

Characterization of a novel DNA-sensing protein with links to innate immunity in planaria

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by

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Under the guidance of

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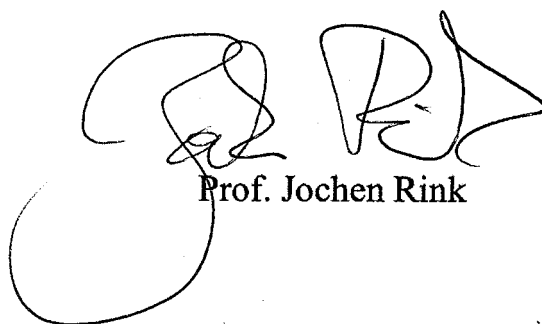
Max Planck Institute for Multidisciplinary Sciences

From June 2024 to March 2025

INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH PUNE

Certificate

This is to certify that this dissertation entitled *Characterization of a novel DNA-sensing protein with links to innate immunity in planaria* towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Somdatta Naskar at Max Planck Institute for Multidisciplinary Sciences under the supervision of Prof. Jochen Rink, Department of Tissue Dynamics and Regeneration, during the academic year 2024-2025.



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This thesis is dedicated to my friends and family.

Declaration

I hereby declare that the matter embodied in the report entitled *Characterization of a novel DNA-sensing protein with links to innate immunity in planaria* are the results of the work carried out by me at the Department of Tissue Dynamics and Regeneration, Max Planck Institute for Multidisciplinary Sciences, under the supervision of Prof. Jochen Rink, and the same has not been submitted elsewhere for any other degree. Wherever others contribute, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions.

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Abstract

Schmidtea mediterranea is a planarian flatworm that is receiving growing attention as a model organism for immunological research due to its remarkable regenerative abilities, simple yet evolutionarily conserved immune system, and genetic tractability, offering insights into innate immunity and host-pathogen interactions. In an ongoing effort to identify and characterize components of the planarian innate immune system, the lab identified a potential sensor of foreign DNA that is called 2100. It seems to have evolved from a prokaryotic ATP nucleosidase protein called Cap 17 which is involved in a bacterial defense system and triggers bacterial cell death upon phage infection. This study shows that 2100 is essential for the survival of *S. mediterranea* since the knockdown of 2100 leads to progressive lesions and death of the organism. Despite having a lethal phenotype, there was no major change at the transcriptome level indicating that 2100 acts on the protein level. Potential interactors of 2100 were identified by immunoprecipitation followed by mass spectrometry proteomics. These data, together with super-resolution microscopy of 2100 localization as well as data from RNA sequencing, suggests that 2100 might act during endocytosis or autophagocytosis. Unfortunately, there are no established methods and assays for studying pathogen infections in planaria. Therefore, to take advantage of the extensive genetic toolset available in *Drosophila*, bacterial infection experiments were performed in flies with knockdown of 2100 in immune cells, suggesting a role of 2100 during innate immune system activation. 2100 is, therefore, likely a novel component of the invertebrate innate immune system with ancient roots in prokaryotic antiviral defense.

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Contributions

Contributor name	Contributor role
Dr. Leonard Drees, Somdatta Naskar	Conceptualization Ideas
Somdatta Naskar	Methodology
-	Software
Somdatta Naskar	Validation
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Somdatta Naskar	Investigation
Prof. Jochen Rink	Resources
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Somdatta Naskar	Writing - original draft preparation
Dr. Leonard Drees, Somdatta Naskar	Writing - review and editing
Somdatta Naskar	Visualization
Dr. Leonard Drees	Supervision
Prof. Jochen Rink	Project administration
Prof. Jochen Rink	Funding acquisition

This contributor syntax is based on the Journal of Cell Science CRediT Taxonomy¹.

¹ <https://journals.biologists.com/jcs/pages/author-contributions>

Chapter 1 Introduction

1.1 *Schmidtea mediterranea* - a versatile model organism

Schmidtea mediterranea is a free-living freshwater planarian that belongs to the phylum of Platyhelminthes and the order Tricladia. It has a three-branched gastrovascular cavity and uses the pharynx, a muscular appendage that opens on the ventral side, for both food intake and excretion. Its nervous system consists of a pair of cerebral ganglia functioning as a primitive brain in the anterior part and two nerve cords present along the entire length of the animal (Sluys & Riutort, 2018). It also has eyes composed of photoreceptive and pigment cells (Pineda *et al.*, 2000). The body wall of the animal consists of muscular layers and an epidermis that is covered by a thick layer of mucus. They do not have any dedicated circulatory or respiratory systems, and the exchange of gases is based on simple diffusion through their surface (Lewallen & Burggren, 2020). The network formed by the gut branches, in addition to its function in digestion, also serves as a circulatory system, by facilitating the distribution of nutrients throughout the animal's body. Protonephridia is located within the mesenchyme that functions as the excretory system in planaria and plays a crucial role in osmoregulation and waste removal (Rink *et al.*, 2011). They consist of branched tubules that are capped by so-called flame cells, which provide cilia-driven ultrafiltration (Vu *et al.*, 2015).

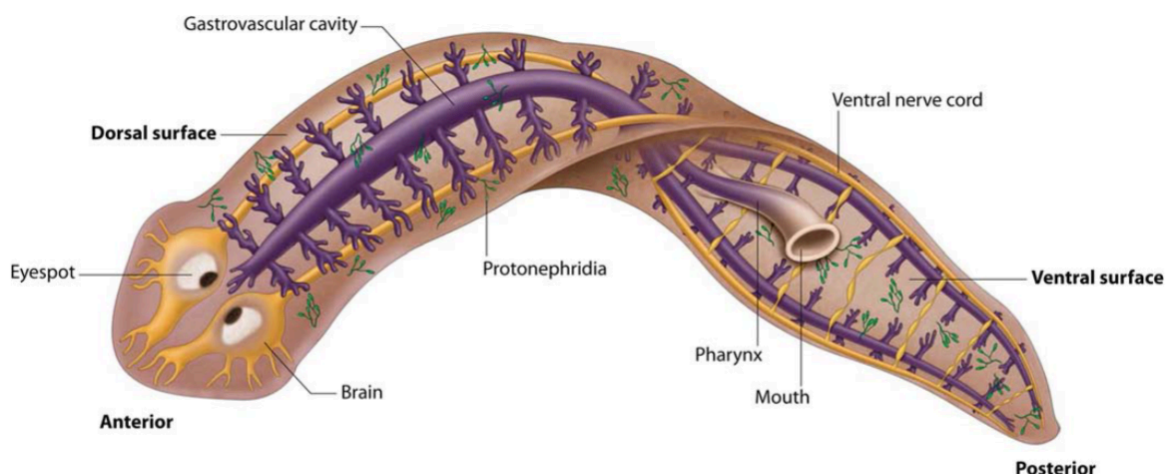


Fig. 1.1 - External view of *Planaria* adapted from Pagan, 2014

Planaria have pluripotent stem cells called neoblasts, which are the only dividing cells. They are present throughout the body and have the ability to self-renew and differentiate into all cell types of the organism. Hence, they are often used as a model organism for understanding regeneration, stem cell biology, cellular turnover, and aging (Newmark and Alvarado, 2002; Oviedo *et al.*, 2008). Neoblasts constitute a heterogeneous cell population, with some being truly pluripotent stem cells capable of fully repopulating organisms lacking stem cells (Wagner *et al.*, 2011). Other subsets of neoblasts express specific transcription factors, indicating their commitment to particular cell lineages (Fincher *et al.*, 2018; Plass *et al.*, 2018). Despite this heterogeneity, all neoblasts are characterized by the expression of germline related genes such as *piwi*, *vasa*, *bruno* and others. Among these, *piwi* family genes serve as the hallmark markers of neoblasts (Reddien *et al.*, 2005). Asexual strains of planaria reproduce only by fission and hence never mix genetic material between individuals. This can potentially lead to accumulation of mutations over generations and in turn occurrence of uncontrolled growth or cancer, as is the case in many animals. However, this has never been reported to occur in planaria indicating that there should be a very good quality control mechanism or removal of compromised cells.

Planaria also don't have a fixed body size. They can grow in size when food is abundant by increasing neoblast proliferation and scaling their internal organs to maintain body proportions. While, they shrink during starvation without losing functionality for extended amounts of time (Takeda *et al.*, 2009). It has been shown that there is a steady turnover of cells. They can utilize energy from digesting old cells with the help of intestinal phagocytes (Lee *et al.*, 2023; Lindsay-Mosher *et al.*, 2024). This again suggests that there must be highly controlled mechanisms of cell removal or cell turnover during shrinking and regeneration.

S. mediterranea can regenerate an entirely new body from a small tissue fragment within just two weeks (Elliott & Alvarado, 2013). Following amputation, the organism initiates an initial wave of apoptosis at the wound site, peaking approximately 1 to 4 hours post-injury, likely to facilitate wound healing (Pellettieri *et al.*, 2010). Mitotic cell division begins around 48 hours after injury in proximity to the regeneration site, known as the blastema. Subsequently, a second wave of apoptosis occurs

throughout the entire body around 72 hours post-injury, suggesting a systemic response to regeneration (Pellettieri *et al.*, 2010; Karge, 2020).

To establish planaria as a suitable model organism, various methods have been developed and optimized for their use. RNA interference (RNAi) is one of the widely used methods in planaria to knock down specific genes and assess their functions. This technique involves feeding, injecting, or soaking planaria with double-stranded RNA (dsRNA) corresponding to a target gene, leading to its degradation and suppression. RNA sequencing is also used to analyze gene expression profiles across different conditions. For further analysis, quantitative PCR (qPCR) is often done to quantify gene expression levels and hence validate the effectiveness of RNAi-mediated knockdown. Additionally, specific gene expression patterns can be analyzed using fluorescent in situ hybridization (FISH). Likewise, protein expression patterns can also be analyzed using immunofluorescence staining using antibodies, providing insights into protein localization and function. However, antibody availability is often limited in planaria, requiring the development and validation of custom-made antibodies for specific targets. Confocal and electron microscopy are also widely used to examine cellular structures in high resolution. Regeneration assays play a crucial role in identifying factors that control stem cell proliferation and differentiation. Further, an annotated genome library is also available which serves as an important resource for investigating gene functions (Rozanski *et al.*, 2018; Ivanković *et al.*, 2024). This database includes genome sequences, gene annotations, and functional insights, which allows genetic data analysis. However, proteomics approaches such as mass spectrometry-based protein analysis are not commonly used in planaria. Transgenesis is also not established in planaria due to its high regenerative capacity and lack of efficient germline transmission of transgenes. CRISPR-Cas9 gene editing is also less effective than in other organisms due to low DNA repair efficiency and high cell turnover from constant regeneration.

1.2 Planarian immune system

The rapid, scarless wound healing of planaria is interesting since, with open wounds, it is also vulnerable to many foreign pathogens. However, planarians seem to overcome the problem of exposing their inner organs to the environment during

amputation efficiently. This means that they must have an immune system efficient enough to protect them from microbes in their immediate environment.

Planarians do not possess an adaptive immune system like the vertebrates and hence solely rely on innate immune responses to fight against infections. The planarian immune system exhibits a high degree of evolutionary conservation with the human innate immune system (Maciel *et al.*, 2019). Like humans, planarians can recognize pathogen-associated molecular patterns (PAMPs) and produce antimicrobial peptides and proteins (Gao *et al.*, 2017). Their simplicity, remarkable regenerative capacity, and genetic tractability make them an excellent model for studying host-pathogen interactions and immune responses (Kangale *et al.*, 2021). Research on planarians could provide valuable insights into fundamental immune mechanisms that are conserved across diverse life forms.

Three types of immune cells are suspected to contribute to the planarian innate immune system - reticular cells, neoblasts (stem cells), and intestinal phagocytes (Kangale *et al.*, 2021). The reticular cells in *Dugesia dorotocephala* can internalize dead microbes, such as dead *M. tuberculosis*, as shown before (Morita, 1991), but no more information is available. The role of stem cells in invertebrate immunity is not well studied. But stem cells do play a key role in the vertebrate immune system as they are equipped with Pathogen Recognition Receptors (PRRs), including Toll-like receptors (TLR) (Pevsner-Fischer *et al.*, 2007; Liotta *et al.*, 2008). TLRs are widely conserved across several animal species, but Platyhelminthes (including planarians) do not possess TLR orthologues. However, Toll/Interleukine-1 receptor (TIR) domain-containing proteins are still present in these animals (Leulier and Lemaitre, 2008; Coscia and Giacomelli, 2011; Peiris *et al.*, 2014; Gerdol *et al.*, 2017). In vertebrates, stem cells also exhibit both immunosuppressive and inflammatory properties (Machado *et al.*, 2013), as well as antibacterial functions (Krasnodembskaya, *et al.*, 2017). Phagocytes are also known to express PRRs and eliminate pathogens across various species, including insects, mollusks, echinoderms, and chordates. Previous studies have shown the presence of phagocytic cells in planaria, but the specific expression of PRRs has not been identified.

However, a recent study identified an atypical *RIG-I* gene (Retinoic acid-inducible gene I) in planarian *Dugesia japonica*. *RIG-I* gene is a dsRNA sensor with a conserved structure that activates the host interferon (IFN) system upon recognizing PAMPs, triggering the production of IFNs and cytokines to eliminate pathogens (Yoneyama *et al.*, 2004; Yoneyama and Fujita, 2009). However, the biological functions and regulatory mechanisms of *RIG-I* in the innate immune response of invertebrates remain unknown. *DjRIG-I* was demonstrated to be a highly conserved molecular structure throughout species evolution which is expressed in the pharynx. The results indicated that *DjRIG-I* activates intracellular signaling cascades independently of the classical CARD domains and plays a crucial role in immune-related signaling pathways triggered by viruses and bacteria (Li *et al.*, 2019).

S. mediterranea is a well-established model organism in various research areas, and it has been shown to be resistant to a broad spectrum of pathogens. *S. mediterranea* can eliminate *Staphylococcus aureus* within 6 days post-infection (Tsoumtsia *et al.*, 2017). *C. albicans*, one of the most common fungal infections in humans, can infect, grow, and invade tissues in *S. mediterranea* and the planarian responds to it by activating components of its innate immune system to clear and repair host tissues (Maciel *et al.*, 2019). Hence, *S. mediterranea* is a suitable model organism for innate immune studies even though the majority of its innate immune pathways has yet to be explored.

1.3 Conserved innate immune pathways

The innate immune system typically initiates upon sensing the pathogen-associated molecular patterns (PAMPs) during an infection detected by immune cells having PRRs (Janeway, 1992). The host might also be able to recognize target modifications that helps in distinguishing self from non-self, thereby sensing foreign pathogens like in the case of restriction-modification systems (Murray, 2002). Once a foreign invader is detected, the sensor may produce a nucleotide second messenger (such as an oligonucleotide), or the sensor may use oligomerization to trigger the activation of effector domains which leads to inflammation, production of cytokines, and the activation of other immune cells (Fischer *et al.*, 2019; Hornung *et al.*, 2014). The activation of these pathways leads to the recruitment of immune cells to the site

of infection in order to restrict pathogen replication. Pathogen restriction can happen either by direct targeting of a pathogen or by destroying specific host molecules that are essential for pathogen replication, or the infected cells can undergo regulated cell death (or abortive infection) to limit the pathogen's replicative niche.

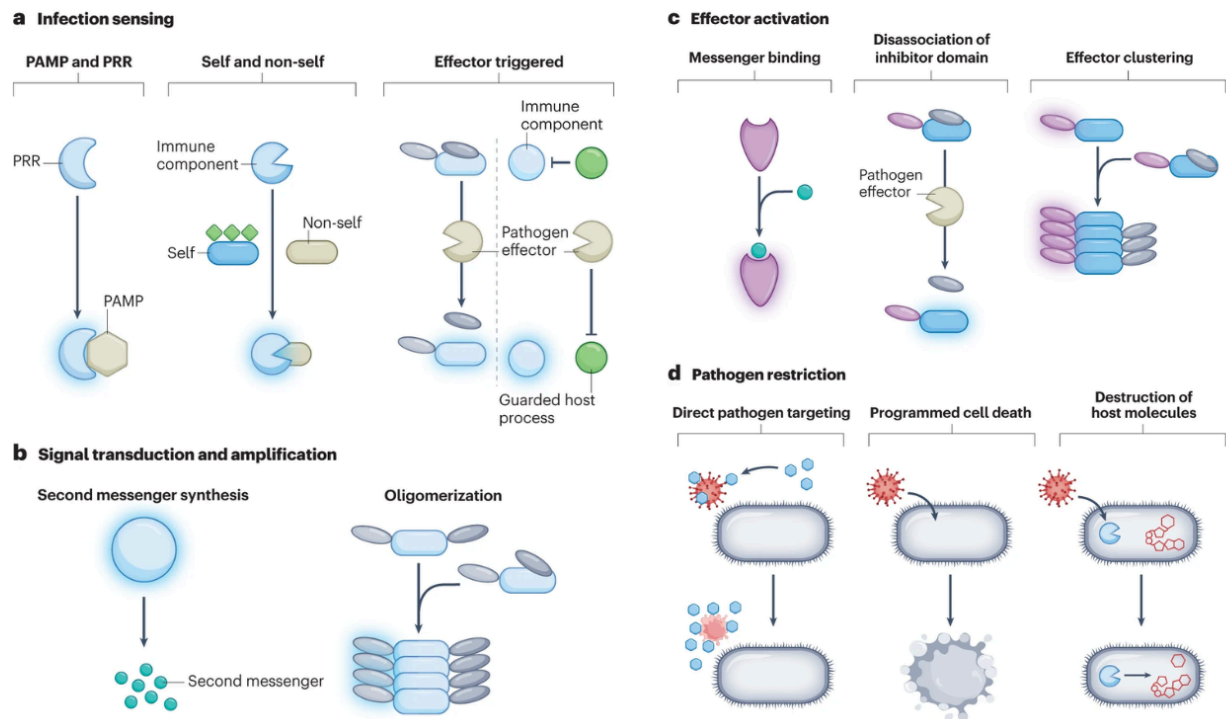


Fig. 1.2 - Innate immune signalling pathways adapted from Ledvina & Whiteley, 2024

One of the most common and conserved innate immune signaling pathways is the Toll-like receptor (TLR) mediated NF- κ B activation. TLRs are transmembrane proteins expressed on various immune cells, including macrophages, neutrophils, and dendritic cells, recognizing specific PAMPs. For example, TLR4 detects lipopolysaccharide (LPS) from Gram-negative bacteria, while TLR5 recognizes bacterial flagellin (Takeda *et al.*, 2003). Following the detection of a pathogen, TLRs recruit adaptor proteins like myeloid differentiation primary response 88 (MyD88) and TIR-domain-containing adapter-inducing interferon- β (TRIF). Most TLRs, except TLR3, utilize the MyD88-dependent pathway, leading to the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and mitogen-activated protein kinases (MAPKs). While, TLR3 and, in certain conditions, TLR4 utilize the TRIF-dependent pathway, which activates interferon regulatory factors (IRFs) and induces the production of type I interferons (Kawasaki and Kawai, 2014). The

activation of NF- κ B and MAPKs leads to the transcription of pro-inflammatory cytokines and chemokines (Iwasaki *et al.*, 2004). This results in the recruitment and activation of additional immune cells to the site of infection hence amplifying the immune response. The recruited immune cells, such as neutrophils and macrophages, phagocytose and destroy invading microbes. However, Platyhelminthes (including planarians), as mentioned before, do not possess TLR orthologues.

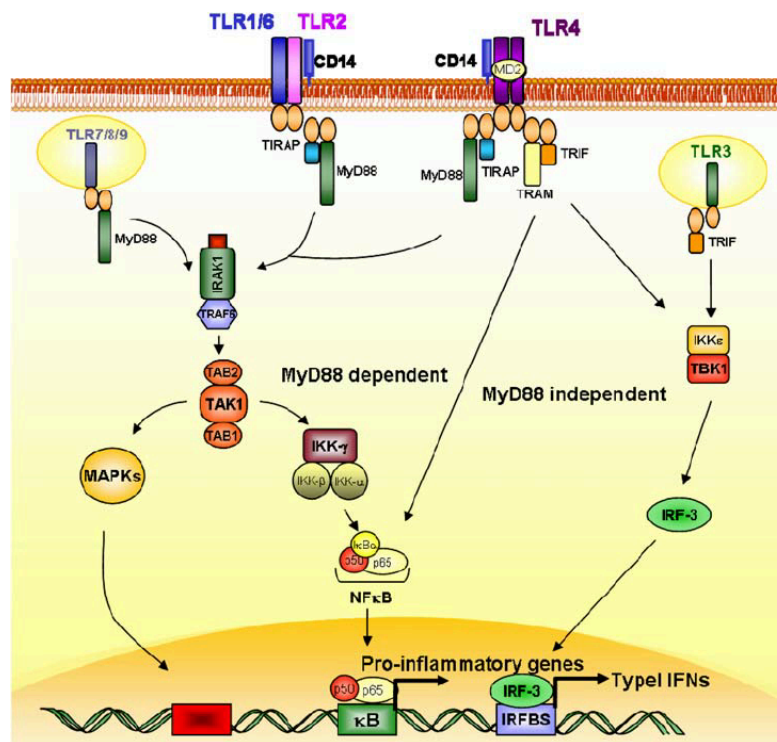


Fig. 1.3 - Toll-like receptor signaling pathway adapted from Ropert *et al.*, 2008

It has been shown that over 70% of prokaryotic genomes encode systems that use regulated cell death as the strategy for pathogen restriction (Rousset and Sorek, 2023). One common and conserved regulated cell death pathways from the prokaryotes to the eukaryotes is the cGAS/CD-NTases and STING pathway. This involves sensing of double stranded DNA of foreign invader by cytosolic PRR-metazoan cyclic GMP-AMP synthase (cGAS) and bacterial cGAS/dinucleotide cyclase in *Vibrio* (DncV)-like nucleotidyltransferases (CD-NTase) family enzymes to produce nucleotide second messengers in response to infection (Kranzusch, 2019). These second messengers activate effector molecules that lead to the activation of stimulators of interferon genes (STING) that ultimately leads to cell death (Chen *et al.*, 2016; Cohen *et al.*, 2019). In eukaryotes, STING furthermore activates

pro-inflammatory signaling via activation of IRF-3 transcription factor. However, planaria lack the cGAS STING pathway but there are different types of effector domains leading to different pathways like DNA degradation, NAD⁺ degradation, ATP degradation, or membrane disruption, which causes cell death.

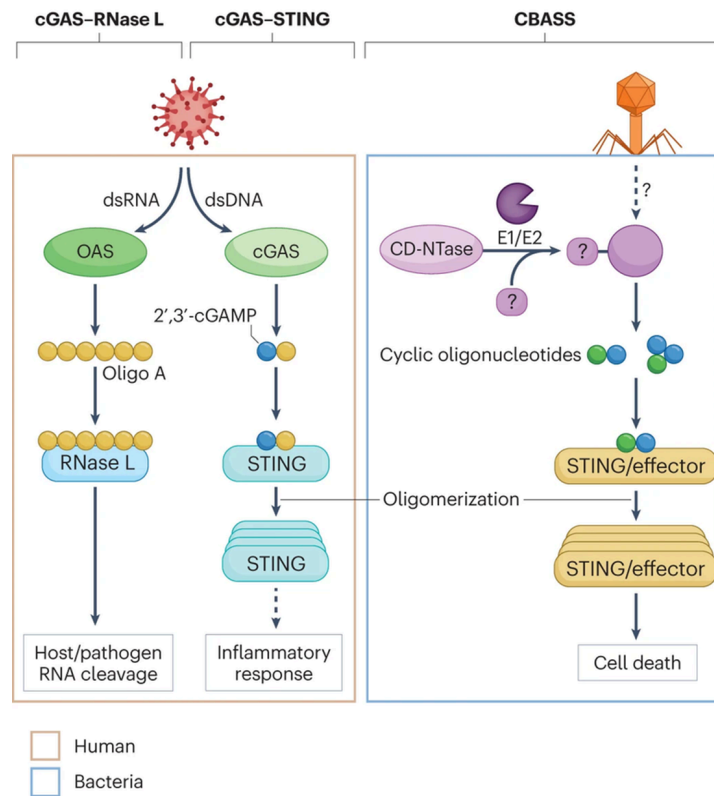


Fig. 1.4 - cGAS/CD-NTase and STING pathway adapted from Ledvina & Whiteley, 2024

1.4 Innate immune effector domain - PNP domain

One of the most common and conserved effector domains found in eukaryotes and is likely to be obtained from the prokaryotes is the PNP (Purine Nucleoside Phosphorylases) domain which leads to ATP degradation depriving the foreign invader of energy and hence restricts its ability to replicate and infect other cells. In a recent study, a novel innate immune defense protein named Cap17 was described in bacteria, which is an ATP nucleosidase and contains a PNP domain. It was shown to cleave ATP and dATP molecules into adenine and sugar moieties in case of bacteriophage infection, thus preventing bacteriophage propagation (Rousset *et al.*, 2023).

Previous work in our lab found a single planarian protein named 2100 to be the single protein to share homologies with the PNP domain. 2100 has a PNP domain, a domain of unknown function, and a Z-DNA binding domain, unlike the cyclic oligonucleotide domain present in the Cap17 protein.

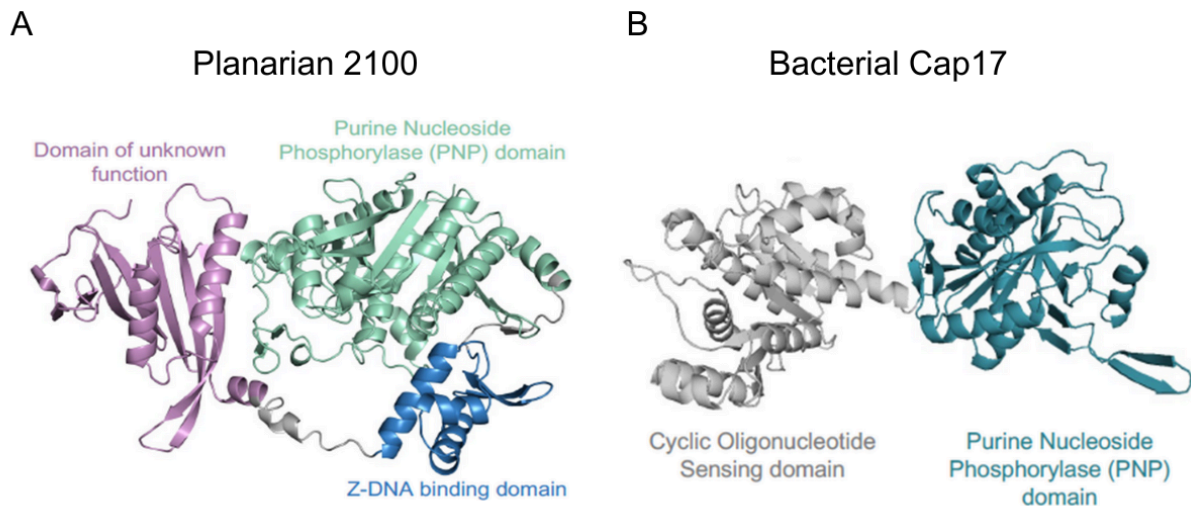


Fig. 1.5 - The AlphaFold-2 prediction of 2100. A) Planarian *dd_Smed_v6_2100* protein 3D structure. B) Bacterial Cap17 protein adapted from Rousset et al., 2023

The current hypothesis is that once the Z-DNA binding domain is triggered, it potentially activates the PNP domain, which initiates the depletion of cellular ATP molecules required for pathogen propagation. This study aims to functionally analyze this ancient immune defense system in planaria and further in *Drosophila* to test whether the function is conserved from flatworms to insects (or if there are differences) to strengthen conclusions about the role of 2100 in animals. Studying the novel planarian innate immunity protein 2100 could offer valuable insights into the mechanisms of the planarian innate immune system and the factors contributing to its remarkable resilience.

Chapter 2 Materials and Methods

2.1 Planarian Maintenance Conditions

Schmidtea mediterranea (GOE00071) was maintained in boxes with planarian water (1x PW) containing 1.6 mM NaCl, 1.2 mM NaHCO₃, 1.0 mM CaCl₂, 1.0 mM MgSO₄, 0.1 mM MgCl₂, and 0.1 mM KCl. In addition, 5 µg/mL of antibiotic Ciprofloxacin was added to prevent the growth of bacteria unless specified otherwise. The boxes were kept at 20°C in the dark and cleaned once a week. The flatworms were fed with homogenized calf liver and starved for 3-4 weeks prior to any experiments.

2.2 RNA interference-mediated knockdowns

2.2.1 dsRNA synthesis and purification

For most genes, templates for dsRNA synthesis were available from a genome-wide library in the Rink lab. For the genes that were not present in that library, the DNA template was amplified from pPRT4P plasmids with the target gene using T7-AA18 and pR244 primers and Q5® DNA Polymerase according to the producer's protocol at an annealing temperature of 68°C. Following PCR purification after successful DNA amplification (verified by running a 1% TAE agarose gel), dsRNA was synthesized from these amplified target DNA products by performing in vitro transcription overnight using T7 DNA polymerase with the standard protocol for IVT (Thermo Fisher, EP07112). The synthesized RNA was annealed by heating the samples at 70°C for 3 mins and then left to cool down to room temperature. The dsRNA was then precipitated using one volume of NaCl/PEG-8000 precipitation solution (2.5 M NaCl, 20% (w/v) PEG-8000, 10 mM Tris-HCl pH 8.0) followed by centrifugation at 16,000 x g at 4°C for 20 mins. The resulting pellet was washed with 70% ethanol, centrifuged again for 5 mins, and left to air dry for a 1 min. Then it was resuspended in 200 µL of nuclease-free water. A 1:100 dilution of the obtained dsRNA was made, and its concentration was determined using the Nanodrop. The diluted dsRNA was also run on an agarose gel to verify the length and integrity of the synthesized constructs. The dsRNA was then aliquoted in 30 µL liver in PCR tubes at a final concentration of 2 µg/mL unless specified otherwise and stored at -80°C.

Table 2.1 - Primers used in this study

Identifier	Sequence	Application
T7-AA18	GAAATTAATACGACTCACTATAGGGAGCCACCGGTT CCATGGCTAGC	amplification of templates for dsRNA synthesis
PR244	GAGGCCCAAGGGGTTATGTG	
616V	AGAGTTTGATYMTGGCTC	amplification of bacterial 16s for rRNA sequences
1492R	GGYTACCTTGTTACGACTT	

Table 2.2 - Plasmids used in this study

Identifier	Application	Source
<i>pPRT4P-eGFP</i>	amplification of <i>eGFP</i> dsRNA synthesis template	Rink lab
<i>pPRT4P-2100</i>	amplification of <i>2100</i> dsRNA synthesis template	Rink lab
<i>pPRT4P-prosaposin</i>	amplification of <i>prosaposin</i> dsRNA synthesis template	Rink lab
<i>actin5C-Gal4</i>	Activation of UAS-construct expression in cultured cells	Usui et al. 1999
<i>pUAST-eGFP</i>	UAS-controlled expression of eGFP	Rink lab
<i>pUAST-CG12065::eGFP</i>	UAS-controlled expression of a CG12065::eGFP fusion protein	Rink lab

2.2.2 RNAi feeding protocol

Before feeding, worms were transferred to petri dishes. Liver aliquots with dsRNA were left to thaw on ice and then mixed properly by pipette mixing and were given a short spin to avoid air bubbles. Worms were allowed to feed for about 1.5 hrs in the dark. The remaining liver was taken out, and worms were washed and stored again at 20°C. Worms were always washed the following day of feeding. RNAi feeding was always done on Monday and Thursday till the number of desired feedings was achieved.

2.3 Regeneration assays

Worms were placed on a cooling plate at 4°C to immobilize them temporarily. Two amputations were made - above and below the pharynx with a scalpel. Cut pieces were transferred to a fresh petri dish. Cut pieces were always washed the following

day of amputation. Images were taken on a stereoscope to check for regeneration efficiency.

2.4 Protein Extraction and Western Blot

2 or 3 worms were taken in two replicates for each group in 1.5 mL microcentrifuge tubes. 1 mL of Urea Lysis buffer (9 M Urea, 100 mM NaH₂PO₄, 10 mM Tris-NaCl pH8, 2% SDS, 130 mM DTT, 1mM MgCl₂) was added to each tube. The worms were homogenized with a pestle first and then sonicated in a sonicator bath in the cold room for 20 seconds. 1 µL benzonase was added to each sample and was incubated at 50°C for 20 mins until no solid chunks were present. The samples were centrifuged at 21,000 x g for 1 min. Protein concentration of 1:10 dilutions was measured in the Nanodrop (protein A280) using a 1:10 dilution of the urea lysis buffer as blank. A final concentration of 1x LDS buffer (12% LDS, 0.06% Bromophenol Blue, 50% glycerol, 600 mM DTT, 60 mM Tris-NaCl pH 6.8) was added to the protein lysate samples and stored at -20°C.

2.4.1 SDS-PAGE

Proteins were separated using NuPAGE 4-12 % BisTris Protein Gels at 125 V for 90 mins in 1x NuPAGE™ MES SDS Running Buffer (Thermo Scientific, Cat n°: NP0002) using the XCell SureLock™ Mini-Cell electrophoresis system (Thermo Scientific, Cat n°:EI0001). A total of 20 µg of proteins per sample were loaded on the gel unless specified otherwise and together with the PageRuler™ Plus Pre-stained Protein Ladder (Thermo Scientific, Cat n°: 26619).

2.4.2 Western blot

Proteins were transferred onto 0.2 µm nitrocellulose Amersham Protran membrane at 30 V for 90 mins in transfer buffer (1x NuPAGETM transfer buffer (Thermo Scientific, Cat n°: NP00061), 20% Methanol) at 4°C. Membranes were blocked for 1h in blocking solution (5% Soy protein in 1x PBS) at room temperature and continuous agitation. The membranes were incubated overnight with primary antibodies (0.5% Soy protein, 1x PBS, 0.1% Tween 20) at 4°C. The primary antibody anti-2100 (from rabbit) was used at a dilution of 1:10000, and anti-ef1alpha (from mouse) was used at a dilution of 1:20000. Next day, membranes were rinsed thrice and washed 3

times for 15 mins each with 1x PBS, 0.1% Tween 20. The membranes were incubated for 1.5 hrs with secondary antibodies (5% Soy protein, 1x PBS, 0.1% Tween 20) at room temperature. Secondary antibodies anti-rabbit-CF770 (from donkey) and anti-mouse-CF647 (from goat) were used at a dilution of 1:10000. The membranes were rinsed thrice and washed thrice for 15 mins each with 1x PBS, 0.1% Tween 20, followed by washing twice with 1x PBS for 15 mins each. The membranes were dried and imaged with Amersham Typhoon instrument. All western blot images were quantified using ImageJ and normalized with ef1alpha.

Table 2.3 - Antibodies used in this study

Sl No.	Antibody	Dilution	Host	Supplier	Used for
1	anti-2100	1:250	Rabbit	Rink Lab	Confocal+STED
2	anti-Z-DNA/Z-RNA	1:500	Mouse	ZMS1185 Sigma Aldrich	Confocal+STED
3	anti-Bcat2	1:1000	Mouse	Rink Lab	STED
4	anti-rabbit-AF647	1:1000	Goat	A21245 Thermo Fisher Scientific	Confocal
5	anti-mouse-AF594	1:1000	Goat	A-11032 Thermo Fisher Scientific	Confocal+STED
6	anti-rabbit-StarRed	1:500	Goat	STRED-1002-500UG Abberior	STED
7	anti-2100	1:10000	Rabbit	Rink Lab	Western Blotting+IP
8	anti-eF1alpha	1:20000	Mouse	Rink Lab	Western Blotting
9	anti-rabbit-CF770	1:10000	Donkey	20484 Biotium	Western Blotting+IP
10	anti-mouse-CF647	1:10000	Goat	SAB4600351-125UL Merck	Western Blotting+IP
11	anti-RFP	1:10000	Rabbit	600-401-379 Thermo Fisher Scientific	IP

2.5 Whole-mount antibody staining and Whole-mount Fluorescent *in situ* hybridization (FISH)

2.5.1 Fixation

Worms were put into 5 mL microcentrifuge tubes (<20 worms) or 15 mL falcon tubes (>20 worms). 1x PW was replaced with 0.5% Neutralized N-acetyl-L-cysteine (in H₂O, 20 mM HEPES-NaOH, pH 7.25) and incubated on a nutator for 10 mins. It was then replaced with 5% N-acetyl-L-cysteine (in 1x PBS) and incubated on the nutator for 5 mins. It was then replaced with 4% paraformaldehyde (in 0.5% 1x PBS, 0.3% Triton) and left on the bench for 30 mins with occasional rocking (every 5-10 mins). The samples were washed twice with 1x PBS, 0.3% Triton for 5-10 mins each on the

nutator. It was then replaced with reduction solution (1% NP-40, 0.5% SDS, 50 mM DTT, 1X PBS) for 5 mins at 37°C with occasional rocking. Samples were again washed twice with 1x PBS, 0.3% Triton for 5-10 mins each on the nutator. It was then replaced with 50% methanol for 5-10 mins and then was replaced with 100% methanol and stored at -20°C.

2.5.2 Bleaching and Staining of Worms for Whole-mount Antibody Staining

100% methanol was replaced with 50% methanol, followed by two washes with 1x PBS, 0.3% Triton for 5-10 mins each on the rotator. The samples were bleached overnight with 6% H₂O₂, 1x PBS, 0.3% Triton under direct light. Next day, samples were washed with 1x PBS, 0.3% Triton for 5-10 mins each. Proteinase K treatment (2 µg/mL, 1:10,000) in 1x PBS + 0.1% SDS was given for 10 mins at room temperature. Proteinase K solution was replaced with 4% PFA in 1x PBS, 0.3% Triton for 10 mins. Followed by two more washes with 1x PBS, 0.5% Triton for 5-10 mins each, blocking solution (5% horse serum in 1x PBS, 0.5% Triton) was added, and the samples were incubated on the rotator for 1 hr. The blocking solution was replaced with primary antibodies in 5% horse serum in 1x PBS, 0.5% Triton overnight at 4°C. Next day, followed by 6 washes for 2-3 hrs with 1x PBS, 0.5% Triton, samples were incubated with secondary antibodies along with DAPI (2.5 µg/mL) in 5% horse serum in 1x PBS, 0.5% Triton overnight at 4°C. Next day, samples were again washed 6 times for 2-3 hrs with 1x PBS, 0.5% Triton. Then they were incubated in 4% PFA in 1x PBS, 0.3% Triton for 10 mins, followed by 2 washes with 1x PBS, 0.3% Triton. Next, they were put in Scale S4 (10% Glycerol, 15% DMSO, 40% Sorbitol, 4M Urea, 0.1% TritonX-100, 2.5% DABCO) clearing medium at 4°C. Samples were then mounted between long (24mmx60mm) and short (22mmx22mm) coverslips using 0.1 mm or 0.25 mm spacers based on the size of the samples.

2.5.3 Bleaching and staining for FISH

100% methanol was replaced with 50% methanol, followed by two washes with 1x PBS, 0.3% Triton for 5-10 mins each on the rotator. Samples were rinsed with 1x SSC (Sigma-Aldrich, Cat n°, 1.06009.2511), 0.1% Tween 20 for 5-10 mins. 1x SSC was replaced with bleaching solution (5% non-deionized formamide; 1.2% H₂O₂; in 0.5x SSC) under direct light for 1.5 hrs. Samples were again rinsed with 1x SSC for

5–10 mins followed by two washes with 1x PBS, 0.3% Triton (1x PBST) for 5-10 mins each on the rotator. Proteinase K treatment (2 µg/mL, 1:10,000) in 1x PBS + 0.1% SDS was given for 10 mins at room temperature. Proteinase K solution was replaced with 4% PFA in 1x PBST for 10 mins. Followed by two more washes with 1x PBST for 5-10 mins each, wash was given 1:1 1xPBST:PreHybe (50% formamide, 5X SSC, 1x Denhardtts (Invitrogen, Cat n°: 750018), 100 µg/µL Heparin (Sigma, Cat n°: H3393-1MU), 1% Tween 20, 0.25 mg/mL Torula yeast RNA (Sigma-Aldrich, Cat n°: R6625-25G), 50 mM DTT) solution for 10 mins. 1:1 1xPBST:PreHybe was replaced with PreHybe for 2 hrs at 58°C. Riboprobe (already available from previous work in the lab) was heat-denatured in 700 µL Hybe buffer (50% formamide, 5X SSC, 1x Denhardtts, 100 µg/µL Heparin, 1% Tween 20, 0.25 mg/mL yeast RNA (Roche, Cat n°: 10109223001), 50 mM DTT, 5% Dextran sulfate) at 70°C for 3 mins and then put at 58°C. PreHybe was replaced, and samples were incubated with this Riboprobe mix for 16 hrs at 58°C. The next day, blocking was performed for 2 hrs in TNTx (0.1 M Tris pH 7.5, 0.15 M NaCl, and 0.3% Triton X-100) + 5% sterile horse serum + 0.5% Roche western blocking reagent, at room temperature with rocking. The blocking was replaced with anti-DIG-POD (Roche, Cat n°: 11207733910) at 1:1000 in 5% sterile horse serum + 0.5% Roche western blocking reagent in TNTx, and incubated overnight at 4°C. Next day, samples were washed 6 times with TNTx for 2-3 hrs and incubated in 500 µL of Rhodamine-Tyr (1:2000) in TSA buffer (2 M NaCl, 0.1 M Boric acid pH 8.5, 0.02% (v/v) H₂O₂, 20 µg/µL 4-IPBA) for 30 mins at room temperature. Samples were washed twice with TNTx for 20 mins, followed by incubation in 200 mM of sodium azide (in TNTx) for 1 hr to kill peroxidase activity. Samples were again washed 6 times with TNTx for 2-3 hrs.. Samples were incubated in a DAPI staining solution (2.5 µg/mL DAPI, 1x TNTx) for 1 hr followed by two washes in TNTx. Samples were then put in ScaleS4 clearing medium at 4°C overnight and then mounted between long (24mmx60mm) and short (22mmx22mm) coverslips using 0.1 mm or 0.25 mm spacers based on the size of the worms.

2.5.4 Imaging and image processing

Images were acquired on an Olympus IX83 spinning disc system unless specified otherwise. Images were then processed using ImageJ (Java 1.8.0_172) and samples stained for the same marker were set to the same brightness and contrast

to allow comparison between different groups. All the figures were then prepared using Affinity Designer 2 (2.3.1).

2.6 Whole-mount lipid droplet staining

Worms were starved for 4 days post-last feeding and put into 2 mL microcentrifuge tubes. 1x PW was replaced with 5% N-Acetyl-Cysteine (NAC) in 1x PBS and incubated on the nutator for 5 mins. NAC was replaced with 4% paraformaldehyde (in 0.5% 1x PBS, 0.3% Triton) and left on the bench for 30 mins with occasional rocking (every 5-10 mins). The samples were washed twice with 1x PBS, 0.3% Triton for 5-10 mins each on the nutator. The samples were bleached overnight with 6% H₂O₂, 1x PBS, 0.3% Triton under direct light. Next day, samples were washed with 1x PBS, 0.3% Triton for 5-10 mins each. Samples were incubated with LD540 (3 µg/mL) and DAPI (1 µg/mL) in PBS overnight at 4°C. Next day, two washes were given with 1x PBS. Samples were then mounted in 60% glycerol H₂O between long (24mmx60mm) and short (22mmx22mm) coverslips using 0.25 mm spacers.

2.7 RNA Sequencing

2.7.1 Total RNA Isolation

30 similar-sized worms were taken for each group - *eGFP* RNAi (negative control) and *2100* RNAi. These were given two feedings with aliquots of calf liver mixed with double-stranded RNA (dsRNA prepared by in-vitro transcription) at a final concentration of 2 µg/µL. Two worms in three replicates per group were taken in 2 mL microcentrifuge tubes on the 3rd day after each feeding. Planarian water was replaced with 1 mL Trizol and then stored at -80°C. During the time of RNA extraction, samples were thawed on ice for about 20 mins. 3-4 metal beads were added to each tube and samples were homogenized at a frequency of 30/s for 2 mins at 4°C until the samples were completely homogenized. Samples were then incubated for 5 mins at room temperature for complete nucleoprotein-complex dissociation. 200 µL Chloroform (1:5 ratio to Trizol) was added to each tube, inverted-mixed for 15 sec, and incubated for 2-3 mins at room temperature. Samples were then centrifuged at 12,000 x g for 15 mins at 4°C. The upper aqueous phase was then removed into a new 1.5 mL microcentrifuge tube. The aqueous phase was

then added to 250 μ L RNase free Isopropanol and 250 μ L high-salt precipitation solution (0.8 M Sodium-citrate and 1.2 M NaCl). The samples were then inverted-mixed 15-20 times. The mixture was transferred to Zymo-Spin Column II CG and centrifuged for 1 min at 10,000 x g. The flowthrough was discarded, and 400 μ L RNA-Wash was added and centrifuged for 30 s at 10,000 x g. The flowthrough was discarded, followed by adding 80 μ L DNase solution (75 μ L DNA-Digestion buffer, 5 μ L DNase) and incubating for 15 mins. 700 μ L RNA-PreWash was added and centrifuged for 30 s at 10,000 x g. This step was repeated again, and samples were centrifuged for 2 mins to remove residual buffer. The column was transferred to a new RNase-free 1.5 mL microcentrifuge tube. For elution, 25 μ L nuclease-free water was added, and after waiting for 1 min for binding, samples were centrifuged for 1 min at 10,000 x g.

2.7.2 Integrity check and sample preparation for RNA Sequencing

Agarose gel electrophoresis was performed to confirm the integrity and size of the samples. 3 μ L of the sample were taken in a PCR tube and denatured at 70°C for 2 mins. 2 μ L of RNA loading dye was mixed, and the samples were run in 1% TTE Agarose gel at 90 V for 30 mins. The concentration of the samples was further checked in the Nanodrop (A260nm). polyA enriched library preparation and paired-end 150bp RNA sequencing with 40 million reads was done per sample.

2.8 Immunoprecipitation

3-4 medium-sized worms (6-8 mm) were used as input. Worms were put into 1.5 mL microcentrifuge tubes, and planarian water was removed. 150 μ L ice-cold lysis/wash buffer of kit supplemented with HALT protease inhibitor cocktail to 1x concentration was added, and worms were immediately lysed with a pestle. 650 μ L ice-cold lysis/wash buffer was added (to 800 μ L total volume). Worm lysates were incubated on ice for 10 mins with pipetting up and down 10 times every 3 mins. Worm lysates were centrifuged at 13,000 x g for 10 mins at 4°C. The supernatant was moved to a fresh microcentrifuge tube. 37.5 μ L of the lysate was moved to a fresh microcentrifuge tube and mixed with 10 μ L 5x SDS sample buffer and 2.5 μ L 1 M DTT (lysate samples). The supernatant was split into two fresh microcentrifuge tubes (400 μ L each). 5 μ g antibodies were added to the lysates (anti-RFP and anti-2100

antibody). Samples were incubated for 1.5 h at room temperature on a spinning wheel. 25 μ L protein A/G magnetic beads were moved to fresh microcentrifuge tubes. 175 μ L lysis/wash buffer was added to the beads and mixed by pipetting. Beads were separated on the magnetic rack, and the supernatant was removed. 1 mL lysis/wash buffer was added to the beads and inverted-mixed for 1 min. Beads were separated on the magnetic rack, and the supernatant was removed. 500 μ L samples were added to the beads and incubated for 1 h at room temperature on a spinning wheel. Beads were separated on the magnetic rack. 37.5 μ L supernatant was moved to a fresh microcentrifuge tube and mixed with 10 μ L 5x SDS sample buffer and 2.5 μ L 1 M DTT (supernatant samples). The remaining supernatant was removed, and 500 μ L ice-cold lysis/wash buffer was added and mixed by pipetting. The washing steps were repeated another two times (three washed with lysis/wash buffer in total). Beads were separated on the magnetic rack, the supernatant was removed, and 500 μ L ice-cold nuclease-free ddH₂O was added. Beads were again separated on the magnetic rack, the supernatant was removed, and 60 μ L 1x SDS sample buffer with 50 mM DTT was added to the beads and mixed by pipetting. Supernatant and magnetic bead samples in SDS sample buffer were heated to 95°C for 8 mins. Beads were separated on the magnetic rack, and immunoprecipitated (IP) samples were moved to a fresh microcentrifuge tube. All lysate, supernatant, and IP samples were stored at -20°C. The samples were run on NuPAGE 4-12 % BisTris Protein Gels at 70 V for 5 mins.

2.9 Identification of Bacteria Found in Planaria

Asexual *Schmidtea mediterranea* were starved for 3 weeks and stored in a box with 1x PW without antibiotics for 5 days. From this box, planarian water of various concentrations (1 mL, 500 μ L, 100 μ L, and 10 μ L) was plated in an LB agar plate without antibiotics. 3 biological replicates of mucus samples were prepared by putting each worm in 500 μ L LB medium, vortexing, and plating the same in LB agar plates without antibiotics. 3 biological replicates of worm lysate were prepared by washing each worm three times with fresh 1x PW. It was then lysed with a pestle in 100 μ L LB medium and made to pass through a 50 μ m cell strainer. Three concentrations (5 μ L lysate + 495 μ L LB medium, 2.5 μ L lysate + 495 μ L LB medium, and remaining lysate + 400 μ L LB medium) of each worm lysate were plated in LB

agar plates without antibiotics. Plates were incubated at 37°C for 2-3 days till the appearance of colonies. Colonies with different morphologies from all the groups were inoculated in 6 mL LB media at 20°C at 140 rpm for 1-2 days till all the cultures grew. 200 µL cultures from each were taken into microcentrifuge tubes and centrifuged at 11,000 rpm for 1 min at 4°C. After discarding the supernatant, the pellet was resuspended in 500 µL 1x PBS and centrifuged at 11,000 rpm for 1 min at 4°C. After discarding the supernatant, the pellet was resuspended in 200 µL Elution Buffer (from QIAquick PCR purification kit). The samples were heated at 95°C for 5 mins. 3 µL of this sample was used as the template for the PCR reaction of 50 µL. 616V and 1492R primers (primers for 16sRNA), along with Q5® DNA Polymerase, were used according to the producer's protocol at an annealing temperature of 60°C. Samples were sent for sequencing with both the primers.

2.10 Challenging Planaria with Bacteria

Bacterial strains found from planaria were incubated for 1-2 days in 6 mL LB medium at 20°C at 140 rpm till the cultures grew. *Bacillus subtilis* was also grown overnight in 6 mL LB medium at 37°C at 140 rpm. Cultures were harvested by centrifugation at 11,000 x g for 1 min at 4°C. The supernatant was discarded, and bacterial pellets were resuspended in 1 mL 1x PW. Samples were again centrifuged, the supernatant discarded, and resuspended in 500 µL 1x PW. The OD₆₀₀ was measured for each culture and accordingly, different concentrations were prepared. 6 worms for each group were taken in 30x40 mm plates and were incubated in planarian water mixed with bacteria with concentrations of O.D.=0.1 and O.D.=0.4. 10 worms for each group were also taken in 94 mm plates and were fed with calf liver mixed with bacteria with a total concentration of O.D.=1 and O.D.=4. The planarian water was replenished with fresh bacteria, and worms were fed with fresh bacteria following 1 week. After 3 days, 3 worms from each group were cut into 3 pieces each and were checked for their regeneration ability. The rest of the worms were kept under observation. The bacterial strains that seemed to have an effect on the worms were again cultured. 60 worms for each bacterial strain were taken in 60mm plates and incubated in planarian water mixed with bacteria of O.D.=0.4. After 4 days, half of the worms from each group were washed and incubated in fresh petri dishes with 1x PW without antibiotics. Western blot samples were taken for 1 day, 7 days, and 11 days

post-first incubation for groups with continuous incubation with bacteria. Western blot samples were taken for 7 days and 11 days post-first incubation for groups that did not have continuous incubation with bacteria.

2.11 Challenging *Drosophila* with Bacteria

20 freshly hatched female flies (not older than a week) were taken for each group and placed into 5-6 flies per vial at 25°C. Bacteria that seemed to have an effect on planaria were cultured, and 1 mL of O.D.=1 was pelleted down and resuspended in 70 µL 1x PBS and used for infection. Flies were anesthetized using CO₂ and poked once in the thorax with a needle after dipping it in the bacteria every time before poking. For the control group, flies were only poked without dipping in any bacteria. Flies were then incubated at 25°C and kept under observation.

Table 2.4 - *Drosophila* strains used in this study

Identifier	Genotype	Strain ID	Source
Hml-Gal4	w[1118]; P{w[+mC]=Hml-GAL4.Delta}2, P{w[+mC]=UAS-2xEGFP}AH2	30140	BDSC
UAS-CG12065 TRiP RNAi	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01111}attP2	34636	BDSC
UAS-CG12065 KK RNAi	P{KK105204}VIE-260B	104144	VDRC
UAS-CG12065 GD RNAi	w[1118]; P{GD6740}v31121	31121	VDRC
UAS-Arl8 RNAi	w[1118]; P{GD10612}v26085	26085	VDRC
UAS-Drp1 (Dynamin) RNAi	w[1118]; P{GD10456}v44156/TM3	44156	VDRC
UAS-Sapr (Saposin related)	w[1118] P{GD3133}v51130	51130	VDRC

2.12 Extraction of hemocytes and staining for lysosome

10 adult male flies not more than 1 week old were taken per group and immobilized on a cold plate (4°C). 100 µL of Schneider's Medium with 10% FBS was added as a drop on the cold plate. The flies were ripped apart with the help of forceps to allow the hemolymph to mix in the Schneider's Medium. The hemolymph was then transferred to a glass bottom dish, and the hemocytes were allowed to adhere to the dish for 20 mins. Following this, 400 µL of Schneider's Medium with 20% FBS was added to cover the whole area of the dish. The LysoTracker (SCT138, Sigma-Aldrich, BioTracker NIR633 Lysosome Dye) and MitoTracker (MitoTracker™

Deep Red FM, Cat n°: M22426) were added in the dilution of 1:1000 and pipette-mixed slowly such that the cells remained adhered. Following 15 mins of incubation, a coverslip was placed, avoiding any bubbles and cells were imaged at 100x.

2.12 S2R+ cell culture and transfection

Drosophila cell line S2R+ cells were cultured in Schneider's Medium with 10% FBS and 1x Pen Strep solution (15070063 Thermo Fisher) and incubated at 25°C. Cells were passaged and split in regular intervals to maintain a healthy population. Before the day of transfection, cells were split into 1:5 dilution and 200 µL of cells were seeded in each well in a 96 well plate. Transfection mix was freshly prepared on the day of transfection by keeping the amount of nucleic acids the same for each condition and transfection was performed by using the standard transfection protocol of the Effectene reagent (301425 Qiagen).

Table 2.5 - Transfection mix preparation for each group

Group	dsRNA	Actin-Gal4	UAS-eGFP	EC buffer	Enhancer	Effectene
eGFP RNAi UAS-eGFP	150 ng/ 1.5 µL	75 ng/ 0.16 µL	75 ng/ 0.16 µL	21.5 µL	2.5 µL	2.5 µL
CG12065 RNAi UAS-eGFP	150 ng/ 1.5 µL	75 ng/ 0.16 µL	75 ng/ 0.16 µL	21.5 µL	2.5 µL	2.5 µL
UAS-eGFP	-	75 ng/ 0.16 µL	75 ng/ 0.16 µL	21.5 µL	2.5 µL	2.5 µL
UAS-CG12065-sfGFP	-	75 ng/ 0.16 µL	75 ng/ 0.16 µL	21.5 µL	2.5 µL	2.5 µL

Chapter 3 Results

3.1 Phenotype of 2100 RNAi

To analyze the function of 2100 in *S. mediterranea*, RNAi-mediated knock-down experiments were conducted. 20 similar-sized (3-4 mm) worms were taken for each group - eGFP (negative control), 2100 and Bcl2-1 per concentration. These were fed with aliquots of calf liver mixed with double-stranded RNA (dsRNA prepared by in-vitro transcription) at a final concentration of 1 $\mu\text{g}/\mu\text{L}$, 2 $\mu\text{g}/\mu\text{L}$, and 4 $\mu\text{g}/\mu\text{L}$ for each respective gene. Only two feedings were given to the Bcl2-1 groups, whereas three feedings were given to eGFP and 2100 groups. Progressive lesions and, ultimately, death of the flatworms were observed in the 2100 RNAi group (Fig. 3.1A). The number of surviving worms (Fig. 3.1B) and the number of healthy worms without lesions (Fig. 3.1C) were scored for each group and plotted for comparison. The effect in 2100 RNAi was weaker but similar to the Bcl2-1 group. Bcl2-1 is a conserved anti-apoptotic regulator which is described in planaria (Pellettieri *et al.*, 2010). This shows that 2100 is essential for the survival of the *S. mediterranea* and possibly have a role in apoptosis or cell death regulation.

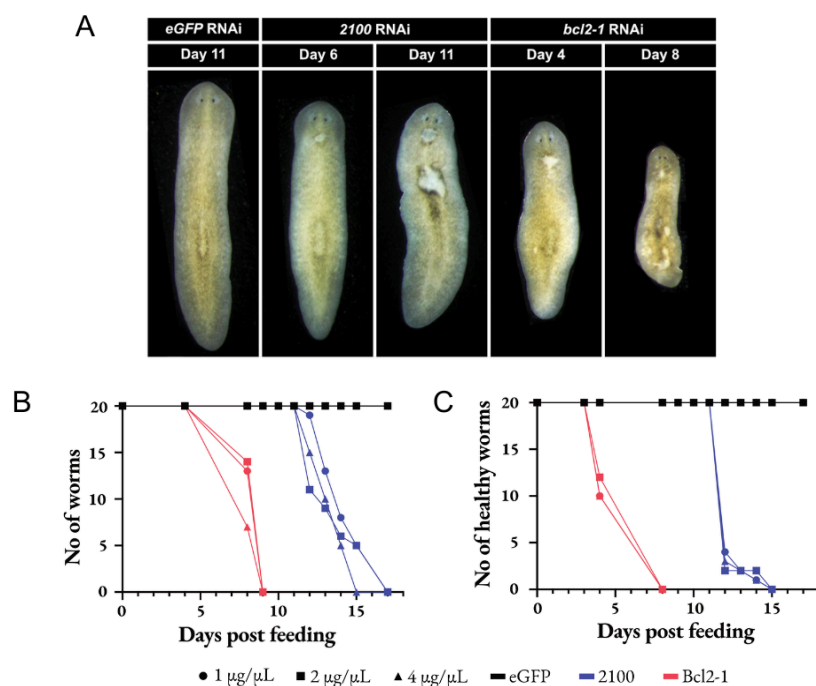


Fig. 3.1 - Phenotypic comparison of 2100 RNAi with bcl2-1 RNAi and eGFP RNAi in *Schmidtea mediterranea*. A) Worms fed with a final concentration of 4 $\mu\text{g}/\mu\text{L}$ dsRNA showing lesions in 2100 RNAi similar to that in bcl2-1 RNAi. B) Survival plot showing the number of surviving worms. C) Survival plot showing the number of healthy worms without lesions.

3.2 Expression pattern of 2100 protein

To visualize the expression pattern and subcellular localization of 2100, whole-mount immunostainings were performed. 10 similar-sized (3-4 mm) flatworms of each group, eGFP and 2100, were injected with 2 $\mu\text{g}/\mu\text{L}$ of dsRNA to prevent the background from the liver feed in the stainings. Worms were injected twice with an interval of 3 days between the two injections. All worms were fixed after 1 week from the first injection. Worms were then bleached and stained with custom-made antibody against 2100 and DAPI. 2100 showed to be enriched at the epidermis in the eGFP dsRNA injected group while it was also present inside the tissue. However, 2100 was highly depleted in the 2100 dsRNA injected group validating the specificity of the custom-made antibody and efficient knockdown of 2100 (Fig. 3.2).

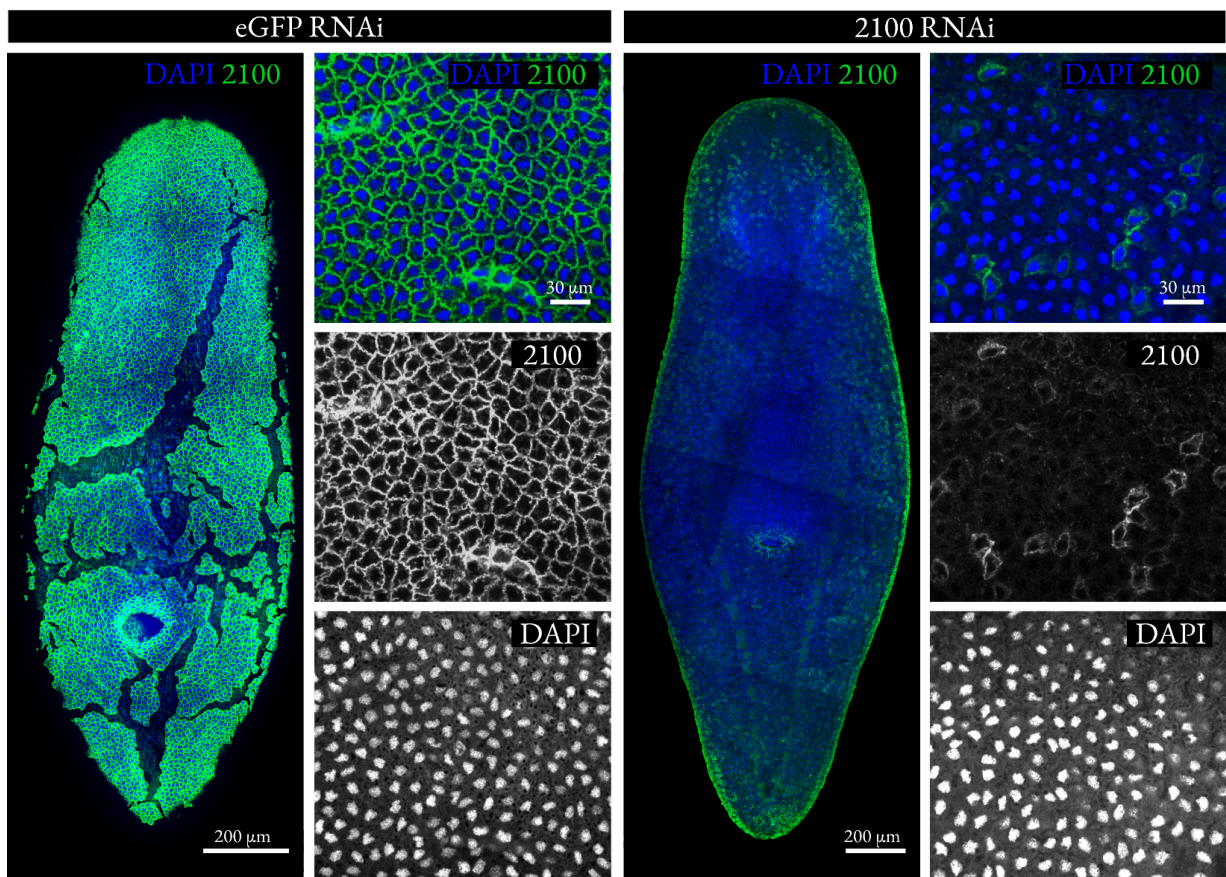


Fig. 3.2 - Whole-mount immunostaining with anti-2100 antibody and DAPI showed enrichment of 2100 at the epidermis. 2100 RNAi showed depleted expression of 2100 validating the specificity of the custom-made antibody and efficient knockdown of 2100. Maximum z projection of whole worms (left) and magnified regions (right) are shown in the panel. Images were taken at 30x in a confocal microscope.

3.3 RNA sequencing of *2100* RNAi vs *eGFP* RNAi

To identify the potential mechanisms and pathways involved in the phenotype of *2100* knockdown, RNA sequencing was performed for transcriptome comparisons of *2100* RNAi worms and *eGFP* RNAi worms (control) following both, 1 and 2 dsRNA feedings. For 3 biological replicates per group, total RNA isolation followed by polyA enriched library preparation was done, and samples were sent for paired-end 150 bp RNA sequencing with 40 million reads per sample (Fig. 3.3A). In the PCA plot, two main clusters were observed along the PC1 axis, which were separated based on the two feeding statuses. However, the separation between the two knockdown conditions was small. This suggests that the difference was greater between the two feeding statuses rather than the two RNAi conditions (Fig. 3.3B). According to the volcano plots following both 1 (Fig. 3.3C) and 2 feedings (Fig. 3.3D), transcripts of very few genes were differentially expressed. However, the *2100* transcript was significantly lowered in the *2100* RNAi group, proving that the knockdown of the gene was successful. This indicates that *2100* is acting at the protein level and not at the transcript level. A cutoff of $\text{padjust} < 0.5$, $-1 < \text{Log}_2\text{FC} > 1$ was set to define the differentially expressed genes (DEGs). Prosaposin was the only DEG (more than $\log_2=1$ fold change) with a high significance. Following the 1st feeding, most DEGs identified were unknown (no homologies in other organisms) or non-coding RNAs, and some localized in the cytoplasm, extracellular, endoplasmic reticulum, mitochondria, membrane, and nucleus. Following the 2nd feeding, most DEGs identified were associated with the membrane and extracellular, while some non-coding RNAs and the rest were in the cytoplasm, nucleus, lysosome, and endoplasmic reticulum (Fig. 3.3E). The only DEG present when comparing *eGFP* RNAi versus *2100* RNAi after 1 feeding or 2 feedings or when comparing *eGFP* RNAi versus *2100* RNAi from both feedings together was the RNAi target *2100* (Fig. 3.3F). All other genes were either unique to the comparison of 1 feeding samples or 2 feedings samples (Fig. 3.3F), indicating that there is no consistent trend of gene expression regulation on the transcript level during *2100* RNAi. However, the DEGs from the 2 feedings samples are more likely to be caused by the *2100* RNAi as the worms are starting to show the specific phenotype at this stage. A cell-type expression pattern analysis of the DEGs after 2 feedings reveals, that many of these DEGs, including prosaposin, are specifically expressed in phagocytes, while some

are also present in various parenchymal cells (which could also be phagocytic) (Fig. 3.3G). This suggests that 2100 might have a role in phagocytic cells.

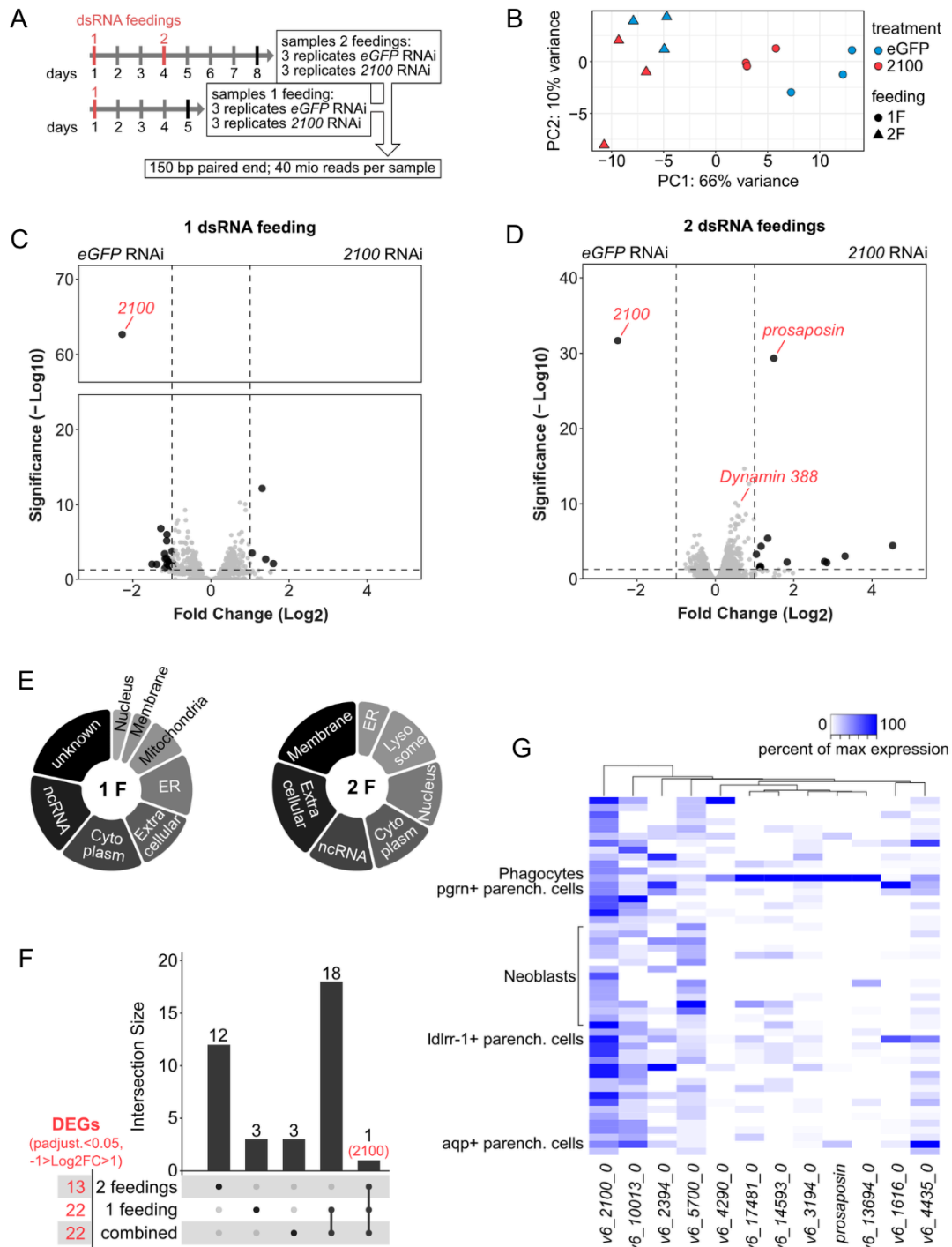


Fig. 3.3 - RNA sequencing of 2100 RNAi and eGFP RNAi. A) Experimental Setup B) PCA plot showing two main clusters along PC1 separated based on feeding statuses. Volcano plot of C) 1 dsRNA feeding D) 2 dsRNA feeding shows changes in the transcript level of very few genes. E) Subcellular localization of DEGs identified post each feeding status F) Upset plot showing that 2100 is the only DEG present in all comparisons (eGFP RNAi vs. 2100 RNAi) after 1 feeding, 2 feedings, or both combined. G) Cell Type Expression pattern of DEGs post 2 dsRNA feedings shows that many are specifically expressed in phagocytes.

3.4 Analysis of the role of 2100 in gut morphology

While 2100 RNAi worms show a visible phenotype in the epidermis (lesions), it is unclear to what extent other organs are affected. RNA-sequencing data showed that most of the DEGs are expressed in phagocytes. Phagocytes are one of the major gut cell types. Also, the gut is the first to encounter pathogens through feeding. Hence, gut morphology was analyzed in 2100 RNAi compared with eGFP RNAi. Whole-mount fluorescent RNA *in situ* hybridization was performed using riboprobes for phagocyte cell marker (dd_75) and lateral goblet cell marker (dd_131). However, no significant difference was observed with either of the gut markers following the 2100 knockdown in comparison with the eGFP knockdown worms (Fig. 3.4). This indicates that 2100 does not have any effect on the gut morphology.

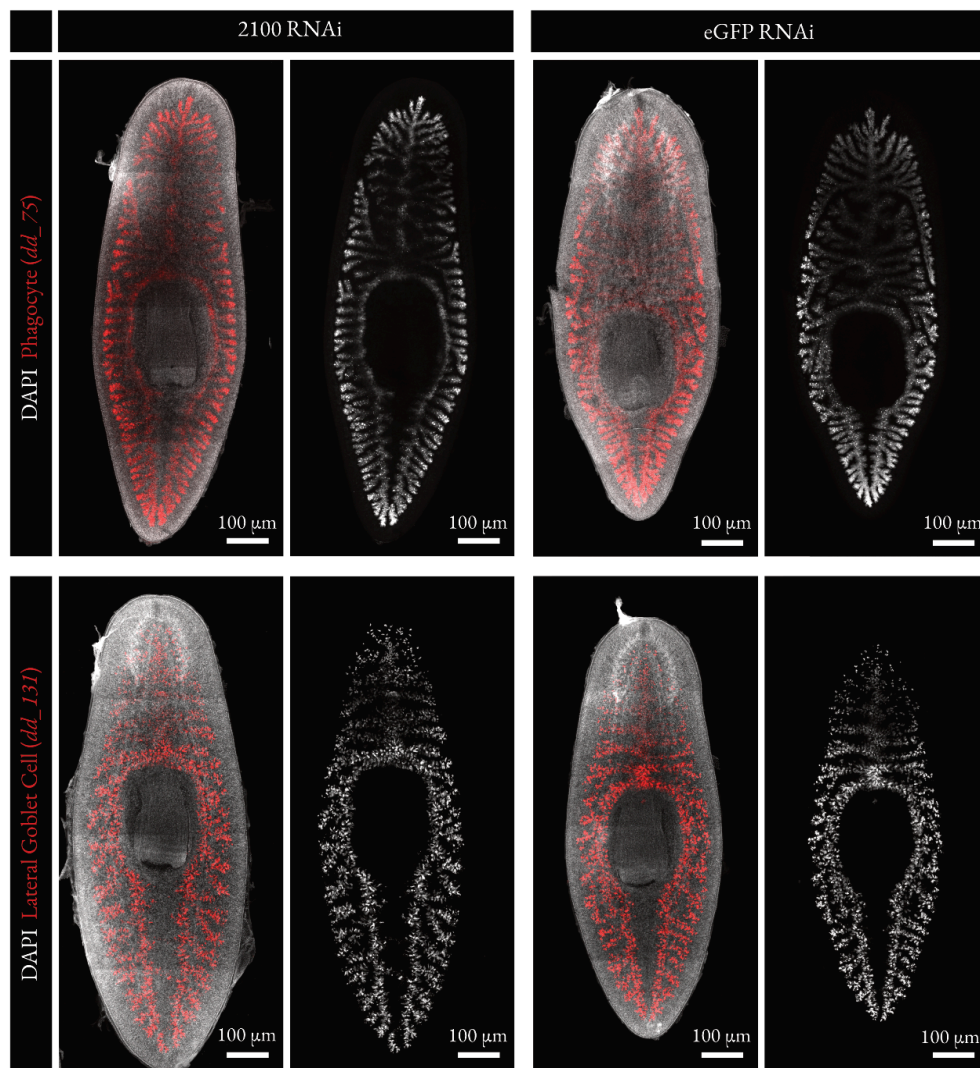


Fig. 3.4 - Whole-mount *in situ* hybridization for Phagocyte (upper) and lateral goblet cell (down) markers in 2100 RNAi (left) and eGFP RNAi (right) shows no difference in the gut morphology. Images were taken at 20x in a confocal microscope.

3.5 Analysis of the effect of 2100 RNAi on lipid levels

The RNA sequencing data showed that the only differentially expressed gene in 2100 RNAi with a high significance was *prosaposin* (Fig. 3.3 A). The activated form of Prosaposin, Saposin (sphingolipid activator proteins), enters lysosomal membranes to induce bilayer disintegration and lipid extraction. It is known to have roles in lipid antigen presentation, processing of apoptotic bodies, and antibiotic function. Mutations in *prosaposin* have been reported in many clinical diseases like Parkinson's disease (He *et al.*, 2023), Gaucher Disease (Varacco *et al.*, 2010), etc., due to the accumulation of lipids. Hence, the levels of lipids were analyzed in 2100 RNAi worms compared to *eGFP* RNAi by performing whole-mount lipid staining with LD540. However, no significant change was observed between the two knockdown conditions. (Fig. 3.5) This indicates that 2100 RNAi does not cause a major change in the lipid levels.

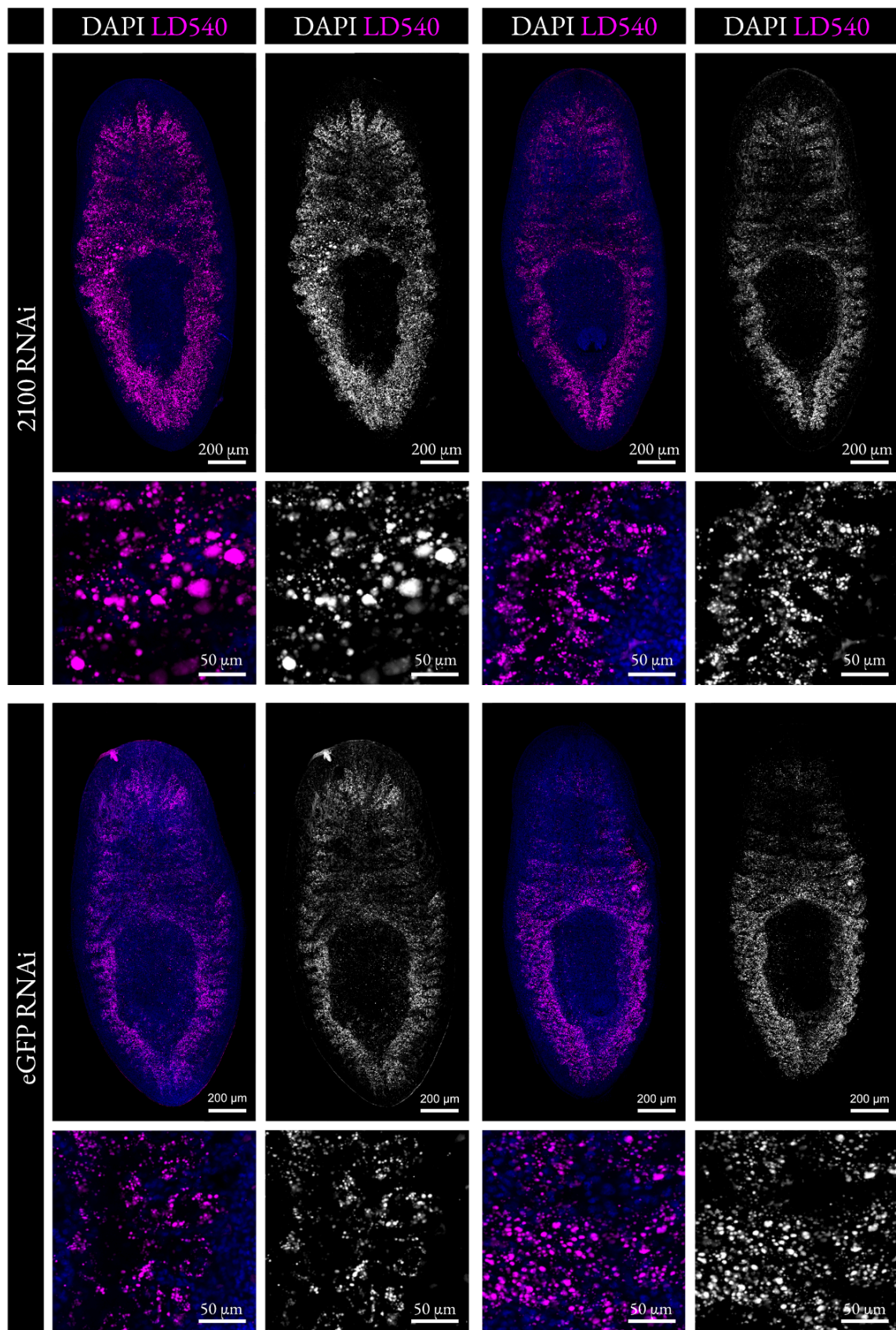


Fig. 3.5 - Whole-mount lipid staining with LD540 in 2100 RNAi and eGFP RNAi does not show major change in lipid levels. Maximum z projection (upper) and single z plane (down) are shown in the panel. Images were taken at 20x in a confocal microscope.

3.6 Pull-down of 2100 from worm lysate followed by Mass Spectrometry

3.6.1 Interactors identified from pull-down of 2100

To identify the potential interaction partners of 2100, an immunoprecipitation (IP) of 2100 from wildtype worm lysate using the anti-2100 antibody was done with anti-RFP antibody as a control. 2100 showed the highest abundance in the IP using anti-2100 antibody, confirming successful pull-down and antibody specificity (Fig. 3.6A). The pull-down furthermore identified 44 potential interactors (Table 3.1) of 2100, including Arl8-B, Peroxiredoxin, Dynamin-1-like, antiviral Mx Dynamin, Calpain-B, and Endophilin B1 amongst others. It also identified Prosaposin, which was also found in the RNA-sequencing data as a DEG (Fig. 3.6A). Most of these interactors are associated with the cytoplasm, cell membrane, nucleus, mitochondrial membrane, extracellular matrix, cytoskeleton, endoplasmic reticulum membrane, cell cortex, endosome, lysosome, and others (Fig. 3.6B). The cell type expression pattern reveals that a lot of these interactors are present in the phagocytes and parenchymal cells, similar to the DEGs in *2100* RNAi shown by the RNA sequencing data (Fig. 3.6C).

Table 3.1 - Potential interactors of 2100 identified from immunoprecipitation of anti-2100 antibody. The table shows the gene ID and the knockdown phenotype in homeostasis, regeneration, and anti-2100 staining.

dsRNA No.	Gene Name	ID	Homeostasis phenotype	Regeneration phenotype	anti-2100 staining
1	eGFP	NA	wt	wt	wt
2	2100	dd_Smed_v6_2100_0_1	lethal	lethal	depleted
3	Arl8B	dd_Smed_v6_2667_0_1	wt	wt	wt
4	Mx884	dd_Smed_v6_884_0_1	wt	wt	wt
5	Mx3169	dd_Smed_v6_3169_0_1	wt	wt	wt
6	Tudor-SN	dd_Smed_v6_906_0_1	wt	wt	wt
7	RecQ DNA helicase	dd_Smed_v6_7318_0_1	wt	wt	wt
8	Peroxiredoxin	dd_Smed_v6_3414_0_1	wt	wt	wt
9	Mx1074	dd_Smed_v6_1074_0_1	wt	wt	wt
10	slc1a	dd_Smed_v6_7570_0_1	wt	wt	wt
11	CAD	dd_Smed_v6_7167_0_1	wt	wt	wt
12	CDP synthase	dd_Smed_v6_4856_0_1	wt	wt	wt
13	Calpain-B	dd_Smed_v6_1971_0_1	wt	wt	wt
14	prosaposin	dd_Smed_v6_215_0_1	wt	wt	wt
15	ATP synthase	dd_Smed_v6_428_0_1	head regression, lesions	no regeneration	wt
16	adducin 1	dd_Smed_v6_3074_0_1	wt	wt	wt
17	adducin 1	dd_Smed_v6_1425_0_1	wt	wt	wt
18	Dynamin-1-like	dd_Smed_v6_388_0_1	-	-	-
19	quinolinate phosphoribosyltransferase	dd_Smed_v6_2522_0_1	-	-	-
20	erythrocyte membrane protein band 4.1 like 2	dd_Smed_v6_2032_0_1	-	-	-
21	tRNA methyltransferase 1	dd_Smed_v6_10289_0_1	-	-	-
22	myosin heavy chain, non-muscle	dd_Smed_v6_791_0_1	wt	wt	wt
23	NADH dehydrogenase (ubiquinone) Fe-S protein 5, 15kDa	dd_Smed_v6_1674_0_1	wt	no regeneration	wt
24	polynucleotide kinase 3'-phosphatase	dd_Smed_v6_1211_0_1	wt	wt	wt
25	NA	dd_Smed_v6_313_0_1	wt	wt	wt
26	dumpy	dd_Smed_v6_1748_0_1	wt	wt	wt
27	coactosin like F-actin binding protein 1	dd_Smed_v6_63_0_1	wt	wt	wt
28	KHD-1	dd_Smed_v6_294_0_1	wt	wt	wt
29	signal sequence receptor subunit 3	dd_Smed_v6_1265_0_1	-	-	-
30	NA	dd_Smed_v6_8_0_1	-	-	-
31	Myosin light chain cytoplasmic	dd_Smed_v6_559_0_1	lethal	wt	wt
32	NA	dd_Smed_v6_199_0_1	wt	wt	wt
33	SH3-domain GRB2-like endophilin B1	dd_Smed_v6_4055_0_1	wt	wt	wt
34	CDGSH iron sulfur domain 2	dd_Smed_v6_2098_0_1	wt	wt	wt
35	myosin VI	dd_Smed_v6_4503_0_1	wt	wt	wt
36	adducin 1	dd_Smed_v6_3064_0_1	-	-	-
37	collectin-11-like	dd_Smed_v6_10_1_1	-	-	-
38	Pterin-binding domain	dd_Smed_v6_1111_0_1	wt	wt	wt
39	NA	dd_Smed_v6_359_0_1	wt	wt	wt
40	Ast5	dd_Smed_v6_497_0_1	wt	wt	wt
41	NA	dd_Smed_v6_1360_0_1	wt	wt	wt
42	apolipoprotein O-like	dd_Smed_v6_2953_0_1	wt	wt	wt
43	succinate dehydrogenase complex iron sulfur subunit B	dd_Smed_v6_925_0_1	wt	wt	wt
44	echinoderm microtubule-associated protein like 1	dd_Smed_v6_958_0_1	wt	wt	wt
45	Tropomyosin 1	dd_Smed_v6_759_0_1	wt	wt	wt
46	Tropomyosin 1	dd_Smed_v6_1790_0_1	wt	wt	wt

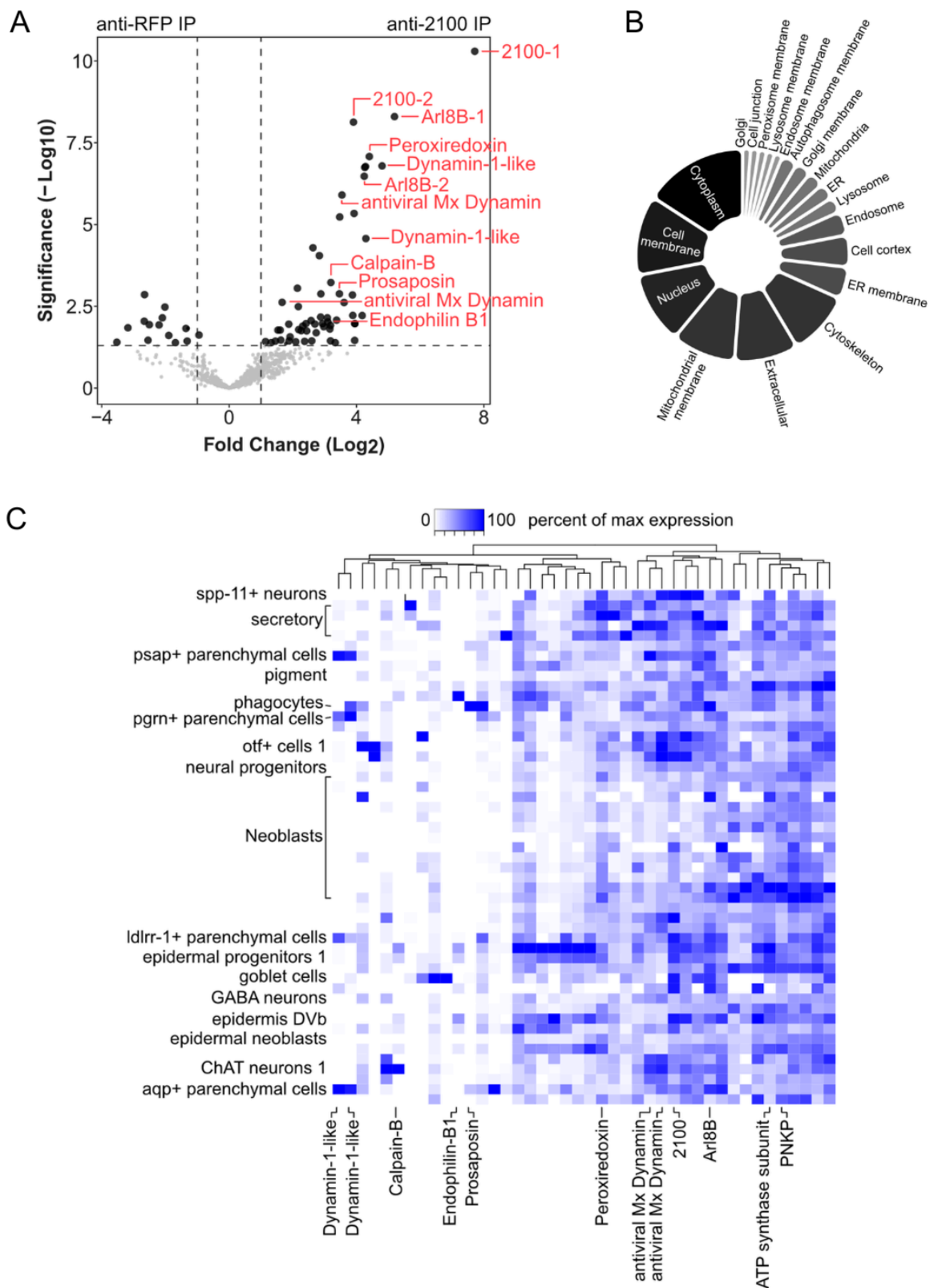


Fig. 3.6 - Immunoprecipitation of 2100 with anti-2100 antibody from whole worm lysate. A) Volcano plot highlighting some of the potential interactors of 2100 B) Subcellular localization of DEGs C) Cell Type Expression pattern of DEGs shows that many are expressed in phagocytes.

3.6.2 Analysis of knockdown of potential interactors of 2100

In order to analyze the function of the interactors in *S. mediterranea* and draw inferences about their association with 2100, knockdown experiments of all the interactors, except for a few (due to unsuccessful cloning of PCR template represented by '-' in Table 3.1), have been done. These RNAi experiments were done to find out whether knockdown of the interactors creates a similar phenotype to 2100 RNAi, affects 2100 localization, and whether it creates any other phenotypes. While none of the knockdown conditions showed a phenotype similar to 2100 knockdown, RNAi of some genes caused different phenotypes. Knockdown of ATP synthase (no. 15, Table 3.1) showed head regression and few lesions but only post 3 weeks after last dsRNA feeding. Knockdown of myosin light chain (no. 31, Table 3.1) showed an edema effect and proved lethal post 1 week after last dsRNA feeding. Knockdown of all the other genes did not cause any phenotype in homeostasis. Regeneration assays have also been conducted following knockdown of each gene. Worms with knockdown of ATP synthase and NADH dehydrogenase (no. 23, table 3.1) lost the ability to regenerate and the cut un-regenerated pieces still survived for a month (Fig. 3.7A). Worms with knockdown of myosin light chain showed early regeneration but died similarly to the intact worms. Worms with knockdown of all the other genes showed normal regeneration patterns (Fig. 3.7A). Whole-mount immunostaining for 2100 using anti-2100 antibody along with DAPI was also conducted for the knockdowns of all the genes. The worms were imaged from both the ventral and dorsal sides since localization of 2100 shows differences when comparing ventral and dorsal epidermis. In control worms with knockdown of eGFP, 2100 was enriched in the membrane, as was observed before. The presence of sub-cellular vesicle-like structures was also observed, which were present more on the dorsal side. However, no change in localization of 2100 was identified for worms with knockdown of any of the interactors. The sub-cellular vesicle-like structures were present in the dorsal side and no difference in the abundance of these intracellular structures could be accounted for (Fig. 3.7B). This indicates that the main function of 2100 is not dependent on forming a functional complex with other proteins since none of its potential interactors showed a phenotype similar to the knockdown of 2100.

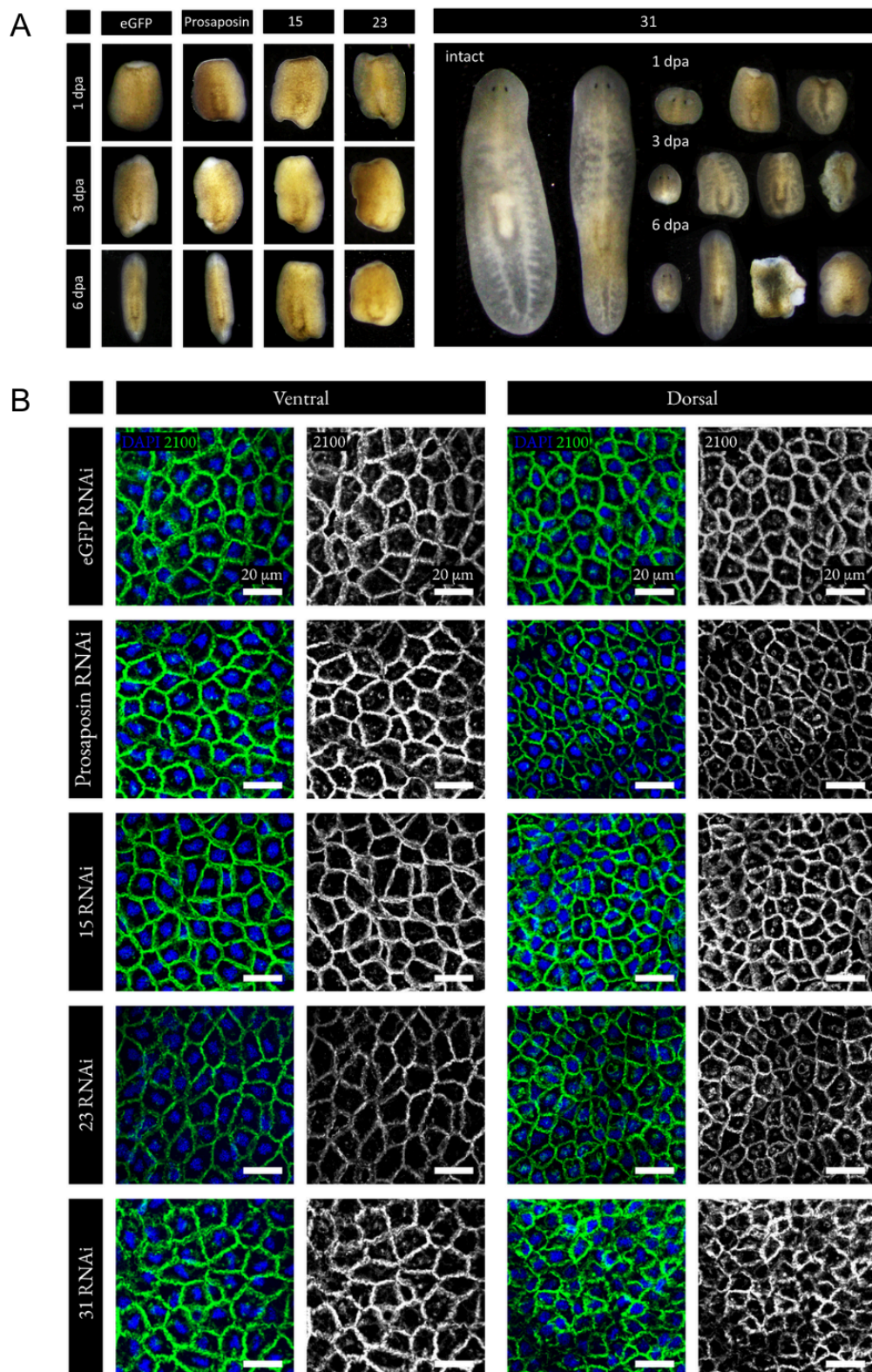


Fig.3.7 - Representation of knockdown of a few of the potential interactors of 2100 - prosaposin, ATP synthase (15), NADH dehydrogenase (23), and myosin light chain (31). A) Regeneration assay showing normal regeneration in eGFP (control) and prosaposin, while no regeneration in 23 and 31 and an edema effect in 31. B) Whole-mount immunostaining for anti-2100 antibody shows no difference in localization of 2100 across the RNAi groups. The dorsal side shows more abundance of the intracellular structures. Maximal z projection of only the epidermis from both ventral and dorsal sides is shown. Images were taken at 60x in a confocal microscope.

3.6.3 Double-knockdown of *prosaposin* followed by 2100

Since *prosaposin* was identified both from the RNA sequencing and the immunoprecipitation, further analysis was done for this gene. It was hypothesized that 2100 might be inhibiting *prosaposin*, and as a result, the knockdown of *prosaposin* does not have a similar effect as the knockdown of 2100. This would mean that a double knockdown of *prosaposin* followed by 2100 should rescue the phenotype of knockdown of 2100. This was tested with three appropriate control groups - eGFP-eGFP, eGFP-2100, and *Prosaposin*-eGFP. 3 dsRNA feedings were given for the first round of knockdown, whereas 2 dsRNA feedings were given for the second round of knockdown. However, the double knockdown of *prosaposin* followed by 2100 showed a similar effect to the double knockdown of eGFP followed by 2100. While the eGFP-eGFP and *Prosaposin*-eGFP showed a 100% survival rate as expected (Fig. 3.8). This disproves the hypothesis that the absence of a similar phenotype in the knockdown of *prosaposin* is due to 2100 inhibiting *prosaposin*.

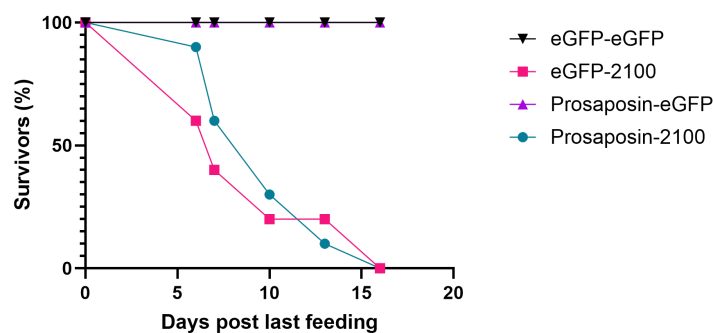


Fig. 3.8 - Survival plot showing similar effect for knockdown of *prosaposin* followed by 2100 (*Prosaposin*-2100) and knockdown of eGFP followed by 2100 (eGFP-2100). 3 dsRNA feedings were given for the first knockdown while 2 dsRNA feedings were given in the second knockdown.

3.7 Characterizing localization of Z-DNA in *S. mediterranea*

2100 has a Z-DNA binding domain instead of a cyclic oligonucleotide sensing domain present in bacterial Cap 17. Z-DNA (the left-handed form of DNA) and Z-RNA can be present in many cellular contexts, e.g., at active sites of transcription, in RNA of stress granules, or in cells that are infected by pathogens (Shin *et al.*, 2016; Deigendesch *et al.*, 2006; Kim *et al.*, 2003). To check for the localization pattern of Z-nucleic acids in *S. mediterranea*, whole-mount immunostaining was performed in wild-type worms with anti-Z-DNA/Z-RNA monoclonal antibody along with anti-2100 antibody and DAPI. Z-DNA was shown to be present in the nuclei of

cells from all sections of the worm (epidermis and cross-section, as shown in Fig. 3.9). It was also enriched in many cells with weak DAPI staining (Fig. 3.9). Hence, analysis of the role of Z-DNA can also be interesting for checking gene expression and cell survival patterns.

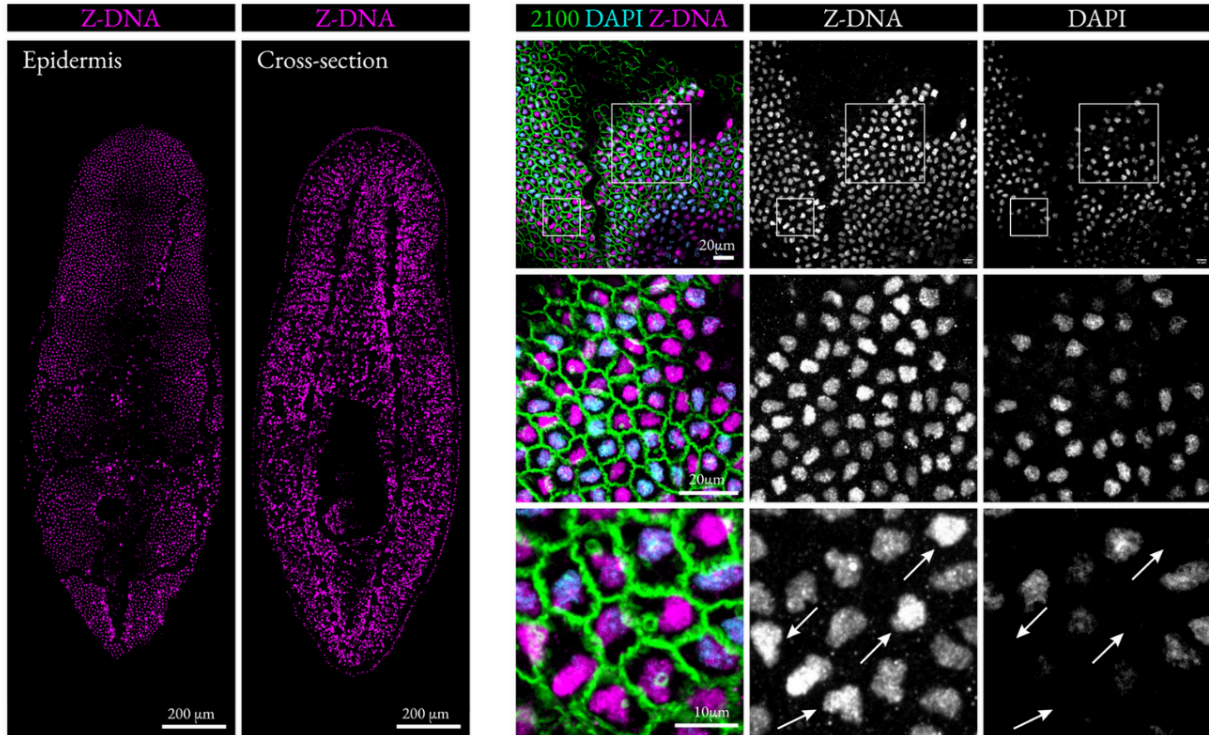
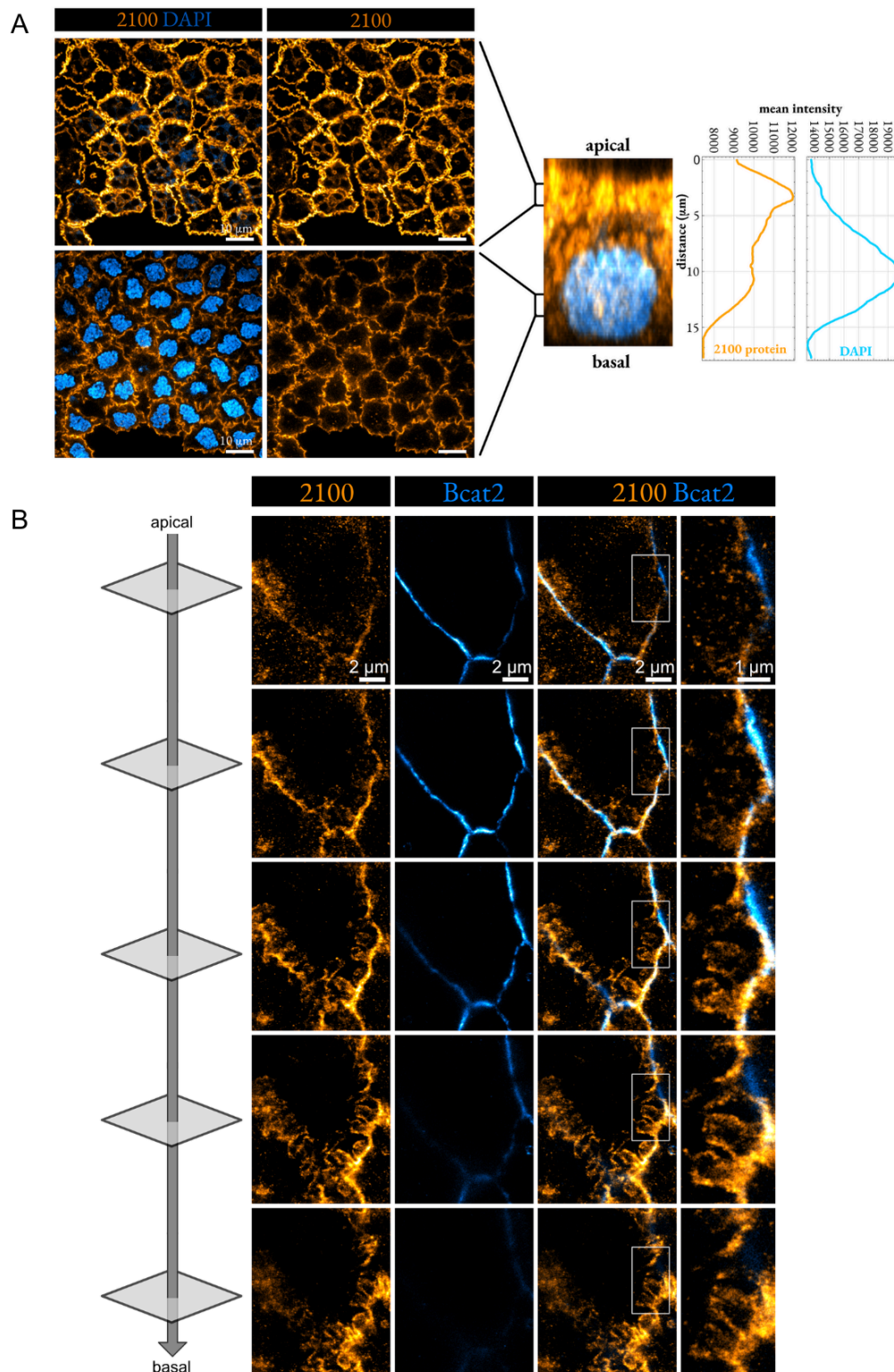


Fig. 3.9 - Expression pattern of Z-DNA in *S. mediterranea*. A) Single z plane of whole worms taken at 20x and B) Single z plane image taken at 60x magnification in a confocal microscope. Arrows showing enrichment of Z-DNA and weak DAPI staining.

3.8 Super-resolution microscopy for sub-cellular localization of 2100

Confocal microscopy at 60x showed the presence of sub-cellular vesicle-like structures formed by 2100. To further analyze these structures, stimulated emission depletion (STED) microscopy was performed at 100x in whole-mount immunostainings. Stainings with anti-2100 antibody and DAPI confirmed that 2100 is localized on the apical side of the cell, as the peak of the mean intensity of DAPI is more inside the tissue than that in 2100 (Fig. 3.10A). Stainings of anti-2100 antibody and beta-catenin 2, adherent junction marker confirmed that 2100 is present just below the adherent junction, forming vesicle-like structures that seem to bud off from the membrane (Fig. 3.10B). In invertebrates, this is the region - below the adherent junction and within the septate junction where endocytosis and exocytosis occurs. Further stainings with DAPI and anti-2100 and anti-Z-DNA/Z-RNA antibodies

showed the presence of intracellular compartments formed by 2100, which only contains DAPI and no Z-DNA (Fig. 3.10C). Since Z-DNA marks for active transcription, this could indicate the internalization of dead cells, and hence account for the absence of Z-DNA in these compartments. This suggests that 2100 might have a role in autophagocytosis.



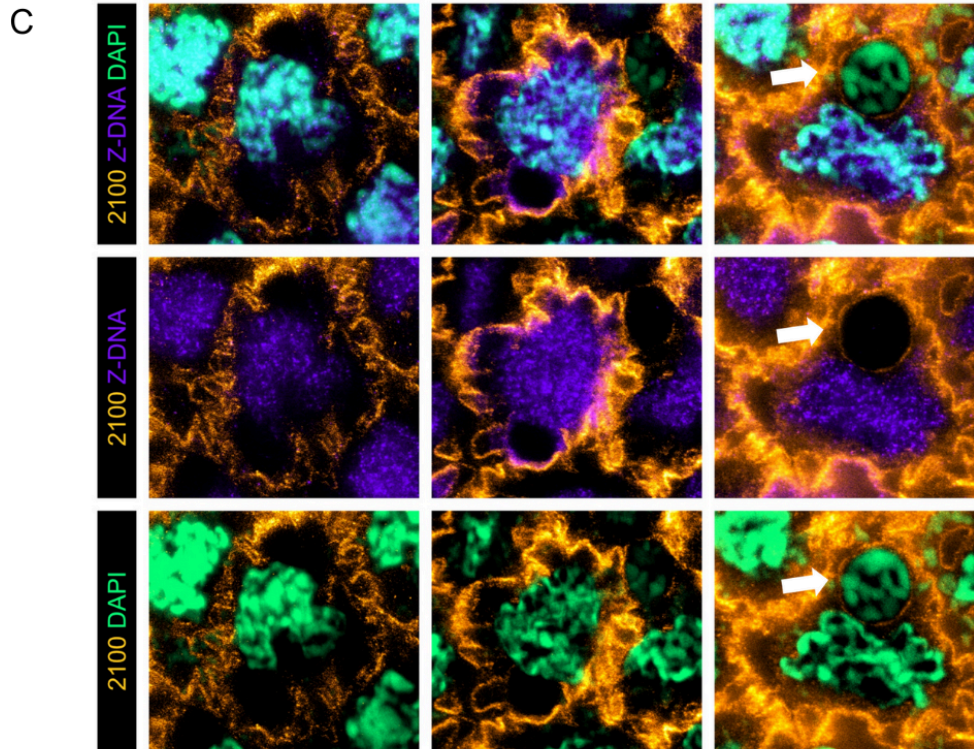


Fig. 3.10 - STED microscopy of whole-mount immunostaining for subcellular localization of 2100. A) Stainings with anti-2100 antibody and DAPI at 60x showing localization of 2100 at the apical side of the cell. B) Stainings with anti-2100 antibody and beta-catenin 2 (or Bcat2- adherent junction) marker at 100x showing localization of 2100 just below the adherent junction and vesicle-like structures budding off from the membrane. C) Stainings with anti-2100 antibody, anti-ZDNA/RNA antibody, and DAPI at 100x showing intracellular compartments formed by 2100. The arrow is showing the presence of DAPI but no Z-DNA inside the compartment.

3.9 Analyzing the effect of inhibition of autophagocytosis in 2100 localization

Since the subcellular localization of 2100 suggested that 2100 might play a role in autophagocytosis, wildtype *S. mediterranea* was treated with 1 μ M, 2 μ M, and 4 μ M Bafilomycin A1 along with a DMSO-treated group as a control. Bafilomycin A1 is an autophagy inhibitor that interacts with V-ATPase and hence inhibits the acidification of the lysosome. This prevents the ability of lysosomal proteases to degrade engulfed cargo (Shacka *et al.*, 2006). Worms treated with Bafilomycin A1 were affected and started dying in a concentration-dependent manner, whereas DMSO-treated worms did not show any effect (Fig. 3.11A). However, whole-mount immunostaining with the anti-2100 antibody did not show any effect on the localization of 2100 (Fig. 3.11B).

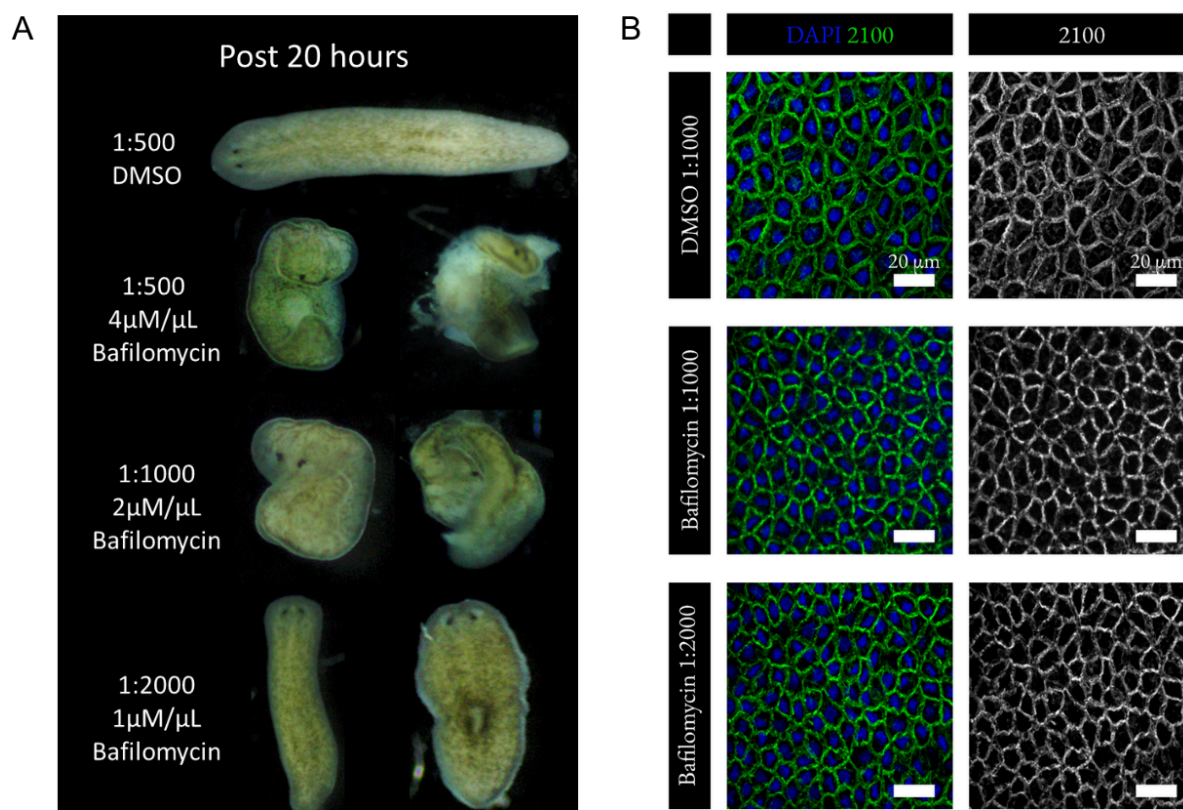


Fig. 3.11 - Treatment with Bafilomycin A1. A) Phenotype comparison of DMSO-treated worms with Bafilomycin A1 post 20 hours of treatment shows lethal phenotype in Bafilomycin A1-treated worms in a concentration-dependent manner. B) Whole-mount immunostaining post 15 hours of treatment from the ventral side shows no difference in 2100 localization. The maximal z projection of the epidermis is shown. Images were taken at 60x in a confocal microscope.

3.10 Analyzing the localization of 2100 in response to foreign DNA

In order to check whether 2100 localization changes in response to foreign DNA, plasmid labelled with DIG (20 μL OptiMeM, 2.5 μL DIG-labeled plasmid (100 ng/μL), 1 μL TransIT-X2 transfection reagent) was injected in wildtype *S. mediterranea* in various regions near the tail and the gut. Worms were fixed immediately post-injection along with a 1x PBS injected group as control. Whole-mount immunostainings were performed with anti-2100 antibody along with DAPI. DIG-labelled plasmid could be visualized in parts of the area of injection and also spread through the gut. 2100 was enriched in the epidermis as observed before, but the stainings were not very clear inside the tissue due to reduced penetration of the antibody. In some cases, there was co-localization of 2100 with the labelled plasmid near the gut beside the lumen (Fig. 3.12). However, due to the low enrichment of 2100 in these regions, no conclusion could be made.

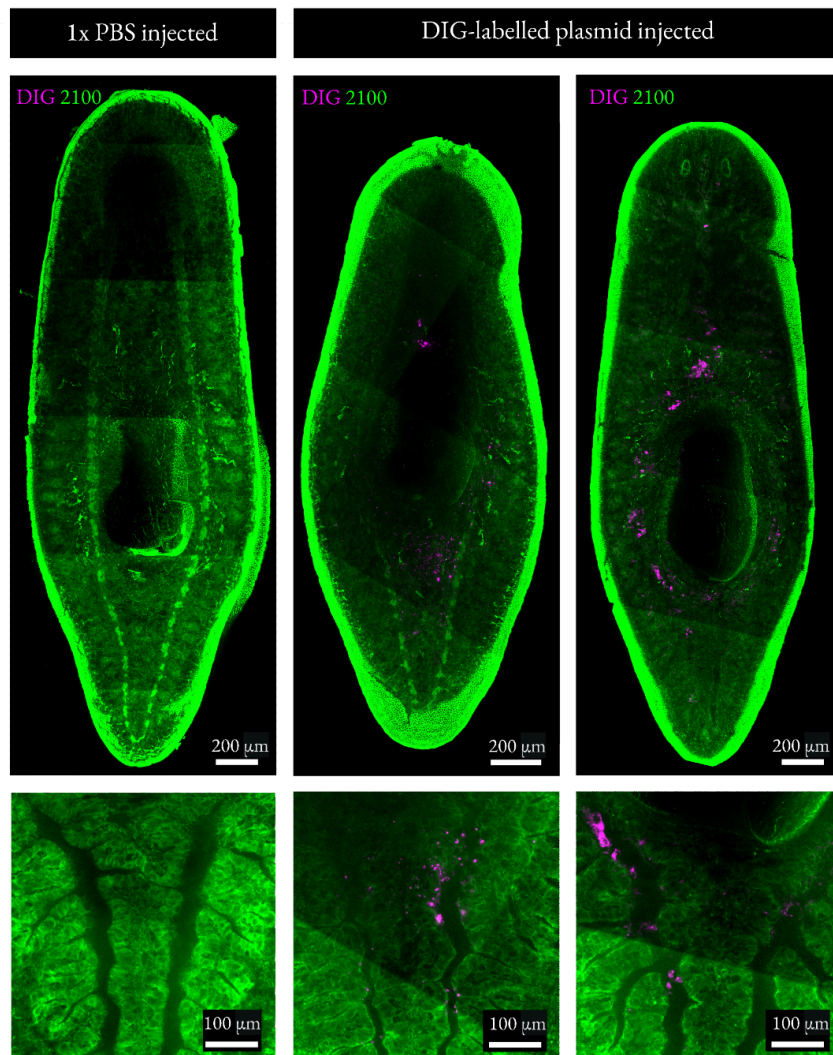


Fig. 3.12 - Whole-mount immunostaining for anti-2100 antibody in response to foreign DNA. 2 representative worms for DIG-labelled plasmid injections- image taken from the ventral side (middle) and image taken from the dorsal side (right). Maximum z projection of planes having plasmid DNA (upper) and single z plane (down) are shown. Images were taken at 60x in a confocal microscope.

3.11 Characterizing the pathogenicity of different strains of bacteria found in *S. mediterranea*

Previous work in the lab suggested that *Bacillus subtilis* and *Pseudomonas japonica* are pathogenic to *S. mediterranea*. In order to find more bacterial strains that could be used to challenge *S. mediterranea*, worms were kept in antibiotics-free 1x PW for 5 days. Mucus samples, worm lysate, and water from the culture box were plated on LB agar plates and left to grow for 2-3 days. Colonies with all different types of morphologies were grown in LB medium. Genotyping was done by PCR amplification with 16srRNA followed by Sanger sequencing. 5 different strains of bacteria were found - *Staphylococcus cohnii*, *Pedobacter* sp., *Chryseobacterium* sp.,

Stenotrophomonas sp., and *Pseudomonas gessardii*. In order to identify if any of these bacteria can be pathogenic to worms, worms were challenged with bacteria of all these 5 strains. Worms were fed with calf liver mixed with bacteria with a total concentration of O.D.=1 and O.D.=4, along with a control group fed with just calf liver. Worms were also incubated in antibiotics-free 1x PW mixed with bacteria of O.D.=0.1 and O.D.=0.4 just for 4 days, and then incubated in antibiotics-free 1x PW. Worms were kept under observation, and a regeneration assay was performed. However, worms from all groups seemed unaffected and also regenerated normally.

Worms were also left to be incubated with bacteria of O.D.=0.1 and O.D.=0.4 continuously. *Pseudomonas gessardii* seemed to be the most pathogenic, with the cut pieces dying after 1 dpa in both O.D.=0.4 and O.D.=0.1 and intact pieces dying after 9 days post-infection. *Pedobacter sp.* was the second most pathogenic, with the cut pieces in O.D.=0.4 dying after 1 dpa, while the cut pieces in O.D.=0.1 showed signs of regeneration but started dying after 6 dpa. The intact pieces also seemed affected after 9 days of incubation in O.D.=0.4. *Chryseobacterium sp.* and *Stenotrophomonas sp.* showed moderate pathogenicity, with the cut pieces in O.D.=0.4 dying after 6 dpa in both cases, and the intact worms seemed affected only after 11 days post-infection. *Staphylococcus cohnii* seemed to be the least pathogenic, with the cut pieces regenerating normally, and worms seemed affected in O.D.=0.4 only after 11 days.

Table 3.2 - Different strains of bacteria identified from *S. mediterranea*. The table shows the morphology, sequence ID from NCBI, and pathogenicity of each strain.

SI No.	Morphology	Strain	Sequence ID	Pathogenicity
1	white, small, smooth	<i>Staphylococcus cohnii</i>	JN128237.1	Low
2	brown, small, smooth	<i>Pedobacter sp.</i>	LS453293.1	Moderate
3	orange, big, smooth	<i>Chryseobacterium sp.</i>	KY056237.1	High
4	yellow, big, smooth	<i>Stenotrophomonas sp.</i>	MW905624.1	Moderate
5	brown, big, rough	<i>Pseudomonas gessardii</i>	MH712957.1	High

3.12 Analyzing the abundance and localization of 2100 in *S. mediterranea* in response to bacteria

In order to analyze if 2100 levels are changed during bacterial challenge, western blot analysis was done post-bacterial challenge. Worms were incubated in 1x PW

with *Staphylococcus cohnii* (low pathogenicity), *Pedobacter* sp. (moderate pathogenicity), *Pseudomonas gessardii* (high pathogenicity), and *Pseudomonas japonica* (moderate pathogenicity according to previous work in the lab) of O.D.=0.4. Half of the worms from each group were moved to new petri dishes without bacteria post 4 days. Western blot samples were taken for both groups - worms that continued in bacteria (post 1 day, 7 days, and 11 days) and worms that were incubated in bacteria for 4 days (post 1 day and 7 days) along with wild-type worms as control. However, there was no significant difference in the levels of 2100 in any of the groups in response to any of the bacterial strains compared to the control. In order to analyze whether 2100 localization changes after challenging worms with bacteria, whole-mount antibody staining was performed with anti-2100 along with DAPI. However, no change in the localization of 2100 was observed with respect to the control.

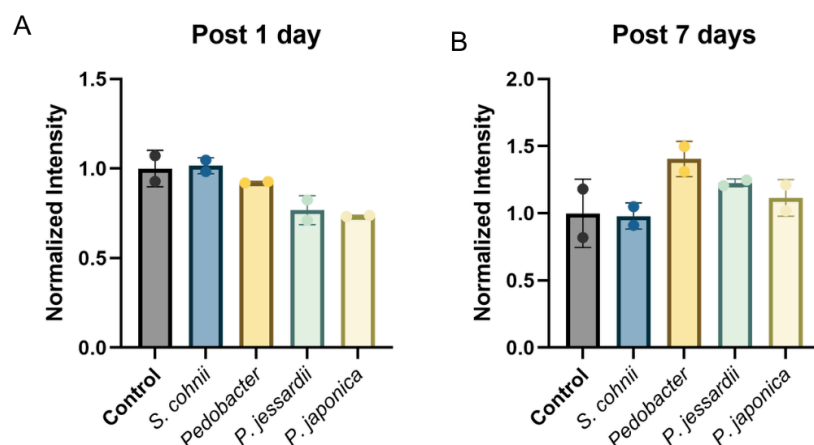


Fig. 3.13 - Western blot analysis post A) 1 day and B) 7 days for 2100 levels normalized to eF1α from worms that remained in bacteria.

3.13 Pull-down of 2100 from worm lysate post bacterial challenge followed by Mass Spectrometry

To check whether 2100 has a role in an immune response post-bacterial challenge, another immunoprecipitation was performed to identify if there are any new potential interacting partners of 2100. Worms stored in 1x PW without antibiotics for 5 days were incubated in 1x PW with *Pseudomonas japonica* of O.D.=0.4. Worm lysates were prepared following 4 days along with wild-type worms, and immunoprecipitation of anti-2100 antibody was done along with anti-RFP antibody. The pull-down identified 19 potential interactors of 2100 in the wild type group while 31 for worms

challenged with bacteria. Both the groups include dynamin-1-like, calpain-B, antiviral Mx dynamin, Arl8B, and RecQ helicase, which were identified in the previous pull-down experiment as well (Fig. 3.6). The sequencing depth of this pull-down is lower than the previous experiment as the samples were run in the gel for a shorter period of time (5 mins) before giving for mass spectrometry analysis. However, many new genes were identified from both the wildtype control and bacteria challenge groups. Some genes that were unique in the bacteria challenge group were also identified, including piwi-2 and TIR domain-containing. It would be interesting to conduct knockdown experiments on these new genes to analyze their role in *S. mediterranea* and draw comparisons with 2100.

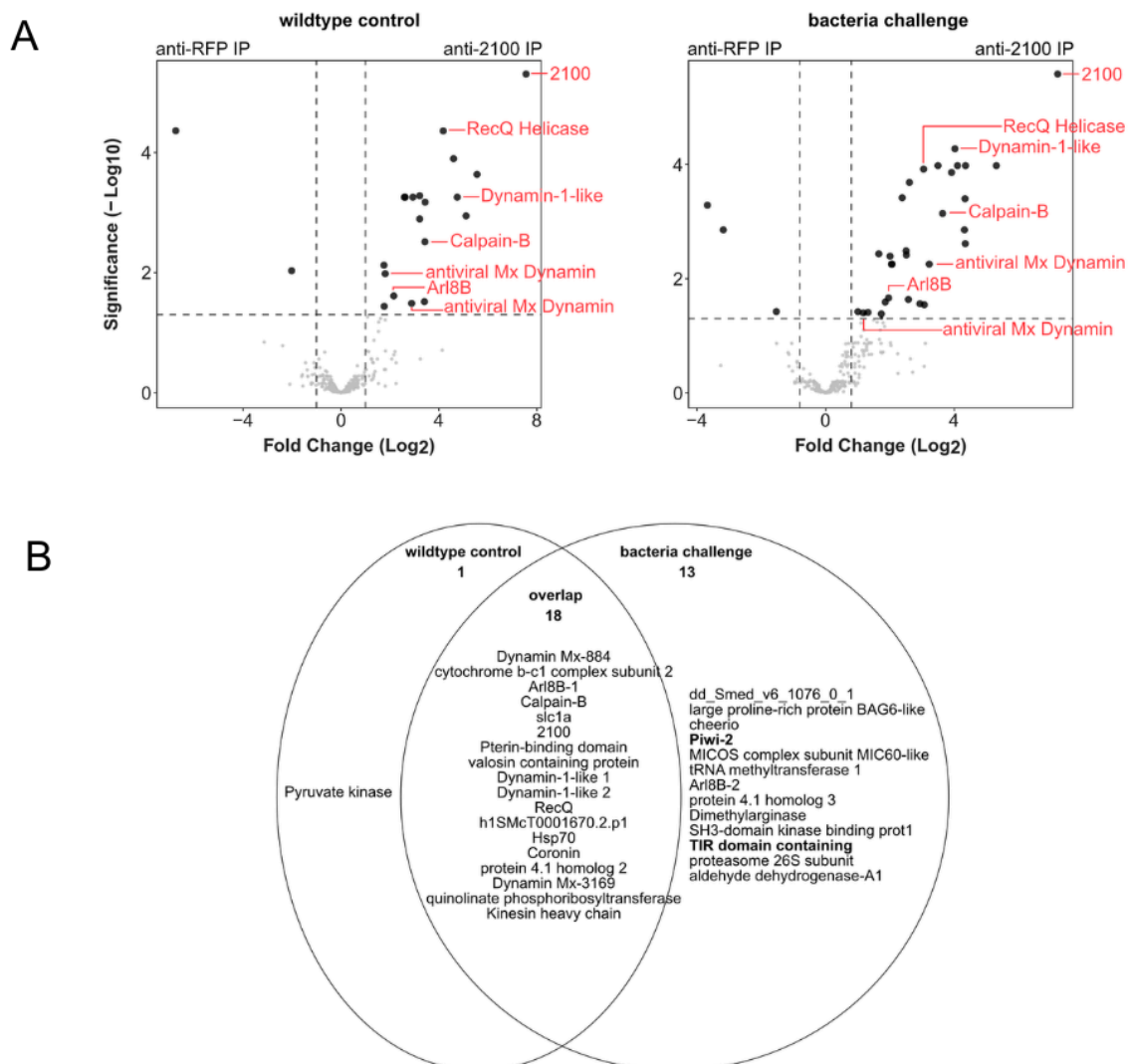


Fig. 3.14 - Immunoprecipitation of anti-2100 antibody from whole worm lysate after challenging with bacteria. A) Volcano plots of wild-type worms and worms challenged with bacteria. Common interactors from previous pulldown experiment is marked. B) Venn diagram of the genes showing the overlap and unique genes identified in each group.

3.14 Analyzing the effect of 2100 knockdown in sub-cellular localization of hemocytes (immune cells) from *Drosophila*

An orthologue of 2100 was also found in *Drosophila melanogaster* - CG12065. In order to do further analysis to find the role of 2100 and also to comment on the conservation of the protein, *Drosophila* was used as the next model organism since it has a large genetic toolset available. *Drosophila* lines with CG12065 RNAi were taken from three different RNAi libraries - KK_CG12065, GD_CG12065, and TriP_CG12065 as controls to rule out off target effect. These RNAi constructs were made to express specifically in the hemocytes, which are considered to be the immune cells in *Drosophila*, by crossing the RNAi responder strains with the Hml-Gal4 strain (Sinenko and Mathey-Prevot, 2004), creating offspring with hemocyte specific knockdown: Hml-KK_CG12065, Hml-GD_CG12065 and Hml-TriP_CG12065.

Experiments in *S. mediterranea* suggested that 2100 might have a role during phagocytosis or endocytosis. Lysosomes play a major role in these processes. Both the RNA sequencing data and pull-down of 2100 from worm lysate suggest that 2100 has associations with lysosomes. Prosaposin, the DEG with the highest significance and also identified as an interactor of 2100 from the pull-down of 2100, has associations with lysosomes. Arl8B, one of the potential interactors of 2100 identified from the pull-down, is a GTPase that helps phagosomes fuse with lysosomes and helps in the trafficking of endocytosed materials to lysosomes. Mitochondria helps in the biogenesis of lysosomes, and it also releases reactive oxygen species (ROS) that enhance the activity of lysosomes. The pull-down of 2100 from worm lysate also identified interactors of 2100 (ATP synthase, NADH dehydrogenase, amongst others) that are associated with mitochondria. Dynamin-1, identified both from the RNA sequencing and pull-down data, has a role in endocytosis, vesicle budding from golgi and mitochondrial fission, and mitochondrial fusion. Hence, the morphologies of lysosomes and mitochondria were analyzed in strains with hemocyte-specific knockdown of CG12065 (orthologue of 2100) and knockdown of its potential interactors (Prosaposin, Arl8B, and Dynamin) along with the control Hml-Gal4. Hemocytes were extracted from male flies not more than a week old and stained with LysoTracker (for visualizing lysosomes) and MitoTracker (for visualizing mitochondria). Stainings with MitoTracker produced a lot of

background. While stainings with LysoTracker showed that lysosomes are present all across the cell except for the nuclei. There was a high degree of variance in the abundance of lysosomes within a single group. However, no significant differences across the different knockdown strains could be identified (Fig. 3.15). Additionally, no difference in the abundance of hemocytes was observed across the knockdown strains.

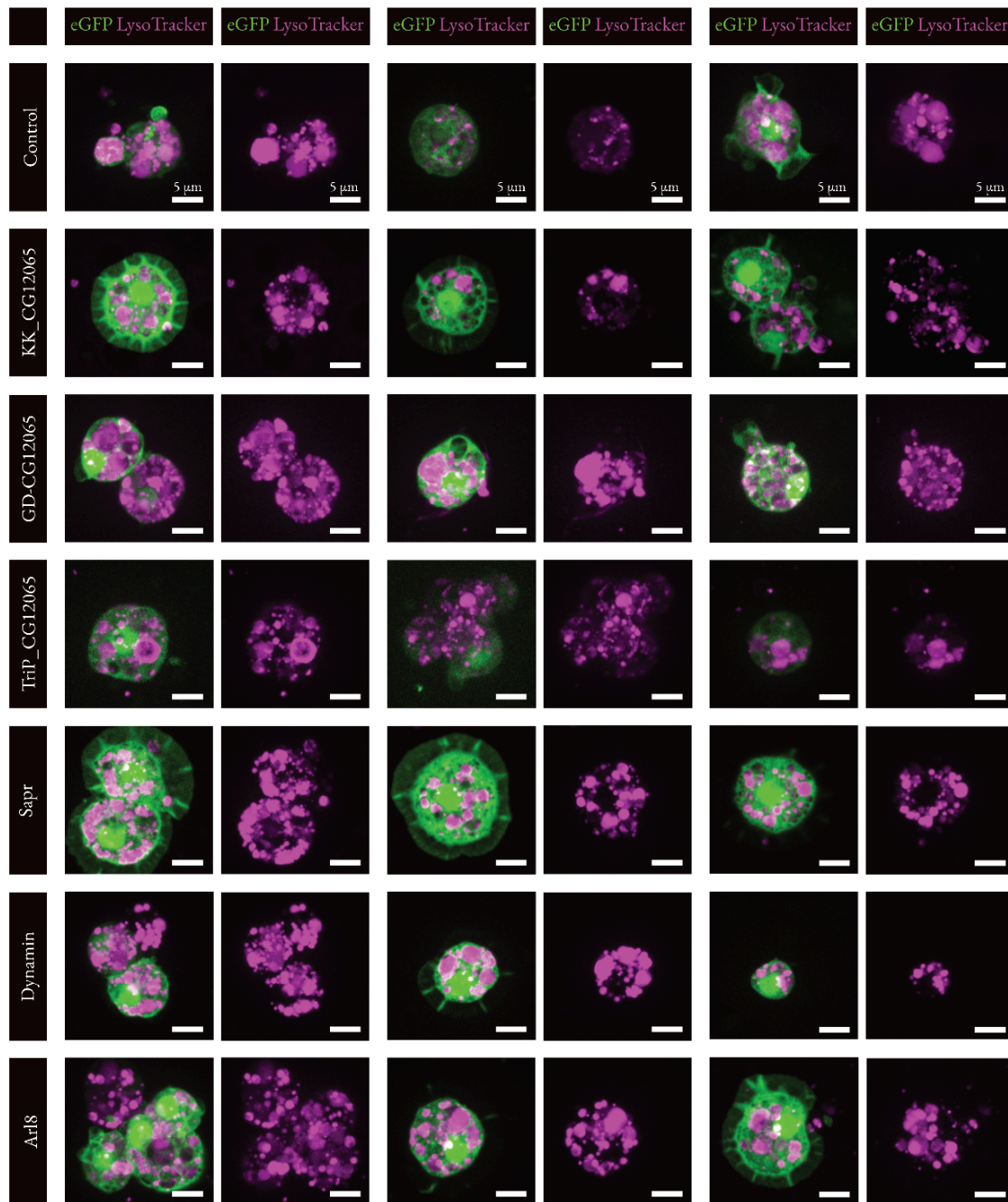


Fig. 3.15 - Lysosome staining in hemocytes from *Drosophila* knockdown strains targeting the orthologue of 2100 (CG12065) and its potential interactors identified from IP. *Hml-Gal4* was used as the control. No difference were observed in the lysosomal localization across all groups. 3 representative images are shown per group. Images were taken at 100x in a confocal microscope.

3.15 Analyzing the effect of 2100 knockdown in *Drosophila* after bacterial infection

In order to analyze whether 2100 has a role in the innate immune system in *Drosophila*, infection assays were performed. Hml-KK_CG12065, Hml-GD_CG12065 and Hml-TriP_CG12065, having the hemocyte specific knockdown of CG12065 were taken along with their respective controls KK_CG12065, GD_CG12065, and TriP_CG12065. The hemocyte specific knockdown strain of Arl8 was also taken along with control Hml-Gal4. Three different bacterial strains were used for the infection assay - *Bacillus subtilis* (well-studied bacteria for infection in *Drosophila* and showing moderate pathogenicity in *S. mediterranea* according to previous work in the lab), *Pseudomonas gessardii* (bacteria isolated from *S. mediterranea* showing high pathogenicity), and *Chryseobacterium sp.* (bacteria isolated from *S. mediterranea* showing moderate pathogenicity). Infection was done in 20 female flies per group by pricking the thorax with a needle after dipping it in bacteria. A control group for each *Drosophila* line was used where pricking was done without any bacteria.

The survival curves show that the setting up of the infection assay was successful since the control groups for all the strains had a high survival rate after pricking, implying that the death of flies is due to the pricking with bacteria and not because of the physical injury made by pricking itself. Both *Chryseobacterium sp.* and *Pseudomonas gessardii* seemed highly pathogenic for all the strains, with flies dying within 1 day. *Bacillus subtilis* showed to be a good bacterial strain for this experiment with moderate pathogenicity. In the GD and TriP RNAi groups, no significant difference was found in the strains expressing dsRNA compared to their respective controls. However, in the KK RNAi (phiC31-based transgenes with a single and defined insertion site) strains for CG12065, Hml-KK_CG12065 had more than 40% greater survivability than KK_CG12065 and Hml-Gal4 (Fig. 3.16A). Hml-Arl8 also had a 25% greater survivability than Hml-Gal4 (Fig. 3.16D). This implies that knocking down CG12065 and Arl8 increased the survival rate of the flies.

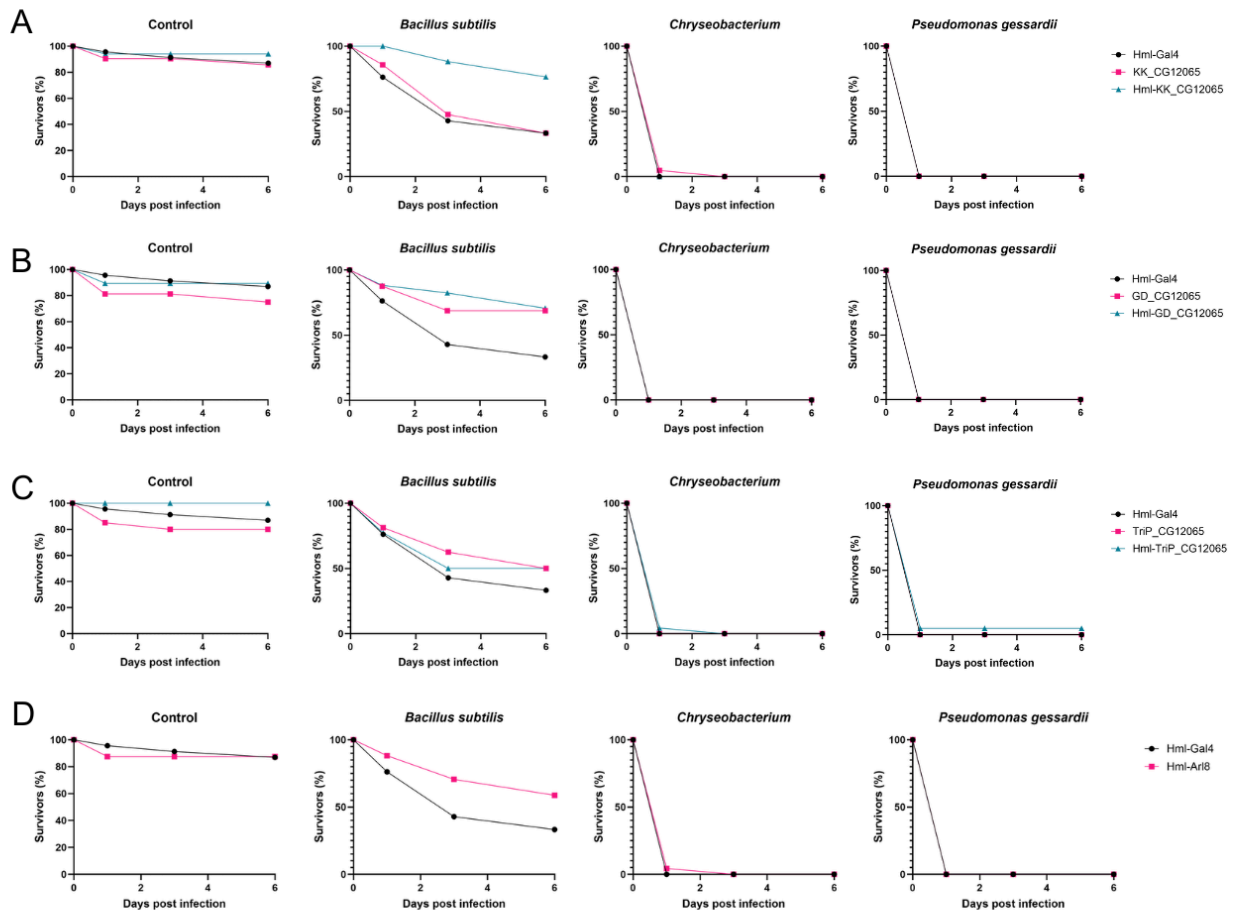


Fig. 3.16 - Survival plots of *Drosophila* post bacterial challenge. *Chryseobacterium* and *Pseudomonas gessardii* proved highly pathogenic while the control showed at least 80% survival rate for all group. For *B. subtilis* A) Hml-KK_CG12065 has more than 40% greater survivability than both KK_CG12065 and Hml-Gal4 B) No significant difference between Hml-GD_CG12065 and GD_CG12065 C) No significant difference between Hml-TriP_CG12065 and TriP_CG12065 D) Hml-Arl8 has 25% greater survivability than Hml-Gal4

3.16 Analyzing the effect of 2100 knockdown in sub-cellular organization of S2R+ cells

In order to draw comparisons between knockdown and overexpression of CG12065 (orthologue of 2100 in *Drosophila*) on the morphology of lysosomes and mitochondria, S2R+ cells (*Drosophila* cultured cell line) were used. For knockdown of CG12065, cells were transfected with plasmids for eGFP expression (to mark transfected cells) and CG12065 dsRNA. As a positive control for the RNAi effect, one batch of cells were transfected with plasmids for eGFP expression and eGFP dsRNA. The eGFP RNAi control showed reduced eGFP signal within 3 days proving efficiency of the knockdown method. Cells were stained with LysoTracker and MitoTracker post 4 days transfection. Both stainings of lysosomes (Fig. 3.17A) and

mitochondria (Fig. 3.17B) showed their presence all across the cells except for the nuclei. There was a high degree of variance in the abundance of lysosomes within a single group in both the stainings. However, no significant differences across the different knockdown strains could be identified.

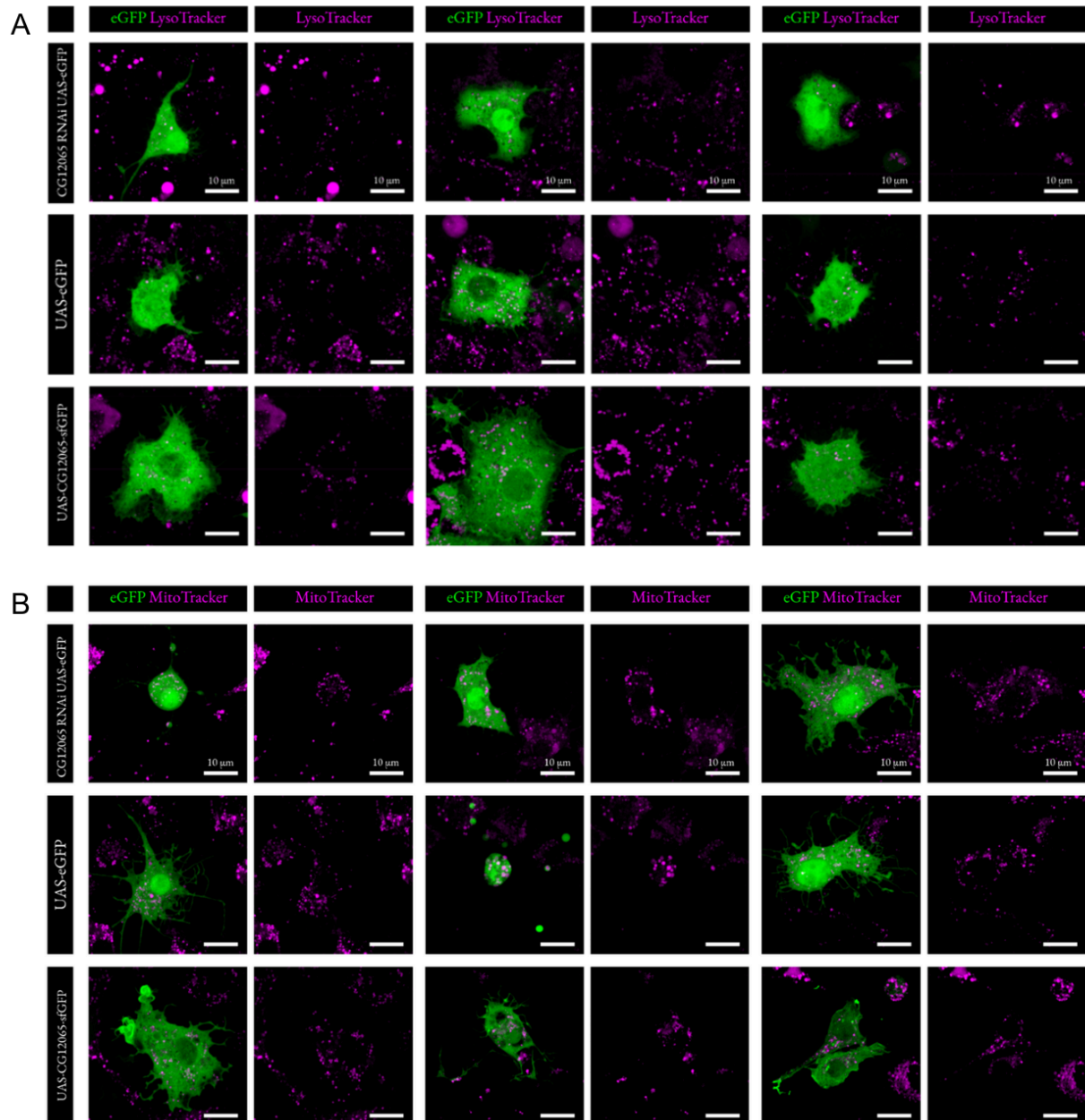


Fig. 3.17 - Knockdown of CG12065 (top), control (middle), and overexpression of CG12065 (bottom)- A) lysosome and B) mitochondria in S2R+ cells. No difference was observed in either of the stainings across all groups. 3 representative images are shown per group. Images were taken at 100x in a confocal microscope.

Chapter 4 Discussion

This study shows that 2100, suspected to be an immune effector protein, is essential for the survival of *Schmidtea mediterranea* since knockdown of the gene leads to progressive lesions and death, similar as seen in the case of Bcl2-1, which is an anti-apoptotic gene (Fig. 3.1). However, RNA sequencing data does not show a major change on the transcriptome level (Fig. 3.3). This is surprising because the worms begin developing lesions shortly after the day the samples from two feedings were collected for RNA sequencing. The change in the 2100 level after the 1st feeding itself was significantly high (Fig. 3.3C), proving that the knockdown of 2100 by RNA interference was successful. This suggests that 2100 is acting on the protein level rather than going through translational regulation. This could be helpful if its function has an immediate and direct role, as is the case for many ribosomal proteins (Luan *et al.*, 2022). The differentially expressed genes (DEGs) identified from the RNA-seq data were mostly expressed in the phagocyte (Fig. 3.3G). Phagocytes are mostly enriched in the gut, which is one of the first cells to encounter pathogens through feeding. However, 2100 knockdown does not affect the gut morphology (Fig. 3.4).

The pull-down experiment of 2100 from whole worm lysates identified 44 potential interactors, many of which are linked to different membranes, as well as endosomes and lysosomes, with Prosaposin, Arl8-B and dynamins being the most significant ones (Fig. 3.6). However, the knockdown of these interactors did not show a similar phenotype as 2100 and also did not have a major effect on the regeneration assay and 2100 localization (Fig. 3.7). This shows that 2100 does not form a functional protein complex. The cell-type expression pattern revealed clustering of many genes, which could suggest redundancy and potentially explain the lack of a phenotype following the knockdown of individual genes. Prosaposin, identified from both RNA sequencing and the pull-down experiment, was further analyzed to check if 2100 has an inhibitory effect on prosaposin in order to explain the lack of phenotype upon knockdown of prosaposin (Fig. 3.8). However, a double knockdown of prosaposin followed by 2100 did not rescue the phenotype, hence disproving the hypothesis. Lipid stainings were also done in whole worms since prosaposin is linked

with lipid metabolism (Fig. 3.5). However, 2100 RNAi did not cause a change in lipid levels.

Whole-mount immunostainings showed that 2100 protein is expressed in many cell types all across the animal but is mostly enriched in the epidermis and associated with the cell membrane (Fig. 3.2). This could be a strategic location for 2100 if it indeed has a role in innate immunity since this region is most susceptible to pathogen entry. 2100 also formed intracellular structures that were more abundant on the dorsal side (Fig. 3.7B). Super-resolution microscopy showed that 2100 is present on the apical side and just below the adherent junction markers, the region where endocytosis occurs in invertebrates (Kowalczyk *et al.*, 2012). It also showed that 2100 forms vesicle-like structures that seem to bud off from the membrane. Staining of 2100 with Z-DNA and DAPI revealed the formation of compartments that contained only DAPI staining and no Z-DNA staining, indicating the internalization of dead cells and suggesting autophagy (Fig. 3.10). However, 2100 localization did not have a major change upon inhibition of autophagocytosis (Fig. 3.11).

To assess the immune function of 2100, *S. mediterranea* was challenged with various bacterial strains. However, no changes in the localization or expression of 2100 were observed after infection, suggesting that 2100 does not play a significant role during bacterial infection (Fig. 3.13). Unfortunately, there are no established methods and assays for studying pathogen infections in planaria. Therefore, to take advantage of the extensive genetic toolset available in *Drosophila* and to explore the conservation of the protein's role, strains with hemocyte (immune cells of *Drosophila*) specific knockdown of the *Drosophila* orthologue of 2100 (CG12065), as well as knockdown strains of three of 2100's interactors - Prosaposin, Arl8-B, and Dynamin, were created. The abundance of hemocytes were not affected in any of the knockdown strains when compared with the control (Hml-Gal4). Prosaposin and Arl8 are lysosome associated proteins and Dynamins can regulate lysosomal and mitochondrial morphology. As a result, localization of lysosome and mitochondria was also analyzed across the strains. Lysosome localization in hemocytes was not affected in any of the knockdown strains (Fig. 3.15) while mitochondria stainings produced a lot of background. The overexpression and knockdown of CG12065 in S2R⁺ cells also did not change the localization of lysosomes and mitochondria (Fig. 3.17). However, challenging *Drosophila* with *B. subtilis* lead to increased survival of

the flies in the case of Hml-KK_CG12065 and Hml-Arl8-B (Fig. 3.16). This could be explained because of reduced immunopathology since immune response could often be the reason for death rather than the infection itself as has been shown in many studies (Cervantes *et al.*, 2023 and Scumpia *et al.*, 2017). This is further supported in a recent study which showed that *B. subtilis* was cleared within 3 days after infection while the flies continued to die (Vaibhvi *et al.*, 2022). Also, the immune response typically activates different pathways in response to various stimuli. Inhibiting a specific pathway that is triggered unnecessarily during infection could help in a more directed pathogen restriction mechanism, potentially improving the chances of survival. Similarly, 2100 might be involved in innate immunity, potentially functioning in alternative pathways such as antiviral defense, rather than in response to bacterial infections. So, the next goal is to investigate the role of 2100 in response to viral particles. However, the greater survivability of worms with knockdown of CG12065 was observed only in the Hml-KK_CG12065. As a result, the experiment should be conducted again in the future, followed by a qPCR analysis to determine the efficiency of CG12065 knockdown and thereby, assess its effect on fly survival.

Since 2100 has a Z-DNA binding domain unlike the oligonucleotide sensing domain in Cap17, Z-DNA expression was analyzed, which showed an increased Z-DNA level in many cells but reduced DAPI expression (Fig. 3.9). This could also be due to a competition between the two stainings, and further experiments need to be done to rule this out. But regardless it will be interesting to identify factors that change Z-DNA levels. 2100 localization post foreign DNA injection was also performed but due to low expression of the protein inside the tissue, no conclusions could be made (Fig. 3.12). Further experiments need to be conducted to show if 2100 is not showing enrichment inside the tissue due to reduced penetration of the antibody or if it indeed does not have high expression inside the tissue. The pull-down of 2100 from worms after the bacterial challenge identified many new genes in both the wildtype control and bacterial challenge groups. Additionally, many genes were identified that were specific to the bacterial challenge group such as Piwi-2 and TIR-domain containing. Piwi-2 regulates silencing of transposon in neoblasts while TIR-domain genes are involved in immune response and pathogen detection. Hence these newly identified potential interactors of 2100 should be analyzed in the future.

In summary, 2100 is essential for the survival of *S. mediterranea*. It acts on the protein level but does not form a functional complex with other proteins. It most likely acts during endocytosis or autophagocytosis. It might be a novel component of the invertebrate innate immune system with ancient roots in prokaryotic antiviral defense but further experiments need to be conducted to test this.

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