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Honor Thesis:

Elucidating the Role of HNF4 in Intestinal Regeneration in the Sea Cucumber *Holothuria glaberrima*

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Abstract: Although transcription factors (TFs) are known to be involved in cellular plasticity, their regulatory roles during regenerative processes remain poorly understood. Echinoderms such as *Holothuria glaberrima* regenerate the intestine after evisceration, providing a deuterostome model to investigate transcriptional control of cellular plasticity. Here, we focus on Hepatocyte Nuclear Factor 4 (HNF4), a TF that maintains epithelial identity and regulates gastrointestinal metabolism and homeostasis. In *H. glaberrima*, transcriptomic data indicate downregulated HNF4 during early intestinal regeneration, suggesting a transient reduction in its activity may enable dedifferentiation, an initial regenerative step. This thesis tests the hypothesis that HNF4 regulates key cellular processes in early regeneration. HNF4 activity was pharmacologically modulated using the agonist NCT and antagonist BIM5078. Regenerative outcomes were assessed through cell proliferation (BrdU), dedifferentiation (phalloidin), extracellular matrix (ECM) remodeling (E6D9), and blastema morphometrics, analyzed via one-way ANOVA. HNF4 activation significantly decreased muscle cell dedifferentiation. Blastema size and cell proliferation showed non-significant decreasing trends, while ECM remodeling remained unchanged. These findings support a process-specific role for HNF4. It may indirectly influence regenerative progression, but its primary regulatory role occurs during dedifferentiation. This work aims to clarify how TFs regulate epithelial identity during regeneration by defining HNF4's role in a non-vertebrate deuterostome.

Introduction:

Regeneration is a process with which every human is familiar, at least to some degree. For example, most people have a general understanding that lizards can regenerate a lost tail. From epic ancient stories to modern films, it is often associated with fiction, assuming that humans or other organisms would be incapable of such a feat. Nonetheless, regeneration, meaning the ability to restore damaged cells, tissues, or organs to their original functionality, is a process conserved across all living organisms. (NIGMS, 2023; Quispe-Parra et al., 2021).

To study the process of regeneration, a variety of model organisms, such as planarians, axolotls, hydras, mice, zebrafish, and echinoderms like sea stars, sea urchins, and sea cucumbers, are employed (Tanaka et al., 2011; Antes et al., 2001; NIGMS, 2023; García-Arrarás et al., 2018). Apart from elucidating the cellular events needed for regeneration to take place, many researchers have turned to understanding and defining the molecular components and mechanisms that regulate and coordinate the cellular events necessary for regeneration to occur. Key events include rapid cell proliferation, programmed cell death or apoptosis (Reyes-Rivera, 2024), dedifferentiation (García-Arrarás et al., 2011), and extracellular matrix (ECM) remodeling (Quiñones et al., 2002). Each one of these cellular processes is regulated by specific gene networks that are in turn regulated by proteins such as transcription factors. These proteins recognize distinct DNA sequences, and this sequence specificity underlies the diversity of transcription factors and of their actions. The present project uses the sea cucumber *Holothuria glaberrima*, an echinoderm that shows remarkable regeneration abilities, including regeneration of the full digestive tract after evisceration (García-Arrarás et al., 1998; 2018), to study whether the transcription factor hepatocyte nuclear factor 4 (HNF4) plays a role in the cellular events necessary for regeneration to occur in this animal.

HNF4 is a highly conserved member of the nuclear receptor (NR) superfamily that regulates a wide array of biological processes, including embryonic development, growth, metabolism, and adult tissue homeostasis (Beinsteiner et al., 2023; Vemuri et al., 2023). It has also been seen as a proliferation suppressor and essential to maintaining cell differentiation (Bonzo et al., 2012; Huck et al., 2019; Lazarevich et al., 2004). HNF4 plays diverse roles in metabolic regulation and development across species such as *Drosophila melanogaster* (FlyBase/SDB, 2025) and *Schmidtea mediterranea* (Lobo et al., 2016; Scimone et al., 2014). In mammals, particularly mice, HNF4 promotes differentiated cell identity in the liver (Bonzo et al., 2012; Huck et al., 2019) and intestine (Vemuri et al., 2023; Chen et al., 2020). Also, the loss or downregulation of HNF4 is associated with dedifferentiation and increased proliferation, as seen in liver regeneration, cancers, and inflammatory intestinal conditions (Bonzo et al., 2012; Huck et al., 2019; Lazarevich et al., 2004; Chen et al., 2020; Vemuri et al., 2023). Since dedifferentiation is essential for regeneration to proceed, and HNF4 maintains differentiation, we hypothesize that HNF4 downregulation is required for dedifferentiation to take place.

To test our hypothesis, we pharmacologically modulated HNF4 levels in *H. glaberrima*, using NCT (N-trans-caffeoyl tyramine) as an agonist and BIM5078 as an antagonist (Lee et al., 2021, 2022; Kiselyuk et al., 2012). We then evaluated cell proliferation, dedifferentiation, ECM remodeling, and blastema formation. Given HNF4's role (Beinsteiner et al., 2023; Vemuri et al., 2023), we anticipate that agonist treatment will delay dedifferentiation, while antagonist treatment will enhance it. If confirmed, this would provide important information on whether HNF4 has a role in the process of intestinal regeneration of *H. glaberrima*.

Literature Review:

Regeneration is a biological process present in many organisms through which they restore damaged or missing tissues. This process involves complex signaling pathways and transcriptional regulation. In addition to rapid cell proliferation, key cellular events include apoptosis, or programmed cell death (Reyes-Rivera et al., 2024); dedifferentiation, where cells revert to a less specialized state, and in some cases, one cell type becomes a different one, and/or differentiation, where dedifferentiated or stem cells give rise to cells with a specific identity (García-Arrarás et al., 2011). Also critical is extracellular matrix (ECM) remodeling, in which enzymes such as matrix metalloproteinases degrade existing ECM so that new components can be synthesized, deposited, or modified, essential in wound healing, organ formation, and cell migration (Quiñones et al., 2002). Finally, cellular memory enables precise tissue patterning and structural restoration (Reizel et al., 2021).

Among animals, this process varies in complexity, with some organisms possessing more extensive regenerative abilities than others. For example, planarians can regenerate a whole organism from a piece of head, trunk, or tail. Hydras can regenerate two whole individuals after being cut in half. Others, like zebrafish, can regenerate their heart, pancreas, eyes, brain, kidney, and spinal cord. In addition, regeneration may be mediated by different mechanisms depending on the organism. In many species with higher regenerative capacity, regeneration after amputation, damage, or injury involves the formation of an anlage or blastema, a specialized bud of dedifferentiating cells that restores missing tissue (Figure 1). This blastema-based mechanism is widespread across taxa: zebrafish regenerate injured fins, axolotls can regenerate nearly any organ or body part, and echinoderms such as brittle stars and sea urchins restore arms or spines through this same process. However, as organismal complexity increases, regenerative capacity generally decreases. Thus, the lack of blastemas has been associated with incomplete or a lack of regeneration. In this context, humans and other complex animals do not form blastemas; instead, they develop scars to heal wounds. They can regrow hair and skin and repair bone fractures, but their overall regenerative capacity is limited. In humans, this is often associated with disease and aging, making it a focus of developmental biology (Tanaka et al., 2011, NIGMS, 2023).

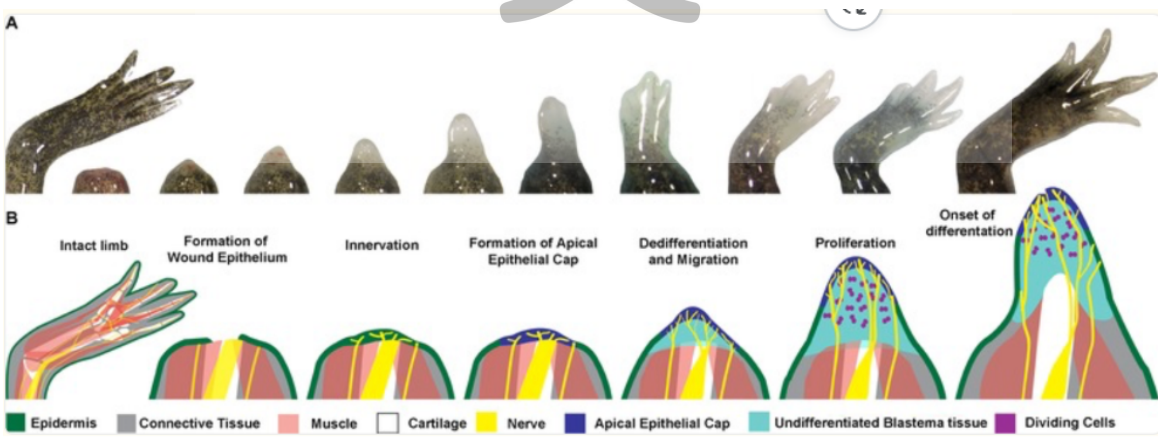


Figure 1 Blastema mediated Axolotl arm regeneration*. Figure obtained from: McCusker C et al. (2015) Regeneration 2(2):54–71.*

In echinoderms, particularly in the subclade Echinozoa, regeneration is especially extensive. Specifically, from the class Holothuroidea, animals such as *Holothuria leucospilota*, *Apostichopus japonicus*, and *Holothuria glaberrima* are sea cucumbers that have been used in regeneration studies as Pacific, Asian,

and Caribbean models, respectively (García-Arrarás et al., 2018). Holothurians belong to the deuterostome lineage, which includes humans. This makes them a suitable model for research with implications beyond developmental biology (García-Arrarás et al., 2018). Within its clade, *H. glaberrima* stands out as a particularly valuable model system. First, its remarkable regenerative capacity is evidenced as it restores its intestines, muscle bands, skin, radial nerve cords, and ambulacral feet after injury (García-Arrarás et al., 2018; San Miguel-Ruiz, 2007). Second, its accessible anatomy makes it practical for experimental manipulation and molecular approaches (García-Arrarás et al., 2018). Third, *H. glaberrima* shares about 7,203 orthogroups with model species such as *Danio rerio*, *Homo sapiens*, *Mus musculus*, and *Strongylocentrotus purpuratus*, highlighting its phylogenetic importance (Medina-Feliciano et al., 2021). Fourth, it conserves key signaling pathways fundamental to regenerative processes such as Wnt/β-catenin, retinoic acid, and ubiquitin–proteasome (Alicea-Delgado et al., 2021; Quispe-Parra et al., 2021; Pasten et al., 2012). Finally, studies in *H. glaberrima* have contributed to the current understanding of cellular dedifferentiation, epithelial-to-mesenchymal transitions, and extracellular matrix remodeling, which are fundamental biological processes of animal repair and restoration (García-Arrarás et al., 2011; Quiñones et al., 2002).

Most notably, *H. glaberrima* has the capacity, after ejecting its internal organs, including the intestine, through a process called evisceration, of completely regenerating them in the span of approximately a month (García-Arrarás et al., 1998) as shown in Figure 2. This short timeline is advantageous for experimental research. Intestinal regeneration occurs first, and it includes the complete restoration of the small and large components, which account for much of the holothurian digestive tract. This makes for a particularly powerful model for studying regenerative mechanisms (García-Arrarás et al., 2018). The process of intestinal regeneration in *H. glaberrima* consists of three key stages: an initial degradation phase, repopulation, and redifferentiation (García-Arrarás et al., 1998). The cellular mechanisms behind these stages include dedifferentiation of mesothelial cells and proliferation of epithelial and mesenchymal cells (García-Arrarás et al., 2011).

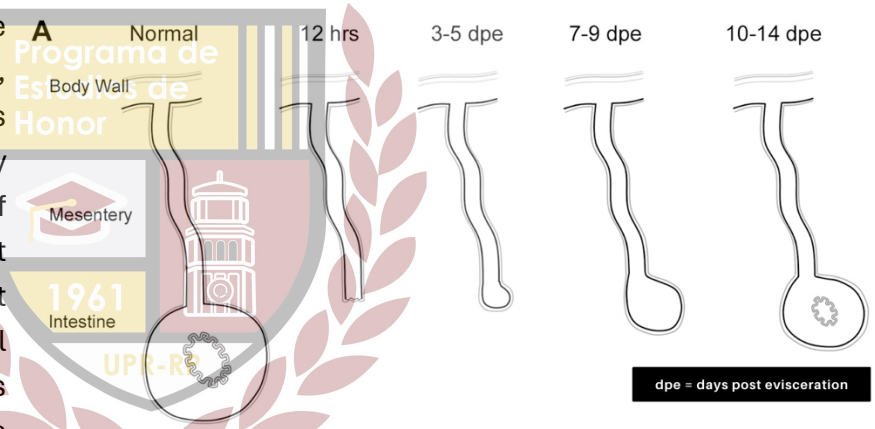


Figure 2 Intestinal regeneration timeline in Holothuria glaberrima. *Transverse cut of a normal intestine attached to the animal's body wall through the mesentery. In the span of 14 days after evisceration, dedifferentiating cells form the blastema that later gives way to a smaller yet functional intestine. Figure adapted from García Arrarás et al.*

In *H. glaberrima*, intestinal muscle cell dedifferentiation has been identified as one of the earliest events in the regenerative response timeline. During this process, muscle cells compact their contractile apparatus and expel it as small, elongated spindle-like structures (SLS), leaving behind a smaller dedifferentiated cell with increased redifferentiation

Figure 3 Intestinal muscle cell dedifferentiation schematic. *Green represents the muscle cell's contractile apparatus and red the cell nucleus. Figure adapted from García Arrarás et al, 2011.*

capacity as shown in Figure 3. Dedifferentiation progresses gradually, starting near the rudiment and extending along the mesentery toward the body wall. Muscle cell dedifferentiation (MCD) is commonly assessed using fluorescent phalloidin staining to visualize actin organization and the formation of spindle-like structures (SLS), which are characteristic of dedifferentiating muscle cells. MCD is typically quantified using semi-quantitative scoring based on the spatial distribution of SLS in the blastema relative to the animal's body wall. As seen in Figure 4 it begins during the first day post-evisceration (dpe) in the region adjacent to the intestinal rudiment and peaks around the fifth dpe, before the reported peak in cell proliferation at approximately eight dpe. The spatial and temporal progression of this process has been used as an indicator of the regenerative stage. Because this event occurs early in the regeneration timeline, intestinal muscle cell dedifferentiation is considered a major early contributor to regeneration and an initial cellular source for intestinal rudiment formation. At present, ongoing studies aim to determine whether these dedifferentiated muscle cells redifferentiate into intestinal muscle cells or transdifferentiate into another cell type, as this remains unknown. Later stages rely more heavily on cell proliferation to expand the regenerating intestine. However, these visible cellular events, depicted in Figure 5, represent only part of the overall regenerative program, which operates under molecular control. These processes involve conserved molecular signaling pathways and likely depend on transcriptional regulators, whose roles remain to be elucidated (Vemuri et al., 2023). Therefore, an important question emerges: what is occurring at the cellular and molecular level in organisms with robust regenerative ability that likely does not occur in humans? (NIGMS, 2023)

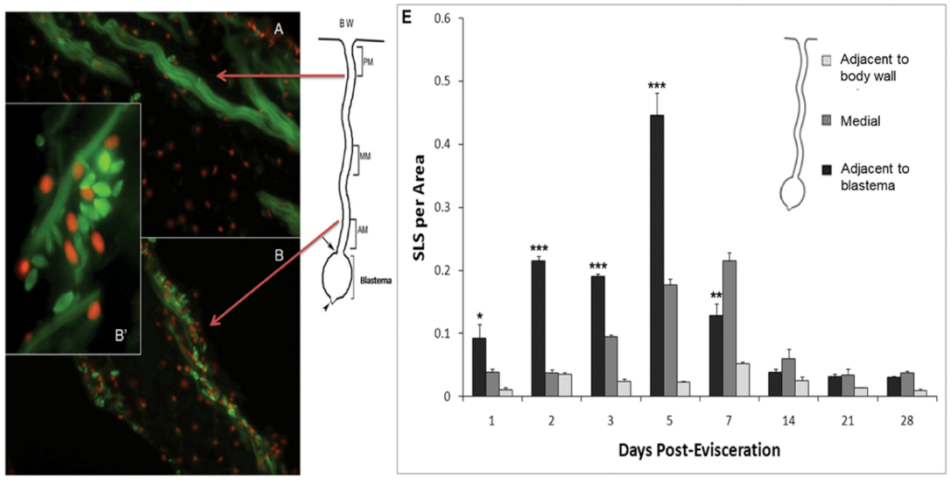


Figure 4 Spatiotemporal progression of SLS formation during intestinal regeneration of H. glaberrima. *Double labeling of muscle fibers and SLS with rhodamine-labeled phalloidin (green) and cell nuclei with DAPI (red) (A-B') Spatiotemporal pattern of SLS formation during intestinal regeneration in H. glaberrima, assessed within distinct regions of the regenerating rudiment (E). Figure adapted from García Arrarás et al, 2011.*

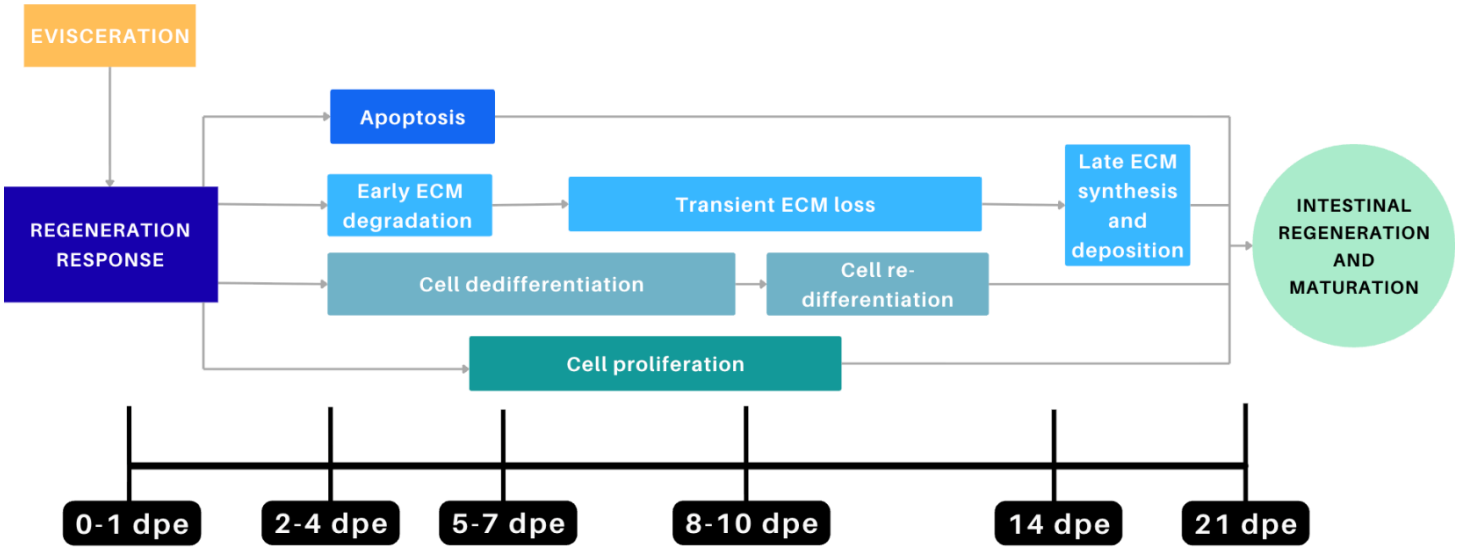


***Figure 5 Timeline of cellular events that are part of the regeneration response in H. glaberrima**. Early events include apoptosis, degradation of the extracellular matrix (ECM), and cell dedifferentiation. Following these events, cell proliferation has been observed to peak during day 8 post-evisceration. Lastly, later in the regeneration response, cell redifferentiation and late extracellular matrix synthesis and deposition occur, leading to intestinal regeneration and maturation.*

Many researchers, in the quest to answer these questions, have focused on searching for the molecular components driving the cellular events associated with the process of regeneration (Quispe-Parra et al., 2021; Tanaka et al., 2011). Among these are cellular receptors and their ligands that may mediate these processes, as well as key enzymes associated with driving the cellular signaling that produces the final biological response (Quispe-Parra et al., 2021). Nonetheless, these essential factors are ultimately regulated by a fundamental aspect: the genetic code in each cell. This is why researchers have turned to learning which genes are responsible for coordinating and regulating the various stages in the regeneration process and what sets these genes apart as "regeneration-associated genes" rather than simply genes that are turned on during any biological process (Scimone et al, 2014; Quispe-Parra et al., 2021).

As is known, genes are discrete sequences of nucleotides that encode specific information, ultimately determining how cellular events take place. They are associated with a myriad of processes, coordinating how cellular events occur. Some genes encode proteins called transcription factors that act as master regulators or molecular switches by turning gene expression ON and OFF. Specifically, transcription factors regulate and mediate the process whereby other genes become mRNA transcripts and therefore end as protein products. Many studies in organisms such as planarians (Scimone et al, 2014), zebrafish (Tanaka et al., 2011), and echinoderms (García-Arrarás et al., 2018). have revealed a variety of transcription factors involved during the process of regeneration. For example, RNA sequencing of neoblasts from injured planarians that were screened for expression patterns demonstrated 33 transcription factors that were identified as being transcribed in specific differentiated cells and in small fractions of neoblasts during regeneration. Specifically, functional validation experiments within the study found that RNA inhibition of the transcription factor FoxA disrupted planarian's pharynx regeneration and healing of dorsal lesions (Scimone et al, 2014).

In *H. glaberrima*, molecular analyses following evisceration have revealed that regeneration engages conserved signaling pathways. Wnt/β-catenin signaling regulates cell proliferation, while retinoic acid signaling is associated with proliferation, muscle differentiation, and rudiment size during intestinal regeneration (Alicea-Delgado et al., 2021; Quispe-Parra et al., 2021). These observations were supported by functional studies, where pharmacological inhibition of Wnt reduced cell proliferation and retinoic acid antagonists diminished proliferation and blocked muscle dedifferentiation (Alicea-Delgado et al., 2021; Quispe-Parra et al., 2021). Similarly, components of the ubiquitin–proteasome system were found to be upregulated during regeneration (Pasten et al., 2012). Beyond these targeted approaches, broader transcriptomic analyses have also revealed differential expression of additional signaling components, transcription factors, and other cellular regulators, including members of the BMP/TGF-β family (Quispe-Parra et al., 2021; Quispe-Parra and Medina-Feliciano, 2021). More recently, single-cell RNA sequencing uncovered the pluripotency of the coelomic epithelium and identified multiple distinct cell populations contributing to regeneration (Medina-Feliciano et al., 2025).

Despite these advances, the transcription factors orchestrating these gene expression programs remain poorly characterized in *H. glaberrima*. Among these, Hepatocyte Nuclear Factor 4 (HNF4) has emerged as a particularly relevant candidate to be explored given its conserved roles in epithelial identity and regeneration, while its function in *H. glaberrima* remains unexplored. What is especially striking is that transcriptomic analyses indicate its expression is strongly downregulated during the early stages of intestinal regeneration in *H. glaberrima,* as is shown in Figure 6, highlighting a potentially critical role that is yet to be explained (Quispe-Parra and Medina-Feliciano, 2021). While HNF4 was not specifically discussed in the published scRNA-seq analysis, transcriptomic bulk data indicate its downregulation during early regeneration, supporting its relevance. Its cell-type-specific expression in scRNA-seq datasets remains to be fully resolved and may require reanalysis of raw data.

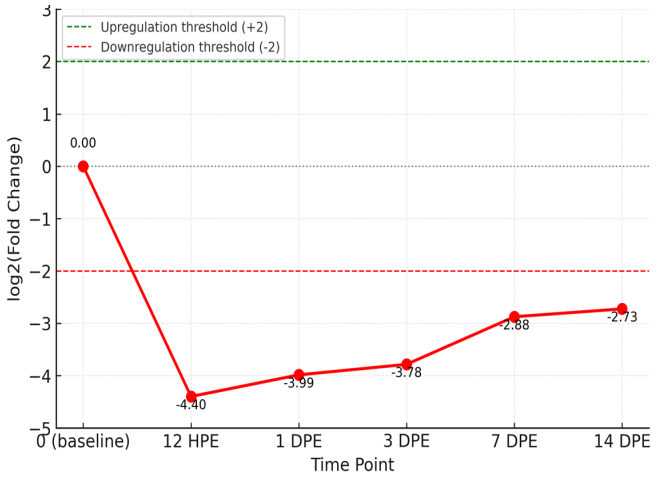


Figure 6 Differential Gene Expression Data of HNF4 during Holothuria glaberrima's intestinal regeneration. *(HPE: hour post evisceration, DPE: day/s post evisceration)*

HNF4 was first identified and cloned by Francis M. Sladek and colleagues in 1990 as a novel liver-enriched transcription factor belonging to the nuclear receptor superfamily. HNF4 is highly conserved among different species and is known to regulate a wide array of biological processes, including embryonic development, growth, metabolism, and adult tissue homeostasis (Sladek et al.,1990; 1991; Vemuri et al., 2023). Since its discovery, it has been determined that paralogous genes HNF4A and HNF4G exist in mammals. These genes code for the HNF4α and HNF4γ paralogous nuclear receptors that arose from a duplication of an ancestral HNF4 gene. In other species, like some teleost fish lineages in addition to the previously mentioned genes, another paralog, HNFB, is present and codes for HNF4β, which also arose from a duplication event. However, it remains poorly characterized due to its low expression (Heppert et al., 2022; Beinsteiner et al., 2023; Vemuri et al., 2023). In vertebrates, gene duplication has resulted in the previously mentioned three HNF4 paralogs, which have diverged to regulate distinct but overlapping transcriptional programs. In contrast, echinoderms, including *Holothuria glaberrima*, retain only a single HNF4 ortholog, annotated as HNF4γ-like, suggesting that this gene represents the ancestral unduplicated form of the family (Medina-Feliciano et al., 2021).

Regarding functions associated with HNF4, it has been demonstrated that HNF4α suppresses excessive proliferation and acts as a tumor suppressor in various epithelial cancers (Lazarevich et al., 2004). In addition, overexpression of HNF4α in adult mice suppresses hepatocyte proliferation, suggesting a conserved mechanism of growth control (Bonzo et al., 2012). In the gastrointestinal context, it has been reported that HNF4 regulates fatty acid oxidation and is essential for the renewal of intestinal stem cells, further emphasizing its importance in epithelial regeneration (Chen et al., 2020). In addition, it is vital for the proper functioning of the intestinal epithelium, including barrier maintenance, lipid metabolism regulation, and intestinal stem cell homeostasis (Vemuri et al., 2023). It has been observed that HNF4 acts in synergy with other transcription factors to enhance gene expression in intestinal cells, providing a basis for understanding its regulatory roles in epithelial differentiation (Antes et al., 2001). Additionally, there

have been recent discoveries regarding HNF4's cofactor associations and tissue-specific actions, reinforcing its versatility in different contexts (Beinsteiner et al., 2023; Vemuri et al., 2023). Moreover, HNF4's regulatory plasticity may depend on cofactor interactions and tissue context (Beinsteiner et al., 2023; Vemuri et al., 2023). For example, interplay between HNF4 and pioneer transcription factors has been observed: FoxA-dependent DNA demethylation initiates epigenetic memory of cellular identity, suggesting a mechanism by which HNF4 and FoxA could cooperate in regeneration (Reizel et al., 2021). This hypothesis is further supported by studies which show that FoxA2 is regulated post-transcriptionally by insulin, indicating that HNF4-related pathways may be subject to hormonal control as well (Howell et al., 2009)

Although HNF4 has been extensively studied in the gastrointestinal system, its role in regeneration remains poorly defined. It has been found that activation of HNF4α is essential for successful completion of liver regeneration, a process involving the re-establishment of quiescence and the suppression of further proliferation (Huck et al., 2019). In planarians, it was predicted *in silico* and validated *in vivo* that HNF4 is a regulatory gene in planarian regeneration (Lobo et al., 2016). Furthermore, research reveals that c-Myc modulates the transcriptional activity of HNF4 during liver regeneration, supporting the notion that HNF4's function is embedded within broader regulatory networks (Kotulkar et al., 2024). Together, these findings position HNF4 as a key factor capable of balancing both proliferation and differentiation in regeneration.

Altogether, the scientific literature, ranging from classical histological observations to modern transcriptomic and structural studies, not only justifies the focus on HNF4 in this project but also offers a theoretical scaffold for hypothesizing that HNF4 is involved with both proliferation and redifferentiation phases in the intestinal regeneration of *H. glaberrima*. These insights guide the experimental design and underscore the relevance of transcriptional regulation in regenerative biology.

Theoretical Framework:

This study is based on fundamental scientific principles. As established by cellular biology, a myriad of coordinated events, including dedifferentiation, proliferation, and extracellular matrix remodeling must occur to restore lost or damaged tissue (García-Arrarás et al., 2011; Reyes-Rivera et al, 2024; Quiñones et al., 2002). These processes are rooted in the principle that DNA is read by cellular machinery and subsequently genes are expressed to modulate biological responses. During this process, transcription factors act as molecular "switches" that regulate gene expression (Vemuri et al., 2023). Regarding HNF4, when it is active it promotes and maintains differentiated cellular states, as shown in the cartoon in Figure 7 (Bonzo et al., 2012; Chen et al., 2020; Huck et al., 2019; Vemuri et al., 2023). Conversely, when HNF4 expression is suppressed or absent it is associated with cellular dedifferentiation and proliferation (Lazarevich et al., 2004; Bonzo et al., 2012; Huck et al., 2019; Vemuri et al., 2023). This principle reflects the role of transcription factors as molecular "switches" coordinating gene expression

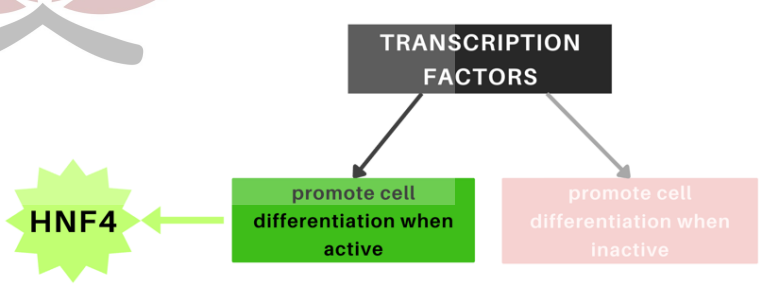


Figure 7 HNF4 is a transcription factor that has been seen to promote cell differentiation when active.

and, in turn, controlling the cellular events necessary for regeneration (Tanaka et al., 2011; Vemuri et al., 2023; NIGMS, 2023)

In addition, HNF4 stands out as a transcription factor with a biochemical nature as a homodimeric protein possessing ligand and DNA binding sites (Beinsteiner et al., 2023; Vemuri et al., 2023). This structural property makes it central to the regulation of differentiation and dedifferentiation and it provides the basis for pharmacological modulation through activation and inhibition experiments (Lee et al., 2021; 2022; Kiselyuk et al., 2012). The interaction between proteins, ligands, and nucleic acids is governed by biochemical affinity, which explains how HNF4 and its ligand's function and how drugs influence its activity (Beinsteiner et al., 2023; Vemuri et al., 2023). Conceptually, this research perceives HNF4 as a molecular regulator of the cellular events that underlie intestinal regeneration in *Holothuria glaberrima* (Quispe-Parra and Medina-Feliciano, 2021; Medina-Feliciano et al., 2025; Quispe-Parra et al., 2021; Reyes-Rivera et al, 2024; Vemuri et al., 2023; Chen et al., 2020). While the literature review details the experimental findings that support this view, the theoretical foundation rests on understanding regeneration as a process driven by gene expression control, transcriptional regulation, and molecular interactions (Tanaka et al, 2011, Quispe-Parra et al., 2021; NIGMS, 2023).

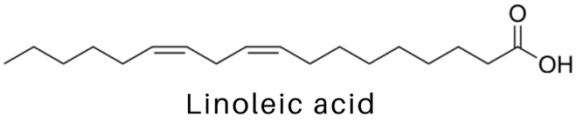


Figure 8 HNF4 is constitutively active and functions with a reversible interaction with an endogenous ligand, the essential fatty acid linoleic acid. *This makes it a potential target for pharmacologic modulation. (Beinsteiner et al., 2023)*

Justification:

Metabolic and digestive diseases remain a major cause of mortality and disease in humans, largely due to the limited regenerative capacity of the human gastrointestinal system (NIGMS, 2023, Tanaka et al., 2011). While some advances have been made in promoting repair of specific epithelial layers, complete organ regeneration remains elusive in vertebrates (NIGMS, 2023, Tanaka et al., 2011). In contrast, echinoderms such as *Holothuria glaberrima* exhibit remarkable regenerative potential, capable of regenerating their entire digestive tract following evisceration (García-Arrarás et al., 1998; 2018). This ability makes them particularly valuable for studying the cellular and molecular foundations of regeneration (García-Arrarás et al., 2018).

The purpose of this research is to establish whether the modulation of HNF4, through its activation with an HNF4 agonist (NCT) or its inhibition with an antagonist (BIM5078), affects the initial phases of intestinal regeneration in the sea cucumber, *H. glaberrima*. The focus is on the impact on initial dedifferentiation, examining its repercussions on other cellular processes such as proliferation, extracellular matrix remodeling, and blastema size. By investigating whether HNF4 functions as a regulator of cellular differentiation involved during the regeneration process, the current study seeks to bridge a critical gap in our understanding of how intestinal regeneration occurs in deuterostomes (Quispe-Parra et al., 2021; Medina-Feliciano et al., 2025; Quispe-Parra and Medina-Feliciano, 2021). This analysis aims to provide a deeper understanding of the molecular processes that control tissue regeneration in an alternative model, thus expanding the paradigm of developmental biology and regeneration beyond traditional organisms (García-Arrarás et al., 2018; Tanaka et al., 2011; NIGMS, 2023).

Importantly, HNF4 is not only relevant in regenerative contexts but also has broad biomedical significance. HNF4 is a conserved transcription factor that plays central roles in epithelial identity, metabolic regulation, and gastrointestinal homeostasis, and its dysregulation has been associated with multiple disease contexts, including hepatocellular, gastric, and colon cancers; MODY1 and type 2 diabetes; fatty liver disease, obesity, inflammatory bowel disease, and several hepatic and renal disorders (Vemuri et al., 2023). Despite extensive study in homeostatic and disease settings, far less is known about how HNF4 activity is dynamically modulated during whole-organ regeneration. Studying HNF4 regulation in a highly regenerative model therefore helps address this gap and may provide insight into how transcriptional control of cell identity is temporarily relaxed and later restored during tissue rebuilding.

Although initially associated with the liver, HNF4 is now known to be expressed not only in the liver but also in other organs such as the pancreas, intestine, kidney, and stomach, as well as in epithelial tissues (Vemuri et al., 2023). With this in mind, we found HNF4 naturally expressed in *H. glaberrima*'s intestinal tissue, present throughout the first two weeks following evisceration in the transcriptomic data (Quispe-Parra and Medina-Feliciano, 2021; Medina-Feliciano et al., 2025), which allows us to see a "snapshot" of gene expression and its relative abundance. However, as shown previously in Figure 6, HNF4's expression is noticeably downregulated during the early stages of intestinal regeneration in *H. glaberrima* (Quispe-Parra and Medina-Feliciano, 2021) persisting through day 14 post evisceration.

This temporal pattern coincides with the window during which dedifferentiation occurs in *H. glaberrima*'s intestinal regeneration (Figure 3). As established in the literature, HNF4 is closely linked to the maintenance of cellular differentiation, while its suppression allows for dedifferentiation (Vemuri et al., 2023; Bonzo et al., 2012; Huck et al., 2019; Lazarevich et al., 2004; Chen et al., 2020). Also, in *H. glaberrima*, apoptosis is one of the first cellular events after evisceration (Reyes-Rivera et al, 2024), followed by dedifferentiation, a key process that initiates the regenerative cascade (García-Arrarás et al., 2011). Since dedifferentiation is essential for regeneration to proceed in *H. glaberrima*, and HNF4 maintains cell differentiation, we hypothesize that HNF4 downregulation is required for dedifferentiation to take place.

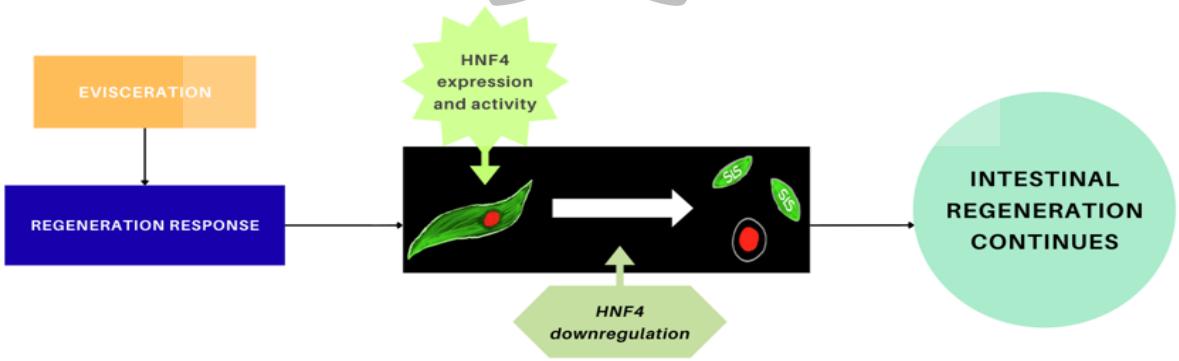
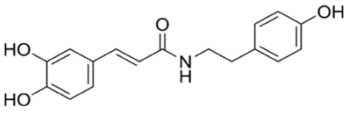


Figure 9 Hypothesis. *Regeneration requires cells to differentiate. HNF4 maintains cells in a differentiated state. Therefore, we propose that HNF4's downregulation is needed for dedifferentiation to occur.*

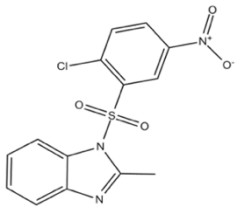
To evaluate this, we experimentally manipulated HNF4 activity in *H. glaberrima* with pharmacological drugs. For the activation, we used the small-molecule agonist NCT (N-trans-Caffeoyl Tyramine). NCT has been utilized to achieve pharmacological activation of HNF4 in in vivo mouse models, as well as in mouse and human organoids, specifically targeting Paneth and epithelial cells. As a potent activator, NCT has been proven to be more effective than other agonists, such as alverine, a weak activator, and NFT, a

derivative that differs from NCT by a single methyl group (Lee et al., 2021; 2022). These studies indicate that NCT provides new insights into HNF4 biology by stabilizing the receptor for longer periods, thereby extending its activity and boosting downstream gene expression.

For inhibition of HNF4 function, we employed the small-molecule antagonist BIM5078 (1-(2'-chloro-5'-nitrobenzenesulfonyl)-2-methylbenzimidazole). This compound was selected based on its demonstrated ability to suppress HNF4 target gene expression via direct binding to the receptor. BIM5078 exhibits higher binding affinity to HNF4 than related derivatives, effectively preventing the receptor from engaging DNA and activating downstream transcriptional programs (Kiselyuk et al., 2012; Kim et al., 2019; Vemuri et al., 2023). Both BIM5078 and its analog, BI6015, have been shown to potently reduce HNF4 activity in vivo in mice as well as in a range of tumorigenic cell lines, highlighting their utility as effective HNF4 antagonists.



NCT



BIM5078

Figure 10 HNF4 can be modulated by small molecules. *HNF4's agonist, NCT and its antagonist BIM5078.*

Compounds such as BIM5078 and NCT interact with the ligand-binding domain of HNF4α by occupying the hydrophobic pocket that normally binds fatty acids. Within this pocket, the ligands are stabilized through hydrophobic interactions with residues lining the cavity and hydrogen-bond interactions with polar residues that help anchor the ligand (Beinsteiner et al., 2023; Kiselyuk et al., 2012). These interactions influence the receptor's conformation and can modulate its transcriptional activity. (Vemuri et al., 2023; Sladek et al., 2024)

Although the structure of HNF4 has not yet been experimentally resolved in echinoderms, HNF4 is an evolutionarily conserved nuclear receptor, and the structural features that define its ligand-binding domain are broadly conserved across metazoans (Beinsteiner et al., 2023; Sladek et al., 2024; Heppert et al., 2022). Even when specific amino acids differ between species, substitutions that preserve similar physicochemical properties, such as hydrophobicity or charge, often maintain the overall structure and function of the domain. Therefore, this structural conservation suggests that compounds such as BIM5078 and NCT could potentially interact with HNF4 orthologs in echinoderms.

Lastly, to determine the pharmacological effects of the drugs, we analyzed the key cellular events involved in intestinal regeneration in *H. glaberrima*: cell dedifferentiation, ECM remodeling, cell proliferation, and blastema formation.

Methodology:

Specimens were collected from Puerto Rico's northeast rocky shore and maintained in tanks of filtered seawater to ensure water cleanliness. Four pre-washed tanks were prepared and labeled for the experimental groups: no-injection control, DMSO control, NCT injections, and BIM5078 injections. Each tank housed four animals, for a total of sixteen.

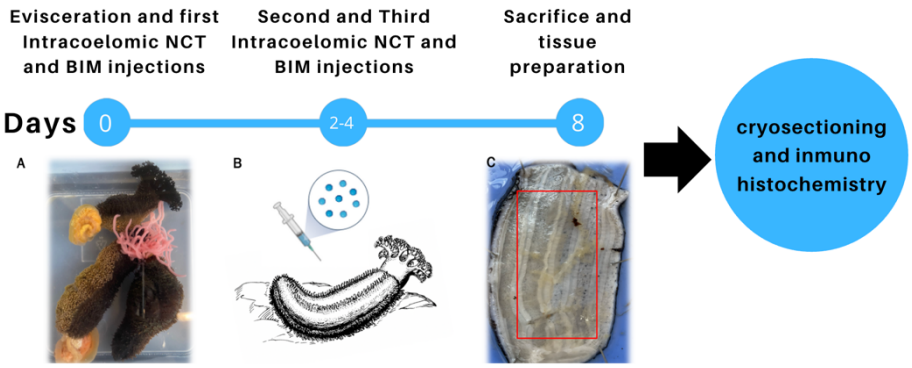


Figure 11 Experimental design of pharmacological modulation of HNF4's activity

On day 0, animals were eviscerated by injecting 3–4 mL of 0.35 M KCl into the coelom using a 5 mL syringe, as described previously (García-Arrarás et al., 1998). 1-hour post-evisceration (hpe), the radial nerve cord (RNC) was injured along the ventral (V) and dorsal (D) axes. Although a fellow student will analyze the regenerating RNC, this project focuses solely on intestinal regeneration.

After evisceration, animals were placed in their respective tanks and water changes occurred every 2 days. Injections took place on day 0-, 2-, and 4-days post-evisceration (dpe). The DMSO control group received a 200 µL injection of the vehicle used for the drugs (DMSO diluted in seawater). HNF4 activation was induced by injecting 200 µL of 0.167 mM NCT, and inhibition was achieved by injecting 200 µL of 1.775×10^{-6} M BIM5078, doses that have proven effective in various models (Lee et al., 2021; 2022; Kiselyuk et al., 2012). Animals at 7 dpe were injected with BrdU at a concentration of 0.5 mg/100 µL per kg (animal wet weight) in a 400 µL injection and sacrificed at 8 dpe, corresponding to the peak of proliferative activity identified in the lab database.

Animals were anesthetized for 30 minutes to 1 hour, then sacrificed, and the intestinal tissue was dissected from the most posterior part of the intestine, just above the cloaca. During dissection, the tissue was pinned flat in a petri dish, covered with 4% paraformaldehyde (PFA) for at least 5 minutes, and transferred to labeled, pre-washed vials to remain submerged in PFA overnight. The following day, tissues underwent three 10-minute PBS washes. Vials were filled with 40% sucrose and stored at 4 °C until cryosectioning. For cryosection preparation, the bottom of a mold was coated with OCT embedding medium (TissueTek OCT; Miles Inc.). Tissues were removed from sucrose solution, placed ventral side down, fully embedded in OCT, and rapidly frozen in a pre-cooled cryostat. Once frozen, the block was wrapped in paraffin film for protection and stored in −20 °C freezer until use. Tissue sections (25 µm thick) were cut using a Leica CM1850 cryostat at −35 °C, then transferred to Poly-L-lysine-coated slides, air-dried for 1 hour, and stored in slide boxes until further use for histochemical and immunostaining procedures (García-Arrarás et al., 1998). For each animal, fifteen slides were prepared (sixty per group), with four tissue sections per slide. To ensure representative sampling, two slides per animal for the proliferation assay (8 slides per group per assay) and one slide per animal for dedifferentiation and ECM remodeling (4 slides per group per assay) were analyzed. Fluorescence imaging was performed using a Nikon Eclipse DS-Qi2 microscope. A solution consisting of

50 µL of 10 mg/mL DAPI stock diluted into 24 mL of 0.1 M PBS and 25 mL of glycerol was prepared to counterstain nuclei and mount slides. Finally, blastema size was quantified using ImageJ software at 10–20× magnification.

Dedifferentiation (Phalloidin): Fluorescently labelled phalloidin was used to assess cell dedifferentiation. Phalloidin is a toxin that binds polymerized actin, a molecule present in muscle cells. In dedifferentiating cells this polymerized actin is expelled in spindle-like structures (SLS). Thus, muscle fibers and SLS were visualized following the procedure of Reyes-Rivera and colleagues (2023), of submerging slides in PBS for 15 minutes in a humid chamber. Phalloidin-TRITC (1:1000; Sigma P1951) was then added and incubated for 1 hour After three 15-min PBS washes, DAPI mounting media was added before coverslipping. Slides were dried overnight, sealed with nail polish, and air-dried again. Slides were observed under 40X magnification and a semi-quantitative scale (0–5) was used to assign a muscle cell dedifferentiation score (MCD score) based on the spatial distribution of SLS in the blastema relative to the animal's body wall. We averaged the four section scores into one slide score; because there is one slide per animal, that score will represent the animal (Reyes-Rivera et al, 2024).

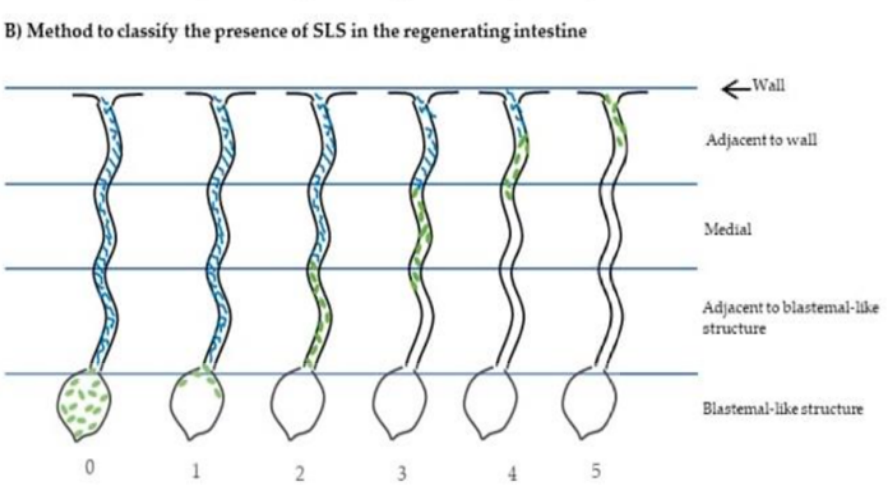


Figure 12 A Semi-Quantitative Scale to Score Dedifferentiation

ECM Remodeling (E6D9): Extracellular matrix remodeling was analyzed by immunolabeling with the anti-collagen antibody, E6D9 (Díaz-Díaz et al., 2021), using CY3-conjugated secondary antibody as the fluorescent marker (GAM-Cy3, 1:1000; Jackson ImmunoResearch). Slides are air-dried on a cool setting for 1 hour. Then, the centrifuged cultured supernatant of antibody E6D9 producing clones was applied overnight in a humid chamber. The next day, three 15-minute PBS (0.1 M) washes were performed before incubation with the CY3-conjugated secondary antibody followed by DAPI mounting and coverslipping. Slides dried overnight, were sealed with nail polish, and air-dried again. A semi-quantitative scale (0–5) under 10–20× magnification was used, with scoring and averaging as in the Phalloidin assay. Collagen was selected due to its established association with ECM remodeling during *H. glaberrima* regeneration (Quiñones et al., 2002).

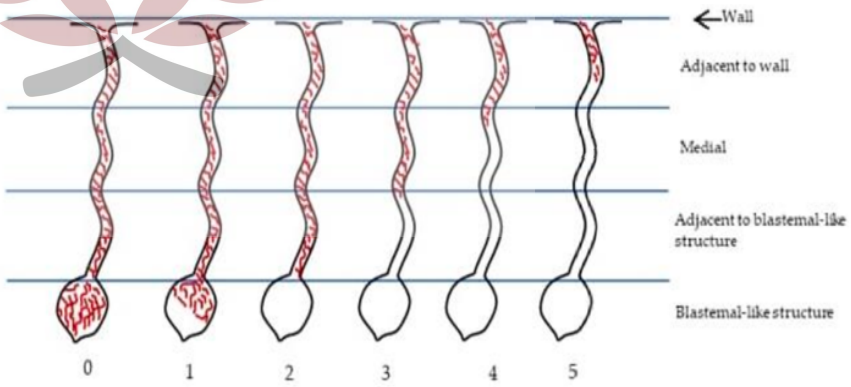


Figure 13 A Semi-Quantitative Scale to Score Extracellular Matrix Remodelling

Cell Proliferation (BrdU): Animals at 7 dpe were injected with BrdU at a concentration of 0.5 mg/100 µL per kg (animal wet weight) in a 400 µL injection, and sacrificed at 8 dpe, corresponding to the peak of proliferative activity (García-Arrarás et al., 2011). Slides were first washed for 5 minutes in PBS. They were then incubated for 1 hour. in 0.05 M HCl, followed by two additional 15-minute PBS washes. The primary antibody anti-BrdU (1:5; GE Healthcare RPN202) was applied and incubated overnight. The following day, slides underwent three 15-minute PBS washes before incubation with the CY3-conjugated secondary antibody as the fluorescent marker (GAM-Cy3, 1:1000; Jackson ImmunoResearch) for 1 hour. This was followed by three final 15-minute. PBS washes. Nuclei were counterstained with DAPI, and slides were left to dry overnight, sealed with nail polish, and air-dried again prior to imaging (Reyes-Rivera et al, 2024)

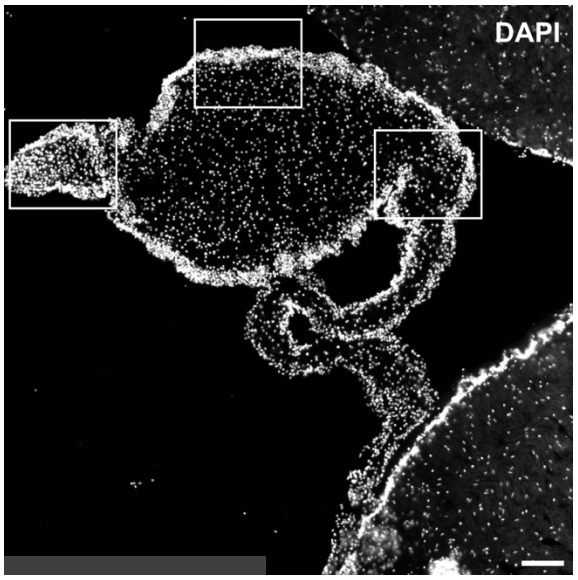


Figure14 Three anatomical quadrants in the blastema used to analyze cellular proliferation. *Left corner quadrant: tip, top middle qudrant: lateral blastema, and right quadrant: proximal mesentery. Bar = 100µm*

Regenerating blastemas were examined under a fluorescence microscope at 60× magnification across three anatomical quadrants: tip, lateral, and proximal mesentery. In each quadrant, DAPI labeled nuclei and DAPI–BrdU colocalized nuclei were counted in the four tissue sections on each of two slides (eight sections total). These counts were divided by eight to yield the animal's mean BrdU count per quadrant. Mean values from the four animals were averaged to generate the group's proliferation index for each quadrant. A total blastema proliferation index was obtained by summing the three quadrant means.

Blastema Size Analysis: Blastema size was quantified using images obtained with a Nikon Eclipse microscope equipped with a DS-Qi2 camera at 10–20× magnification. ImageJ software tools were used to determine the blastema area using the bordering tool, measuring the entirety of the blastema up to the proximal side, excluding the mesentery.

Statistical Analysis: All quantitative data, blastema size, dedifferentiation (Phalloidin) scores, ECM remodeling (E6D9) scores, and BrdU proliferation indices were analyzed by one-way ANOVA with treatment and region (or time point) as the two factors. Post-hoc pairwise comparisons used Tukey's test. Graphs illustrating mean ± SEM for each group and factor combination were generated.

Antigen Table

Antigen	Host & Type	Immunogen Source	Dilution Used
Anti-BrdU	Mouse monoclonal	Proliferative cells (Sigma GERPN202)	1:4
E6D9	Mouse monoclonal	Collagen fiber (Dr. García-Arrarás lab)	1:4

Results:

HNF4 activation seems to decrease blastema size: Representative DAPI-stained transverse sections at 10X magnification illustrate intestinal formation in Control, DMSO, NCT, and BIM5078-treated animals (Figure 15A–D). Control and DMSO-treated animals displayed well-defined rudiments consistent with previously described 7-dpe morphology, including a rod-like structure and organized thickening at the distal mesentery (García-Arrarás et al., 2011). In contrast, NCT-treated animals exhibited increased structural variability, with some specimens presenting reduced rudiment thickness or less consolidated organization. Overall blastema morphology and cellular distribution appeared comparable across groups. Quantification of blastema area (Figure 15E) was analyzed using a one-way ANOVA followed by Tukey's multiple comparisons test. No significant differences in blastema area were detected among treatment groups. Although NCT-treated animals exhibited a trend toward reduced blastema area, this difference did not reach statistical significance. These results indicate that pharmacological modulation under these conditions did not significantly alter overall blastema size.

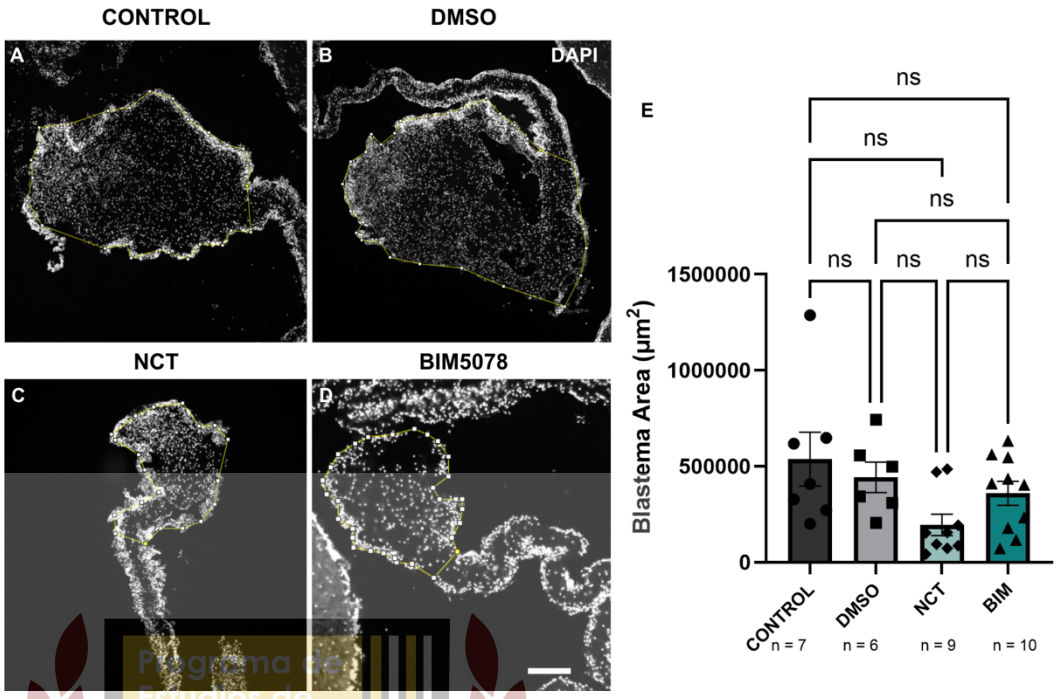
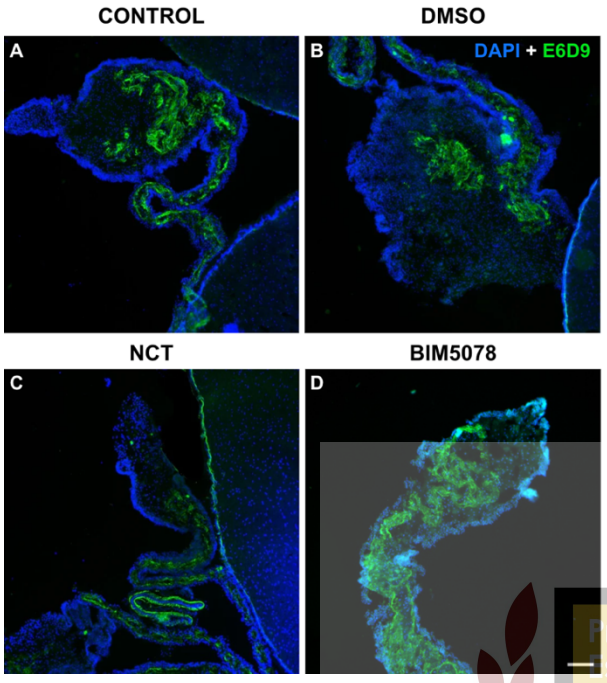


Figure 15 Effect of HNF4 activation on blastema size. *Transverse sections of Control (A), DMSO (B), NCT (C), and BIM (D) treated animals at 8 dpi, showing intestinal regeneration. (E) Quantification of blastema size between treatments data are presented as mean ± SD. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test. Comparisons are indicated as: P ≥ 0.05, not significant (ns). DAPI labels cell nuclei. Bar = 100µm*

HNF4 modulation does not alter ECM remodeling: Extracellular matrix remodeling was examined by immunolabeling fibrous collagen using the E6D9 antibody and applying a semi-quantitative scoring system. Representative transverse sections at 10X magnification illustrate the blastema structure outlined by DAPI stained nuclei in blue and the fibrous collagen in green marked by the E6D9 antibody in Control, DMSO, NCT, and BIM5078-treated animals (Figure 16A–D). Overall, the presence of fibrous collagen inside the blastema appeared comparable across groups. Quantification of the extracellular matrix score (Figure 16E) was analyzed using a one-way ANOVA followed by Tukey's multiple comparisons test. No statistically significant differences in ECM remodeling scores were detected among treatment groups. However, marked variability was observed within the NCT-treated group, with individual intestines displaying heterogeneous collagen organization and remodeling patterns across sections. In contrast, ECM scores in Control, DMSO and BIM5078-treated intestines were more tightly clustered, indicating greater consistency among animals despite the absence of a significant difference relative to NCT-treated animals. These

results indicate that pharmacological modulation under these conditions did not significantly alter overall extracellular matrix remodeling.



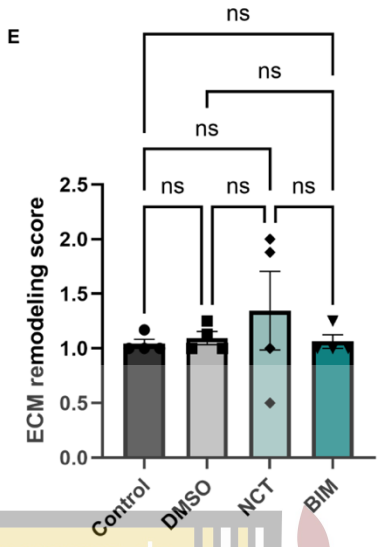


Figure 16 Effect of HNF4 activation on extracellular matrix remodeling. *Transverse sections of Control (A), DMSO (B), NCT (C), and BIM (D) treated animals at 8 dpi, showing intestinal regeneration. (E) Classification of ECM remodeling between treatments data are presented as mean ± SD. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test. Comparisons are indicated as: P ≥ 0.05, not significant (ns). DAPI labels cell nuclei and E6D9 labels collagen fibers. Bar = 100µm*

HNF4 activation decreases muscle cell dedifferentiation: Cellular dedifferentiation during intestinal regeneration was assessed using phalloidin staining to visualize spindle-like structures (SLS) associated with actin reorganization. Representative transverse sections at 10X and 40X magnification illustrate the blastema structure outlined by DAPI stained nuclei in blue and the SLS in green marked by phalloidin in Control, DMSO, NCT, and BIM5078-treated animals (Figure 17A–D and A'-D'). Quantification of muscle cell dedifferentiation scores assigned based on the semiquantitative scale in Figure 12 (Figure 17E) was analyzed using a one-way ANOVA followed by Tukey's multiple comparisons test. Semi-quantitative analysis revealed a significant reduction in dedifferentiation scores in intestines treated with the HNF4 agonist (NCT) relative to Control, DMSO, and BIM5078-treated animals. These results indicate that pharmacological modulation under these conditions significantly altered muscle cell dedifferentiation.

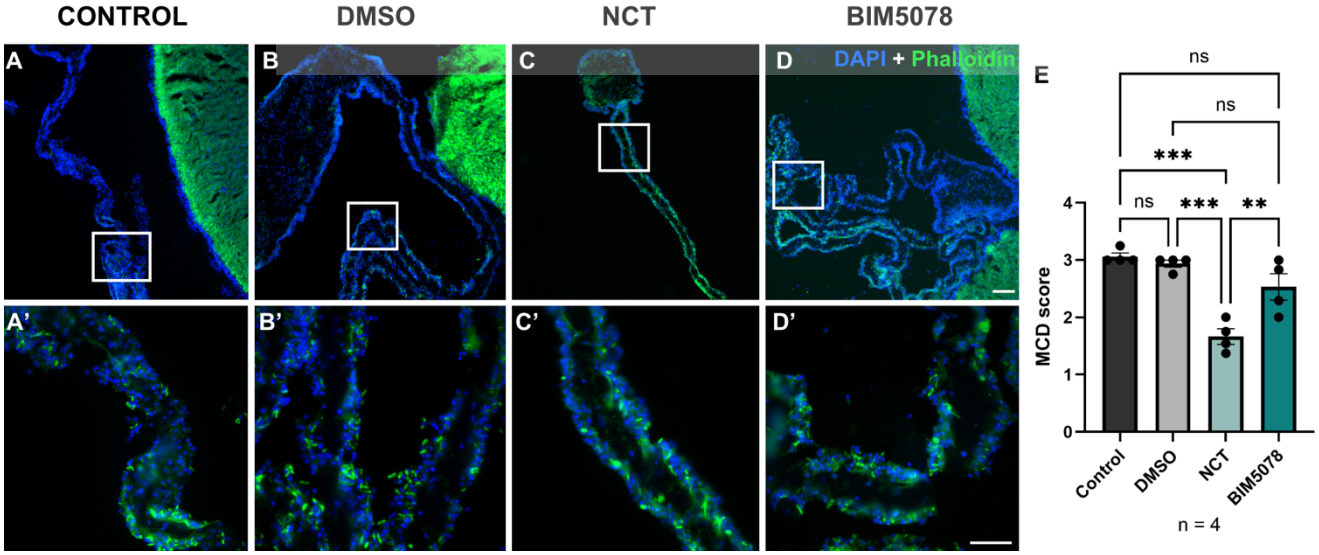


Figure 17 Effect of HNF4 activation on cellular dedifferentiation. *Transverse sections of Control (A, A'), DMSO (B, B'), NCT (C, C'), and BIM (D, D') treated animals at 8 dpi, showing intestinal regeneration. (E) Classification of SLS between treatments data are presented as mean ± SD. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test. Comparisons are indicated as: P ≥ 0.05, not significant (ns); P < 0.005 (**); P < 0.001 (***); P < 0.0001 (***). DAPI labels cell nuclei and phalloidin labels spindle-like structures. Bar = (A-D)100µm, (A'-D') 50µm*

HNF4 activation does not seem to alter cell proliferation: Representative DAPI-stained transverse sections at 60X illustrate the blastema structure outlined by DAPI stained nuclei in blue and proliferating cells (BrdU+) marked in green in Control, DMSO, NCT, and BIM5078-treated animals (Figure 18A, B, D, E). Quantification of proliferative cells (Figure 18E) was analyzed using a one-way ANOVA followed by Tukey's multiple comparisons test. Cell proliferation during intestinal regeneration was quantified by BrdU incorporation at 8 days post-evisceration. BrdU-positive nuclei were counted, and the percentage of BrdU-labeled cells per blastema was calculated. No statistically significant differences in proliferation indices were detected among treatment groups. These results indicate that pharmacological modulation under these conditions did not significantly alter cellular proliferation.

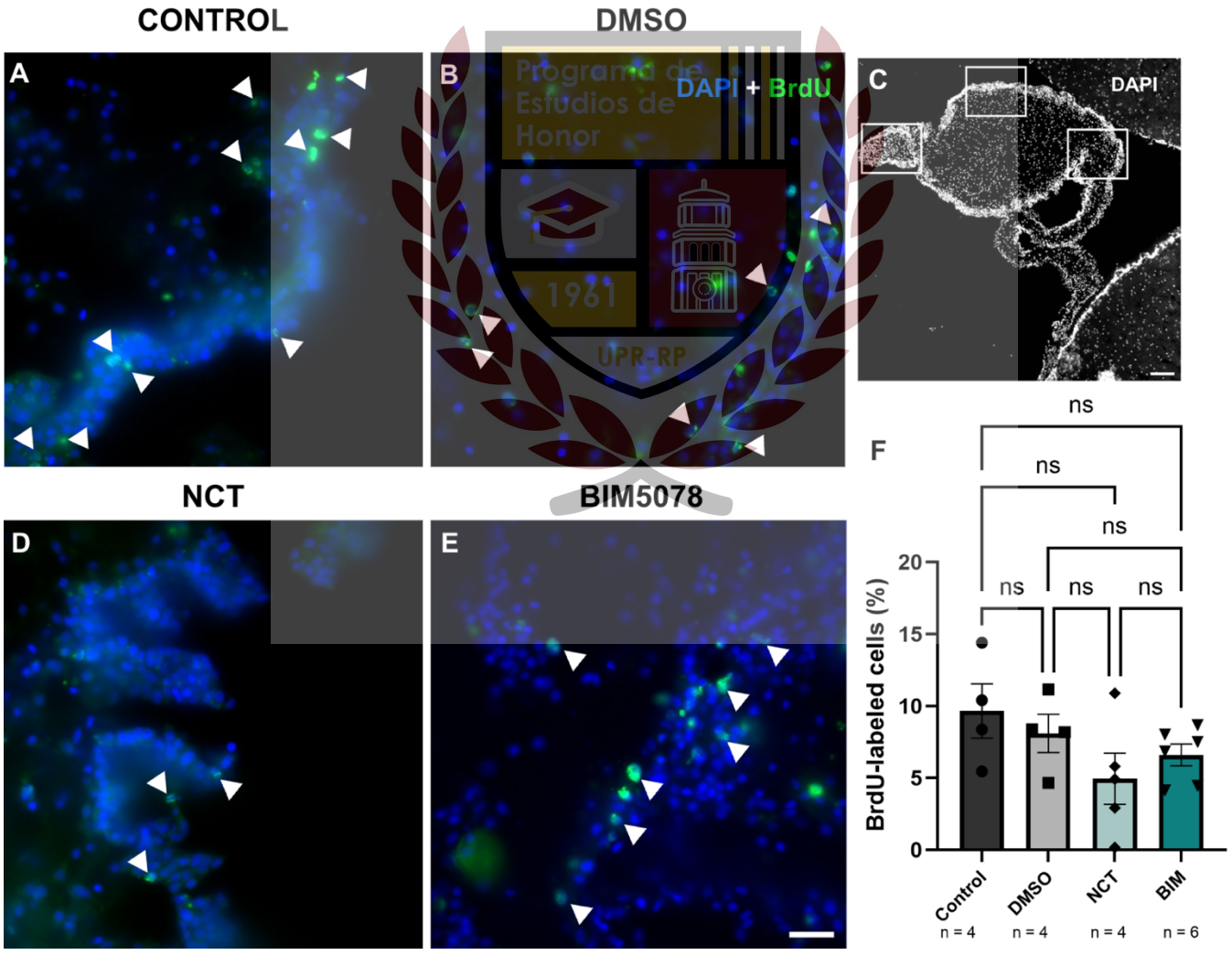


Figure 18 Effect of HNF4 activation on cell proliferation. *Transverse sections of Control (A), DMSO (B), NCT (D), and BIM-treated blastema (E). (C) Regions analyzed. (F) Quantification of cell proliferation across three regions data are presented as mean ± SD. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test. Comparisons are indicated as: P ≥ 0.05, not significant (ns). DAPI labels cell nuclei. Bar = 25µm*

Discussion:

HNF4 Modulation and the Role of Dedifferentiation in Intestinal Regeneration: Because HNF4 is a well-established regulator of epithelial identity and cellular differentiation, we hypothesized that HNF4 downregulation is required for dedifferentiation to occur during intestinal regeneration in *Holothuria glaberrima*. Since intestinal muscle cell dedifferentiation is an essential early step in holothurian intestinal regeneration, characterized by loss of mesothelial identity and acquisition of a more pluripotent state (García-Arrarás et al., 1998; 2011; Quispe-Parra et al., 2021) we predicted that HNF4 activation would delay regeneration, whereas its inhibition would enhance early regenerative events. We further expected downstream effects on proliferation, as cellular dedifferentiation precedes and supports proliferative expansion during regeneration (Reyes-Rivera et al., 2024).

Blastema Size Analysis and Structural Variability: Although no statistically significant differences in blastema area were detected among treatment groups at 8 dpe, qualitative morphological differences were evident. The increased structural variability in NCT treated animals is a feature resembling earlier described regenerative stages characterized by a less developed connective tissue thickening and dispersed mesenchymal cells (Reyes-Rivera et al., 2024). The increased heterogeneity observed in NCT-treated animals may reflect delayed structural maturation or altered tissue consolidation rather than an overall reduction in blastema size. Such variability could also contribute to the absence of statistical significance in area measurements. Nonetheless, a tendency toward a smaller rudiment may be observed, which suggests delayed regeneration.

Extracellular Matrix Remodeling Following HNF4 Modulation: Pharmacological modulation of HNF4 activity did not result in significant alterations in ECM remodeling at 8 dpi, suggesting that collagen degradation during early intestinal regeneration is not markedly disrupted by changes in HNF4 signaling at this stage (Vemuri et al., 2023). However, the marked variability observed within the NCT-treated group, resembled early-stage regeneration in which some regions show a tendency toward organized tissue while others remain disorganized. Additionally, ECM turnover during regeneration is mediated by tightly regulated molecular programs, including metalloproteinase activity and mesothelial restructuring (Quiñones et al., 2002; García-Arrarás et al., 1998, 2018), raising the possibility that HNF4 is not a primary upstream regulator of early collagen breakdown. Instead, HNF4 may contribute to other aspects of regenerative progression, such as cellular organization, metabolic regulation, or structural maturation of the rudiment (Alicea-Delgado & García-Arrarás, 2021; Medina-Feliciano & García-Arrarás, 2021; Tanaka & Reddien, 2011). Compensatory transcriptional networks may preserve ECM remodeling in the presence of pharmacological perturbation (Vemuri et al., 2023), reflecting the robust and multilayered control of regenerative dynamics.

Effects of HNF4 Modulation on Cellular Dedifferentiation: Clear effects were observed at the level of cellular dedifferentiation. Animals treated with the HNF4 agonist (NCT) exhibited decreased dedifferentiation, consistent with HNF4's established role in maintaining differentiated epithelial states (Beinsteiner et al., 2023; Vemuri et al 2023), whereas inhibition of HNF4 (BIM5078) produced minimal changes relative to baseline. This pattern suggests that HNF4 functions as a modulatory factor capable of suppressing dedifferentiation when active. These observations support our central hypothesis that downregulation of HNF4 is necessary to permit the loss of cellular identity required for regenerative

reprogramming, positioning HNF4 as a molecular gatekeeper of cellular differentiation during holothurian intestinal regeneration. The relevance of HNF4 to regenerative programs is further supported by studies in other regenerative systems, including planarians and vertebrates, where HNF4 family members have been implicated in tissue regeneration and cellular identity control (Lobo et al; 2016; Huck et al., 2019; Heppert et al., 2022). Overall, these findings do not indicate a global delay in regeneration but rather suggest that HNF4 activation selectively modulates early regenerative processes related to cellular identity, while the robustness of dedifferentiation is maintained through additional regulatory networks (García-Arrarás et al., 2011; Alicea-Delgado & García-Arrarás, 2021; Lobo et al., 2016; Medina-Feliciano & García-Arrarás, 2021).

Cell Proliferation and Temporal Regulation of Regenerative Responses: In contrast to dedifferentiation, no significant differences were observed in cell proliferation between experimental groups, although the NCT group exhibited an overall BrdU-positive cell percentage of approximately 5%. This value is lower than typical proliferation indices reported in regenerating intestines of *Holothuria glaberrima*, which generally show higher BrdU labeling under normal conditions at approximately 7 to 9 dpe (García-Arrarás et al., 1998; Alicea-Delgado & García-Arrarás, 2021; Quiñones et al., 2002). Interestingly, we initially hypothesized that proliferation would be highly sensitive to HNF4 modulation, given that it occurs downstream of dedifferentiation; however, our results suggest that although it was not significantly affected, there is a tendency toward reduced cell proliferation. It is also important to note that we did not measure cell migration, which can contribute to apparent proliferation indices, and that our sample size was relatively small, which may have limited the detection of subtle effects. Collectively, these findings indicate that early regenerative proliferation, although a prioritized process, may be affected in the context of pharmacological perturbation of HNF4.

Limitations, Proof-of-Concept Nature, and Biological Complexity: Importantly, this study should be regarded as a proof-of-concept investigation. While preliminary, our findings support a role for HNF4 in regulating dedifferentiation. These results highlight HNF4 as a candidate regulator in *Holothuria glaberrima* regeneration, since pharmacological activation or inhibition of its activity seems to impact important cellular processes such as dedifferentiation. Importantly, regeneration is orchestrated by highly complex and interconnected gene regulatory networks (Kotulkar et al., 2024; Sladek et al., 2024) and HNF4 likely represents only one component of a broader molecular system coordinating cellular identity, tissue remodeling, and growth. Nonetheless, the observed effects of HNF4 modulation provide promising initial evidence that this transcription factor participates in intestinal regenerative programs and that investigation of HNF4 in *H. glaberrima* is a fruitful avenue for identifying molecular regulators of regeneration.

Overall Implications and Future Directions: Collectively, these findings indicate that pharmacological modulation of HNF4 selectively influences key regenerative processes in *Holothuria glaberrima*, particularly those related to cellular identity and dedifferentiation. Given the conserved roles of HNF4 in intestinal biology and regeneration across species (Chen et al., 2020; Heppert et al., 2022; Vemuri et al., 2023) this work highlights its potential relevance to regenerative medicine, wound healing, and gastrointestinal repair strategies. (Kiselyuk et al., 2012 ; Kim et al., 2019 ; Vemuri et al., 2023 ; Lee et al., 2021 & 2022)

Several future directions emerge from this study. Although the effects observed with NCT were subtle in some analyses, trends toward lower values and increased variability were evident in parameters such as rudiment area and cell proliferation. Because BIM5078 acts as an antagonist of HNF4, we initially expected it to produce effects opposite to those observed with NCT. Specifically, inhibition of HNF4 activity was predicted to accelerate regenerative responses by further reducing the availability of active HNF4 molecules. However, this pattern was not observed, and BIM-treated groups remained comparable to controls across the analyses performed. These findings raise several possibilities that warrant further investigation.

Dose selection for NCT and BIM5078 was based on previously published concentrations and adapted to the physiological context of *H. glaberrima*, including dilution within the coelomic cavity and repeated administration to account for potential compound loss due to water exchange and filtration. Given that both drugs were tested in mouse models, dose–evaluation experiments using increased concentrations of BIM5078 may help clarify the sensitivity of regenerative processes to HNF4 inhibition in *H. glaberrima*. It is also possible that a biological "carrying capacity" or saturation effect exists, whereby further inhibition of HNF4 activity becomes limited due to the already reduced levels of the transcription factor during regeneration. Alternatively, the limited response to BIM could reflect molecular characteristics of the HNF4 ligand-binding domain, including specific amino acid residues that influence antagonist binding or activity. However, the overall conservation of key amino acids and structural motifs, such as the π-helical turn in helix 7 of the LBD, across metazoan species makes major functional differences unlikely (Beinsteiner et al., 2023). Ultimately, the only way to definitively assess this is to resolve the structure of HNF4 in echinoderms, specifically in *Holothuria glaberrima*. Another possibility is that the antagonist may exert limited functional effects in this experimental context and a different drug should be explored. Conversely, additional dose–response experiments with NCT may help determine whether the concentration currently used in *H. glaberrima* represents an optimal dose for modulating HNF4 activity.

At the cellular level, further analyses will also be informative. A supplementary approach to evaluating the role of HNF4 in dedifferentiation is to study its expression within cells presenting a transcriptional state associated with dedifferentiation. This way, aside from relying on morphological markers such as SLS, cells can be classified based on the expression of genes associated with progenitor-like states such as Wnt pathway targets, proliferative markers, or other dedifferentiation-associated transcription factors. Such cells would represent a dedifferentiating or transitional population, and the expression of HNF4 within these cells could then be assessed using single-cell RNA sequencing. A steady reduction of HNF4 within this population, specifically along a dedifferentiation trajectory, would reinforce the hypothesis that HNF4's downregulation is necessary for intestinal muscle cells to dedifferentiate. This approach would provide a transcriptional perspective to support the morphological observations during intestinal regeneration in *Holothuria glaberrima*. Because specific molecular markers of dedifferentiation are not well defined in *H. glaberrima's* intestinal regeneration, this process is instead inferred from morphological and broader transcriptional indicators of cellular plasticity. Nonetheless, current single-nuclei RNA sequencing (snRNA-seq) studies, in addition to resolving cell-type-specific expression profiles, aim to identify candidate dedifferentiation markers and to more precisely characterize the cellular populations undergoing dedifferentiation during intestinal regeneration.

Additionally, TUNEL assays will allow evaluation of apoptosis, providing insight into tissue turnover dynamics, which are known to interact closely with dedifferentiation and proliferation during holothurian regeneration (Reyes-Rivera et al., 2024). In addition, spatiotemporal analysis of HNF4 expression using HCR-FISH will further define when and where HNF4 acts during regeneration. Previous HCR-FISH experiments conducted in our laboratory have shown that HNF4 is expressed in the luminal epithelium and, notably, in the coelomic epithelium, suggesting potential roles in both epithelial identity and mesothelial dynamics. In addition, Ultimately, this work establishes a conceptual and experimental foundation for studying HNF4 in *H. glaberrima* regeneration and supports a model in which HNF4 constrains dedifferentiation without serving as a primary regulator of ECM remodeling or proliferative expansion.

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