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Developing a Gene Editing System to Study Haplodiploidy in the Jewel Wasp, Nasonia Vitripennis

Emily A. Muller
Scripps College

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Keck Science Department

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ABSTRACT

Hymenopteran insects, which include all ants, bees and wasps, reproduce through a poorly understood form of reproduction known as haplodiploidy. A promising experimental system for understanding this developmental process is the jewel wasp, *Nasonia vitripennis*. A critical aspect of using *Nasonia* as a model is establishing an effective means for editing specific genes of interest so that their functions can be studied through genetic means. For my thesis research, I performed a pilot study of the gene editing method known as CRISPR in *Nasonia*. I targeted the single heterochromatin protein 1 (HP1) gene present in the *Nasonia* genome in order to assess the feasibility of this gene editing approach. Targeting HP1 would provide a clear phenotype when this gene is mutated due to its essential functions in early development known from studies in other eukaryotes. Additionally, creating a mutant of this gene will provide a means for studying the role of HP1 in wasp spermatogenesis, an aim that interlinks with the broader chromatin-based goals of our laboratory. Through this study I worked out a streamlined procedure for injecting CRISPR molecules into young wasp embryos, conducting genetic crosses with injected wasps, and screening through their progeny for potential mutants. I observed no mutant phenotypes in injected wasps, but instead, I isolated four potential mutants in F1 progeny. My work has helped to create a solid framework for improving this procedure in *Nasonia*, and they allow for a better overall understanding of the limitations of producing mutants through CRISPR gene editing in non-model organisms such as *Nasonia*.

INTRODUCTION

Much of what is known about fundamental cell and developmental processes and their relevance to disease stems from experimental work performed in model organisms. Some of the most commonly used eukaryotic model organisms include *Drosophila melanogaster* (fruit fly), *C. elegans* (roundworm), *Arabidopsis thaliana* (thale cress), *Danio rerio* (zebrafish), and *Mus musculus* (mouse). A traditional genetic approach to studying biological processes in these organisms is to generate random mutations in genes throughout the genome and subsequently screen through these mutants for phenotypes of interest. This process then leads to the identification of the underlying genes, making their further study possible. The generation of gene mutations has been conducted mostly through x-rays, UV radiation, nitrous acid, and hydroxylamine (Malling, 2004). These approaches involve numerous research hours and are uncertain for uncovering genes of interest in an efficient manner. However, in recent years, several new methods have been developed that allow for the precise generation of mutations in specific genes of interest. These methods include TALENS (Boch, 2011) (Tesson et al., 2011), zinc-finger nucleases (Klug, 2010) (Santiago et al., 2008), and now more recently CRISPR (Jansen, Embden, Gaastra, & Schouls, 2002). This thesis explores the use of CRISPR in directed gene editing (mutant generation in specific genes) in *Nasonia.*

Important Aspects of Hymenopteran Insect Biology

The insect order Hymenoptera, which includes all known ants, bees, and wasps, is the most prominent insect group in the world, comprising the larges biomass of any animal group (Beukeboom, Kamping, & van de Zande, 2007). Hymenopteran insects represent a wide range of interesting biological characteristics including eusociality and social caste systems, unique ecological niches, and host-parasite interactions (Whiting, 1967). Additionally, many hymenopteran insects such as honeybees are enormously important for agricultural purposes, such as natural crop pollination (Roubik, 2001).

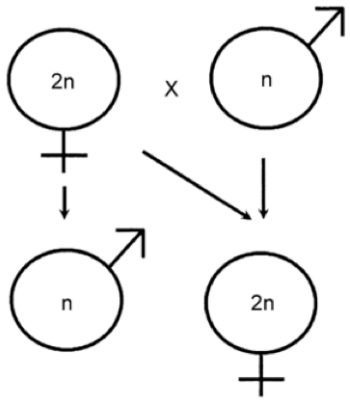


Fig 1. Haplodiploid sex determination. Females are diploid and produce haploid eggs. Males are haploid and produce haploid sperm. Unfertilized eggs develop into haploid males and fertilized eggs into diploid females (figure from Beukeboom, Kamping, & van de Zande, 2007).

Perhaps one of the most compelling and poorly understood developmental characteristics of Hymenopteran insects is their haplodiploid mode of reproduction. In this system, females develop as diploids from fertilized eggs while males arise as haploids from unfertilized eggs (figure 1) (Trivers & Hare, 1976). *Nasonia* males have 5 chromosomes while females have 10 chromosomes (Werren & Loehlin, 2009). In particular, male haploid development is remarkable because several cellular and developmental adaptations are required to allow haploid male development to occur. For example, male meiosis, required for producing functional sperm, must be unique since normal meiosis in diploids involves homologous

chromosome pairing; in haploid males, there are no homologous chromosome pairs, making it necessary that there be some alteration of meiosis to account for this difference (Paccagnini, De Marzo, Giusti, & Dallai, 2006) (Werren & Loehlin, 2009) (Beukeboom et al., 2007). Additionally, unfertilized eggs that are destined to form males must obtain their centrosomes from a source other than the sperm (fertilized eggs get their centrosome from the sperm, while unfertilized eggs do not) (Tram & Sullivan, 2000). Currently very little is known about the molecular and cellular aspects of these and other important developmental phenomena relating to haplodiploidy. An important aspect of investigating these processes is the existence of a tractable Hymenopteran insect for performing laboratory-based genetic experimentation.

Nasonia vitripennis as a Model Insect for Hymenopteran Biological Studies

Perhaps the best candidate for an amenable genetic Hymenopteran insect model is *Nasonia vitripennis*. In nature, this wasp is cosmopolitan in its distribution, being found in all continents in the northern hemisphere (Whiting, 1967). An important reason for this wide range is the fact that *Nasonia* are parasitoids of

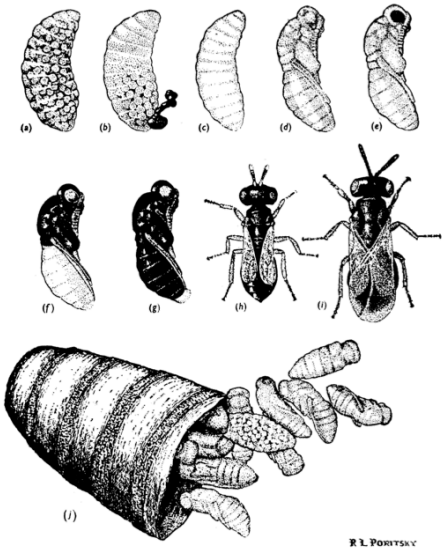


Fig 2. (a) Diapausing larva. (b) Defecating larva. (c) Early pre-pupa. (d) Pink pupa. (e) Red eyes. (f) Black head and thorax. (g) All black. (h) Adult male. (i) Adult female. (j) Sarcophaga puparium broken open to reveal enclosed diapausing larvae and pupae of Mormoniella. The size of the larva is 2.2 mm (figure from Whiting, 1967).

numerous blowfly species, which are present across the world (Whiting, 1967). *Nasonia* hold very strong promise as a rising model organism for a number of reasons. First, *Nasonia* are easily reared in the laboratory and their blowfly hosts are commercially available at an inexpensive price. Additionally, female wasps produce small (0.33mm long and 0.12mm in diameter) eggs that can be collected in large numbers and are incredibly clear (lipid-free), making them well-suited for high quality microscopic analyses (Whiting, 1967). The wasp lifecycle is relatively short, being approximately 14-16 days from egg to adult (similar to other model insects such as *Drosophila melanogaster*) (figure 2) (Whiting, 1967).

Currently, high quality genome sequences and web genome browsers exist for *N. vitripennis* and its sibling species, *N. giraulti* and *N. longicornis*, making possible comparative genomic studies and identification of individual genes for directed gene studies. The Ferree lab recently identified about 300 genes that are exclusively produced in the male testis of *Nasonia* (unpublished findings). These genes may ultimately help fill in the gaps of understanding how haploid male development occurs in the jewel wasp. However, what is currently lacking is an efficient way to perform directed gene mutagenesis in *Nasonia* for functional genetic studies. The CRISPR gene editing system holds strong promise for achieving this goal.

CRISPR as an Effective System for Gene Editing in Eukaryotic Organisms

Several gene-editing tools have been developed, including TALENs and zinc-finger nucleases (Klug, 2010) (Tesson et al., 2011). These tools have proven to be

effective across a wide range of different animals and even plants. However, they require a substantial amount of time and resource investment to generate specific reagents for making a given mutation. Additionally, they can be expensive. A more recently developed alternative for gene editing is CRISPR (clustered regularly interspaced short palindromic repeats) (Larson et al., 2013). This system promises to be more straightforward because of the greater ease with which mutagenesis reagents can be made, requiring only simple PCR and transcription kits. Below I first describe the natural (bacterial) origins and biological function of CRISPR. Subsequently I detail how CRISPR has already been engineered for gene editing purposes in model organisms. I then propose how this system might be adapted for use in *Nasonia*, and I describe some potential gene targets relating to chromosome/chromatin biology and how mutations in these genes will further promote *Nasonia* as a rising model organism. Finally, I outline the general steps I have taken to test for the first time CRISPR gene editing in *Nasonia*.

The Bacterial Origins of CRISPR

Natively, CRISPR is a type of immune response system present in bacteria and archaea (Ishino, Shinagawa, Makino, Amemura, & Nakata, 1987). Interestingly, CRISPR is the only adaptive immune and heritable system found in prokaryotes (Westra et al., 2012). The system functions by breaking down invading DNA such as from bacteriophages with enzymes. Interestingly, remnants of degraded invading DNA are saved and integrated into the bacterial genome for use as an immune

response in case a second invasion by the same phage occurs (Horvath & Barrangou, 2010).

The natural CRISPR response occurs in three steps: (*i*) the adaptive phase, (*ii*) the expression phase, and (*iii*) the interference phase (Jinek et al., 2012). The adaptive phase occurs when the host bacterium responds to a viral DNA invasion by integrating short fragments of the invaders DNA sequence into the host CRISPR array (figure 3). Each integrated foreign DNA is called a proto-spacer (Jinek et al., 2012), and the entire CRISPR array contains multiple integrated proto-spacers (Jansen et al., 2002). The expression phase occurs when the proto-spacers are transcribed into precursor CRISPR RNA (pre-crRNA) as a response to the same or similar virus. These transcripts are processed into mature crRNAs, also known as 'guide RNAs' (figure 3), which are complementary to invading viral DNA (Jinek et al., 2012). In the final phase of interference, the guide RNAs are matched to the foreign DNA and guide proteins called Cas proteins to these DNA-RNA hybrid duplexes. Cas9 is an

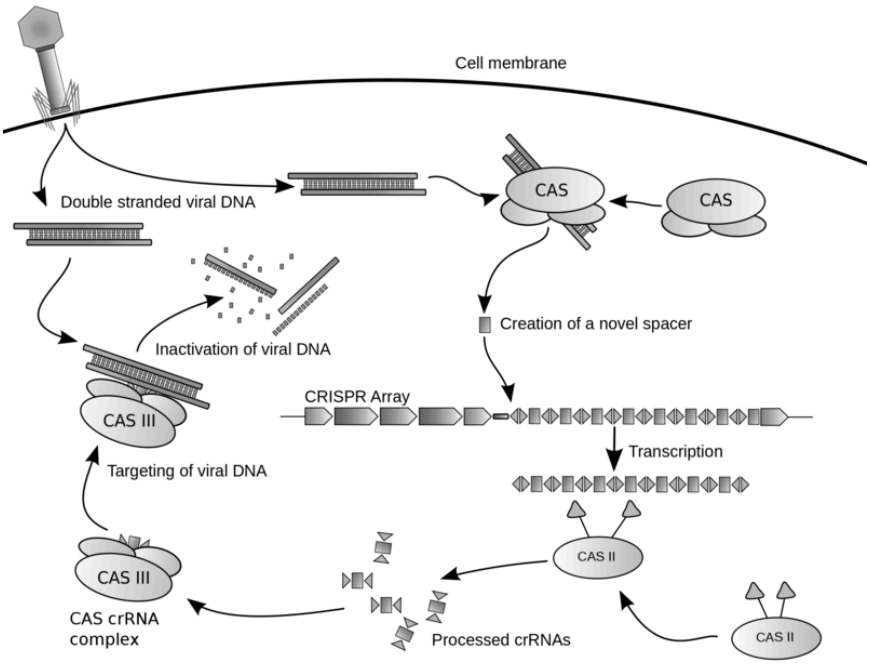


Fig 3. Model of the natural CRISPR/Cas9 system (figure from Horvath & Barrangou, 2010).

enzyme that induces double-strand breaks in the invading viral DNA, resulting in its degradation. An essential aspect of this process is that Cas9 properly forms a special protein/RNA complex that allows Cas9 to induce double-stranded breaks in the viral DNA (figure 3) (Jinek et al., 2012).

CRISPR CAS9 as a Method for Gene Editing in Eukaryotic Model Organisms

Over the past several years, the CRISPR system from the bacterium *Streptococcus pygenes* has been successfully engineered for use as a gene-editing tool in eukaryotic organisms (Westra et al., 2012) (Horvath & Barrangou, 2010) (Larson et al., 2013). There are three components for an effective gene editing event using CRISPR: (*i*) a unique gene sequence, (*ii*) a guide RNA (or several guide RNAs) complementary to a region uniquely present within the target gene sequence, and (*iii*) the Cas9 protein (Cho et al., 2014) (Sampson, Saroj, Llewellyn, Tzeng, & Weiss, 2013). One of the main requirements for functionality of this system in a non-native genome is that the specific targeted region within the gene to be mutated must end in an NGG nucleotide sequence known as the PAM sequence (figure 4) (Bolotin,

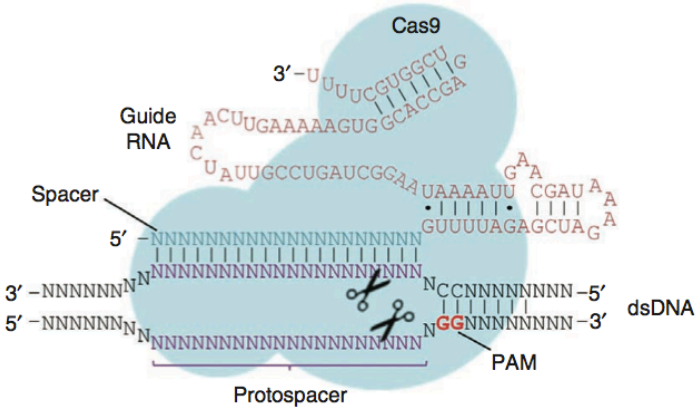


Fig 4. The basic *S. pyogenes* Cas9-gRNA nuclease complex for eukaryotic genome engineering. Target recognition and cleavage require protospacer sequence complementary to the spacer and presence of the appropriate NGG PAM sequence at the 3′ of the protospacer (figure from Mali, Esvelt, & Church, 2013).

Quinquis, Sorokin, & Ehrlich, 2005). Fortunately, this simple sequence is present widely within eukaryotic genes; thus, nearly any gene in principle can be effectively targeted. In one of the most basic approaches for performing CRISPR editing, *in vitro* transcribed guide RNA(s) can be co-injected into young embryos along with either mRNA containing the open reading frame for Cas9 or, alternatively, commercially available Cas9 protein (figure 5) (Jinek et al., 2012). The guide RNA(s) and Cas9 RNA (or pure Cas9 protein) are taken up by the precursors to the germ line stem cells, where they can together induce cleavage of the targeted DNA sequence at a site immediately upstream of the PAM sequence (figure 5) (Larson et al., 2013). This cleavage causes a double-stranded break at the cleavage site (figure 4) (Larson et al., 2013). Improper repair by non-homologous end joining (NHEJ), a process by which the cell will attempt to repair the cleaved ends of DNA by re-ligating the cleaved ends of DNA together without using a homologous DNA template as guidance (Lieber, Ma, Pannicke, & Schwarz, 2003). Improper repair following CRISPR-induced cleavage of target DNA results in the loss of one or more nucleotides at the 3' end of broken DNA, thus frequently causing a shift in the reading frame of the targeted gene (Mali, Esvelt, & Church, 2013) (Santiago et al., 2008). If, however, multiple guide RNAs are introduced simultaneously, then cleavage at each targeted site can lead to loss of entire DNA sequences between cleavage sites (Cho et al., 2014). One important consideration is that the presence of genome-wide sequences that are similar to the targeted site can lead to multiple, off-target mutations, which may cloud interpretations of intended mutational effects (Cho et al., 2014).

Therefore, care must be taken to best insure that the targeted sequence is unique to minimize off-target hits.

CRISPR-induced gene editing has been successfully conducted in a number of

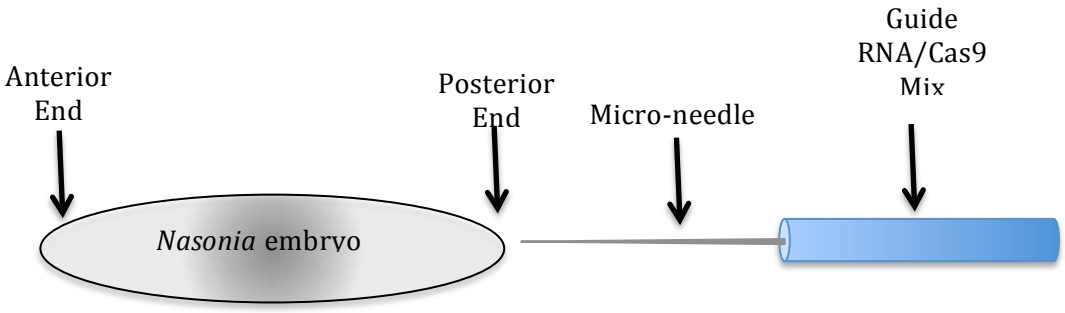


Fig 5. The guide RNA and Cas9 enzyme are injected with a micro-needle into the embryo at the posterior end under a microscope.

model organisms, although the efficiencies of mutagenesis vary from organism to organism. For example, CRISPR induces mutation rates of around 10% in the tissues of Zebra fish (Hwang et al., 2013). In fruit flies the yellow gene in the germ line cells was targeted with CRISPR and 59-68% of the offspring of injected flies were yellow mutants (Yu et al., 2013). Promising results are being reported for other model organisms including *A. thaliana* and *M. musculus* (Feng et al., 2013) (Wang et al., 2013). Taken together, the wide success of CRISPR in these organisms strongly argues that this method will be an effective way for precise gene editing in *Nasonia*.

Using CRISPR to Target Heterochromatin Genes in *Nasonia*

As much as 50% of the genome in higher eukaryotes consists of heterochromatin, a type of chromatin that is highly condensed, transcriptionally quiescent, and gene poor (Gokhman & Westendorff, 2000) (Brown et al., 1997)

(Wakimoto & Hearn, 1990). Heterochromatin resides primarily in large (mega-base pair) regions surrounding the centromeres and telomeres of the chromosomes (Fanti, Giovinazzo, Berloco, & Pimpinelli, 1998). These regions are typically known to be gene-poor and consist primarily of highly repetitive non-coding DNAs including satellite DNA repeats and transposable elements (Wakimoto & Hearn, 1990). Previous experiments have shown that deletion of heterochromatic regions results in severe genome instability, strongly arguing that heterochromatin is very essential for transmission of the hereditary material (Singh et al., 1991).

An important component of heterochromatin is its high level of compaction, a quality that primarily stems from function of the heterochromatin protein 1 family proteins (Lomberk, Wallrath, & Urrutia, 2006). Much of what is known about HP1-family proteins stems from studies performed in *D. melanogaster* (James & Elgin, 1986). This species contains three major HP1 family genes – HP1a, HP1b, and HP1c – although recent studies have revealed a number of other genes belonging to this family in *D. melanogaster* and other closely related fly species (Clark et al., 2007)(Adams et al., 2000). Mutational analyses in *D. melanogaster* have shown that loss of HP1a results in severe mitotic abnormalities during early embryogenesis, under-condensed heterochromatic regions, and death (Jones, Cowell, & Singh, 2000). In wild type *D. melanogaster* tissues including polytene salivary glands, ovary and testis, HP1a localizes broadly across the centromeric and telomeric sequences (Lomberk et al., 2006) (Fanti et al., 1998). The other two major HP1 family genes, HP1b and HP1c, have more restricted localization patterns, suggesting that the primary heterochromatin protein in *D. melanogaster* is HP1a (Adams et al.,

2000). All other examined eukaryotes have at least one counterpart to HP1a, further arguing its central role in heterochromatin function (Lomberk et al., 2006).

The Ferree research group recently performed bioinformatical analyses in order to identify all HP1 family genes in *N. vitripennis* and its sibling species. This work has confirmed that these *Nasonia* species have a single HP1 gene, arguing that this gene serves many/all functions that the multiple HP1-family genes perform in flies and other eukaryotes (unpublished findings). A strong prediction is that one of these functions is to properly compact all repetitive sequence regions around the centromeres and telomeres into heterochromatin (the main function of HP1a) (Eissenberg & Elgin, 2000). Also, this single HP1 gene in *Nasonia* likely performs important heterochromatin function in all tissues, including the testis (our lab's work has confirmed that this gene is expressed in the testis and ovary). Understanding the functional role and dynamics of HP1 in *Nasonia* will provide a platform for exploring chromatin dynamics relating to haploid male development in this organism.

Experimental Objectives

In this study I performed a pilot study to determine if the CRISPR system can be used as an effective/efficient means for making site-specific gene mutations in the *Nasonia* genome. I used the HP1 gene in *Nasonia* as a target for site-specific mutation due to our lab's interest in chromatin dynamics and haploid male development. This work stands as the first known attempt to explore CRISPR in Nasonia.

METHODS

Summary of the Methodological Approach

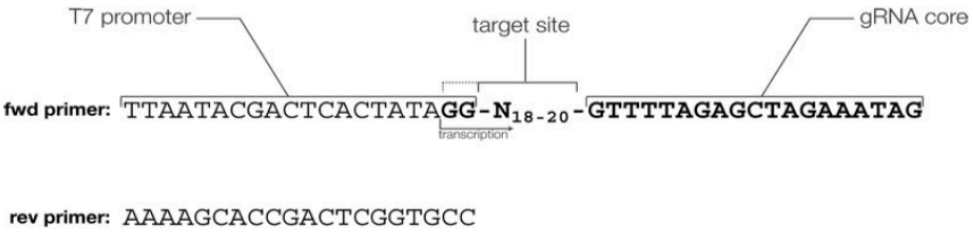
Here I describe in detail the methodological approach I used to test the CRISPR system in *Nasonia*. Specifically, I explain (*i*) making the guide RNAs, (*ii*) injecting guide RNAs and CAS9 protein into *Nasonia* embryos, and (*iii*) screening through progeny to identify possible mutants.

Preparation of the Guide RNAs

The means for generating guide RNAs is based on *in vitro* transcription from a PCR product. I first selected proper target sites near the beginning of the coding region of *Nasonia* HP1. Two target sites in the first exon were chosen based on their proximity to the beginning of the open reading frame for better insuring strong disruption of the gene and also because target sites must include 18-20 nucleotides preceding an NGG site (also known as PAM site). I then designed two different forward primers, each corresponding to one of these target sites. Each primer includes the T7 bacterial RNA polymerase promoter sequence followed by sequence complementary to the 18-20 bases of the target site, and finally a region of 19 bases that is complementary to a portion of the core (invariable) region of guide RNA sequence (figure 5). A single reverse primer was generated that amplifies the 3' end of the guide RNA invariable core. Each forward primer was used in PCR with the common reverse primer to amplify a PCR product from a plasmid containing the

sequence to the core of the guide RNA. Each of these PCR products provided a template for guide RNA production through *in vitro* transcription.

Fig 5. Schematic of the guide RNA design, the target side indicates the location of the target HP1 gene sequence, the gRNA core and the T7 promoter are the Guide RNA scaffolding.



PCR Conditions for Products as Templates for Guide Transcription

One µl of plasmid template was used for each PCR reaction. Thermocycler reaction parameters were as follows: 2 minutes at 92˚C for once cycle, then 92˚C, 57˚C, and 72˚C all for 30 seconds each for 35 cycles, then 72˚C for 5 minutes for 1 cycle, and then left at 10˚C until removal from PCR machine.

The PCR products were then run on a 1.0% agarose gel. The bands were excised from the gel and purified by using the QiaQuick Gel Extraction kit. I quantified the concentrations of the cleaned PCR products using a Nanodrop spectrophotometer. Each of the products following purification was 72ng/µl. The recommended target concentration for guide RNA based on CRISPR fly design (Laboratory of Molecular Biology and Division of Cell Biology of Cambridge) is 60 ng/µl or more.

In vitro Transcription and Purification

The Riboprobe® T7 *In vitro* Transcription System was used to generate guide RNAs from the purified PCR products. Eight µL of each PCR template was used in each transcription reaction (figure 5). Transcriptions were conducted exactly as recommended by the manufacturer's instructions. Once produced, the guide RNAs were column purified by using the Ambion® MEGAClear kit following directions as provided by the company. After purification we obtained about 50µl of 390.7ng/µl of injection grade Guide RNA as determined by the Nanodrop spectrophotometer.

Injection of Guide RNAs into wasp Embryos

Following transcription, we purified the guide RNAs using the Ambion® MEGAClear kit following directions as provided by the company. After purification we obtained about 50µl of 390.7ng/µl of injection grade Guide RNA as determined by the Nanodrop spectrophotometer. Proper size of the transcripts was determined by agarose gel electrophoresis.

Injection of the Guide RNA and Cas9 Enzyme

The careful preparation and timing of embryo collection and injections is very important because embryos must be injected before the formation of the pole cells, the future germ line stem cells of the animal. The idea is that guide RNAs and CAS9, once injected into young embryos, will be taken up by the pole cells, the future germ line stem cells, as they form from a population of nuclei at the posterior pole of the embryo. This uptake can result in editing of target genes in some portion

of germ line stem cells of the injected animal. These mutations can then be passed on to progeny produced from gametes of these animals.

Collecting Embryos for Injection

For embryo collection, 10-25 wild type females were mated to 20-30 males (all 2-4 days old). Animals were allowed to mate for 24 hours in the presence of 50% honey water. Subsequently the wasps were separated into vials containing 2-3 females and 4-6 males, and these vials were capped with a Styrofoam plug, each containing a blowfly pupa inserted into a small hole in the plug. The pupa was inserted into the plug so that the rounded closed end was exposed to the laying females. The purpose of this step was to direct female egg-laying in this particular region of the pupa, making it easy to locate embryos following egg laying.

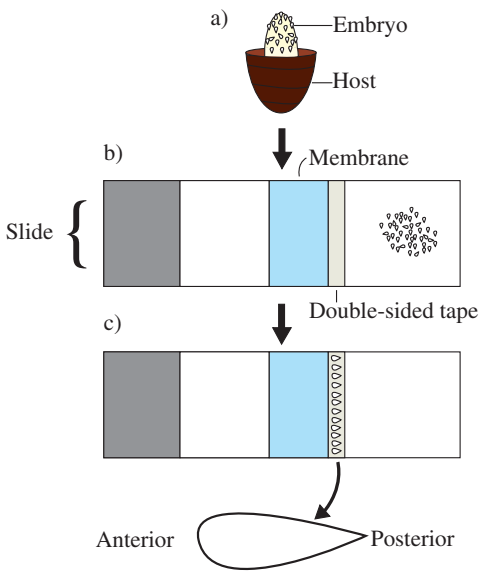


Fig 6. Overview of embryo collection and lining up process before injections

My initial embryo collections and injections were performed in the Desplan Lab at NYU. There, embryo collections from the hosts were conducted in an 18˚C room so that the embryos development was slightly slowed. However, subsequent collections and injections at Keck were done at room temperature; at this temperature, care must be taken to insure that the procedure occurs in an efficient

manner because the embryos develop slightly faster. In either case, female wasps were allowed to lay embryos within host pupae for 1 hour. Subsequently, using a dissecting microscope the shell of the hosts were removed carefully so as not to burst the soft yellow pupal skin. An egg pick was used to carefully remove the clutch of embryos from each host pupa. Wasp embryos were then placed onto a microscope slide containing a small layer of polycarbonate membrane (5μM pore size; figure 6). This membrane was fixed onto the slide with a very narrow strip of double-sided tape (figure 6). Approximately 200 embryos were placed onto a given slide (figure 6). At this point, the embryos were repositioned in an organized row along the tape so that their posterior (more pointed) ends were facing the membrane side (figure 6). This orientation allowed for injection into the posterior pole in a streamlined manner. Using this approach, several slides were prepared for one injection session; .the total time for each session is about 20-35 minutes.

Preparing the gRNA and Cas9 for Injections

Before injections, the gRNA and Cas9 enzyme were mixed and divided into aliquots for multiple different injections. The Cas9 enzyme was commercially purchased from PNA Bio Inc. This enzyme (50 ug) was hydrated with nanopure water in order to achieve a concentration of 5000ng/μl (10 uL total volume). Our guide RNA mixture for HP1 targeting was 390.7ng/μl. We added CAS9 and guide RNA proportions to achieve a recommended ration of ~1:2, respectively. Specifically, we combined 9μl of gRNA and 1μl of Cas9 for each of 5 aliquots. Each

aliquot was good for 1 injection session and was enough to inject 10-15 slides of embryos.

Injecting and Transferring Embryos

For injections, the slides with aligned embryos were slightly dried in a desiccation chamber for 15-20 minutes prior to injections. This step helps to prevent embryos from bursting during injection. While the embryos dried, a 5µL aliquot of guide gRNA/CAS9 mixture was thawed (if bringing from the freezer) and diluted with 5µl of nanopure water. After the embryo desiccation period, pre-pulled micro-needles were filled with the entire aliquot of the gRNA/CAS9 mixture (~9µl) using eppendorf microloader tips and the embryos were injected during a 10-20 minute period under a compound microscope and a micro-injector (can be done at 18^{o}C if possible). We used a picospritzer III machine with a release time between 10 -40 milliseconds, depending on the flow rate of the needle. Water was used for unclogging the needles, as well as breaking off the tips of needles if severely clogged.

After the injections were completed, the strip of tape with the injected embryos was very carefully removed from the slide and placed into a petri dish containing 1% agarose (in 1xPBS). The agar plate was placed in a humidity chamber at 25˚C overnight. Embryos were checked for hatch at 24 hours post injection. Hatched larvae were transferred into a pre-stung host with the part of the upper shell removed so that the host pupa was exposed (wasp larvae can feed only on pre-stung hosts that have been stung by a female). About 30-45 larvae were transferred into each pre-stung host. These hosts containing the newly hatched larvae were

then placed upright in a large, covered petri dish with dampened paper towels in order to maintain a moist environment for the larvae. The embryos were checked every 2 hours, during which any hatched larvae were transferred to pre-stung hosts. This operation was performed until no more embryo hatch occurred. The agarose plates with wasp larvae were held in a 25˚C incubator for 3 to 4 days until they reached the red eye pupae stage. The wasp pupae were then removed from the blowfly hosts and sorted by gender. Females were separated and labeled into individual vials while the males were discarded. I chose to focus on females instead of males because females can be scored for hatch of their male progeny (mutant males will immediately show developmental defects because their single HP1 gene would be affected) and then mated to wild type males for mutation propagation.

Screening F1 and F2 Progeny for Mutations

Expectations for Mutant Frequency as Measured by Hatch Rate

We hypothesized that the hatch rates of embryos from injected females would not necessarily show strong reduction in hatch rate. This logic is based on formation of the future germ line stem cells in *Nasonia.* By injecting the embryos at the posterior end very early in embryogenesis, we hoped to induce mutations in the pole cells, which are precursors to the germ line stem cells (figure 7). At the most conservative level, a mutation in the target gene could occur in only one of the ~16 pole cells that bud off from the blastula very early on in embryo development. Moreover, one of the two HP1 gene homologs could incur a mutation (figure 7).

Eventually these pole cells migrate to the mid-gut and the ovaries (or testis) form around them (figure 7). By this time in development the pole cells have become the germ line stem cells, which divide and produce the future gametes (eggs in the case of females). If a mutation occurred in one of the original pole cells, and in only one of two homologous HP1 copies, the cell would produce haploid eggs of which half would carry the mutation. However, these mutant eggs would only make up a fraction of total mutant eggs because most other eggs would come from non-mutant stem cells. Specifically, if only one HP1 copy of a single germ line stem cell harbored a CRISPR-induced mutation, then there would be a 1/32 chance that one embryo contained

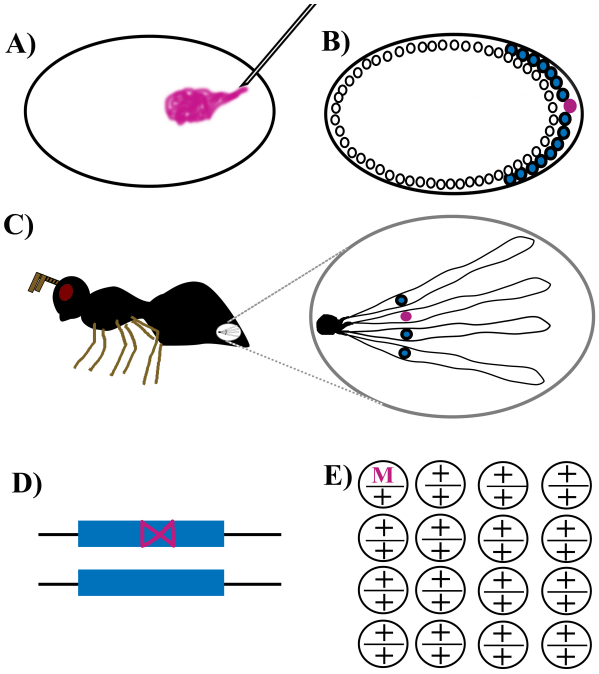


Figure 7. Model for HP1A mutation in F1 injected females with CRISPR. A) CRISPR/Cas9 is injected within the first 1.5 hours after embryo is laid, before pole cell formation. B) The HP1A mutation, shown in pink, occurs is one of the 16 pole cells that bud off from the blastula in early embryo development. C) Inside the mature female wasp the pole cells, now called germ line stem cells, have migrated into the posterior mid-gut and the ovary has formed around them. There is one ovary for each stem cell, one of which contains an HP1A mutation. D) The HP1A mutation would likely only occur in a copy of the gene on chromosome. E) Only one our of 16 germ line stem cells may carry the mutation, and therefore at most only 1/32 haploid eggs would carry the mutation if each germ line stem cell makes an equal amount of embryos

the HP1 mutation, assuming that each germ line stem cell is equally represented in the embryos laid by the female wasp (figure 7). Such a small percentage of mutant eggs/embryos laid would be difficult to see by monitoring hatch rates in F1 progeny of injected females. Based on this rationale (the most conservative case) we would expect to see minimal visible reduction in viability of F1 progeny from injected females. However, more efficient gene editing would result in a higher likelihood of seeing reduced viability in F1 progeny.

In contrast, we reasoned that F1 progeny of the injected females carrying a mutation in their HP1 gene would show much clearer reduction in viability of their F2 offspring. Approximately one out of every 32 F1 progeny from an injected female carrying an HP1 mutation in one of her germ line stem cells would inherit the mutation. An F1 mutant individual, which would be heterozygous for the mutation, should yield 50% mutant and 50% non-mutant progeny. I predict that because HP1 is known to show very strong hatch rate reduction in flies because this gene is so essential for chromosome segregation during all life stages, then any haploid progeny carrying a strong HP1 mutation will not hatch from embryo into larva. I therefore assayed for HP1 mutants by scoring embryonic hatch rates initially of injected females, and then of a subset of F1 females arising from these injected females.

Determining Injected Female Wasp Hatch Rates

Prior to measuring hatch rates of injected females, I carefully scored the hatch rates of 20 un-injected wasps to obtain a maximum expected hatch rate for

the wild type state. Hatch rates were measured only from virgin (unmated) females since all of their progeny would be haploid males; thus any mutation would be manifested directly in hatch of these individuals. If an individual wasp displayed a significant difference in hatch rate compared to the wild type hatch rate, then such an individual would be considered as a strong candidate for carrying a CRISPR-induced mutation.

All hatch rates were monitored and determined by placing individual virgin females (either injected or un-injected) with hosts for 3-6 hours as described above. After 3-6 hours the hosts were collected and the host pupal shells were removed at the region containing wasp embryos. The newly laid embryos were removed and lined up on slides, and subsequently counted. The slides were placed in a humid room temperature chamber for 36-48 hours, after which all embryos were scored for hatch. This process was repeated 3 to 4 times per individual wasp in order to accurately assess whether a given female harbored a possible mutation in HP1.

Hatch rates were examined for each female and compared with the average un-injected hatch rate for any significant differences using a chi-square test.

F1 Female Progeny Hatch Rates

Following measurement of embryonic hatch, all females were mated with wild type males in order to propagate any mutations. The F1 progeny were sorted by gender during the pupae stage and separated into individual vials. As before, the male F1 progeny were discarded. I then assessed the hatch rates of the F1 females with the same method stated above. The hatch rate of embryos produced from each

F1 female was compared to the hatch rate of embryos from un-injected wild type wasps by using a chi-square test.

H3K9 Tri-methyl Antibody Staining as a Proxy for the HP1 Gene

H3K9 Tri-methyl Antibody Stain Predictions

An important goal for assessing the developmental effects of an HP1 mutation is to understand the normal function of this gene/protein during wild type development. HP1a in most/all eukaryotes so far examined associates with methylated histone H3. This association is thought to play a large role in compacting chromatin that carries H3K9me into condensed heterochromatin. Thus, permanent heterochromatin in the cell exhibits an overlap of HP1a and H3K9me across heterochromatin. An important goal is to directly visualize the nuclear-wide distribution of the single HP1 gene in Nasonia. However, currently our group does not have an antibody that will allow us to achieve this goal. Instead, there are many commercially available antibodies that recognize H3K9me marks broadly across the eukaryotes because histones and their post-translational marks are highly conserved. In this project, I used an antibody against H3K9me3 (Active Motif, Inc.) to visualize this mark in *Nasonia* testis and ovary cells; this mark serves as a proxy for reflecting where HP1 should be across the genome (Ushijima et al., 2012).
I therefore stained wild type *Nasonia* testis and ovaries in order to begin understanding the wild type distributions of H3K9me and HP1 so that comparisons can be made in future HP1 mutants. Because H3K9me patterns have been

extensively worked out in *D. melanogaster*, I can use these patterns as a baseline for comparison to H3K9me patterns found in *Nasonia*.

H3K9 Tri-methyl Antibody Staining Process

Wild type testis (from red eye-yellow body-stage males) and ovaries were dissected in 1xPBS and fixed in 600µl heptane and 200µl of 4% para-formaldehyde for 10 minutes. The fixed tissues were then washed three times with 1xPBT. Tissues were then stored in the fridge until antibody staining could be conducted. Fixed tissues were always used within three days post-fixation in order to insure that tissues would be intact.

Fixed tissues were stained with a 1/300 dilution of rabbit anti-histone H3 trimethyl lysine (H3K9) at 4 degrees overnight, and subsequently they were stained with a 1/200 dilution of anti-rabbit secondary antibody conjugated with Cy3 (Molecular Probes) for 1 hour at room temperature. Three ten minute washes were performed after each antibody staining. After washings, the tissues were mounted in Vectashield with DAPI (Vector Labs, Inc) and imaged with a Zeiss DMIRB confocal miscroscope (Harvey Mudd College, Biology Department). All microscopy images were processed by using Adobe Photoshop.

RESULTS

Identifying possible HP1 mutants in CRISPR-injected wasps

A total of ~800 embryos were injected with CRISPR reagents (guideRNA targeting HP1 and CAS9). From these injected individuals, 27 females survived to adulthood. In order to screen for possible mutants, I scored embryo hatch rates for each injected female. The distributions of hatch rate for embryos from control (uninjected) and injected females were very similar (figure 8A). There were no obvious outliers showing dramatically reduced hatch. This pattern strongly argues that either the mutation efficiency is very low under the conditions used for this pilot study, or that any mutations do not cause strong loss-of-function effects (or both). This finding matches our most conservative expectation for screening embryo hatch from injected females (see Methods).

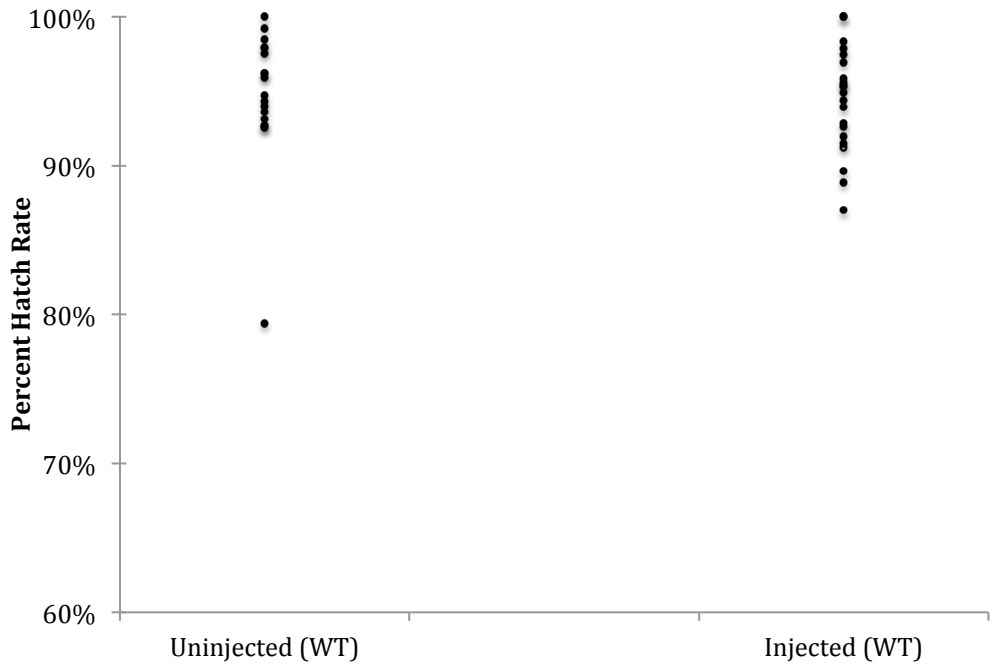
We next examined the hatch rate of embryos produced from a subset of F1 females (daughters of injected females) for further screening for HP1 mutants. To do this, all injected females were mated to wild type males, and a subset of their F1 daughters was allowed to lay embryos as virgins (all of their progeny would be haploid males, which would manifest the effects of any mutation). From this round of screening, I found four F1 females whose embryos showed a substantial decrease in embryo hatch rate when compared to hatch of embryos from control females (table 1) (figure 8B). Thus, these four females are strong candidates for carrying a CRISPR-induced mutation in HP1. This finding is in agreement with our expectation

that the HP1 mutation would be easier to identify in F1 progeny if CRISPR-induced gene editing is inefficient (see methods for further explanation).

Table 1. Significant differences in the hatch rates of four F2 female progeny when compared with un-injected expected hatch rate (Chi-Square 'Goodness of Fit').

F2 Progeny	P Value	Chi Square	Average Hatch Rate
14 E	<0.0001	0.08	67.4%
14G	<0.0001	0.06	70.6%
14N	<0.0001	0.06	70.6%
18L	<0.0001	0.05	73.1%

A)



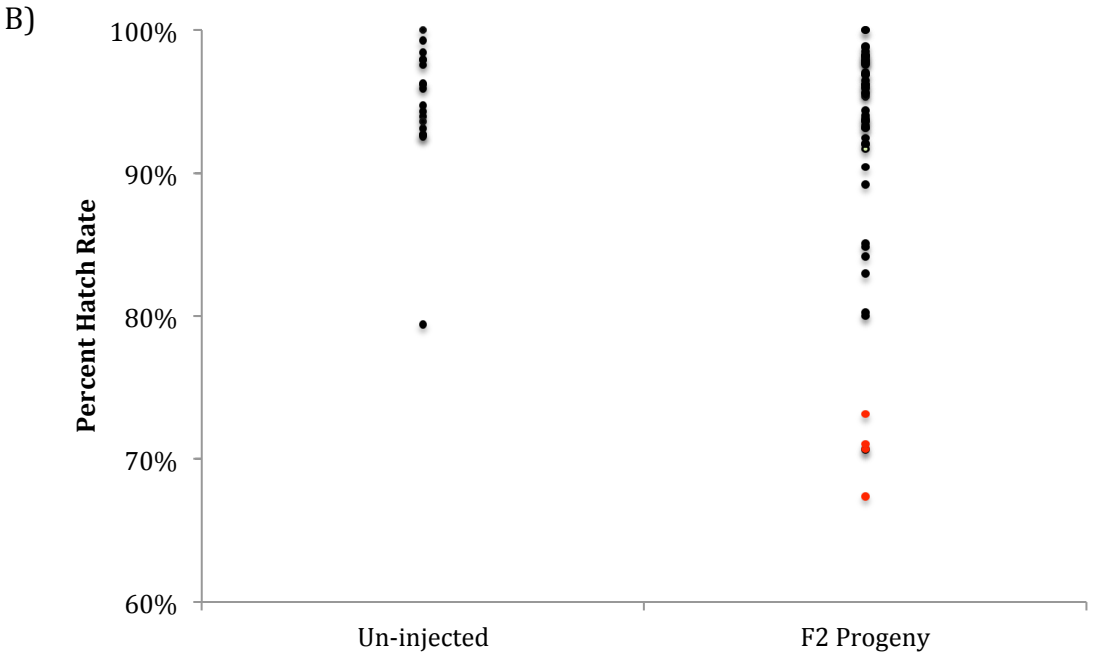


Fig 8. A) Average embryo hatch rates for wild type (WT) injected wasps and wild type un-injected wasps. B) Hatch rates for wild type female wasps and F1 progeny females from injected wasps. Red points indicate the four possible F1 HP1 mutants.

Characterizing Wild Type H3K9me Patterns in Testis and Ovary cells

In order to begin to understand the normal patterns of heterochromatin in *Nasonia*, I performed antibody staining on both wild type adult wasp ovaries and wild type male testes. This goal is important for discerning any effects of HP1 mutants on heterochromatin structure and function.

In the wasp ovary, there are three major types of cells: (*i*) the oocyte or future egg, (*ii*) the nurse cells, which are very large due to polyploidization, and are physically connected to the oocyte in a structure called the egg chamber, and (*iii*)

somatic follicle cells, which are slightly polyploidy and surround the oocyte within the egg chamber (figure 9). During oogenesis, the nurse cells 'feed' the oocyte with mRNA and proteins that are required for the first few ours of development during embryogenesis. However, during middle stages of oogenesis, the nurse cells degenerate, donating all of their cytoplasm to the oocyte, thus transforming the multinuclear egg chamber into a single cell (egg) with one nucleus (Bate & Arias, 2009)

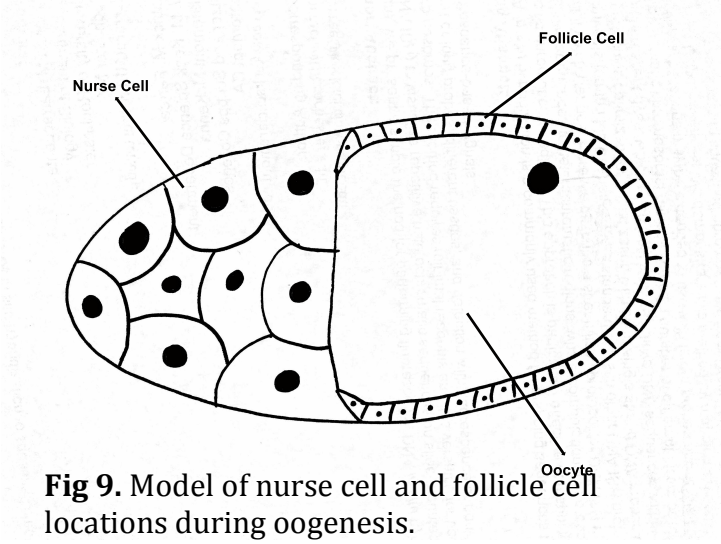


Fig 9. Model of nurse cell and follicle cell locations during oogenesis.

During the very early stages of oogenesis, there was very low H3K9me staining in any cells within the egg chamber (figure 10). However, during later stages, H3K9me became much more prominent within all cell types (figure 10A,C). In particular, H3K9 was observed in distinct regions that comprised approximately 50% of each nucleus. This amount matches the estimated proportion of heterochromatin known to comprise the genome of *Nasonia (Gokhman & Westendorff, 2000)*. Interestingly, H3K9me became especially bright in the nurse cell nuclei just before they become degraded in late stages of oogenesis (figure 9B).

Within the testis, there are two major cell types: (i) pre-sperm cells (spermatocytes), which are organized into multicellular cysts, and highly polyploid somatic cells (figure 11). Unlike in cysts in the ovary, in which only one of many

cells will become an oocyte, all germ cells in each cyst in the testis will become sperm. Similar to what was observed in the ovary, I found that approximately half of all chromatin within each nucleus, regardless of cell type, was positive for H3K9me (Figure 11A,C). The H3K9me staining appears to concentrate around the outside edge of each nucleus suggesting that this is where heterochromatin resides within these nuclei (Figure 11A, C). Additionally as the cysts mature and enter into late stages of spermatogenesis, the cells and their nuclei become highly elongated, and it is widely known that in most eukaryotes the histones are stripped away from DNA and it is repackaged with special proteins known as protamines. In agreement with this information, I found that H3K9me staining began to disappear in nuclei as they underwent elongation (Figure 11B). In general, there is more H3K9me staining in less mature spermatocytes than in later stages (Figure 11A).

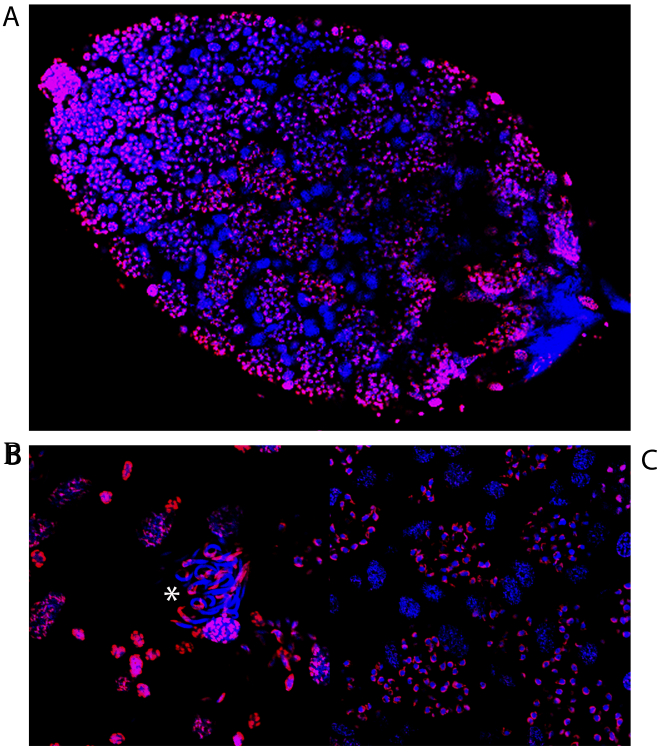


Fig. 10 Adult *Nasonia* testes stained with H3K9 methyl. Red indicates H3K9 methyl, blue shows Cytox G DNA stain. About half of the nuclei that make up the cysts of developing sperm show H3K9 methyl staining (A, C). Additionally H3K9 begins to disappear as spermatocytes undergo elongation (white asterisk) (B).

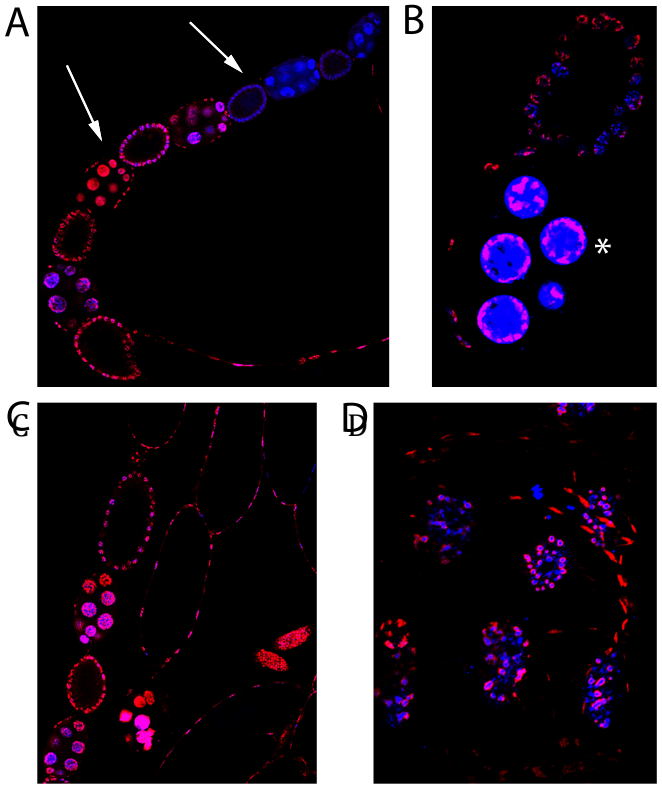


Fig. 11 Adult *Nasonia* ovaries stained with H3K9 methyl. Red indicates H3K9 methyl, blue shows Cytox G DNA stain. The presence of H3K9 is in a gradient pattern (white arrows) along the ovariole, with more H3K9 present in more mature oocytes (A). There is more H3K9 present in nurse cells (white star) when the oocyte is closer to maturation (B, C). Around half of the nuclei of follicle cells shows H3K9 methyl staining (D).

DISCUSSION

Assessment of CRISPR in *Nasonia*: Detection of Possible HP1 Mutants

In this pilot study I sought to test the effectiveness of the CRISPR/Cas9 system as a method of directed gene editing in *Nasonia vitripennis*. For this purpose, I chose to target the single full-length HP1 gene in this organism. I predicted that it could be challenging to detect any HP1 mutations carried in the germ line of injected females, especially if the gene editing efficiency is low under the conditions chosen for this pilot (see methods for expansion of this logic). However, mutations would be expected to show up more clearly by looking at hatch rates of haploid (unfertilized)

embryos produced from F1 females (daughters of injected females) because they would be heterozygous for a mutant allele of HP1, thereby giving rise to ~50% progeny that fail to hatch (those that inherit the mutant allele from the mother).

Through my work I identified four F1 females that exhibited substantially reduced hatch rates. However, none of these females produced embryos that failed to hatch at the expected 50% level. Instead, hatch rates produced by these females ranged from 67-73% (Table 1). This observation could be explained by the fact that any mutation(s) produced by CRISPR resulted in weak mutations that do not completely inhibit function of the HP1 gene. Such mutations may cause defects at the chromatin/chromosome level that end up making the carrying progeny sick, but not strong enough to kill them. Another possibility is that the mutations are strong loss-of-function mutations but that some other gene(s) compensate for the HP1 mutation. Currently no functional studies of HP1 have been performed in *Nasonia*. Interestingly, although there is only one full-length HP1 gene in this organism, there is one half-length HP1 gene (unpublished findings, Ferree lab). It is possible that the protein of this half-length HP1 gene could, working with other protein(s), function in a parallel manner to the full HP1 gene product.

A final possibility is that the CRISPR system did not cause any mutations in the HP1 gene, but instead, in other off-target genes, which may give rise to the reduced hatch rates that I have observed. The more unique the target DNA sequence used to make the guide RNA is, the less likely it is for an off-target mutation to occur (Cho et al., 2014). We may have caused a mutation in the partial HP1 gene or a completely different gene that has some homology to the targeted region of full

length HP1. However, this scenario is unlikely because I used genome-wide BLAST searching to choose targeted regions that had poor homology to any known gene sequences in *Nasonia*.

My current plans are to test progeny of the four potential mutant females obtained in this pilot study for carrying a mutation in the HP1 gene by performing PCR and sequencing of this gene. I will examine the upstream regulatory region of this gene, including promoter, as well as the sequence around the coding region that was targeted, for any lesions. In this way I will be able to confirm whether this gene was in fact edited. If not, then we will rethink about how to re-adjust our injection approach in order to improve efficiency of CRISPR in *Nasonia*.

H3K9me Patterns in the *Nasonia* Ovary and Testis

An important goal for assessing the cellular effects of any HP1 mutations generated by CRISPR in *Nasonia* is to first understand the normal distribution of heterochromatin in this organism. The most ideal marker for heterochromatin would be the full HP1 protein, especially since strong mutants in the HP1 gene would be expected to have severely reduced or absent HP1 protein. Moreover, HP1 is absolutely essential in a number of other organisms for formation and maintenance of heterochromatin (Lomberk et al., 2006). Therefore, loss of HP1 by strong mutation would likely severely disrupt heterochromatin. Having a clear idea of normal heterochromatin distribution in wild type *Nasonia* cells would serve as an important baseline for comparison to mutant phenotypes in this gene.

Unfortunately no antibodies exist that specifically recognize Nasonia HP1 (the Ferree lab has tested a number of antibodies to *Drosophila* HP1a, but these reagents do not work in *Nasonia* tissues, probably due to the divergence of wasp HP1 from fly HP1a). As an alternative, I used an antibody that recognizes tri-methylated histone H3, post-translational mark/protein that is highly conserved across animals, in order to visualize heterochromatin patterns in the major cell types of *Nasonia* ovary and testis tissues. In the wasp testis, I found that half of the chromatin within each spermatocyte nucleus stains positive for H3K9me3, strongly suggesting that this is the normal pattern of heterochromatin in these cells. Additionally, this pattern is very likely to be the same distribution of HP1, since there is a near-perfect overlap in HP1a and H3K9me3 patterns in flies and other higher eukaryotes. This 50% value matches the approximate estimate of total heterochromatin as measured by DAPI bright regions (unpublished results). This finding argues that the H3K9me pattern may serve as a proxy for analyzing visually any harmful effects to chromatin caused by HP1 mutants once they are confirmed (and until our lab can make an antibody against Nasonia HP1). Additionally, we found that during spermatocyte elongation during late spermatogenesis, the H3K9me signal decreased within the elongating nuclei and completely disappeared in fully elongated sperm nuclei. It will be interesting to determine if H3K9me, HP1, and heterochromatin structure in general are important for normal sperm formation in the testis, something that will be able to be addressed with HP1 mutants that stem from our CRISPR experiments.

Interestingly, we found a dynamic pattern of heterochromatin, as measured by H3K9me distribution, in the wasp ovary. During early oogenesis, there was little H3K9me staining in the oocyte nucleus and also in nurse cell nuclei. However, during middle stages the nurse cell nuclei became especially enriched in H3K9me. This enrichment occurs in the time leading up to when the nurse cells break down. The nurse cells would have denser and denser staining of H3K9 until right before they were broken down and the oocyte was almost completely mature. Further examination of how HP1 mutants undergo oogenesis is necessary to fully characterize the role of HP1. However, the prevalence of H3K9 staining during oogenesis indicates that embryo formation would likely be very abnormal in mutant HP1 lines. The nurse cells provide much of the nutrients and developmental factors necessary for the survival of embryos, and because a dense amount of H3K9 staining is found in the nurse cells, HP1 mutants would likely form inadequate nurse cells and therefore lead to embryos that are unable to fully develop when laid.

Future Steps

Although it is likely the four significantly different hatch rates in the F2 progeny are due to a mutation caused by the CRISPR/Cas9 system, this needs to be confirmed by examining their genomic DNA and mapping for mutations using PCR. This would allow for complete certainty of the possible mutation.

If, after examination of the genomic DNA, the CRISPR/Cas9 system has successfully caused a mutation in the HP1A gene, we would likely to characterize the mutation by staining the ovaries and embryos carrying the mutation with the

H3K9 and HP1 antibody stain and compare the visible differences with the antibody staining we performed on non-mutated wasps.

Another step would be to target another more phenotypically recognizable gene with the CRISPR/Cas9 system. The Cinnabar gene is the specific gene we would like to target. A mutation in the cinnabar gene is well known to cause a change in eye color to become reddish. A cinnabar mutant lacks Kynurenine hydroxylase activity, causing the reddish hue (Ghosh & Forrest, 1967). Because a mutation in the cinnabar gene causes a phenotypic change, it is often used as a marker for mutational success. In order to easily determine the efficiency of mutation caused by CRISPR Cas9, we would like to target a commonly recognizable phenotype. If CRISPR Cas9 successfully mutates the Cinnabar gene in *Nasonia*, we would expect the progeny to have red eyes.

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