Using CRISPR/Cas9 mediated genome editing to probe appendage morphogenesis in *Parhyale hawaiensis*

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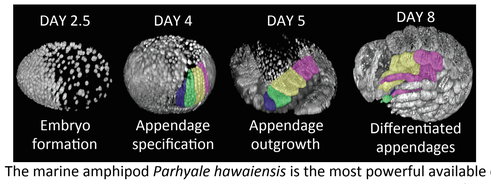
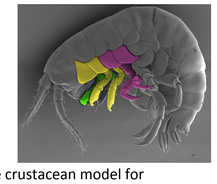
Abstract

The crustacean amphipod *Parhyale hawaiensis* has recently emerged as a powerful new model system to study the molecular and cellular basis of organ morphogenesis and diversification. So far, all imaging studies exploring appendage morphogenesis have been carried out with wild-type embryos. Here, we describe and characterize the use of the CRISPR/Cas9 based genome editing system in *Parhyale* for the first time. By knocking out the *Distal-less* leg patterning gene, we are able to probe how it governs appendage morphology and cell behavior in a developing embryo. Early stage *Parhyale* embryos were microinjected with either Cas9 protein or mRNA and one of several short guide RNAs (sgRNAs) targeting the *Distal-less* gene. After embryogenesis, we were able to observe mutant organisms with truncated appendage morphology at rates above 70%. Subsequent mutant genotyping experiments confirmed the high prevalence of *Distal-less* out-of-frame alleles and indicated high overall Cas9 mutagenesis rates. By conducting scanning electron microscopy and multi-view light sheet microscopy on the *Distal-less* mutants, we were also able to show that *Distal-less* is specifically required for development of the medial and distal appendage structures but not for the proximal appendage structures in *Parhyale*. Further acquisition of light-sheet microscopy data over the course of embryogenesis is underway to assess the impact of the *Distal-less* knockout on single cell behaviors. Our results here pave the way for G0 mutagenesis screens in *Parhyale* and for comparative studies of tissue and organ morphogenesis between wild-type and genetically perturbed organisms in the near future.

Background & Experimental Design

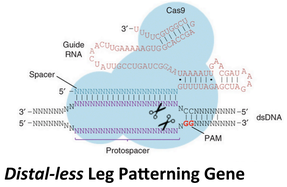
Appendage morphogenesis in WT *Parhyale* embryos





The marine amphipod *Parhyale hawaiensis* is the most powerful available crustacean model for developmental studies. *Parhyale* is a direct developer. Most aspects of the adult body plan are established during the 10 days of embryogenesis (at 25˚C) when fluorescently labeled embryos can be imaged at high spatial and temporal resolution with multi-view light-sheet microscopy. Like other crustaceans (e.g. lobsters), *Parhyale* are living swiss army knives exhibiting a remarkable morphological diversity along their anterior-posterior body axis. Each embryo develops a series of appendages that are specialized for different functions (sensation, feeding, grasping, locomotion) and that differ in size, shape and pattern. Therefore, the *Parhyale* body plan offers exceptional material to study the molecular and cellular basis of organ specification, growth and diversification by comparing neighboring appendages in a single embryo.

CRISPR/Cas9 Genome Engineering Tools



The CRISPR genome engineering system consists of a Cas9 protein and a short RNA transcript. The Cas9 nuclease is targeted using this RNA transcript termed the short guide RNA (sgRNA). Once bound to the target, the Cas9 nuclease makes a double stranded blunt cut. Non-homologous end joining (NHEJ) repair machinery repairs the break and often introduces insertions and deletions at the target site (introducing frameshift mutations for knockouts).

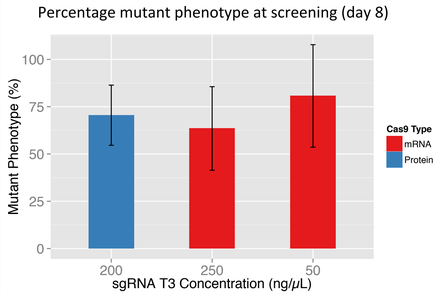
Distal-less Leg Patterning Gene

We employed the CRISPR/Cas system to knock out the leg patterning gene *Distal-less* (*Ph-Dll)* in *Parhyale. Ph-Dll* is expressed in all appendage primordia (shown in red) and is required for normal patterning of the proximal-distal axis.

We designed and in vitro transcribed 4 sgRNAs targeting different parts of the *Ph-Dll* coding sequence. Based on in vitro activity assays (data not shown), we selected 2 of these sgRNA, sgRNA1 and sgRNA3, for downstream analysis (cut sites shown below).

Results and Discussions

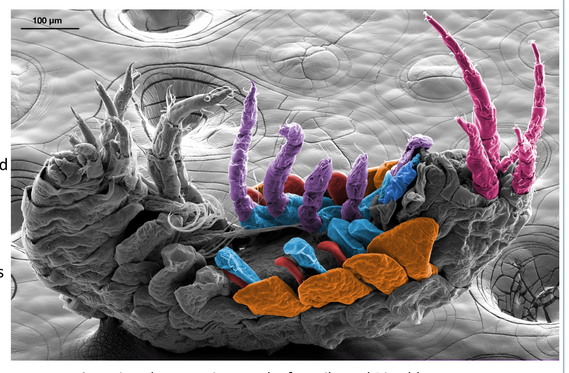
Injections & Mosaicism



The CRISPR/Cas9 system knocks out *Distal-less* with high-efficiency in *Parhyale*. Injection data showed that sgRNA 3 worked most efficiently (not shown). Different concentrations of sgRNA3 were co-injected with either Cas9 protein or Cas9 mRNA into 1-cell embryos. The graph on the left shows that injections performed well with an average mutant phenotype rate between 60 and 80% at the point of screening 7-8 days after injection (when appendage morphogenesis is almost complete). Further analysis shows that 30-50% of embryos both survive **and** are mutant on day 8 out of the total embryos that exist on day 4 (day before *Distal-less* is expressed). This shows that we can pick an embryo for live imaging on day 4 and still have a reasonable chance that it will both survive and be mutant on day 8. This demonstrates CRISPR's efficacy for mutant live imaging in *Parhyale.*

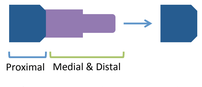
Distal-less Knock-out Mutant Morphology

Scanning electron microscopy of a unilaterally affected *Distal-less* mutant shows specific loss of the medial and distal appendage structures (purple) on the affected side. All the proximal appendage structures including the gill (red), cuticle plates (orange), and proximal segments (blue) appear unaffected. The antennae also show notable appendage truncation (pink).

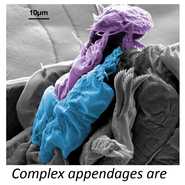


Scanning electron micrograph of a unilateral Distal-less mutant

Examples of color coded WT Appendage morphology



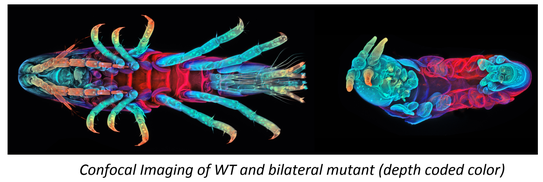
Ph-DII mutation truncation paradigm



Complex appendages are also affected

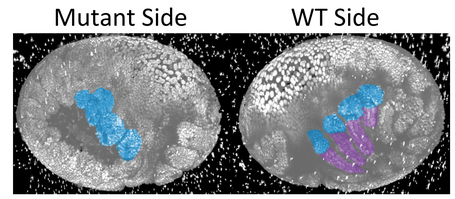
Bilateral Mutants

The vast majority of mutant embryos from single-cell injections surprisingly turn out to be unilateral mutants as shown in the SEM above. Rare bilateral mutants that survive to screening have notable gut abnormalities and had to be dissected out of the egg for imaging (below). We hypothesize that a large proportion of bilateral mutants may die early on during development due to tissue abnormalities that unilateral mutants are able to tolerate. 2-cell-injected embryos can also give rise to mutant appendages on a single side, but are not discussed here. Also, it is expected that a biallelic loss-of-function modification is required to evoke the *Ph-Dll* mutant phenotype.



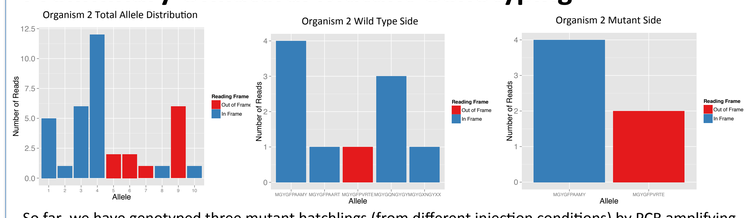
Confocal Imaging of WT and bilateral mutant (depth coded color)

Multi-view light-sheet microscopy of mutant embryos



SIMView multiview lightsheet microscopy allows us to live image the developing mutant embryos at single cell resolution in three dimensional space. This allows for comparative studies between cell behavior in mutant tissue and wildtype tissue. Quantitative parameters such as directionality and rate of cell divisions can be extracted from this data. This allows us to connect specific genetic perturbations to the roles they play in cell behavior during development. Above is a SIMview 3D reconstruction of a single time point of a mutant embryo. This transgenic fluorescent embryo was imaged over several days after it was determined to be a mutant. In the future, random injected embryos will be placed and imaged to capture single cell behaviors during early development.

Preliminary Unilateral Mutant Genotyping

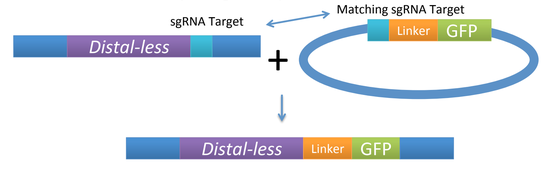


So far, we have genotyped three mutant hatchlings (from different injection conditions) by PCR amplifying from genomic DNA and sequencing (40 reads). These experiments show that Cas9 activity is highly specific for each tested sgRNA. The majority of sequenced alleles (70%-90%) were modified by Cas9. Multiple alleles were detected in each genotyped animal, indicating that Cas9 acts after the single cell stage. In addition, each unilateral mutant was microdissected into wild type and mutant sections that were each genotyped separately. The wild type side shows several modified alleles, but 90% are in-frame. The mutant side shows both out-of-frame and in-frame modifications. To confirm and explore some of these initial observations, however, more reads from more animals are necessary. A python script was written to fully automate sequence processing and analysis, which should speed up more in depth future analysis.

Future Directions

Distal-less GFP Knock-In

We are currently testing a CRISPR/Cas9 knock-in approach to generate a live Ph-Dll gene expression reporter. Our approach, shown below, involves the co-inection of Cas9+sgRNA and a tagging plasmid that is cut by Cas9 and incorporated into the genomic target site by NHEJ. With an endogenous reporter, levels of induction of GFP and other targets can be seen over time and space during live imaging and specific cell behaviors and responses can be tied to the sets of tagged. This allows us to link temporal gene expression dynamics to cell and tissue behaviors.



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Citations

1. Esvelt, K. K. M., Church, G. G. M. & Mali, P. Cas9 as a versatile tool for engineering biology. Nat. Methods 10, 957–963 (2013).
2. Liubicich, D. M. et al. Knockdown of Parhyale Ultrabithorax recapitulates evolutionary changes in crustacean appendage morphology. Proc. Natl. Acad. Sci. U. S. A. 106, 13892–6 (2009).