UNIVERSITY OF CALIFORNIA SAN DIEGO

Imaging and CRISPR mediated genetic modification of pathways involved in sea urchin primordial germ cell migration and protection.

A dissertation submitted in partial satisfaction of the
requirements for the degree of Doctor of Philosophy

in

Marine Biology

by

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The dissertation of Jose Antonio Espinoza is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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DEDICATION

To Kim, the best partner I could ever ask for, I love you more everyday. To Mateo and Lucas, I love you more than you'll ever know, and I can't wait to see who you will become.

EPIGRAPH

"Everything that's alive is made of tiny bags of water…these bags are full of smaller bags. Life uses lots of bags. All life is made from different kinds of water, and a bag keeps the stuff inside it from touching the stuff on the outside. By using bags, living things can keep different kinds of water in one place without it all coming together."

Randal Munroe
Thing Explainer: Complicated Stuff in Simple Words

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When I was a kid jumping around the tide pools of Crescent City I would never have guessed the course my life would take. The end of this dissertation is the culmination of a long academic career (for me at any rate; for the graduate-students-yet-to-come reading this may your embryos fluoresce and you applications be funded). I'd like to thank all the generations of Hamdoun Lab members who made our little corner of Hubbs Hall a family; Amro for always believing in me, even when I didn't; Lauren and Joe, I came into the lab interested in science, and you turned me into a scientist, even if Amro had to polish off the rough edges; Hannah and Wei, muddling through teaching you how to "scientist" was my favorite thing about lab; Kate, there's no one I'd rather be a grad student with, you helped me get through; Cat, Tufan, Sascha, and Himanshu, y'all were the craziest bunch of postdocs and always had the right advice to keep me level; Gary, you inspired me to be better at the bench; Vic, you've helped me and championed my work in every way you could.

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ABSTRACT OF THE DISSERTATION

Imaging and CRISPR mediated genetic modification of pathways involved in sea urchin primordial germ cell migration and protection.

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Jose Antonio Espinoza

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Professor Amro Hamdoun, Chair

This thesis concerns aspects of the development of the sea urchin *Lytechinus pictus*, a species that can be cultured from egg to breeding adult in six months. The first experimental portion of this thesis (Chapter 2) examines the developmental role of the enzyme phosphatidic acid phosphatase 2B (Pap2b). Fluorescent in situ hybridization demonstrated that Pap2b is expressed ubiquitously in the larval skeletogenic mesenchyme, and not in migrating primordial germ cells where expression was initially expected by analogy with studies on flies and zebrafish. The specific inhibitor Propranolol was used to reduce the function of Pap2b. At a low dose (0.5 μM) the body rods and ventral transverse rods of the

larval skeleton failed to fuse normally; higher concentrations resulted in stunted embryos with fragmented, unpatterned skeletal elements.

The second major experimental part of this thesis (Chapter 3) concerns the adoption of CRISPR-Cas9 gene editing in *L. pictus.* The majority of sea urchin CRISPR studies have been done in species that cannot be easily cultured, and have therefore relied on analysis of mosaic F_0 embryos. I used CRISPR to create a homozygous knockout sea urchin line, an accomplishment so far attained by only one other laboratory in the world. I mutated the ATP binding cassette (ABC) transporter ABCB1, a small molecule transporter involved in cell signaling, environmental chemical uptake, and immune biology. *L. pictus* embryos were injected with two guide RNAs against *Lp-ABCB1*, and genotyping of post-metamorphic juveniles confirmed their activity. Subsequently, non-lethal somatic genotyping was used to identify mutant post-metamorphic individuals. F_0 mosaic animals were outbred to wild-type animals to generate F_1 heterozygotes, and these were subsequently inbred to produce homozygous F_2 mutants. These results provide a foundation for further studies on the function of ABCB1, and more generally for the broader adoption of genetic engineering in sea urchin research. Finally, Chapter 4 of this thesis presents several topics, generally on insertion of transgenes using gene editing, which do not merit a whole chapter individually. These observations may serve as a starting point for future researchers interested in expanding the repertoire of gene editing available in *L. pictus*.

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Chapter One

General introduction

1.1 Background

Emerging technologies and developmental biology. Our understanding of the natural world is inextricably linked to our senses, and our sight in particular. In the biological sciences we constantly seek to "visualize" phenomena, from the location of genes on chromosomes to the movements of cells during epiboly. Advances in technology allow more accurate, more detailed, and in some cases simply numerically more observations to be obtained. The science of developmental biology has particularly strong ties to technological advances. The roots of modern developmental biology lie in van Leeuwenhoek's microscopic observations of sperm in the 1670s (1), when it first became possible to observe the earliest stages of life. Advances in optics, new chemical dyes, and the advent of film all contributed to the explosion of research at the beginning of the modern era of developmental biology in the late 1800s and early 1900s (2–4). At the time, developmental models were chosen in part due to the technical limitations they were best suited to overcome. Ease of imaging made sea urchins an attractive model for watching embryogenesis led to discoveries such as the first description of the centrosome (5), while the ease of laboratory culture made animals such as flies and mice attractive for genetic studies (6). While valuable insights can be gained from a wide variety of organisms, funding, research effort, and research techniques have focused on a handful of model organisms that are seen as easy to work with, with a major focus on those that can be cultured in the laboratory (7–9). Most technical innovations in the last thirty years, such as fluorescent protein technology, advances in microscopy, or genome sequencing, were developed for use in model organisms before being adapted to other systems.

The sea urchin has been an experimental organism since at least the 1870s, and is prized for its fecundity, the optical clarity of eggs and embryos, and the developmental

synchrony of embryos during early development (10–12). The study of sea urchins has included everything from the regulation of maternal RNAs to the dynamics of cell cycle control (13,14). Sea urchin researchers were early adopters of fluorescent proteins, confocal microscopy, and genome sequencing (15–17). Some aspects of development are more comprehensively understood in sea urchins than in any other organism. For example, sea urchins have the most well described developmental gene regulatory network of any organism, and the most well described expression and functional data on ATP binding cassette transporters of any developmental system (18–20). To date these achievements have all been carried out using transient approaches, such as microinjection of sea urchin zygotes, and establishment of laboratory culture of sea urchins has remained elusive.

In the past decade two technologies that are poised to change the reliance on transient methods in sea urchin research have come into their own; next generation sequencing and CRISPR. Short for clustered regularly interspersed short palindromic repeats, CRISPR is a genome editing system that is comprised of the RNA guided nuclease Cas9 (CRISPR associated protein 9) and the targeting guide RNA (a duplex of a target specific CRISPR RNA and structural tracr RNA) (21). Target sites for this system consist of twenty nucleotide sequences upstream of a protospacer adjacent motif (PAM), which for Cas9 has the sequence NGG (21). The gRNA scaffolds a conformational change in the Cas9 protein that brings active sites into position and hybridizes with the genome, but Cas9 can only open the double helix at PAM sites, making this system highly customizable and specific (22,23). Both gRNAs and Cas9 are cheaply available for multiple delivery modalities, such as microinjection of mRNA and gRNA or transfection of plasmids that express the gRNA and Cas9. The promise of CRISPR was embraced by scientists in many fields, but especially by developmental biologists who saw the advent of CRISPR as an opportunity to expand functional genetics into more experimental organisms (24,25). CRISPR knockouts were

reported in a plethora of organisms, including *Strongylocentrotus purpuratus* in 2015 (26). In general CRISPR in the sea urchin has been used to study injected, or “crispant”, embryos, and in only one study has CRISPR been used to create a homozygous mutant (27). Developing this approach will be the focus of Chapter 3, and will be discussed in more detail there.

In addition to CRISPR, next generation sequencing (NGS) has radically changed biology since its widespread adoption in the last decade. NGS works under the principle of “numerically more” mentioned above. Millions of short (generally about 100-400 base pair) DNA reads are generated during an NGS experiment, although the different methodologies vary in how exactly these reads are produced, and these millions of reads are computationally aligned to generate a genome or transcriptome (28). Widespread adoption and technical innovation has led to the sequencing of over 400 thousand genomes (mostly of bacterial) since the introduction of NGS products in 2005 (29). NGS projects continue to increase in ambition, from 10,000 human genomes in a 2016 study to over 50,000 in a single 2021 study (30,31), and projects in progress aim to sequence whole taxa, ecosystems, and even all eukaryotes in the near future (32–34).

The genomic revolution hit echinoderms relatively early, with a Sanger sequencing based genome for the purple sea urchin, *Strongylocentrotus purpuratus*, being completed in 2006 (35). This genome has gone through several refinements since then, and is on par with the quality of many more heavily studied model organisms (36). NGS modalities have allowed genomes and transcriptomes (of varying quality) to be assembled for several dozen echinoderm species, including high quality assemblies of non-echinoid species such as the crinoid *Anneissia japonica* and the crown-of-thorns sea star (37–40). Relevant to this dissertation, the genome of *Lytechinus pictus*, a small sea urchin native to the west coast of North America, was recently assembled at chromosome level coverage using a combination

of NGS modalities (41). As will be discussed in more detail in Chapter 3, the life history of *L. pictus* makes it a good candidate for multigenerational laboratory culture (42,43).

Primordial germ cells and their migration. Primordial germ cells (PGCs) are the stem cell population of the gametes, and are formed via induction or preformation (i.e. inherited germplasm) in the early embryo (44). PGCs are molecularly quiescent, with minimal transcription or translation, in order to maintain their very particular cell identity (45,46). Despite this necessary quiescence, PGCs generally form far from the site of the nascent gonad and must actively migrate to their final niche (47,48). PGCs that become lost during migration will either lose their potential to become germ cells or become germ cell tumors (46,49).

Given the necessity of establishing a germline, and the consequences of aberrant migration, it is no surprise that PGC migration involves complex cell-to-cell signaling. For example, zebrafish PGCs are specified by inheritance of maternal mRNAs during the cleavage stages (50). These cells aggregate into clusters and then begin to actively migrate during somite formation (51). At this point multiple levels of control mechanisms are activated to guide PGCs to the gonad; the PGCs must migrate over a highly specific extracellular matrix (52); specific chemokines act as a positive migration cue, and the levels and distribution of chemokine are controlled by internalization in the surrounding soma (53); Chemokine signaling sustains PGC polarity and motility behaviors such as filopodia production (54,55); and repulsive lipid signaling acts to further constrain PGCs along the appropriate path (56). Final commitment to the germ cell fate only occurs after reaching and responding to cues from the somatic gonad (57). Similarly, the sea urchin PGCs form during cleavage stages, protect maternally deposited mRNAs and proteins while the rest of the embryo degrades them, and undergo an active migratory phase exhibiting cellular behaviors like filopodia and blebs (58–60). However, the signaling pathways involved in these

processes remain largely unidentified. Further development of transgenic lines may address some of these outstanding questions.

ATP binding cassette (ABC) transporters. There are around 800 membrane transporters encoded in the human genome, of which there are around 50 ABC transporters (61,62). In humans there are seven ABC transporter subfamilies (A-G), while most non-mammalian animals have an additional H subfamily (62–64). A smaller subset of the ABC transporters in the ABCB, C, and G subfamilies are small molecule transporters that exhibit polyspecificity (65,66). These transporters distribute nutrients and signaling molecules and offer protection from harmful secondary metabolites, heavy metals, and man-made synthetic chemicals (66,67) One of the best characterized ABC transporters is ABCB1, also known as the permeability-glycoprotein (P-gp), which was first described as the mechanism by which colchicine resistant cancer cells develop resistance to structurally unrelated pharmaceuticals (68). In addition to overexpression in cancers, ABCB1 is implicated in chemosensitivity disorders such as ivermectin sensitivity in collies or human inflammatory bowel disease (69,70).

ABC transporters, PGC migration, and the sea urchin, *Strongylocentrotus purpuratus*. In recent years the purple sea urchin, *Strongylocentrotus purpuratus*, has become one of the most well characterized embryological systems for studying ABC transporters. Around 20 ABC transporters within the ABCB, ABCC, and ABCG subfamilies are expressed during early sea urchin development, including the sea urchin P-gp paralog ABCB1a (19). The evolution of ABC transporters (71), ABCB1 transporter activity at fertilization and during early development (72–74), the role of ABC transporters in heavy metal defense (75), and the role of ABC transporters in developmental signaling of gut morphogenesis have all been examined in purple sea urchin embryos (76). Current research

in purple sea urchins has clarified the ontogeny of protective transporters and established methods for robust F0 analysis of ABC transporter crispants (20,77).

One of the most interesting findings from this work was the discovery that sea urchin PGCs are formed without ABCB1 activity (72). In fact the PGCs' parent cells have reduced ABCB1 activity during the cell cycle immediately preceding the birth of the PGCs, but immediately regain ABCB1 activity after that cell division (72). This finding was intriguing for a few reasons. First, the PGCs are important to the survival of species as the stem cell population for the gametes. Why then would an ABC transporter with demonstrated roles in chemosensitization be removed from the membrane of these important cells? In mouse PGCs mutations in Wnt, chemokine, TGF-beta, and several other signaling pathways lead to failure to successfully integrate in the gonadal niche (78). Similarly, an as yet unrecognized signaling pathway that is sensitive to ABCB1 activity may be involved in sea urchin PGC homeostasis or migration. The second reason this finding is interesting is that ABC transporters excrete PGC guidance cues in fruit fly and colonial tunicates, and have been implicated in zebrafish PGC migration (79–81). More generally, recent evidence has pointed to cell-to-cell signaling and homeostasis, especially in stem cell populations, as roles for ABC transporters that are equally as important as their chemoprotective functions (82–85). Similar to the PGCs of other organisms, sea urchin PGCs tightly regulate transcription and are translationally quiescent (86,87). Our lab has demonstrated that sea urchin PGCs undergo an active migratory phase, while another lab has recently shown that ectopic sea urchin PGCs will home to the appropriate niche in the left coelomic pouch (60,88). The signaling networks that underlie homeostasis and migration in sea urchin PGCs have not yet been uncovered, but one possibility is that ABCB1 activity would interfere with these processes.

1.2 Outline of the Dissertation

At the outset of this dissertation sea urchin research was limited to transient genetic manipulations, such as fluorescent protein overexpression, but was otherwise fully integrated into the modalities of developmental biology research in the 21st century. In the two years prior to starting my dissertation research the seminal work of the Doudna and Charpentier labs showed the potential of CRISPR as a genome editor, and the technology had been demonstrated in developmental models such as mouse, *C. elegans*, *Drosophila*, and zebrafish (21,89–92). Within a year of starting my disseration the technology had been demonstrated in *S. purpuratus* for the first time (26).

Former students in our lab had raised pressing questions about the regulation of ABC transporters and the migration of primordial germ cells, and I intended to follow this line of research. The research presented here includes projects aimed at furthering these lines of investigation, as well as projects aimed at adapting various aspects of genome engineering to the sea urchins *S. purpuratus* and *L. pictus*. The research for this dissertation is organized as follows:

Chapter 2: An investigation of *S. purpuratus* primordial germ cell migration, with a focus on high resolution imaging of cellular behaviors over time. Based on these data I pursued the characterization of a candidate repulsive cue in the lipid phosphate phosphatase family of proteins, *Sp-Pap2b*. I found that this gene is strongly expressed in the skeletogenic mesenchyme, but did not elucidate a role in PGC migration.

Chapter 3: In these experiments I identified and performed an initial characterization of the *L. pictus* homolog of ABCB1. Subsequently, I used a CRISPR/Cas9 strategy to generate *Lp-ABCB1* mutants, and raised these through multiple generations.

Chapter 4: In this chapter I present the use of sea urchin embryos for heterologous expression of coral proteins, and additional experiments relating to insertional mutagenesis in both *S. purpuratus* and *L. pictus.*

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Chapter Two

Primordial germ cell migration and lipid phosphate phosphatase expression in the purple sea urchin, *Strongylocentrotus purpuratus*

2.1 Abstract

This project began as an extension of the primordial germ cell (PGC) migration project that had been a focus of the lab for several years (1,2). At the time, a number of studies had shown that sea urchin PGCs respond to unknown external stimuli to home to the left coelomic pouch (CP). Our previous studies indicated that there was likely an ABC transporter mediated mechanism guiding the PGCs into the left and right CPs, however the nature of this signal was unclear. This project aimed to identify these migration cues. First, I took a high-resolution imaging approach to try to identify cell behaviors during PGC migration that could correspond to different types of signaling. These data indicated that there was likely a mix of chemoattractant and chemorepellant cues during the active phase of migration. Based on recent evidence from PGC migration in zebrafish (3), I then investigated the expression of *Sp-Ppap2b,* a member of the lipid phosphate phosphatase/phosphotransferase (LPT) family of enzymes, as a candidate repulsive cue. Unexpectedly, this enzyme was expressed in the skeletogenic mesenchyme. Chemical inhibition of *Sp-Ppap2b* leads to decreased skeletogenesis and abnormal development in pluteus larvae, suggesting that PGC migration is not the primary purpose of this gene in early development.

2.2 Introduction

Primordial germ cells. Primordial germ cells (PGCs) are the progenitors of the gametes, and for this reason are vital to the survival of the species. While gametes were some of the first samples investigated by Hooke and van Leeuwenhoek in the 1600s, it wasn't until the improvement of optics and the formalization of the discipline of developmental biology at the turn of 1900s that PGCs were discovered. Studying the differentiation of cells during development in tractable model organisms such as *Ascaris* and

the Mediterranean sea urchin *Paracentrotus lividus* led to the idea of the cellular fate map, with the implication that all tissues could be traced back to their developmental origins (4). The germ line is distinct from the somatic gonad, and their progenitor population is the primordial germ cells. As the 20th century progressed, studies of the PGCs of more animals, from mouse and chick to fly and nematode, revealed commonalities and differences in PGC biology. For example, most PGCs express a similar suite of transcription factors and transcriptional and translational repressors to maintain their PGC identity (5). However, PGC specification varies amongst animals. Some animals, such as sea urchins and zebrafish, segregate germ line determinants in the egg, while other animals, such as mice, induce PGCs to form from non-PGC tissues (6,7). Although the developmental timing and method of specification of PGCs varies greatly across taxa (8,9), most PGCs undergo an active, guided migration to the somatic gonad or its primordium (10,11).

While PGC migration is well characterized in *Drosophila* and zebrafish, sea urchin primordial germ cell migration was only recently described (1). The PGCs of the sea urchin, also known as the small micromeres, are formed from an asymmetric division in the vegetal pole at the 5th cleavage (12). Sea urchin small micromeres exhibit many of the conserved characteristics of PGCs, such as contributing to the germline, undergoing restricted cell division, expression of germline specific transcription factors, mRNA sequestration, and transcriptional quiescence (13–16). Sea urchin PGCs travel with the elongating archenteron before undergoing an active migratory phase (1). However, it remains unclear what signaling processes guide sea urchin PGC migration and how those signals are interpreted.

Lipid phosphate phosphatase/phosphotransferases (LPTs). Phospholipids are one class of candidate signaling molecules. Phospholipids are a diverse class of extracellular signaling molecules with many important developmental functions. During development most extracellular signals are long-lived proteins, such as nodal, that self-regulate, and interact

with other protein signals to form spatiotemporal gradients (17,18). In contrast to this, phospholipids are typically short-lived molecules that have both homeostatic and patterning functions (19). Phospholipids can alter cellular behaviors, such as motility, and change cell fate by acting as anti-apoptotic cues (20). In order to perform developmental functions, phospholipids are globally synthesized (21) and then locally controlled by tissue specific expression of phosphatases in the lipid phosphatase/phosphotransferase (LPT) family of enzymes (22).

The LPT superfamily of membrane proteins are the major regulators of signaling phospholipids such as lysophosphatidic acid (LPA) *in vivo*, and they play an evolutionarily conserved role in creating barriers to primordial germ cell migration (23). The members of the LPT super family are integral membrane proteins that act primarily as extracellular regulators of LPA and other phospholipids (24). In general the LPTs have six membrane spanning domains and three conserved catalytic domains on the second and third extracellular loops (25). Two subfamilies of LPTs, the lipid phosphate phosphatases (LPPs) and the plasticity related genes (PRGs), can bind signaling lipids (25). The LPPs alter the spatiotemporal availability of signaling lipids by enzymatic degradation, while the PRGs lack key catalytic residues and their role in signaling lipid regulation remains unresolved.

LPA has been shown to induce cell survival, angiogenesis, and motility under normal physiological and developmental conditions (26,27). These responses have been coopted in several cancers, including ovarian and cervical cancer, and correlate with poor clinical outcomes. For example, ovarian cancer cells from LPA-high backgrounds have higher expression levels of pluripotency and motility genes (28,29). Similarly, cervical tumor cells from patients with high LPA levels showed increased expression of pro-angiogenic factors that promote tumor survival (30). LPA signaling also plays a role in fetal hydrocephalus. In a study designed to mimic the effect of a ruptured blood vessel in the embryo, researchers

found that increased LPA acts through LPAR1, one of several LPA responsive receptors, to initiate changes in neural progenitor cell mitosis and patterning, leading to matrix integrity defects in the neural ventricle and ultimately to ventricular occlusion and the formation of hydrocephaly (29).

LPA and LPTs in development. LPA signaling plays a role in reproduction and is necessary for implantation and survival in mammalian embryos (31), however a clear understanding of the role of LPA in early development has been complicated by the fact that null mutations in the LPA synthesis pathway are embryonically lethal (32). Many researchers instead study the LPT superfamily of genes, as they have been shown to regulate LPA levels *in vivo*. In mammals there are three *Lpp* genes, *Lpp*1-3 expressed during embryonic development. In mice *Lpp* 1 and 2 are generally expressed, while *Lpp* 3 is expressed in spatially and temporally restricted patterns, beginning in the extra-embryonic tissue (E6.5) and subsequently in the allantois (E8.5) and the somites (E9.5) (22). Mice with homozygous knockout of *Lpp*1 or *Lpp*2 do not show morphological or reproductive abnormalities (33,34). *Lpp*3 homozygous mutations are embryonically lethal, as embryos lack a chorionic-allantoic placenta and have abnormal yolk sac vascularization at E9.5 (22). This corroborates the embryonic lethality observed in LPA synthesis knockout mice, which exhibit catastrophic, general defects such as lack of yolk sack vascularization prior to resorbtion at E10.5 (32). This suggests that there are specific, critical roles for LPA signaling during development that are mediated by the *Lpp* genes.

A developmental role for LPPs in several species is control of primordial germ cell (PGC) migration. In *Drosophila* two LPP homologs, *Wunen* and *Wunen*-2, are expressed in the gut and central nervous system and knockout of both genes leads to ectopic PGCs (23,35). In zebrafish the PGCs of LPP knockouts move away from their normal migratory path along the yolk sac towards the somites, the region that normally expresses LPP

transcript (3). This suggests that one function of LPPs in development is to maintain spatiotemporal gradients of phospholipids that act as barriers, preventing the PGCs from accessing the wrong embryonic compartments.

Experiments included in this chapter. In this chapter I used high resolution imaging of *S. purpuratus* PGC migration to identify when signaling to the PGCs may be occurring. I then determined the *S. purpuratus* complement of LPT genes using common bioinformatics approaches. I then used *in situ* hybridization to look at the expression of LPTs during sea urchin PGC migration. Finally, I used chemical inhibitors to show that LPT activity is necessary to formation of the larval skeleton.

2.3 Material and Methods

Culture and injection of sea urchin embryos. Sea urchins (*Strongylocentrotus purpuratus*) were collected sub-tidally from San Diego, California, and kept in flowing seawater aquaria at 16 °C. Animals were spawned by injection with 0.55 M KCl and sperm was collected undiluted and stored on ice.

Overexpression of PGC markers and live imaging of PGC migration. For imaging experiments two females and one male were spawned for paired observations to increase coverage of each stage. Embryos were microinjected as previously described (36) with 250 ng/μL mCherry Vasa (PGCs), 150 ng/μL mCerulean PH-domain (PGC membrane), and 50 ng/μL of mCitrine Life-Act (actin) mRNAs. Beginning at the desired time point z-stacks were acquired of two embryos from each female every hour on a Leica SP-8 laser scanning confocal. Whole embryos were imaged in the pre-gastrula stages. During gastrulation a single color, single plane overview image and a zoom in z-stack of the PGCs at the tip of the archenteron were acquired. Images were processed using Image J (37).

Reciprocal BLAST search for sea urchin LPTs. Sigal et al define the human complement of LPTs, and these protein sequences were accessed using the GenBank accession numbers provided by the authors (25). BLASTp searches were performed against the sea urchin peptide database (echinobase.org). Candidate sea urchin genes were BLAST searched against the human genome (GRCh38) via Ensembl.org (38).

Evaluation of sea urchin LPT topology and consensus catalytic domains (CCDs). The topology of the candidate sea urchin LPTs was determined using the TOPCONS topology predictor (39). To determine the sequence of the sea urchin CCDs each sea urchin LPT was aligned with its nearest homolog, as well as the mammalian LPP consensus CCDs, using Clustal Omega (40). Finally, a

LPP expression and localization. The expression of the sea urchin LPT candidates was retrieved from Echinobase (41). Only the candidate LPPs, *Sp-Ppap2b* and *Sp-Ppapdc1* were generated as *in situ* probes. Digoxin labeled antisense and sense RNA probes were generated using a Roche DIG labeling kit (Basel, Switzerland) as previously described (42). Fluorescent ISH was performed as per Shipp et al. 2015 for both Papdc1 and Pap2b.

Inhibition of LPP activity with Propranolol. Propranolol was obtained from Sigma Aldrich (St. Louis, MO) and rehydrated in dimethylsulfoxide (DMSO). Embryos were exposed to 0.5, 1, or 5 μM Propranolol or an equivalent DMSO control at hatching, 24 hours post fertilization (n=4 batches, 140-210 embryos/batch). Embryos were scored based on the presence of skeletal elements, as compared to the DMSO control. In a second experiment embryos were exposed to 0.5 μM propranolol or DMSO control and scored based on amount of skeletalization (absent, partial, or complete; n=2 batches, 260-265 embryos/batch). Embryos were imaged on a Zeiss LSM 700 (Jena, Germany). In each experiment propranolol exposures were compared to control measurements using a type-2 T-Test.

2.4 Results

High resolution PGC migration time series. Two time series, an early time series from 24 to 36 hpf (Fig2.1 A-F) and a late time series 36 to 64 hpf (Fig2.1 G-P), with four total batches of embryos (two/time series) were used to construct a combined series of PGC behavior between hatching and the end of PGC migration. In the early time series PGCs do not exhibit membrane extrusions characteristic of migratory cells. Instead, they are tightly clustered at the vegetal plate (Fig2.1 A-D) before clustering at the tip of the archenteron (Fig2.1 E-F).

This quiescent membrane behavior continues until the end of archenteron elongation between 44 and 46 hpf (Fig2.1 G). The PGCs remain tightly clustered but begin to produce blebs and filopodia (Fig2.1 H-K). At the onset of active migration the PGCs remain associated with neighboring PGCs, but the cluster as a whole loses cohesion, and larger blebs and filopodia are evident (Fig2.1 L-M). Finally, the PGCs extend large filopodia towards first the apical ectoderm (Fig2.1 N), and then the lateral ectoderm (Fig2.1 O), before moving into the right and left coelomic pouches (Fig2.1 P).

Identification and characterization of sea urchin LPTs. The sea urchin complement of LPTs, including the LPPs, has not been identified previously. I used reciprocal BLAST search to identify seven unique candidate LPTs in four of the six recognized LPT subfamilies (Table 2.1). Using Clustal Omega I generated a phylogenetic tree of sea urchin (Sp), human (Hs), zebrafish (Dr), and yeast (Sc) LPT amino acid sequences (Fig2.2). *Sp -Pap2b* and *-Papdc1* are the most similar proteins to the human, zebrafish, and yeast LPPs.

Although *Sp-Pap2b* and *Sp-Papdc1* are the most similar to the LPPs, there is no guarantee that overall homology is matched with conservation of important residues and motifs. I therefore compared the candidate LPT amino acid sequences to two defining

characteristics of the LPP subfamily, their topology and their Consensus Catalytic Domains (CCDs). I found that *Sp-Pap2b*, *-Papdc1*, *-LPPR*, and *–Pap2dl* fit the predicted 6 transmembrane domain structure (Fig2.3 A, dark grey), while *Sp-sgpp2* has the predicted 8 transmembrane domains (Fig2.3 A, light grey). *Sp-G6P* and *–G6P-1*, appear to both be partial annotations, and don't contain a full six transmembrane domains as expected from the human G6P. The LPTs are integral membrane proteins, and the three CCDs are dispersed in extracellular loops two (CCD1 and 2) and three (CCD 3) regardless of the number of transmembrane domains. The candidate sea urchin LPTs fit this predicted distribution (Fig2.3 A, red rectangles).

For an additional check I aligned the sea urchin and human LPT sequences to the mammalian consensus CCD sequences for the LPP subfamily (Fig2.3 B, red residues) (24,26,43). Both *Sp-Pap2b* and *–Papdc1* have 100% conservation of consensus CCD residues. The remaining sea urchin LPTs showed similarity to their predicted subfamilies, but did not share the same level of CCD conservation as the putative sea urchin LPPs.

Expression and localization of *Sp-Pap2b*. I expected that LPPs would be involved in the Left/Right migration of sea urchin PGCs, which occurs between 44 and 62 hours post fertilization (1). The sea urchin developmental transcriptome (41), accessed from Echinobase.org, indicates that *Sp-Pap2b* is expressed at close to five times the peak level of *Sp-Papdc1* expression, and is most highly expressed during PGC migration (Table 1).

I cloned *in situ* fragments from cDNA samples at the peak of *Sp-Pap2b* and *Sp-Papdc1* gene expression. Papdc1 antisense probe binding was indistinguishable from sense controls at all time points tested (data not shown). In contrast, *Sp-Pap2b* showed a clear pattern of localization in a cell population in the vegetal plate and blastocoel at 24 HPF (Fig. 4-A). These cells appear to be Primary Mesenchyme Cells (PMCs), as they take on the characteristic vegetal ring formation (36 HPF) before extending towards the animal pole (52

hpf) (44). Expression appears concentrated in the ventral lateral clusters, the origin of the first triradiate spicules (45), however this is likely an artifact of the cell density and syncytial nature of this cellular cluster.

Functional characterization of Pap2b. Propranolol is a non-selective beta-adrenergic receptor inhibitor that has been shown to also block LPP activity in vertebrates. I applied 1 and 5 μM propranolol or an equivalent DMSO control to batches of embryos starting at hatching (24 hpf), and observed the skeleton at 72 hpf (Fig 2.4 A). At this point in the control embryos the initial triradiates have elaborated into the pluteus skeleton with a complex arrangement of skeletal rods. While 1 μm treated embryos were often significantly smaller than control embryos, most of these had some skeletal rod formation, but these were not properly patterned (Fig2.4 A-B, n=4 batches, 140-210 embryos/batch). In 5 μM treated embryos only a few embryos with initial triradiates were observed in a small percentage of embryos.

In a second experiment 0.5 μM propranolol was applied at hatching. At this concentration most of the embryos maintained normal proportions, however the majority of the embryos were incompletely skeletonized (Fig2.5 C-D, n=2 batches, 260-265 embryos/batch).

2.5 Discussion

Time lapse imaging was essential to proving that the sea urchin PGCs actively migrate along the archenteron (1), however this approach necessarily reduces image resolution to improve speed. To complement this approach I took a high-resolution time series using still micrographs taken every two hours. This had the advantage of improving resolution but reduced the speed of acquisition to around 15 minutes per micrograph. To obtain the best spread of stages I used two batches of embryos in each time series and imaged two embryos from each batch at each time point. While embryos within batches are

synchronous in early development, there is heterogeneity between embryos of different batches.

I took an initial time series beginning at 34 hours post fertilization (hpf) and ending at 60 hpf. I anticipated that this would cover the inactive and active phases of migration (1), however in these batches of embryos the archenteron was already fully elongated by 34 hpf (data not shown). I therefore used these embryos to construct a time series of post gut elongation stages, of which representative images are shown in Figure 2.1 G-P. A second time series beginning at 22 hpf and continuing through 38 hpf was conducted with two additional batches of embryos, and representative images presented in Figure 2.1 A-F. Two interesting observations can be made from these time series. The first is that prior to the active phase of migration the PGCs tightly adhere to one another, to the extent that they even seem to be pushed out of the main epithelial layer as a group at the onset of gastrulation (Fig2.1 B-D). The second, and most relevant to this chapter, is that the PGCs produce filopodia in a stereotyped fashion, first extending towards the oral apical ectoderm (Fig2.1 N) while the PGCs are loosely arranged along the top of the archenteron. The PGCs then begin to move left and right, extending filopodia towards the ectoderm in the direction of travel (Fig2.1 O). Evidence suggests that developmental signaling molecules can be sensed using specialized filopodia (46,47), and I hypothesized that this was the function of the filopodia seen during active PGC migration.

In most systems studied the regulation of PGC migration contains multiple complementary components that act in concert to guide migration. Based on the evidence from the migration time series, I began investigating possible signaling mechanisms that were identifiable in the sea urchin genome and expressed at the appropriate time. Lipid phosphate phosphatases (LPPs) were identifiable in the genome and expressed at the

correct time (Table 2.1), and had recently been shown to act as repulsive cues in zebrafish PGC migration (3).

Of the seven candidate genes (Table 2.1) in the broader lipid phosphate phosphatase/phosphotransferase (LPT) superfamily, only two clustered phylogenetically with mammalian LPPs (Fig2.2) and had the correct topology and catalytic domains to be LPPs (Fig2.3), *Sp-pap2b* and *Sp-papdc1*. *Sp-pap2b* is expressed at nearly 5 times the level of *Sp-papdc1* and was therefore considered the main candidate LPP.

Based on the reported role of LPPs zebrafish and *Drosophila* PGC guidance(3,23), I expected that *Sp-pap2b* would be expressed on the archenteron or in the areas of the ectoderm that PGC filopodia touch during migration. Unexpectedly, I found that the major sea urchin *Sp-Pap2b* is expressed strongly and specifically in the primary mesenchyme cells (PMCs) during the PGC migration (Figure 2.4). These cells are spatially distant from the tip of the archenteron and the PGCs. The PMCs form a syncytial, precisely patterned network of biomineralizing cells that give structure to the embryo through a series of elaborate skeletal rods (48). There are no clear homologous functions in the LPP literature that would explain this expression.

In order to determine the functions of *Sp-pap2b* I used the small molecule LPP inhibitor propranolol. The substrates of LPPs are long chain phospholipids, and propranolol is one of the few chemicals that can effectively inhibit the catalytic action of LPPs, up to 75% of LPP enzymatic activity in one study (49). Skeletal elements were absent in 96% of embryos treated with 5 μM propranolol, while 85% of embryos exposed to 1μM had some skeletal elements, although they were much reduced (Fig2.5 A-B). In contrast, at an exposure of 0.5 μM propranolol the embryos maintain a normal shape and appear to have normally spaced PMCs, but have stunted and unconnected skeletal elements as compared to controls (Fig2.5 C-D). One possibility that should be further explored is that LPP activity is necessary for

maintaining the syncytium between different PMC subtypes (44). This could explain why *Sp-pap2b* is uniformly expressed in the PMCs, even though it does not have a biomineralization function.

2.6 Conclusions

The phases of sea urchin PGC migration have previously been described by our lab (1), however a high resolution time series at each phase of the PGC migration had not been performed previously. With these data I identified two phenomenon that warrant further study; the extrusion of the PGCs from the epithelium during primary invagination, and the possible role for filopodia in detecting signaling molecules during the active phase of migration. Based on recent evidence that LPPs act as a repulsive cue in zebrafish PGC migration, I identified candidate members of the sea urchin LPT super family and singled out *Sp-pap2b* as the most likely LPP to be involved in sea urchin PGC migration. *Sp-pap2b* is strongly expressed in the primary mesenchyme cells from hatching through the pluteus stage, and its activity is necessary for proper skeletogenesis.

PGC extrusion is a good candidate for further mechano-biological studies to understand the forces, cell adhesion pathways, and cell-to-cell signaling that allow this process to precisely occur during primary invagination. Further study of the role of LPPs during sea urchin skeletogenesis is also warranted. In many ways the PMCs are not analogous to tissues where LPPs have been previously described, making it difficult to hypothesize about what role they may play in this system. *Sp-pap2b* may be necessary for maintaining the PMC syncytium, but it could also be linked to PMC patterning, similar to how mammalian LPPs help regulate proliferation and cell migration during vascularization (22). It would be useful to apply the genetic engineering approaches described in Chapter 3 to create *Sp-pap2b* null mutants. Finally, this study has not yielded a promising candidate for a

PGC guidance mechanism. Newly available single cell transcriptome data (50) may yield insights into candidate signaling pathways expressed in the archenteron, coelomic pouches, and the ectoderm regions contacted by PGC filopodia.

2.7 References

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2.8 Chapter Two Figures

Figure 2.1 Time series of sea urchin primordial germ cell (PGC) migration. In the blastula stages (A-D) and early gastrula stages (E-F) the PGCs, labeled with Vasa (red) and a PGC enhanced membrane marker (cyan), do not exhibit membrane activity associated with motile cells, such as filopodia or blebbing. In contrast, cells adjacent to the PGCs in the early gastrula stages (E and F) extend large lamellipodia as part of the active pulling stage of gut elongation. After the gut is fully extended the PGCs remain in a tight “grape bunch” formation (G). The PGCs remain tightly grouped but begin extending first blebs and then filopodia (H-K), before dissociating from each other (L and M). The PGCs then extend filopodia towards the oral ectoderm (N) and then towards the lateral ectoderm (O), before moving into the coelomic pouches (P).

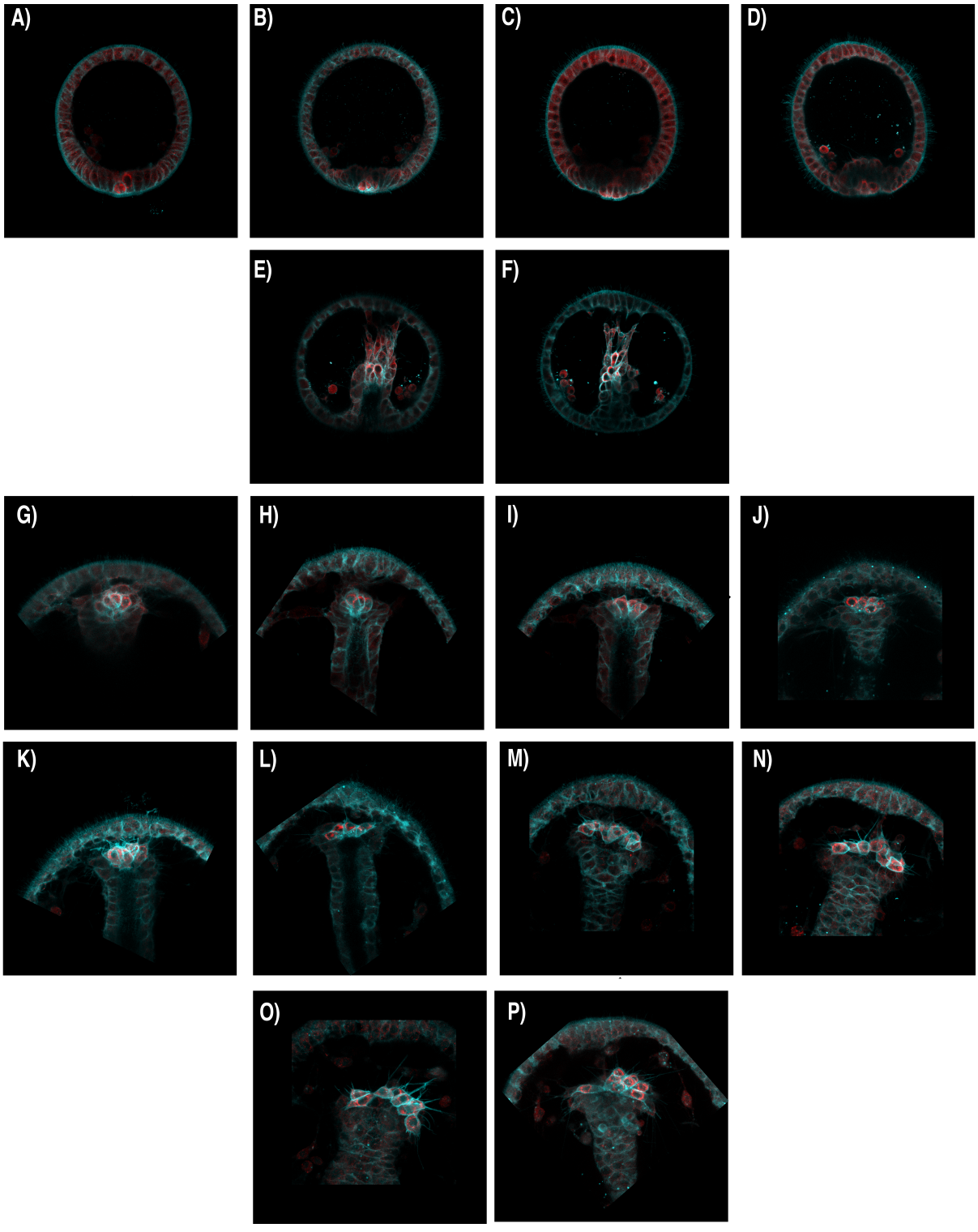

A)
B)
C)
D)
E)
F)
G)
H)
I)
J)
K)
L)
M)
N)
O)
P)

Table 2.1 Identification of sea urchin LPTs and their categorization into subfamilies by homology, topology, and conserved catalytic domains. Candidate sea urchin LPTs were identified by reciprocal BLAST of the sea urchin and human genome, using accession numbers of human LPTs identified by Sigal et al. The seven candidates were separately aligned with the human LPTs (as peptides) using Clustal Omega, and genes with the highest identity were assigned as their human homolog. Topology was assessed using the Topcons topology predictor. LPPs and LPPRs were expected to have six TMs, while G6Ps and SGPPs were expected to have eight TMs. Gene expression for each candidate LPT was determined from the developmental transcriptome data on Echinobase (Tu et al. 2014). Sp-Pap2b is expressed approximately five times higher than Sp-Papdc1.

Gene ID	Gene Name	Subfamily	Human Homolog (% Identity)	Predicted TMs/ Homolog TMs	Peak Expression
SPU_011127	Sp-Pap2B	LPP	Hs-PAP2B (38.91%)	Six/Six	56 HPF (4256)
SPU_023631	Sp-Papdc1	LPP	Hs-PAP2A (28.27%)	Six/Six	24 HPF (875)
SPU_000491	Sp-Pap2cl	LPPR	Hs-PLPPR-1/ Hs-PLPPR-5 (19.9%/19.9%)	Three/Six	Egg (21)
SPU_016994	Sp-Pap2dl	LPPR	Hs-PLPPR-5 (29.15%)	Six/Six	18 HPF (15,866)
SPU_028336	Sp-Lppr	LPPR	Hs-PLPPR-1 (36.68%)	Six/Six	48 HPF (78)
SPU_012921	Sp-Sgpp2	SGPP	Hs-SGPP-2 (34.87%)	Nine/Eight	10 HPF (3294)
SPU_008734	Sp-G6pc	G6P	Hs-G6PC (31.8%)	Seven/Eight	10 HPF (3130)

Figure 2.2 Neighbor joining tree of selected LPT amino acid sequences. Putative sea urchin LPTs were determined by reciprocal BLAST against the Human genome, and representatives of four of six LPT subfamilies were found. These are Lipid Phosphate Phosphatases (LPPs; *Sp-Pap2b* and *–Papdc1*), LPP related genes (LPPRs; *Sp-Pap2dl* and -*Lppr*), Sphingosine Phosphate Phosphatases (SGPPs; *Sp-Sgpp2*), and Glucose-6 Phosphate phosphatases (G6Ps; *Sp-G6P*). Representative amino acid sequences were collected for Human (Hs), Zebrafish (Dr), Yeast (Sc), and Sea Urchin (Sp) LPTs (Hs- and Dr- from Ensembl database, Sc- from the Saccharomyces Genome Database, Sp- from Echinobase). Amino acid sequences were aligned and a phylogenetic tree created using the Clustal-Omega multiple sequence alignment tool (EMBL-EBI). Results are color coded by subfamily. Sea urchin sequences are denoted with a black sea urchin figure. The subfamily of an additional LPT, *Sp-Pap2cl*, was not resolved using this method.

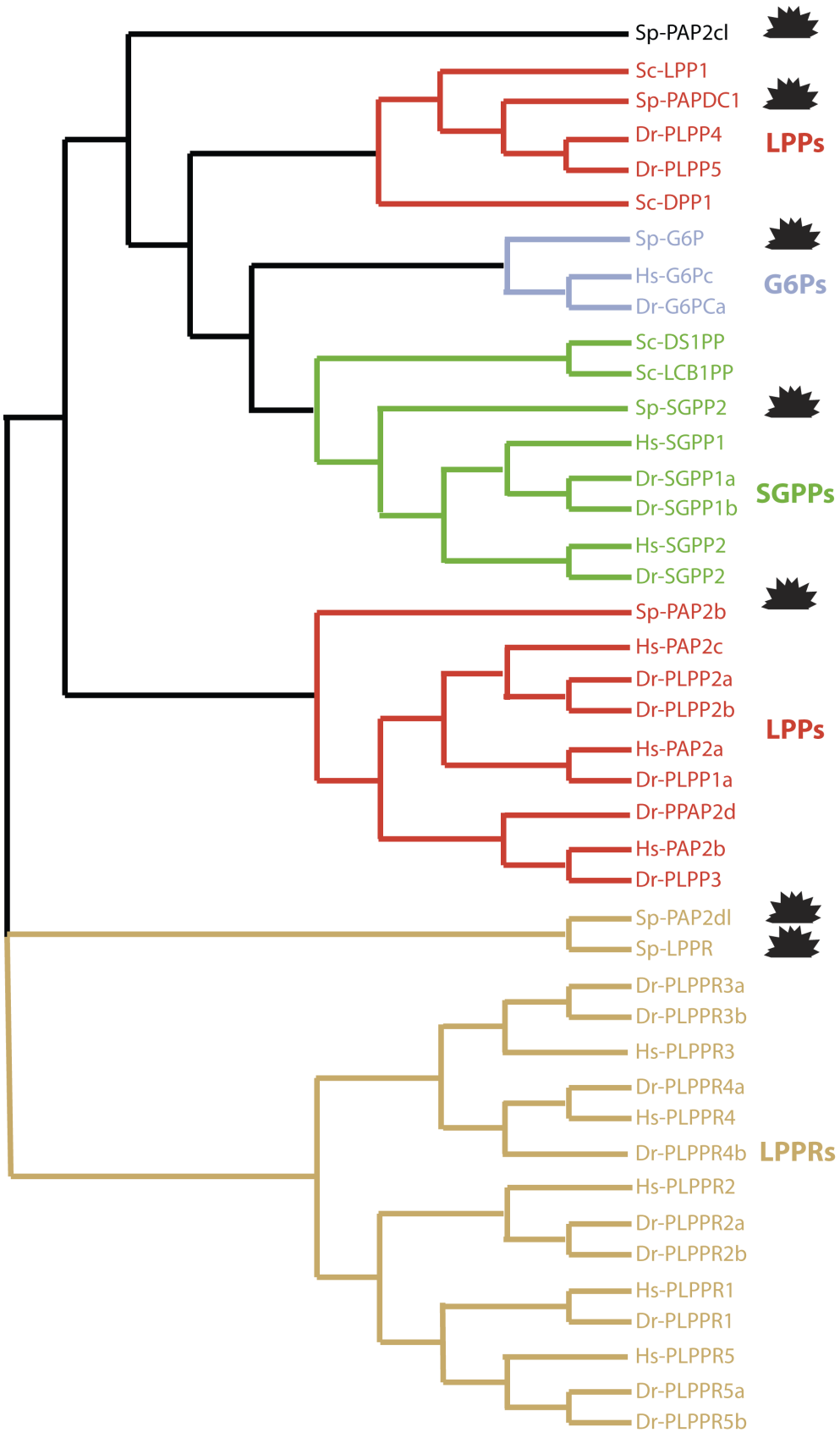
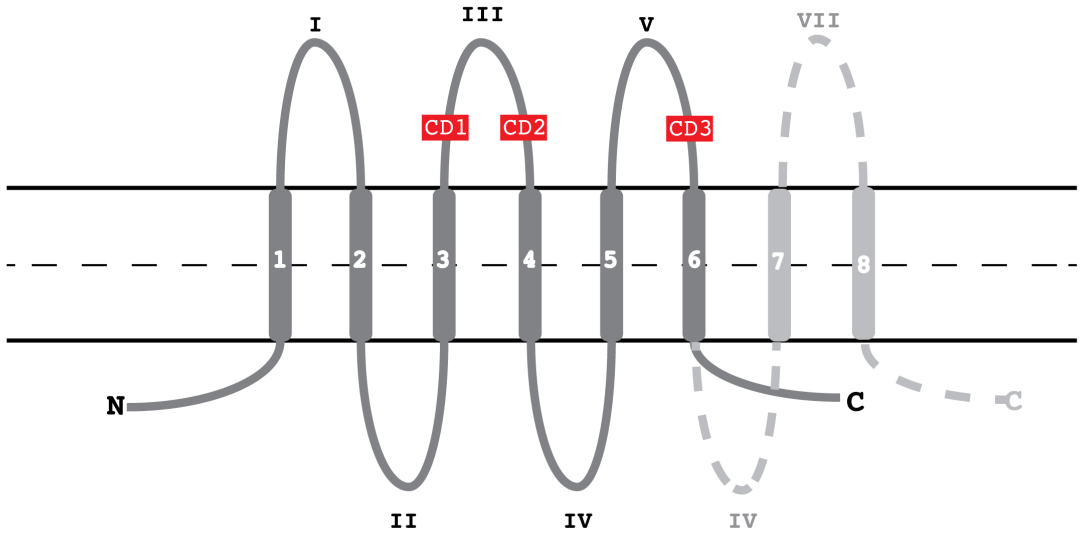

Sp-PAP2cl
Sc-LPP1
Sp-PAPDC1
Dr-PLPP4
Dr-PLPP5
Sc-DPP1
LPPs
Sp-G6P
Hs-G6Pc
Dr-G6PCa
G6Ps
Sc-DS1PP
Sc-LCB1PP
Sp-SGPP2
Hs-SGPP1
Dr-SGPP1a
Dr-SGPP1b
SGPPs
Hs-SGPP2
Dr-SGPP2
Sp-PAP2b
Hs-PAP2c
Dr-PLPP2a
Dr-PLPP2b
Hs-PAP2a
Dr-PLPP1a
LPPs
Dr-PPAP2d
Hs-PAP2b
Dr-PLPP3
Sp-PAP2dl
Sp-LPPR
Dr-PLPPR3a
Dr-PLPPR3b
Hs-PLPPR3
Dr-PLPPR4a
Hs-PLPPR4
Dr-PLPPR4b
LPPRs
Hs-PLPPR2
Dr-PLPPR2a
Dr-PLPPR2b
Hs-PLPPR1
Dr-PLPPR1
Hs-PLPPR5
Dr-PLPPR5a
Dr-PLPPR5b

Figure 2.3 Predicted topology and consensus catalytic domain alignment of sea urchin LPTs. A) Sea urchin LPPs and LPPRs have six transmembrane domains (dark grey), while sea urchin SGPPs and G6Ps are predicted to have 8 transmembrane domains (dark grey + light grey). Predicted topology is based on TopCons predictions (http://topcons.cbr.su.se/) and published literature. Catalytic domains (CD1-3) are located in loops III and V. B) Alignments of the catalytic domains from Human and Sea Urchin LPTs. Consensus catalytic domains 1-3 (CD1-3) are shown with conserved residues in red. Histidine residues (H's) in CD2 and 3 bind and separate phosphate residues from acyl chains in phospholipid substrates. Sea urchin G6Ps are truncated compared to Human homologs and lack CD1.

A)



B)

		CD1		CD2		CD3	
		K X X X X X X R P		S G H		S R X X X X X H X X X D	
	Sp-Ppapdc1	K L I I A R P R P	106	S G H	142	S R T A D Y R H H Y E D	202
	Sp-Ppap2b	K N V V G R L R P	137	S G H	185	S R I S D Y K H H W S D	241
	Hs-Pap2b	K V S I G R L R P	156	S G H	199	S R V S D H K H H P S D	255
	Hs-Pap2c	K Y M I G R L R P	125	S G H	168	T R V S D Y K H H W S D	224
	Hs-Pap2a	K Y S I G R L R P	128	S G H	171	S R V S D Y K H H W S D	227
	Sp-Lppr	Q R V T G I Q T P	160	S L H	203	T R A S K Y R H H W S D	259
	Sp-Pap2dL	Q L I L S H P A P	153	S L Y	192	Q Q I A A H R A H W G D	248
	Hs-PRG-1	Q L S T G Y Q A P	146	S Q H	198	T R I T Q Y K N H P V D	245
	Hs-PRG-2	Q L A T G Y H T P	148	S Q H	207	T Q I T Q Y R S H P V D	254
	Hs-PRG-3	Q V V T G H L T P	152	S K H	205	N R V S E Y R N H C S D	260
	Hs-PRG-4	Q V V T G N P T P	197	C K D	250	V R V A E Y R N H W S D	296
	Hs-PRG-5	Q V V T G N L A P	141	S K E	193	N R V A E Y R N H W S D	240
	Sp-Sgpp2	K E I I R W P R P	160	S T H	182	S R L Y K G M H Y I L D	233
	Hs-Sgpp1	K D I I R W P R P	186	S T H	208	S R I Y M G M H S I L D	259
	Hs-Sgpp2	K D V L K W P R P	144	S T H	166	S R L Y T G M H T V L D	217
	Sp-G6P	- - - - - - - - -	---	S G H	031	S R V Y T A T H F P H Q	092
	Sp-G6p-1	- - - - - - - - -	---	S G H	031	S R V Y T A T H F P H Q	092
	Hs-G6pC	K W I L F G Q R P	084	S G H	119	S R I Y L A A H F P H Q	180

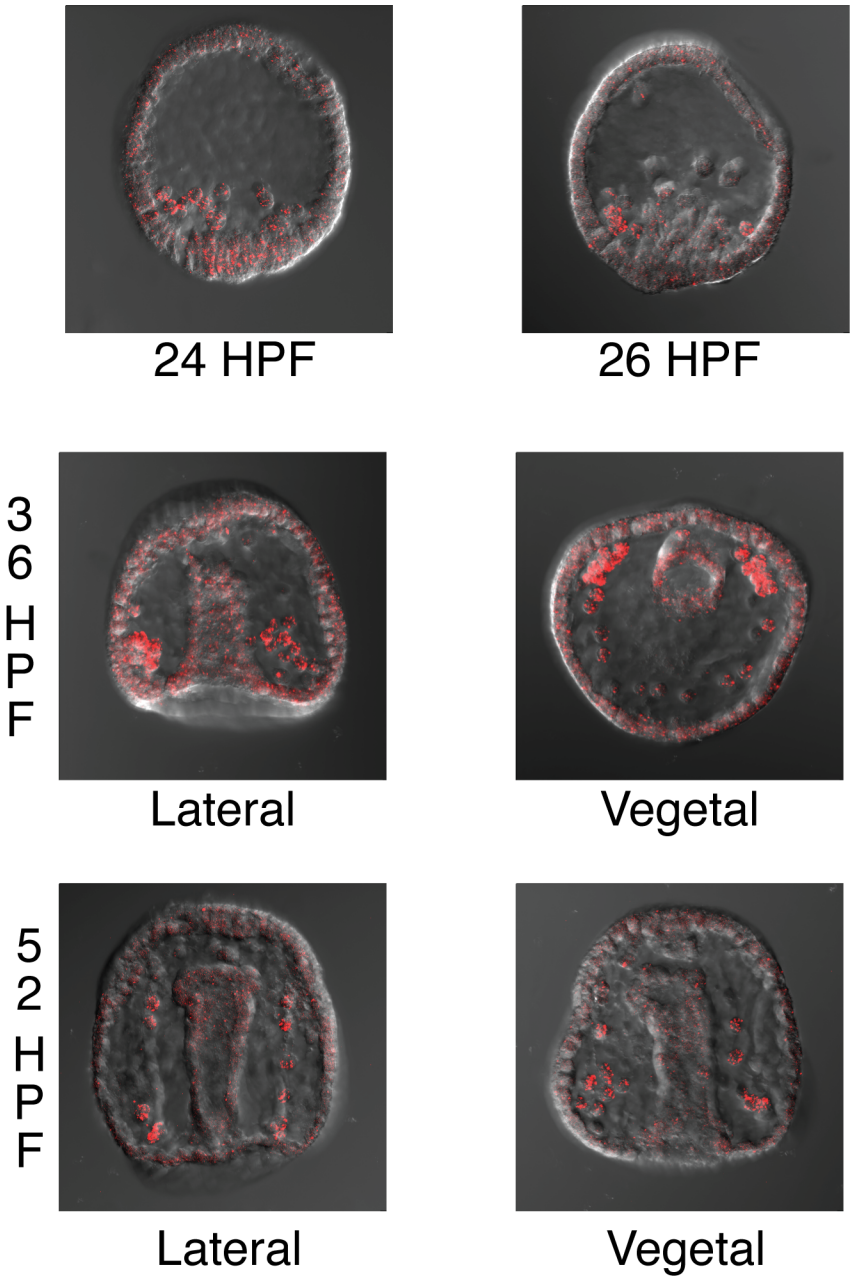


Figure 2.4 Localization of *Sp-Pap2b* during the period of PGC migration. In Situ Hybridization (ISH) of an antisense DIG-labeled RNA probe encoding bases 48-687 of the *Sp-Pap2b* open reading frame. *Sp-Pap2b* expression is concentrated in the primary mesenchyme cells (24 and 26 HPF) and their descendants, the skeletogenic mesenchyme (36 and 52 HPF).

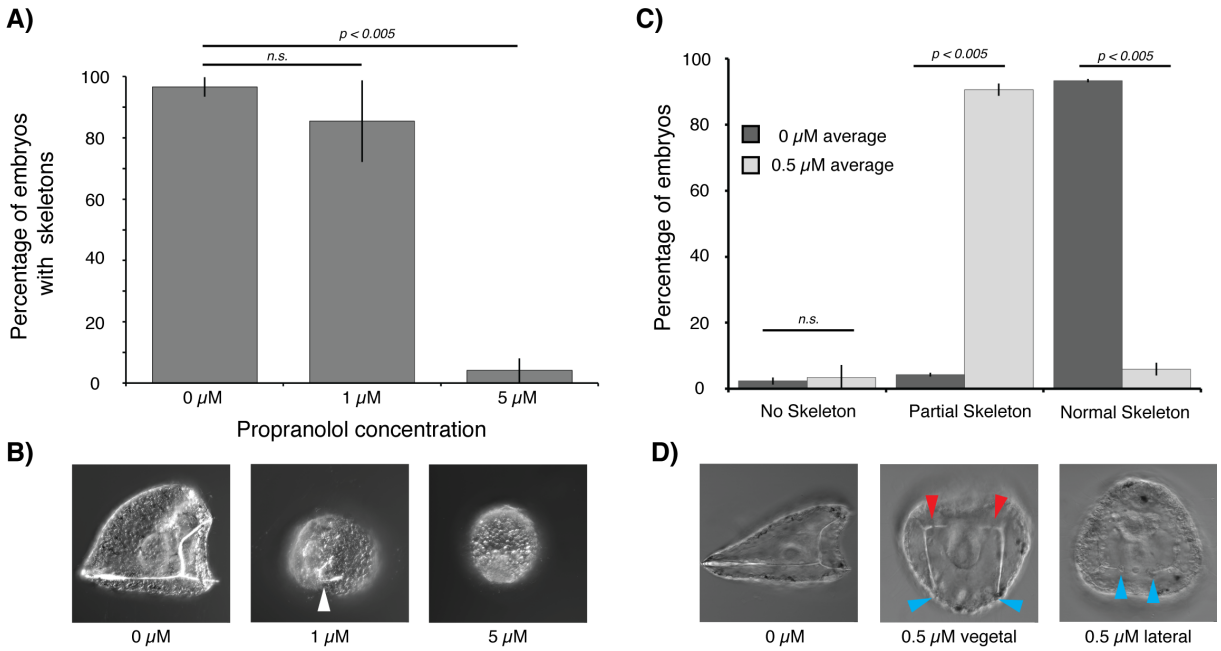


Figure 2.5 Effect of *Sp-pap2b* inhibition with propranolol on skeletal growth. A) 5 μM propranolol exposure resulted in the majority of embryos did not form skeletal elements, while at 1 μM the majority had some skeletal elements. B) Example images of 0, 1, and 5 μM propranolol exposed embryos. White arrow indicates position of a partial skeletal element. C) A 0.5 μM propranolol exposure causes most embryos to develop a partial skeleton. D) Examples of 0.5 μM propranolol treated with breaks (red arrows) and absent mineralization between skeletal elements (blue arrows). All treatment groups were exposed at 24 hours post fertilization. All experimental groups were compared to control using a type 2 t-test.

Chapter Three

Generation of homozygous ABCB1 knockouts in the sea urchin, *Lytechinus pictus*, using CRISPR-Cas9 technology

3.1 Abstract

ATP-binding cassette transporters are well studied for their roles in human cancers, particularly ABCB1, also known as P-gp. However, relatively few studies have been done on the roles of ABCB1 in development or ecotoxicology. Sea urchins have been used to address these questions in previous studies, leading to insights about ABCB1's roles in early development (1–4). However the utility of sea urchins for this work has been limited by genetic manipulation options available in common laboratory species. We have used *Lytechinus pictus*, a sea urchin with a short reproductive cycle, to generate mutant, laboratory-cultured sea urchin lines. In this study we report the development and initial characterization of a CRISPR knockout of the *L. pictus* P-gp homolog *Lp*-ABCB1a through the second generation.

3.2 Background

Sea urchins as model organisms. Model organisms enable us to understand complex biological principles by studying them first in animals well suited to our questions, be that drosophila for classical genetics or mice for human disease research (5–7). Sea urchins have been a developmental and cellular biology model for over 100 years. Rapid, synchronous development and the ability to procure many thousands of gametes led to foundational research on the cell biology of fertilization and inheritance in the 1890's and early 1900's (8). In the later half of the 20th century research on sea urchins led to the discovery of cyclins and the biochemical basis of fertilization and speciation (9,10). Since the publication of the purple urchin genome in 2006 the sea urchin has become the preeminent model for gene regulatory analysis (11,12). While many types of F_0 genetic analyses are well vetted in sea urchins, such as introduction of reporters and BACs, the largest drawback to the sea urchin system remains the inability to generate stable genetic modifications, as

utilized in other model organisms (13,14). Many commonly used species of sea urchins, such as the purple urchin *(Strongylocentrotus purpuratus)*, take several years to reach sexual maturity (15) and are thus unsuited to the multigenerational breeding schemes necessary to isolate and analyze homozygous mutant phenotypes.

CRISPR gene editing, and its use in sea urchins. The introduction of RNA guided mutagenesis using CRISPR has fundamentally altered precise genome editing and rapidly changed many disciplines of biology. In the past fifteen years two methods for precise genome editing using programmable DNA binding domains became available, Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) (16). These methods are difficult to use, as they rely on protein engineering to change target specificity. Despite these disadvantages, these systems were a great improvement over existing mutagenesis techniques and were widely applied, including in sea urchin research (17,18).

In contrast to these methods, CRISPR uses RNA molecules as a targeting system in complex with a nuclease, most commonly the CRISPR associated protein 9 (Cas9) (19). Commonly, the multiple components of the CRISPR RNA are synthesized as a single guide RNA (sgRNA), a single molecule that performs both the targeting and scaffolding functions of the CRISPR RNA (20).

CRISPR has mostly been used in sea urchin research to study the role of known transcriptions factors such as Nodal, Delta-Notch, and Alx1, using F_0 analysis (21–23). In addition, some work has gone into developing genetic knockout of the gene polyketide synthase 1 (PKS1) as a marker gene, as this knockout leads to an albino animal (24–26). This approach was recently successful in generating homozygous pigment mutants in *Temnopleurus reevesii* (27). In this study we have utilized the advantages of CRISPR to create ATP Binding Cassette transporter mutants.

ABC transporters and environmental defense. The environment is filled with chemicals, natural and manmade, that can be harmful to organisms. While the immune system deals with biotic insults, chemical insults are dealt with using cellular defenses. At the front lines of this defense are transport mechanisms that efflux toxic compounds from cells. The ATP binding cassette (ABC) transporters are a superfamily of integral membrane proteins that use ATP hydrolysis to efflux substrate molecules from cells. There are 49 human ABC transporters in seven subfamilies, ABCA to ABCG, that have diverse roles in cell physiology and human health (28). Unlike most transport mechanisms, the ABC transporter superfamily is unusual in that many of its members exhibit polyspecificity, or specific binding and efflux of structurally unrelated molecules (29).

One of the best characterized ABC transporters is ABCB1, also known as the permeability-glycoprotein (P-gp; P-glycoprotein), which was first described as the mechanism by which colchicine resistant cells develop resistance to structurally unrelated pharmaceuticals (30). Subsequent research showed that many ABC transporters in the ABCB, ABCC, and ABCG subfamilies contribute to this phenotype in humans and animals (31,32). While the role of P-gp and its overexpression phenotype are well studied in cancer cells, the roles of P-gp in ecotoxicology and embryonic development are less well understood.

Sea urchin ABC transporters. Sea urchins are model species for both ecotoxicology and developmental studies due to their rapid, synchronous development and the ability to procure thousands of gametes with ease. Sea urchin embryos are one of the most well characterized models for studying the roles of ABC transporters in development and ecotoxicology. Sea urchin larvae have been used to study toxicity of everything from heavy metals to nanoplastics (33–36). At least 20 ABC transporters within the ABCB, ABCC, and ABCG subfamilies are expressed during early sea urchin development, including the sea

urchin P-gp paralog ABCB1a (4). The evolution of ABC transporters (37), the ontogeny of ABCB1a transporter activity at fertilization and through early development (1–3), and the role of ABC transporters in developmental signaling of gut morphogenesis have been examined in the embryos of the purple sea urchin (*Strongylocentrotus purpuratus*) (38). Current research in purple sea urchins has clarified the ontogeny of ABCB1a and established methods for robust analysis of ABC transporter crispants (CRISPR-generated mutants; (39,40).

***Lytechinus pictus* ABCB1 knockout sea urchins.** In order to extend the utility of the sea urchin for studying ABC transporters we have established a knockout sea urchin model for the P-gp paralog ABCB1a. Recently, we described updated culturing methods for the sea urchin *Lytechinus pictus* as a species that is easy to raise in laboratory conditions (41,42). The purple sea urchin has many resources, such as a well annotated genome and BAC library, but this species takes several years to reach sexual maturity, while *L. pictus* can reach sexual maturity in four months (15,42). New high-quality genomic and transcriptomic data for *L. pictus* have made this species more amenable to CRISPR experiments (43). Utilizing these culturing and bioinformatics data we report here the identification and initial characterization of the *L. pictus* P-gp paralog *Lp*-ABCB1, the F_0 phenotype of *Lp*-ABCB1a CRISPR, and the expansion of F_0 crispants to F_2 homozygous mutant animals. With this transgenic line we will be able to probe new questions as to the roles of P-gp in development and ecotoxicology that we would otherwise be unable to address.

3.3 Materials and Methods:

Adult *L. pictus* husbandry, gamete collection, and microinjection. Adult *L. pictus* were collected off San Diego, CA, and housed in flowing seawater aquaria at 22 ˚C. Adults were injected with 0.55 M KCl. Females were spawned into natural seawater and the eggs

washed four to six times in 0.22 µm filtered seawater (FSW) at room temperature. Sperm was collected undiluted in 1.75 mL tubes and stored on ice. Eggs were prepared for microinjection as previously described (15).

Culturing *L. pictus* larva and juveniles. Larval *L. pictus* were cultured in 1, 2, or 8L containers, based on the size of the cultures, with motorized paddles providing water movement. Larvae were *Rhodomonas lens* at ~3000 cells/mL every other day, and water was changed prior to each feeding. Post metamorphic juvenile adult *L. pictus* were grown in 150 mm diameter petri dishes at 22 ˚C until one month post metamorphosis (mpm), when they were transferred to 10 L aquaria. Juvenile adults were fed a mixture of diatoms (*Nitzschia alba)* and sea lettuce (*Ulva lactuca*) in petri dishes. Following transfer to 10 L aquaria, juveniles were fed *U. lactuca.* Between 5 and 6 mpm the juveniles were transitioned to kelp (*Macrocystis pyrifera*), with additional supplementation of market squid (*Doryteuthis opalescens*) every 2-3 weeks. Juvenile adults were spawned by trans-peristomal injection of 0.55 M KCl.

Identification of *L. pictus* ABCB1/P-gp orthologs. Structurally, ABCB transporters are classified as either full transporters, with at least two membrane-spanning domains (MSDs) of six transmembrane helices each, and two nucleotide binding domains (NBDs), or half transporters, which have one MSD and one NBD. Half transporters must dimerize to be functional. ABCB1/P-gp is a full transporter with two MSDs and two NBDs.

Previously, we identified three paragolous P-gp like ABCB transporters in the purple sea urchin; ABCB1a, ABCB1b, and ABCB4a (4,44,45). A fourth P-gp like transporter, ABCB1c, was identified during the course of this work. The four paralogs share the same topology as P-gp. Functionally, Sp-ABCB1a is the most similar to mammalian P-gp in subcellular localization and substrate preference (44). In addition to *S. purpuratus* sequences, we used sequences of known ABCB1 and ABCB4 transporters from vertebrates

and invertebrates (Table S1) to identify the ABCB1a ortholog of *L. pictus*. Topology and domain architecture was inferred using Topcons and ScanProsite (46,47).

Peptide sequences were aligned in Geneious (v11.1.5) using the ClustalW alignment method with default parameters. The resultant alignment was run through ProtTest (v.3.4.2) to predict the best fit model for tree construction. A maximum likelihood tree (RaxML-HPC2 on XSEDE) with 1000 bootstraps was then produced running the LG+G model, with yeast selected as an outgroup, through the CIPRES Science Gateway (v.3.3) (48). Remaining parameters were used with default settings. The resulting tree was exported and alterations to the tree format were performed using FigTree (v1.4.4). Separately, the NBDs were aligned using Clustal Omega, and Walker A, Walker B, Q-loop, and LSQQG motifs were identified.

Design, storage, and analysis of sgRNAs. In vitro mutations that affect the catalytic ability of the NBDs block efflux activity of P-gp (49). We therefore aimed to design a CRISPR knock-out (KO) schema that would truncate the protein before the 1st NBD, as this should completely block P-gp efflux activity. The first cytoplasmic domain (located between MSD1 and MSD2 and containing the first NBD) begins in *Lp-ABCB1* exon 10, and the first NBD begins in exon 11, based on the comparison of the *Lp-DN66174* open reading frame and the genomic alignment described above (data not shown).

Several methods exist for choosing guide RNAs (gRNAs) for use with Cas9. We used the web application ChopChop v.2 (https://chopchop.cbu.uib.no/, (50)), which ranks potential gRNAs on metrics such as predicted efficiency and predicted number of off target cut sites. Crucially, both *S. purpuratus* and *Lytechinus variegatus* genomes are available for off target analysis at the time our experiment was designed. While these genomes only provide a rough approximation of potential off target effects in *L. pictus*, it is considerably more useful than the mammalian genomes that are often the only available options on many bioinformatics tools. Of the several tens of gRNA options predicted by ChopChop, we

considered those with high predicted efficiency and no or few predicted off target sites. Of these similar options one gRNA in each exon was chosen based on proximity to the beginning of the cytoplasmic domain (exon 10) or the first NBD (exon 11). These gRNAs will be referred to as Ex10+76 and Ex11-152, with +/- indicating orientation relative to the ORF and the number representing the base adjacent to the PAM site in the orientation of the ORF.

Recent evidence suggests that synthetic single guide RNAs (sgRNAs) are more effective in transient experiments than two component synthetic gRNAs or in vitro transcribed sgRNAs (51). We ordered the sgRNAs used in this study from Synthego (Redwood City, CA, USA). The sgRNAs were rehydrated in nuclease free water to 3300 ng/μL and stored in aliquots at -80 ˚C. Individual aliquots were diluted to 900 ng/μL (6x) and stored at -80 ˚C. To maintain sgRNA consistency, aliquots of 6x stocks were defrosted and used only once. Cas9 mRNA and sgRNAs were injected at 750 ng/μL and 150 ng/μL, respectively, as per previously established protocols (21). When both sgRNAs were injected they were each injected at 150 ng/μL (300 ng/μL total of sgRNA). An injection marker, mCherry-LCK, was injected at 25 ng/μL in all CRISPR experiments.

Genomic DNA samples were extracted using a gDNA extraction buffer adapted from Sambrook (52) composed of 50 nM NaCl, 50 nM Tris pH 7.5, 5 mM EDTA pH 8.0, 0.5% SDS, and 200 μg/mL Proteinase K. Samples were incubated at 55 ˚C for 1 hour (larvae) or overnight (juveniles/tube-feet/gametes), followed by heat inactivation at 95 ˚C for 10 minutes and RNAse A (10 μg/mL) incubation for 1 hour at 37 ˚C. Two extractions were performed with 25:24:1 Phenol:Chloroform:Isoamyl Alcohol, followed by overnight precipitation in 2.5 volumes 100% Ethanol and 0.1 volumes 3 M Sodium Acetate.

All larval stage DNA extractions were mixed from 40-80 injected (RFP positive) larvae at 48 hpf (hours post fertilization) two-arm pluteus stage, prior to feeding. Individual one-week post metamorphosis juvenile adults were homogenized in gDNA extraction buffer using

a glass rod. Samples from juvenile adults >3 months post metamorphosis were obtained from tube foot clips. To perform tube foot clips juveniles were removed from aquaria and transferred to a 35 mm diameter petri dish. When placed on a dissecting microscope the juveniles moved away from the light source by extending tube feet. Sharp forceps are used to grab an extended tube foot. Gently pulling away from the animal as the animal pulls toward itself causes the distal section of tube foot to detach, and these segments were used for gDNA isolation. Gamete samples were concentrated by centrifugation, seawater was removed, and the same extraction procedure was used as described above.

For all analyses the ~3kb region between Exon 8 and Exon 12 of Lp-ABCB1 was PCR amplified (Ex8-Forward1, tacggcaagaacttggatgaagctaa; Ex12-Reverse1, cacggatgtcgattccgtcaatcttg) using PrimeStar high-fidelity DNA polymerase (Takara Bio, Shiga, Japan). A nested PCR was performed with Exon 9 and 11 specific primers with m13 tails (LpB1-Ex9-m13Forward, tgtaaaacgacggccagtacggcacagttctttatttagatggtga; lpB1-Ex11-m13Reverse, caggaaacagctatgacgataataatgatcatgatggtagtaatgatgac). These reaction products were gel purified and cloned into pMiniT 2.0 using the NEB PCR cloning kit (Ipswich, MA, USA) and sequenced as plasmid or purified single colony PCR product. Identification of mutant alleles and alignments were generated using Sequencher (v.5.0.1) and Snapgene (5.1.4.1).

Cloning *Lp-DN66174* and in vitro mRNA synthesis. Total RNA was isolated from gastrula stage (24 hpf) embryos, and converted into cDNA using the SMARTer PCR cDNA synthesis kit (Takara). The *Lp-DN66174* transcript was amplified using UTR to UTR primers (lpB1-5utr-1, ccacgttattatctgcggcacc; lpB1-3utr-1, ctgggaaccaatctatgcggttcttt) and PrimeStar high fidelity DNA polymerase. The open reading frame was sub-cloned into the PCS2+8 NmCherry plasmid using In-Fusion cloning (Takara Bio, Shiga, Japan), as described previously (44,45).

In vitro transcription of tagged Lp-ABCB1 mRNA, as well as Cas9 and the injection markers mCherry LCK and mCherry Histone H2B, was performed as previously described using the mMessage mMachine kit (Thermofisher, Waltham, MA, USA, (44)).

Fluorescent substrate efflux assays. Calcein-acetoxymethyl esther (C-AM) was purchased from Thermofisher (Waltham, MA, USA). PSC-833 was purchased from Tocris (Minneapolis, MN, USA). For blastula stage efflux assays embryos were injected with 250 ng/μL of mCherry-*LpABCB1* or 50 ng/μL of H2B-RFP mRNA on delta-T glass bottom dishes as previously described (37,44). Five hours post fertilization (blastula stage) 250 nM Calcein-AM was added for one hour, after which cross sections of overexpressing and uninjected embryos were imaged on a Zeiss LSM 700 confocal microscope (Jena, Germany). Individual fluorescence measurements were normalized to the average wild-type fluorescence of each mate pair, and the average percent change compared to control calculated for each treatment. Statistics were performed using JMP statistical software (JMP Pro v15, SAS, Cary, NC, USA).

To evaluate the effect of *Lp*-ABCB1 CRISPR, we injected embryos with exon 10 and 11 sgRNAs (150 ng/μL each), Cas9 mRNA (750 ng/μL), and 25 ng/μL of the membrane marker mCherry-LCK. At 23 hpf (mid gastrula stage) the embryos were exposed to 250 nM Calcein-AM for one hour. Imaging and fluorescence analysis were performed the same as for the blastula stage embryos.

3.4 Results:

Identification of the *S. purpuratus* ABCB1a homolog in *L. pictus.* There are four P-gp like ABC transporters in *S. purpuratus*: *Sp-ABCB1a*, *Sp-ABCB1b*, *Sp-ABCB1c,* and *Sp-ABCB4a* (4,44,45). These transcripts were used to search the *L. pictus* transcriptome, identifying three candidate P-gp like transporters, *Lp-66174*, *-67108*, and *-60219*. These six

sequences were aligned with P-gp transporters from twelve additional organisms and used to create a neighbor-joining phylogenetic tree (Fig3.1 A,). This analysis shows that transcript *Lp-66174* is the closest homolog to *Sp-ABCB1*, and will be referred to as *Lp-ABCB1*. More high quality basal deuterostome genomes are now available for analysis than were available when the purple urchin P-gp genes were identified (2). With these additional sequences we were able to identify two trends in the phylogeny of P-gp like transporters. First, the ABCB1 containing node clusters more closely with the vertebrate chordate like P-gp genes, while the ABCB4 node clusters more strongly with the protostome P-gp genes. This is supported by analysis of conserved motifs within the NBDs (Fig3.1 B). The second residue of the Walker A domain of NBD2 is fixed in the ABCB1/vertebrate clade as a serine, while in the ABCB4/invertebrate clade this residue is either a glutamic acid, proline, or histidine. Secondly, there was an expansion of ABCB4 in echinoderms, including at least three coding genes and one pseudogenes, and at least one of these duplicated genes has evolved from an apically localized ancestor into a basolaterally localizing protein (45). These analyses support assignment of *Lp-66174* as the sole ABCB1 homolog in *L. pictus,* and the reassignment of the previously identified *S. purpuratus* ABCB genes as a single ABCB1 (formerly ABCB1a) and three ABCB4 homologs (formerly ABCB1b, ABCB1c, and ABCB4).

Subcellular localization and calcein efflux by *Lp-ABCB1*.Overexpression of ABC transporters in sea urchin blastulae is an established method for characterizing substrate selectivity and subcellular localization (37,44). At the blastula stage the cells of the embryo form a single layer of columnar epithelium, allowing for easy distinction between localization in different membrane compartments. NmCherry-*Lp-ABCB1a* localizes to the apical plasma membrane, similar to *Sp-ABCB1a* (Fig3.2 A-B). Embryos over expressing NmCherry-*Lp-ABCB1a* accumulated ~40% less intracellular calcein compared to uninjected embryos, while

RFP-H2B overexpressing controls were not significantly different than uninjected embryos (Fig3.2 C-D).

Knockout strategy and validation. BLAST search of *Lp-ABCB1a* against the draft *L. pictus* genome identified scaffold 62667 as the likely genomic locus. This scaffold covers 39 megabases. *Lp-ABCB1a* covers approximately 150 kb of sequence in 27 exons (26 coding and 1 non-coding) (Fig3.3 A). The first NBD of *Lp-ABCB1a* begins in the 3' end of exon 11 (Fig3.3 B). Mutants truncated before this point should lack the ATPase activity necessary for substrate efflux. We therefore chose to test two sgRNA targets, Exon 10+76 (Ex10+76) and Exon 11-152 (Ex11-152) (Fig3.3 B). To test their efficacy these guides were injected along with Cas9 mRNA and a membrane marker, and genomic DNA extracted at 48 hours post fertilization (HPF). Example indels (Fig33 C) show that both small indels at each target site and large deletions between the target sites occur at the larval stage. Finally, we used a CAM efflux assay in gastrula embryos, which we have recently shown to be an effective stage for looking at the effects of mutation in the purple urchin (39), to test the effect of this CRISPR scheme on transporter function. We found that CAM accumulation increased 60% relative to uninjected controls (Fig3.3 D; n=9 females, 70-87 embryos per treatment).

Generation of F_0 knockout animals. To create mutant founders for an *Lp-ABCB1a* transgenic lines two batches of *L. pictus* adults were injected with both Ex10+76 and Ex11-152 (150 ng/μL of each sgRNA, 750 ng/μL of Cas9 mRNA) and raised through to settlement (Fig3.4 A). In Batch-1 235 embryos were injected, and 28 settled at four weeks post fertilization (wpf; ~12% metamorphosis). In Batch-2 280 individuals were injected and 20 settled by four wpf (~7%).

CRISPR mutagenesis produces edited alleles that are both heterogeneous and mosaicly distributed in the embryo. During the larval stage the rudiment forms from a subset of larval tissues, primarily from the left coelomic pouch. It is unclear whether the

heterogeneity and mosaicism present in the larval stage would be present in the juveniles. To address this question we sacrificed two juveniles from each cross at one-week post fertilization for genomic DNA isolation, and cloned the knockout region. Between 12 and 22 clones were analyzed from each individual (Fig3.4 B). In three of the four individuals a wide range of mutant alleles (5-11 different alleles) were detected and no WT alleles were observed. In contrast, individual #4 showed only one mutant allele in 22 clones.

Of the 24 total mutant alleles observed three contained large deletions that would disrupt splicing between exons 10 and 11, and 11 alleles resulted in frame shift mutations without interrupting the intron. The remaining 10 mutant alleles would preserve the correct reading frame. None of the frame shift alleles would produce proteins with NBDs; all of the mutant alleles that preserve the correct reading frame would produce two NBDs, despite the close proximity of the NBD coding sequence to the Ex11-152 sgRNA site.

Test cross of crispant juveniles. Sexual maturity in sea urchins is a function of both size and age (42). At five months post settlement we removed the distal portion of 2-3 tube feet from 17 juvenile crispants, and observed 0% mortality after 30 days. At seven months post settlement 14 individual F_0 juveniles were measured for test diameter, had tube foot clips performed, and were injected with 0.55 M KCl to induce spawning. Sequencing was performed as above, and clones were characterized as frameshift (Fs) or non-frameshift (Nf) mutations, or as wild-type (Wt). We sequenced 188 clones from 13 individuals (10-17 per individual) and found that nine individuals had at least one observed Fs allele. Individuals were observed with 100% Fs, Nf, or Wt alleles, and with a mix of all three types (Table3.1). The animals' test diameter ranged in size from 3 to 11 mm.

Individual #3 and Individual #8, a male and female respectively, spawned following injection with 0.55 M KCl. Gametes from individuals #3 and #8 were outcrossed to wild-type

and also crossed together, (~200 embryos per cross). The remaining gametes were used for genomic DNA extraction.

A second spawn was conducted at 11 months post metamorphosis on six juvenile adults with identified mutant alleles. Individual #4, a male, and individual #8, a female, both spawned, and their gametes were outcrossed to Wt gametes. Additional sperm from individual #4 was used for genomic DNA extraction. A third spawn was conducted at 17 months post metamorphosis of Individuals #5 and #6, both male.

Gamete mutant clones, 17-50 from each animal that spawned, were compared to the somatic clones from the same individual (Fig3.5). The proportion of mutant to Wt clones was similar between the somatic and gamete samples. F_0 Individual #5 was chosen to propagate the F_1 generation due to the presence of a single mutant allele, an 800 base pair deletion, in the sequenced sperm sample.

Establishment of F_2 *Lp-ABCB1* null mutants. Approximately 7000 F_1 larvae were obtained after crossing F_0 Individual #5 with two wild-type females, and 305 of these metamorphosed after six weeks (Fig3.6 A). 33 reproductively sized adults were genotyped five months after metamorphosis. Beginning at seven months post fertilization sibling crosses were carried out to generate the F_2 generation. PCR amplification of the ABCB1 target region in F_2 larvae resulted in three distinct banding patterns that were confirmed to be homozygous wild-type, heterozygous, and homozygous mutant by Sanger sequencing (Fig3.6 B-C). Based on these results 119 larvae were genotyped showing a distribution of 26.9% Homozygous wild-type, 45.4% Heterozygous, and 27.7% Homozygous mutant (Fig3.6-D).

3.5 Discussion

Previously, we identified three P-gp like ABCB transporters in the purple sea urchin, of which *Sp*-ABCB1a is most similar to human ABCB1 (2). To identify the *L. pictus* ABCB1a

homolog we compared candidate *L. pictus* P-gp like genes to a similar set of P-gp like genes, and found that transcript *Lp-66174* is the *L. pictus* ABCB1 homolog (Fig 1A). We found that the expansion of the ABCB4 lineage into ABCB4 and ABCB1b and ABCB1c occurs in all of the sampled echinoderms, suggesting that ABCB1b arose from a gene duplication event in the echinoderm's last common ancestor. Additionally, we found that the sea urchin ABCB1a genes are more similar to chordate ABCB transporters, while echinoderm ABCB4/ABCB1b/ABCB1c are more similar to protostome and non-bilaterian ABCB transporters. Evidence of this split can be seen in conserved motifs of the NBDs, where the Walker A site of the ABCB1 clade contains a serine at residue two, while the ABCB4a clade does not have a fixed residue at that position (Fig 1B). Its likely that other such conserved sequence differences will become clear with future analysis. Sea urchins are basal invertebrate deuterostomes, and along with hemichordates form the ambulacraria, the sister clade to chordates (53). This suggests that while the last common ancestor of the deuterostomes had two P-gp like ABCB transporters, only one of the genes gave rise to the pair of chordate P-gp like ABCB transporters seen in most vertebrates. Further assembly of high quality invertebrate deuterostome genomes may allow us to elucidate this transition more accurately.

ABC transporters often have conserved subcellular localization to apical, basolateral plasma membranes, or internal membranes. Blastula stage sea urchin embryos form a single layered epithelium that is useful for studying the subcellular localization of ABC transporters (44). We cloned the full length *Lp-ABCB1a* transcript into the N-mCherry PCS2+8 plasmid, and used this template to synthesize N-mCherry-*LpABCB1a* mRNA. In microinjected embryos the tagged *Lp-ABCB1a* localizes to the apical plasma membrane (Fig 2C). To evaluate the efflux activity of *Lp-ABCB1a* we exposed these embryos to Calcein-AM (CAM), a substrate predominantly effluxed by ABCB1a in sea urchin embryos, which fluoresces

when the –AM moiety is cleaved by intracellular esterases (44,54). *Lp-ABCB1a* overexpressing embryos efflux significantly more CAM, a ~40% reduction in intracellular fluorescence, while the injection control, N-mCherry Histone H2B, had no effect (Fig 2C, D). These findings support a similar substrate profile between echinoderm *ABCB1a* genes and chordate *ABCB1* genes. Future work will characterize other potential substrates of *Lp-ABCB1a,* and in particular studies using homozygous mutants will allow us to broaden our understanding of the physiological substrates of *Lp-ABCB1a*.

Lp-ABCB1a is encoded by 28 exons spanning ~150 KB of scaffold 62667 of the *L. pictus* genome (Fig 3A). This is a similar genomic structure to human ABCB1 (55). We designed our CRISPR knockout strategy to generate edits in exons 10 and 11, that encode the cytoplasmic domain prior to the first nucleotide binding domain (Fig 3B). This schema resulted in both small indels contained within each intron and large deletions spanning between exon 10 and 11 (Fig 3C). Interestingly, in several clones large deletions were generated from either target site, while the second site contained only a small indel. Many studies have shown that unexpectedly large deletions can come from individual sgRNAs, in some cases up to several hundred kilobases, as well as complex rearrangements from homolog crossover or other forms of homology dependent DNA repair (56–58). In multiplexed CRSIPR experiments only a small percentage of cutting and repair events happen simultaneously (59). Large deletions were seen in both the whole animal lysis (Fig 4B) and somatic genotyping, which indicates that large deletions are not deleterious in the F_0 generation.

We assessed *Lp-ABCB1a* activity in crispant plutei larvae using the cell-permeable ABCB substrate Calcein-AM, as described above. Crispant larvae accumulate 60% more Calcein after a 60 minute exposure, as compared to control larvae. This is a smaller effect than that observed in purple urchin *ABCB1a* crispants (39), and further studies with

homozygous mutants will be needed to elucidate if this is due to a difference in the two *ABCB1a* paralogs' affinity for CAM, or a difference in gene expression changes in crispant embryos between the two species.

Having confirmed that our CRISPR schema would generate a variety of alleles and have a functional consequence on *Lp-ABCB1a* efflux activity, we set out to generate crispant founders for an *Lp-ABCB1a* knockout line. We injected two batches of *L. pictus* zygotes with both sgRNAs, ~250 zygotes each, of which 49 metamorphosed into juvenile adults by four weeks post fertilization (wpf; Fig 4A). Reported genotypes of crispant sea urchin juvenile adults have generally shown between 3 and 5 mutant alleles per individual, but these early studies did not use a multiplexing approach (25,26). At one month post metamorphosis (mpm) four individuals, two from each batch, were sacrificed for whole animal lysis and genomic DNA extraction, and 12 to 22 clones sequenced from each (Fig 4B). All four individuals had indels, however they were otherwise different from each other. A single allele in Individual #2 accounted for 66% (8/12) of observed clones, while in Individuals #1 and #3 no allele accounted for more than 30% of the observed clones. In all three individuals only mutant clones were observed. In contrast, Individual #4 had a single observed clone with a mutant allele, while the rest were wild-type (21/22). It is possible that the genomic locus or the multiplexing approach account for the discrepancy between the heterogeneity we observed, although it is also possible that more experimental effort was expended in this study to observe the mutations in the F_0 generation.

At seven months post metamorphosis we used tube foot clips, a non-lethal somatic genotyping method, to genotype 14 animals with a test diameter of over 3 mm (Table 1). Nine individuals had frameshift alleles (Fs), which were prioritized for spawning. Five animals spawned, Individuals #3-6 and #8. Gametes from these animals were used to create F_1 larvae. The sea urchin germline originates from eight progenitor cells known as small

micromeres, which are necessary for both somatic gonad and germline cells in the adult (60). Given the heterogeneity of the crispant juvenile adults, we decided to genotype the gamete samples obtained from each individual and compare them to the corresponding somatic gonad samples (Fig3.5). While the trend in mutation frequency was similar between the two sample types (majority mutant or majority wild-type), in most individuals the observed clones were different between the somatic and gamete samples, confirming that CRISPR editing continued after the split between these cell types.

Propagation of F_1 and F_2 animals. F_0 Individual #5 was used to create two outcrossed batches of F_1 animals at 17 months post fertilization. These crosses resulted in 33 genotyped heterozygotes of reproductive size and age, and siblings were crossed to create F_2 larvae for long term culturing. Importantly, the F_1 juveniles began to give gametes after injection with KCl at six months post fertilization, and large-scale cultures were started at 7 months post fertilization. This represents a 2-3 times decrease in generation time between the F_0 and F_1 generations. In all likelihood this is due to an improvement in water quality associated with the move from aerated 8L aquaria to a recirculating seawater system. This makes the propagation of both future generations of the ABCB1 mutant line and new mutant lines increasingly feasible.

F_0 Individual #5 had a single allele present in the sequenced sperm sample, an 800 base pair deletion that was also present in the tube feet (Fig3.6). We predicted that F_2 larvae could be genotyped by PCR analysis due to the significant change in amplicon size between wild-type and mutant alleles, and Sanger sequencing of representative samples confirmed this approach was valid (Fig3.7 B-C). Sampling of over one hundred larvae revealed that each genotype occurs at an almost perfect 1:2:1 Mendelian ratio (Fig3.7 D), supporting our hypothesis that *Lp*-ABCB1 mutations would not be embryonically lethal.

Future Directions: Characterization of ABCB1 Mutants. While we have confirmed that we can generate F_2 homozygous ABCB1 mutants, we have not yet characterized an associated phenotype. The phenotype most often observed in P-gp mutants is chemosensitization, or susceptibility to an otherwise tolerable xenobiotic. In border collies and related dog breeds, a naturally occurring loss-of-function P-gp mutation has no effect until the application of the common deworming medicine Ivermectin, at which point life threatening neurotoxicity occurs (61–63). One of the main mechanisms of this chemosensitization is the absence of P-gp from the blood-brain barrier, one of the critical roles of P-gp in many mammals (64,65). Interestingly, many synonymous and missense P-gp polymorphisms in humans also cause a chemosensitization phenotype. For example, both the non-synonymous 2677G>T/A (SNP rs2032582) and synonymous 3435C>T (SNP rs1045642) polymorphisms sensitize patients to chemotherapeutic toxicity, even as both variants create full-length proteins (66). In sea urchins *ABCB1a* is expressed in the larval ectoderm and the gut, important barrier tissues between the sea urchin larvae and the environment (4,40). One area of future study will be assessing the sensitivity of *L. pictus* ABCB1a mutants to environmental chemicals. We have recently shown that many persistent organic pollutants found in food fish and human blood samples act as inhibitors of P-gp (67). With a marine P-gp knockout model we will be able to investigate the pathways of absorption for these toxic compounds.

In this study we demonstrated that we could identify specific targets in the *L. pictus* genome and reliably use CRISPR to induce mutations at desired loci. We further showed that juvenile sea urchins can be non-lethally genotyped using tube foot clips, a procedure analogous to fin or ear clips, but with the added advantage of using a regenerative tissue. Finally, we showed that CRISPR induced mutations in *Lp-ABCB1a* could be propagated to the F_2 generation in homozygous mutants. These knockout lines will enable us to perform

further targeted studies on the roles of ABCB transporter activity in early development, gut differentiation, and pollution sensitivity.

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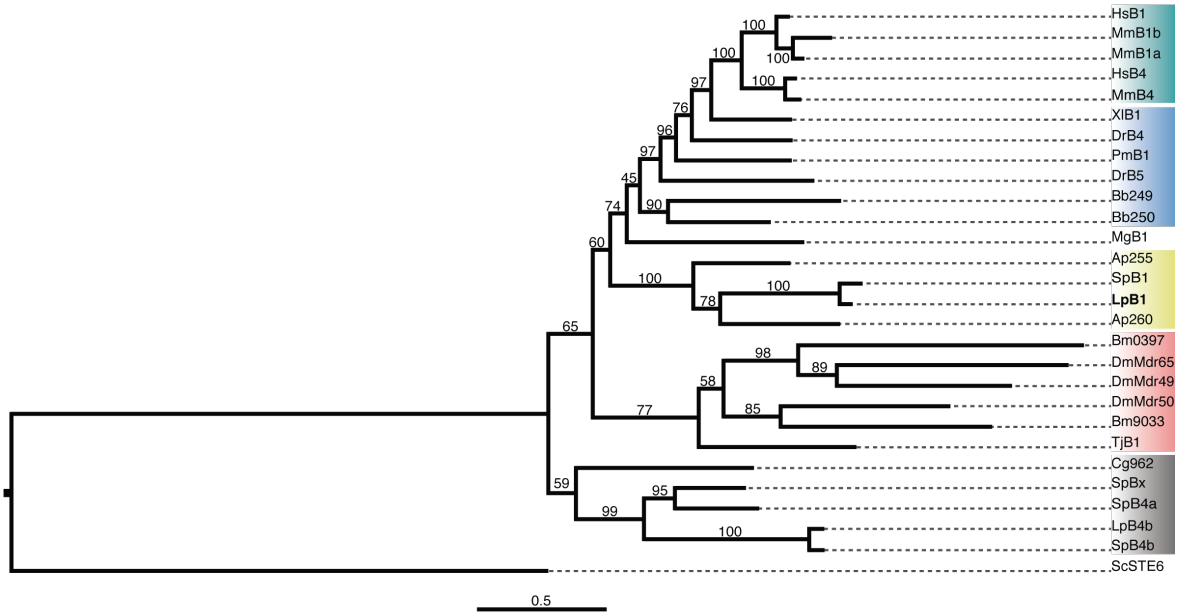
3.8 Chapter Three Figures

Figure 3.1 Identification of transcript *Lp-66174* as the *L. pictus ABCB1a* gene. A) *Lp*-66174 is the most similar *L. pictus* ABCB transporter to purple urchin ABCB1a. The echinoderm ABCB1a clade is most similar to the chordate ABCB1/B4 genes, while the echinoderm ABCB4 clade is more similar to invertebrate ABCB1/B4 genes. Sequences of known ABCB1 and ABCB4 transporters using ClustalW were aligned and used to construct a neighbor-joining tree using the Jukes-Cantor model. Bootstrap values represent the support level after 1,000 replicates. Yeast (*Saccharomyces cerevisiae,* Sc) was chosen as the out-group. B) Alignment of known conserved domains in nucleotide binding domains (NBDs) 1 and 2 reveals a conserved serine in the Walker A domain of NBD2 in the ABCB1a like clade. The ABCB4 like clade lacks a consistent residue at this position, suggesting that the sequence became fixed after the evolutionary split between the two clades. Grey residues represent conservative amino acid changes, black residues represent non-conservative changes, and dashes indicate incomplete sequences. Additional abbreviations; Ap, *Acanthaster plancii*; Bb, *Branchiostoma belcherii*; Dm, *Drosophila melanogaster*; Dr, *Danio rerio*; Eb, *Eptatretus burgeri*; Et, *Eucidaris tribuloides*; Hs, *Homo sapiens*; Lp, *Lytechinus pictus*; Mm, *Mus musculus*; Nm, *Nematostella vectensis*; Pm, *Petromyzon marinus*; Sp, *Strongylocentrotus purpuratus*; Xl, *Xenopus laevis.*

A)


HsB1
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MmB1a
HsB4
MmB4
XlB1
DrB4
PmB1
DrB5
Bb249
Bb250
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Ap255
SpB1
LpB1
Ap260
Bm0397
DmMdr65
DmMdr49
DmMdr50
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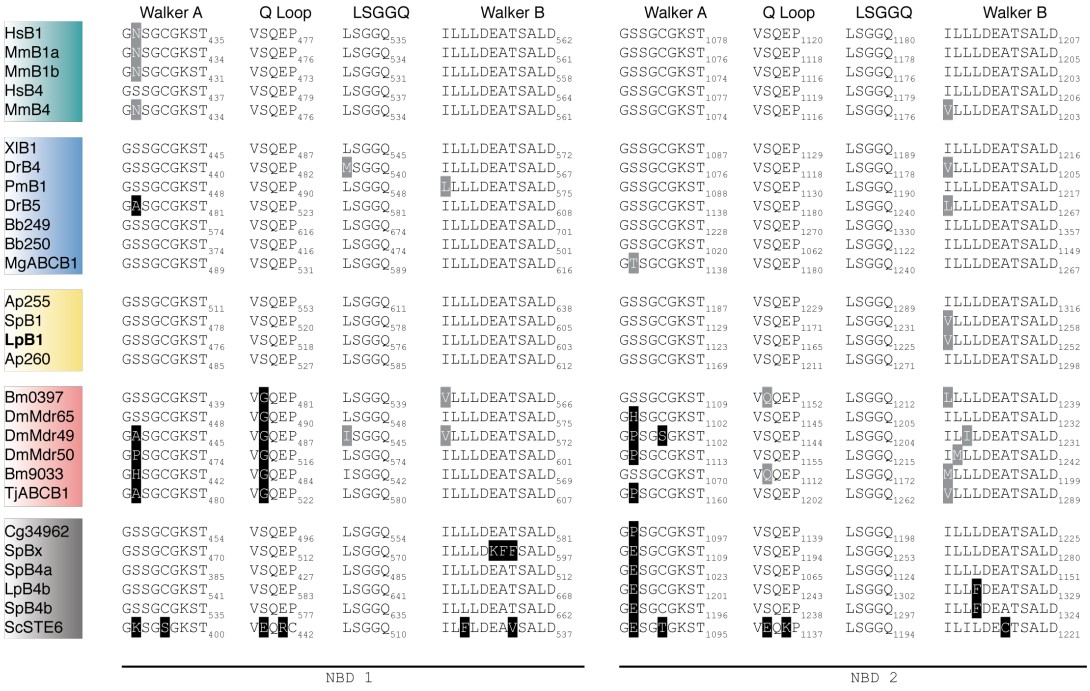
LpB4b
SpB4b
ScSTE6
0.5

B)


Walker A
Q Loop
LSGGQ
Walker B
Walker A
Q Loop
LSGGQ
Walker B
HsB1
MmB1a
MmB1b
HsB4
MmB4
XlB1
DrB4
PmB1
DrB5
Bb249
Bb250
MgABCB1
Ap255
SpB1
LpB1
Ap260
Bm0397
DmMdr65
DmMdr49
DmMdr50
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LpB4b
SpB4b
ScSTE6
NBD 1
NBD 2

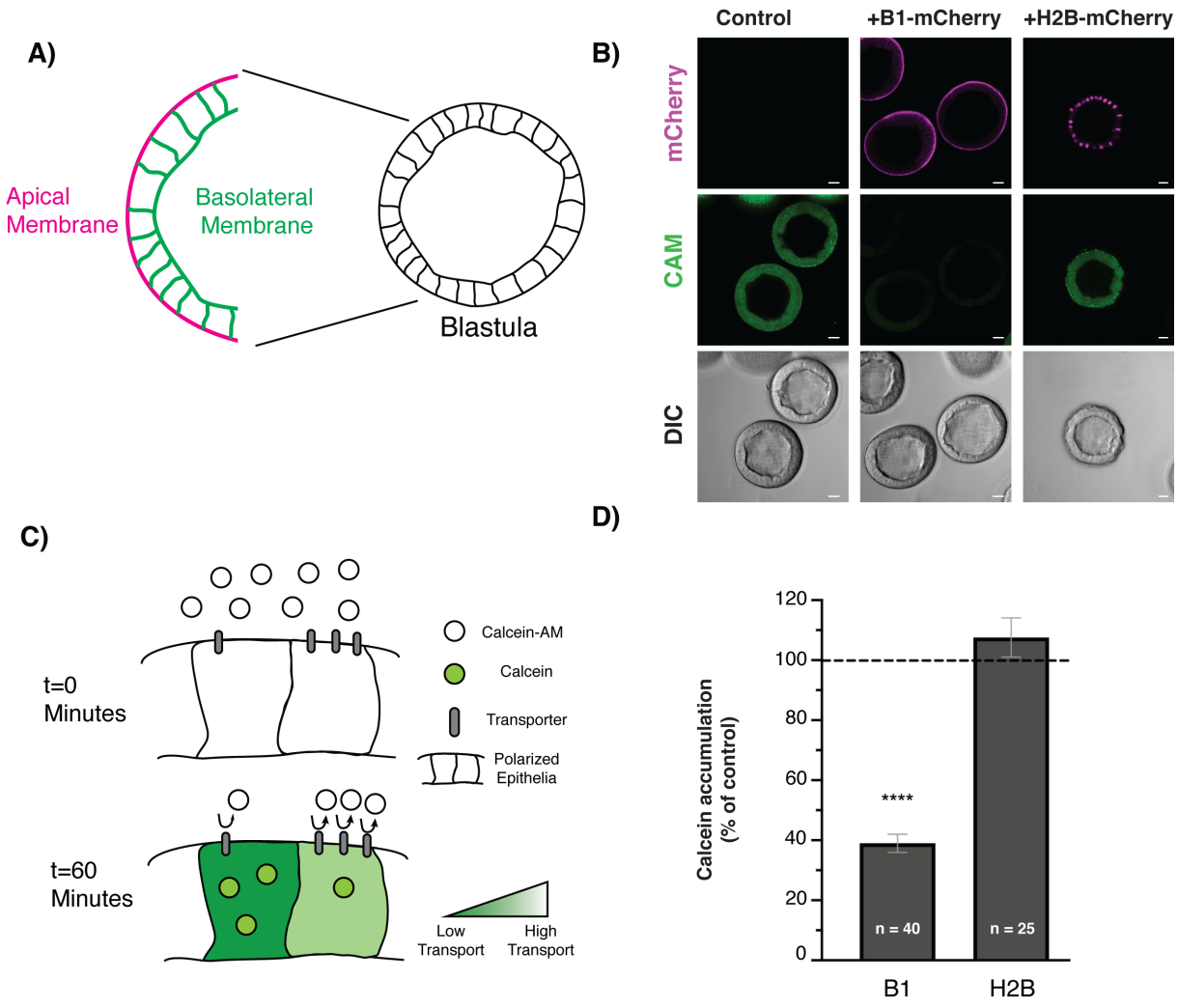


Figure 3.2 *Lp-ABCB1a* has similar subcellular localization and substrate preferences to *Sp-ABCB1a.* A) Representation of a blastula stage *L. pictus* embryo, with apical (magenta) and basolateral (green) plasma membranes highlighted B) Close-up of an epithelial cells demonstrating the effect of Calcein-AM incubation at t=0 minutes and t=60 minutes. In cells with high ABC transporter activity (right) less Calcein-AM enters the cell to be converted to fluorescent calcein, as compared to cells with low ABC transporter activity (left). C) Example micrographs showing calcein accumulation in uninjected, mCherry-ABCB1a, or H2B-mCherry injected embryos. *Lp*-ABCB1a localizes to the apical plasma membrane. D) Quantification of CAM accumulation in mCherry-ABCB1a and H2B-mCherry embryos relative to uninjected embryos. Scale bars are 20 μM.

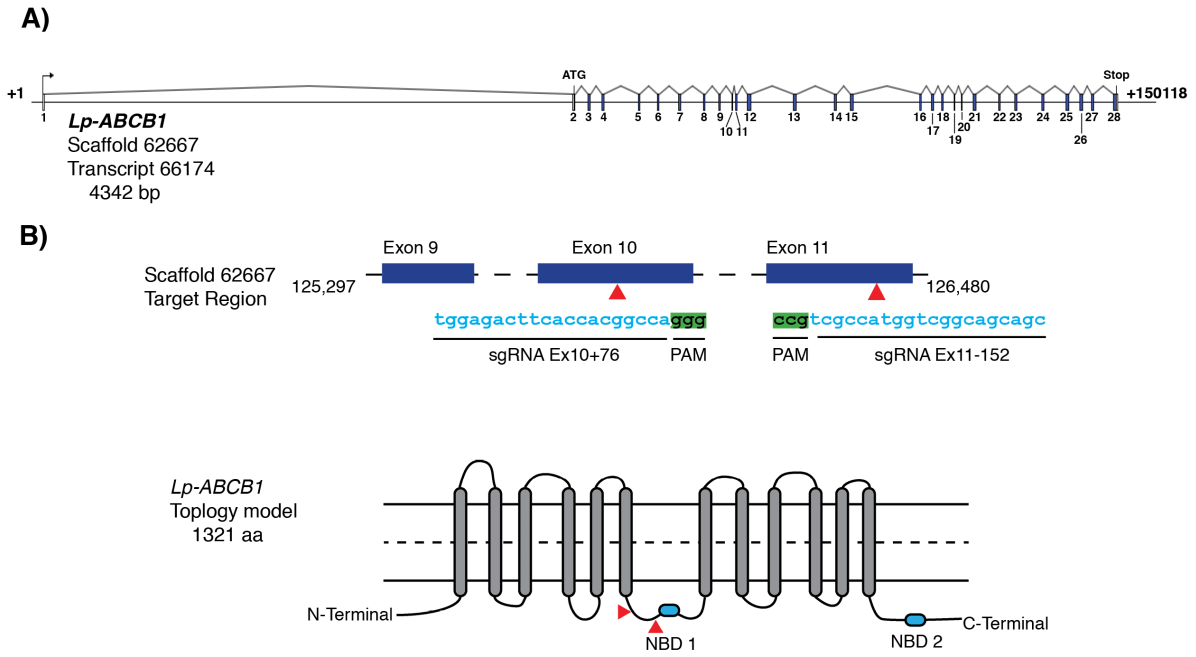
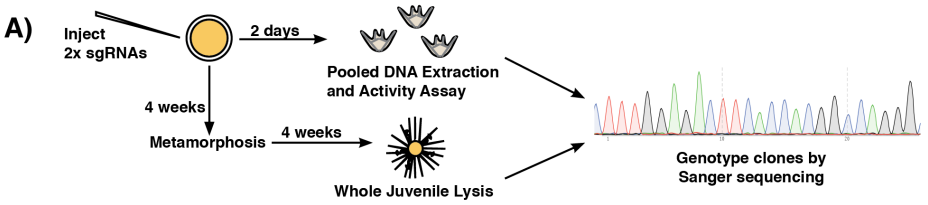


Figure 3.3 Generation and characterization of *Lp-ABCB1a* crispant larvae. A) The Lp-ABCB1a transcript (4342 bp) covers 150 kb of scaffold 62667 in 28 exons. B) The cytoplasmic loop prior to the first nucleotide binding domain (cyan oval) is encoded by exons 10 and 11. Guide RNA target sites Ex10+76 and Ex11-152 are indicated by red triangles, as are their corresponding locations in the Lp-ABCB1a topology model. C) Alignment of recovered genomic DNA clones from 2-day old larvae co-injected with Ex10+76 and Ex11-152 sgRNAs. Both small indels at either target site (middle set), as well as large indels that span between the target sites (lower set) were recovered. The target sites are indicated as cyan nucleotides and the PAM sequence is highlighted in green. Insertions are upper case magenta bases, and deletions as magenta dashes. The total change at each target site is indicated in the table at the right. The distance between the 5' end of Ex10+76 and the 3' end of Ex11-152 is 831 bases in the reference genome. D) Embryos were co-injected with Ex10+76 and Ex11-152 sgRNAs and a membrane marker, and ABCB1a activity was measured at 24 hours post fertilization using a Calcein-AM accumulation assay. Crispant embryos (ΔABCB1a) were visually brighter than control embryos and showed no morphological differences compared to control embryos. Crispant embryos (ΔABCB1a, n=70) accumulated on average 60% more Calcein than control embryos (n=87).

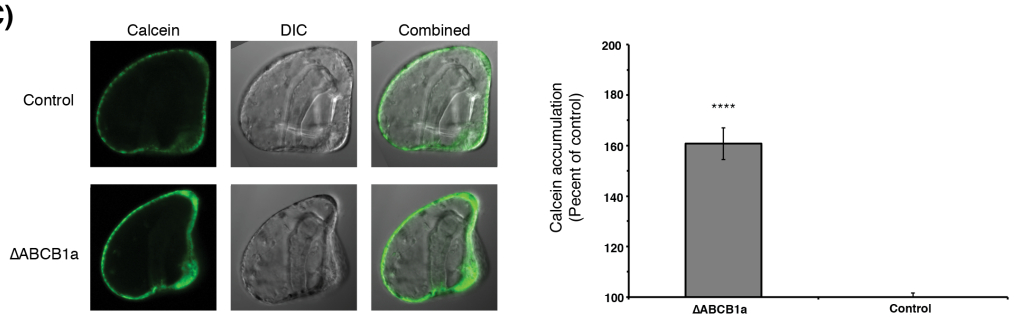
Figure 3.4 Genotype analysis of F_0 post-metamorphosis crispant juvenile adults. A) Two batches of embryos were injected with both sgRNAs and raised through metamorphosis. Genomic DNA samples were obtained from either whole animal lysis at one week post metamorphosis (wpm) or from tube foot clips after one month post metamorphosis (mpm). Plasmids containing PCR amplicons were sequenced via Sanger sequencing. B) At one-week post metamorphosis four individuals, two from each batch, were randomly selected for sequencing by whole animal lysis. Between 12 and 22 clones were sequenced from each individual, and all four individuals genotyped had at least one mutation. The target sites are indicated as cyan nucleotides and the PAM sequence is highlighted in green. Insertions are upper case magenta bases, and deletions as magenta dashes.



B)

	Exon 10 Target Site												Exon 11 Target Site									Exon 10	Exon 11
Reference Genome	F	G	D	F	T	T	A	R	A	A	K	T	V	A	M	V	G	S	S	G			
	ttt	gga	gac	ttc	acc	acg	gcc	agg	gct	gca	...aag	acc	gtc	gcc	atg	gtc	ggc	agc	agc	ggg			
Indels within Exons	ttt	gga	gac	ttc	acc	acg	gcT	Gca	ggg	ctg	...---	---	---	---	---	--g	gtc	ggc	agc	agc	ggg	+2	-18
	ttt	gga	gac	ttc	acc	ac-	---	agg	gct	gca	...aag	acc	gtc	g--	---	---	-gc	agc	agc	ggg		-4	-9
	ttt	gga	gac	ttc	acc	a--	---	-gg	gct	gca	...aT-	---	---	gcc	atg	gtc	ggc	agc	agc	ggg		-6	-7 (-8+1)
Indels Spanning Intron	---	---	---	---	---	---	---	---	---	---	...aag	acc	gt-	---	--g	gtc	ggc	agc	agc	ggg		-670	-6
	ttt	gga	gac	ttc	acc	acg	g--	---	---	---	...---	---	---	---	---	-tc	ggc	agc	agc	ggg		-401	-402
	ttt	gga	gac	ttc	acc	acT	TCA	CAT	TC-	---	...---	---	---	---	-cc	atg	gtc	ggc	agc	agc	ggg	-391 (-400+9)	-399
	---	---	---	---	---	---	---	---	---	---	...---	---	---	-cc	atg	gtc	ggc	agc	agc	ggg		-415	-283 (-290+7)

C)



D)

Experiment	# Clones	Exon 10 Target Site												Exon 11 Target Site									Exon 10	Exon 11
Reference Genome	-/-	F	G	D	F	T	T	A	R	A	A	K	T	V	A	M	V	G	S	S	G			
		ttt	gga	gac	ttc	acc	acg	gcc	agg	gct	gca	...aag	acc	gtc	gcc	atg	gtc	ggc	agc	agc	ggg			
Juvenile 1	5/17	ttt	gga	gac	ttc	CAC	---	---	---	---	---	...aag	acc	gtc	gcc	atg	gtc	ggc	agc	agc	ggg	-23 (-26+3)	0
	4/17	ttt	gga	gac	ttc	acc	acg	g--	---	gct	gca	...a--	---	---	---	-tg	gtc	ggc	agc	agc	ggg	-5	-12
	3/17	ttt	gga	gac	ttc	acc	a--	---	-gg	gct	gca	...aag	acc	gtc	gcc	atg	gtc	ggc	agc	agc	ggg	-6	0
	2/17	ttt	gga	gac	ttc	CAC	---	---	---	---	---	...aag	a--	---	---	-tg	gtc	ggc	agc	agc	ggg	-23 (-26+3)	-8
	1/17	ttt	gga	gac	ttc	acc	acg	g--	---	gct	gca	...aag	acc	gtc	--c	atg	gtc	ggc	agc	agc	ggg	-5	-2
	1/17	ttt	gga	gac	ttc	acc	acg	gc-	---	--t	gca	...aag	acc	gtc	gcc	atg	gtc	ggc	agc	agc	ggg	-6	0
	1/17	---	---	---	---	---	---	---	---	---	---	...aag	acc	gt-	---	--g	gtc	ggc	agc	agc	ggg	-74	-6
Juvenile 2	8/12	ttt	gga	gac	ttc	acc	acg	gc-	---	--t	gca	...aag	acc	---	---	atg	gtc	ggc	agc	agc	ggg	-6	-6
	1/12	ttt	gga	gac	ttc	acc	ac-	---	agg	gct	gca	...aag	acc	---	---	atg	gtc	ggc	agc	agc	ggg	-4	-6
	1/12	ttt	gga	gac	ttc	acc	acg	g--	---	gct	gca	...aag	a--	---	---	---	---	---	---	---	---	-5	-36
	1/12	ttt	gga	gac	ttc	acc	acg	g--	---	---	---	...aag	acc	gtc	--c	atg	gtc	ggc	agc	agc	ggg	-26	-2
	1/12	ttt	gga	gac	ttc	acc	acg	g--	---	GTt	gca	...aag	acc	---	---	atg	gtc	ggc	agc	agc	ggg	-5 (-7+2)	-6
Juvenile 3	3/21	ttt	gga	gac	ttc	acc	a--	---	-gg	gct	gca	...aag	acc	gtc	g--	---	---	-gc	agc	agc	ggg	-6	-9
	3/21	ttt	g--	---	---	---	---	---	---	---	-ca	...aag	acc	gtc	gcc	atg	gtc	ggc	agc	agc	ggg	-24	0
	3/21	ttt	gga	gac	ttc	acc	ac-	---	agg	gct	gca	...aag	acc	g--	---	---	gtc	ggc	agc	agc	ggg	-4	-8
	2/21	ttt	gga	gac	ttc	acc	acg	gGC	TGC	AGC	ACG	...aag	acc	gtc	g--	---	---	-gc	agc	agc	ggg	+7 (-4+11)	-9
	2/21	ttt	gga	gac	ttc	acc	acg	g--	---	gct	gca	...aag	acc	gtc	g--	---	---	-gc	agc	agc	ggg	-5	-9
	2/21	ttt	gga	gac	ttc	ac-	---	---	---	--t	gca	...aag	acc	gtc	g--	---	---	-gc	agc	agc	ggg	-12	-9
	2/21	ttt	gga	gac	ttc	acc	ac-	--c	agg	gct	gca	...aag	acc	gtc	gcc	atg	gtc	ggc	agc	agc	ggg	-3	0
	1/21	ttt	g--	---	---	---	---	---	---	---	-ca	...aag	acc	gtc	g--	---	---	-gc	agc	agc	ggg	-24	-9
	1/21	ttt	gga	gac	ttc	aAA	AGT	C--	---	---	---	...aag	acc	---	--c	atg	gtc	ggc	agc	agc	ggg	-17 (-23+6)	-5
	1/21	ttt	gga	gac	ttc	acc	a--	---	-gg	gct	gca	...aag	acc	gtc	g--	atg	gtc	ggc	agc	agc	ggg	-6	-449 (-447/-2)
	1/21	ttt	gga	gac	ttc	acc	a--	---	-gg	gct	gca	...aag	acc	gtT	GGA	AAG	ACC	GTC	TTT	GA-	---	-6	-123 (-141+18)
Juvenile 4	1/22	ttt	gga	gac	ttc	acc	ac-	--c	agg	gct	gca	...aag	acc	gtc	gcc	atg	gtc	ggc	agc	agc	ggg	-3	0
	21/22	ttt	gga	gac	ttc	acc	acg	gcc	agg	gct	gca	...aag	acc	gtc	gcc	atg	gtc	ggc	agc	agc	ggg	0	0

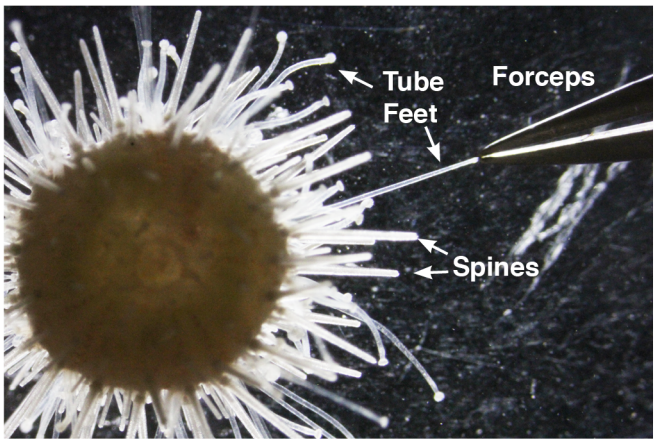


Figure 3.5 Tube foot clip of juvenile sea urchin. Forceps are used to grasp fully extended tube feet. The animal will react by attempting to retract its tube feet, and gentle pressure in the opposite direction will liberate a fragment several millimeters long that can be used for genotyping.

Table 3.1 Characterization of mutant alleles from F_0 juvenile adult somatic tissue. At seven months post metamorphosis 14 crispant juvenile adults were measured, genotyped, and spawned. The test diameters ranged from 3 to 11 mm. Alleles were categorized as frameshift (Fs), non-frameshift (Nf), or wild-type (Wt), and animals were ordered by the number of recovered Fs alleles. 10 to 17 clones were sequenced from each somatic genomic DNA sample.

Individual	Size (mm)	Variant	Copies	Exon 10	Exon 11	Total Change	Mutation Type
1	10	1	11	-394	0	-394	Fs
		2	1	-394	-1	-395	Fs
		3	1	-394	-21	-415	Fs
		4	1	-5	0	-5	Fs
		5	1	-5	-9	-14	Fs
2	6	1	8	-5	-9	-14	Fs
		2	4	-5	-3	-8	Fs
		3	1	+1 (+4-3)	-9	-8	Fs
		4	1	-5 (+1-6)	-9	-14	Fs
		5	1	-5	0	-5	Fs
3	9	1	10	+28 (+30-2)	-9	+19	Fs
		2	3	-353 (+1-354)	-450	-803	Fs
4	11	1	4	-660	-9	-669	Fs
		2	3	-5	-18 (+8-26)	-23	Fs
		3	2	-5	-1	-6	Fs
		4	1	-5	-18 (+8-26)	-23	Fs
		5	1	-345	0	-6	Nf
5	4	1	3	-345	-460	-805	Fs
		2	3	-344	-456	-800	Fs
		3	2	-103	0	-103	Fs
		4	2	+21 (+61-40)	0	+21	Nf
6	5	1	14	-3	-9	-12	Nf
		2	2	+2 (+5-3)	0	+2	Fs
7	6	1	14	-6	0	-6	Nf
		2	1	-6	0	-6	Nf
		3	1	-5	0	-5	Fs
		4	1	-344	-459	-803	Fs
8	9	1	17	0	0	0	Wt
		2	1	-338 (+9-347)	-453	-791	Fs
9	5	1	12	-6	-6	-12	Nf
		2	1	+40	-6	+34	Fs
10	3	1	10	-6	0	-6	Nf
		2	3	-18	0	-18	Nf
		3	1	-18	0	-18	Nf
		4	1	-3	0	-3	Nf
11	4.5	1	16	0	0	0	Wt
12	5	1	15	0	0	0	Wt
13	4	1	16	0	0	0	Wt
14	5	1	10	0	0	0	Wt

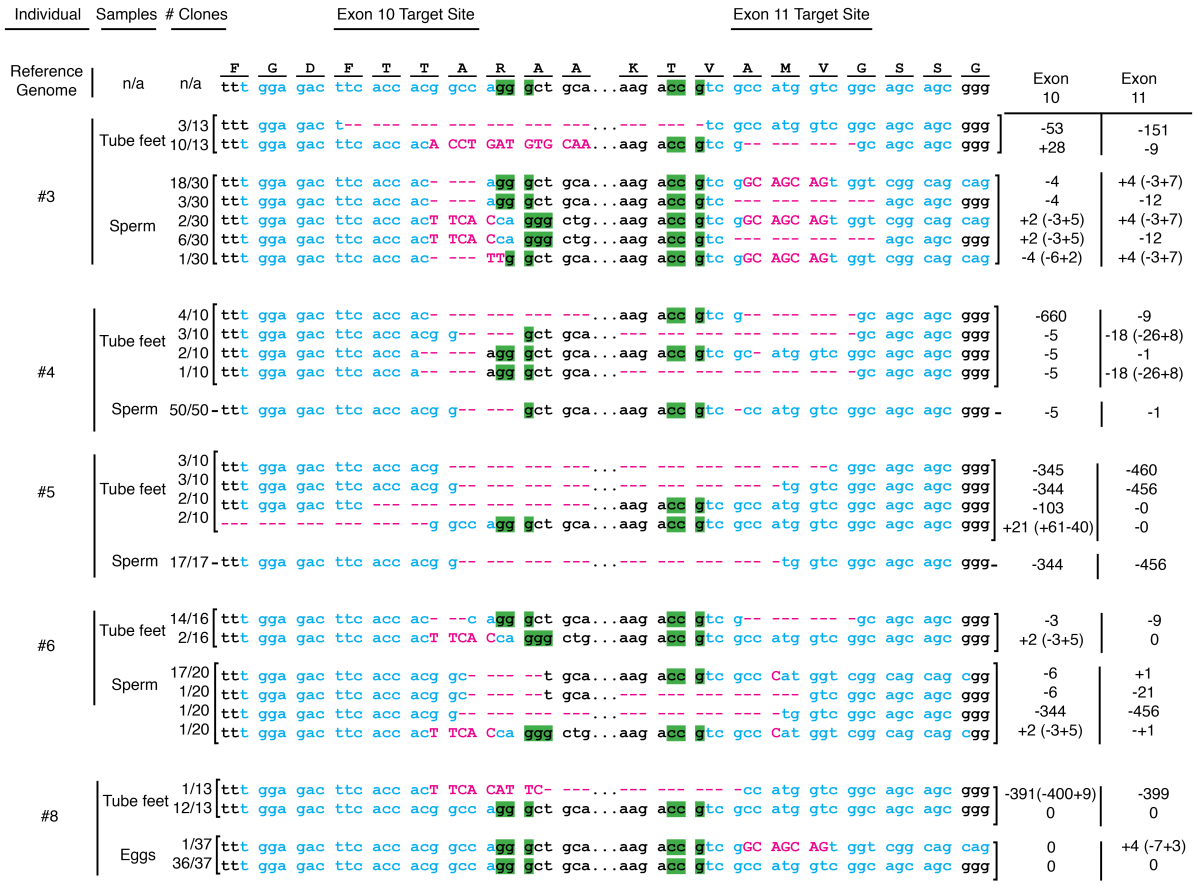
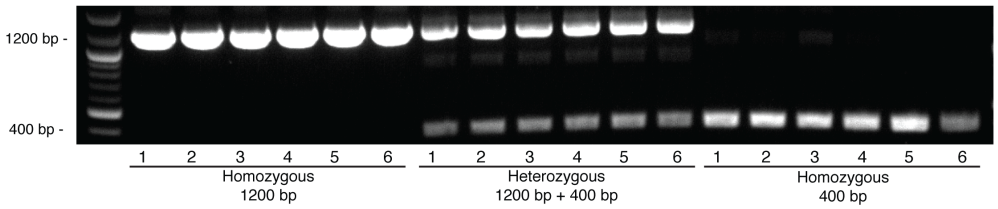


Figure 3.6: Comparison of recovered mutations from somatic tissue and gametes. Four males, individuals #3-6, and one female, individual #8, spawned between 7 and 17 months post fertilization. Between 11 and 18 clones were sequenced from each somatic sample, and between 17 and 50 clones from each gamete sample. The target sites are indicated as cyan nucleotides and the PAM sequence is highlighted in green. Insertions are upper case magenta bases, and deletions as magenta dashes. The total change at each target site is indicated in the table at the right.

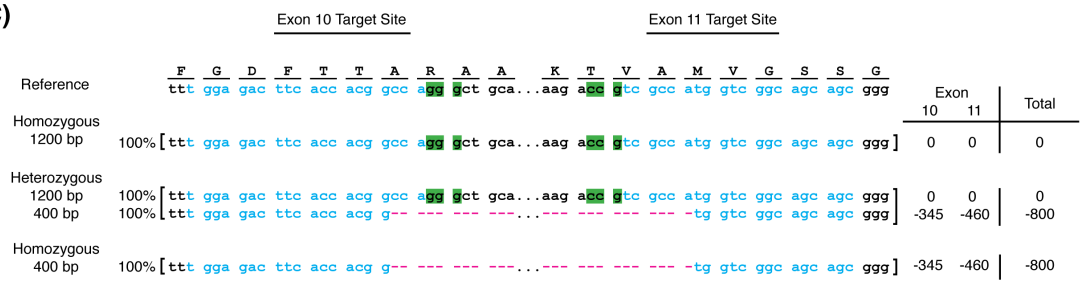
A)

	Larvae	Juveniles	Genotyped Juveniles	Heterozygous mutants
Ind. #5 x WT1	4400	245	30	14
Ind. #5 x WT2	2500	105	39	19
Total	6900	305	69	33

B)



C)



D)

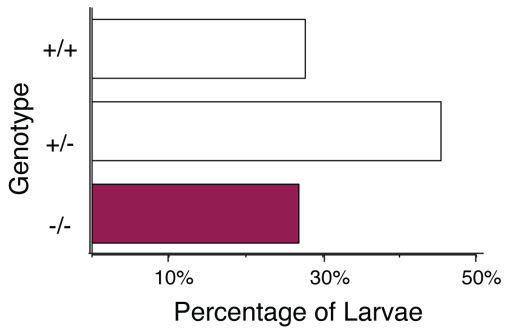


Figure 3.7 Generation of ABCB1 homozygous F2 larvae. A) Two cultures of F_1 animals were generated from F_0 individual #5, resulting in 33 genotyped, sexually mature heterozygous juveniles. B) Representative PCR alleles obtained from F_2 incross showing three distinct genotypes. C) Alignment of sequenced representative PCR bands from part B. D) Quantification of the genotypes of F2 larvae based on PCR genotyping (n=119, +/+ 1200 bp band, -/- 400 bp band, +/- 1200 and 400 bp band).

Chapter 4
The sea urchin as a heterologous expression system and preliminary experiments towards insertional mutagenesis

4.1 Abstract

In the course of this dissertation several different avenues of research relating to high-resolution imaging or sea urchin transgenesis were undertaken that on their own do not merit an entire chapter. Some were unsuccessful due to time or experimental constraints, while others resulted in co-authorships. In general, they represent approaches or study organisms that were unusual in our lab, and can act as a stepping-stone for future researchers. Specifically, the data presented in this chapter pertain to studies with coral membrane transporter localization and strategies for knock-in of fluorescent protein reporters in both *S. purpuratus* and *L. pictus* sea urchins. These strategies included both CRISPR mediated targeted transgenesis and Tol2 transposase mediated random transgenesis.

4.2 Background

Heterologous expression in animal models. As discussed previously in this manuscript, certain species have characteristics that make them easy to study, such as optical clarity or abundance. But this does not mean that other animals don't deserve or need to be studied. Heterologous expression is one way to address the imbalance between animals that researchers wish to study and those that are easy to study. In these approaches a gene of interest is expressed, either transiently or permanently, in a model organism or cell line that is used to visualize protein localization, screen chemical effectors, or synthesize the protein in sufficient quantities for purification(1–4).

Sea urchin blastula stage embryos form a single layer polarized epithelium (5). Our lab has used this stage extensively to study the subcellular localization of both sea urchin and human ABC transporters (6–8). In addition to localization, it is also possible to discern the direction of transport either apically or basolaterally (7,8). Heterologous expression in this

stage can therefore be a useful tool for studying the localization and function of genes, particularly if the genes are difficult to study in their native environment.

Sodium/Calcium exchanger and calcification in corals. Coral reefs form over time from the calcium carbonate skeletons of Scleractinian corals. In the coral body an epithelial layer, known as the calicoblastic epithelium, forms over a medium, known as the subcalicoblastic medium, where calcium carbonate deposition onto the skeleton occurs (9). Numerous parameters, such as dissolved inorganic carbon and pH, must be precisely regulated in order for calcification to occur, and in large part this is accomplished by different transporters (10,11). These transporters can differ by species and physiological condition, such as day/night cycle or temperature, but in general are meant to create conditions favorable for calcification in the subcalicoblastic medium (12–14). In other words, transporters on both the apical and basolateral membranes are presumably acting in concert to facilitate flow into and out of the subcalicoblastic membrane, much like the blood brain barrier or placental epithelium in humans (15). However, the nature of the calicolastic epithelium (single layered, following the shape of the skeleton/polyp and in close proximity to skeleton) makes it difficult to definitively assign subcellular localization to proteins in this cell type.

The sodium calcium exchanger (NCX) is a member of the Slc8 family of solute carrier (SLC) membrane transporters (16,17). The SLC superfamily is the second largest membrane protein family, with around 400 genes in the human genome (18). Slc8 genes encode a protein with 10 transmembrane domains and localizes mainly to basolateral membranes. In the model coral *Acropora yongei* NCX (AyNCX) is localized in the calicoblastic epithelium (19). This would imply that NCX is involved in the accumulation of calcium ions in the subcalicoblastic medium, however without knowing the subcellular localization of AyNCX it is difficult to determine the genes' true function. The geometry of the calicoblastic epithelium

makes it difficult to determine the subcellular localization of AyNCX. In the data presented here I made use of transient heterologous expression of AyNCX in sea urchin embryos to determine the likely subcellular localization of AyNCX.

Mutagenesis as a biological tool. Transient heterologous expression is a related approach to permanent insertional mutagenesis (also known as transgenesis). Mutagenesis, or the altering of genes, is one of the most commonly used techniques in modern biology, and is closely tied to our understanding of natural variation and genetics. Studying natural variation has been a staple of biology research since modern biology emerged as a discipline, exemplified by the natural pea plant variations studied by Mendel. At the turn of the 1900s genetics was established as a discipline based on understanding the inheritance of natural variations, such as eye color mutants in *Drosophila* (20,21). Chemical and radiological means of inducing random mutations followed in the mid 1900s and were widely used in model organisms, such as the screens for pattern mutants in *Drosophila* and zebrafish (22,23). While random mutagenesis has been a useful tool, the ability to introduce novel DNA remained elusive outside of microbes until the end of the century. By the year 2000 several transgenesis modalities, such as random DNA integration and homologous recombination, were in widespread use in animal models. These technologies were precursors to transposon based random insertions and targeted insertions utilizing CRISPR and homology directed repair.

Precursors to modern gene editing. Random insertion of a DNA transgene was first described in the 1980s, when viral gene fragments and the Rabbit β-Globin gene were injected as linear DNA into mouse oocytes (24,25). This technique was quickly adopted in sea urchins, which are particularly amenable to microinjection (26,27). These studies found that linear DNAs form large concatamers that are expressed and replicated in the larvae, but are rarely passed on to juvenile animals. In sea urchins the adult rudiment forms from the left

coelomic pouch, and most of the larval cells do not contribute to this structure (28). This means that low frequency integrations would be infrequent in the juvenile animal post metamorphosis, and perhaps even more rare in the germline. Given this limitation, random DNA insertion in sea urchins has been limited to reporter constructs used during embryogenesis and the larval stages.

In retrospect, one of the most important uses of random DNA insertion for the development of modern gene editing was the large-scale use of trapping vectors in mice. The most common of these vectors, the gene trap, contains a splice acceptor site fused with a marker gene, such as the neomycin resistance gene or *LacZ* gene, that will only express when integrated into the intron of an actively transcribed gene (29). Since these vectors disrupt the endogenous gene function researchers can both localize gene expression and study a loss of function mutant. However in rare cases these insertions lead to ubiquitous reporter expression and normal development, such as insertion into the *ROSA26* locus of mouse (30). These loci are known as “safe harbor” regions and can accommodate large insertions with little discernible effect on the animals (31). For example, many mouse lines with fluorescent reporters, including complex conditional reporters such as the brainbow system, use the *Rosa26* locus as the reporter integration site (32). As yet, there have been no safe harbor sites identified in sea urchins. New chromosome level assemblies in multiple sea urchin species may make this an achievable goal in the near future.

In contrast to random reporter integration, homologous recombination (HR) was developed as a method for making targeted mutations or insertions (33). This method requires long homology arms (~10 kb or larger in total), and these are often generated as restriction fragments, although even longer homology arms (~100 kb total) are sometimes generated using bacterial artificial chromosomes (BACs) (34,35). A similar approach, known as homology directed repair (HDR) is commonly applied now with short homology arms

(1000 bp or less). HR and HDR both work because double strand breaks trigger a homology based DNA repair pathway (36,37). However, HR is predicated on random double strand breaks triggering recombination, whereas HDR depends on targeted breaks from guided nucleases.

Despite the widespread use of BAC reporters in sea urchins there have not been any reports of transgenic mutants generated using this approach (38). In sea urchins HDR has been demonstrated using zinc finger nucleases, a protein-guided programmable nuclease (39,40). However, as is detailed in Chapter 3, the combination of the RNA-guided nuclease Cas9 and lab culture of *Lytechinus pictus* has made creating precise double stranded breaks feasible, which raises the possibility that HDR could be used for insertional transgenesis in sea urchins.

Transposon based DNA insertions. As discussed above, foreign DNA can form concatamers and randomly integrate into the host genome. While targeted insertion with HDR can be useful, certain applications, such as transcriptionally self-sufficient fluorescent reporters, don't necessarily need to integrate into specific loci to be functional. In fact, as noted above, there are no defined safe harbor sites in the sea urchin genome, so it is unclear where these types of insertions would even be targeted to in the genome using HDR. But random integration of foreign DNA does not persist past metamorphosis in great frequency (27). One approach to increase the frequency of random DNA integration is to employ a transposon integration system.

Transposons are selfish genetic elements that were first described by Barbara McClintock in maize, where transposon activity leads to chromosome breakage (41). There are many types of transposons that have important roles in evolution, there are at least several dozen eukaryotic transposon derived genes for example, but for genetic engineering the most relevant transposons are cut-and-paste DNA transposons (42). In these systems

the transposase excises DNA flanked by repetitive sequences known as inverted terminal repeats (ITRs) and then pastes that DNA into a new location (42). The integration site preference, composition of the ITRs, and the size of the insert that can be handled by the transposase are dependent on the particular transposon system employed. One of the most commonly used transposon systems for transgenesis is the Tol2 transposon, an autonomous transposable element isolated from Medaka, that does not have a defined integration site and can handle inserts of approximately 10 kb without a reduction in efficiency (43,44). Tol2 is widely used in zebrafish and has been used in human cells, mice, frogs and chicks (45,46). While use of Tol2 has never been reported in sea urchins, activity of other cut-and-paste transposons has been reported in sea urchin embryos (47).

Experiments included in this chapter. This chapter includes three sets of experiments. In the first, sea urchin embryos are used as a heterologous expression system for a coral transmembrane protein, the sodium/calcium exchanger. In the second set of experiments, I attempted to use CRISPR guided homology directed repair to insert foreign DNA into the genomes of *Strongylocentrotus purpuratus* and *Lytechinus pictus*. Finally, in the third set of experiments, I describe the use of Tol2 transposase as a method for integrating reporter constructs into *Lytechinus pictus*. The first experiment was published as apart of "A vesicular Na^{+}/Ca^{2+} exchanger in coral calcifying cells" in *PLoS ONE* and has been reformatted for this dissertation.

4.3 Materials and Methods

Sea urchin husbandry and gamete collection. Adult *Strongylocentrotus purpuratus* and *Lytechinus pictus* were collected in San Diego, California, and held in 16˚C or 18˚C, respectively, in flowing seawater aquaria. Animals were spawned by intra-coelomic injection of 0.55 M KCl. Gametes were prepared for injection as previously described (6,48).

Heterologous expression of *Ay*-NCX in sea urchin embryos. The plasmids encoding N and C terminal mCherry and mCerulean fusions of *Ay*-NCX were prepared by Dr. Megan Barron, who also synthesized the mRNA (19). *Sp*-ABCC9a and LCK (membrane anchoring domain of lymphocyte tyrosine kinase, LCK) fusion protein mRNAs were synthesized using the SP6 mMessage mMachine kit (Thermo Fisher) as previously described (7,49,50). For injections, 100 ng/μL *Ay*-NCX and 50 ng/μL LCK or 500 ng/μL *Sp*-ABCC9a were injected in *S. purpuratus*.

Cloning of homology directed repair (HDR) donors. In general, the homology arms for the various HDR donors attested to in this section were cloned out of purified genomic DNA. In both *S. purpuratus* and *L. pictus* these genomic DNAs were purified according to the genomic DNA extraction protocol described in Chapter 3. Several primer pairs were ordered for each gene, and fragments between 2 and 4 kb were amplified using a high fidelity polymerase, either Phusion (NEB) or PrimeSTAR (Takara), and cloned into the pMiniT-2.0 plasmid using the NEB PCR cloning kit. These fragments were then Sanger sequenced using a commercial service. These verified sequences were used to design CRISPR guide RNAs in ChopChop (51). Finally, desired homology arms were subcloned into PCS2+8 using In-Fusion cloning (Takara), in frame with a mCitrine or mCherry fluorescent protein. Single guide RNAs (sgRNAs) were generated using the EnGen sgRNA synthesis kit (NEB).

In a few cases different cloning approaches were used. The donors for *S. purpuratus* ABCB1a and β-catenin were ordered as synthesized as gBlocks DNA fragments (IDT). In the case of ABCB1a only the homology arms were ordered, while the entire donor (homology arms plus fluorescent protein) were ordered for β-catenin. The donors for *L. pictus* metallothionein were designed using the HITI approach, which relies on non homologous end joining to essentially blunt end ligate the donor into the target locus (52). Briefly, short

oligos containing the Cas9 target sites were subcloned 5' and 3' of the fluorescent protein donor, creating a donor cassette that is excisable from the plasmid with the same gRNA that targets the genome.

Injection of HDR donors. In all experiments Cas9 mRNA was injected at 750 ng/μL along with sgRNAs at 150 ng/μL, 120 mM KCl to promote DNA stability, and either 1.2 μg/mL tetramethylrhodamine (TRITC) or 25 ng/μL LCK (either mCherry or mCitrine) as an injection marker. Donors were prepared as circular plasmids (10-100 ng/μL), linear purified PCR products (50-200 ng/μL), or long single strand DNA (ssDNA) prepared using the Guide-IT ssDNA kit (Takara, 100-500 ng/μL).

Tol2 donors and injections. The plasmid containing the minimal Tol2 inverted terminal repeats (ITRs) and the plasmid containing the Tol2 transposase were gifts from Dr. Vanessa Barone. Early histone H3 (ehH3) and the metallothionein promoter were cloned from *L. pictus* gDNA using reported sequences from the literature and Genbank (53–55). The *S. purpuratus* Hatching Enzyme promoter was subcloned from the *Sp*HE-eGFP reporter (56). Similarly, the 2.5 kb *Sp*PKS1 promoter was subcloned from the plasmid described by Calestani and Rogers (57). The pleckstrin homology (PH) domain from Human ATK1 eGFP fusion was sublconed from Addgene plasmid #18836 (58). All other fluorescent protein markers were subcloned from plasmids previously reported from our lab (6,49,59).

Injection mixes contained Tol2 mRNA at 500 ng/μL, 120 mM KCl, and 10-100 ng/μL of plasmid DNA, and were injected in one-cell zygotes. In most experiments 25 ng/μL LCK mCherry was also injected as an injection marker, however this was omitted from experiments with donors that express membrane markers (i.e., LCK and PH domain donors).

For the Tol2 excision assay, embryos were injected as described above, and then genomic DNA from ~10 embryos was extracted. PCR was first performed using the standard

m13 forward and reverse primers, followed by a second nested PCR with primers to the ITRs. Samples were analyzed using gel electrophoresis with a 1% agarose gel.

Microscopy of sea urchin embryos. Embryos were screened for fluorescence on a Leica fluorescent dissection microscope (Wetzlar, Germany). Confocal images were acquired on a Zeiss LSM 700 (Jena, Germany) or Leica Sp8 confocal microscope and images were processed using ImageJ (60).

4.4 Results

Co-Expression of *Ay*-NCX and LCK or *Sp*-ABCC9a in sea urchin embryos. In initial experiments the fluorescence of *Ay*-NCX appeared to be localized in small vesicles near the plasma membrane (data not shown). In order to further investigate this two markers, the general membrane marker LCK and the vesicle specific *Sp*-ABCC9a (Fig4.1 A, C, D), were separately co-injected with *Ay*-NCX. Sea urchin embryos at the blastula stage form a spherical epithelium (Fig4.1 B). *Ay*-NCX does not colocalize with LCK, but is distributed near the apical plasma membrane in small vesicles (Fig4.1 C). In embryos co-expressing *Sp*-ABCC9a and *Ay*-NCX there are many times more ABCC9 positive vesicles than NCX positive vesicles, however all observed NCX vesicles were ABCC9 positive as well (Fig4.1 E).

Design and injection of CRISPR HDR donors. A total of 20 different HDR donors targeting eight genes (1-6 donors/gene) were generated (Table 4.1). Between 1 and 4 sgRNA target sites were selected from each gene, and each target had a single HDR donor designed for the target site (Table 4.1). In 15 of the donors a traditional HDR approach was used. The approach for *Sp*-ABCB1a is outlined in Figure 4.2. The *Sp*-ABCB1a locus covers ~38 kb of sequence in 27 exons (Fig4.2 A). A guide RNA target site beginning at base 9 of the first exon (ABCB1a1.9) had previously been identified by the lab (target sequence and

position relative to the start codon shown in the upper portion of Fig4.2 B). Two homology arms were ordered as synthesized double stranded DNA from IDT, and together with the DNA encoding an mCherry fluorescent protein and a flexible linker region compose the donor cassette (Fig4.2 B). The 5' homology arm is 728 bp long and covers part of exon 1, the 5' untranslated region, and ~600 bp of the upstream noncoding region. The 3' homology arm covers the remainder of exon 1 and most of intron 1. This donor took ~3 weeks to construct; a few days to finalize a design for the in-frame donor, ~10 days to order and receive the homology arms, and another 4-5 days to clone, maxiprep, and sequence the donor.

A total of 49 crosses with different target sites and donor preparations were made, including 18 with the ABCB1a donor. In all, fluorescence was only observed in the promoter/reporter HDR donors designed for *S. purpuratus* Nodal. However, injections of the plasmid donors without Cas9 or sgRNAs resulted in similar levels of expression (data not shown). In no experiments involving donors without promoters was fluorescence observed.

Design and injection of Tol2 donors. Tol2 donors were constructed by subcloning the promoter of interest and the reporter/fluorescent protein fusion in between the minimal Tol2 inverted terminal repeats (Fig4.3 A). The majority of the donors constructed for this project contain the *Sp*-Hatching Enzyme promoter (Table 4.2). The *Sp*-Hatching Enzyme donor variants were rapidly constructed in parallel using the same restriction sites. When injected at 25 ng/μL each donor strongly expressed in gastrula stage *L. pictus* embryos (Fig4.3 B).

Injection of Tol2 mRNA and the *Lp*-Metallothionein-mCherry-ehH3 donor at low (10 ng/μL) and high (25 ng/μL) levels of DNA both resulted in large, clonal mCherry-ehH3 expression, particularly in the ectoderm (Fig4.3 C). However, this expression disappeared by 4 days post fertilization (data not shown). A DNA excision assay was used on both the high

and low donor amount samples, and in both cases the major product band shifted from ~3000 bp to ~200 bp, indicating active excision of the donor cassette (Fig4.3 D).

4.5 Discussion

Heterologous expression of coral *Ay*-NCX in sea urchin embryos. One of the main arguments for model organism research is that problems can be addressed in systems that are best suited for measuring the answer. In this case, the calicoblastic epithelium, due to its structure, cannot be imaged in living organisms and is generally a difficult tissue to study by light microscopy. In contrast, blastula stage sea urchin embryos are well suited to assaying subcellular localization of membrane proteins, and fluorescent protein techniques have been in use for nearly a quarter of a century (7,61).

In the present study, it was known that the sodium/calcium exchanger was part of the calcifying machinery in the calicoblastic epithelium, but its exact role was unknown. Does NCX import calcium from the overlying layers, or does NCX export calcium into the subcalicoblastic medium? In order to answer this question we overexpressed *Ay*-NCX in purple sea urchin embryos, and initial experiments seemed to indicate that *Ay*-NCX localized in spherical vesicles (data not shown). *Sp*-ABCC9a is similarly expressed in vesicles, while LCK only localizes in the plasma membrane (Fig4.1B, D). Accordingly, we co-expressed LCK or *Sp*-ABCC9a with *Ay*-NCX, and found that *Ay*-NCX localizes to *Sp*-ABCC9a positive vesicles near the apical membrane (Fig4.1 C, E). These results informed further experiments in *Acropora*, particularly immunofluorescence and electron microscopy examining vesicles in the calicoblastic epithelium (19).

This study highlights some of the advantages of the sea urchin as a model organism. The main advantages for this study were the ease and accuracy of heterologous expression, and the background of sea urchin specific research. The first of these is useful, in that one

can generally expect exogenous proteins, either transients as in this case or permanent transgenes, to maintain their characteristics in sea urchin embryos. Vesicular *Ay*-NCX and membrane associated LCK both localize in a reproducible manner. The second advantage, a deep knowledge base, is evident in the fact that a plasma membrane marker and vesicle marker that were vetted in sea urchins were ready to "pull off the shelf" for these experiments. More generally, the wealth of sea urchin studies should translate into opening more avenues of research with new genetic tools.

Challenges in CRISPR mediated DNA insertions. The newest tool transforming biological research is CRISPR RNA guided gene editing. As outlined in Chapter 3, we have successfully used CRISPR to generate ABCB1 homozygous mutants in *Lytechinus pictus*. In this chapter I attempted to use CRISPR mediated DNA insertion using a homology directed repair (HDR) strategy. HDR is an accurate method for precise insertion, however the tradeoff is that HDR happens infrequently (62). Target choice, cell type, cell cycle stage, and format of the donor and Cas9 all influence the rate of HDR (63–65). There are no reliable methods, at least in a marine model, to predict the efficiency of HDR at a specific locus.

Given these limitations, I chose to use *S. purpuratus* for most of the donors in this study and chose multiple guide RNA targets for most genes. The benefit of *S. purpuratus* was the quality of the purple urchin genome, but the tradeoff to this choice was that we couldn't reasonably expect to grow multiple generations of transgenic animals. In other words, we could accurately design donors but we had to select targets that would be easy to observe in the larval stages. *CyIIIb, Slc25a4,* and *Mt1* are some of the most highly expressed genes in the larval stages (66), whereas *β-catenin* and *Cpa2L* have very specific localizations to the vegetal pole or gut, respectively (67,68). *ABCB1a* is well studied in our lab, and would have had the most benefit to the lab group. In addition, a gRNA target site in the first exon of ABCB1a had been shown to work with what seemed like high efficiency by

other members of the lab. However subsequent investigation revealed that ABCB1a1.9 was the least consistent of the tested gRNAs against *ABCB1a* (69).

Suspecting that gRNA inefficiency may have been a factor, I also designed donors to a gRNA target site in the *Nodal* gene that had previously been demonstrated to work efficiently (70). Disrupting *Nodal* results in a radially symmetric embryo due to failed left-right patterning (71). There should therefore be a double signal: a radialized embryo indicating gRNA cutting, and the fluorescent protein marker of the promoter-reporter fusion in the donor. In practice, the nodal donors fluoresced regardless of whether they were co-injected with Cas9 and gRNA.

Given the limited scope of these experiments it is impossible to say exactly why I never saw evidence of integration. However, three things are apparent for future students attempting to use this technique. Firstly, screening in the crispant generation doesn't appear to be a viable strategy. Using *L. pictus* and screening for fluorescent F1s would be the natural method to try. Secondly, gRNAs should be thoroughly vetted in vivo prior to use for HDR experiments. All of the gRNAs I used scored high according to the metrics of the design software (in this case ChopChop v2.0), however we have seen variability even amongst similar gNRA (69). More vetting in vivo for high efficiency guides should reduce the amount of cloning (i.e. only clone donors for the best target site) and increase the number of DNA cleavage events, which should in turn increase the rate of HDR. Finally, more screening should be done with PCR or other molecular based approaches. Integration of fluorescent proteins may not occur in very many cells, and most likely only needs to happen in one of the primordial germ cells in order for the integration to be inherited. Additionally, some genes may not have high enough expression to see fluorescence in heterozygous F1 animals. Molecular methods, such as PCR with primers inside and outside of the donor cassette, could more accurately screen animals for evidence of integration.

Tol2 Insertional mutagenesis. Based on the findings above, i.e. good expression of reporters and little to no evidence of integration, I pursued a transposon-based method for DNA integration in *L. pictus*. Transposons have higher rates of integration as compared to homology based methods, and can insert large sequences (up to ~10 kb for Tol2) without a significant drop in efficiency (72). However, the integration site is random. Therefore, I decided the most practical approach would be to use a constitutive, highly expressing promoter-reporter donor.

The *S. purpuratus* hatching enzyme promoter proved to be effective at producing high levels of expression with several fluorescent protein reporter combinations (Fig4.3-B). However, none of these constructs had visible fluorescence after 4 days post fertilization (data not shown). To determine if this was the result of using the *S. purpuratus* promoter in *L. pictus* I cloned out a previously reported *Lp*-Metallothionein promoter fragment with strong, constitutive expression during early development (53,54), and coupled it with a UTR-to-UTR *Lp*-ehH3-mCherry reporter (Fig4.3 C). This donor was highly expressed out to the early larvae stages, however visible fluorescence was gone by day 4. Interestingly, an excision assay, which measures the relative amount of Tol2 activity, indicated that the donor had been excised from the injected plasmid (Fig4.3 D). Both the Hatching Enzyme and Metallothionein promoters are small segments of larger cis-regulatory regions controlling gene regulation. One likely scenario is that the embryo specific regulatory elements in the promoters are no longer activated around day 4, possibly due to the accelerated growth of *L. pictus* embryos relative to *S. purpuratus* embryos. From each batch of injected embryos I saved as many injected larvae as possible after imaging, typically the majority of the injected embryos, and raised these as described in Chapter 3. Only five of these animals reached metamorphosis, and those died within a few days. These animals were spawned in late fall

(October-December) well after the peak breeding season, which may explain the failure of the cultures.

In general, Tol2 mediated insertions seem like a promising future direction. The donors, at least in these early stages, are easy to design and clone. There appears to be Tol2 activity in *L. pictus*. As with HDR insertions, the best path forward is likely injection followed by long term culturing and retrieval of heterozygous F1 animals. One interesting challenge for future investigators will be attempting to discover *L. pictus* safe harbor sites. Safe harbor sites are commonly used to create complex transgenic lines, i.e. conditional or stochastic mosaic lines such as the brainbow lineage tracing system (32). Indeed, whether techniques like Cre-Lox recombination, the basis of brainbow, will work in *L. pictus* remains to be seen. These data will hopefully support the establishment of fluorescent reporter sea urchin lines in the near future, and with them the expansion of *L. pictus* into a fully realized genetic model system.

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4.8 Chapter Four Figures

Figure 4.1 AyNCX localizes in intracellular vesicles in sea urchin embryos. Schematic of the fluorescent protein fusions used in these experiments (A). Protein colors match the fluorescence in micrographs B-D. The sea urchin embryo at ~20 hours post fertilization is a hollow, spherical, epithelial ball approximately 80 μm wide, and LCK is a cell plasma membrane marker (B). Two representative embryos expressing AyNCX and LCK (C). Upper row: an equatorial cross section showing AyNCX vesicles towards the apical surface of the cells. Lower row: Tangential section showing AyNCX vesicles predominantly at the apical vertices between cells. An example of an ABCC9 expressing embryo (surface projection) and a zoomed in cross-section with vesicles labeled with white arrows (D). ABCC9 localizes to vesicles, which colocalize with AyNCX (white arrowhead, E).

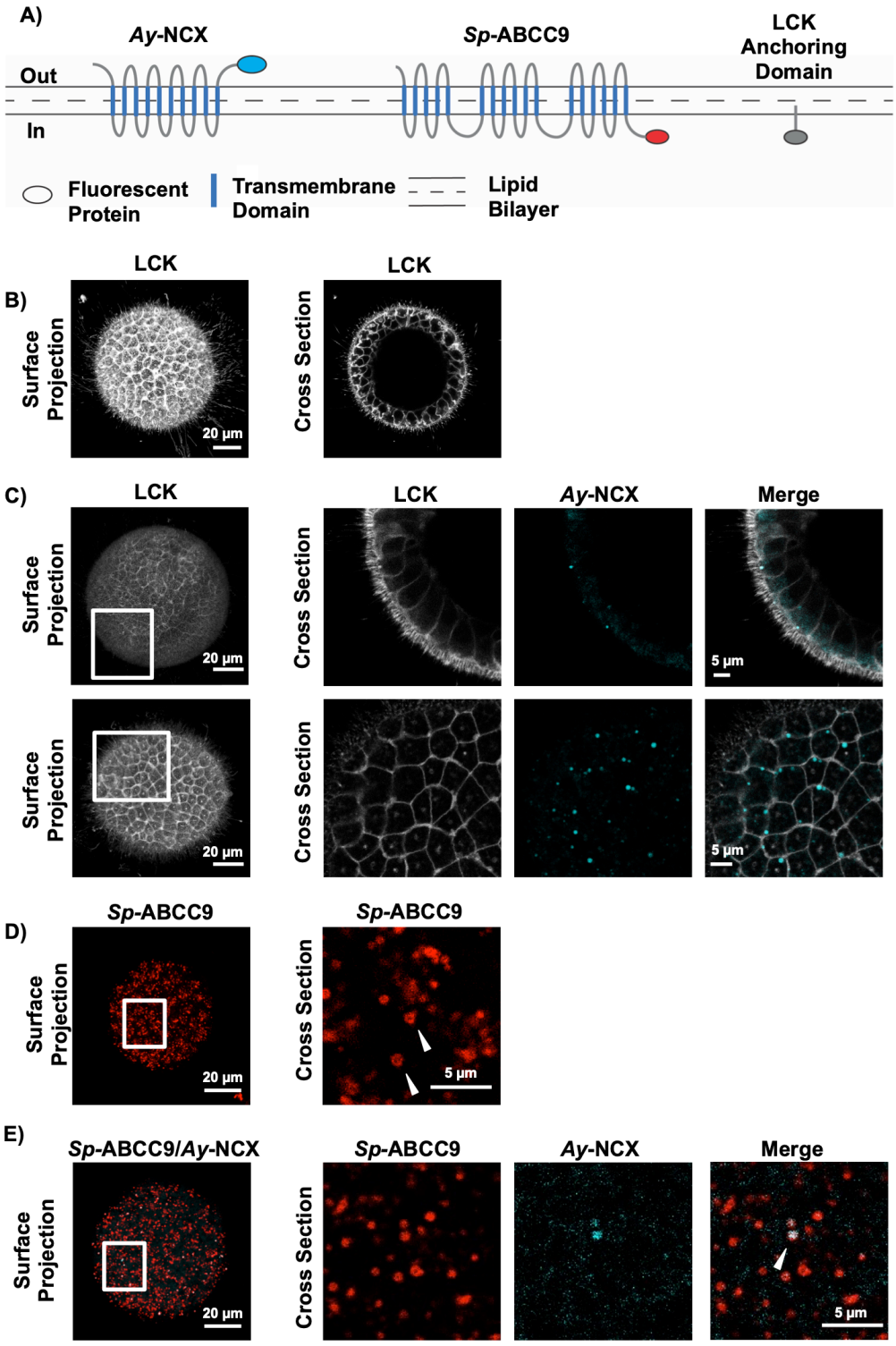
A)
Ay-NCX
Sp-ABCC9
LCK Anchoring Domain
Out
In
Fluorescent Protein
Transmembrane Domain
Lipid Bilayer
B)
LCK
LCK
Surface Projection
Cross Section
20 µm
C)
LCK
LCK
Ay-NCX
Merge
Surface Projection
Cross Section
20 µm
5 µm
D)
Sp-ABCC9
Sp-ABCC9
Surface Projection
Cross Section
20 µm
5 µm
E)
Sp-ABCC9/Ay-NCX
Sp-ABCC9
Ay-NCX
Merge
Surface Projection
Cross Section
20 µm
5 µm

Table 4.1 Description of homology directed repair donors for CRISPR mediated insertional mutagenesis. A total of 8 gene loci were targeted with 1-4 gRNAs and 1-6 donor plasmids. Six of the eight loci were in *S. purpuratus* and two in *L. pictus*. Genes were chosen based on expression level, expression pattern, and/or subcellular localization. 49 different batches of embryos were injected, the majority with plasmid DNA as the donor. HDR, homology directed repair; TF, transcription factor; NHEJ, non-homologous end joining.

Gene name	Common Name	sgRNA Targets	Species	Embryonic Localization	Subcellular Localization	Methods Used	N (Injected Crosses)	Donors Made
ABCB1a	ABCB1a	1	*S. Purp.*	Ectoderm and Gut	Apical Membranes	HDR with plasmid/ long ssDNA/oligo ssDNA	18	1
Ctnnb	β-Catenin	1	*S. Purp.*	Vegetal Pole Domain	Nuclei (TF) Junctions	HDR with plasmid and long ssDNA	10	1
Nodal	Nodal	1	*S. Purp.*	Right Lateral Ectoderm	Nuclear	HDR of Promoter/ Reporter with plasmid, ssDNA	7	5
CyIIIB	Ciliary Band Actin	2	*S. Purp.*	Ciliary Band	Actin Filaments	HDR with plasmid	2	2
Slc25a4	Adenine nucleotide translocator	2	*S. Purp.*	Ubiquitous	Mitochondria	HDR with plasmid	2	2
Cp2aL	Carboxy-peptidase A2	2	*S. Purp.*	Stomach (midgut)	Secreted	HDR with plasmid	2	2
Mt1	Metallothioein	4	*L. pictus*	Cytoplasmic	Golgi Apparatus	HDR with plasmid, NHEJ insertion,	4	6
EhH3	Early Stage Histone 3	1	*L. pictus*	All cells	Nuclei	HDR with plasmid	4	1

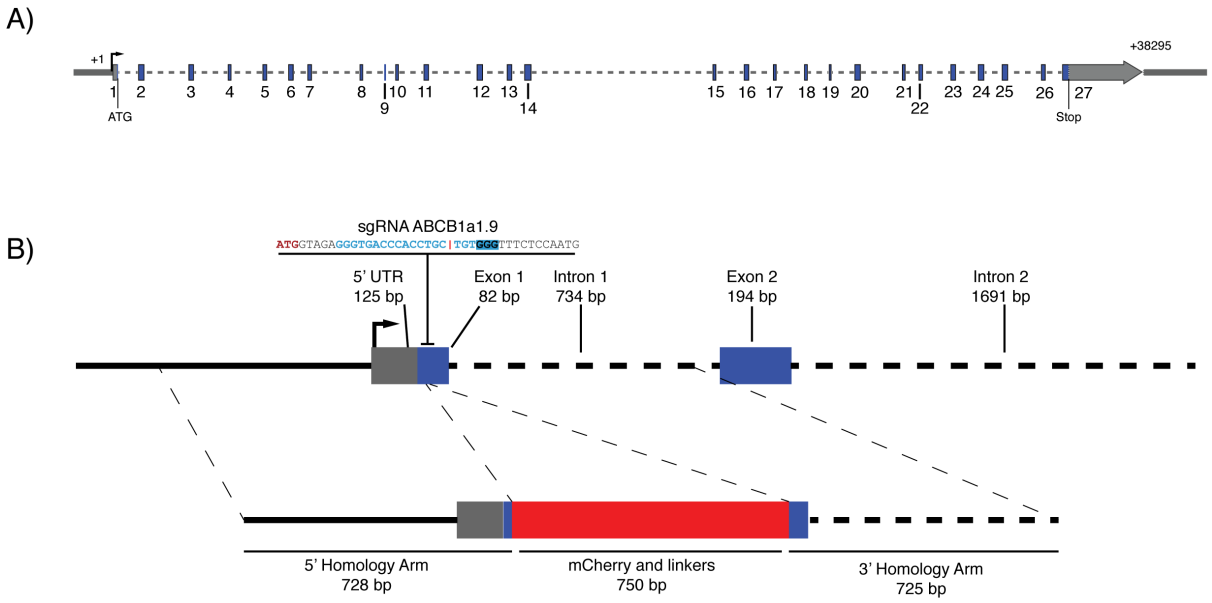

Figure 4.2 Homology directed repair donor design for the *S. purpuratus* ABCB1a locus. *Sp-ABCB1a* consists of 27 exons spanning 38 kb, with a short 5' UTR (A). N-terminal fluorescent protein fusions recapitulate *Sp-ABCB1a* subcellular localization and function. A guide RNA targeting the 5' end of the first exon (ABCB1a1.9) is the optimal predicted guide RNA for making an N-terminal fusion with HDR (B). The donor is constructed to cover ~725 bp on either side of the target site, delivering an in-frame mCherry coding sequence and flexible linker.

Table 4.2 Summary of Tol2 donors used in *L. pictus* embryos. Tol2 donors were all promoter-reporter constructs flanked with minimal Tol2 recognition sites, subcloned into the PCS2+8 plasmid backbone. Seven *S. purpuratus* Hatching Enzyme promoter constructs expressing different membrane or nuclear markers were cloned, along with one *L. pictus* Metallothionein donor, one *S. purpuratus* Polyketide Synthase 1 donor, and one gene trap donor. Donors ranged in size from 2-3kb. UTR, untranslated region; CDS, coding DNA sequence; pA, polyadenylation sequence.

Promoter	Species	Reporter Fusion	Expected signal	Insert size
Hatching Enzyme	*S. purp.*	mCherry::*L.p.*-ehH3 CDS::pA	Ubiquitous red nuclei	2754 bp
Hatching Enzyme	*S. purp.*	mCherry::5'UTR::*L.p.*-ehH3::pA	Ubiquitous red nuclei	2835 bp
Hatching Enzyme	*S. purp.*	LCK::mCherry::pA	Ubiquitous red membrane	2306 bp
Hatching Enzyme	*S. purp.*	LCK::mCitrine::pA	Ubiquitous green membrane	2315 bp
Hatching Enzyme	*S. purp.*	PLCδ-PH-Domain::mCitrine::pA	Ubiquitous red membrane	2754 bp
Hatching Enzyme	*S. purp.*	AKT-PH::eGFP::pA	Ubiquitous green membrane	2757 bp
Hatching Enzyme	*S. purp.*	2xNLS::mCerulean::pA	Ubiquitous blue nuclei	2412 bp
Metallothionein	*L. pictus*	5'UTR::mCherry::*L.p.*-ehH3::3'UTR	Ubiquitous red nuclei, Cadmium sensitive	2252 bp
Polyketide synthase 1	*S. purp.*	GFP::pA	Cytoplasmic GFP in pigment cells	3128 bp
n.a	*n.a.*	SA:IRES::mCherry:*L.p*-ehH3::pA	Gene trap, red nuclei	2078 bp

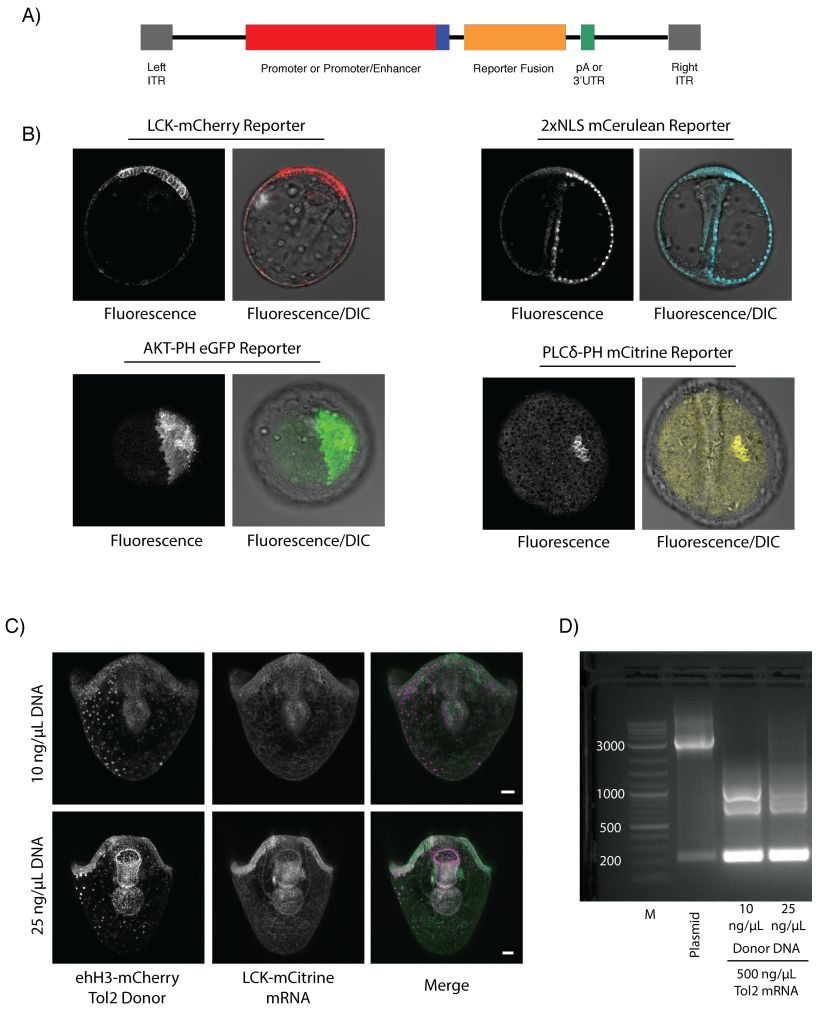

Figure 4.3 Expression of Tol2 donor constructs in *L. pictus* embryos. The Tol2 donors used in these experiments consist of a promoter from *S. purpuratus* or *L. pictus* directly 5' of a fluorescent protein reporter and 3' UTR or polyadenylation site, and this cassette is flanked on either end by minimal Tol2 inverted terminal repeats (A). The *S. purpuratus* Hatching Enzyme promoter drives ubiquitous expression of membrane and nuclear reporters during gastrulation (B). The *L. pictus* Metallothionein promoter drives similar expression of *Lp-ehH3*-mCherry reporter at low (10 ng/µL) or high (25 ng/µL) concentrations of donor plasmid (C). DNA recovered from injected embryos at both levels of donor injection shows excised plasmid (200 bp band) indicative of Tol2 activity, as opposed to the plasmid only control (D).

Chapter Five

Concluding Remarks

5.1 Concluding Remarks

As I have demonstrated above, the advent of easy and economical genome editing changed the course of my dissertation and the field of biology writ large. Biologists will be grappling with the implications of CRISPR for years to come, but this body of work demonstrates just how disruptive the advent of CRISPR has been for developmental biology in particular.

This thesis begins with an investigation of lipid phosphate phosphatases (LPPs) as possible regulators of primordial germ cell migration in *Strongylocentrotus purpuratus* (Chapter 2). I found that the main LPP expressed during the migration period is actually expressed in the skeletal cells, and chemical inhibition supports a role for this gene in skeleton formation.

At this point my research focus shifted to adapting CRISPR gene editing for use in sea urchins (Chapter 3). I primarily used this technique to create a line of *Lytechinus pictus* with mutant ABCB1. These animals developed normally and had normal fecundity, however homozygous ABCB1 knockouts were more sensitive to ABC transporter substrates.

Finally, I used CRISPR and the Tol2 transposon system to attempt insertional mutagenesis (Chapter 4). While neither approach produced adult transgenic animals, there is evidence that the Tol2 transposase was active in *L. pictus* embryos.

The experiments described in this dissertation have set up a number of research avenues for future trainees. How does loss of ABCB1 affect tolerance of environmental chemicals? Can safe-harbor sites be identified in *L. pictus* using Tol2, given the current resolution of the genome? More fundamentally, how will new methods of transgenesis change types of research that can be done in the sea urchin? Answering these questions will build on over a century of research sea urchin research and may keep sea urchins relevant for another hundred years.