et al., 2019; Kiseleva et al., 2014; O'Connor et al., 2021; Ziegler et al., 2009; Zugasti et al., 2014). For example, the Protease Activated Receptor 1 (PAR1) GPCR promotes wound healing in murine skin by stimulating production of keratinocytes (Kiseleva et al., 2014). Downstream heterotrimeric G proteins also modulate regeneration. Gα class subunits from several families have been shown to promote *or* inhibit axon regeneration in vertebrates (Bates & Meyer, 1996; S. Li, Yang, et al., 2016) and *C. elegans* (Shimizu & Hisamoto, 2020). However, roles for GPCR pathways have not yet been explored thoroughly in organisms that complete robust, whole-body regeneration. Studying GPCR signaling in highly regenerative models could reveal new roles for these pathways in regeneration of complex tissues.

Freshwater flatworms called planarians provide an appealing model for investigation of mechanisms underlying robust regeneration. After nearly any injury, planarians produce a

blastema in which differentiating cells accumulate and mature to reconstruct missing structures (Baguna et al., 1989). Regeneration proceeds through key events that include wound detection (Wenemoser et al., 2012; Wurtzel et al., 2015), activation of pluripotent adult stem cells (Raz et al., 2021; Wagner et al., 2011), and polarity re-establishment (Reddien, 2018; Witchley et al., 2013). Through these processes, planarians regenerate all tissues and complex organs *de novo,* including a brain. How planarian cells detect injury, reinterpret polarity axes, and mount the correct regenerative response after injury remain key areas of investigation. Because regeneration requires multifaceted, fine-tuned coordination of cellular responses after injury and because GPCR signaling functions in diverse aspects of cell biology and healing in other animals, we hypothesized that GPCR pathways play key roles in planarian regeneration that have yet to be discovered.

Currently the genome of *Schmidtea mediterranea* is predicted to contain 566 GPCR-encoding genes (Saberi et al., 2016; Zamanian et al., 2011). Very few of these genes are functionally characterized, with the identified GPCRs promoting posterior identity, supporting planarian locomotion, coordinating germline differentiation and maintenance, facilitating reproductive system development and repair, or impacting eye regeneration (Lozano, 2015; Pascual-Carreras et al., 2021; Saberi et al., 2016; Zamanian, 2011). Characterization of planarian heterotrimeric G protein subunits is also limited. *gpas* is expressed in the brain branches and pharynx (Cebrià et al., 2002; Iglesias et al., 2011), while 4 other G protein subunit genes (*gna-q, gna-o, gnb,* and *gnc*) are highly expressed in photoreceptors (Lapan & Reddien, 2012). Work among planarian species assessed function for a handful of specific GPCR/G protein pathways (Chan et al., 2015, 2016; Zamanian, 2011; Zamanian et al., 2012). However, a comprehensive analysis of heterotrimeric G protein function could help indicate the extent to which GPCR

pathways regulate tissue regeneration and help uncover new roles for GPCR pathways in planarians.

As an essential first step toward pursuing our hypothesis that GPCR signaling promotes regeneration, we characterized heterotrimeric G proteins in the planarian *Schmidtea mediterranea*. In this work, we identified and characterized 38 predicted heterotrimeric G protein subunit-encoding genes, which includes highly conserved homologs of described vertebrate G protein families and divergent subunits. We show that 7 G protein subunit-encoding genes—*Gαs1, Gαs2, Gαq1, Gαq2, Gαo2*, *Gα-like6,* and *Gβ1-4a*—promote planarian regeneration. 2 of the identified genes, *Gαq1* and *Gβ1-4a*, are essential for promoting the late phase of anterior-posterior axis reestablishment, likely by influencing production of *follistatin*$^{+}$ anterior pole cells. We also show that 5 genes—*Gαs1, Gαs2, Gαq1, Gβ1-4a,* and *Gβx2*—are required for planarian movement. To illustrate the utility of our G protein-centered approach to identifying key GPCRs, we further identified a GPCR-encoding gene, *gcr052* (Saberi et al., 2016), as a potential partner of *Gαs2* and *Gβx2*. Taken together, our results reveal new functions for heterotrimeric G protein signaling in the highly regenerative planarian model. Our data further provide a much-needed starting point for identifying GPCRs with roles in regeneration.

Results

Identification of the planarian G protein subunit repertoire

To better understand G protein-coupled receptor signaling in planarians, we identified 38 G protein subunit homologs (26 Gα subunits, 7 Gβ subunits, and 5 Gγ subunits) in *S. mediterranea* transcriptomes based on the presence of key domains (Brandl et al., 2016; Rozanski et al., 2019) (Figure 3.1B, Sup Table S3.1). This list included all 5 previously identified planarian G protein

subunit genes (Cebrià et al., 2002; Iglesias et al., 2011; Lapan & Reddien, 2012). Both numbers and proportions of subunits are consistent with those found in other animals, including humans (Syrovatkina et al., 2016), *C. elegans* (Jansen et al., 1999), and *Drosophila* (Malpe et al., 2020). These results suggest that planarians utilize a typical repertoire of heterotrimeric G protein subunits.

We next classified planarian heterotrimeric subunit homologs into families using phylogenetic analysis. We classified 7 Gαi/o homologs, 4 Gαs homologs, 2 Gαq/11 homologs, 2 Gα12/13 homologs, 3 Gβ1-4 subgroup homologs, and 1 Gβ5 homolog (Figure 3.1B; Sup File S3.1; S3.2). 1 Gα homolog and 3 Gβ homologs contained all functional domains (Sup File S3.1) but did not cluster with a specific family (Sup File S3.2). We therefore designated these genes as "Gαx" or "Gβx" (Figure 3.1B). Additionally, 10 Gα class homologs retrieved in our search were truncated, preventing accurate classification (Sup File S3.3). We designated these genes "Gα-like" (Figure 3.1B). Lastly, due to the divergent nature of Gγ homologs, we were unable to classify them into families, so we designated them as "Gγ-like" (Figure 3.1B; Sup Files S3.1; S3.2). Our phylogenetic analysis suggests that the Gα class homolog *gpas* (Cebrià et al., 2002; Iglesias et al., 2011) was previously misclassified, and the name *Gαi2* more accurately represents this subunit's classification.

After defining the *S. mediterranea* heterotrimeric G protein complement, we next sought to characterize the expression patterns of these genes, to potentially provide insight into tissue-specific roles and possible heterotrimer combinations. We observed broad expression for 9 G protein subunit homologs (Figure 3.1C; Sup Figure S3.1). However, many subunits showed tissue-specific enrichment in the nervous system (Figure 3.1D; Sup Figure S3.1) or the intestine (Figure 3.1E). Lastly, we detected no expression pattern for 2 subunits through ISH (Sup Figure S3.1). In

addition to our observations, we determined that 32 of the 38 subunits are expressed within stem cells based on available transcriptomic resources (Fincher et al., 2018; Labbé et al., 2012; Plass et al., 2018; A. Zeng et al., 2018) (Sup Table S3.1). Our results suggest that *S. mediterranea* heterotrimeric G proteins likely function in many different tissue types, including stem cells and a diverse set of neural cell types.

Elucidation of roles for heterotrimeric G proteins in planarian behavior

We next performed, to our knowledge, the first comprehensive investigation into roles for planarian heterotrimeric G proteins. We completed an RNAi screen by feeding dsRNA and assessing behavioral and regenerative phenotypes (Figure 3.2A). We evaluated the penetrance of RNAi for a sampling of 5 genes in these screens using Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR) and observed knockdown efficiency ranging from 92 to 98% (Sup Figure S3.2A). Specificity of knockdown was examined with the two subunits with the most similar DNA sequence, *Gβ1-4a* and *Gβ1-4b* (58% identity, with stretches of up to 20 identical base pairs). We note that we did observe some degree of cross-reactivity for the dsRNA of these subunits at the level of RT-qPCR but did not see overlap in phenotypes after RNAi (Sup Figure S3.2B).

Though our ultimate focus was on regeneration, during our screens we incidentally observed that knockdown of 5 G protein subunit-encoding genes caused behavioral phenotypes. The strongest behavior we documented was reduced movement and paralysis in *Gαs1(RNAi)* animals, which was most clear when animals were placed on their dorsal sides (Sup Video S3.1). All control animals righted themselves after being placed on their dorsal side, taking an average of 27.35 seconds (Figure 3.2B-C). In contrast, 5 of 20 *Gαs1(RNAi)* animals failed to flip onto their

ventral side within 5 minutes. The remaining *Gαs1(RNAi)* animals took an average of 168 seconds to flip (Figure 3.2B-C). These results indicate that *Gαs1* is required for the righting response and gross movement in planarians. Although we saw reduced movement prior to amputation, the paralysis and flipping phenotypes were enhanced after amputation or long-term RNAi, which may suggest that the movement phenotype results from loss of a slow-turnover cell type (Sup Table S3.2).

Inhibition of any of 4 genes—*Gαs2*, *Gβx2, Gβ1-4a, and Gαq1*—resulted in decreased gliding movement, which leads to "inching" behavior (Glazer et al., 2010). The quickest effects were seen following RNAi of *Gαs2* or *Gβx2*. We first documented the inching after amputation (Fig 2D), but the phenotype was nearly identical in intact worms (Sup Figure S3.3A; Sup Table S3.2; Sup Videos S3.2-S3.3). Movement defects in *Gαs2(RNAi)* and *Gβx2(RNAi)* animals resulted in reduced distance traveled over time (Figure 3.2E; Sup Figure S3.3B). *Gαq1(RNAi)* animals also appeared to move slower than controls in short-term RNAi paradigms, and amputation marginally increased this phenotype (Sup Table S3.2). After long-term RNAi, *Gαq1(RNAi)* animals displayed labored movement (Sup Figure S3.3C; Sup Video S3.4). *Gβ1-4a(RNAi)* animals alternated between inching and gliding, most perceptibly after amputation or long-term RNAi (Sup Figure S3.3C; Sup Table S3.2; Sup Video S3.4). An assay documenting negative phototaxis in these animals also demonstrated slow movement to a dark area of a dish, with the strongest effects resulting from perturbation of *Gβx2* (Sup Figure S3.3D). Finally, we also note that *Gαq1(RNAi)*, *Gβ1-4a(RNAi)*, *Gαs2(RNAi)*, and *Gβx2(RNAi)* animals spent a noticeable amount of time raising and turning their heads, which may be indicative of additional sensory or movement dysfunction.

Locomotion of *Gαs2(RNAi)* and *Gβx2(RNAi)* animals was indistinguishable, which led us to hypothesize that *Gαs2* and *Gβx2* might be operating in the same cells. We noted that *Gαs2* is

expressed in a head margin pattern consistent with putative peripheral sensory neurons (K. G. Ross et al., 2018) (Figure 3.1D, Figure 3.2F). *Gβx2* is expressed in a similar pattern, but also in cells of the intestine (Figure 3.1E, Figure 3.2F). We further validated the coexpression of *Gαs2* and *Gβx2* in cells at the tip of the head through fluorescent ISH (FISH) (Figure 3.2G). The colocalization of *Gαs2* and *Gβx2* transcripts supports the hypothesis that they could potentially work in the same cells.

Our goal in focusing on heterotrimeric G proteins was to uncover roles for GPCRs. As proof-of-principle, we next sought to identify the GPCR that works with *Gαs2* and *Gβx2*. We identified and screened 8 GPCR-encoding genes enriched in the same cell clusters as *Gαs2* or *Gβx2* in available single cell sequencing datasets (Fincher et al., 2018; Plass et al., 2018) (Figure 3.2H; Sup Table S3.3). Using this method, we identified a putative serotonin receptor, *gcr052* (the homolog of *DtSER-1* (Zamanian et al., 2012), *S7.1R* (Chan et al., 2015, 2016), and *Smed-ser85* (Zamanian, 2011) in planarian literature), for which knockdown caused inching indistinguishable from that displayed by *Gαs2(RNAi)* and *Gβx2(RNAi)* animals (Figure 3.2I-J; Sup Videos S3.2-S3.3; Sup Figure S3.3A-B).

gcr052 is expressed broadly throughout the CNS (Figure 3.2K). Using FISH, we detected coexpression of *gcr052* with *Gαs2* and *Gβx2* in many cells, including clusters at the anterior (Figure 3.2L). While targeting *Gαs2*, *Gβx2*, and/or *gcr052* in combination did not noticeably exacerbate the phenotype (Sup Figure S3.3E-F) (Sup Video S3.5), some GPCR research indicates that loss of one component can prevent the assembly of the receptor/trimer complex (Dupré et al., 2009; Smrcka, 2008). We thus hypothesize that Gαs2 and Gβx2 act downstream of the GCR052 receptor to support gliding motion.

In summary, our results show that 5 planarian heterotrimeric G proteins are essential for normal animal movement. Additionally, our identification of GCR052 provides proof-of-principle that the heterotrimeric G proteins characterized in this work can accelerate planarian GPCR research.

Planarian heterotrimeric G proteins function in regeneration

Over the course of our functional analysis, we knocked down each G protein subunit and assessed the degree of brain regeneration after amputation (Figure 3.3A-E; Sup Table S3.4), because brain size is a highly robust way of detecting regeneration defects (Roberts-Galbraith et al., 2016). After screening 37 of the 38 predicted subunit genes, we found 7 genes for which RNAi caused significant reduction in brain regeneration (Figure 3.3B-E). Of these candidates, RNAi targeting *Gαs1, Gαs2, Gαo2, Gαq2,* or *Gα-like6* produced modest effects (Figure 3.3B-E). RNAi targeting *Gαq1* or *Gβ1-4a* caused a strong reduction of brain regeneration (Figure 3.3B-E). Of these genes, knockdown of three candidate subunits, *Gαs1, Gαq1*, or *Gβ1-4a*, also caused reduction in tail regeneration (Sup Figure S3.4A-C). These results show that multiple Gα class and one Gβ class subunit play roles in planarian regeneration.

Interestingly, we detected no significant regeneration phenotypes after RNAi targeting individual Gγ subunit genes (Figure 3.3E). To account for potential functional redundancy among Gγ subunits, we observed brain regeneration after combinatorial RNAi targeting all identified Gγ class subunit genes (Sup Figure S3.4D). Indeed, targeting these genes concurrently produced a significant reduction in regenerated brain size (Sup Figure S3.4D). Furthermore, RNAi of *Gγ-like1*, *Gγ-like4*, and *Gγ-like5* together caused a severe reduction in brain regeneration (Figure 3.3F-

G). These results indicate that Gγ subunits are likely functionally redundant and have cooperative roles in regeneration.

Due to the strong roles for *Gαq1* and *Gβ1-4a* in regeneration, we sought to further identify the cell types that express *Gαq1* and *Gβ1-4a* and determine whether these genes are expressed in overlapping cells. Based on the colorimetric ISH expression patterns, the transcripts of *Gαq1* and *Gβ1-4a* appear to both be particularly enriched in the central nervous system and eyespots (Figure 3.1D). Additionally, based on published sequencing datasets, these genes are also detected in muscle and at low levels in stem cells (Sup Table S3.1). We confirmed expression of *Gαq1* and *Gβ1-4a* in the brain branches and eyespots through FISH (Figure 3.3H). Additionally, due to the highly enriched expression in the eyespots, we took a closer look at these cells and saw that *Gαq1* and *Gβ1-4a* transcripts indeed colocalize (Figure 3.3H). Although we require biochemical analyses to prove functional pairing, these results show that *Gαq1* and *Gβ1-4a* are expressed in overlapping cell populations.

Additionally, we considered that *Gαq1* or *Gβ1-4a* could impact regeneration by affecting the timing of tissue regrowth. To determine whether the phenotypes we saw were due to delays in regeneration, we observed brain regeneration at 14 days post-amputation (dpa) in knockdown animals. *Gαq1(RNAi)* and *Gβ1-4a(RNAi)* animals showed partial recovery of regenerated brain size with additional time (Sup Figure S3.4E). However, we note that the distribution of brain regeneration is not the same in *Gαq1(RNAi)* and *Gβ1-4a(RNAi)* animals. A small proportion of *Gαq1(RNAi)* animals failed to initiate any regenerative response, and while the rest of the animals regenerated the expected brain size, brain morphology appeared more collapsed toward the midline relative to control brains (Sup Figure S3.4E). In contrast, all *Gβ1-4a(RNAi)* animals regenerated a reduced, but otherwise normal, bilobed brain structure (Sup Figure S3.4E). Our results at 14 dpa

support the notion that *Gβ1-4a* promotes the speed of brain regeneration, whereas *Gαq1* shows a more complex role in brain regeneration including initiation of regenerative response and proper morphology of the mature CNS.

Through these studies, we find that multiple heterotrimeric G proteins promote regeneration, with *Gαq1* and *Gβ1-4a* playing especially critical roles. Further, although we saw overlap in roles for regeneration and behavior after perturbation of some genes (*Gαs1, Gαs2, Gαq1* and *Gβ1-4a*), some genes specifically impact regeneration (*Gαo2, Gαq2, and Gα-like6*) or behavior (*Gβx2)* (Figure 3.2, 3.3, and Sup Table S3.2).

Gβ1-4a promotes mitotic response after amputation and long-term survival

Our next goal was to understand why *Gαq1* and *Gβ1-4a* are critical for regeneration. We first considered whether *Gαq1* and *Gβ1-4a* affect initial response to wounding. We examined the expression of *Gαq1* and *Gβ1-4a* after injury. Indeed, we found that *Gαq1* and *Gβ1-4a* are upregulated at the amputation site at both 6 hours post-amputation (hpa) and 3 dpa (Sup Figure S3.5A). Planarians initiate a molecular wound response program during this time that includes upregulation of genes like *follistatin, jun-1, inhibin,* and *wnt1* (Wenemoser et al., 2012; Wurtzel et al., 2015). We determined that *Gαq1(RNAi)* and *Gβ1-4a(RNAi)* animals expressed wound-induced genes normally at 6 hpa (Sup Figure S3.5B-C). The only significant difference we observed was a mild increase in *follistatin* transcripts in *Gαq1(RNAi)* animals, detected through RT-qPCR (Sup Figure S3.5C). These results suggest that while *Gαq1* and *Gβ1-4a* are upregulated during regeneration, they are dispensable for early injury response.

Next, we investigated whether the regeneration defects observed after *Gαq1(RNAi)* or *Gβ1-4a(RNAi)* result from perturbed stem cell maintenance or differentiation. We looked at expression

of a stem cell marker (*Smedwi-1* (Reddien, Oviedo, et al., 2005)) and epidermal progenitor markers (*prog-1* and *AGAT-1* (Eisenhoffer et al., 2008; Tu et al., 2015)) after head regeneration. We did not see depletion of stem cell or progenitor markers through ISH (Figure 3.4A-C). However, transcript abundance of *Smedwi-1* showed modest or mild reduction through RT-qPCR after RNAi of *Gαq1* or *Gβ1-4a*, respectively (Figure 3.4D).

We also examined mitotic activity of stem cells in *Gαq1(RNAi)* and *Gβ1-4a(RNAi)* animals. Planarian stem cells divide at a regular rate in intact animals, and after amputation two primary waves of mitosis occur: one at ~6 hpa that is body-wide and one at ~48 hpa that is localized to the amputation site (Baguna et al., 1989; Wenemoser & Reddien, 2010). To investigate the rates of stem cell division in *Gαq1(RNAi)* and *Gβ1-4a(RNAi)* animals, we performed an antibody stain for a histone modification associated with mitosis (phospho-histone-H3-Ser10) (Hendzel et al., 1997; Newmark & Sánchez Alvarado, 2000). We detected a significant decrease in proliferative cells in *Gβ1-4a(RNAi)* animals at 48 hpa, but otherwise the mitotic activity in *Gαq1(RNAi)* and *Gβ1-4a(RNAi)* animals appeared comparable to controls (Figure 3.4E-F). We also found that *Gβ1-4a* is not strongly co-expressed with *Smedwi-1* in intact or regenerating animals, but we did see *Gβ1-4a*$^{+}$ and *Smedwi-1*$^{+}$ cells near one another in regenerating tissue (Sup Figure S3.6A-B), suggesting that any effect of Gβ1-4a signaling on stem cells might be non-cell autonomous. We conclude that *Gαq1* and *Gβ1-4a* may play subtle roles in stem cell maintenance, differentiation, or division, but that these defects are likely insufficient to explain the severe regenerative phenotypes seen in RNAi animals.

Finally, we asked whether the roles of *Gαq1* and *Gβ1-4a* were exclusive to regeneration or whether either gene also functioned during homeostasis. We performed longer term RNAi and measured animal growth and survival over time (Sup Figure S3.6C). *Gβ1-4a(RNAi)* animals

ceased growth after day 21 and we halted the growth measurements of *Gαq1(RNAi)* animals at that time point because they began to fission (Sup Figure S3.6D). Long-term RNAi targeting *Gβ1-4a* was lethal, with animals showing head lysis and dying near day 40 (Sup Figure S3.6E-F). We also noted postural changes without change in viability in *Gαq1(RNAi)* animals, suggesting that *Gαq1* promotes head regeneration but is not required for head maintenance (Sup Figure S3.6E-F). Intriguingly, *Smedwi-1*$^{+}$ cells remained abundant at later timepoints of RNAi (Sup Figure S3.6G-H), suggesting that the stem cells are maintained even as *Gβ1-4a(RNAi)* animals begin to lyse.

To summarize, our data indicate that *Gαq1* is essential for regeneration but not strictly required for wound response induction, mitosis, or stem cell maintenance. Long-term inhibition of *Gβ1-4a* is lethal, but other than modestly promoting the late wave of mitotic response after amputation, we did not detect strong impacts of *Gβ1-4a* perturbation on stem cell regulation. These results may suggest that *Gβ1-4a* supports viability through additional means. Importantly, several results indicate key differences in function for *Gαq1* and *Gβ1-4a,* despite the two genes having similarly important roles in regeneration. Because the impacts we saw for both subunits in stem cell biology were mild, we sought to examine influences of *Gαq1* and *Gβ1-4a* on other physiological processes that contribute to regeneration.

Gαq1 and Gβ1-4a support the late phase of anterior-posterior polarity reestablishment

Early in planarian regeneration, tissues reorganize to pattern body axes using conserved developmental signaling pathways (e.g., Wnt/β-catenin (Gurley et al., 2008; Iglesias et al., 2008; Petersen & Reddien, 2008)). We next considered whether regeneration failures after *Gαq1(RNAi)* or *Gβ1-4a(RNAi)* occur due to abnormal body polarity. During the early phase of polarity reestablishment, the remaining tissue determines which end of the animal is anterior and which is

posterior (Figure 3.5A) (reviewed in (Owlarn & Bartscherer, 2016)). To determine whether *Gαq1(RNAi)* and *Gβ1-4a(RNAi)* animals correctly complete the initial anterior-posterior decision, we examined *notum* and *wnt1* expression 18 hpa (Figure 3.5B). *notum* expression resumed normally at the anterior in *Gαq1(RNAi)* and *Gβ1-4a(RNAi)* animals (Figure 3.5C). *Gαq1(RNAi)* animals also expressed bipolar *wnt1*, but over half of *Gβ1-4a(RNAi)* animals displayed posterior-enriched expression of *wnt1* (Figure 3.5C). These results suggest that *Gαq1* is not involved in early polarity decisions, but *Gβ1-4a* might affect anterior wound-induced *wnt1* expression.

After re-initiation of axial polarity, anterior and posterior poles form at the distal ends of the planarian body (Figure 3.5A). To determine whether pole formation was disrupted in *Gαq1(RNAi)* and *Gβ1-4a(RNAi)* animals, we analyzed *notum* and *wnt1* expression at 3 dpa (Figure 3.5B). 50% of *Gαq1(RNAi)* animals and all *Gβ1-4a(RNAi)* animals lacked anterior *notum* expression (Figure 3.5D). Additionally, *Gαq1(RNAi)* animals displayed an asymmetric *wnt1* pattern or absent *wnt1* in the posterior domain, and *Gβ1-4a(RNAi)* animals regenerated with a broadened and/or asymmetrical domain of *wnt1* expression (Figure 3.5D). Our results indicate that *Gαq1* and *Gβ1-4a* impact pole formation at both anterior and posterior ends of the animal.

During the late phase of polarity reestablishment, anterior and posterior poles further coalesce and mature (Figure 3.5A). To investigate whether *Gαq1* and *Gβ1-4a* support the maturation of the key polarity domains, we examined expression of *notum* and another posterior marker, *wnt11-2* (Gurley et al., 2008), in knockdown animals at 7 dpa (Figure 3.5B). We observed a lack of *notum* staining at the anterior pole in ~25% of *Gαq1(RNAi)* animals (Figure 3.5E), and we confirmed this pattern by using a second pole marker, *sFRP-1* (Gurley et al., 2008; Petersen & Reddien, 2008) (Sup Figure S3.7A). Posterior pole maturation was also disrupted in ~33% of *Gαq1(RNAi)* animals, which displayed broader and more diffuse *wnt11-2* expression (Figure

3.5E). All *Gβ1-4a(RNAi)* animals recovered anterior *notum* expression by 7 dpa, although the domains appeared less consolidated than in control animals, which could signify slower maturation (Figure 3.5E, Sup Figure S3.7A). The formation of an anterior pole domain at a slower pace is consistent with our previous results suggesting that *Gβ1-4a* largely affects the speed of head regeneration rather than ultimate success (Sup Figure S3.4E). Strikingly, most *Gβ1-4a(RNAi)* animals expressed posterior *wnt11-2* asymmetrically, with staining on either side of the animal's midline (Figure 3.5E). Notched tails were also commonly seen after *Gβ1-4a(RNAi)* (Sup Figure S3.4C), though our data did not support the presence of a secondary anterior domain, as has been seen after other RNAi treatments (Sup Figure S3.7B-C) (Cloutier et al., 2021).

Taken together, we conclude that *Gβ1-4a* supports the speed of anterior pole reestablishment and promotes proper midline placement of the posterior pole. Our data also support a role for *Gαq1* in promoting robust anterior pole formation, though this phenotype was limited to a minority of animals. Interestingly, while both *Gαq1* and *Gβ1-4a* function during regeneration and influence anteroposterior polarity, the precise phenotypes seen after RNAi of *Gαq1* and *Gβ1-4a* are distinct.

Gαq1 promotes head regeneration through production and activity of follistatin^{+} anterior pole cells

The anterior pole is established and maintained through two mutually dependent signaling proteins, Notum and Follistatin (Gaviño et al., 2013; Petersen & Reddien, 2011; Roberts-Galbraith & Newmark, 2013). *notum* and *follistatin* encode key extracellular inhibitors of posterior-promoting Wnt and Activin pathways, respectively (Kakugawa et al., 2015; Nakamura et al., 1990). We noted several similarities between the phenotypes caused by *follistatin(RNAi)* and those

caused by *Gαq1(RNAi)* or *Gβ1-4a(RNAi)*. Similarities include: strong impacts on head and brain regeneration; reduced or delayed *notum* expression in the regenerating head; unaffected expression of early wound response genes; and subtle impacts on stem cells (Gaviño et al., 2013; Roberts-Galbraith & Newmark, 2013; Tewari et al., 2018).

Based on phenotypic similarities, we sought to determine whether RNAi of *Gαq1* or *Gβ1-4a* impacts *follistatin* expression during regeneration (Figure 3.6A). We detected no change in *follistatin* expression at 12 hpa after perturbation of *Gαq1* or *Gβ1-4a* (Figure 3.6B). We similarly saw equivalent or higher *follistatin* transcripts 6 hpa through RT-qPCR (Sup Figure S3.5C). These results indicate that regeneration failure in *Gαq1(RNAi)* and *Gβ1-4a(RNAi)* animals is not correlated with a reduction of wound-induced *follistatin* expression.

To determine whether *Gαq1* and *Gβ1-4a* support *follistatin* expression in the anterior pole, we examined *follistatin* expression during pole formation (Figure 3.6A). At 3 dpa, most *Gαq1(RNAi)* and all *Gβ1-4a(RNAi)* animals had absent *follistatin* expression at the anterior pole (Figure 3.6C). At 7 dpa, ~36% of *Gαq1(RNAi)* animals still lacked *follistatin*$^{+}$ anterior pole cells (Figure 3.6D). However, all *Gβ1-4a(RNAi)* animals established *follistatin*$^{+}$ pole cells by 7 dpa (Figure 3.6D), reflecting a similar delay in anterior pole formation seen with other markers (Figure 3.5 and Sup Figure S3.7).

Both *notum* and *follistatin* expression in anterior pole cell progenitors relies on a key transcription factor, encoded by *foxD* (Roberts-Galbraith & Newmark, 2013; Scimone, Lapan, et al., 2014; Vogg et al., 2014). To investigate whether Gαq1 could modulate *follistatin* through FoxD, we examined *foxD* expression following *Gαq1* knockdown. Indeed, anterior *foxD* expression was absent in 50% of *Gαq1(RNAi)* animals at 3 dpa and ~36% of animals at 7 dpa (Sup Figure S3.7D-E). We confirmed this significant reduction of *foxD* expression through RT-qPCR

(Sup Figure S3.7F). *Gβ1-4a(RNAi)* animals displayed absent *foxD* anterior pole expression 3 dpa and most animals resumed *foxD* expression by 7 dpa (Sup Figure S3.7D-E). Thus, our data suggest that impacts on *follistatin* expression could be mediated by *foxD.* Alternatively, the lack of these anterior pole markers could result from a failure to produce and/or specify anterior pole progenitors, resulting in fewer pole cells.

Previous work characterizing the Follistatin/Activin and Notum/Wnt pathways determined that reduction of the antagonistic posterior-promoting ligands rescued head regeneration (Gaviño et al., 2013; Petersen & Reddien, 2011; Roberts-Galbraith & Newmark, 2013). The similarities between *Gαq1(RNAi)* and *follistatin(RNAi)* phenotypes and the impact of *Gαq1(RNAi)* on *follistatin* expression led us to hypothesize that *Gαq1* functions to promote Follistatin signaling from the pole. To test this hypothesis, we performed RNAi targeting *Gαq1* with *activin(RNAi)*, *wnt1(RNAi)*, or *bmp4(RNAi)* (a TGF-beta ligand that impacts dorsoventral polarity) (Gaviño et al., 2013; Reddien et al., 2007; Roberts-Galbraith & Newmark, 2013; Tewari et al., 2018). *activin(RNAi)* significantly rescued *Gαq1(RNAi)*-induced brain regeneration defects (Figure 3.6E-F; Sup Figure S3.8A-E). *wnt1(RNAi)* also partially rescued *Gαq1(RNAi)* (Figure 3.6E-F; Sup Figure S3.8A-E). As expected, *bmp4(RNAi)* failed to rescue regeneration in *Gαq1(RNAi)* animals (Sup Figure S3.8A-B). Our results were also confirmed in a second experiment that showed equally strong *Gαq1* knockdown efficiency in double RNAi conditions (Sup Figure S3.8C-E). Incidentally, though we primarily focused on a functional connection between Gαq1 and Follistatin, we also found that *activin* inhibition modestly restored brain regeneration in *Gβ1-4a(RNAi)* animals (Sup Figure S3.8F-G). We conclude that Gαq1 function specifically supports Follistatin signaling from the anterior pole during head regeneration.

Discussion

The vast number of GPCRs hinders progress in understanding the function of this fascinating receptor family in planarian regeneration and stem cell biology. In this work, we take a step toward investigating planarian GPCR signaling by identifying and functionally characterizing the heterotrimeric G protein subunit complement in behavior and regeneration. We characterized 38 heterotrimeric G protein homologs, of which 23 were conserved enough to categorize. Through our functional screens, we identified 5 subunit genes required for proper planarian movement (Figure 3.7). Using these data as a starting point and relying on single cell sequencing data, we identified a putative serotonin receptor (GCR052) that could function with Gαs2 and Gβx2 in movement. Through our brain regeneration screen, we identified 7 genes with roles in regeneration, with *Gαq1* and *Gβ1-4a* having especially significant effects (Figure 3.7). We determined that *Gαq1* and *Gβ1-4a* promote successful regeneration and establishment speed of the anterior pole, respectively. Our findings indicate new pathways active in planarian regeneration and behavior and support the hypothesis that GPCR signaling is likely to be involved in key molecular events that drive and coordinate planarian regeneration.

Due to the functionally overlapping, but nonidentical effects of *Gαq1* or *Gβ1-4a*, we reason that these subunits could be activated downstream of a common GPCR but stimulate different downstream pathways to support tissue regeneration (Brock et al., 2003; Inglese et al., 1995; Tang & Gilman, 1991). This model is supported by coexpression of *Gαq1* and *Gβ1-4a* through FISH and in single cell sequencing data (Fincher et al., 2018) (Figure 3.3H; Sup Tables S3.1 and S3.3). Additionally, we show through RT-qPCR that while targeting *Gβ1-4a* does not impact expression of *Gαq1,* expression of *Gβ1-4a* is significantly reduced in *Gαq1(RNAi)* animals (Sup Figure S3.2C). We also demonstrated phenotypes for *Gβ1-4a(RNAi)* but not *Gβ1-4b(RNAi)*, despite some

cross-targeting of dsRNA (Sup Figure S3.2). This indicates that the relationships between G protein subunits could involve additional redundancy or regulatory elements.

Gαq1 provides a putative connection between planarian GPCR signaling and defined polarity axes

The phenotypic similarities between *Gαq1(RNAi)* and *follistatin(RNAi)* animals and the ability to rescue phenotypes via *activin* double knockdown indicate that the Gαq1 protein likely cooperates with Follistatin during regeneration. Our results suggest that Gαq1 could function upstream to promote *follistatin* expression at the anterior pole. How Gαq1 promotes *follistatin* expression, and whether this results from a failure to specify early anterior pole progenitors or a failure to turn on key gene networks in differentiating anterior pole cells, remains to be determined. Alternatively, Activin signals belong to the transforming growth factor-β (TGF-β) family, and recent work describes the potential for GPCRs to modulate TGF-β pathways through transactivation (Burch et al., 2012; Hinck et al., 2016; A. E. Schafer & Blaxall, 2017). Therefore, Gαq1 could potentially influence the Activin/Follistatin axis through a noncanonical mechanism. Further exploring relationships between *Gαq1* and pathway components will help define the nature of the Gαq1/Follistatin cooperation.

Additionally, because *Gαq1(RNAi)* animals displayed functional wound-induced *follistatin* expression, our results also support the notion that *follistatin* expression from the anterior pole is specifically needed to drive successful head regeneration (Gaviño et al., 2013; Roberts-Galbraith & Newmark, 2013; Tewari et al., 2018). Therefore, results from future work with *Gαq1* could inform the nature of anterior identity establishment. Potential roles for *Gαq1* (and GPCRs) in

modulating the Activin pathway and promoting polarity reestablishment will require further investigation.

The relationship between Gαs2, Gβx2, and gcr052 suggests complexity of serotonin's role in planarian locomotion

In addition to characterizing planarian heterotrimeric G proteins with roles in regeneration, this work also contributes to knowledge of mechanisms governing planarian movement. The current model for planarian gliding is that serotonergic neurons directly innervate ventral epidermal cells and coordinate the beating of motile cilia (Currie & Pearson, 2013; März et al., 2013). Furthermore, experiments with mianserin, a pharmacological inhibitor of serotonin receptors, also implicated GPCRs in cilia coordination in *S. mediterranea* (Currie & Pearson, 2013; Kuang et al., 2002). In this work, we identify 2 G protein subunits (*Gαs2* and *Gβx2*) that similarly affect locomotion in *S. mediterranea* (Figure 2). We further identified *gcr052* (Saberi et al., 2016), which encodes a putative serotonin GPCR, as a potential specific mediator of gliding motion. Homologs of the receptor *gcr052* have well documented roles in movement among planarian species, with coupling validation to Gαs protein family subunits (receptor referred to in literature as *DtSER-1* (Zamanian et al., 2012), *S7.1R* (Chan et al., 2015, 2016), and *Smed-ser85* (Zamanian, 2011)). We identified the receptor through our study of G proteins, displaying the usefulness of our pipeline method.

Further supporting the notion that Gαs2 and Gβx2 operate together and downstream of GCR052, we identified cells that are enriched with *Gαs2/Gβx2*, *Gαs2/gcr052*, and *Gβx2/gcr052* through FISH (Figure 2). These cells are patterned similarly to cells of the *soxB1-2*$^{+}$ dorsal ciliated stripes of sensory neurons in the peripheral nervous system (K. G. Ross et al., 2018). While these cells were the most identifiable localization of all three transcripts, we note that additional cell

types also appeared enriched for one or more of these genes. For example, we also observed high levels of *gcr052* in putative epidermal cells at the periphery of the animal, potentially supporting the model that serotonin directly influences ciliary coordination on the epidermal cells via this receptor (Figure 2L). However, *Gαs2* and *Gβx2* transcripts were not highly enriched in these cells, suggesting that serotonin signaling to other cells, such as the putative neurons described here, may also be important for planarian locomotion. Future work further characterizing the specific cells in which *Gαs2*, *Gβx2*, and *gcr052* operate *in vivo*, along with detailed documentation of how these genes affect planarian motile cilia, could elucidate the mechanisms regulating neural control of cilia-based gliding.

Furthermore, additional assays may reveal new roles of heterotrimeric G proteins in behavior and sensation. G proteins act in diverse biological processes, such as sensation, in other animals (D. T. Jones & Reed, 1989; Wong et al., 1996; Yarfitz & Hurley, 1994) and 8 planarian G protein subunits show expression enrichment in sensory structures called the brain branches (Agata et al., 1998; Okamoto et al., 2005), further supporting this notion (Figure 3.1; Sup Figure S3.9). Using the G protein group as a primary screening strategy may be a beneficial starting point for future study of GPCRs in planarian sensory neurobiology or other aspects of planarian physiology.

Planarian heterotrimeric G proteins can suggest candidate receptors for future planarian GPCR research

Because GPCRs represent one of the largest receptor families in many organisms, including humans (Fredriksson et al., 2003) and planarians (Saberi et al., 2016; Zamanian et al., 2011), approaches to accelerate identification of relevant GPCRs for a given process can prove to be

valuable. Our investigation into planarian heterotrimeric G protein subunits produced functionally distinct and measurable phenotypes, supporting the idea that planarian heterotrimeric G protein subunits could provide a practical first step for identifying and studying roles of GPCRs.

To develop a G protein subunit-driven candidate approach, we formulated a pipeline that identifies candidate GPCR genes using phenotypes from our work along with published single cell sequencing datasets (Figure 3.2H; Sup Table S3.3) (Fincher et al., 2018; Plass et al., 2018; Swapna et al., 2018). Our work with *Gβx2* and *gcr052* demonstrates the utility of characterizing heterotrimeric G proteins as a first step in identifying relevant GPCRs and understanding the cellular mechanism (Civelli et al., 2013; Ngo et al., 2016; Oh et al., 2006; Wise et al., 2004). In the future we plan to apply this approach to identify candidate GPCRs that work with heterotrimeric G proteins to promote polarity establishment and successful regeneration. Identification of novel signaling pathways with key roles in regeneration will help us understand how information about injury is converted into cellular responses to coordinate and drive planarian regeneration.

Materials and Methods

Animal Maintenance: Planarians from an asexual strain of the species *S. mediterranea* (CIW4; (Alvarado et al., 2002)) were kept in 1X Montjuïc salts (1.6 mmol/L NaCl, 1 mmol/L $CaCl_2$, 1 mmol/L $MgSO_4$, 0.1 mmol/L $MgCl_2$, 0.1 mmol/L KCl and 1.2 mmol/L $NaHCO_3$ prepared in ELGA PURELAB [ELGA LabWater, Woodridge, IL] ultrapure water) (Cebrià & Newmark, 2005) at 18°C in the dark. Animals were fed beef liver puree weekly or biweekly. Animals were cut periodically to expand their numbers and generate properly sized (~2-5 mm) individuals for experiments. Animals were starved for a minimum of one week before experiments.

Gene Identification: Gα subunit-like transcripts were mined using the Guanine nucleotide-binding domain (PF00503 (D. Coleman et al., 1994)), Gβ subunit-like transcripts were mined using the WD40-repeat-containing domain preceded by N-terminal alpha helix (IPR001632 (Wall et al., 1995)), and Gγ subunit-like transcripts were mined using the GGL domain (PF00631 (Snow et al., 1998)). Each relevant functional domain (from Pfam (El-Gebali et al., 2019) or InterPro (Paysan-Lafosse et al., 2023)) was searched within the translated *S. mediterranea* transcript dataset dd_Smed_v6 (Brandl et al., 2016; Rozanski et al., 2019), then redundant transcripts were removed. To ensure the retrieved Gγ subunit-like sequences were not Regulator of G protein Signaling (RGS) proteins, the absence of an RGS domain (PF00615 (Chen et al., 2001; Longenecker et al., 2001)) was confirmed (Sup Table S3.1).

Protein alignment and Phylogenetic Analysis: Amino acid sequences were predicted using the web-based translation tool Swiss ExPASy (Expert Protein Analysis System) Molecular Biology Server (Swiss Institute of Bioinformatics, University of Lausanne, Switzerland) (Gasteiger et al., 2003). Protein sequences were aligned to reference sequences from other animals (Sup Table S3.5) using Clustal Omega O(1.2.4) (Sievers & Higgins, 2014) and secondary structures were predicted with ESPript3.0 (Robert & Gouet, 2014), using well-characterized structure examples (PDB ID: 1GP2). Phylogeny was analyzed using Phylogeny.fr (Dereeper et al., 2008). The “a la carte” option was selected with MUSCLE for alignment (Edgar, 2004) and PhyML for construction of the phylogenetic tree (Guindon et al., 2010). For the PhyML analysis, 100 bootstrap replicates were performed, and the WAG model of amino acid substitution was applied.

Molecular cloning: For genes of interest, primers were designed using Primer3 (Rozen & Skaletsky, n.d.) to amplify an ~700 bp region of the corresponding gene from asexual *S. mediterranea* cDNA (Sup Table S3.6). PCR products were cloned into the vector, pJC53.2 (Collins et al., 2010) using standard molecular biology protocols.

RNA interference (RNAi) experiments: dsRNA was transcribed in vitro from PCR products amplified from pJC53.2 using standard molecular methods (Collins et al., 2010; Rouhana et al., 2013). Concentration of dsRNA was determined using either a Nanophotometer NP80 (Implen, Munich, Germany) or by band intensity after gel electrophoresis. For a typical experiment, 10-12 animals were fed 1-3µg dsRNA mixed in ~30µL food (beef liver paste, 4:1 liver:salts mixture), and 1 µL green food dye was added to validate that the animals ate. The mixture was doubled for larger experiments. Negative control worms were fed dsRNA matching *green fluorescent protein* (*GFP)* or bacterial genes (*Chloramphenicol resistance gene* [Cm^R] and *toxin CcdB* [*ccdB*]). Animals were kept in 60-100 mm Petri dishes. After eating, the animals were washed, transferred to fresh dishes, and salts were supplemented with 1:1000 gentamicin sulfate (50 mg/mL stock [Gemini Bio, West Sacramento, CA]). Animals were fed dsRNA ~once per week for three total feedings (more feedings given in long-term RNAi experiments [Figure 2; Sup Figure S3.6]) then were processed. Live images during experiments were obtained using a Zeiss Axiocam 506 Color camera mounted on a Zeiss Axio Zoom.V16 microscope (ZEISS Microscopy, Jena, Germany). Live images and video were also captured on an iPhone 6 and/or SE and processed in iMovie (Apple Inc., Cupertino, California).

Behavior Assays: For the flipping assay (Figure 2B-C), live recordings were captured for up to 5 minutes after each animal was put on its dorsal side. We observed how long it took each animal to flip to its ventral side. For locomotion studies (Figure 3.2; Sup Figure S3.3), animals were recorded in 13x13 mm/square grid dishes (VWR International, Radnor, PA) for at least 15 minutes. Velocity was quantified for 8-12 individual animals while they showed forward movement over at least a 40 second timespan. Distance was tracked using BioTracker (Mönck et al., 2018), then velocity was calculated for intervals of 4 seconds. Average velocities were determined from the values of 11 successive intervals. For negative phototaxis assays, animals were put in 13x13mm/square grid dishes (VWR International, Radnor, PA) with lids half-covered with black electrical tape. This produced an uncovered/light side and covered/dark side of the dish. Animals were placed in the far-left corner of the uncovered region, then recorded for at least 10 minutes. For each 60 second interval, the number of animals visible in the uncovered region was documented.

In situ hybridization (ISH): Single-stranded antisense riboprobes were transcribed with Digoxygenin (Dig-11-UTP) (Sigma-Aldrich, St. Louis, MO) using standard molecular methods (Collins et al., 2010). Animals were fixed, hybridized with riboprobes, and stained as previously described (King & Newmark, 2013), with the following modifications: animals were killed in a 10% N-Acetyl Cysteine solution and treated with a 2 μg/mL Proteinase K solution. Regenerating animals were treated with the Proteinase K/post fixation steps (as opposed to a boiling step). After the hybridization step, 56 °C washes were as follows: one 20-minute Wash hyb (25% Formamide, 3.5X SSC [0.15 M NaCl, 0.015 M Na Citrate], 0.1% Triton X-100, and pH 7.0) wash, three 20-minute 2X SSCx (2X SSC and 0.1% Triton X-100) washes, four 20-minute 0.2X SSCx (0.2X SSC and 0.1% Triton X-100) washes. We also replaced MABT with TNTx (0.1 M Tris pH 7.5, 0.15 M

NaCl, and 0.3% Triton X-100). After antibody incubation, animals were washed in TNTx for 5 minutes (1 wash), 10 minutes (1 wash), and six 20-minute washes. The fixation step after sample development was omitted. Other key reagents include anti-digoxygenin conjugated with an alkaline phosphatase (Anti-Dig-AP 1:2000 dilution), nitro-blue tetrazolium (NBT), and 5-bromo-4-chloro-3'-indolyphosphate (BCIP) (all Sigma-Aldrich, St. Louis, MO). Animals were mounted in 80% glycerol and imaged with a Zeiss Axiocam 506 Color camera mounted on a Zeiss Axio Zoom.V16 microscope (ZEISS Microscopy, Jena, Germany).

Fluorescent ISH (FISH): Single-stranded antisense riboprobes were synthesized with Digoxygenin (Dig-11-UTP) (Sigma-Aldrich, St. Louis, MO), Fluorescein isothiocyanate (FITC-12-UTP) (Roche, Basel, Switzerland), or 2,4-dinitrophenol (DNP) (PerkinElmer, Inc., Waltham, MA) using standard molecular methods (Collins et al., 2010). Riboprobes were detected using Anti-Dig-POD (1:1000; Sigma-Aldrich, St. Louis, MO), Anti-FITC-POD (1:1000; Sigma-Aldrich, St. Louis, MO), or Anti-DNP-HRP (1:3000; Vector Laboratories, Burlingame, CA). Tyramide-conjugate signal amplification was performed as previously described (King & Newmark, 2013). The final incubation was with DAPI [10 μg/mL] (1:1000; Thermo Fisher Scientific, Waltham, MA). Animals were mounted in VECTASHIELD (Vector Labs, Burlingame, CA) for imaging.

Immunofluorescence (IF): Immunofluorescence was adapted from existing protocols (Forsthoefel et al., 2014; K. G. Ross et al., 2015). Planarians were killed in 2% HCl for 5 min with alternating 1-min incubations on ice and gently inverting at room temperature. The HCl step was followed by three 5-minute washes in PBS (Phosphate Buffered Saline: 137 mM NaCl, 2.7 mM

KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.4) at room temperature. Animals were then fixed for 15 minutes in 4% formaldehyde solution in PBS, then shaken in PBSTx (PBS and 0.3% Triton X-100) for 10 minutes three times at room temperature. The animals were bleached under light overnight in 6% H_2O_2 in PBSTx. Bleaching was followed by 2 10-minute PBSTx washes at room temperature. Animals were then blocked (PBSTx and 1% Bovine Serum Albumin [Jackson ImmunoResearch Laboratories, Inc., West Grove, PA]) for at least 4 h. Blocking solution was replaced with a solution containing primary antibody anti-phospho-Histone H3 (Ser10) (1:1600 [Cell Signaling Technology, Danvers, MA]) to mark cells in the process of mitosis and were incubated gently shaking at 4°C overnight. The next day animals were incubated in PBSTx at room temperature eight times for 30 minutes. Then animals were incubated in blocking solution for 1 hour. Blocking solution was replaced with a solution containing the secondary antibody, goat-anti-mouse IgG+IgM-horse radish peroxidase (1:1000, [Sigma-Aldrich, St. Louis, MO]) and animals were shaken gently at 4°C overnight. Afterward, animals were washed for 30 minutes eight times in PBSTx at room temperature. The samples were then shaken for 30 minutes at room temperature in PBSTi (PBSTx and 10 mM imidazole) wrapped in foil (foil remained until mounting). The samples were then developed for 5 minutes through tyramide signal amplification (TSA reaction) using FITC-Tyramide (1:1000 in PBSTi and .015% H_20_2) The samples were then shaken at room temperature in PBSTx 3 times for 10 minutes, then 2 times for 30 minutes. The final incubation was in DAPI solution (0.5 μg/mL in PBSTx) overnight at 4°C. Samples were then mounted in VECTASHIELD (Vector Labs, Burlingame, CA) for imaging.

Confocal Image Acquisition: Confocal images were obtained for FISH and IF samples using Zen black 2.3 SP1 software on a Zeiss LSM 710 AXIO Observer Z1 inverted microscope or Zeiss 880

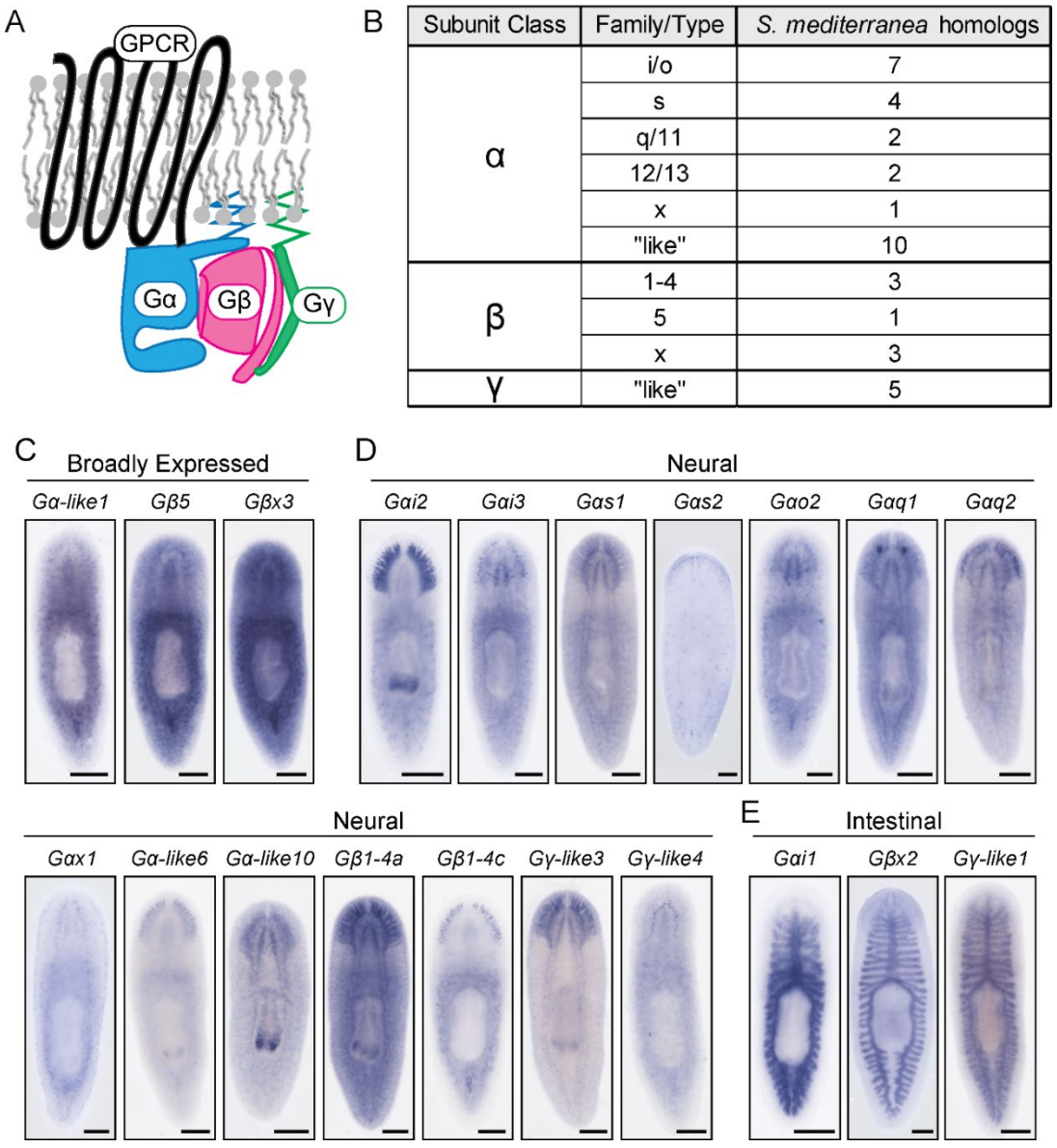
AXIO Imager Z2 microscope (ZEISS Microscopy, Jena, Germany). The details for FISH images are as follows: Figure 2G and 2L are single slice images using a 20X objective (Numerical Aperture [NA] 0.8), the head region images in Figure 3.3H are Max Intensity Projections of 10 z-sections (9.72µm sections) taken with a 10X objective (NA 0.3), the zoomed eyespot images in Figure 3.3H are single slices captured with a 40X objective (NA 1.4), Sup Fig S3.6A are Max Intensity Projections of 12 z-sections (1µm sections) taken with a 20X objective (NA 0.8), and Sup Fig S3.6B are single slices taken with a 20X objective (NA 0.8). For H3P IF, images of four tiles and ~30 z-sections (1µm sections) capturing the anterior half of the animals were taken with a 10X objective (NA 0.3). For post-processing, tiles were stitched with Imaris (Oxford Instruments, Abingdon, United Kingdom) or FIJI (Fiji is just ImageJ) (Schindelin et al., 2012) software.

Image Quantification: For regeneration assays, areas of the brains (Figure 3.3; 3.6 and Sup Figures S3.4; S3.8) or blastemas (Sup Figure S3.4) were measured from fixed sample images by tracing the structures with FIJI imaging software (Schindelin et al., 2012), and normalized as described previously (Roberts-Galbraith et al., 2016) (Sup Table S3.4). Brain measurements were traced around the outer boundary of the brain, encompassing the entire structure including the brain branches. For growth assays (Sup Figure S3.6), animal lengths were measured from live images in FIJI (Schindelin et al., 2012). Data were statistically analyzed and visualized using Prism - GraphPad Version 7.0 software (GraphPad Software, San Diego, CA). Specific tests employed are found in the corresponding figure legends.

For H3P analysis, Imaris software (Oxford Instruments, Abingdon, United Kingdom) was used for quantification of $H3P^{+}$ cells in the body volume of the anterior half of 4-5 animals per RNAi

treatment. The spots function of the software was employed to detect green cells. After automated counting, spots were manually checked and adjusted. The surfaces function was employed to measure the body volume captured in each z stack. Mitosis/mm^3 was calculated from the total number of $H3P^+$ cells in a given volume.

Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR): RNA was extracted from animals using Trizol Reagent (Thermo Fisher Scientific, Waltham, MA) as per the manufacturer's protocol (Liu & Rink, 2018). Samples were treated with RQ1 RNase-free DNase (Promega Corporation, Madison, WI) for 15 minutes at 37°C. cDNA was synthesized from RNA using an iScript kit (Bio-Rad, Hercules, CA). RT-qPCR reactions were completed using SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA) in a QuantStudio® 3 real-time PCR system (Applied Biosystems, Foster City, CA). Primers were generated in Primer3 (Rozen & Skaletsky, n.d.) and targeted sequences ~100 bp in length (Sup Table S3.6). RT-qPCR primers were designed to match a region of the transcripts not included in dsRNA constructs using Benchling software (Benchling, San Francisco, CA). Transcript abundance for genes of interest was normalized using the control gene, *β tubulin* (Collins et al., 2010). Experiments were performed in biological and technical triplicate (n=12 animals per biological replicate). Data were statistically analyzed and visualized using Prism - GraphPad Version 7.0 software (GraphPad Software, San Diego, CA).



Subunit Class	Family/Type	*S. mediterranea* homologs
α	i/o	7
	s	4
	q/11	2
	12/13	2
	x	1
	"like"	10
β	1-4	3
	5	1
	x	3
γ	"like"	5

Figure 3.1 Planarians possess diverse heterotrimeric G proteins. (A) Graphical summary of a typical heterotrimeric G protein complex associated with a GPCR (black). The heterotrimer is composed of Gα (blue), Gβ (magenta), and Gγ (green) subunits that are activated upon ligand binding to the receptor. **(B)** Table depicting *S. mediterranea* homologs for heterotrimeric G protein subunits. Representative images of G protein subunit expression patterns categorized by the most visually enriched tissue type into broad **(C)**, neural **(D)**, and intestinal **(E)** patterns. Scale bars = 200 μm. The anterior of the animals is oriented toward the top of the page in all figures.

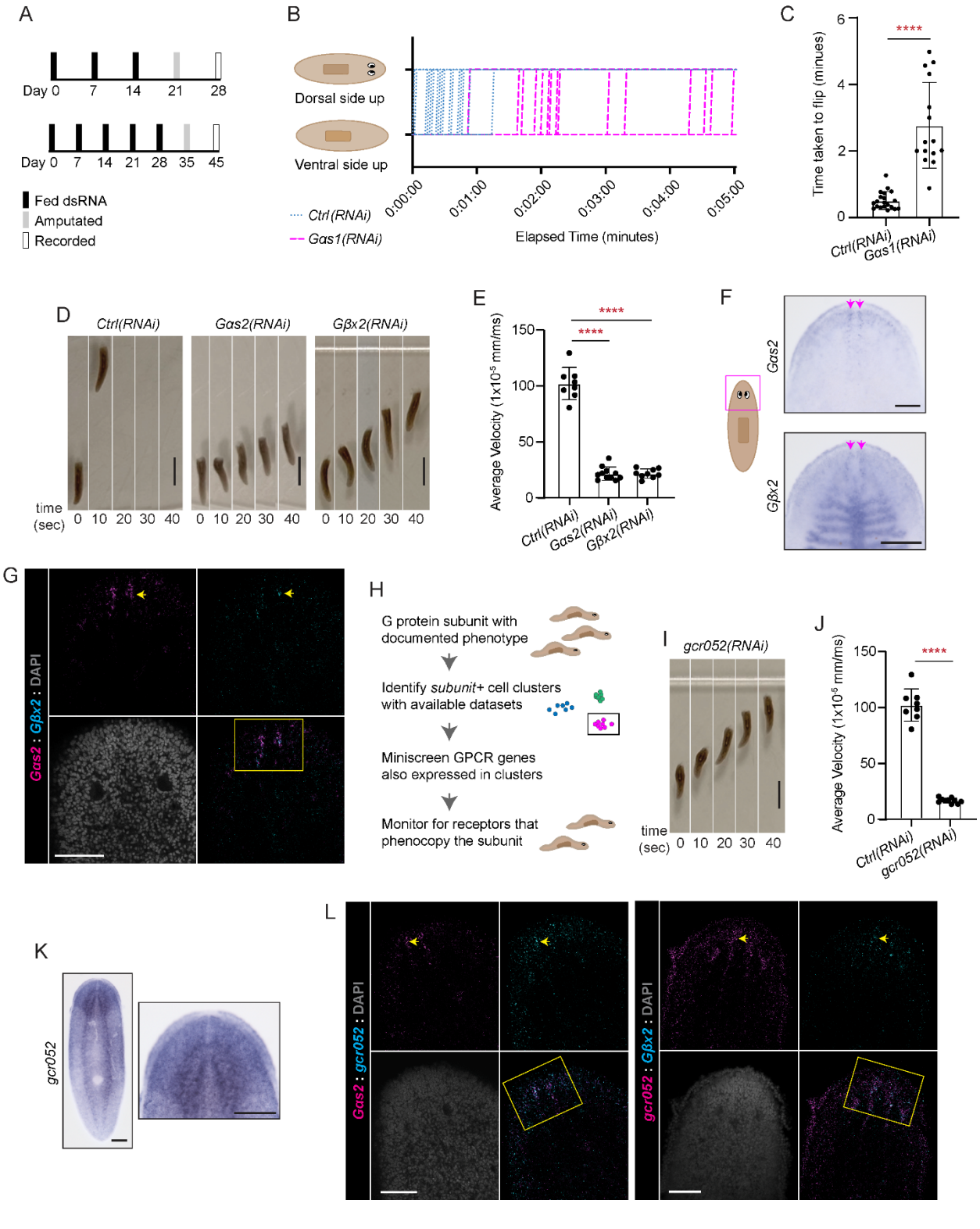

A
Day 0 7 14 21 28
Day 0 7 14 21 28 35 45
Fed dsRNA
Amputated
Recorded
B
Dorsal side up
Ventral side up
Ctrl(RNAi)
Gαs1(RNAi)
0:00:00 0:01:00 0:02:00 0:03:00 0:04:00 0:05:00
Elapsed Time (minutes)
C
Time taken to flip (minues)
****
Ctrl(RNAi)
Gαs1(RNAi)
D
Ctrl(RNAi)
Gαs2(RNAi)
Gβx2(RNAi)
time (sec)
0 10 20 30 40
E
Average Velocity (1x10⁻⁵ mm/ms)
****
Ctrl(RNAi)
Gαs2(RNAi)
Gβx2(RNAi)
F
Gαs2
Gβx2
G
Gαs2 : Gβx2 : DAPI
H
G protein subunit with documented phenotype
Identify subunit+ cell clusters with available datasets
Miniscreen GPCR genes also expressed in clusters
Monitor for receptors that phenocopy the subunit
I
gcr052(RNAi)
time (sec) 0 10 20 30 40
J
Average Velocity (1x10⁻⁵ mm/ms)
****
Ctrl(RNAi)
gcr052(RNAi)
K
gcr052
L
Gαs2 : gcr052 : DAPI
gcr052 : Gβx2 : DAPI

Figure 3.2 Planarian heterotrimeric G proteins and GCR052 promote animal movement. (A) RNAi paradigms used during initial regeneration screens (top) and follow up, longer-term experiments (bottom). Data from (B-C) resulted from the top paradigm and data from (D-E and I-J) resulted from the bottom paradigm. **(B)** Flip assay used to document paralysis in *Gas1(RNAi)* animals. The graph includes flipping data for 20 animals per RNAi condition. **(C)** Bar graph showing times taken for animals to flip over to a correct ventral-down posture (excluding 5 non-flipping *Gas1(RNAi)* animals), displayed as mean and standard deviation. Differences were analyzed with Unpaired T-Test with Welch's correction. **** = P-value ≤ 0.0001. **(D)** Image stills from videos capturing locomotion displayed by regenerating control, *Gas2(RNAi)*, and *Gβx2(RNAi)* animals 10 dpa. **(E)** Results from quantification of average velocity over a 40 second timespan in regenerating control, *Gas2(RNAi)*, and *Gβx2(RNAi)* animals, displayed as mean and standard deviation. **(F)** Images of *Gas2* and *Gβx2* zoomed colorimetric ISH showing the clusters of cells at the anterior tip of the animals, indicated with magenta arrowheads. **(G)** *Gas2* and *Gβx2* dFISH images of the head region. Yellow arrowhead indicates an example of a cell enriched with both transcripts. The yellow box indicates the region of interest where the anterior clusters are found. **(H)** Graphical scheme showing the method used to identify candidate GPCRs for G protein subunits with documented phenotypes. **(I)** Image stills from videos capturing locomotion displayed by regenerating *gcr052(RNAi)* animals. **(J)** Results from quantification of average velocity over a 40 second timespan in intact control and *gcr052(RNAi)* animals, displayed as mean and standard deviation. Data displayed in (I-J) are from the same experiment as shown in (D-E). Differences in average velocities were analyzed with Brown-Forsythe and Welch ANOVA with multiple comparisons. **** = Adjusted P-value ≤ 0.0001. **(K)** Images showing the expression pattern of *gcr052* through colorimetric ISH. **(L)** *gcr052* dFISH images with *Gas2* or *Gβx2* in the head region. The yellow box indicates the region of interest where the anterior clusters are found. Yellow arrowheads indicate an example of a cell enriched with both transcripts. Scale bars in (D) and (I) = 2 mm. Scale bars in (F) and (K) = 200 μm. Scale bar values for FISH data are indicated on the images.

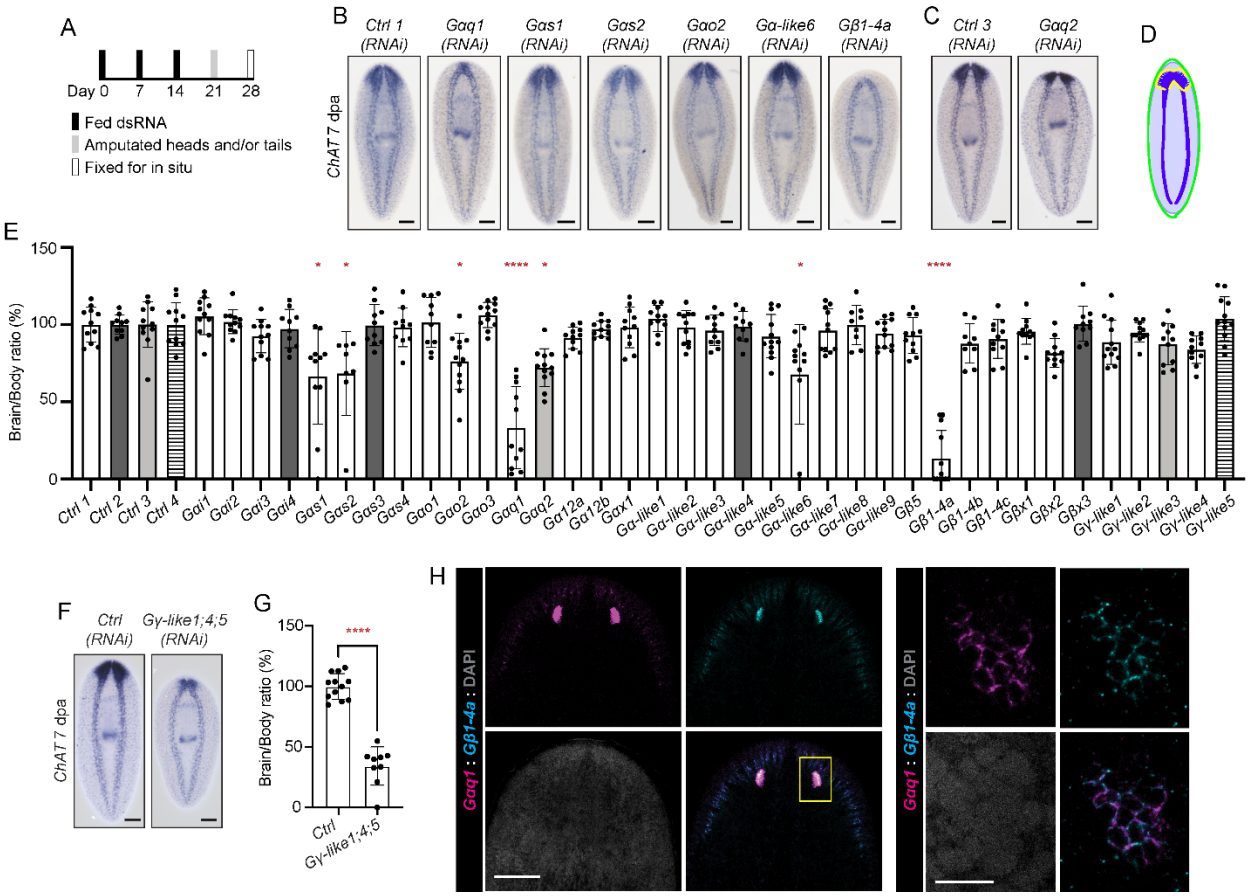


Figure 3.3 Specific planarian heterotrimeric G protein genes promote brain regeneration. **(A)** RNAi paradigm used for initial regeneration screens. **(B and C)** Representative images showing animals treated with RNAi targeting genes that reduced brain regeneration along with corresponding controls. **(D)** Visual schematic displaying our method for brain regeneration quantification. From *ChAT* ISH images, the area of the brain (yellow) and body (green) for each animal are used to calculate brain/body ratios (Roberts-Galbraith et al., 2016). **(E)** Bar graph of data from quantification of brain/body ratios after RNAi, displaying mean and standard deviation. Bars are color coded to match samples to controls from the same experiment. Differences were analyzed using Brown-Forsythe and Welch ANOVA with multiple comparisons. * = Adjusted P-value $\leq$ 0.05. **** = Adjusted P-value $\leq$ 0.0001. **(F)** Representative images showing brain regeneration in control and combinatorial *Gγ-like 1;4;5(RNAi)* animals. **(G)** Bar graph of quantified brain/body ratios in Gγ-like combinatorial RNAi, displayed as mean and standard deviation. Differences were analyzed using Unpaired T-Test. **** = P-value $\leq$ 0.0001. **(H)** *Gαq1* and *Gβ1-4a* dFISH images focusing on the head. The yellow box indicates the region shown in the next, zoomed image of the eyespot, validating coexpression. Scales in (B), (C), (F) and the head region image in (H) = 200 µm. Scale in the eyespot image of (H) = 20 µm.

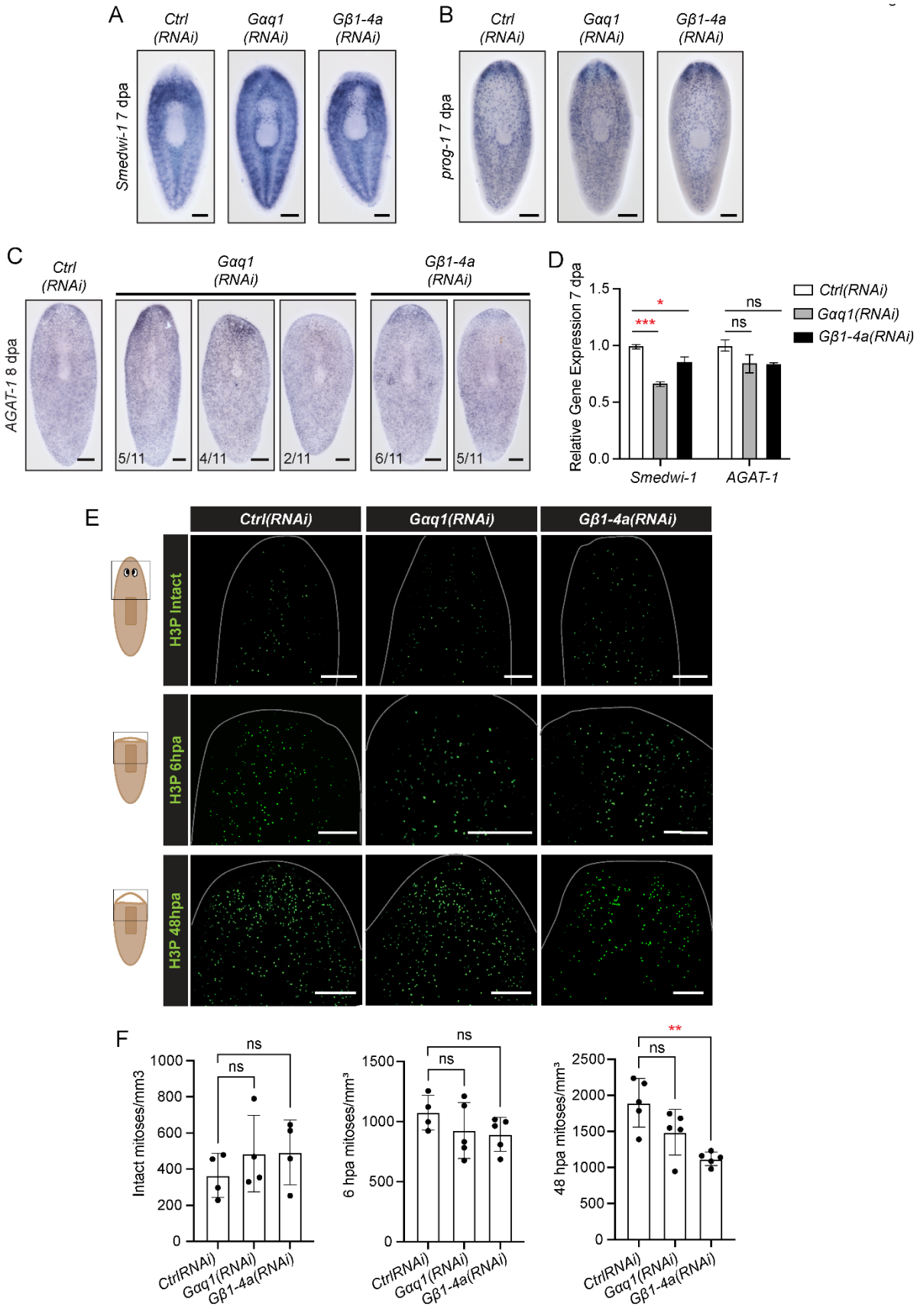

A
Ctrl (RNAi)
Gαq1 (RNAi)
Gβ1-4a (RNAi)
Smedwi-1 7 dpa
B
Ctrl (RNAi)
Gαq1 (RNAi)
Gβ1-4a (RNAi)
prog-1 7 dpa
C
Ctrl (RNAi)
Gαq1 (RNAi)
Gβ1-4a (RNAi)
AGAT-1 8 dpa
5/11
4/11
2/11
6/11
5/11
D
Relative Gene Expression 7 dpa
1.5
1.0
0.5
0.0
*
***
ns
ns
Ctrl(RNAi)
Gαq1(RNAi)
Gβ1-4a(RNAi)
Smedwi-1
AGAT-1
E
Ctrl(RNAi)
Gαq1(RNAi)
Gβ1-4a(RNAi)
H3P Intact
H3P 6hpa
H3P 48hpa
F
Intact mitoses/mm3
1000
800
600
400
200
0
ns
ns
6 hpa mitoses/mm³
1500
1000
500
0
ns
ns
48 hpa mitoses/mm³
2500
2000
1500
1000
500
0
**
ns
CtrlRNAi)
Gαq1(RNAi)
Gβ1-4a(RNAi)

Figure 3.4 *Gαq1* and *Gβ1-4a* are not required for stem cell maintenance. Representative images of **(A)** *Smedwi-1*, **(B)** *prog-1*, and **(C)** *AGAT-1* ISH in regenerating animals after RNAi targeting *Gαq1* and *Gβ1-4a*. **(D)** Relative transcript abundance of stem cell markers, measured by RT-qPCR. Differences were analyzed with One-way ANOVA with multiple comparisons. Error bars represent standard error. * = Adjusted P-value ≤ 0.05. *** = Adjusted P-value ≤ 0.0005. **(E)** Representative images of proliferative cell detection (anti-H3P) at the anterior region of intact, 6 hours regenerating, and 48 hours regenerating RNAi animals. **(F)** Results from quantification of $H3P^+$ cells detected in the anterior region of the animals at each timepoint, displayed as mean and standard deviation. Differences were analyzed with Brown-Forsythe and Welch ANOVA with multiple comparisons. ** = Adjusted P-value ≤ 0.01. Scale bars = 200 μm.

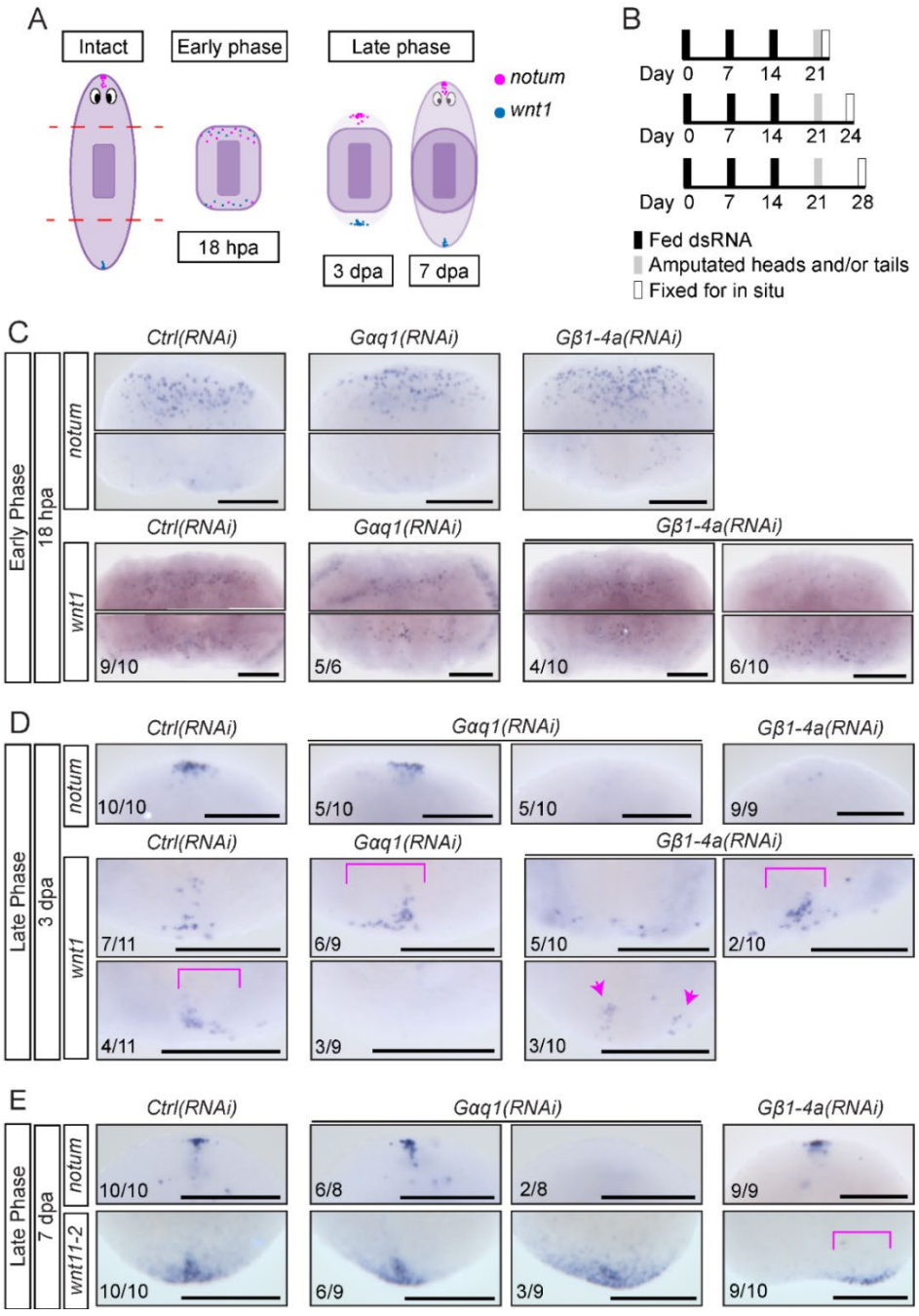


Figure 3.5 *Gαq1* and *Gβ1-4a* support the late phase of anterior and posterior pole regeneration. **(A)** Graphic summary depicting phases of polarity reestablishment after head and tail amputation, as summarized in (Owlarn and Bartscherer, 2016). **(B)** RNAi paradigms for 18 hpa (top), 3 dpa (middle), and 7 dpa (bottom). The following images are zoomed to focus on the regenerating head or tail blastemas for each stage. Representative images of anterior *notum,* and posterior *wnt1* or *wnt11-2* expression at **(C)** 18 hpa, **(D)** 3 dpa, and **(E)** 7 dpa of heads (pointing upward) and/or tails (pointing downward). Magenta brackets denote non-medial expression domains. Magenta arrowheads indicate multiple expression domains. Scale bars = 200 μm.

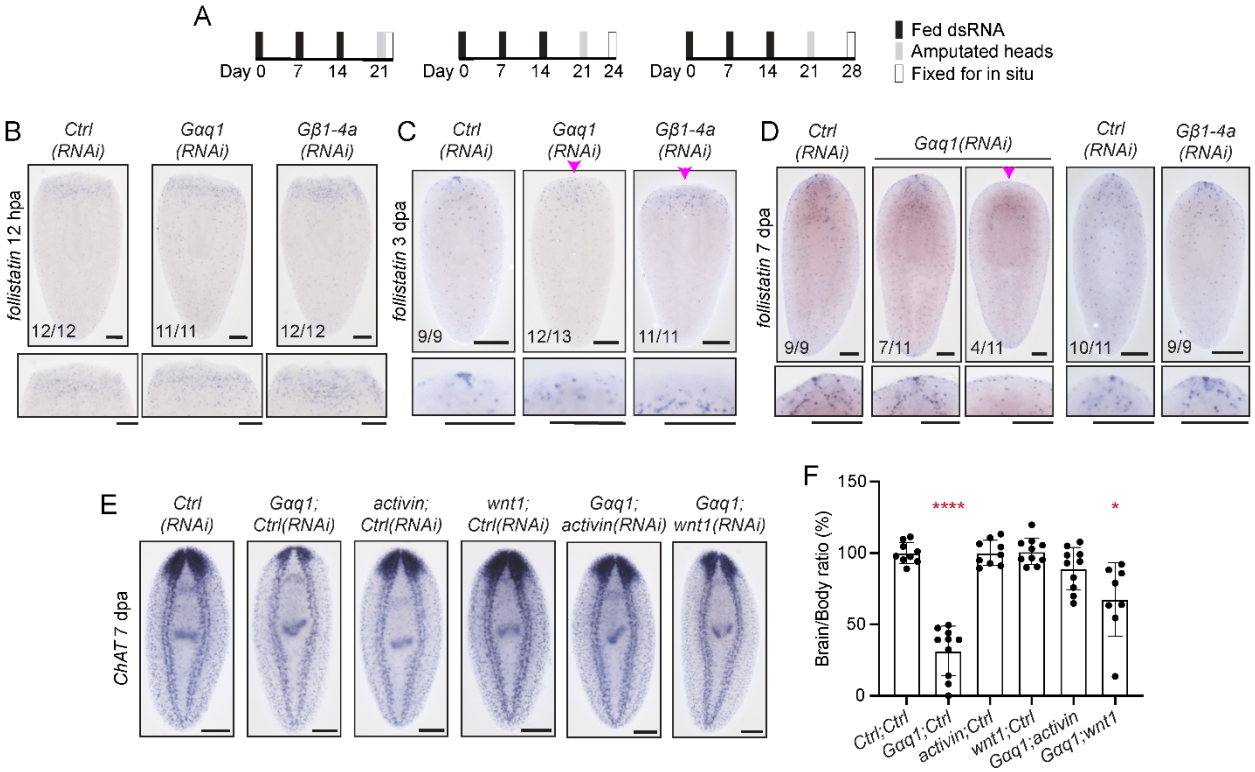


Figure 3.6 *Gαq1* supports head regeneration through production of *follistatin*$^{+}$ anterior pole cells. (A) RNAi paradigms used for data presented at 12 hpa (left), 3 dpa (middle), and 7 dpa (right). Representative images of *follistatin* expression at **(B)** 12 hpa, **(C)** 3 dpa, and **(D)** 7 dpa in *Gαq1(RNAi)* and *Gβ1-4a(RNAi)* animals. Magenta arrowheads indicate absent anterior pole expression domain. Insets show close-up images of the animals above. (**E**) Representative images showing *ChAT* expression from rescue experiments 7 dpa. (**F**) Bar graph showing results from quantification of brain/body ratios in rescue experiments, displayed as mean and standard deviation. Differences were analyzed using Brown-Forsythe and Welch ANOVA with multiple comparisons. * = Adjusted P-value ≤ 0.05 and **** = Adjusted P-value ≤ 0.0001. Scale bars = 200 µm.

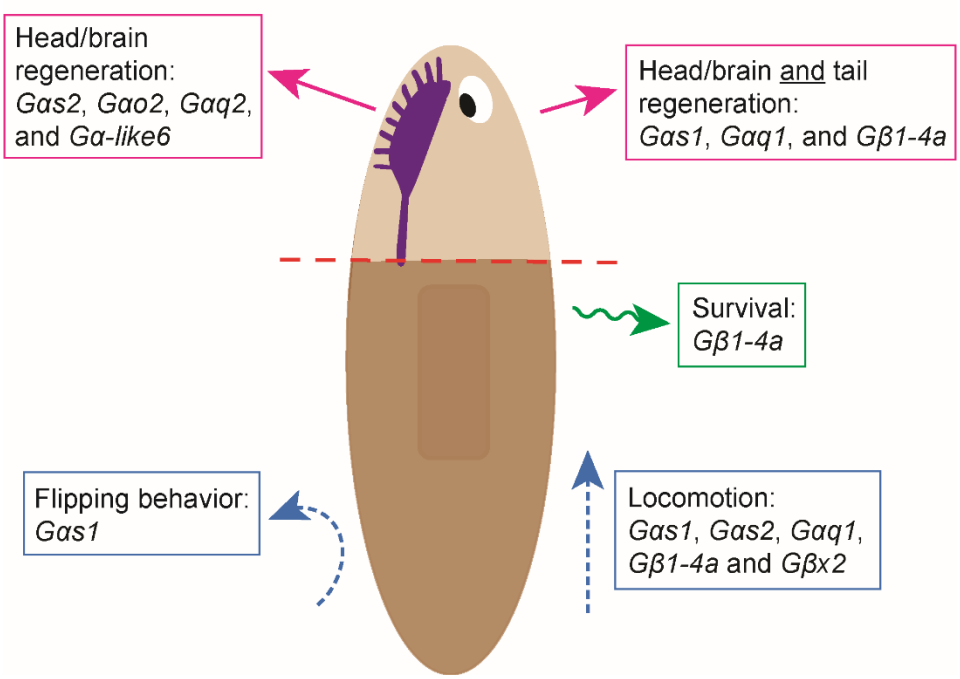
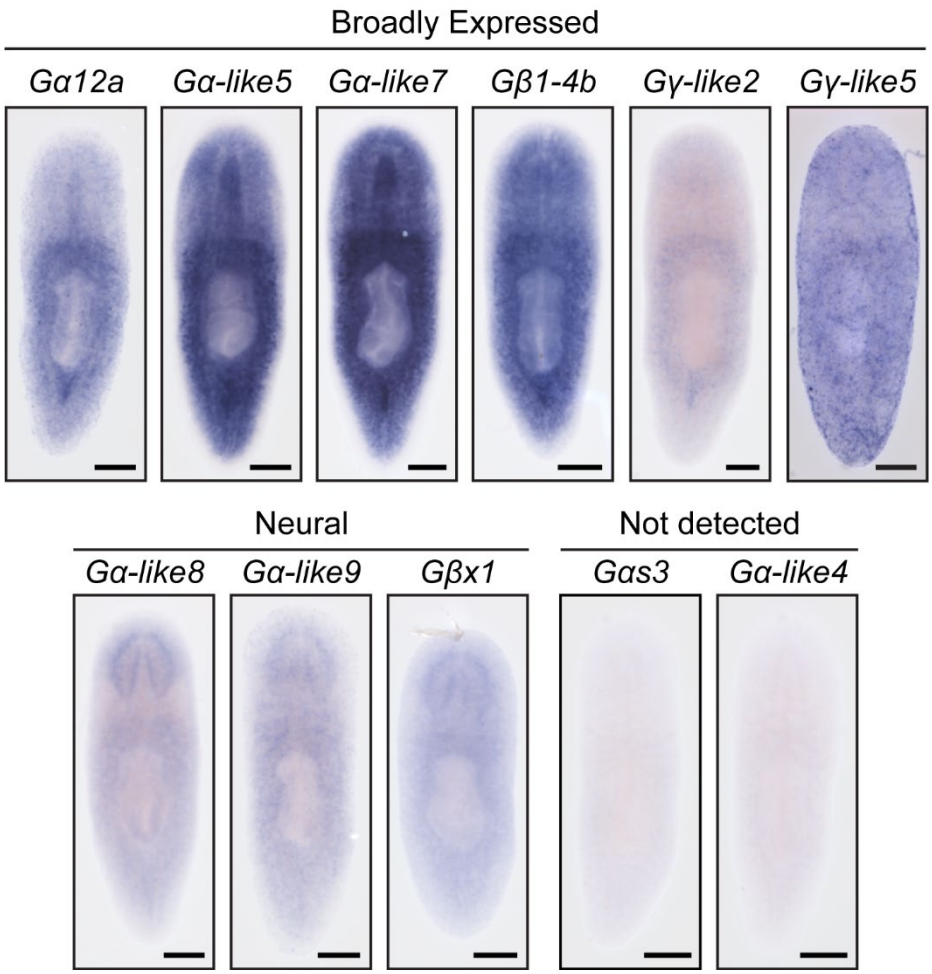
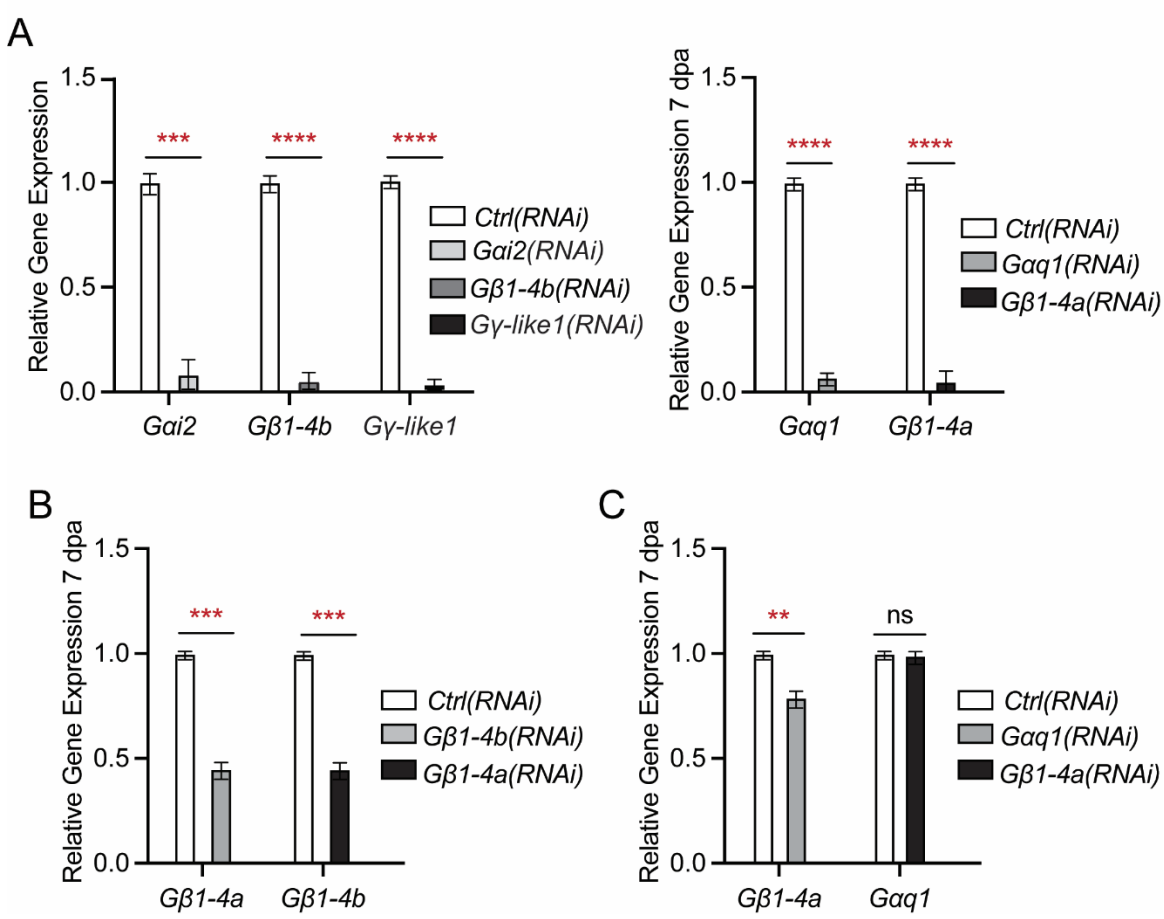


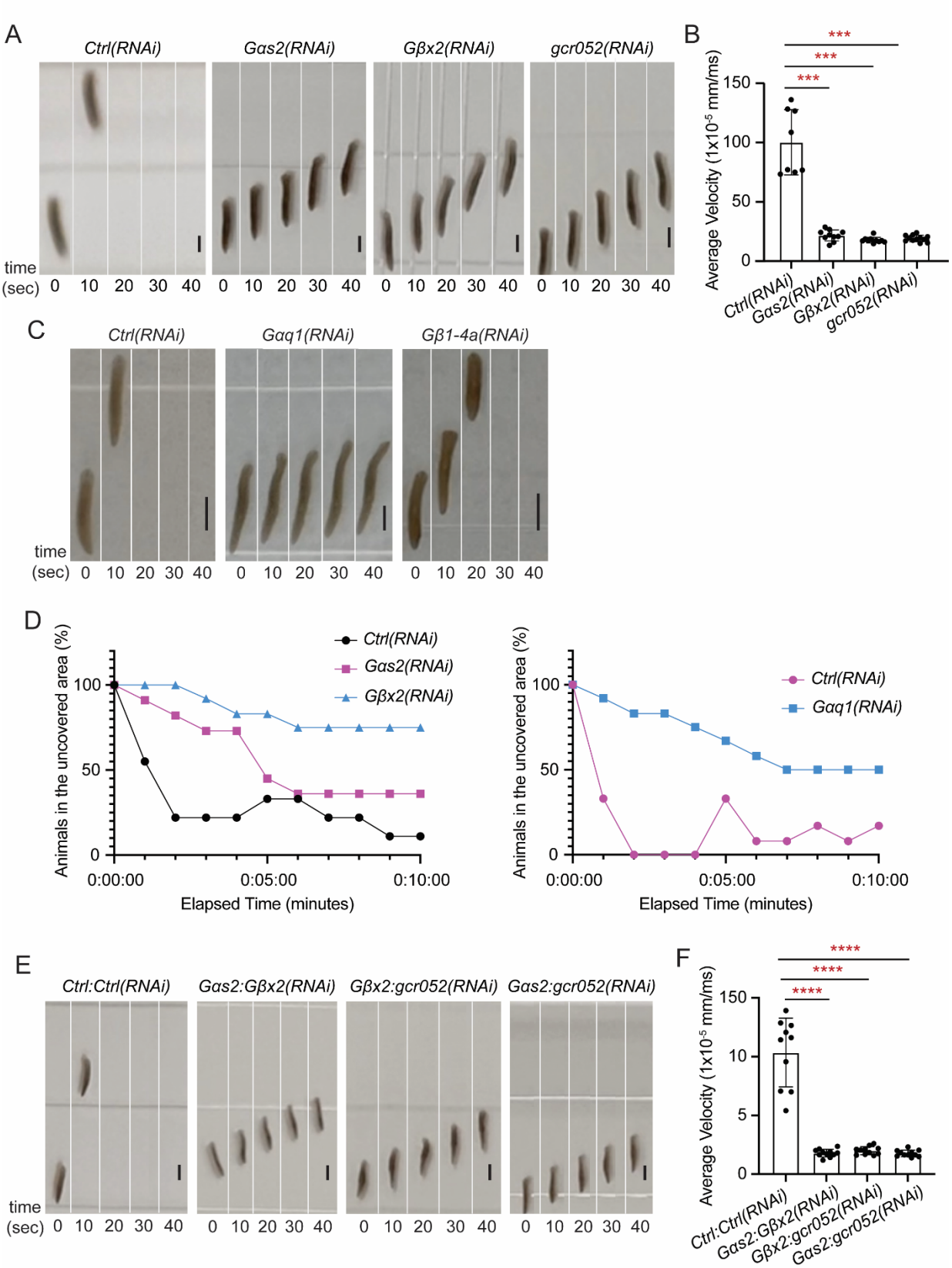
Figure 3.7 Planarian heterotrimeric G proteins play diverse roles in regeneration, physiology, and behavior. Graphical summary of roles described in this work for heterotrimeric G proteins in *S. mediterranea*.



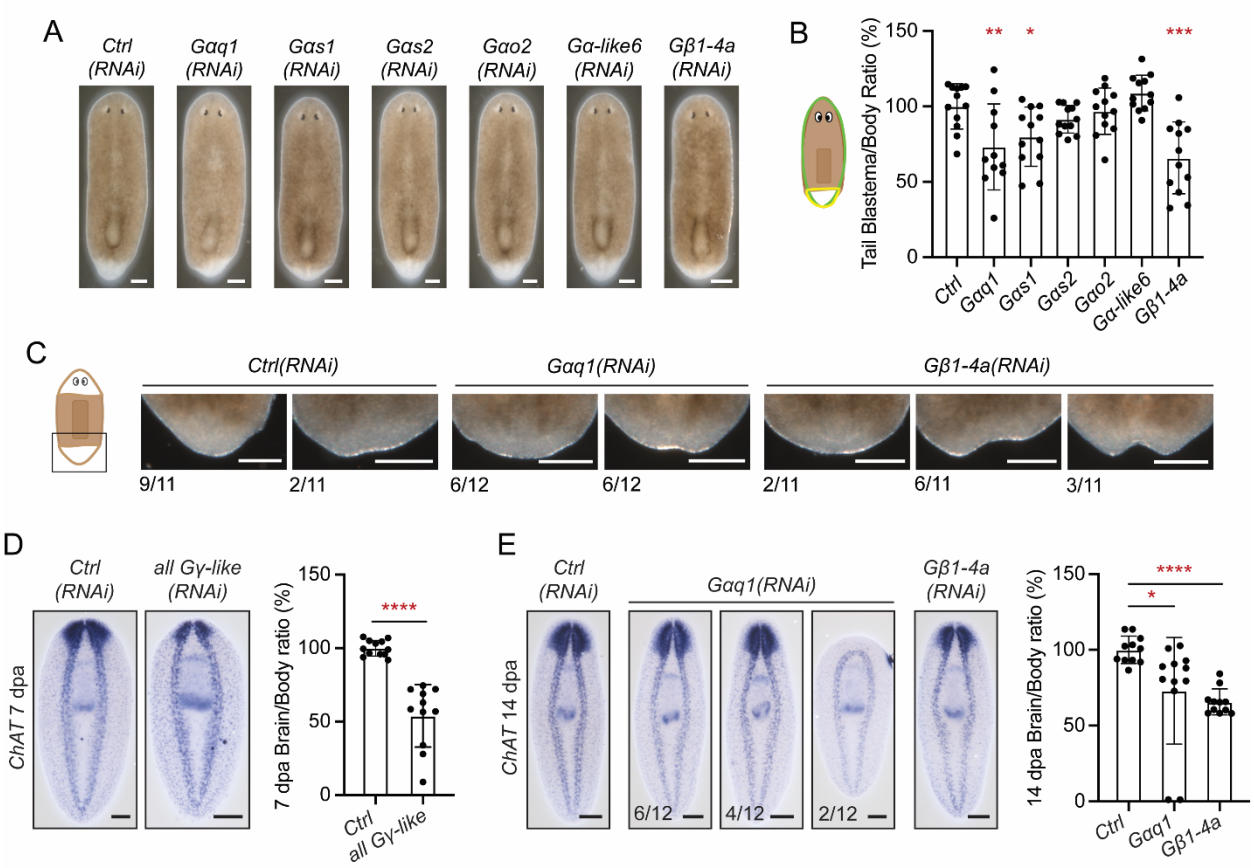
Supplemental Figure S3.1 Additional G protein subunit expression patterns. Representative images of G protein subunit ISH that were not included in Figure 1 of the main text. Images are grouped by the most visually enriched tissue type. Scale bars = 200 μm.



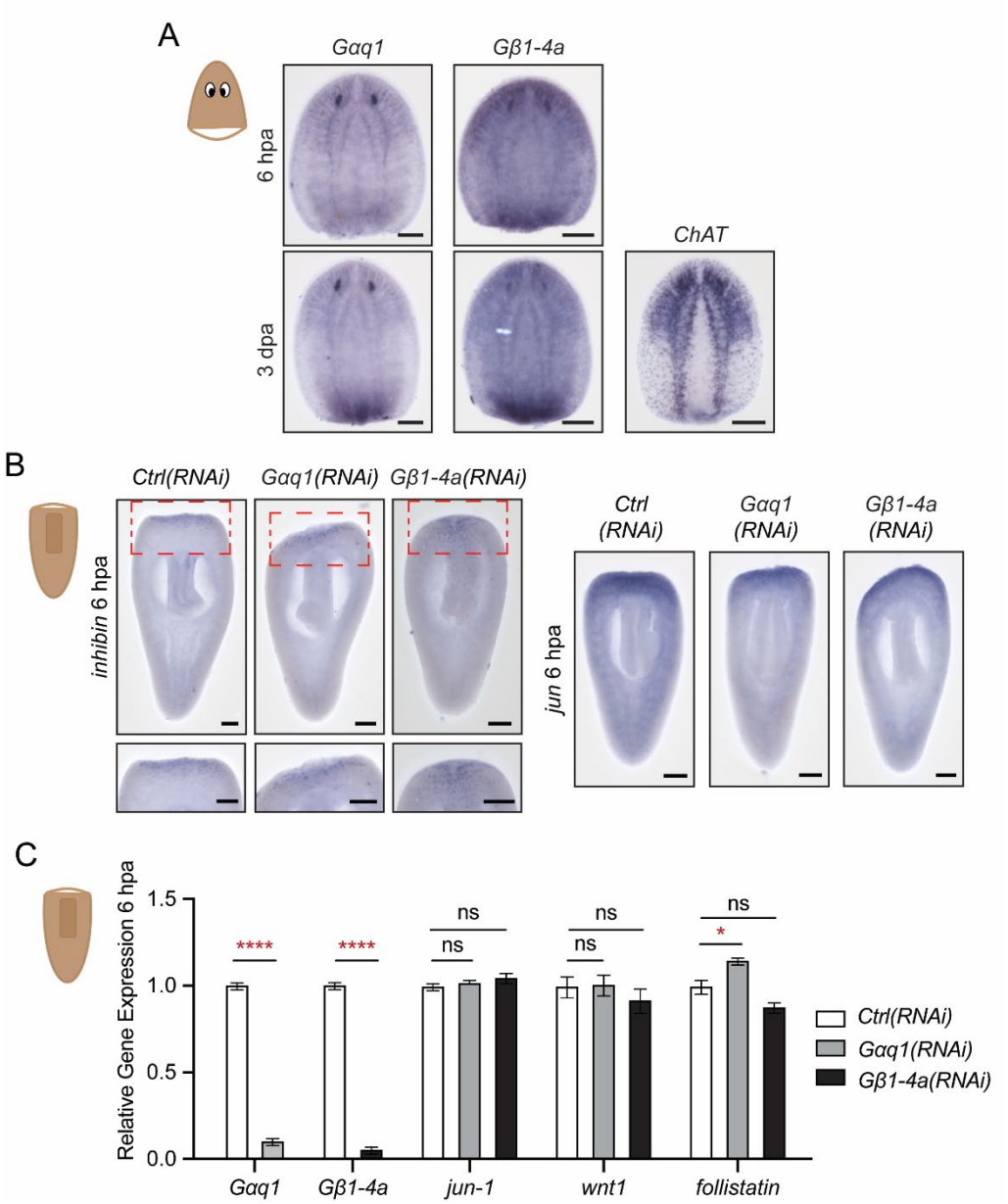
Supplemental Figure S3.2 Quantitative assessment of RNAi efficiency and specificity. (A) Relative expression levels of *Gαi2, Gβ1-4b, Gγ-like1, Gαq1,* and *Gβ1-4a* after targeting the corresponding gene with RNAi, measured by RT-qPCR. Values are grouped by experiment. **(B)** Relative expression levels of *Gβ1-4a* and *Gβ1-4b* after targeting the opposite gene with RNAi. **(C)** Relative expression levels of *Gβ1-4a* and *Gαq1* after targeting the opposite gene with RNAi. Error bars represent SEM. Differences in sample means were statistically analyzed with Unpaired T-Tests. ** = P-value ≤ 0.01. *** = P-value ≤ 0.0005. **** = P-value ≤ 0.0001. 7 dpa RT-qPCR experiments throughout this work utilize the *Gαq1(RNAi)* or *Gβ1-4a(RNAi)* cDNA used to produce the data displayed here.


A
Ctrl(RNAi)
Gαs2(RNAi)
Gβx2(RNAi)
gcr052(RNAi)
time
(sec)
0 10 20 30 40
B
Average Velocity (1x10-5 mm/ms)
150
100
50
0
***
***
***
Ctrl(RNAi)
Gαs2(RNAi)
Gβx2(RNAi)
gcr052(RNAi)
C
Ctrl(RNAi)
Gαq1(RNAi)
Gβ1-4a(RNAi)
time
(sec)
0 10 20 30 40
D
Animals in the uncovered area (%)
100
50
0
Ctrl(RNAi)
Gαs2(RNAi)
Gβx2(RNAi)
0:00:00
0:05:00
0:10:00
Elapsed Time (minutes)
Animals in the uncovered area (%)
100
50
0
Ctrl(RNAi)
Gαq1(RNAi)
0:00:00
0:05:00
0:10:00
Elapsed Time (minutes)
E
Ctrl:Ctrl(RNAi)
Gαs2:Gβx2(RNAi)
Gβx2:gcr052(RNAi)
Gαs2:gcr052(RNAi)
time
(sec)
0 10 20 30 40
F
Average Velocity (1x10-5 mm/ms)
150
10
5
0
****
****
****
Ctrl:Ctrl(RNAi)
Gαs2:Gβx2(RNAi)
Gβx2:gcr052(RNAi)
Gαs2:gcr052(RNAi)

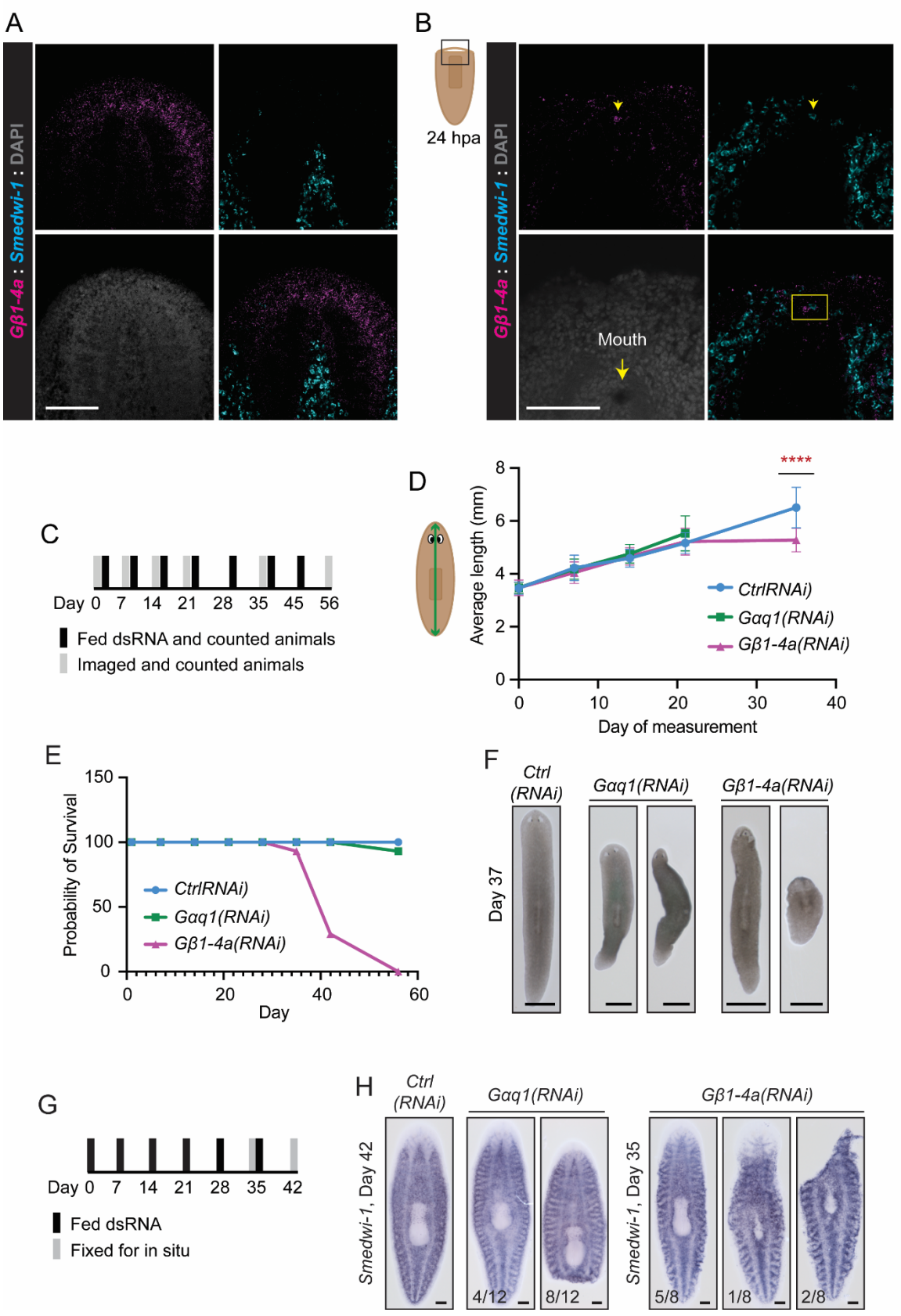
Supplemental Figure S3.3 Additional behavior phenotypes. (A) Image stills from videos capturing locomotion displayed by intact control, *Gas2(RNAi)*, *Gβx2(RNAi)*, and *gcr052(RNAi)* animals. **(B)** Results from quantification of average velocity over a 40 second timespan in intact knockdown animals. Each dot represents an individual animal, and mean and standard deviation are displayed. **(C)** Image stills from videos taken during the long-term assay in Sup Figure S3.6 capturing locomotion displayed by intact control, *Gaq1(RNAi)*, and *Gβ1-4a(RNAi)* animals after 28 days of RNAi. **(D)** Results from negative phototaxis assays, displaying the percentage of animals residing in the uncovered area after each minute. Data are grouped by experiment. Poor animal health prevented the analysis of negative phototaxis in *Gβ1-4a(RNAi)* animals. **(E)** Image stills from videos capturing locomotion displayed by intact control, *Gas2:Gβx2*, *Gβx2:gcr052*, and *Gas2:gcr052* double knockdown animals. **(F)** Results from quantification of average velocity over a 40 second timespan in double knockdown animals, displayed as mean and standard deviation. Differences in both regenerating and double knockdown experiments were analyzed with Brown-Forsythe and Welch ANOVA with multiple comparisons. *** = Adjusted P-value ≤ 0.0005. **** = Adjusted P-value ≤ 0.0001. Scale bars = 2 mm.



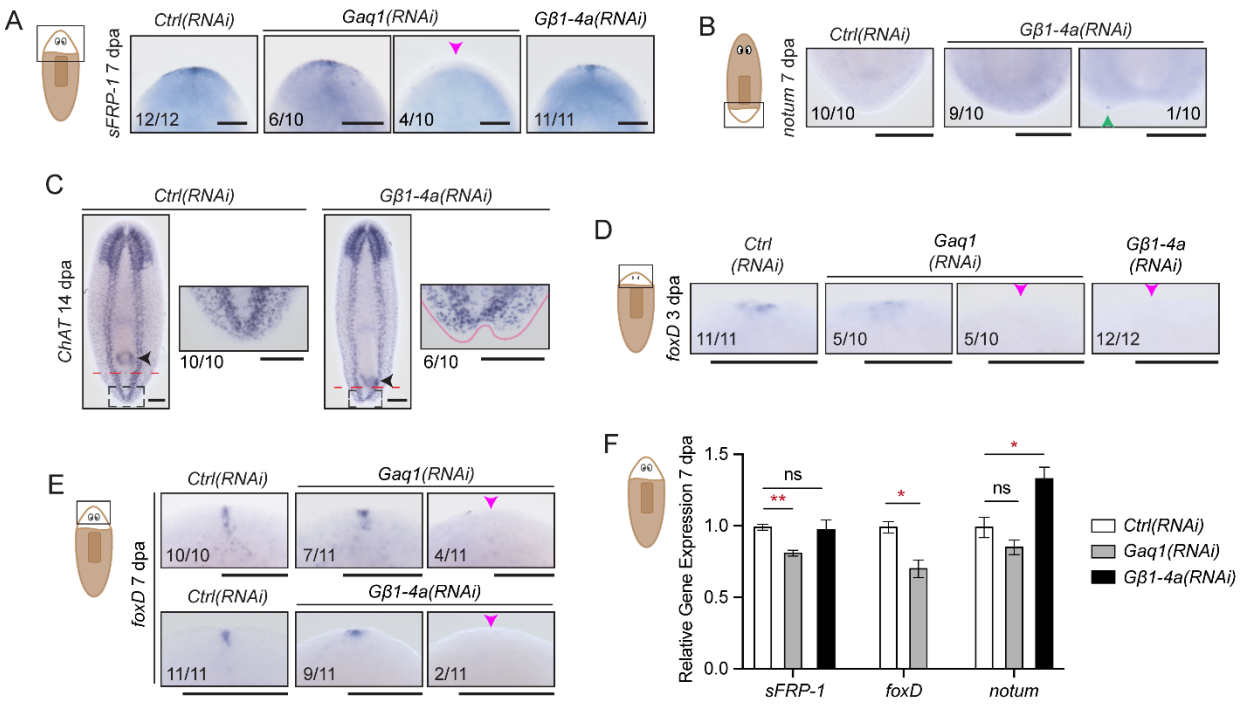
Supplemental Figure S3.4 Additional regeneration phenotypes. (A) Representative images showing animals treated with RNAi 7 days after tail amputation. **(B)** Bar graph with quantification of tail blastema/body ratios, using the quantification method referred to in Main Figure 3 applied to blastemas instead of brains. Results are displayed as mean and standard deviation. Differences were statistically analyzed with One-Way ANOVA with multiple comparisons. * = Adjusted P-value ≤ 0.05. ** = Adjusted P-value ≤ 0.005. *** = Adjusted P-value ≤ 0.0005. **(C)** Representative images showing asymmetrical and notched tail blastemas in *Gαq1(RNAi)* and *Gβ1-4a(RNAi)* animals 7 dpa. Representative images and quantification of brain regeneration in **(D)** combinatorial RNAi of all predicted Gγ-like subunits and **(E)** *Gαq1(RNAi)* and *Gβ1-4a(RNAi)* animals 14 dpa. Bar graphs are displayed as mean and standard deviation. Differences were analyzed with Unpaired T-Tests with Welch's correction in (D). **** = P-value ≤ 0.0001. Differences were analyzed with Brown-Forsythe and Welch ANOVA with multiple comparisons in (E). * = Adjusted P-value ≤ 0.05. **** = Adjusted P-value ≤ 0.0001. Scale bars = 200 μm.



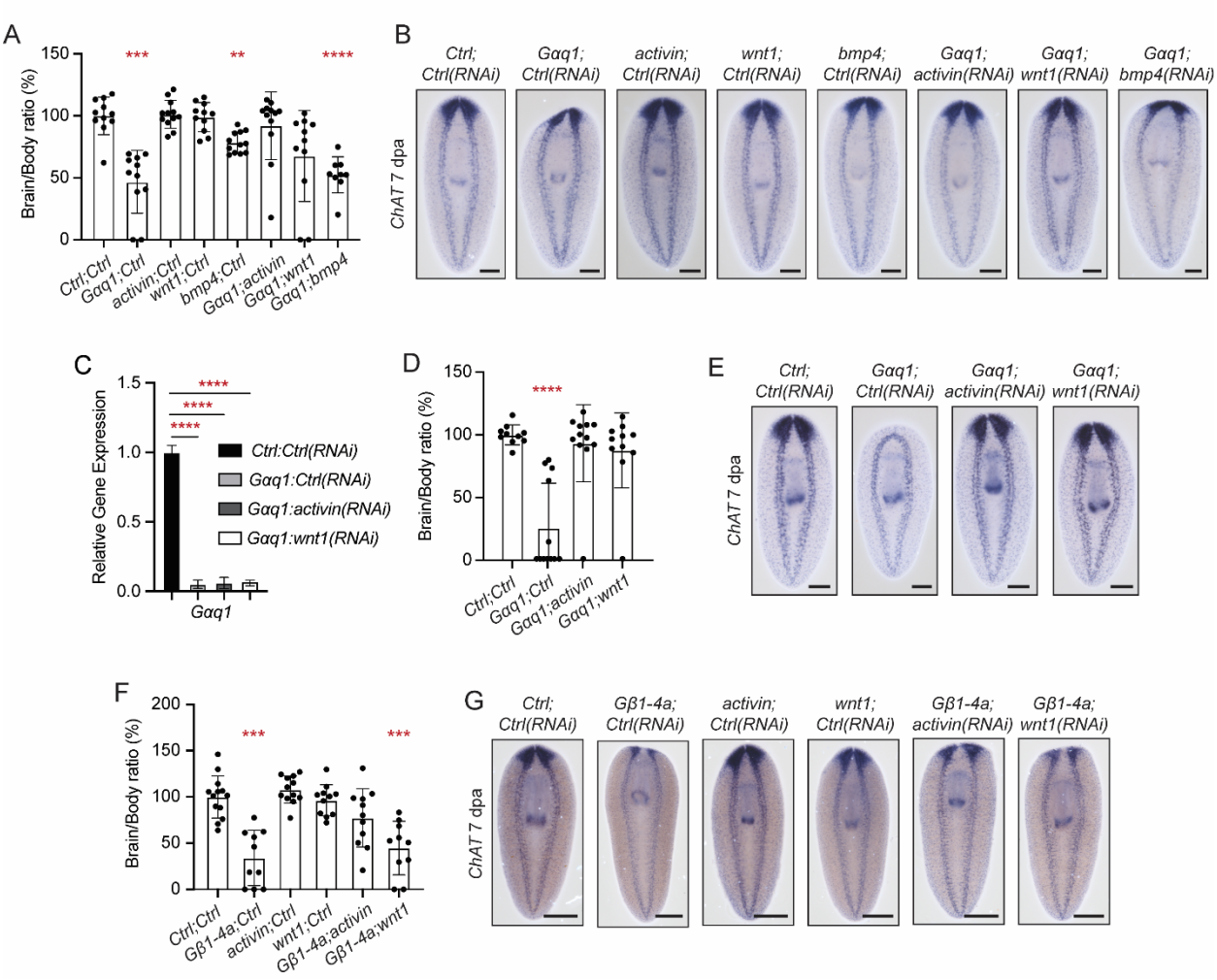
Supplemental Figure S3.5 *Gαq1* and *Gβ1-4a* are upregulated after injury but are not required for described wound response programs. (A) Representative images of *Gαq1* and *Gβ1-4a* ISH in untreated animals 6 hpa and 3 dpa. Example image of *ChAT* ISH at 3 dpa, for reference. **(B)** Representative images of *inhibin* and *jun-1* ISH in *Gαq1(RNAi)* and *Gβ1-4a(RNAi)* animals 6 hpa. Zoomed images are the red-dashed boxes in the top, whole-body images. **(C)** Relative expression levels of wound response markers 6 hpa, measured by RT-qPCR. Knockdown strengths for each target gene are included. Error bars represent SEM. Differences in *Gαq1* or *Gβ1-4a* transcripts were measured with Unpaired T-Tests. Differences in wound response markers were analyzed with One-Way ANOVA with multiple comparisons. * = Adjusted P-value ≤ 0.05. **** = P-value ≤ 0.0001. Scale bars = 200 μm.


A
Gβ1-4a : Smedwi-1 : DAPI
B
24 hpa
Gβ1-4a : Smedwi-1 : DAPI
Mouth
C
Day 0 7 14 21 28 35 45 56
Fed dsRNA and counted animals
Imaged and counted animals
D
****
Average length (mm)
Day of measurement
CtrlRNAi)
Gαq1(RNAi)
Gβ1-4a(RNAi)
E
Probability of Survival
Day
CtrlRNAi)
Gαq1(RNAi)
Gβ1-4a(RNAi)
F
Ctrl (RNAi)
Gαq1(RNAi)
Gβ1-4a(RNAi)
Day 37
G
Day 0 7 14 21 28 35 42
Fed dsRNA
Fixed for in situ
H
Ctrl (RNAi)
Gαq1(RNAi)
Gβ1-4a(RNAi)
Smedwi-1, Day 42
Smedwi-1, Day 35
4/12
8/12
5/8
1/8
2/8

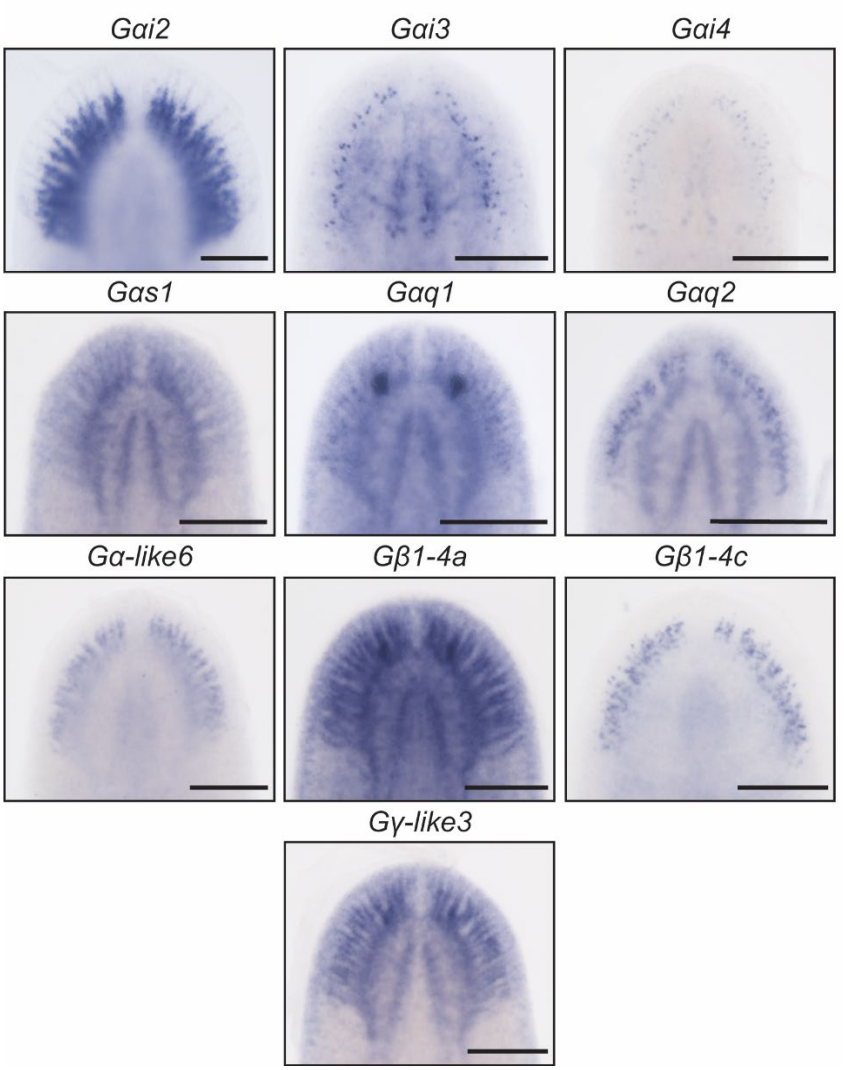
Supplemental Figure S3.6 *Gβ1-4a* likely influences mitosis in a cell non-autonomous manner and promotes survival. *Smedwi-1* and *Gβ1-4a* dFISH showing **(A)** the head region of intact, untreated animals or **(B)** the head-facing region of amputated animals 24 hpa. Yellow arrowheads indicate examples of cells enriched for each marker, and the yellow box highlights the close association between some *Smedwi-1*$^+$ and *Gβ1-4a*$^+$ cells near the amputation site. **(C)** Homeostatic roles for *Gαq1* and *Gβ1-4a* were determined through long-term RNAi paradigms. **(D)** Growth curve data showing mean animal length of 10-12 animals/RNAi treatment over time, displayed with standard deviation. Differences were analyzed with One-Way ANOVA with multiple comparisons for timepoints with values for all three RNAi treatments and an Unpaired T-Test for the last timepoint with only control and *Gβ1-4a(RNAi)*. **** = P-value ≤ 0.0001. **(E)** Survival curve showing the relative percentage of surviving animals after each week of RNAi. **(F)** Representative images of homeostatic phenotypes on day 37 of RNAi. **(G)** The RNAi paradigm used to observe *Smedwi-1* expression after longer-term RNAi. *Gβ1-4a(RNAi)* animals were fixed 1 week earlier due to highly penetrant lysing. **(H)** Representative images of Smedwi-1 ISH in *Gαq1(RNAi)* and *Gβ1-4a(RNAi)* after 42 or 35 days of RNAi. Animals were not amputated, but 8/12 *Gαq1(RNAi)* animals fissioned and 2/8 *Gβ1-4a(RNAi)* animals showed head lysis. Scale bars = 200 μm.



Supplemental Figure S3.7 Additional characterization of polarity in *Gαq1(RNAi)* and *Gβ1-4a(RNAi)* animals. (A) Representative images of *sFRP-1* ISH in animals treated with RNAi targeting *Gαq1* or *Gβ1-4a* 7 dpa. **(B)** Representative images of *notum* ISH in the posterior of *Gβ1-4a(RNAi)* animals 7 days after tail amputation. The green arrowhead indicates potential expression of *notum* in the regenerating tail region in 1/10 animals. **(C)** Representative images of *ChAT* ISH in *Gβ1-4a(RNAi)* animals 14 days after tail amputation. The red dashed lines indicate the amputation site and the black arrowheads indicate expression in the pharynx. Representative images of *foxD* ISH in regenerating heads at **(D)** 3 dpa and **(E)** 7 dpa in *Gαq1(RNAi)* and *Gβ1-4a(RNAi)* animals. Magenta arrowheads indicate absence of *foxD* expression in anterior pole domains. **(F)** Relative expression of anterior pole markers 7 dpa, measured by RT-qPCR and displayed as mean and standard error. Data for *foxD* was analyzed with Unpaired T-Test. * = P-value ≤ 0.05. Data for *sFRP-1* and *notum* were analyzed with One-way ANOVA with multiple comparisons. * = Adjusted P-value ≤ 0.05. ** = Adjusted P-value ≤ 0.005. Scale bars = 200 μm.



Supplemental Figure S3.8 *Gαq1* and *Gβ1-4a* regeneration phenotypes are rescued to varying degrees by co-targeting of posterior-promoting pathway signals. (A) Bar graphs showing results from quantification of brain/body ratios in rescue experiments with *Gαq1(RNAi)* animals, including *bmp4* controls. Error bars represent standard deviations. **(B)** Representative images showing *ChAT* expression in 7 dpa regenerated animals from rescue experiments in (A). **(C)** Relative expression levels of *Gαq1* in animals from rescue experiments, measured by RT-qPCR and displayed as mean and standard error. **(D)** Bar graph showing results from quantification of brain/body ratios from rescue experiments performed alongside animals destined for RT-qPCRs in (C). **(E)** Representative images showing *ChAT* expression 7 dpa from rescue experiments in (D). **(F)** Brain/body ratios from rescue experiments performed with *Gβ1-4a(RNAi)*, displayed as mean and standard deviation. **(G)** Representative images showing *ChAT* expression 7 dpa from rescue experiments in (F). Differences in sample means for (A), (D), and (F) were statistically analyzed with Brown-Forsythe and Welch ANOVA with multiple comparisons. Differences in transcript abundance in (C) were analyzed with One-Way ANOVA with multiple comparisons. ** = Adjusted P-value ≤ 0.006. *** = Adjusted P-value ≤ 0.0006. **** = Adjusted P-value ≤ 0.0001. Scale bars = 200 μm (B and E) and 500 μm (G).



Supplemental Figure S3.9 Many heterotrimeric G protein subunit-encoding genes in *S. mediterranea* display expression enriched in the brain branches. Images from the head region of ISH images showing G protein subunit gene expression in brain branches. Staining patterns displayed include a variety of brain branch patterns, ranging from broad to narrow expression in these structures. Scale bars = 200 μm.

Supplemental File S3.1 Alignment and secondary structure predictions for *Schmidtea mediterranea* Gα, Gβ, and Gγ class subunits. (Page 1) Secondary structure assignments are taken from *Rattus norvegicus* Gαi1, *Bos taurus* Gβ1, and *Bos taurus* Gγ2 (PDB ID: 1GP2). For Gα class subunits, triangles denote residues that contact Gβ, and stars represent the residues involved in GTP hydrolysis and switch II residues (Lys209, Trp211, Ile212, and Phe215) that interact with Gβ1. Highly truncated *S. mediterranea* Gα sequences were excluded (see Sup File S3.3). (Page 5) For Gβ class subunits, triangles denote residues that contact Gγ, diamonds represent regions where contacts form with the switch regions in Gα, and stars indicate the Gβ1 residues (Trp99, Asp228, and Asp246) that interact with the Gαi1 switch II helix. (Page 8) For Gγ class subunits, triangles denote residues that contact Gβ and the star represents the Gγ prenylation site.

Supplemental File S3.2 Phylogenetic trees for *S. mediterranea* Gα, Gβ, and Gγ class subunits. Branch support values displayed in red.

Supplemental File S3.3 Alignment and secondary structure predictions for *S. mediterranea* Gα class subunits including truncated sequences. Secondary structure assignments are taken from *Rattus norvegicus* Gαi1 (PDB ID: 1GP2).

Supplemental Video S3.1 *Gαs1* is required for planarian flipping behavior. A 10 second video showing different reactions when placed dorsal side down displayed by *Ctrl(RNAi)* and *Gαs1(RNAi)* animals 7 dpa, on day 28 of RNAi. Playback set to 8X speed.

Supplemental Video S3.2 Behavior displayed by intact control, *Gαs2(RNAi), Gβx2(RNAi),* and *gcr052(RNAi)* animals. A 10 second video showing locomotion of intact knockdown animals. Playback set to 20X speed. Each grid square = 13x13 mm.

Supplemental Video S3.3 Behavior displayed by regenerating control, *Gαs2(RNAi), Gβx2(RNAi),* and *gcr052(RNAi)* animals. A 10 second video showing locomotion of knockdown animals 10 dpa. Playback set to 20X speed. Each grid square = 13x13 mm.

Supplemental Video S3.4 Behavior displayed by intact control, *Gαq1(RNAi)*, and *Gβ1-4a(RNAi)* animals. A 10 second video showing locomotion of knockdown animals on day 28 of the long-term RNAi paradigm in Supplemental Figure S3.6C. Playback set to 20X speed. Each grid square = 13x13 mm.

Supplemental Video S3.5 Behavior displayed by intact double knockdown animals. A 10 second video showing locomotion of intact control, *Gαs2:Gβx2(RNAi)*, *Gαs2:gcr052(RNAi)*, and *Gβx2:gcr052(RNAi)* animals. Playback set to 20X speed. Each grid square = 13x13 mm.

CHAPTER 4

DISCUSSION

The incredible regenerative ability of planarian flatworms has fascinated people across cultures and time (Elliott & Sánchez Alvarado, 2013). In recent years, planarian research has shown a resurgence due to the development of more complex molecular tools, re-emphasizing the ability of this animal to provide a unique *in vivo* model with which to study regeneration and stem cell regulation (Fincher et al., 2018; Ivankovic et al., 2019; Newmark & Alvarado, 2002; Plass et al., 2018; Rouhana et al., 2013). With more sophisticated tools, the planarian research community has described key traits required to support the regenerative ability of these animals: a genetic wound response program, dynamic polarity, and a population of functionally pluripotent adult stem cells (Ge et al., 2022; Reddien, 2018). However, the underlying cell signaling mechanisms that coordinate the required responses after amputation for robust regeneration of all tissues remain key areas of investigation. Looking to work among other organisms, roles for neuropeptide- and GPCR-related pathways are emerging in regulation of stem cells, wound response, and regeneration (Choi et al., 2015; Czarnecka et al., 2019; Doze & Perez, 2013; S. Li, Yang, et al., 2016; Malva et al., 2012; Nässel & Zandawala, 2019; Ulum et al., 2020). The aim of this thesis was to determine whether neuropeptide- and GPCR-related pathways may function as pro-regenerative and/or instructive signaling mechanisms in planarian regeneration.

PC2 function promotes regeneration and stem cell biology

In Chapter 2, I investigated my hypothesis that neuropeptides are pro-regenerative signals in planarians by targeting an enzyme required to produce mature neuropeptides, PC2 (Collins et al., 2010; Steiner, 1998). I show strong reduction in planarian whole-body regeneration after knocking down *pc2* by RNAi. I also show that planarians die after long-term inhibition of *pc2*. Finally, both the regeneration and survival defects coincide with depletion of the planarian stem cell population. These findings support my hypothesis that neuropeptide-related signaling promotes planarian regeneration, likely through supporting stem cell behaviors like self-renewal or production of progeny.

pc2(RNAi) leads to strong effects on regeneration and stem cells, which is consistent with a previous screen that reported perturbed tissue maintenance and blastema formation in our model species, *S. mediterranea* (Reddien, Bermange, et al., 2005). Additionally, evidence in sexual *S. mediterranea* showed regulatory roles for *pc2* and a downstream neuropeptide (*npy-8*) in germline differentiation and/or maintenance. However, work in a different planarian species, *D. japonica*, reported no effect on regenerative ability after RNAi of *pc2* (Nogi et al., 2009). It is unclear at this time whether the discrepancy of roles for *pc2* between these studies is a function of variable knockdown strength or a true difference between the closely related planarian species. Regarding work in *S. mediterranea*, my results further verified regeneration and tissue maintenance phenotypes in *pc2(RNAi)* animals and provide the first steps of investigation into a mechanism behind these functional outcomes after perturbation of planarian *pc2*.

While neuropeptides play described roles in stem cell regulation in other species (Geloso et al., 2015; Nässel & Zandawala, 2019; Park et al., 2015; Peng et al., 2017; Ulum et al., 2020), a universal role for PC2 in stem cell regulation is not supported. The occurrence of peptide signaling

and *pc2* expression has been validated across many animal phyla (Braks et al., 1992; Cummins et al., 2009; Gómez-Saladín et al., 1994; Nagle et al., 1995), but research assessing the animal-wide roles of *pc2* is less common. The reported work in mice and *Drosophila* indicates specific roles for *pc2* after development, relating to defects in sugar homeostasis (Rhea et al., 2010; Scamuffa et al., 2006). This contrasts with the regulatory roles of neuropeptide Y (NPY), a neuropeptide processed by PC2, in rodent neurogenic niches (Agasse et al., 2008; Malva et al., 2012) and a related peptide, neuropeptide F (NPF), which regulates germline stem cells in *Drosophila* (Ameku et al., 2018).

Why might the phenotypes of *pc2* mutant mice and flies be less severe and/or less relevant to stem cells than when individual neuropeptides are lost? While prohormone convertases show substrate promiscuity *in vitro*, the convertase substrates are more specific *in vivo,* and different cell types may express specific combinations of the processing enzymes (N. Seidah et al., 2013; N. G. Seidah et al., 1990, 1994). For example, many neuropeptide processing enzymes are expressed in the rat central nervous system alongside *pc2*, so the prohormone convertases might have more possible redundant function in processing the neuropeptide prohormone substrates expressed in the central nervous system enzymes (Mesnard et al., 2011; Roebroek et al., 2004; M. Schafer et al., 1993; N. Seidah et al., 2013). PC2-encoding genes also show specific enrichment in endocrine cells, such as the glucagon-producing pancreatic alpha-cells in mice (Furuta et al., 1997, 2001) and the endocrine corpora cardiaca cells (considered homologous to mammalian alpha-cells) in *Drosophila* (Rhea et al., 2010). In these cells, the neuropeptide prohormone substrates may require more specific processing by PC2.

Differences in PC2 function in other animals could also relate to the number of prohormone convertase genes within an animal genome, wherein individual convertases may show more

functional specificity. There are 9 prohormone/proprotein convertase family members identified in mice (Garten, 2018). In contrast, *pc2* is the only currently characterized prohormone convertase in *S. mediterranea*, and the genome encodes three additional predicted convertase genes (Collins et al., 2010). Therefore, individual prohormone convertases in *S. mediterranea* may show broader function and greater substrate cleavage. Finally, work in rodents indicates that a single prohormone convertase can process peptides with opposite and/or antagonistic effects (e.g., products from proneuropeptide Y stimulate feeding whereas products from proopiomelanocortin inhibit feeding behavior), and the reduction of both peptides simultaneously might buffer the resulting phenotypes (Beck, 2006; Benjannet et al., 1991; Paquet et al., 1996; Thomas et al., 1991; Tung et al., 2006).

Limitations of our study of PC2 function

One of the main limitations of this study is the highly pleiotropic phenotype of *pc2* knockdown, which makes it difficult to decouple one phenotype from another. For example, *pc2(RNAi)* leads to reduced animal health and eventual lethality. While I show significant reduction in regeneration, it is unclear whether the regeneration defects reflect an additional role for PC2-processed signals. Because I saw strong effects on stem cell and progenitor populations before the animals appear ill, I believe that stem cell maintenance defects lead to lethality. However, I recommend that potential roles in planarian wound response (Wenemoser et al., 2012; Wenemoser & Reddien, 2010; Wurtzel et al., 2015) and regeneration be further explored.

Considering our results regarding stem cells, another limitation to this study is that we only observed molecular markers for a sample of stem cell progeny. Our results would be strengthened by investigation into markers for additional populations to better understand whether the effects to stem cells are global or specific to certain lineages. Some lineages that I would recommend include

those of the pharynx, due to phenotypes seen after *pc2* perturbation. For example, I show that *pc2(RNAi)* animals eject their pharynges. A similar pharynx ejection phenotype is seen in planarians after RNAi targeting *FoxA*, a transcription factor expressed in the pharynx itself (Adler et al., 2014). Therefore, pharyngeal progenitors may be a logical population to investigate. Once we have a clear understanding of the range of stem cell populations that are affected by *pc2(RNAi)*, the next logical step would be to determine whether the remaining stem cells are still functional.

My work looking at progenitor populations in *pc2(RNAi)* animals also suggests that differentiation is affected in these animals. Once future researchers determine whether the progenitor population reductions are specific or broad, thymidine analogs (e.g., bromodeoxyuridine, BrdU) could be used to determine whether the remaining differentiating cells can integrate into mature tissues (Newmark & Sánchez Alvarado, 2000; van Wolfswinkel et al., 2014). Because stem cells are also lost, we should investigate division particularly because neuropeptides act as mitogenic signals in many animals. Therefore, assessing the level of mitoses (through anti-phospho histone H3 (Newmark & Sánchez Alvarado, 2000)), normalized to the number of stem cells remaining in *pc2(RNAi)* animals, could reveal whether the stem cells are diving at the expected rates in homeostasis and during regeneration. If mitotic rates are normal but there is still a broad reduction of stem cells and progenitors, this could indicate higher rates of cell death. Whole-mount terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays can detect apoptotic cells in planarians (Pellettieri et al., 2010). However, newly developed fluorescence activated cell sorting (FACS) methods can also measure cells undergoing necrotic death (and mitotic cells), making FACS a potentially strategic method to utilize (Peiris et al., 2016).

Finally, when considering my original hypothesis that neuropeptide signaling promotes planarian regeneration, a broader limitation to my study is that, while highly likely, I cannot say for certain that neuropeptides mediate the roles I described for *pc2*. Therefore, to understand the mechanisms at work in *pc2(RNAi)* animals, future researchers should identify and characterize the specific downstream proteins processed by PC2 (Figure 4.1). An additional benefit to characterizing neuropeptides is that such work could decouple the multifaceted phenotype after *pc2* perturbation and clarify the primary outcomes. My RNAi screening of the currently identified neuropeptide prohormones yielded mild results, indicating that future researchers may need to employ more aggressive screening strategies (e.g., stronger RNAi paradigms, more thorough behavior analysis, etc.) and/or target multiple prohormone-encoding genes to account for potential functional redundancy. Future researchers may also consider strategically narrowing the list of prohormone genes to study more thoroughly. For example, Collins and colleagues showed through mass spectrometry that *pc2* knockdown reduced the production of a specific set of peptides (Collins et al., 2010). Therefore, aggressive investigation of the prohormone genes for the set of validated PC2-processed neuropeptides may be a logical strategy to find the neuropeptides responsible for the animal roles of *pc2*. Finally, more complete sequenced genomes for *S. mediterranea* have been developed since the primary characterization of planarian neuropeptides (Grohme et al., 2018; O. Nishimura et al., 2015; Robb et al., 2015). Therefore, reinvestigating the peptidomic data with these more complete references could uncover more potential candidate neuropeptides downstream of PC2.

Neuropeptides in regeneration in a broader context

Once the limitations are addressed, our work in planarians can contribute greatly to the broader field of research on neuropeptide signaling in tissue repair. Neuropeptide signaling appears to be an ancient form of cell communication, with conserved neuropeptide precursors and enzymes important for neuropeptide processing found in non-metazoan species such as unicellular green algae (D. Kumar et al., 2016; Luxmi et al., 2018; Yañez-Guerra et al., 2022). Therefore, fascinating ventures to explore are the evolution of complex neuropeptide repertoires in metazoans, the specific utilization of peptide signaling by neural tissue, and the multitude of roles neuropeptides play in metazoans. Planarians are part of the lophotrochozoan/spiralian clade, which has historically been underrepresented in animal research but is gaining representation as molecular tools are established for new animal models (Bleidorn, 2019; Davison & Neiman, 2021; Ivankovic et al., 2019; Özpolat & Bely, 2016). Planarians also have a unique repertoire of neuropeptide prohormone-encoding genes, with genes showing high homology to both vertebrate and invertebrate counterparts, making the potential implications of results using these animals intriguing (Collins et al., 2010; C. M. Miller & Newmark, 2012; Ong et al., 2016; Shimoyama et al., 2016). How similarly are neuropeptides utilized in planarians compared to other animals?

The stem cell maintenance phenotypes that I documented in *pc2* knockdown animals could indicate that planarians utilize neuropeptides broadly for stem cell regulation. I find this idea particularly exciting, because roles for neuropeptides in stem cell regulation is gaining attention in broader resaerch. Evidence is established for stem cell regulatory roles of neuropeptides in rodent models (Asrican et al., 2020; Y. Zhang et al., 2021), *Drosophila* (Nässel & Zandawala, 2019), and *Hydra* (Takahashi et al., 2000). Rodents, *Drosophila*, *Hydra*, and planarians represent distinct phylogenetic positions (deuterostomia, ecdysozoa, cnidaria, and lophotrochozoa/spiralia,

respectively). Therefore, further exploring phenotypes in planarians could help indicate the degree of conservation for the relationship between neuropeptides and stem cells. For example, do planarian neuropeptides influence many different stem cell behaviors? Can planarian neuropeptides act directly and/or indirectly on stem cells, as seen in *Drosophila* (Nässel & Zandawala, 2019)? Are planarian neuropeptides exerting a mitogenic effect like that seen for stem cell populations in mammals (Malva et al., 2012; Peng et al., 2017) and *Drosophila* (Nässel & Zandawala, 2019)? If future researchers document similar effects in planarians and other animals, will the peptide mediators be homologous? Or will planarians utilize different neuropeptides for a similar physiological process?

Along with the stem cell roles for planarian *pc2*, I also documented strong behavior phenotypes, including some not described in previous literature (e.g., ejecting pharynges). These results indicate that planarians utilize neuropeptides for regulation of many animal behaviors, which appears to be a highly conserved role for neuropeptides in metazoans (Bhat et al., 2021; Kash et al., 2015; Nässel & Zandawala, 2019). Therefore, other exciting avenues for future investigators would be to determine the specific neuropeptide mediators of each *pc2*-connected behavior, discover additional behavior phenotypes for neuropeptides, and elucidate their molecular mechanisms of action. In other vertebrate and invertebrate models, neuropeptides show diverse mechanisms of signaling, including neuromodulation of other small molecule transmitters in processes such as learning and memory (Cropper et al., 2018; van Damme et al., 2021; van den Pol, 2012). A hormonal signaling role for planarian neuropeptides is hypothesized (Collins et al., 2010; C. M. Miller & Newmark, 2012; Shimoyama et al., 2016), but it is unclear whether planarian neuropeptides can also act as neuromodulators. Single-cell sequencing data indicates that planarian neurons can express *pc2* along with other neurotransmitter-producing enzymes and peptide

receptors along with small molecule receptors, which may indicate the potential for cotransmission and neuromodulation (Wyss et al., 2022). However, *in vivo* evidence for these mechanisms of action is not yet available. Understanding how planarian neuropeptides act in neural circuits and exert their control on behavior could help clarify the diversity of ways neuropeptides act in planarians and suggest whether these mechanisms are shared in other animal species.

Heterotrimeric G proteins promote regeneration and polarity re-establishment

In Chapter 3, I investigated my hypothesis that GPCR-related signaling promotes planarian regeneration. After a comprehensive functional screen of planarian heterotrimeric G protein subunits, I showed that multiple G protein subunits play roles in regeneration. Further, I identified two subunits, Gαq1 and Gβ1-4a, that had highly significant effects. I later determined that these subunits both promote successful re-establishment of the anterior and posterior poles. Finally, I determined that loss of Follistatin signaling from the anterior pole appeared to be a key outcome causing these regenerative defects. My results support the hypothesis that GPCR-related signaling plays important roles in promoting and coordinating established processes in planarian regeneration.

To my knowledge, this is the first functional investigation into regenerative roles for heterotrimeric G proteins in our model, *S. mediterranea*. Additionally, work in other planarian species typically validates G protein subunit coupling to a candidate GPCR through pharmacological studies instead of targeting individual subunit genes. Through these pharmacological methods, coupling was validated between Gαs family subunits and a GPCR that affected regenerative polarity in *D. japonica* (called *S7.1R* (Chan et al., 2015, 2016)). Our results also support a role for Gαs class subunits in regeneration, with two of these subunit genes (*Gαs1*

and *Gαs2*) having significant effects. Our results also indicate that Gαq class subunits play key roles, with both Gαq genes (*Gαq1* and *Gαq2*) having significant effects and *Gαq1* being the Gα class subunit with the strongest roles in regeneration in my screen. Gαq family subunits promote axon regeneration in both mice and *C. elegans*, which may indicate that Gαq subunits are utilized in multiple regeneration scenarios (S. Li, Yang, et al., 2016; Shimizu et al., 2022; Shimizu & Hisamoto, 2020).

Limitations of our studies of planarian heterotrimeric G proteins

We are still not clear about how Gαq1 promotes anterior pole regeneration. Because we show evidence that generic wound response is functional in these animals, and because the regeneration of a new anterior pole requires proper stem cell specification and differentiation, we believe that the defect arises in the production of new anterior pole cells. A key question remaining for the interpretation of our results is whether the anterior pole cells are specified at all, and whether they fail to turn on the required gene regulatory networks after specification. Whether anterior pole cells are specified may be clarified by quantifying early anterior pole progenitors (e.g., *zic-1*$^{+}$/*Smedwi-1*$^{+}$ cells) 24 hours after head amputation in *Gαq1(RNAi)* planarians (Scimone, Lapan, et al., 2014; Vásquez-Doorman & Petersen, 2014; Vogg et al., 2014).

Do *Gαq1*$^{+}$ cells provide important instructive signaling to the differentiating pole cells? Is *Gαq1* required for differentiating cells to interpret the local polarity signaling environment? Once the early anterior pole cell progenitor dynamics are characterized, to understand why generating these cells is defective, the next step will be to investigate the spatial relationship between *Gαq1* and anterior pole progenitors. These results could inform whether the relationship between Gαq1 signaling and anterior pole regeneration is cell autonomous or cell non-autonomous. Deciphering

how Gαq1 influences the process of anterior pole regeneration is also clouded by the increasingly complex role for *activin* in regenerative polarity (Cloutier et al., 2021). I found difficulty in exploring expression of *Gαq1* through FISH because it appears to be expressed at low levels in many cells, but there is a characterized antibody that could help identify localization with confidence (Shettigar et al., 2021). A deeper understanding of the relationship between Gαq1 and the regenerating anterior pole and components of the Activin pathway could help define mechanisms through which heterotrimeric G protein signaling (and putative GPCRs) may coordinate processes in planarian regeneration.

One of the broader limitations of my study when considering that my original hypothesis was that GPCR-related signaling promotes planarian regeneration is that I did not yet identify specific GPCRs in regeneration and instead investigated potential roles via the heterotrimeric G proteins. With my work with heterotrimeric G proteins in planarian locomotion, I presented proof-of-principle for a G protein subunit-first approach to identify candidate GPCRs using available transcriptome resources. Therefore, I recommend that the same logic be applied to the subunits that had significant regeneration roles (Figure 4.1). I find this avenue to be one of the most exciting potential future directions of this work, because once the receptors are defined, it can accelerate our understanding of important cell types involved and predict upstream ligands. Additionally, if the defined GPCRs are predicted neuropeptide receptors, it could also suggest a connection between my two objectives.

What could stand in the way of identifying candidate GPCRs? One aspect to consider is that my pipeline relies on datasets that examine mRNA transcripts and therefore are looking at the transcription-level regulation of genes. GPCR and heterotrimeric G protein subunit transcripts can be expressed at low levels, potentially preventing detection through single-cell sequencing

techniques if their levels are below threshold (Fredriksson & Schiöth, 2005; Insel et al., 2015; Soave et al., 2021). GPCRs and G proteins also show a high degree of post-translational regulation, which could indicate that future investigations of the dynamics of these pathways may require robust, protein-level approaches (Patwardhan et al., 2021; Soave et al., 2021). GPCRs may also operate in multimer conformations of functional significance, which might add complexity to finding individual receptors for a specific role (Gahbauer et al., 2018; Milligan et al., 2019). Additionally, although less understood, evidence is emerging for GPCR-independent heterotrimeric G protein activity (B. D. Coleman et al., 2016; de Opakua et al., 2017; DiGiacomo et al., 2018; Matúš & Prömel, 2018). For an example confirmed *in vivo*, a cytosolic factor called DAPLE triggers G protein signaling during neurulation in *Xenopus laevis* (Marivin et al., 2019). Furthermore, heterotrimeric G proteins autoactivate in plants (J. C. Jones et al., 2011). Therefore, the regeneration phenotypes presented in my work could conceivably be independent of upstream GPCRs.

In response to these limitations, I suggest an approach utilizing multiple sequencing datasets. My work relied on (Fincher et al., 2018) for candidate GPCR identification, but other datasets such as may provide additional candidate receptors (Benham-Pyle et al., 2021; Molinaro & Pearson, 2016; Plass et al., 2018; Swapna et al., 2018; van Wolfswinkel et al., 2014; Wurtzel et al., 2015, 2017). The investigation may also require knocking down multiple GPCRs at once to account for potential redundancy. For example, in the list of 13 GPCRs pulled from $G\alpha q1^{+}$ and $G\beta 1\text{-}4a^{+}$ cell clusters, 1 GPCR has a close paralog. If these methods do not identify regenerative GPCRs, future researchers could look into sequencing datasets that investigate upregulated genes during regeneration, which may narrow the list of regeneration-affected GPCRs. Finally, one could investigate groups of receptors based on their predicted ligands. For example, there are 50 peptide-

predicted receptors in the rhodopsin-like family of planarian GPCRs (Saberi et al., 2016). Of the 50 peptide GPCRs, 16 are predicted to bind NPY, which is a neuropeptide with documented roles in stem cell regulation in other animal models.

A wider view of GPCR signaling in regeneration

Once candidate GPCRs are identified, the potential implications of my research extend beyond planarians. GPCRs are one of the most druggable families of receptors, with ~30% of current pharmaceuticals targeting GPCRs (Hauser et al., 2017). The qualities of GPCRs that make them ideal drug targets include their extracellular accessibility, presence of allosteric binding sites allowing for fine-tuned regulatory action, and diversity of activating ligands (Christopoulos et al., 2014). Understanding the downstream heterotrimeric G proteins associated with a desired and/or undesired outcome can also prove highly valuable due to the emerging theme of biased agonism (Violin et al., 2014). Therefore, the candidate GPCRs identified in future work may prove suitable for drug targeting. Potential applications of said drugs would rely on the degree of conservation for the GPCRs in planarian regeneration.

Will regenerative planarian GPCRs have conserved mammalian homologs? Analysis of the planarian GPCR-encoding gene repertoire indicated that 91% of planarian GPCRs belong to the five conserved classes (according to the GRAFs system) (Saberi et al., 2016; Schiöth & Fredriksson, 2005). Furthermore, 143 of the rhodopsin-like family GPCRs cluster with vertebrate counterparts, showing planarians harbor many highly conserved receptor-encoding genes (Saberi et al., 2016). If the GPCRs identified in planarian regeneration share homology with GPCRs found in mammalian genomes, future research could investigate whether the mammalian homologs could be modulated to be pro-regenerative. If the GPCRs are also pro-regenerative in mammals, this

would open the exciting possibility of developing therapies that target the receptor to elicit a more efficient regenerative response in humans.

What if the regenerative planarian GPCRs are divergent from mammalian homologs? 318 out of 461 rhodopsin-like family GPCRs in planarians form divergent subfamilies separate from vertebrate homologs (Saberi et al., 2016). Furthermore, ~6 planarian GPCRs were not homologous to previously reported families, which could indicate that planarians also utilize a small list of flatworm-specific GPCRs (Saberi et al., 2016). If key GPCRs are divergent, future work with the pro-regenerative planarian GPCRs might be relevant in closely related parasitic flatworm species. For example, the parasitic flatworm *Schistosoma mansoni* causes schistosomiasis, a devastating parasitic neglected tropical disease (LoVerde, 2019). Studies indicate multiple comparable aspects between the biology of *S. mansoni* and free-living planarian species used in regeneration research, including a population of adult stem cells resembling the stem cells found in planarians (neoblasts) (Collins III et al., 2013; You et al., 2021). Therefore, drugs that inhibit the function of pro-regenerative planarian GPCRs could be an avenue for anthelminthic therapies.

CONCLUSION

Through the body of work presented in this dissertation, I have provided foundational evidence for the key roles that neuropeptide- and G protein-related signaling pathways play in planarian regeneration. Specifically, I have quantified and initiated the first exploration into mechanisms behind the powerful phenotypes of *pc2(RNAi)* animals. I also completed the first comprehensive functional analysis into roles for planarian heterotrimeric G proteins, showing a multitude of regenerative phenotypes for this group of genes. This dissertation addresses some of the current challenges regarding investigation of neuropeptide and GPCR pathways, such as a lack of

knowledge for broad roles of neuropeptide signaling and the vast number of planarian GPCR-encoding genes. My results indicate that there are many exciting new avenues to investigate to understand how these pathways coordinate regenerative processes in planarians. Additionally, with the new broader understanding of neuropeptide- and GPCR-related signaling in planarians, I hope that these results can accelerate additional research on planarian neuropeptides and GPCRs, further making the planarian a powerful model to understand how these pathways promote complex tissue regeneration.

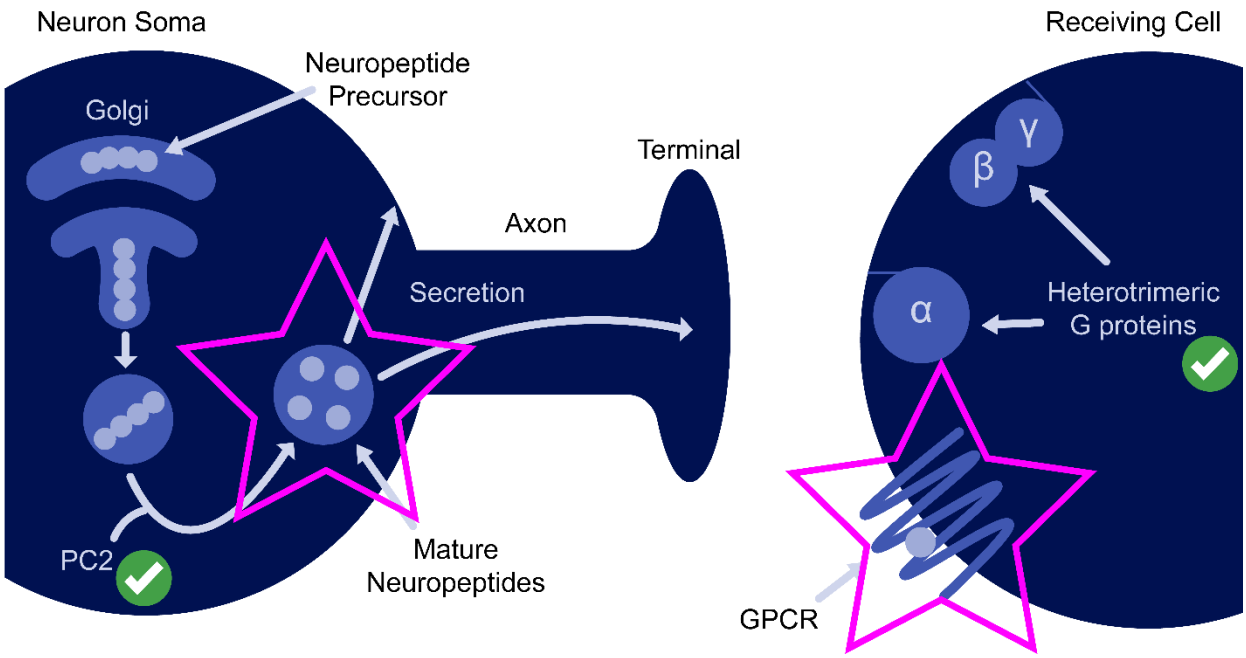


Figure 4.1 Future directions should "work inward" to define the mechanisms for the phenotypes presented in this work. Simplified graphic of a typical neuropeptide signaling pathway. The checkmarks in green represent the completed objectives included in the chapters of my thesis. The magenta stars are highlighting future directions that I believe will help improve the impact of this work, which is identifying the specific neuropeptide signals and GPCRs.

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