PHUYAL, GAURAV M.S. Development of a CRISPR-dCas9 tool to introduce DNA Methylation into a genomic locus *in vivo*. (2023)
Directed by Dr. Ramji K. Bhandari. 55 pp.

The Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated protein 9 (Cas9) system, also called CRISPR-Cas9, has been manipulated for application in a wide variety of biomedical research. The simplicity of the CRISPR system has made it easy for its successful application in introducing both genetic and epigenetic modifications *in vitro* and *in vivo*. While essential for development and differentiation in organisms, epigenetic processes (DNA Methylation, Histone modification, and non-coding RNAs) have been found to be influenced by endogenous, biotic, and abiotic factors. Epigenetic alterations, particularly DNA methylation changes that occur due to early-life environmental exposures have been identified as one of the potential mechanisms underlying the developmental origins of health and disease (DOHaD). The Cas9 protein can be made devoid of its endonuclease activity resulting in a nuclease dead Cas9 (dCas9) molecule that cannot cleave target DNA. Transcriptional effectors are proteins that can activate or repress gene transcription. Although a few experiments have been performed using transcriptional effectors in the Crispr-dcas9 system *in vitro*, an effective and reproducible technique to epigenetically activate/suppress genes using activator and repressor elements *in vivo* has not been developed.

Given that embryos undergo epigenetic reprogramming of parental epigenetic marks during the cleavage stages, it is difficult to determine if epigenome targeting at the one-cell stage can escape genome-wide reprogramming of the embryo occurring at cleavage stages. It is also unknown if *de novo*-established epigenetic alterations are programmed differently from those inherited from parental gametes. To date, there have been no studies demonstrating the fate of epigenetic edits during epigenetic reprogramming *in vivo*. This study examines if the epigenome editing

performed at zygotic stages will alter expression of a heritable epigenetic trait that can withstand the reprogramming window during the subsequent cleavage stages. To this end, we are using a fish model medaka (*Oryzias latipes*) which has been established as an excellent biomedical model given its similarity in germ cell development to mouse and humans and applying CRISPR-dCcas9-DNMT3aa repressor at the first few cell stages. This study will help delineate the applicability of the CRISPR system in introducing epigenetic modifications during the epigenetic reprogramming windows.

DEVELOPMENT OF A TOOL TO INTRODUCE DNA METHYLATION

INTO A GENOMIC LOCUS *IN VIVO*

by

Gaurav Phuyal

A Thesis
Submitted to
the Faculty of The Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Greensboro

2023
Approved by

Dr. Ramji K. Bhandari
Committee Chair

DEDICATION

I want to dedicate my work to my parents, Govinda Prasad Phuyal and Bina Bhattarai Phuyal, my ancestors, Aama Pathivara Devi and my brother, Bidhan Phuyal. I would also like to dedicate it to all teachers and gurus who have guided me to get to this moment.

.

APPROVAL PAGE

This thesis written by Gaurav Phuyal has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

Committee Chair ______________________ Dr. Ramji K. Bhandari

Committee Member ______________________ Dr. John Tomkiel Dean

Committee Member ______________________ Dr. Zhenquan Jia

April 21, 2023
Date of Acceptance by Committee

April 21, 2023
Date of Final Oral Examination

ACKNOWLEDGEMENTS

I would like to express my gratitude to my primary supervisor, Dr. Ramji Bhandari, who guided me throughout this project. I would also like to show my heartfelt appreciation to Dr. Xuegeng Wang, Pooja Bhandari, Sourav Chakraborty, and Dr. Santosh Anand who guided and offered me support throughout my time here at UNCG. I wish to acknowledge the help provided by the undergraduate research students Morgan Rector, Ally Hannon and Shateanu' Bryant.

TABLE OF CONTENTS

LIST OF TABLES vii

LIST OF FIGURES viii

CHAPTER I: INTRODUCTION 1

1.1 Epigenetic Modifications 2

1.2 Epigenetic Alterations and Diseases 3

1.3 CRISPR-dCas9 5

1.4 Epigenetic Reprogramming of Cells 6

1.5 The Medaka Fish Model 9

1.6 The Present Study 9

1.7 Aims and Objectives 11

1.8 Experimental Design 13

CHAPTER II: MATERIALS AND METHODS 15

2.1 Preparation of CRISPR-dCas9-*dnmt3aa* plasmid 15

2.2 Preparation of single guide RNAs (sgRNAs) 17

2.3 Animal Care and Colony Selection 18

2.4 Mating and Offspring Production 19

2.5 Microinjection 20

2.6 DNA isolation 22

2.7 Bisulfite Conversion of the genomic DNA and bsPCR 22

2.8 Targeted Amplicon Library Preparation for Methylome Analysis 23

2.9 Gel Electrophoresis 24

2.10 Sanger Sequencing 24

2.11 Statistical Analysis 24

2.12 Bioinformatics Data Analysis 24

CHAPTER III: RESULTS 26

3.1 Injection of embryos with shorter DNMT3aa Conserved Domain 26

3.2 Sanger Sequencing Results of Experimental and Control Samples 27

3.3 GFP Quantification in 5-day old embryos 28

3.4 Targeted Amplicon Sequencing-MiSeq Results 30
3.4.1 sgRNA4 vs Control 32
3.4.2 sgRNA5 vs Control 33
3.4.3 sgRNA6 vs Control 34
CHAPTER IV: DISCUSSION 36
REFERENCES 45

LIST OF TABLES

Table 1. CRISPR-dCas9-dnmt3aa plasmid verification Sanger sequencing primes. 17

Table 2. Single-Guide RNA sequences. 18

Table 3. crRNA selection criteria[110]. 18

Table 4. Bisulfite DNA amplification primers. 23

LIST OF FIGURES

Figure 1. The mechanism of DNA methylation[7]. 3

Figure 2. DNA methylation reprogramming in an embryo and primordial germ cells[75]. The left panel shows the dynamics of DNA methylation (5mC) in a post-fertilizations stage embryo undergoing cleavage stages. The right panel shows the dynamics of DNA methylation (5mC) in primordial germ cells (PGCs) which are the precursors of eggs and sperm. Similar DNA methylation profiles are maintained in mice and medaka, while a different profile is maintained in zebrafish. 8

Figure 3. (a) Structure of olvas-GFP vector[79] and GFP expression in adult medaka testis and ovary. (b) CRISPR-dCas9-dnmt3aa injection and examination site through the somatic cell reprogramming window. (c) *olvas* promoter with guide RNA target sites for the proposed study. 11

Figure 4. Overall experimental Design 14

Figure 5. pCS2+hSpCas9 CRISPR backbone. 15

Figure 6. (a) Domain structure of the dCas9-DNMT3aa catalytic domain fusion protein; (b) Illustration of the locations of the three sgRNAs designed to target olvas coding sequence. 16

Figure 7. (a) Female medaka(*Oryzias latipes*) with eggs; (b) Early developmental stages in *Oryzias latipes*[100]. 19

Figure 8. (a) Narishige IM-21 microinjector setup; (b) Gel Cast for holding eggs; (c) Microinjection needles; (d) Microinjection with Phenol Red into Yolk. 20

Figure 9. (a) Microscope used for microinjection and GFP quantification; (b) UV light-source 21

Figure 10. GFP Quantification of CRISPR-dcas9-dnmt3aa-gRNA injected samples vs only CRISPR-dcas9-dnmt3aa injected samples. (a) The GFP positive rate of guide-dcas9 vs mock 5dfp. The GFP rate in promoter-targeted embryos was significantly reduced. *: significance. Mean ± SEM. ∇ $p<0.05$. (b) Visual representation of GFP expression in mock vs target samples. The white arrow marks the presumed location of PGCs. 26

Figure 11. qPCR results of MeDIP-Seq DNA of mock vs guide-RNA targeted embryos. The 5mC level in the targeted embryos was ~4 times higher than the mock. 27

Figure 12. Methylation pattern on the targeted site on *olvas* gene at different developmental stages. 28

Figure 13. GFP Quantification of sgRNA injected samples vs control 5 days post-fertilization. (a) The GFP positive rate microinjected embryos vs control 5-dfp. The GFP rate in promoter-targeted embryos was significantly reduced. *: significance. Mean ± SEM. ∇ $p<0.05$. (b) Visual

representation of GFP expression in control vs target samples. Red arrows mark the presumed location of PGCs. 29

Figure 14. Grid Plot of methylation levels of control compared to guide-injected samples. Scale on the right-hand side represents the change in methylation level from NA(not available) to 0 to 1 as gradual color change from light blue to white to black. 31

Figure 15. Grid Plot of methylation levels of 4-cell stage, 8-cell stage and ovary samples. Scale on the right-hand side represents the change in methylation level from NA to 0 to 1 as gradual color change from light blue to white to black. 32

Figure 16. Plot of methylation levels in sgRNA4 microinjected samples compared to control. Significant increase in methylation levels is observed at -261bp and -236 bp in close proximity to sgRNA4 target site. 33

Figure 17. Plot of methylation levels in sgRNA5 microinjected samples compared to control. Observable increase in methylation level is observed in CpGs in close proximity to sgRNA5 target site. 34

Figure 18. Plot of methylation levels in sgRNA6 microinjected samples compared to control. A noticeable decrease in methylation level was observed in CpGs in close proximity to sgRNA6 target site, however, CpGs at -7bp and 25 bp showed a significant increase in methylation. 35

CHAPTER I: INTRODUCTION

The field of epigenetics has undergone a huge evolution compared to its beginning in the late 19th century. Posed with a question: "How does a complex organism with cells of unique phenotypes rise from a single fertilized egg?", scientists set the foundation for our present-day understanding of the relationship between genes and development. Since then, the definition and scope of epigenetics have changed from just ''the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being."[1,2] to "the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence."[3] after the identification of DNA as the primary carrier of genetic information.

Our current understanding of epigenetics has strayed farther from and is not limited to just the study of development. With the recognition of epigenetic modifications such as posttranslational histone tail modifications and covalent modifications of DNA bases, the scope of the epigenetics has expanded to the recognition and study of aforementioned processes in the development of diseases of clinical relevance. Besides that, as we as a species became more exposed to synthetic chemicals because of rapid global industrialization, interest in the health effects of such interactions has led to rapid growth in the sub-field: Environmental Epigenetics.

This sub-field studies Epigenetic modifications that persist through the process of epigenetic reprogramming during early development as a sensitive barometer of environmental interaction. With studies suggesting the presence of epigenetic "memories" in the germline that can increase one's susceptibility to diseases even without direct environmental exposure, the interest in the field has grown rapidly. In addition, the accessibility and simplicity of epigenome

editing with a relatively new CRISPR system have sparked interest in applying this tool to understand epigenetic processes.

1.1 Epigenetic Modifications

Epigenetics is the study of change in gene expression without alterations in the DNA sequence. The three predominant epigenetic mechanisms include DNA methylation, post-translational histone modification and regulation by non-coding RNA. DNA methylation marks are the most studied epigenetic modifications, and largely focus on cytosine methylation at the cytosine-guanine dinucleotide junctions (CpG). The dinucleotide CpG is found to be sparsely spread in the human genome, occurring at only 4% the expected frequency[4]. Vertebrate genomes consist of CpG islands which are regions that contain a large number of CpG dinucleotide repeats. The cytosine at the 5' end of CpG dinucleotide on these sites can be methylated on the fifth carbon and is called 5-methyl cytosine (5-mC). The conversion of cytosine to 5-mC is thought to inhibit the binding of transcription factors and decrease gene expression. Methylation of CpG-dense promoters usually causes transcriptional repression indirectly by recruitment of histone deacetylases to methylated DNA by proteins such as the MDB domain of MeCP2[5] or directly by obstruction of transcription factor binding [6]. The enzymes of the DNMT family such as Dnmt3aa and Dnmt3bb are involved in the conversion process from cytosine to 5-mC by transferring the methyl group onto the target cytosine.

As previously mentioned, epigenetic modifications regulate gene expression by changing DNA accessibility and chromatin structure rather than by making changes in the DNA sequence[8]. Histone modifications and DNA methylation/demethylation are the major epigenetic processes that alter gene transcription[9,10]. While essential for development and differentiation in

organisms, epigenetic processes are influenced by endogenous, biotic, and abiotic factors[11]. These factors may initiate epigenetic changes such as histone modifications, DNA methylation/demethylation and transcription of microRNAs. DNA methylation is one of the major epigenetic factors involved in gene regulation. It regulates gene expression by either inhibiting the binding of transcription factor(s) to DNA or by recruiting proteins involved in gene repression[12]. Amino termini of histones contain a variety of posttranslational modifications[13]. Methylation of histones, by the addition of up to three methylations on a single lysine(K), can cause transcriptional activation or repression depending on the position of lysine residues[14]. Removal of an acetyl group from the lysine residues in histone tails renders chromatin less accessible and is usually associated with transcriptional repression[15]. On the other hand, microRNAs can impact transcription rates by affecting mRNA translation and degradation[16]. All these epigenetic modifications have been linked to promoting health issues in humans.

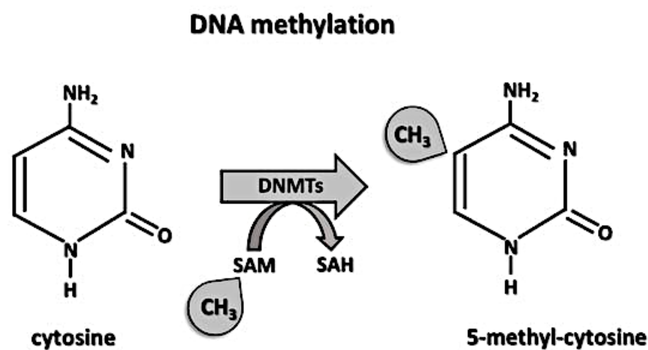

Figure 1. The mechanism of DNA methylation[7].

1.2 Epigenetic Alterations and Diseases

Many human disorders and diseases have been linked to alterations in epigenetic markers[17]. In the past few decades, epigenetic alterations have been implicated in promoting autoimmune disease[17], tumor progression[19], cardiovascular disease[20], neurodegenerative

diseases[21], and behavioral effects of drugs[22]. Additionally, exposure to xenobiotic compounds and chemicals including but not limited to phthalates plasticizers[23,24], bisphenol A[25-27] pesticides[28,29], herbicides[30,31], arsenic[32-35], asbestos[36,37], and pharmaceuticals[38-40] have also been found to cause epigenetic alterations in the germline.

Modifications that occur either in the somatic cells or in the germline epigenome are known as epimutations. Epimutations can appear in the subsequent generations because of direct exposure to environmental conditions, such as xenobiotics, pharmaceuticals, diet, and abiotic stress. Epigenetic mutations that are transferred to subsequent generations via gametes are known as transgenerational epimutations and their inheritance is known as transgenerational epigenetic inheritance. Endocrine-disrupting chemicals (EDCs), which are chemicals that interfere with normal endocrine hormone action, have been implicated in increasing risk for cancer[41] and childhood obesity[42]. Recently, the transgenerational epimutations caused by EDCs, such as BPA, have been extensively studied. Lifelong and transgenerational implications of prenatal exposure to environmental pollutants have also been described[43]. The association of epigenetic alterations with intergenerational[44,45], and transgenerational disease phenotypes[46-49] have been confirmed; however, whether their role in the development of such diseases is causative or correlative in unknown.

To understand the implication of specific epigenetic modifications with respect to disease pathogenesis, DNA binding proteins, namely, transcription activator-like effector nucleases (TALEs), zinc finger proteins (ZFNs), and most recently nuclease-deficient Cas9 fusions (dcas9) have been used. These engineered proteins have made it possible to effectively target and edit individual DNA/histone methylation/acetylation sites. Such studies have been critical in determining the function of those individual residues. Specifically, CRISPR-dCas9 has been

widely adopted for this function because of its simplicity and precise site-specific targeting. Hence, it has been widely utilized in developing gene suppression tools both *in vivo* and *in vitro* and provides an essential means to study the of the role of specific epimutations in transgenerational disease progression.

1.3 CRISPR-dCas9

CRISPR is an acronym for “Clustered Regularly Interspaced Short Palindromic Repeats”, which is a family of DNA sequences found in bacteria and archaea. Cas9 stands for “CRISPR-associated protein 9” which is the most widely studied Cas protein found in the type II CRISPR/Cas system that uses the CRISPR DNA sequence as a guide to recognize and cleave specific DNA segments. The type II CRISPR/Cas system consists of CRISPR loci associated with short CRISPR RNA (crRNAs) composed of small DNA fragments called spacers, which in turn are annealed to trans-activating crRNAs (tracrRNAs). The CRISPR-cas9 system can be modified to target virtually any genomic sequence by simply redesigning the crRNA. The Cas9 nuclease recognizes the PAM (protospacer-adjacent motif) sequence NGG which is 3’ to the target sequence in the genomic DNA, on the non-target strand. The Cas9 protein can be made devoid of its endonuclease activity by introducing two-point mutations into the RuvC and HNH nuclease domains resulting in a nuclease dead Cas9 (dCas9) molecule that cannot cleave target DNA. To date, the CRISPR system has been successfully manipulated to introduce DNA methylations *in vitro*[50], DNA demethylation *in vivo*[51], and histone acetylation *in vitro*[52]. CRISPR-dcas9 has been extensively used in genetic research to regulate transcriptional activation and repression both *in vivo* and *in vitro*. The *DNMT3a* gene has been successfully fused to dcas9 to target DNA methylation for gene suppression *in vitro*[53,54]. RNA-guided dCas9

methyltransferases such as DNMT3A and DNMT3B have been found to successfully methylate specific endogenous CpGs[55]. The dcas9 protein can be fused with a VP64 transcriptional activation domain increasing the expression of endogenous human genes *in vitro*[56]. In human cells, a 2-to-5-fold decrease in transgenic GFP signal was achieved *in vitro* upon transfection of dCas9-repressor fusion targeted to the transgene promoter[57]. The repression of GFP signal up to 15-fold occurred after treatment with specific sgRNAs, while others knocked down the expression by up to 75%. Additionally, the expression of medically relevant genes was successfully activated, and a synergistic effect of co-transfection was established with multiple gRNAs *in vitro*[58]. However, the applicability of CRISPR-dcas9 in establishing epimutations that are transgenerationally inherited is unknown. The process of Epigenetic reprogramming enables an organism's genotype to interact with the environment to produce its phenotype[59]. Given that embryos undergo epigenetic reprogramming of parental epigenetic marks during the cleavage stages, it is unknown if epigenome targeting at the one-cell stage can escape genome-wide reprogramming of the embryo occurring at cleavage stages. It is also unknown if *de novo*-established epigenetic alterations are programmed differently from those inherited from parental gametes.

1.4 Epigenetic Reprogramming of Cells

Epigenetic carried by reproductive cells undergo reprogramming upon fertilization to establish full developmental potency[60,61]. When reprogramming occurs, the DNA methylation patterns of the male and female pronuclei are extremely different. Although previous studies suggested that the mother genome undergoes passive demethylation via DNA replication during cleavage[62] and, only the paternal genome undergoes active demethylation by Tet dioxygenase-

dependent oxidation of 5mC[63], newer studies have suggested the significant presence of active demethylation in maternal genome too[64]. However, the demethylation of the paternal genome is much faster than that of the maternal genome rendering the genome-wide methylation level in male pronuclei lower than that in the female pronuclei after fertilization[65]. This requires the male genome to undergo massive reprogramming compared to the female genome. The methylation pattern in both the paternal and maternal genomes are restored before the blastula stage[66]. Environmentally induced epimutations can be erased and reestablished during the reprogramming of maternal and paternal pronuclei, or they can escape reprogramming in a similar fashion to imprinted loci[67].

Primordial germ cells (PGC) are undifferentiated germline stem cells that eventually differentiate into gamete-producing cells. Before a PGC differentiates into a sex-specific germ cell (spermatogonia or oogonia), it undergoes epigenetic reprogramming during which its DNA methylation profile is completely wiped and reestablished[66,68-70]. This is a crucial phenomenon that, if altered, can adversely affect the health and overall reproductive process of the derived offspring[71]. Reprogramming errors during this stage can result in epigenetic modifications that could be permanently programmed and transmitted to succeeding generations[72,73]. Environmental stressors and lifestyle can alter the epigenetic profile of germ cells by causing epimutations.

The epigenetic reprogramming pattern in zebrafish has been found to be profoundly different than classical mammalian models[74]. Unlike zebrafish, the present animal model medaka (*Oryzias latipes*) shares a common pattern of epigenetic reprogramming with mice and humans[75] (Figure 2). In this organism, the epigenetic memories passed on by parental gametes are reprogrammed in the embryo by the blastula stage. Similar to humans and mice, in medaka the DNA methylation pattern in the paternal genome is erased in the first cell cycle in embryos, and

the global DNA methylation levels gradually increase from 16-cell stages to gastrula stages[76]. DNA methylation marks induced by environmental stressors survive 2 reprogramming windows before being established into the genome. Having similar DNA methylation reprogramming mechanisms to mammals makes medaka an excellent model to study the underlying mechanisms of epigenetic transgenerational inheritance.

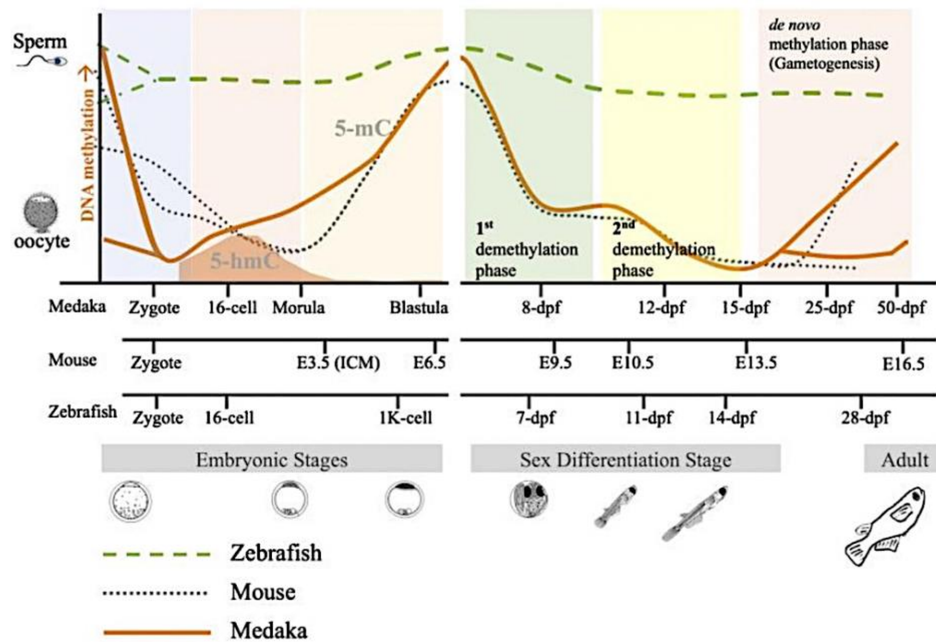

Figure 2. DNA methylation reprogramming in an embryo and primordial germ cells[75]. The left panel shows the dynamics of DNA methylation (5mC) in a post-fertilizations stage embryo undergoing cleavage stages. The right panel shows the dynamics of DNA methylation (5mC) in primordial germ cells (PGCs) which are the precursors of eggs and sperm. Similar DNA methylation profiles are maintained in mice and medaka, while a different profile is maintained in zebrafish.

1.5 The Medaka Fish Model

Accomplishing epigenome editing in mammals requires the arduous task of the injection of fertilized eggs and their transplantation into the host uterus. The process of epigenome editing in a simpler fish model can arguably be as informative provided the model species is similar to mammals with regards to epigenetic inheritance mechanisms. The Bhandari Laboratory has established medaka (*Oryzias latipes*) as an excellent biomedical model organism given its similarity in germ cell development and DNA methylation reprogramming process with mammals[76]. In addition, medaka's short life span and transparent eggs also make it easy to microinject plasmid DNA and follow embryonic development and phenotypic changes throughout embryogenesis. The present study relies on the efficiency of microinjection and non-invasive monitoring of injected embryos at various stages of their embryonic and postnatal development. This makes it a perfect animal model for this study.

1.6 The Present Study

DNA methylation marks on the genome are the earliest known and most studied epigenetic modifications. The relationship between DNA demethylation and gene expression has been explored for over three decades now[77]. Recently, epigenetic alterations, particularly DNA methylation changes that occur due to early-life adverse exposures, have been identified as potential contributors to the developmental origins of health and disease (DOHaD)[78]. Their association with health and disease has been demonstrated[28,29,32]; however, whether these epigenetic alterations are causative has not been experimentally demonstrated. With new disorders and diseases being linked to epigenetic alterations, a toolkit that can effectively target and reverse induced epigenetic alterations (epimutations) could be a saving grace for the modern

human civilization that has been exposed to various environmental biotic and abiotic stressors. Methods for studying the role of genetic factors in diseases and human health have been formulated and refined over the last decade. However, being a budding field, concrete methods for studying the role of epigenetic factors in diseases and human health are yet to be formulated.

The CRISPR-Cas9 system and its ever-expanding toolkit have revolutionized the field of precision genome editing. Although being extensively used to explore physiological relationships in almost every system, collectively, there have been no studies demonstrating the applicability of the CRISPR-Cas9 system to study the fate of epigenetic edits during epigenetic reprogramming *in vivo* and it seems imperative to develop strategies to study it. The catalytically dead Cas9 binds the DNA but does not cleavage it. This dCas9 protein has been fused with the catalytic domains of genes like human acetyltransferase enzyme, p300 and DNA methyltransferases, DNMT3a and DNMT3b, to achieve targeted histone acetylation and DNA methylation of the targeted region, respectively.

Here we describe the development of an epigenome editing technique based on suppression of the germline-specific promoter of *olvas* gene by dcas9-effector complex. The vasa gene (*olvas*) is expressed in primordial germ cells of the medaka embryo. The transgenic line of medaka fish harbors 5.1 kb *olvas* promoter, including exon 1 and 2 and 3' region of *olvas* gene[79]. The transgenic medaka that the present study used contains an olvas-promoter transgene coupled with coding sequences for green fluorescent protein (GFP), which expresses exclusively in PGCs of medaka embryos from day 4 through day 25[79](Figure 3a). Given that converting unmethylated cytosine to 5-mC can inhibit the binding of transcription factors and decrease expression of the target gene, the use of *olvas*-GFP transgenic fish will further allow the characterization and tracing of gene expression following manipulation of DNA methylation on

an *olvas* core promoter region[53,80-82]. This study will examine and optimize the combination of CRISPR-dCas9-dnmt3aa and guide RNAs and determine the most effective dose and combination to effectively induce stable epigenetic modifications leading to transcriptional regulation in the *olvas* promoter in the medaka genome.

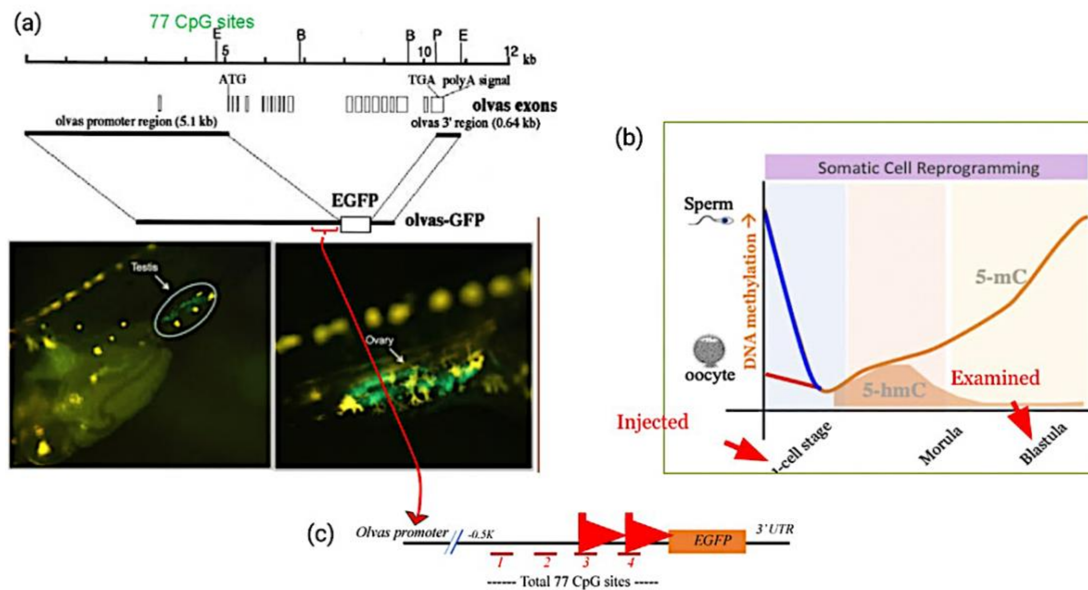

Figure 3. (a) Structure of olvas-GFP vector[79] and GFP expression in adult medaka testis and ovary. (b) CRISPR-dCas9-dnmt3aa injection and examination site through the somatic cell reprogramming window. (c) *olvas* promoter with guide RNA target sites for the proposed study.

1.7 Aims and Objectives

The present study has two aims: a) Prepare CRISPR-dCas9-dnmt3aa and multiple single guide RNAs (sgRNAs) targeting the *olvas* promoter and b) Determine the efficacy of epigenome editing by introducing methyl groups directed by sgRNAs at one cell stage without causing toxicity to the embryo.

To fulfill our aims, we examined the a) effectiveness of CRISPR-dCas9-dnmt3aa in altering the methylation pattern during the first somatic cellular reprogramming window and b)

the effect of the introduced methylation on the *olvas*-GFP expression. These studies are highly significant and timely for the following reasons:

1) ***To determine strategies to mitigate transgenerational epigenetic inheritance:*** It has been established that environmental insults can increase the susceptibility of reproductive[83-86] and metabolic[87,88] diseases. Several studies have suggested changes in DNA methylation patterns in differentially methylated regions (DMRs) may be useful biomarkers to study transgenerational epigenetic inheritance (TEI)[89-93]. TEI has been observed over multiple generations in both animal and human models[94,95]. A major barrier to TEI, however, is germline reprogramming where histone modifications and DNA methylation marks are reset both in the germ line and early in pre-implantation mammalian development. Experimental studies clearly suggest that epigenetic modifications established by many environmental cues or stressors can either withstand the epigenetic resetting process or can reset and reestablish later[96]. It is unclear whether such environmentally established epigenetic alterations are the drivers of the observed phenotypes. To understand the role of epigenetic alterations in the onset or progression of health and disease, it is essential to establish *de novo* epimutations by epigenome editing. So far, there are no reports on studies utilizing the CRISPR-dCas9 system in editing transgenerational methylation marks *in vivo*. There have been a few epigenome editing studies focusing on DNA methylation *in vivo*[97-99]. However, these techniques are yet to be improved and tested in other biomedical disease models. In this study, we focused on the applicability of CRISPR-dCas9-dnmt3aa in editing DNA methylation in medaka (*Oryzias latipes*) to understand its effectiveness to escaping the epigenetic reprogramming window.

2) ***To ascertain how in vivo DNA methylation editing affects phenotype:*** Epigenetic modifications in the germline have been found to alter phenotypes in successive generations[96].

So far, no studies have studied the phenotypic response to CRISPR-dCas9- based DNA methylation editing *in vivo* in a TEI setting. This study observes the response of *olvas*-GFP to site-specific DNA methylation during the first epigenetic reprogramming window.

1.8 Experimental Design

Briefly, the present study examined whether the epigenome editing performed at zygotic stages will result in a heritable trait that can withstand the reprogramming window during cleavage stages. To understand if gene expression can be silenced by employing epigenome editing of the core promoter, we used a CRISPR inhibitor approach by fusing the *dnmt3aa* effector domain into the CRISPR-dcas9 plasmid and tested the efficacy of the plasmid alongside sgRNAs to alter epigenetic marks linked with transcriptional regulation of the *olvas* gene *in vivo.* To do so, we injected multiple combinations of CRISPR-dcas9-dnmt3aa and sgRNAs into the one-cell stage of the medaka embryo and verified the methylation change at the blastula stage by amplicon sequencing on bisulfite-converted DNA. We further made observations about the GFP-positive rate in the microinjected embryos 5 days post-fertilization.

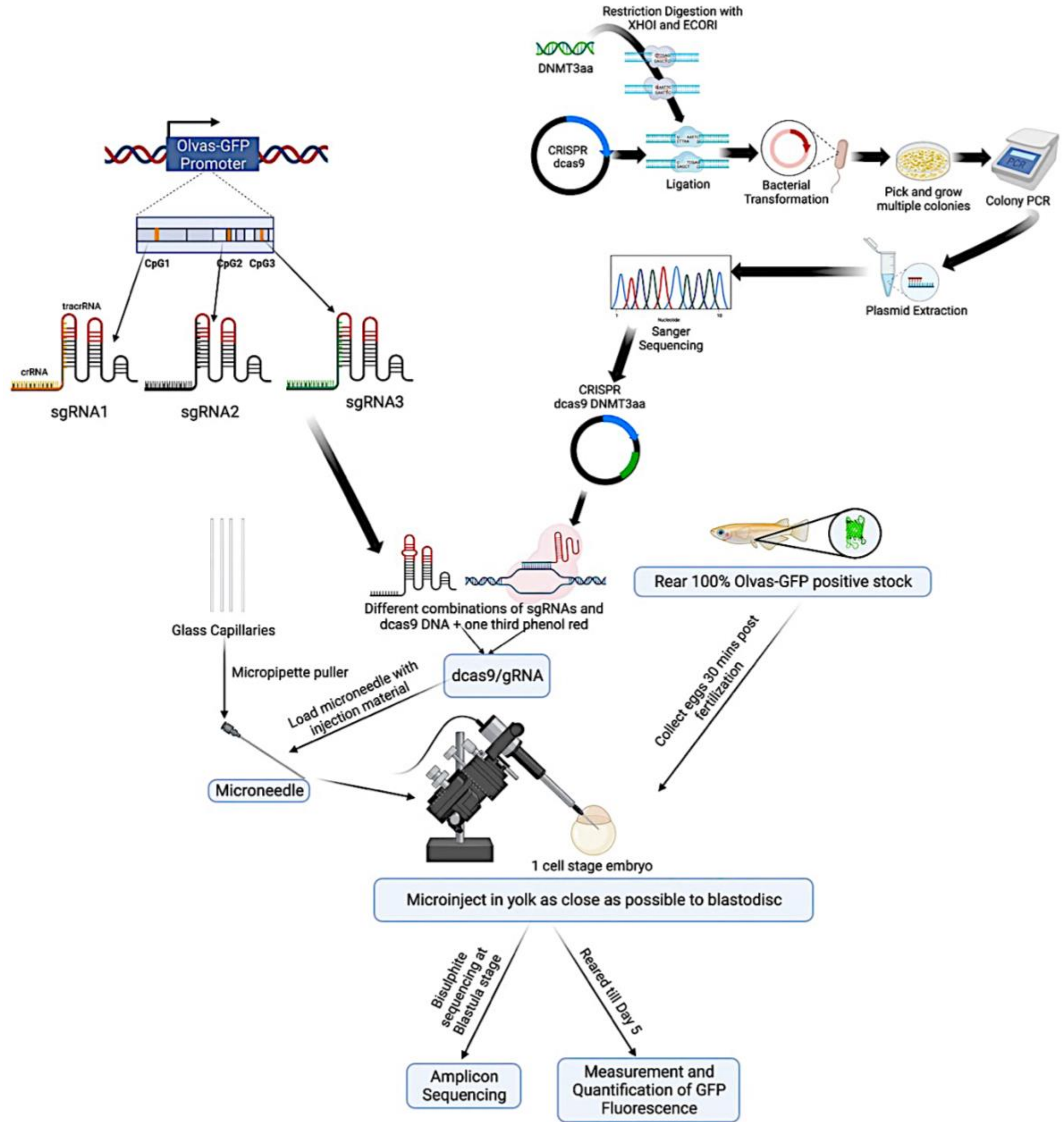

Figure 4. Overall experimental Design

CHAPTER II: MATERIALS AND METHODS

2.1 Preparation of CRISPR-dCas9-*dnmt3aa* plasmid

The CRISPR plasmid backbone pCS2+hSpCas9(Figure 4) was ordered from Addgene (Plasmid #51815) and point mutations (D10A and H840A) were introduced to the cas9 sequence to synthesize a nuclease dead Cas9 (dCas9) molecule by Dr. Xuegeng Wang at the Bhandari Laboratory. A GGGGS linker and multiple clone sites(gaattcgctagcctcgag) were introduced consecutively following the nucleoplasmin Nuclear Localization Signal (NLS) region in the CRISPR plasmid. The mutations were introduced using the Q5® Site-Directed Mutagenesis Kit following the protocol and primers recommended on NEBaseChanger™ website.

Figure 5. pCS2+hSpCas9 CRISPR backbone.

The *dnmt3aa* gene sequence was collected from Ensembl genome browser 107, the conserved domain characterization was done using NCBI Conserved Domain Search. After the

selection of the conserved domain, the gene fragment was designed with EcoRI and XhoI sites and ordered to be chemically synthesized from Integrated DNA Technologies, Inc, USA. The *dnmt3aa* gene fragment was then restriction digested with EcoRI-HF and XhoI enzymes alongside the CRISPR-dcas9 vector plasmid and the product was cleaned and ligated overnight at 4 °C. The reaction mixture was transformed into competent E. coli and grown for 12 hours. Multiple colonies were picked and restreaked to grow overnight. The following day, Colony PCR was performed using back-bone specific primers to verify the presence of the insert. Specific primers in the CRISPR backbone designed using Primer3web were used for Colony PCR which are shown in appendix A. Colonies with bands ≈ 2kb were grown in LB broth overnight and extracted. The extracted colonies were further restriction digested to verify the presence of DNMT3aa and sent for Sanger sequencing at NC state University's Genomic Sciences Laboratory alongside specific primer pairs to identify samples without mutations. The primers are shown in Table 1.

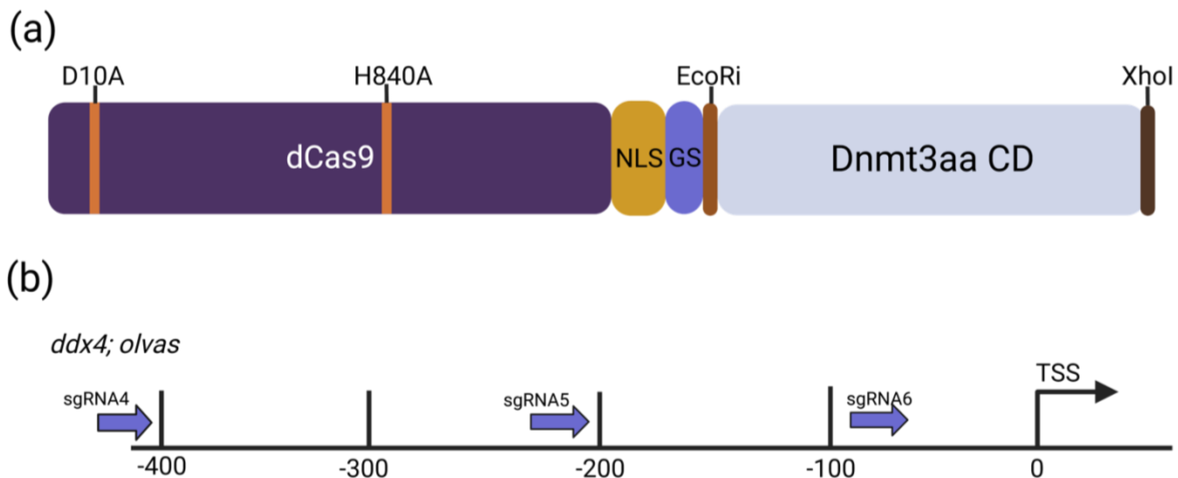

Figure 6. (a) Domain structure of the dCas9-DNMT3aa catalytic domain fusion protein; (b) Illustration of the locations of the three sgRNAs designed to target olvas coding sequence.

Primer Name	Length (bp)	Primer Sequence
CRISPRdcas9-F1	18	GATCCACCAGAGCATCAC
CRISPRdcas9-R2	23	CATGTCTGGATCTACGTAATACG
Dnmt3aa-Fi-1	19	TCCAGAAAGAGGAGGAGCC
Dnmt3aa-Fi-2	20	AAGCCTCAACGTCTCTCTGG
Dnmt3aa-Fi-3	20	TGACCTCTCCATCGTGAACC
Dnmt3aa-Ri-1	20	GAGGTGTGTAGGCTGCTTCA
Dnmt3aa-Ri-2	20	GATGCCACGTACTTGTCCAC
Dnmt3aa-Ri-3	20	GCGAAGAGGTGCCTGATAAC

Table 1. CRISPR-dCas9-dnmt3aa plasmid verification Sanger sequencing primes.

2.2 Preparation of single guide RNAs (sgRNAs)

The crRNAs were designed using online tools; http://crispor.tefor.net/ and https://www.benchling.com/ . The crRNAs with low off-target scores (from CRISPOR.org) and close to the most CpG-rich regions were chosen. 6 crRNAs 50 to 200 bps apart from each other were selected and a scrambled crRNA was designed and verified by BLASTN against Japanese medaka Hd-rR ASM223467v1 (Genomic sequence). The crRNA fragments were exported to Integrated DNA Technologies, Inc, USA to chemically synthesize sgRNAs. The sgRNAs(Table 2) were then dissolved in RNase-free water as 1000 ng/μL stock solution and stored at -80 °C. The sgRNA concentrations were quantified using a Qubit 4 fluorometer. The selection criteria for crRNAs are shown in Table 3 and further elaborated in the discussion section.

Guide Name	DNA Strand	crRNA Sequence (without PAM)
sgRNA1	Forward	5’ TGTGCAAACAAGTTTTCACT 3’
sgRNA2	Forward	5’ ACTGATTGGCTGGCCACAGA 3’
sgRNA3	Reverse	5’ ACTCTGGACAAATGTTTACG 3’
sgRNA4	Forward	5’ AAGGGGAATTCGGACTTTGT 3’
sgRNA5	Forward	5’ TTCAGATCTTTTCACAGAAA 3’
sgRNA6	Forward	5’ GTCAGAACCGGTGTTCTGTG 3’
sgRNA7	Forward	5’ TCGTGTTCATAACGTTGTCA 3’
Scrambled sgRNA	Forward	5’ TTTTTTTTTTTTTTTTTTTT 3’

Table 2. Single-Guide RNA sequences.

Guide Name	MITSpecScore	Off-target Count	Mismatch Count
sgRNA1	88	74	4
sgRNA2	93	32	4
sgRNA3	97	19	4
sgRNA4	93	29	4
sgRNA5	69	154	4
sgRNA6	86	28	3

Table 3. crRNA selection criteria[110].

2.3 Animal Care and Colony Selection

All fish husbandry, tissue collection, germ cell preparation, and DNA/RNA sample preparation will be performed at Bhandari Laboratory, and the procedure for animal care and handling is

approved by Institutional Care Committee of the University of North Carolina Greensboro (#20-002). We reared 100% Olvas-GFP positive stock after careful observation of eggs collected from the original line under the microscope. Fish were fed Otohime C1 medaka food (Reed Mariculture, USA) twice daily. Water temperature was maintained at 26 ±0.5°C and the light: dark cycle was set at 16:8 hours. A total of 3 glass tanks were maintained. Water quality was monitored daily (dissolved oxygen, temperature, pH) and weekly (ammonia and hardness). Unused food and debris were siphoned out from the tanks daily.

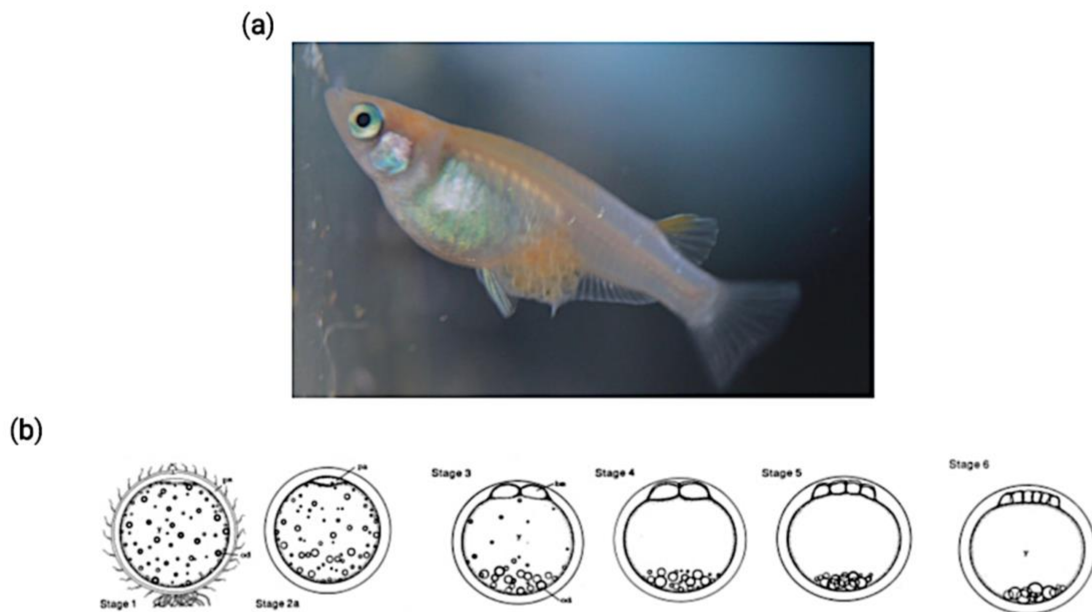

Figure 7. (a) Female medaka(*Oryzias latipes*) with eggs; (b) Early developmental stages in *Oryzias latipes*[100].

2.4 Mating and Offspring Production

Embryos were collected right after mating between 8 and 8:30 am, cleaned and incubated in 0.001% methylene blue solution on ice. The embryos were staged by developmental time under brightfield microscope. After microinjection, some 40 to 50 eggs were incubated in

0.001% methylene blue at 27 °C until hatching on 5th day for quantification of GFP fluorescence.

2.5 Microinjection

The cast to hold the embryos was prepared setting fine borosilicate tubes at the bottom of the on Petri-dish and pouring 3% molten Agarose (ThermoScientific J32802-36) gel on it. It was let dry for 30 minutes and turned upside down to remove the borosilicate tube to obtain cast to hold the medaka eggs within the groove.

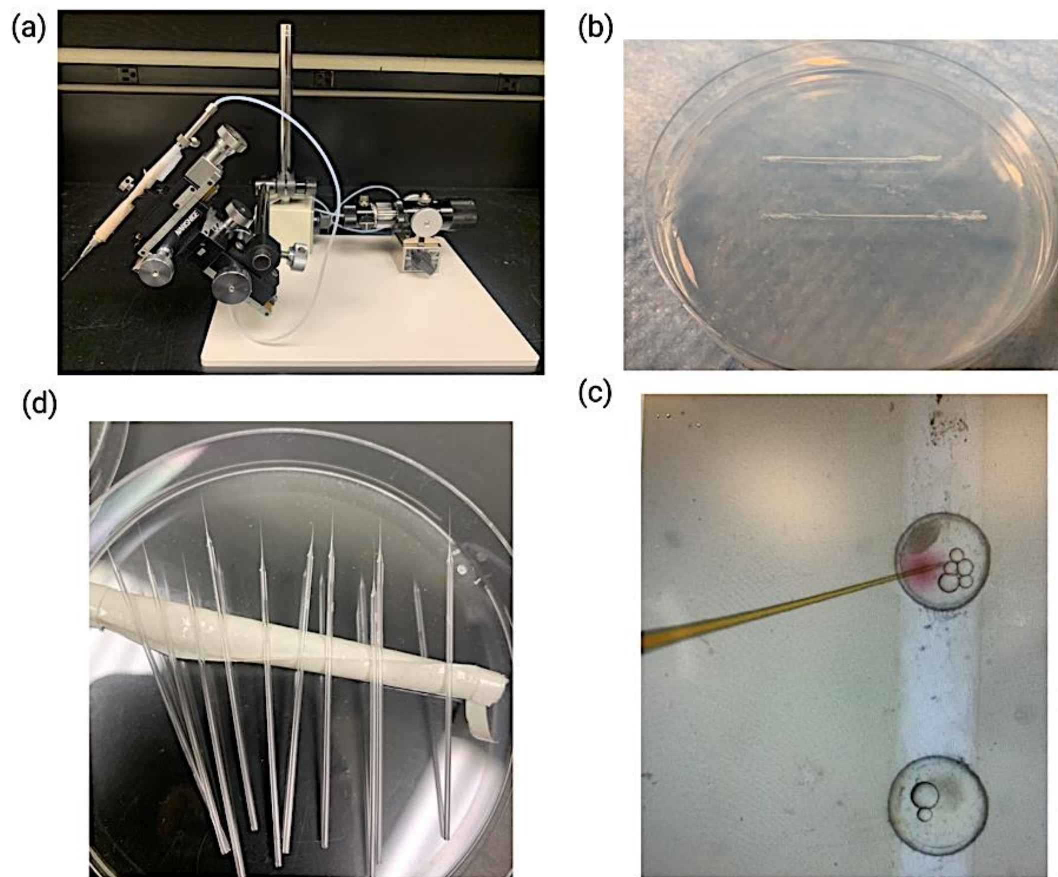

Figure 8. (a) Narishige IM-21 microinjector setup; (b) Gel Cast for holding eggs; (c) Microinjection needles; (d) Microinjection with Phenol Red into Yolk.

The CRISPR-dCas9 DNA and 4 gRNAs were mixed to a final concentration of 300µg/µL and 30µg/µL respectively. 0.3 µL of Phenol Red 0.5% (Sigma-Aldrich #P0290) was added to the solution per µL of mixture to distinguish injected and non-injected eggs. The mixture was stored on ice for 10 to 20 minutes before being micropipetted into the microneedles for injection. The protocol was optimized to microinject 1 nL of solution at the blastodisc of the embryos as 1 cell stage. Scrambled guide RNA and Guides 1 through 6 were separately mixed with CRISPR-dnmt3aa. Each combination of CRISPR-dnmt3aa and sgRNA was microinjected into more than 150 embryos over the course of a month.

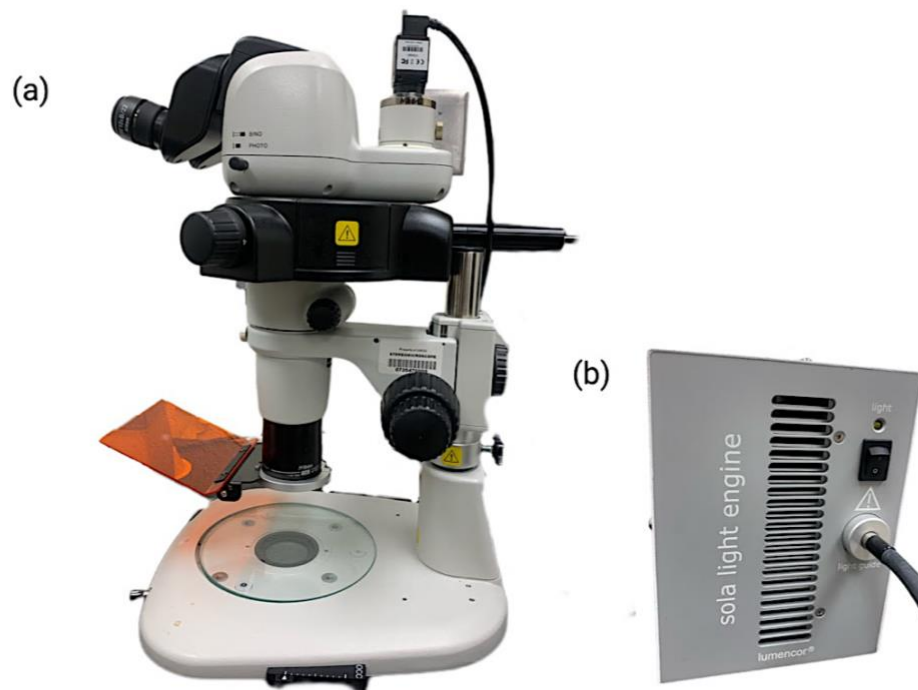

Figure 9. (a) Microscope used for microinjection and GFP quantification; (b) UV light-source

Out of which 80 to 100 surviving embryos were used for DNA extraction and targeted sequencing at blastula stage, 40 to 50 embryos were used for measurement and quantification of GFP fluorescence at Day 5 for each combination of CRISPR-dCas9-dnmt3aa.

2.6 DNA isolation

The DNA was extracted from the injected and uninjected eggs at 4 cell, 8-cell, and blastula stages by collecting 25-30 eggs per replicate. The stages were selected because as depicted in Figure 2, we expect 4- and 8-cell stages of the embryos to have genome to undergo global demethylation, whereas we expect a higher methylation level at the blastula stage. The eggs were collected and directly transferred to -80 C freezer. Zymoresearch Quick-DNA Miniprep Plus Kit (D4068) used to extract DNA according to the manufacturer's instructions. To compare the hypermethylated blastula samples to hypomethylated genome, The DNA from ovary samples was extracted using Zymoresearch Quick-DNA/RNA Microprep Plus kit (D7003. The extracted DNA sample concentrations were quantified using a Qubit 4 fluorometer and stored in a -20 °C freezer in the water until further analysis.

2.7 Bisulfite Conversion of the genomic DNA and bsPCR

The extracted DNA from samples were bisulfite converted with EZ DNA Methylation-Lightning Kit (ZymoResearch, D5030-E) according to the manufacturer's user manual. Hence, the converted DNA was used to amplify the targeted *Olvas* promoter amplicon using ZymoTaq DNA Polymerase Kit (ZymoResearch, E2002) by nested PCR with the following primers (Table 3) and temperature regimes: 10 min at 95°C, 1 cycle; 1 min at 95°C, 1 min at 55°C and 2 min at 72°C, 40 cycles; 7 min at 72°C, 1 cycle followed by a hold at 4°C. The PCR products were purified using the NEBNext Sample Purification Beads by adding beads in a 2:5 and 1:7 ratio in two consecutive cleanup steps following the manufacturer's user manual.

Primer Name	Length (bp)	Primer Sequence
OlvasBBSF1	30	AGAATTTTAATATTTTAAAGAATGTTTTGT
OlvasBBSR1	24	TACAACTACACAACCTCAAAATAC
OlvasBBSF2	28	AGAATGTTTTGTTTTTCCCAGATCATGA
OlvasBBSR2	28	GTGGACCTCCACCCTGACTCTGACTCTG

Table 4. Bisulfite DNA amplification primers.

2.8 Targeted Amplicon Library Preparation for Methylome Analysis

The amplicon library was prepared using NEBNext® Ultra™ II FS DNA Library Prep Kit (NEB, E7805S) using bisulfite-converted amplicon as the starting point. A total of 10 µL of the bisulfite converted amplicon PCR product was validated for the presence of expected amplicon size using gel electrophoresis and cleaned using NEBNext Sample Purification Beads (E7767L) for size selection according to the protocol. The amplicons were then ligated with the methylated adapters by incubating the size-selected amplicon solution, enzyme mix, and adaptor at 20°C overnight. After incubation of the ligation mixture with USER enzyme (NEB, E7338AA) as per the manufacturer's user protocol, NEBNext Sample Purification Beads (E7767L) were used for size selection according to the protocol. DNA fragments were then amplified with Universal and Index PCR primers for 13 cycles and size selected to remove adapters/ primer-dimers using the NEBNext Sample Purification Beads in a one-step cleanup procedure by adding beads in a 9:10 ratio to the PCR reaction solution following the manufacturer's user manual.

2.9 Gel Electrophoresis

2% Agarose was used for gel electrophoresis. DNA samples were mixed with 6x DNA gel loading dye (Thermo Scientific™) prior to loading. The gel was run at 100 volts for 60 minutes. DNA gels were imaged by Biorad Molecular Imager ChemiDoc XRS System.

2.10 Sanger Sequencing

After the blastulas were injected at 1 cell stage, DNA was extracted, bisulfite converted, and PCR amplified. Similar process was taken upon uninjected blastula samples, 4-cell stage, 8 cell stage embryos, and ovary samples. Amplicon-specific primer pairs were designed using Primer3web and are shown in appendix B. The DNA for each sample was quantified to be at least 40 ng in a volume of 10ul and 2ul primer was added before sending NC State University's Genomic Sciences Laboratory for Sanger sequencing.

2.11 Statistical Analysis

Data are expressed as mean ± S.EM. All data were analyzed using GraphPad Prism version 9 (GraphPad by Dotmatics, Boston, MA)[101] with a significant difference established at a P value < 0.05. The unpaired 2-tailed t-test was applied to compare values between groups.

2.12 Bioinformatics Data Analysis

Methylome Data Analysis: Bioinformatic analysis of amplicon Bisulfite Sequencing data was conducted by Dr. Santosh Anand using the pipelines previously developed in our laboratory for medaka epigenome analysis. Briefly, a three-step process was applied: adapter trimming, read alignment, and quantification of methylation levels. Trim Galore (v0.6.7;

https://github.com/FelixKrueger/TrimGalore) was used for adapter trimming as well as quality control of 250bp paired-end read sequences. Trim Galore removes low-quality base calls (Phred score <20) from the 3' end of the reads before adapter removal. The filtered reads thus obtained were mapped to the reference sequence of the amplicon (chr2:945157-945563:-1 medaka_primary_assembly ASM223467v1) using Bismark (version: v0.22.3)[134]. Given that there is only one short reference sequence (407bp) (without adapter) the reads were mapped in single-end mode to maximize mapping efficiency. The methylation levels in each sample at each CpG position was extracted using the bismark_methylation_extractor (v0.22.3) script of Bismark. The methylation data were plotted using Methylation plotter[135] (and custom R/ggplot2 scripts. The Mean quality score of all sample replicates was plotted against their Phred scores using MultiQC[136].

CHAPTER III: RESULTS

3.1 Injection of embryos with shorter DNMT3aa Conserved Domain

Work previously done in the lab analyzed GFP positive rate [(total number of GFP positive eggs/total number of eggs) ×100%] using CRISPR-dCas9 with two *olvas* guide RNAs (Reverse crRNAs: XWgRNA1:GGTATTGAGGTTCTAGATCA and XWgRNA2: AACCTGACCCCACAGAACAC), alongside CRISPR-dCas9-dnmt3aa microinjected egg. The GFP positive rate [(total number of GFP positive eggs/total number of eggs) ×100%], was significantly reduced in the CRISPR-dCas9-guide injected samples compared to the mock.

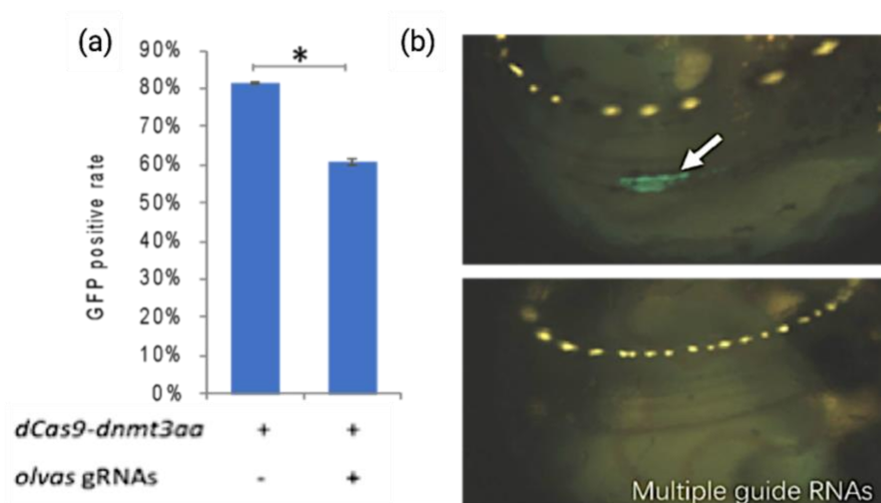

Figure 10. GFP Quantification of CRISPR-dcas9-dnmt3aa-gRNA injected samples vs only CRISPR-dcas9-dnmt3aa injected samples. (a) The GFP positive rate of guide-dcas9 vs mock 5dfp. The GFP rate in promoter-targeted embryos was significantly reduced. *: significance. Mean ± SEM. ∇ $p<0.05$. (b) Visual representation of GFP expression in mock vs target samples. The white arrow marks the presumed location of PGCs.

Work previously done in the lab also subjected the *olvas* promoter region to MeDIP (Methylated DNA Immunoprecipitation) and then analyzed the product by quantitative real-time

qPCR with primers specific for the gRNA targeted region. 5mC was successfully added to the targeted locus of the *olvas*-GFP promoter *in vivo*, which survived epigenetic reprogramming of embryo.

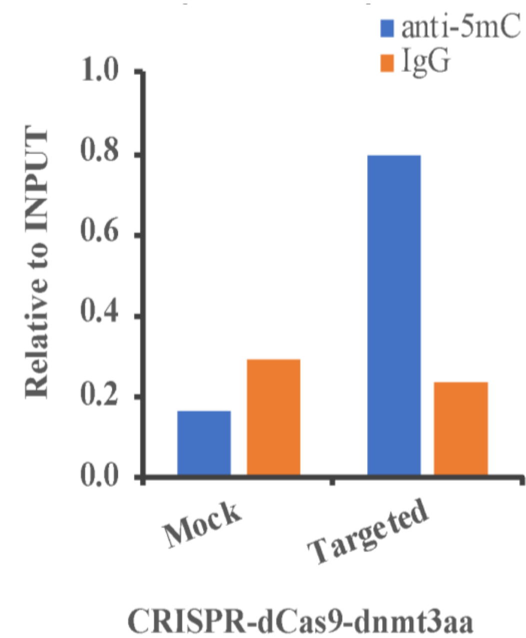

Figure 11. qPCR results of MeDIP-Seq DNA of mock vs guide-RNA targeted embryos**.** The 5mC level in the targeted embryos was ~4 times higher than the mock.

3.2 Sanger Sequencing Results of Experimental and Control Samples

The methylation pattern in the experimental and control samples were deduced by performing Sanger Sequencing with appropriate primers. No significant difference in methylation pattern was seen between samples as shown in Figure 12. The data also suggested gaps in reads which rendered the data incomplete.

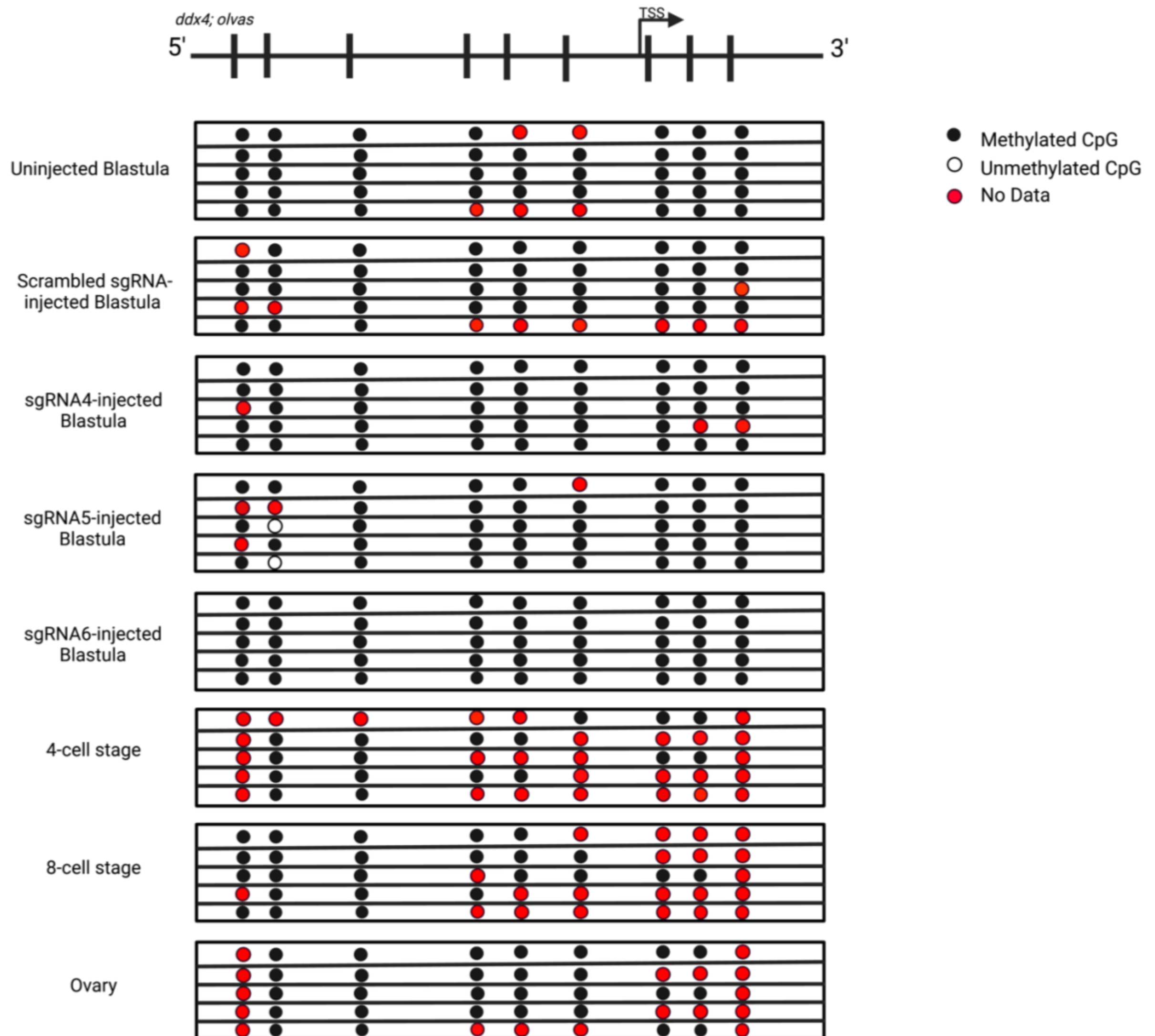

Figure 12. Methylation pattern on the targeted site on *olvas* gene at different developmental stages.

3.3 GFP Quantification in 5-day old embryos

GFP positive rate was analyzed [(total number of GFP positive eggs/total number of eggs) ×100%] in all CRISPR-dCas9-dnmt3aa-guide RNA microinjected and Control eggs. The GFP positive rate was significantly reduced in the microinjected samples compared to the scramble-injected and the control samples(Figure 13).

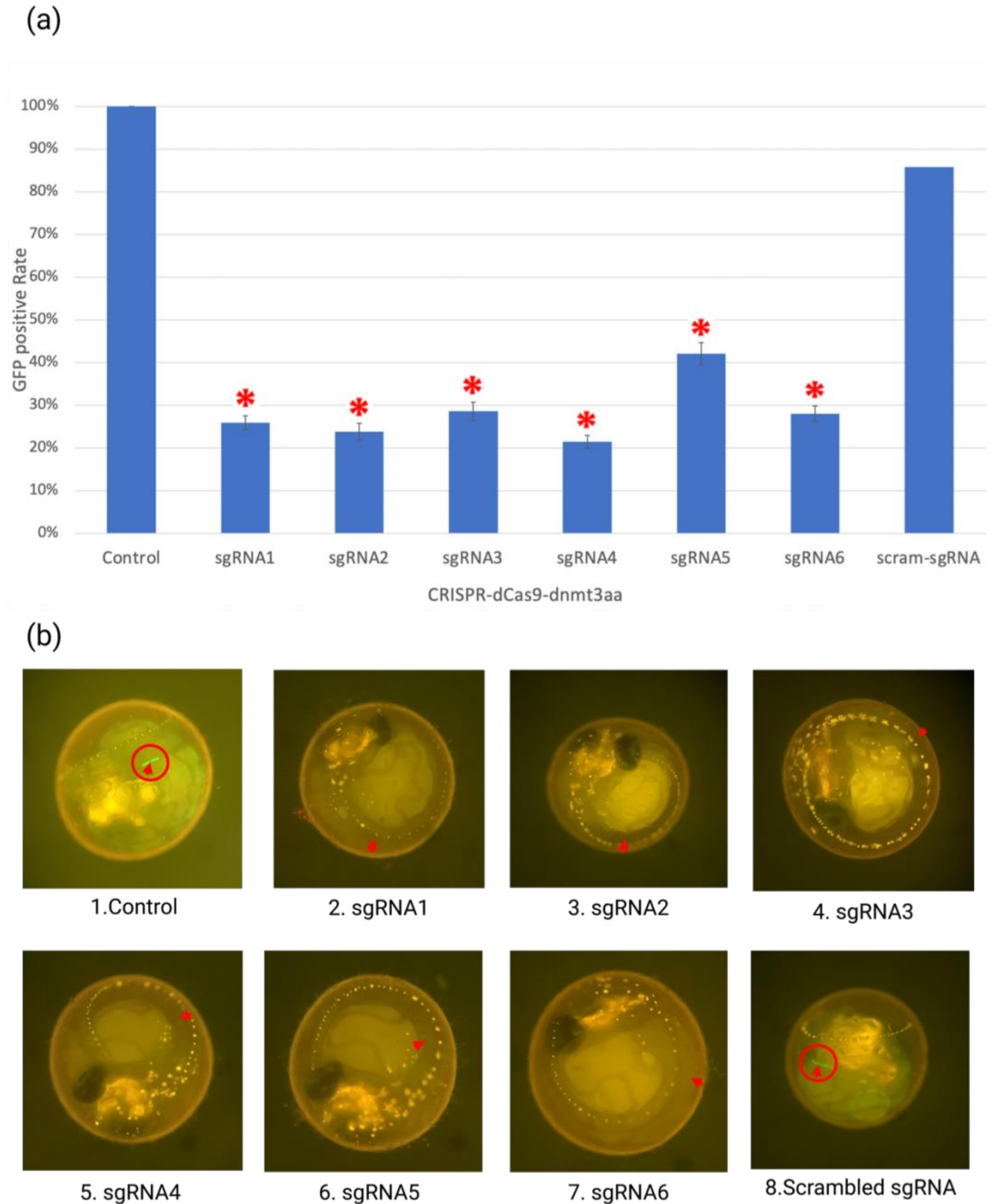

Figure 13. GFP Quantification of sgRNA injected samples vs control 5 days post-fertilization. (a) The GFP positive rate microinjected embryos vs control 5-dfp. The GFP rate in promoter-targeted embryos was significantly reduced. *: significance. Mean ± SEM. ∇ $p<0.05$. (b) Visual representation of GFP expression in control vs target samples. Red arrows mark the presumed location of PGCs.

3.4 Targeted Amplicon Sequencing-MiSeq Results

Final methylation levels at each CpG sites were calculated by Next Generation Sequencing (NGS). We sequenced all the uninjected and CRISPR-DNMT3aa-guideRNA-injected samples at Blastula stage along with 4-cell, 8-cell, and embryo DNA. Between 4 and 16 cell stage, the medaka embryos go through a methylation reprogramming which ends at Blastula. As expected, most reads in the MiSeq result suggest a hypermethylated state of CpGs in Blastula. The data also suggested a considerable difference in methylation pattern between uninjected, scrambled injected and the sgRNA-injected samples. Comparing between the uninjected embryo samples (Control) and the samples injected with gRNA 4, 5 and 6, the CpGs at positions 235 bp downstream, 25, 39 and 6 bp upstream of the transcriptional start site had a lower methylation rate. Reads across samples suggest a hypermethylated state of CpGs in blastula.

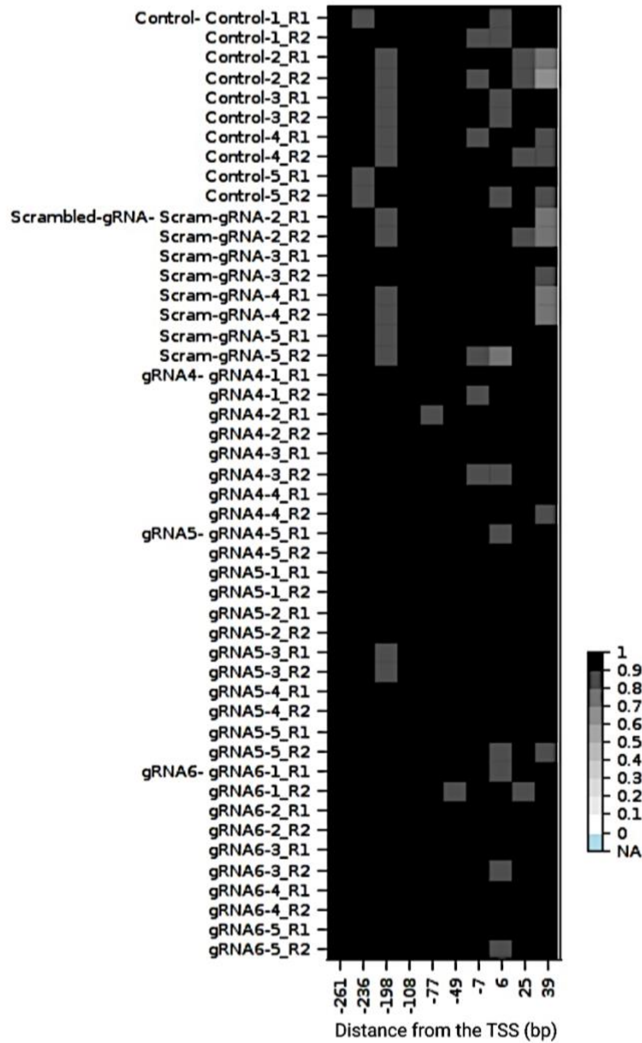

Figure 14. Grid Plot of methylation levels of control compared to guide-injected samples. Scale on the right-hand side represents the change in methylation level from NA(not available) to 0 to 1 as gradual color change from light blue to white to black.

The methylation status of almost of all CpG sites in the ovary DNA are hypomethylated compared to the rest of the experimental data. 8 cell stage DNA also shows hypomethylation status in CpGs closer to the TSS. A large portion of 4-cell stage and Ovary DNA didn't produce any reads.

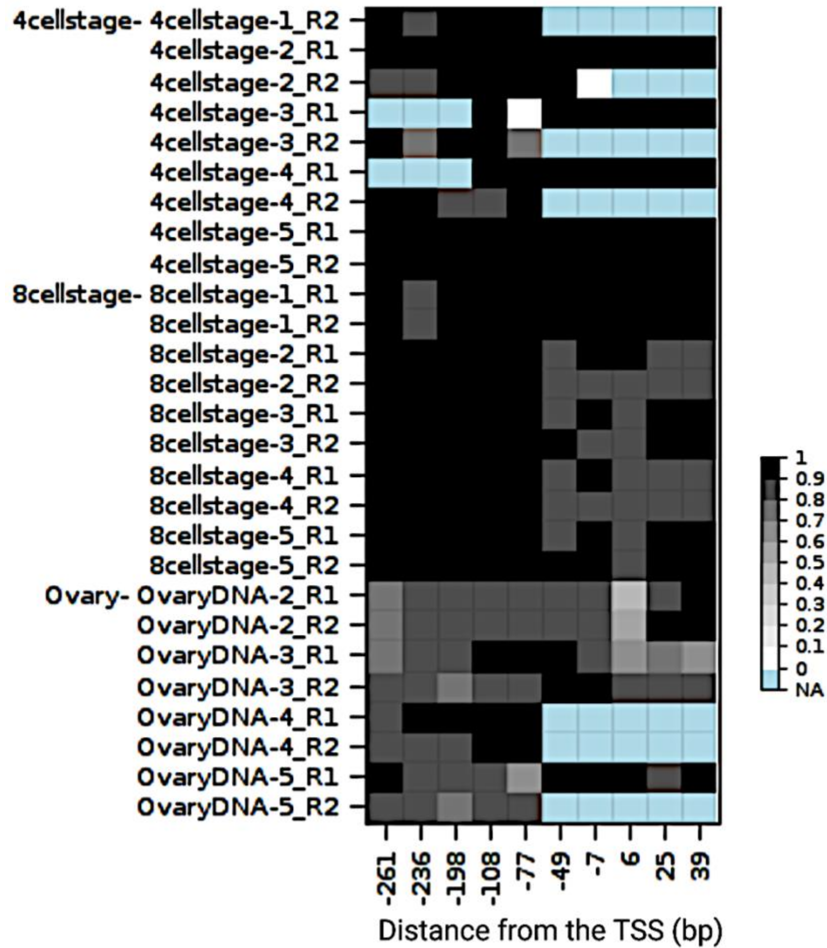

Figure 15. Grid Plot of methylation levels of 4-cell stage, 8-cell stage and ovary samples. Scale on the right-hand side represents the change in methylation level from NA to 0 to 1 as gradual color change from light blue to white to black.

3.4.1 sgRNA4 vs Control

The sgRNA4 targeted at 405 bp downstream of the TSS. The two CpG sites in proximity to the sgRNA4 injected site (-261bp and -236 bp) show a significant increase in the methylation status compared to the CRISPR-guide-uninjected samples. There was also a significant increase in methylation rate seen at +25bp CpG site, which is 430 bp downstream from the gRNA target

site. However, the CpG at -49 bp in the control samples also had a significantly higher methylation rate than the sgRNA4 injected samples.

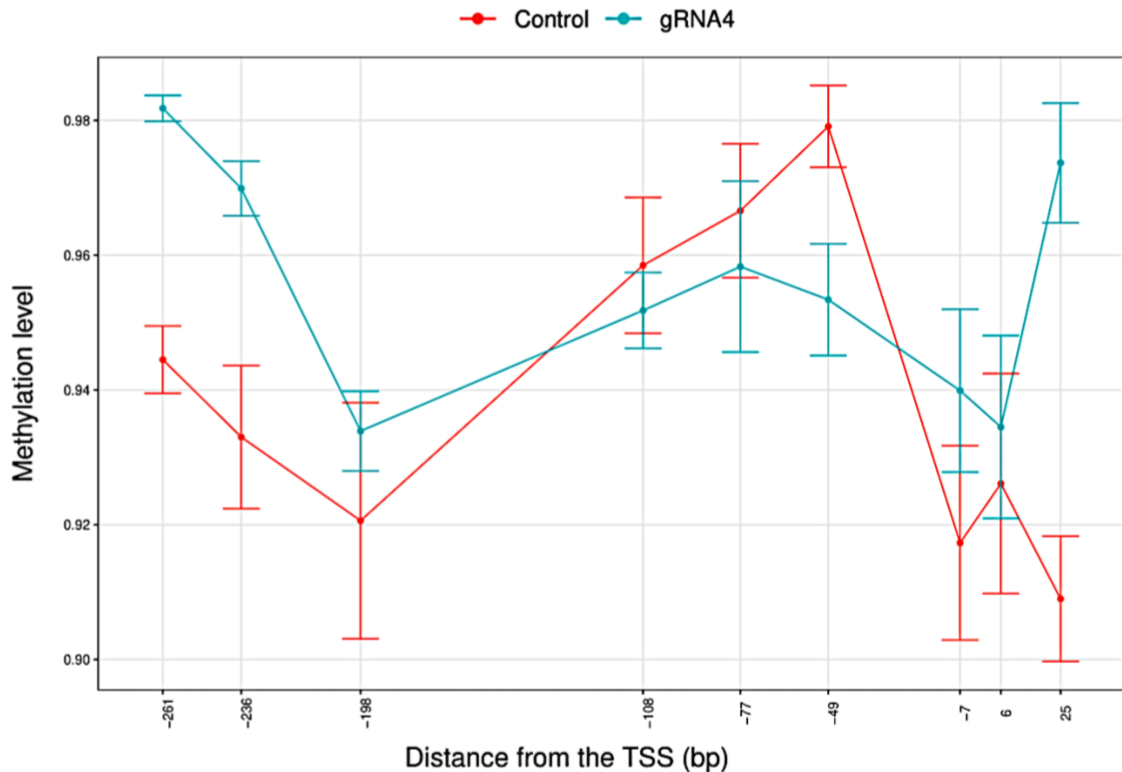

Figure 16. Plot of methylation levels in sgRNA4 microinjected samples compared to control. Significant increase in methylation levels is observed at -261bp and -236 bp in close proximity to sgRNA4 target site.

3.4.2 sgRNA5 vs Control

The sgRNA5 targeted at 206 bp downstream of the TSS. The two CpG sites in proximity to the guide injected site (-261bp and -236 bp) show a significant increase in the methylation status compared to the guide-uninjected samples. CpG at -198 bp, which is only 8 nucleotides apart from the guide attachment site, only showed a non-significant increase in methylation. A significant increase in methylation rate was seen at -7 bp and +25 bp CpG site, which are 211bp

and 244 bp downstream from the gRNA target site. However, the CpGs at -108 bp and -49 bp in the control samples also had a significantly higher methylation rate than the sgRNA5-injected samples.

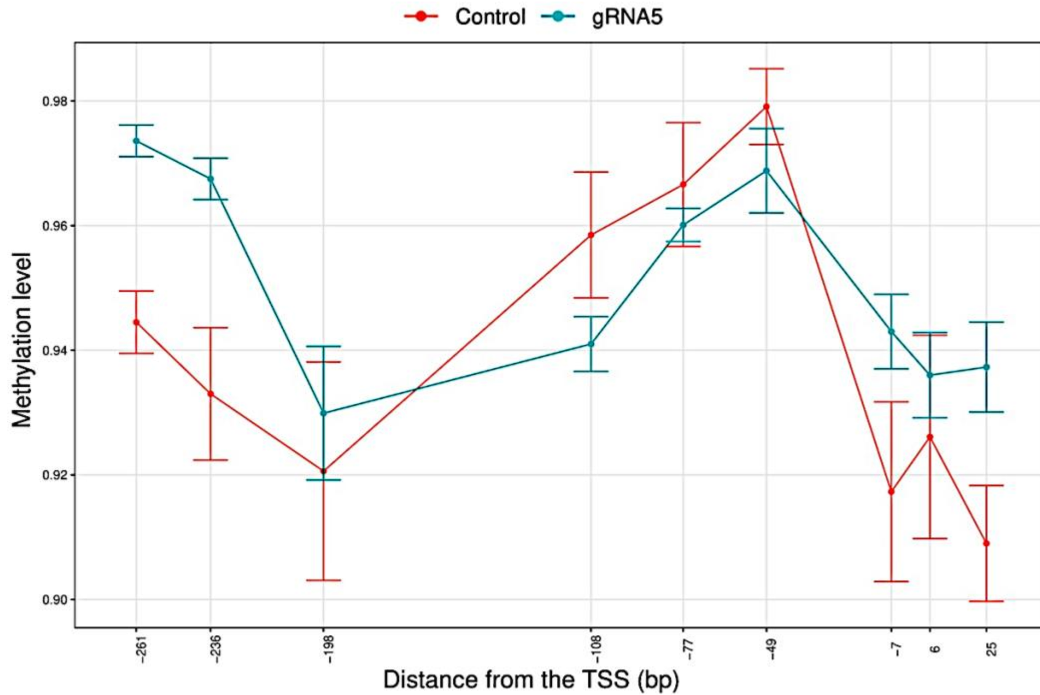

Figure 17. Plot of methylation levels in sgRNA5 microinjected samples compared to control. Observable increase in methylation level is observed in CpGs in close proximity to sgRNA5 target site.

3.4.3 sgRNA6 vs Control

The sgRNA6 targeted at region of the promoter containing CpG site at -77 bp downstream from TSS. The CpGs at -261 bp, -7 bp and+25 bp of TSS were significantly methylated compared to the guide-uninjected samples. Surprisingly, the CpG sites at positions -77bp, -49 bp and 6 bp had a significantly lower methylation status compared to the control.

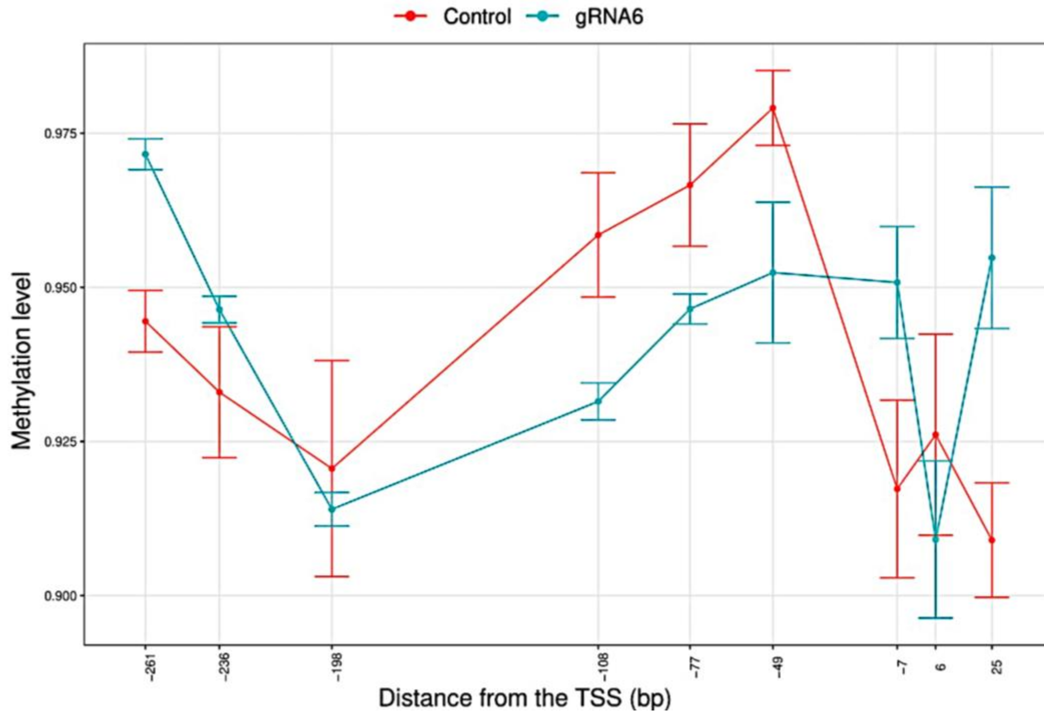

Figure 18. Plot of methylation levels in sgRNA6 microinjected samples compared to control. A noticeable decrease in methylation level was observed in CpGs in close proximity to sgRNA6 target site, however, CpGs at -7bp and 25 bp showed a significant increase in methylation.

In all three sgRNA injected samples, there was a significant increase in methylation level seen at -261 bp and +25 bp CpGs. This suggests an increase in methylation beyond targeted sites which could be secondary effects that occurred in response to alterations at the targeted sites.

CHAPTER IV: DISCUSSION

The main objective of the present study is to develop a DNA methylation editing tool using CRISPR-dCas9-Dnmt3aa system. Using this method, it will be possible to alter the DNA methylation status of a genomic region *in vivo*. The significance of the study lies in the possibility for altering the epigenetic landscape of the genome and studying epigenetic regulation of gene transcription in health and disease in any vertebrate. Literature suggests that an alteration in the epigenetic landscape can promote diseases in humans[17-22].Many xenobiotic chemicals have been implicated in introducing epimutations in the germline and their association with disease traits[23-37].

In vertebrates, the epigenetic landscape goes through 2 reprogramming windows before being established in the genome. First, the gametes undergo massive active demethylation following fertlization[64], which is restored before the blastula stage[66]. Literature suggests that in some cases, epimutations can escape this reprogramming stage in a similar fashion to imprinted loci[67] or can reset and reestablish later[96]. TEI has been observed over multiple generations in both animal and human models[94,95]. The alteration in DNA methylation patterns in differentially methylated regions (DMRs) has been proposed as a potential biomarker to investigate epigenetic inheritance of paternally or ancestrally established disease traits[89-93]. DNA methylation is one of the major epigenetic factors involved in gene regulation[12]. Second erasure and reestablishment of DNA methylation occurs before PGCs differentiate into sex-specific germ cells[66,68-70]. Reprogramming errors during this stage could result in an altered epigenetic landscape being programmed in differentiated germ cells such as spermatogonia and oogonia and transmitted to subsequent generations via gametes[72,73].

The CRISPR-Cas9 system has been widely used for epigenetic research since its discovery. CRISPR-dCas9, devoid of nuclease activity, has been utilized to introduce transcriptional activators and repressors both *in vivo* and *in vitro.* Transcriptional repressors such as *DNMT3a* and *DNMT3b* have already been used to introduce DNA methylation for gene suppression *in vitro*[53,54,55]. Although some epigenome editing studies concentrating on DNA methylation *in vivo* have also been conducted[97-99], there have been no studies using the CRISPR-dCas9 technology to modify transgenerational methylation marks *in vivo*. It is critical to establish *de novo* epimutations or eliminate existing ones using epigenome editing or other appropriate approaches to comprehend the function of epigenetic alterations in the initiation and progression of health and illness. To understand how successful the methylation marks introduced by CRISPR-dCas9-dnmt3aa are at bypassing the epigenetic reprogramming window, the present study targeted a regulatory region of the olvas gene in *olvas*-GFP transgenic medaka.

To fulfill the first aim of the study, an effector domain that would be effective in introducing DNA methylation into our model animal was chosen. Work previously done in the Bhandari Lab elucidated the presence of *de novo* methyltransferases *DNMT3AA* and *DNMT3BB.1* in all stages of early embryonic development[76]. *DNMT3AA* was for this study because of its active methylation activity when coupled with CRISPR-dcas9 as suggested in previous studies[53,54]. At least one study suggested edge of *DNMT3AA* over *DNMT3BB* with regard to CpG methylation *in vivo*[102]. Although fusing only the Cyt_C5_DNA_methylase super family present in the *DNMT3AA* catalytic domain for preliminary studies, the whole protein coding region containing the ADDz superfamily domain (ATRX, Dnmt3 and Dnmt3l PHD-like zinc finger domain) and the chromatin-association PWWP superfamily domain was used for this study following suit with previous studies[53,54]. ADDz superfamily domain consists of a cysteine-

rich domain that binds to the tail of histone H3, which is essential to stimulate the *de novo* DNA methylation activity of Dnmt3a[103]. The PWWP domain is involved in protein-protein interactions in DNA-binding proteins that function as transcription factors regulating developmental processes[104]. A gene fragment of length ~ 2kb was identified as the effector domain.

Continued trial to amplify the *DNMT3AA* from the medaka cDNA was inadvertently met with failure. Primers were designed to include 5-6 nucleotides complementary to the genome followed by switching a couple nucleotides to include EcoR*I* restriction site, and ATG start codon (in the forward primer), and Xho*I* restriction site and TAA stop codon (in the reverse primer) followed by a few nucleotides. To reiterate, primers were designed as follows:
5' Leader sequence (6 nt)---RE site (6nt)---Start/stop codon---Target gene sequence (6-11 nt) 3'
The PCR amplification, done using high-fidelity DNA polymerase, however, was not successful in isolating the effector domain of interest, with no lack of effort. We recognized that the problem could have been resolved using a longer target gene sequence between 12 to 18 bp on the 5' end of the primer[105]. The effector domain was hence chemically synthesized for insertion into the CRIPSR-dCas9 plasmid.

The plasmid-effector ligation /amplification process was fairly straightforward until the time for verification of the final synthesized plasmid. The problem persistent in this part was low quality/short Sanger sequencing reads and misread nucleotide sequences. During the first sequencing run, most of the samples were either not sequenced at all, had less than 100 bp reads or had a lot of nucleotide mismatch. The low quality and short read problem was resolved fairly simply by adding an additional cleanup step with 100% ethanol before sending off the plasmids for sequencing. Since even the samples that produced a result in the first sequencing run had a lot of nucleotide "mutations", we were led to believe these was a problem with our competent cells

in the bacterial transformation step. However, upon using multiple primers with significant overlap during the second sequencing run, these presumed mutations simply turned out to misread nucleotides towards the end of reads through analysis of the corresponding sequencing chromatograms.

Another factor to be considered while designing the study is the selection of the target region in the promoter of the gene of interest. DNA methylation is commonplace at a gene promoter region[106] and has been exhibited to inhibit DNA transcription by preventing the recruitment of RNA polymerases and/or other transcriptional components to the promoter region[107]. CpG dense regions present at gene promoters called CpG islands (CGIs). CGIs' ability to function as transcription start sites is closely related to the absence of DNA methylation at these sites, which has been evolutionarily conserved[108]. In the present study, we used the transgenic line of *Oryzias latipes* and its *vasa* gene coupled with GFP (*olvas*-GFP), where the GFP fluorescence is controlled by the regulatory regions of the *olvas* gene in the germ cells[100]. The *vasa* gene is effectively transcribed in PGCs where the promoter is hypomethylated[109]. Therefore, it's safe to assume the methylation status of the CpG sites in proximity to the transcriptional start site in *olvas*-GFP should be unmethylated. In addition, in medaka, the genome is globally hypomethylated during the first four cleavages after fertilization[76]. Of the 5.1 kb *olvas* promoter, including exon 1 and 2 and 3' region of *olvas* gene[79], 450 bp core promoter consists of 77 CpG sites. Targeting the CpG-rich promoter region of the *olvas* gene during these stages would help us understand the role of DNA methylation in transcriptional control of the gene, which is only transcribed in the hypomethylated promoter state.

The crRNAs to target the *olvas* promoter region were synthetically prepared using the CRISPOR[110,112-115] and Benchling[111] online software. The MITSpecScore, Off-target Count and

Mismatch Count obtained from the CRISPOR[110] software were taken into account(Table 3) to select the crRNAs. The study suggests using a MITSpecScore cutoff score of 70-80[112]. Hence, only guideRNA5 with a lower score of 69 was selected for the study to target the CpG sites on that region. The guides hence selected were further verified using Benchling. At least one study using CRISPR system in medaka selected gRNAs with 3 to 4 mismatch count[116] and a similar strategy was applied in this study. Other CRISPR studies in medaka used CHOPCHOP website [117,118] and CCtop CRISPR/Cas9 target online predictor[118,120] to design guide RNAs.

The next pivotal step in the study is the microinjection of the constructs into the embryos. In this study, we microinjected the prepared construct-guide RNA mix into the blastodisc region of the fertilized embryos at the 1-cell stage. Studies have suggested that microinjecting directly into the cytoplasm at 1-cell stage is the most effective technique in medaka[121,122]. It has also been emphasized that microinjection into eggs after the two-cell stage significantly decreases the effectiveness of injection unless the injection is performed into each cell individually[122]. Unlike zebrafish, microinjection into the yolk is rendered ineffective in medaka[121]. At least one previous study in medaka used electroporation for the transfection of nucleoplasmin into medaka cells effectively[116]. The use of phenol red as a tracer is also discouraged in practical experiments because of the possibility of it adding RNAses or clogging the microinjection needle. In this study, however, an effective, non-toxic dose of the CRISPR-dcas9-phenol red combination was established before injection in the research samples.

The experimental protocol was designed by dividing the promoter region in two regions: A and B, each targeted by 3 sgRNAs. The present study, however, only produced amplicon data for 3 sgRNAs targeting region B closer to the transcriptional start site (TSS), given ongoing complications with the amplification of promoter DNA beyond 320 bp downstream of TSS. The

problem could be resolved by characterizing the whole *olvas* promoter region by Sanger Sequencing followed by preparing primers specific to amplify the Region A (sgRNA1, sgRNA2 and sgRNA3) in future studies.

Furthermore, this study sequenced amplicons using both Sanger sequencing and miSeq technologies. We found almost all CpG sites to be methylated and no significant difference in methylation patterns among the experimental, negative, and positive control samples through Sanger sequencing. Contrary to what was expected, most of the targeted CpGs were hypermethylated even in MiSeq data. Pyrosequencing needs to be done in the whole presumed promoter region of the olvas gene to pinpoint unmethylated CPGs. However, the targeted CpGs were found to be significantly hypomethylated in the ovary (Figure 15). The expression of *olvas*-GFP gradually decreases after the establishment of PGCs, hence this hypomethylated state of ovary reinstates the importance hypomethylated state of *olvas* promoter for its expression. In the uninjected blastula samples, the only relatively hypomethylated CpGs were the farthest from the TSS; -261, -236 and -198 bp (Figure 14 and 16). Two of these three sites were significantly methylated with application of both sgRNA4 and sgRNA5. With both guides in close proximity, the methylation level at these sites could be increased further by using both the guide RNAs together because of the synergetic effect[133] of gRNAs on relatively unmethylated cytosine. Those two sites were also seen to be significantly methylated in sgRNA6 injected replicates, which could be because of proximity of sgRNA targeted site to that section of DNA because of chromatin arrangement. A study using CRISPR-dcas9-DNMT7CD for a similar *in vivo* study has suggested the methylation effect of dCas9-DNMT up to 400 bp away from the protospacer adjacent motif (PAM)[98] (Figure S4). Hence, similar assumption can be made about CpGs at -7bp and 25 bp which also have relatively higher methylation compared to the control in all three

sgRNA-injected samples. Further research needs to be done to summarize the dCas9-dnmt3aa activity in the *olvas* promoter region.

Even though, both Sanger sequencing and NGS employ fundamentally the same concept to identify nucleotide sequences, the critical difference between these technologies is the sequencing volume. NGS technologies like MiSeq sequence millions of fragments simultaneously hence providing higher sensitivity to detect low frequency variants[123]. This enables the identification of novel variants within samples. Sanger sequencing, on the other hand, reads include a single DNA fragment per sample. In the present study, each sample replicate contains bisulfite converted amplicon from 20 to 25 embryos at blastula stage which contain about 1000 cells[100] each. Since Sanger Sequencing provides data for only one read per replicate, it's safe to assume methylation patterns in the rest of the 20 to 25 thousand novel DNA would not be discovered. Therefore, it is evident that in studies like this, it is important to use NGS with higher sequencing depth for increased sensitivity[124] and discovery[125] of unique methylation patterns.

The GFP positive rate in all promoter-targeted embryos was significantly reduced compared to both the control and scrambled-sgRNA-injected embryos (Figure 13). Among the 6 sgRNAs, sgRNA5 has a slightly higher GFP-positive rate, which could be credited to its MITSpecScore being lower than the suggested cutoff score and a high off-target count. A smaller *DNMT3A* effector region (containing only Cyt_C5_DNA_methylase super family domain) significantly reduced the GFP positive rate by 25%. In contrast, in the present study, we designed CRISPR-dCas9-dnmt3aa containing all 3 super families (Cyt_C5_DNA_methylase , ADDz and PWWP) as previously discussed. Using the guides sgRNA4 and sgRNA6 separately to target approximately the same promoter region as the guides used in the previous study

(XWgRNA1 and XWgRNA2), significantly reduced the GFP positive rate by 75% and 67%, respectively, compared to scrambled gRNA injected samples. The MitSpecScore of both XWgRNA1:96 and XWgRNA2:93 is higher than sgRNA4:93 and sgRNA6:86, respectively. However, XWgRNA2:44 has a higher off-target count compared to sgRNA6:28. These data suggest the difference in GFP positive rates is probably not because of inefficiency of guide RNAs and we shift the focus towards the CRISPR plasmid used in these studies. This solidifies the importance of ADDz and PWWP domains in stimulating the *de novo* DNA methylation activity of *DNMT3AA*. Besides that, further research needs to be conducted to examine GFP positive rate in embryos injected with multiple guide RNAs.

In the present study, the results show a strong indication of CRISPR-dcas9-dnmt3aa's transcriptional silencing activity that can escape the reprogramming stages and persist in the offspring somatic genome. Our data suggests a negative correlation between introduced methylation and GFP expression. However, it is important to do further studies targeting the gene promoter with only CRISPR-dCas9 to analyze if our results are because of blockade caused by the cas9 protein. Such blockage could prevent methylating and demethylating enzymes necessary for normal growth thereby resulting in reduced GFP positive rate. Further studies using dCas9 paired with a dead DNMT3aa protein domain would also be necessary to verify the results in this study. It is also important to consider injection of dCas9-effector-guide combination at a different cell stage during early development since the medaka embryos are going through active demethylation until the 4-cell stage. In addition, since the methylation levels reach peak during Blastula, it is difficult to evaluate a methylation increase. Hence, the methylation levels should also be verified at later developmental stages. Furthermore, the impact of sgRNAs present within the cytoplasm on mitosis and its effect on the targeted genome through cell division should also be considered.

It is important to continue this study using other transcriptional repression complexes as effector proteins. Before selecting another transcriptional inhibitor, however, it is imperative to survey the histone acetylation and methylation in the *olvas* promoter loci by performing chromatin immunoprecipitation followed by high-throughput DNA sequencing (ChIP-seq). *HDAC* family of transcriptional inhibitors have been well-established to promote transcriptional repression alongside *DNMT3AA* in sequence-dependent synergetic fashion[126,127]. Studies have shown the function of CRISPR-dcas9-HDAC to significantly modulate gene expression *in vitro*[128,129]. Histone demethylases like *LSD1*[130], Histone methylases like *KRAB*[131] and Histone methyltransferase like *EZH2*[132] have also been fused with CRISPR-dCas9 for successful transcriptional repression *in vitro.* Moreover, using *DNMT3BB* alone or alongside *DNMT3AA* could also aid in successful transcriptional suppression of the *olvas* gene given *DNMT3BB*'s significant involvement in *de novo* methylation during early developmental stages in medaka embryo[76].

In the present study, we observed the first methylation peak -25 bp upstream from the PAM sequence, which is similar to other studies employing DNMT3a reporting peaks at +25 bp downstream[137], +27 bp downstream[54] and +40 bp downstream[98] respectively. However, it is important to note that none of the above studied the effect of CRISPRdCas9-dnmt on DNA methylation during active epigenetic reprogramming window. Here, we showed the phenotypic and methylation-level alteration of *olvas* gene during the early cleavage stage in medaka. Hence, our study sets a benchmark for future studies to develop epigenetic interference tools harnessing the simplicity of CRISPR-Cas9 to further understand the phenotypic and epigenome-level effects of early development epigenetic alteration and the huge significance that entails.

REFERENCES

1 Waddington C. H. (1968). Towards a theoretical biology. *Nature*, *218*(5141), 525–527. https://doi.org/10.1038/218525a0
2 Dupont, C., Armant, D. R., & Brenner, C. A. (2009). Epigenetics: definition, mechanisms and clinical perspective. *Seminars in reproductive medicine*, *27*(5), 351–357. https://doi.org/10.1055/s-0029-1237423
3 Wu Ct, & Morris, J. R. (2001). Genes, genetics, and epigenetics: a correspondence. *Science (New York, N.Y.)*, *293*(5532), 1103–1105. https://doi.org/10.1126/science.293.5532.1103
4 Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., Funke, R., Gage, D., Harris, K., Heaford, A., Howland, J., Kann, L., Lehoczky, J., LeVine, R., McEwan, P., McKernan, K., … International Human Genome Sequencing Consortium (2001). Initial sequencing and analysis of the human genome. *Nature*, *409*(6822), 860–921. https://doi.org/10.1038/35057062
5 Nan, X., Ng, H. H., Johnson, C. A., Laherty, C. D., Turner, B. M., Eisenman, R. N., & Bird, A. (1998). Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature*, *393*(6683), 386–389. https://doi.org/10.1038/30764
6 Dantas Machado, A. C., Zhou, T., Rao, S., Goel, P., Rastogi, C., Lazarovici, A., Bussemaker, H. J., & Rohs, R. (2015). Evolving insights on how cytosine methylation affects protein-DNA binding. *Briefings in functional genomics*, *14*(1), 61–73. https://doi.org/10.1093/bfgp/elu040
7 Ciechomska M, Roszkowski L, Maslinski W. DNA Methylation as a Future Therapeutic and Diagnostic Target in Rheumatoid Arthritis. *Cells*. 2019; 8(9):953. https://doi.org/10.3390/cells8090953
8 Holliday R. (1990). DNA methylation and epigenetic inheritance. Philosophical transactions of the Royal Society of London. Series B, Biological sciences, 326(1235), 329–338. https://doi.org/10.1098/rstb.1990.0015
9 Holliday, R., & Pugh, J. E. (1975). DNA modification mechanisms and gene activity during development. *Science (New York, N.Y.)*, *187*(4173), 226–232.
10 Bannister, A. J., & Kouzarides, T. (2011). Regulation of chromatin by histone modifications. *Cell research*, *21*(3), 381–395. https://doi.org/10.1038/cr.2011.22
11 Jaenisch, R., & Bird, A. (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nature genetics, 33 Suppl, 245–254. https://doi.org/10.1038/ng1089
12 Moore, L. D., Le, T., & Fan, G. (2013). DNA methylation and its basic function. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, *38*(1), 23–38. https://doi.org/10.1038/npp.2012.112
13 Jenuwein, T., & Allis, C. D. (2001). Translating the histone code. *Science (New York, N.Y.)*, *293*(5532), 1074–1080. https://doi.org/10.1126/science.1063127
14 Martin, C., & Zhang, Y. (2005). The diverse functions of histone lysine methylation. *Nature reviews. Molecular cell biology*, *6*(11), 838–849. https://doi.org/10.1038/nrm1761
15 Moazed D. (2001). Common themes in mechanisms of gene silencing. *Molecular cell*, *8*(3), 489–498. https://doi.org/10.1016/s1097-2765(01)00340-9
16 Valencia-Sanchez, M. A., Liu, J., Hannon, G. J., & Parker, R. (2006). Control of translation and mRNA degradation by miRNAs and siRNAs. *Genes & development*, *20*(5), 515–524. https://doi.org/10.1101/gad.1399806

17 Zoghbi, H. Y., & Beaudet, A. L. (2016). Epigenetics and Human Disease. Cold Spring Harbor perspectives in biology, 8(2), a019497. https://doi.org/10.1101/cshperspect.a019497
18 Greer, J. M., & McCombe, P. A. (2012). The role of epigenetic mechanisms and processes in autoimmune disorders. Biologics : targets & therapy, 6, 307–327. https://doi.org/10.2147/BTT.S24067
19 Jones, P., Baylin, S. The fundamental role of epigenetic events in cancer. Nat Rev Genet 3, 415–428 (2002). https://doi.org/10.1038/nrg816
20 Ordovás, J. M., & Smith, C. E. (2010). Epigenetics and cardiovascular disease. Nature reviews. Cardiology, 7(9), 510–519. https://doi.org/10.1038/nrcardio.2010.104
21 Jakovcevski, M., & Akbarian, S. (2012). Epigenetic mechanisms in neurological disease. Nature medicine, 18(8), 1194–1204. https://doi.org/10.1038/nm.2828
22 Koo, J. W., Mazei-Robison, M. S., LaPlant, Q., Egervari, G., Braunscheidel, K. M., Adank, D. N., Ferguson, D., Feng, J., Sun, H., Scobie, K. N., Damez-Werno, D. M., Ribeiro, E., Peña, C. J., Walker, D., Bagot, R. C., Cahill, M. E., Anderson, S. A., Labonté, B., Hodes, G. E., Browne, H., … Nestler, E. J. (2015). Epigenetic basis of opiate suppression of Bdnf gene expression in the ventral tegmental area. Nature neuroscience, 18(3), 415–422. https://doi.org/10.1038/nn.3932
23 Kang, S. C., & Lee, B. M. (2005). DNA methylation of estrogen receptor alpha gene by phthalates. *Journal of toxicology and environmental health. Part A*, *68*(23-24), 1995–2003. https://doi.org/10.1080/15287390491008913
24 Wu, S., Zhu, J., Li, Y., Lin, T., Gan, L., Yuan, X., Xu, M., & Wei, G. (2010). Dynamic effect of di-2-(ethylhexyl) phthalate on testicular toxicity: epigenetic changes and their impact on gene expression. *International journal of toxicology*, *29*(2), 193–200. https://doi.org/10.1177/1091581809355488
25 Weng, Y. I., Hsu, P. Y., Liyanarachchi, S., Liu, J., Deatherage, D. E., Huang, Y. W., Zuo, T., Rodriguez, B., Lin, C. H., Cheng, A. L., & Huang, T. H. (2010). Epigenetic influences of low-dose bisphenol A in primary human breast epithelial cells. *Toxicology and applied pharmacology*, *248*(2), 111–121. https://doi.org/10.1016/j.taap.2010.07.014
26 Avissar-Whiting, M., Veiga, K. R., Uhl, K. M., Maccani, M. A., Gagne, L. A., Moen, E. L., & Marsit, C. J. (2010). Bisphenol A exposure leads to specific microRNA alterations in placental cells. *Reproductive toxicology (Elmsford, N.Y.)*, *29*(4), 401–406. https://doi.org/10.1016/j.reprotox.2010.04.004
27 Doherty, L. F., Bromer, J. G., Zhou, Y., Aldad, T. S., & Taylor, H. S. (2010). In utero exposure to diethylstilbestrol (DES) or bisphenol-A (BPA) increases EZH2 expression in the mammary gland: an epigenetic mechanism linking endocrine disruptors to breast cancer. *Hormones & cancer*, *1*(3), 146–155. https://doi.org/10.1007/s12672-010-0015-9
28 Rusiecki, J. A., Beane Freeman, L. E., Bonner, M. R., Alexander, M., Chen, L., Andreotti, G., Barry, K. H., Moore, L. E., Byun, H. M., Kamel, F., Alavanja, M., Hoppin, J. A., & Baccarelli, A. (2017). High pesticide exposure events and DNA methylation among pesticide applicators in the agricultural health study. *Environmental and molecular mutagenesis*, *58*(1), 19–29. https://doi.org/10.1002/em.22067
29 Rusiecki, J. A., Baccarelli, A., Bollati, V., Tarantini, L., Moore, L. E., & Bonefeld-Jorgensen, E. C. (2008). Global DNA hypomethylation is associated with high serum-persistent organic pollutants in Greenlandic Inuit. *Environmental health perspectives*, *116*(11), 1547–1552. https://doi.org/10.1289/ehp.11338

30 Song, C., Kanthasamy, A., Anantharam, V., Sun, F., & Kanthasamy, A. G. (2010). Environmental neurotoxic pesticide increases histone acetylation to promote apoptosis in dopaminergic neuronal cells: relevance to epigenetic mechanisms of neurodegeneration. *Molecular pharmacology*, *77*(4), 621–632. https://doi.org/10.1124/mol.109.062174
31 Song, C., Kanthasamy, A., Jin, H., Anantharam, V., & Kanthasamy, A. G. (2011). Paraquat induces epigenetic changes by promoting histone acetylation in cell culture models of dopaminergic degeneration. *Neurotoxicology*, *32*(5), 586–595. https://doi.org/10.1016/j.neuro.2011.05.018
32 Chen, H., Li, S., Liu, J., Diwan, B. A., Barrett, J. C., & Waalkes, M. P. (2004). Chronic inorganic arsenic exposure induces hepatic global and individual gene hypomethylation: implications for arsenic hepatocarcinogenesis. *Carcinogenesis*, *25*(9), 1779–1786. https://doi.org/10.1093/carcin/bgh161
33 Marsit, C. J., Eddy, K., & Kelsey, K. T. (2006). MicroRNA responses to cellular stress. *Cancer research*, *66*(22), 10843–10848. https://doi.org/10.1158/0008-5472.CAN-06-1894
34 Zhao, C. Q., Young, M. R., Diwan, B. A., Coogan, T. P., & Waalkes, M. P. (1997). Association of arsenic-induced malignant transformation with DNA hypomethylation and aberrant gene expression. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(20), 10907–10912. https://doi.org/10.1073/pnas.94.20.10907
35 Chanda, S., Dasgupta, U. B., Guhamazumder, D., Gupta, M., Chaudhuri, U., Lahiri, S., Das, S., Ghosh, N., & Chatterjee, D. (2006). DNA hypermethylation of promoter of gene p53 and p16 in arsenic-exposed people with and without malignancy. *Toxicological sciences : an official journal of the Society of Toxicology*, *89*(2), 431–437. https://doi.org/10.1093/toxsci/kfj030
36 Christensen, B. C., Houseman, E. A., Godleski, J. J., Marsit, C. J., Longacker, J. L., Roelofs, C. R., Karagas, M. R., Wrensch, M. R., Yeh, R. F., Nelson, H. H., Wiemels, J. L., Zheng, S., Wiencke, J. K., Bueno, R., Sugarbaker, D. J., & Kelsey, K. T. (2009). Epigenetic profiles distinguish pleural mesothelioma from normal pleura and predict lung asbestos burden and clinical outcome. *Cancer research*, *69*(1), 227–234. https://doi.org/10.1158/0008-5472.CAN-08-2586
37 Kirschner, M. B., Cheng, Y. Y., Armstrong, N. J., Lin, R. C., Kao, S. C., Linton, A., Klebe, S., McCaughan, B. C., van Zandwijk, N., & Reid, G. (2015). MiR-score: a novel 6-microRNA signature that predicts survival outcomes in patients with malignant pleural mesothelioma. *Molecular oncology*, *9*(3), 715–726. https://doi.org/10.1016/j.molonc.2014.11.007
38 Cornacchia, E., Golbus, J., Maybaum, J., Strahler, J., Hanash, S., & Richardson, B. (1988). Hydralazine and procainamide inhibit T cell DNA methylation and induce autoreactivity. *Journal of immunology (Baltimore, Md. : 1950)*, *140*(7), 2197–2200.
39 Arce, C., Segura-Pacheco, B., Perez-Cardenas, E., Taja-Chayeb, L., Candelaria, M., & Dueñnas-Gonzalez, A. (2006). Hydralazine target: from blood vessels to the epigenome. *Journal of translational medicine*, *4*, 10. https://doi.org/10.1186/1479-5876-4-10
40 Phiel, C. J., Zhang, F., Huang, E. Y., Guenther, M. G., Lazar, M. A., & Klein, P. S. (2001). Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. *The Journal of biological chemistry*, *276*(39), 36734–36741. https://doi.org/10.1074/jbc.M101287200

41 Rachoń D. (2015). Endocrine disrupting chemicals (EDCs) and female cancer: Informing the patients. *Reviews in endocrine & metabolic disorders*, *16*(4), 359–364. https://doi.org/10.1007/s11154-016-9332-9

42 Yang, C., Lee, H. K., Kong, A. P. S., Lim, L. L., Cai, Z., & Chung, A. C. K. (2018). Early-life exposure to endocrine disrupting chemicals associates with childhood obesity. *Annals of pediatric endocrinology & metabolism*, *23*(4), 182–195. https://doi.org/10.6065/apem.2018.23.4.182

43 Baccarelli, A., & Bollati, V. (2009). Epigenetics and environmental chemicals. Current opinion in pediatrics, 21(2), 243–251. https://doi.org/10.1097/mop.0b013e32832925cc

44 Stenz, L., Schechter, D. S., Serpa, S. R., & Paoloni-Giacobino, A. (2018). Intergenerational Transmission of DNA Methylation Signatures Associated with Early Life Stress. *Current genomics*, *19*(8), 665–675. https://doi.org/10.2174/1389202919666171229145656

45 Tobi, E. W., Goeman, J. J., Monajemi, R., Gu, H., Putter, H., Zhang, Y., Slieker, R. C., Stok, A. P., Thijssen, P. E., Müller, F., van Zwet, E. W., Bock, C., Meissner, A., Lumey, L. H., Eline Slagboom, P., & Heijmans, B. T. (2014). DNA methylation signatures link prenatal famine exposure to growth and metabolism. *Nature communications*, *5*, 5592. https://doi.org/10.1038/ncomms6592

46 Bhandari, R. K., vom Saal, F. S., & Tillitt, D. E. (2015). Transgenerational effects from early developmental exposures to bisphenol A or 17α-ethinylestradiol in medaka, Oryzias latipes. *Scientific reports*, *5*, 9303. https://doi.org/10.1038/srep09303

47 Franklin, T. B., Linder, N., Russig, H., Thöny, B., & Mansuy, I. M. (2011). Influence of early stress on social abilities and serotonergic functions across generations in mice. *PloS one*, *6*(7), e21842. https://doi.org/10.1371/journal.pone.0021842

48 Crews, D., Gillette, R., Scarpino, S. V., Manikkam, M., Savenkova, M. I., & Skinner, M. K. (2012). Epigenetic transgenerational inheritance of altered stress responses. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(23), 9143–9148. https://doi.org/10.1073/pnas.1118514109

49 Matthews, S. G., & Phillips, D. I. (2012). Transgenerational inheritance of stress pathology. *Experimental neurology*, *233*(1), 95–101. https://doi.org/10.1016/j.expneurol.2011.01.009

50 Huang, YH., Su, J., Lei, Y. et al. DNA epigenome editing using CRISPR-Cas SunTag-directed DNMT3A. Genome Biol 18, 176 (2017). https://doi.org/10.1186/s13059-017-1306-z

51 Morita, S., Noguchi, H., Horii, T. et al. Targeted DNA demethylation in vivo using dCas9–peptide repeat and scFv–TET1 catalytic domain fusions. Nat Biotechnol 34, 1060–1065 (2016). https://doi.org/10.1038/nbt.3658

52 Kwon, D., Zhao, YT., Lamonica, J. et al. Locus-specific histone deacetylation using a synthetic CRISPR-Cas9-based HDAC. Nat Commun 8, 15315 (2017). https://doi.org/10.1038/ncomms15315

53 Liu, X. S., Wu, H., Ji, X., Stelzer, Y., Wu, X., Czauderna, S., Shu, J., Dadon, D., Young, R. A., & Jaenisch, R. (2016). Editing DNA Methylation in the Mammalian Genome. *Cell*, *167*(1), 233–247.e17. https://doi.org/10.1016/j.cell.2016.08.056

54 Aleksandar Vojta, Paula Dobrinić, Vanja Tadić, Luka Bočkor, Petra Korać, Boris Julg, Marija Klasić, Vlatka Zoldoš, Repurposing the CRISPR-Cas9 system for targeted DNA methylation, Nucleic Acids Research, Volume 44, Issue 12, 8 July 2016, Pages 5615–5628, https://doi.org/10.1093/nar/gkw159

55 Lin, L., Liu, Y., Xu, F., Huang, J., Daugaard, T. F., Petersen, T. S., Hansen, B., Ye, L., Zhou, Q., Fang, F., Yang, L., Li, S., Fløe, L., Jensen, K. T., Shrock, E., Chen, F., Yang, H., Wang, J., Liu, X., Xu, X., … Luo, Y. (2018). Genome-wide determination of on-target and off-target characteristics for RNA-guided DNA methylation by dCas9 methyltransferases. GigaScience, 7(3), 1–19. https://doi.org/10.1093/gigascience/giy011

56 Maeder, M. L., Linder, S. J., Cascio, V. M., Fu, Y., Ho, Q. H., & Joung, J. K. (2013). CRISPR RNA-guided activation of endogenous human genes. *Nature methods*, *10*(10), 977–979. https://doi.org/10.1038/nmeth.2598

57 Gilbert, L. A., Larson, M. H., Morsut, L., Liu, Z., Brar, G. A., Torres, S. E., Stern-Ginossar, N., Brandman, O., Whitehead, E. H., Doudna, J. A., Lim, W. A., Weissman, J. S., & Qi, L. S. (2013). CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. Cell, 154(2), 442–451. https://doi.org/10.1016/j.cell.2013.06.044

58 Perez-Pinera, P., Kocak, D. D., Vockley, C. M., Adler, A. F., Kabadi, A. M., Polstein, L. R., Thakore, P. I., Glass, K. A., Ousterout, D. G., Leong, K. W., Guilak, F., Crawford, G. E., Reddy, T. E., & Gersbach, C. A. (2013). RNA-guided gene activation by CRISPR-Cas9-based transcription factors. Nature methods, 10(10), 973–976. https://doi.org/10.1038/nmeth.2600

59 Tang, W. Y., & Ho, S. M. (2007). Epigenetic reprogramming and imprinting in origins of disease. *Reviews in endocrine & metabolic disorders*, *8*(2), 173–182. https://doi.org/10.1007/s11154-007-9042-4

60 Hackett, J. A., & Surani, M. A. (2013). Beyond DNA: programming and inheritance of parental methylomes. *Cell*, *153*(4), 737–739. https://doi.org/10.1016/j.cell.2013.04.044

61 Saitou, M., Kagiwada, S., & Kurimoto, K. (2012). Epigenetic reprogramming in mouse pre-implantation development and primordial germ cells. *Development (Cambridge, England)*, *139*(1), 15–31. https://doi.org/10.1242/dev.050849

62 Rougier, N., Bourc'his, D., Gomes, D. M., Niveleau, A., Plachot, M., Pàldi, A., & Viegas-Péquignot, E. (1998). Chromosome methylation patterns during mammalian preimplantation development. *Genes & development*, *12*(14), 2108–2113. https://doi.org/10.1101/gad.12.14.2108

63 Iqbal, K., Jin, S. G., Pfeifer, G. P., & Szabó, P. E. (2011). Reprogramming of the paternal genome upon fertilization involves genome-wide oxidation of 5-methylcytosine. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(9), 3642–3647. https://doi.org/10.1073/pnas.1014033108

64 Wang, L., Zhang, J., Duan, J., Gao, X., Zhu, W., Lu, X., Yang, L., Zhang, J., Li, G., Ci, W., Li, W., Zhou, Q., Aluru, N., Tang, F., He, C., Huang, X., & Liu, J. (2014). Programming and inheritance of parental DNA methylomes in mammals. *Cell*, *157*(4), 979–991. https://doi.org/10.1016/j.cell.2014.04.017

65 Guo, H., Zhu, P., Yan, L., Li, R., Hu, B., Lian, Y., Yan, J., Ren, X., Lin, S., Li, J., Jin, X., Shi, X., Liu, P., Wang, X., Wang, W., Wei, Y., Li, X., Guo, F., Wu, X., Fan, X., … Qiao, J. (2014). The DNA methylation landscape of human early embryos. *Nature*, *511*(7511), 606–610. https://doi.org/10.1038/nature13544

66 Wang, X., & Bhandari, R. K. (2020). The dynamics of DNA methylation during epigenetic reprogramming of primordial germ cells in medaka (*Oryzias latipes*). *Epigenetics*, *15*(5), 483–498. https://doi.org/10.1080/15592294.2019.1695341

67 Ben Maamar, M., Sadler-Riggleman, I., Beck, D., McBirney, M., Nilsson, E., Klukovich, R., Xie, Y., Tang, C., Yan, W., & Skinner, M. K. (2018). Alterations in sperm DNA methylation, non-coding RNA expression, and histone retention mediate vinclozolin-induced epigenetic transgenerational inheritance of disease. *Environmental epigenetics*, *4*(2), dvy010. https://doi.org/10.1093/eep/dvy010

68 Seisenberger, S., Andrews, S., Krueger, F., Arand, J., Walter, J., Santos, F., Popp, C., Thienpont, B., Dean, W., & Reik, W. (2012). The dynamics of genome-wide DNA methylation reprogramming in mouse primordial germ cells. *Molecular cell*, *48*(6), 849–862. https://doi.org/10.1016/j.molcel.2012.11.001

69 Sasaki, H., & Matsui, Y. (2008). Epigenetic events in mammalian germ-cell development: reprogramming and beyond. *Nature reviews. Genetics*, *9*(2), 129–140. https://doi.org/10.1038/nrg2295

70 Fulka, H., Mrazek, M., Tepla, O., & Fulka, J., Jr (2004). DNA methylation pattern in human zygotes and developing embryos. *Reproduction (Cambridge, England)*, *128*(6), 703–708. https://doi.org/10.1530/rep.1.00217

71 Skinner M. K. (2007). Epigenetic transgenerational toxicology and germ cell disease. *International journal of andrology*, *30*(4), 393–397. https://doi.org/10.1111/j.1365-2605.2007.00796.x

72 Morgan, H. D., Sutherland, H. G., Martin, D. I., & Whitelaw, E. (1999). Epigenetic inheritance at the agouti locus in the mouse. *Nature genetics*, *23*(3), 314–318. https://doi.org/10.1038/15490

73 Skinner, M. K., Guerrero-Bosagna, C., Haque, M., Nilsson, E., Bhandari, R., & McCarrey, J. R. (2013). Environmentally induced transgenerational epigenetic reprogramming of primordial germ cells and the subsequent germ line. *PloS one*, *8*(7), e66318. https://doi.org/10.1371/journal.pone.0066318

74 O'Neill C. (2013). Lessons from zebrafish on reprogramming the epigenetic code after fertilisation. Asian journal of andrology, 15(5), 582–583. https://doi.org/10.1038/aja.2013.91

75 Wang, X., & Bhandari, R. K. (2020). DNA methylation reprogramming in medaka fish, a promising animal model for environmental epigenetics research. *Environmental epigenetics*, *6*(1), dvaa008. https://doi.org/10.1093/eep/dvaa008

76 Wang, X., & Bhandari, R. K. (2019). DNA methylation dynamics during epigenetic reprogramming of medaka embryo. Epigenetics, 14(6), 611–622. https://doi.org/10.1080/15592294.2019.1605816

77 Razin, A., & Cedar, H. (1991). DNA methylation and gene expression. Microbiological reviews, 55(3), 451–458. https://doi.org/10.1128/mr.55.3.451-458.1991

78 Felix, J. F., & Cecil, C. (2019). Population DNA methylation studies in the Developmental Origins of Health and Disease (DOHaD) framework. *Journal of developmental origins of health and disease*, *10*(3), 306–313. https://doi.org/10.1017/S2040174418000442

79 Tanaka, M., Kinoshita, M., Kobayashi, D., & Nagahama, Y. (2001). Establishment of medaka (Oryzias latipes) transgenic lines with the expression of green fluorescent protein fluorescence exclusively in germ cells: a useful model to monitor germ cells in a live vertebrate. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(5), 2544–2549. https://doi.org/10.1073/pnas.041315498

80 Huang, H., Zhang, W., Zhang, J., Zhao, A., & Jiang, H. (2023). Epigenome editing based on CRISPR/dCas9^{p300} facilitates transdifferentiation of human fibroblasts into Leydig-like cells. *Experimental cell research*, *425*(2), 113551. https://doi.org/10.1016/j.yexcr.2023.113551

81 Gemberling, M. P., Siklenka, K., Rodriguez, E., Tonn-Eisinger, K. R., Barrera, A., Liu, F., Kantor, A., Li, L., Cigliola, V., Hazlett, M. F., Williams, C. A., Bartelt, L. C., Madigan, V. J., Bodle, J. C., Daniels, H., Rouse, D. C., Hilton, I. B., Asokan, A., Ciofani, M., Poss, K. D., … Gersbach, C. A. (2021). Transgenic mice for in vivo epigenome editing with CRISPR-based systems. *Nature methods*, *18*(8), 965–974. https://doi.org/10.1038/s41592-021-01207-2

82 Wen, X., Zhang, Y., Cheng, H., An, J., Guo, Y., Wang, L., & Wang, M. (2021). A CRISPR/dCas9-assisted system to clone toxic genes in Escherichia coli. *Biochimica et biophysica acta. General subjects*, *1865*(11), 129994. https://doi.org/10.1016/j.bbagen.2021.129994

83 Anway, M. D., Memon, M. A., Uzumcu, M., & Skinner, M. K. (2006). Transgenerational effect of the endocrine disruptor vinclozolin on male spermatogenesis. *Journal of andrology*, *27*(6), 868–879. https://doi.org/10.2164/jandrol.106.000349

84 Nilsson, E., Larsen, G., Manikkam, M., Guerrero-Bosagna, C., Savenkova, M. I., & Skinner, M. K. (2012). Environmentally induced epigenetic transgenerational inheritance of ovarian disease. *PloS one*, *7*(5), e36129. https://doi.org/10.1371/journal.pone.0036129

85 Manikkam, M., Tracey, R., Guerrero-Bosagna, C., & Skinner, M. K. (2013). Plastics derived endocrine disruptors (BPA, DEHP and DBP) induce epigenetic transgenerational inheritance of obesity, reproductive disease and sperm epimutations. *PloS one*, *8*(1), e55387. https://doi.org/10.1371/journal.pone.0055387

86 Bhandari, R. K., vom Saal, F. S., & Tillitt, D. E. (2015). Transgenerational effects from early developmental exposures to bisphenol A or 17α-ethinylestradiol in medaka, Oryzias latipes. *Scientific reports*, *5*, 9303. https://doi.org/10.1038/srep09303

87 Chakraborty, S., Dissanayake, M., Godwin, J., Wang, X., & Bhandari, R. K. (2023). Ancestral BPA exposure caused defects in the liver of medaka for four generations. *The Science of the total environment*, *856*(Pt 1), 159067. https://doi.org/10.1016/j.scitotenv.2022.159067

88 Dabeer, S., Afjal, M. A., Ahmad, S., Fatima, M., Habib, H., Parvez, S., & Raisuddin, S. (2020). Transgenerational effect of parental obesity and chronic parental bisphenol A exposure on hormonal profile and reproductive organs of preadolescent Wistar rats of F1 generation: A one-generation study. *Human & experimental toxicology*, *39*(1), 59–76. https://doi.org/10.1177/0960327119873017

89 Manikkam, M., Guerrero-Bosagna, C., Tracey, R., Haque, M. M., & Skinner, M. K. (2012). Transgenerational actions of environmental compounds on reproductive disease and identification of epigenetic biomarkers of ancestral exposures. *PloS one*, *7*(2), e31901. https://doi.org/10.1371/journal.pone.0031901

90 Thorson, J. L. M., Beck, D., Ben Maamar, M., Nilsson, E. E., & Skinner, M. K. (2021). Ancestral plastics exposure induces transgenerational disease-specific sperm epigenome-wide association biomarkers. *Environmental epigenetics*, *7*(1), dvaa023. https://doi.org/10.1093/eep/dvaa023

91 Ben Maamar, M., Nilsson, E., Thorson, J. L. M., Beck, D., & Skinner, M. K. (2021). Transgenerational disease specific epigenetic sperm biomarkers after ancestral exposure to dioxin. *Environmental research*, *192*, 110279. https://doi.org/10.1016/j.envres.2020.110279

92 Luján, S., Caroppo, E., Niederberger, C., Arce, J. C., Sadler-Riggleman, I., Beck, D., Nilsson, E., & Skinner, M. K. (2019). Sperm DNA Methylation Epimutation Biomarkers for Male Infertility and FSH Therapeutic Responsiveness. *Scientific reports*, *9*(1), 16786. https://doi.org/10.1038/s41598-019-52903-1

93 Garrido, N., Cruz, F., Egea, R. R., Simon, C., Sadler-Riggleman, I., Beck, D., Nilsson, E., Ben Maamar, M., & Skinner, M. K. (2021). Sperm DNA methylation epimutation biomarker for paternal offspring autism susceptibility. *Clinical epigenetics*, *13*(1), 6. https://doi.org/10.1186/s13148-020-00995-2

94 Pembrey, M. E., Bygren, L. O., Kaati, G., Edvinsson, S., Northstone, K., Sjöström, M., Golding, J., & ALSPAC Study Team (2006). Sex-specific, male-line transgenerational responses in humans. *European journal of human genetics : EJHG*, *14*(2), 159–166. https://doi.org/10.1038/sj.ejhg.5201538

95 Carone, B. R., Fauquier, L., Habib, N., Shea, J. M., Hart, C. E., Li, R., Bock, C., Li, C., Gu, H., Zamore, P. D., Meissner, A., Weng, Z., Hofmann, H. A., Friedman, N., & Rando, O. J. (2010). Paternally induced transgenerational environmental reprogramming of metabolic gene expression in mammals. *Cell*, *143*(7), 1084–1096. https://doi.org/10.1016/j.cell.2010.12.008

96 Nilsson, E. E., Sadler-Riggleman, I., & Skinner, M. K. (2018). Environmentally induced epigenetic transgenerational inheritance of disease. *Environmental epigenetics*, *4*(2), dvy016. https://doi.org/10.1093/eep/dvy016

97 Lei, Y., Zhang, X., Su, J., Jeong, M., Gundry, M. C., Huang, Y. H., Zhou, Y., Li, W., & Goodell, M. A. (2017). Targeted DNA methylation in vivo using an engineered dCas9-MQ1 fusion protein. *Nature communications*, *8*, 16026. https://doi.org/10.1038/ncomms16026

98 Liang, F., Dong, Z., Ye, J., Hu, W., Bhandari, R. K., Mai, K., & Wang, X. (2023). *In vivo* DNA methylation editing in zebrafish. *Epigenetics*, *18*(1), 2192326. https://doi.org/10.1080/15592294.2023.2192326

99 Morita, S., Noguchi, H., Horii, T., Nakabayashi, K., Kimura, M., Okamura, K., Sakai, A., Nakashima, H., Hata, K., Nakashima, K., & Hatada, I. (2016). Targeted DNA demethylation in vivo using dCas9-peptide repeat and scFv-TET1 catalytic domain fusions. *Nature biotechnology*, *34*(10), 1060–1065. https://doi.org/10.1038/nbt.3658

100 Iwamatsu T. (2004). Stages of normal development in the medaka Oryzias latipes. *Mechanisms of development*, *121*(7-8), 605–618. https://doi.org/10.1016/j.mod.2004.03.012

101 Gambardella, J., Kansakar, U., Sardu, C., Messina, V., Jankauskas, S. S., Marfella, R., Maggi, P., Wang, X., Mone, P., Paolisso, G., Sorriento, D., & Santulli, G. (2023). Exosomal miR-145 and miR-885 Regulate Thrombosis in COVID-19. *The Journal of pharmacology and experimental therapeutics*, *384*(1), 109–115. https://doi.org/10.1124/jpet.122.001209

102 Oka, M., Rodić, N., Graddy, J., Chang, L. J., & Terada, N. (2006). CpG sites preferentially methylated by Dnmt3a in vivo. *The Journal of biological chemistry*, *281*(15), 9901–9908. https://doi.org/10.1074/jbc.M511100200

103 Dhayalan, A., Tamas, R., Bock, I., Tattermusch, A., Dimitrova, E., Kudithipudi, S., Ragozin, S., & Jeltsch, A. (2011). The ATRX-ADD domain binds to H3 tail peptides and reads the combined methylation state of K4 and K9. *Human molecular genetics*, *20*(11), 2195–2203. https://doi.org/10.1093/hmg/ddr107

104 Stec, I., Nagl, S. B., van Ommen, G. J., & den Dunnen, J. T. (2000). The PWWP domain: a potential protein-protein interaction domain in nuclear proteins influencing differentiation?. *FEBS letters*, *473*(1), 1–5. https://doi.org/10.1016/s0014-5793(00)01449-6

105 Feeney, M., Murphy, K., & Lopilato, J. (2014). Designing PCR primers painlessly. *Journal of microbiology & biology education*, *15*(1), 28–29. https://doi.org/10.1128/jmbe.v15i1.634

106 Borgel, J., Guibert, S., Li, Y., Chiba, H., Schübeler, D., Sasaki, H., Forné, T., & Weber, M. (2010). Targets and dynamics of promoter DNA methylation during early mouse development. *Nature genetics*, *42*(12), 1093–1100. https://doi.org/10.1038/ng.708

107 Maurano, M. T., Wang, H., John, S., Shafer, A., Canfield, T., Lee, K., & Stamatoyannopoulos, J. A. (2015). Role of DNA Methylation in Modulating Transcription Factor Occupancy. *Cell reports*, *12*(7), 1184–1195. https://doi.org/10.1016/j.celrep.2015.07.024

108 Long, H. K., King, H. W., Patient, R. K., Odom, D. T., & Klose, R. J. (2016). Protection of CpG islands from DNA methylation is DNA-encoded and evolutionarily conserved. *Nucleic acids research*, *44*(14), 6693–6706. https://doi.org/10.1093/nar/gkw258

109 Kitamura, E., Igarashi, J., Morohashi, A., Hida, N., Oinuma, T., Nemoto, N., Song, F., Ghosh, S., Held, W. A., Yoshida-Noro, C., & Nagase, H. (2007). Analysis of tissue-specific differentially methylated regions (TDMs) in humans. *Genomics*, *89*(3), 326–337. https://doi.org/10.1016/j.ygeno.2006.11.006

110 Concordet, J. P., & Haeussler, M. (2018). CRISPOR: intuitive guide selection for CRISPR/Cas9 genome editing experiments and screens. *Nucleic acids research*, *46*(W1), W242–W245. https://doi.org/10.1093/nar/gky354

111 Benchling [Biology Software]. (2022). Retrieved from https://benchling.com.

112 Haeussler, M., Schönig, K., Eckert, H., Eschstruth, A., Mianné, J., Renaud, J. B., Schneider-Maunoury, S., Shkumatava, A., Teboul, L., Kent, J., Joly, J. S., & Concordet, J. P. (2016). Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. *Genome biology*, *17*(1), 148. https://doi.org/10.1186/s13059-016-1012-2

113 El Marjou, F., Jouhanneau, C., & Krndija, D. (2021). Targeted Transgenic Mice Using CRISPR /Cas9 Technology. *Methods in molecular biology (Clifton, N.J.)*, *2214*, 125–141. https://doi.org/10.1007/978-1-0716-0958-3_9

114 Tarverdizadeh, Y., Khalili, M., Esmaeili, S., Ahmadian, G., Golchin, M., & Hajizade, A. (2022). Targeted gene inactivation in Salmonella Typhi by CRISPR/Cas9-assisted homologous recombination. *World journal of microbiology & biotechnology*, *39*(2), 58. https://doi.org/10.1007/s11274-022-03504-0

115 Prajapati, A., & Nain, V. (2021). Screening of CRISPR/Cas9 gRNA for mimicking Powdery Mildew resistant MLO ol-2 mutant. *Bioinformation*, *17*(6), 637–645. https://doi.org/10.6026/97320630017637

116 Liu, Q., Yuan, Y., Zhu, F., Hong, Y., & Ge, R. (2018). Efficient genome editing using CRISPR/Cas9 ribonucleoprotein approach in cultured Medaka fish cells. *Biology open*, *7*(8), bio035170. https://doi.org/10.1242/bio.035170

117 Pan, Q., Luo, J., Jiang, Y., Wang, Z., Lu, K., & Chen, T. (2022). Efficient gene editing in a medaka (*Oryzias latipes*) cell line and embryos by SpCas9/tRNA-gRNA. *Journal of Zhejiang University. Science. B*, *23*(1), 74–83. https://doi.org/10.1631/jzus.B2100343

118 Labun, K., Montague, T. G., Krause, M., Torres Cleuren, Y. N., Tjeldnes, H., & Valen, E. (2019). CHOPCHOP v3: expanding the CRISPR web toolbox beyond genome editing. *Nucleic acids research*, *47*(W1), W171–W174. https://doi.org/10.1093/nar/gkz365

119 Stemmer, M., Thumberger, T., Del Sol Keyer, M., Wittbrodt, J., & Mateo, J. L. (2015). CCTop: An Intuitive, Flexible and Reliable CRISPR/Cas9 Target Prediction Tool. *PloS one*, *10*(4), e0124633. https://doi.org/10.1371/journal.pone.0124633

120 Fukushima, H. S., Takeda, H., & Nakamura, R. (2019). Targeted in vivo epigenome editing of H3K27me3. *Epigenetics & chromatin*, *12*(1), 17. https://doi.org/10.1186/s13072-019-0263-z

121 Porazinski, S. R., Wang, H., & Furutani-Seiki, M. (2010). Microinjection of medaka embryos for use as a model genetic organism. *Journal of visualized experiments : JoVE*, (46), 1937. https://doi.org/10.3791/1937

122 Murakami, Y., & Kinoshita, M. (2018). Medaka-microinjection with an Upright Microscope. *Bio-protocol*, *8*(3), e2716. https://doi.org/10.21769/BioProtoc.2716

123 Rivas, M. A., Beaudoin, M., Gardet, A., Stevens, C., Sharma, Y., Zhang, C. K., Boucher, G., Ripke, S., Ellinghaus, D., Burtt, N., Fennell, T., Kirby, A., Latiano, A., Goyette, P., Green, T., Halfvarson, J., Haritunians, T., Korn, J. M., Kuruvilla, F., Lagacé, C., … Daly, M. J. (2011). Deep resequencing of GWAS loci identifies independent rare variants associated with inflammatory bowel disease. *Nature genetics*, *43*(11), 1066–1073. https://doi.org/10.1038/ng.952

124 Moser, D. A., Müller, S., Hummel, E. M., Limberg, A. S., Dieckmann, L., Frach, L., Pakusch, J., Flasbeck, V., Brüne, M., Beygo, J., Klein-Hitpass, L., & Kumsta, R. (2020). Targeted bisulfite sequencing: A novel tool for the assessment of DNA methylation with high sensitivity and increased coverage. *Psychoneuroendocrinology*, *120*, 104784. https://doi.org/10.1016/j.psyneuen.2020.104784

125 Soto, J., Rodriguez-Antolin, C., Vallespín, E., de Castro Carpeño, J., & Ibanez de Caceres, I. (2016). The impact of next-generation sequencing on the DNA methylation-based translational cancer research. *Translational research : the journal of laboratory and clinical medicine*, *169*, 1–18.e1. https://doi.org/10.1016/j.trsl.2015.11.003

126 Cameron, E. E., Bachman, K. E., Myöhänen, S., Herman, J. G., & Baylin, S. B. (1999). Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nature genetics*, *21*(1), 103–107. https://doi.org/10.1038/5047

127 Griffiths, E. A., & Gore, S. D. (2008). DNA methyltransferase and histone deacetylase inhibitors in the treatment of myelodysplastic syndromes. *Seminars in hematology*, *45*(1), 23–30. https://doi.org/10.1053/j.seminhematol.2007.11.007

128 Kwon, D. Y., Zhao, Y. T., Lamonica, J. M., & Zhou, Z. (2017). Locus-specific histone deacetylation using a synthetic CRISPR-Cas9-based HDAC. *Nature communications*, *8*, 15315. https://doi.org/10.1038/ncomms15315

129 Li, X., Huang, L., Pan, L., Wang, B., & Pan, L. (2021). CRISPR/dCas9-mediated epigenetic modification reveals differential regulation of histone acetylation on Aspergillus niger secondary metabolite. *Microbiological research*, *245*, 126694. https://doi.org/10.1016/j.micres.2020.126694

130 Kearns, N. A., Pham, H., Tabak, B., Genga, R. M., Silverstein, N. J., Garber, M., & Maehr, R. (2015). Functional annotation of native enhancers with a Cas9-histone demethylase fusion. *Nature methods*, *12*(5), 401–403. https://doi.org/10.1038/nmeth.3325

131 Thakore, P. I., D'Ippolito, A. M., Song, L., Safi, A., Shivakumar, N. K., Kabadi, A. M., Reddy, T. E., Crawford, G. E., & Gersbach, C. A. (2015). Highly specific epigenome editing by CRISPR-Cas9 repressors for silencing of distal regulatory elements. *Nature methods*, *12*(12), 1143–1149. https://doi.org/10.1038/nmeth.3630

132 Chen, X., Wei, M., Liu, X., Song, S., Wang, L., Yang, X., & Song, Y. (2019). Construction and validation of the CRISPR/dCas9-EZH2 system for targeted H3K27Me3 modification. *Biochemical and biophysical research communications*, *511*(2), 246–252. https://doi.org/10.1016/j.bbrc.2019.02.011

133 Joberty, G., Fälth-Savitski, M., Paulmann, M., Bösche, M., Doce, C., Cheng, A. T., Drewes, G., & Grandi, P. (2020). A Tandem Guide RNA-Based Strategy for Efficient CRISPR Gene Editing of Cell Populations with Low Heterogeneity of Edited Alleles. *The CRISPR journal*, *3*(2), 123–134. https://doi.org/10.1089/crispr.2019.0064

134 Krueger, F., & Andrews, S. R. (2011). Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. *Bioinformatics (Oxford, England)*, *27*(11), 1571–1572. https://doi.org/10.1093/bioinformatics/btr167

135 Mallona, I., Díez-Villanueva, A., & Peinado, M. A. (2014). Methylation plotter: a web tool for dynamic visualization of DNA methylation data. *Source code for biology and medicine*, *9*, 11. https://doi.org/10.1186/1751-0473-9-11

136 Ewels, P., Magnusson, M., Lundin, S., & Käller, M. (2016). MultiQC: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics (Oxford, England)*, *32*(19), 3047–3048. https://doi.org/10.1093/bioinformatics/btw354

137 Stepper, P., Kungulovski, G., Jurkowska, R. Z., Chandra, T., Krueger, F., Reinhardt, R., Reik, W., Jeltsch, A., & Jurkowski, T. P. (2017). Efficient targeted DNA methylation with chimeric dCas9-Dnmt3a-Dnmt3L methyltransferase. *Nucleic acids research*, *45*(4), 1703–1713. https://doi.org/10.1093/nar/gkw1112