INSTITUTO POLITÉCNICO DE LISBOA

Instituto Superior de Engenharia de Lisboa
Escola Superior de Tecnologia da Saúde de Lisboa

Development of cloning-free protocols for generation of gene knockouts using CRISPR-Cas9 technology in the model organisms *Danio rerio*, *Drosophila melanogaster* and *Mus musculus*

Catarina Filipe da Costa Craveiro

Trabalho Final de Mestrado para obtenção do grau de Mestre em Engenharia Biomédica

Orientadores

Ana Catarina Certal (Fundação Champalimaud)

Isabel Campos (Fundação Champalimaud)

Cecília R.C. Calado (ISEL- Instituto Superior de Engenharia de Lisboa)

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Agradecimentos

Gostaria de agradecer às minhas orientadoras principais, Ana Catarina Certal e Isabel Campos pela oportunidade que me derem para realizar este projeto assim como a confiança que depositaram em mim para obter resultados. Foram o grande alicerce de todo o meu projeto e estiveram sempre disponíveis para colmatar todas as minhas dúvidas.

À minha coorientadora Cecília Calado pelas informações institucionais e também pelo apoio total na elaboração desta tese, mostrando-se também sempre disponível para qualquer questão.

À minha colega Joana Monteiro que se disponibilizou a acompanhar-me na fase inicial deste projeto, nomeadamente em questões relativas à Biologia Molecular, ensinando-me muitas das técnicas aqui aplicadas.

À minha colega Sofia Silva por toda a ajuda relativa aos cruzamentos de moscas, assim como toda a equipa da *Fly Platform* que também sempre me deu o seu apoio e tempo para qualquer questão.

À Plataforma de Biologia Molecular da FC, nomeadamente Ana Cunha e Raquel Tomás, por toda a ajuda e esclarecimento de dúvidas.

Às minhas colegas Andreia Madalena e Natacha Leonardo por serem as colegas mais empenhadas em quererem o resultado positivo deste projeto e também por toda a ajuda delas em questões como recolhas de amostras, superovulação de fêmeas, e muito mais.

Às minhas colegas de faculdade, que apesar de não estarem dentro da engenharia genética se disponibilizaram na ajuda à elaboração desta dissertação.

E por último, ao meu namorado e aos meus pais por todo o apoio, foram essenciais.

Champalimaud Foundation

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2017

This thesis was fully performed at Fish Facility, Fly Facility and Vivarium at Champalimaud Foundation under the direct supervision of Ana Catarina Certal and Isabel Campos, in the scope of the Master Thesis in Biomedical Engineering of the ISEL-Instituto Superior de Engenharia de Lisboa and ESTeSL-Escola Superior de Tecnologia da Saúde de Lisboa.

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List of Abbreviations

ADN – Ácido desoxiribonucléico

ARN – Ácido ribonucléico

Cas - CRISPR-associated

CF – Champalimaud Foundation

CRISPR - Clustered Regularly Interspaced Palindromic Repeats

CrRNA – CRISPR RNA

DNA – Deoxyribonucleic acid

DSB – Double Strand Break

EDTA - Ethylenediaminetetraacetic acid

ES cells - Embryonic stem cells

EtOH - Etanol

gRNA - guideRNA

hCG – Human chorionic gonadotropin

Hpf – hours post fertilization

Hpi – hours post injection

HR – Homologous Recombination

KCl - Potassium chloride

Mgcl2 - Magnesium chloride

MiMIC - Minos Mediated Integration Cassette

mRNA – messenger Ribonucleic acid

NaOH – Sodium hydroxide

NHEJ – Non-Homologous End Joining

PAM - Proto-spacer adjacent motif

PCR – Polymerase Chain Reaction

PMSG – Pregnant’s mare´s serum gonadotropin

RNA – Ribonucleic acid

SRSRs - Short Regularly Spaced Repeats

TracrRNA - Trans-activating RNA

Tris - tris(hydroxymethyl)aminomethane

Tris-HCl - Tris hydrochloride

Tyr - Tyrosinase

ZNFs - Zinc Finger Nucleases

Resumo

Charles Darwin (1809-1882) apresentou a sua teoria da evolução em 1859 quando publicou "Origem das Espécies por Meios de Seleção Natural ou a Preservação das Raças Favorecidas na Luta pela Vida" que indica que todos os seres vivos têm um ancestral comum. Esta teoria leva à conclusão de que a maioria das funções biológicas moleculares e celulares do organismo humano podem ser estudadas de uma forma mais eficiente e simples em organismos não-humanos. A utilização de modelos animais não humanos para determinados estudos de investigação em vez do ser humano traz vantagens a níveis experimentais e, principalmente, a nível ético. A experimentação animal traz benefícios não só ao ser humano mas também aos próprios animais. Organismos Modelo são assim espécies não humanas que são biologicamente estudadas na expectativa de descobrir funções de genes, curas para doenças ou melhorias na qualidade de saúde que podem ser aplicadas a outros organismos. Espécies como *Danio rerio* (peixe-zebra), *Drosophila melanogaster* (mosca-da-fruta) e *Mus musculus* (murganhos), são exemplos de animais usados como organismos modelo pela comunidade científica. Os murganhos por exemplo, constituem o organismo modelo geneticamente mais semelhante ao ser humano, sendo cerca de 85% das regiões codificadoras dos murganhos idênticas à do ser humano, chegando para alguns dos genes mesmo a 99% de semelhança.

Apesar do genoma humano estar completamente sequenciado, para muitos genes ainda é desconhecida a sua função. Para estudar a função dos genes, um organismo *knockout* é essencial porque ao tornar o gene inativo permite quantificar/qualificar a consequência dessa inatividade, e daí inferir a função génica. Um *knockout* pode ser conseguido através de uma mutação no gene. A tecnologia de *CRISPR/Cas9* é um mecanismo encontrado na resposta imunitária das bactérias, que tornou possível provocar mutações dirigidas a genes específicos. Para este sistema funcionar é necessário a proteína CRISPR *associated* 9 (Cas9) (para cortar o ADN), uma região *proto-spacer adjacent motif* (*PAM)* (região no ADN reconhecida pela proteína Cas9) e um *guideRNA* (que guia a Cas9 à região alvo). A proteína Cas9 provoca um corte na dupla cadeia de ADN e a célula tenta reparar esse corte através do mecanismo *Non Homologous End Joining* (*NHEJ)*, mas durante este processo podem ocorrer várias mutações, como deleções ou inserções, provocando uma *frameshift* que, ou produz uma proteína deficiente ou impossibilita a produção da proteína - qualquer das opções é um *knockout* do gene. Não existe um protocolo de produção de *guideRNA* e consequente produção de *knockouts* que seja facilmente intermutável entre os 3 organismos modelo abordados neste projeto, sendo esse o nosso maior objectivo na elaboração deste trabalho.

Para alcançar o objectivo da tese foi usado um protocolo já estabelecido para produção de *guideRNA* e consequente produção de animais mutantes em peixe-zebra: primeiramente como prova de princípio em peixe-zebra e posteriormente em mosca-da-fruta e murganho. Depois de estabelecido

esse protocolo em peixe-zebra e de termos obtido animais mutantes estáveis, tentámos optimizar o mesmo protocolo para mosca-da-fruta e para murganho de acordo com as diferenças de desenvolvimento embrionário inerentes a cada organismo.

Para a realização deste projeto, foram escolhidos genes que provocariam um efeito fenotipicamente visível aquando mutados de modo a facilitar o processo de rastreamento de mutantes. No caso do peixe-zebra e do murganho, o gene escolhido foi *tyrosinase*, envolvido na produção do pigmento preto no corpo e nos olhos dos animais. Para a mosca-da-fruta, o gene escolhido foi o *yellow*, também envolvido na produção do pigmento acastanhado da cutícula deste insecto. Em peixe-zebra, o gene *tyrosinase* foi mutado com sucesso, ficando assim inoperativo. Esta mutação causou mosaicismo fenotípico e genético: algumas células destes animais não tinham pigmento e confirmou-se a presença de diversos alelos mutantes diferentes no genoma.

Exemplo de algumas limitações que existiram na elaboração deste projeto foi, no protocolo de produção de *guideRNA* e produção de animais mutantes e a extração de ARN a partir do ADN transcrito. Para extração de ARN o protocolo utiliza o *Qiagen micro-RNA extraction kit*. No entanto, a quantidade extraída de ARN com recurso a este reagente foi diminuta. Face a estes resultados, fizemos uma comparação direta entre a extração de ARN com esse mesmo *kit* e extração com fenol/clorofórmio a partir do mesmo produto de transcrição. Com o fenol/clorofórmio foi possível extrair quase 10 vezes mais ARN do que com o *kit*. Após estes resultados, todos os outros *guideRNAs* foram extraídos com o método de fenol/clorofórmio.

Outra limitação existente no seguimento do protocolo usado neste projecto, foi a amplificação a partir de ADN genómico extraído de embriões com 24h de peixe-zebra. Para concluir que essa região do gene poderia não estar acessível no estadio de desenvolvimento de embrião de 24h, testámos dois factores: o protocolo de extração de ADN em embriões de 24h e os estadios de desenvolvimento até aos 5 dias de idade. Para testar a extração de ADN em embriões de 24h, comparámos a amplificação a partir de ADN genómico extraído de embriões de 24h para dois genes: *tyrosinase* e *DIA1R* (amplificação deste gene em embriões de 24h já tinha sido anteriormente observada) como controlo. Foi possível observar que para o o gene *DIA1R* continuava a existir amplificação do gene, ao contrário do gene da *tyrosinase*. De seguida, para testar em que estadio de desenvolvimento a amplificação da região pretendida do gene da *tyrosinase* começava a ser observada, extraímos ADN de embriões de 24h, larvas de 72h, larvas com 3 dias e larvas com 5 dias de idade, seguidas de reações de PCR para amplificação dessa mesma região. Amplificação da região pretendida do gene *tyrosinase* a partir de ADN genómico extraído de larvas de 5 dias foi observada, no entanto é uma amplificação muito diminuta.

A microinjeção em mosca-da-fruta de *guideRNA in vitro* ao contrário de em plasmídeo, apesar de ter sido mostrado por outros investigadores, ser mais eficiente, leva a um processo de produção de

guideRNA mais dispendioso e demorado. Ao optimizar este protocolo em mosca-da-fruta estaríamos a ultrapassar essas dificuldades. No entanto, não foi possível terminar a experiência sendo por isso necessária a continuação deste projecto. Pudemos apenas concluir que a co-microinjeção de *guideRNA* com proteína Cas9 não é eficiente, uma vez que a concentração necessária de proteína Cas9 é muito maior do que a que foi possível utilizar neste projeto.

Por último, o protocolo foi utilizado em murganhos e neste caso, obtivemos 41 animais provenientes de microinjeção de *guideRNA* e proteína Cas9, mas nenhum apresentava fenótipo facilmente observável ao nível da pigmentação da pelagem. No entanto, estudos em *tyrosinase* em murganhos mostram resultados de animais sem fenótipo de pigmentação mas que apresentavam mutações quando genotipados, passo essencial para uma conclusão definitiva quanto à aplicabilidade deste método na geração de mutantes em murganho, mas que, infelizmente e por constrangimentos temporais não conseguimos efetuar em tempo útil.

Concluimos que conseguimos reproduzir com sucesso o protocolo em peixe-zebra. Em mosca-da-fruta, o mesmo protocolo de produção e injeção de guideRNA poderá funcionar mas será preciso adpatar a entrega da proteína Cas9. Por útlimo, em murganhos parece que o protocolo a usar poderá ser muito semelhante ao do peixe-zebra, no entanto fica por confirmar o sucesso na produção de mutantes.

Palavras-Chave: CRISPR, mutação, Knockout, mosaico, fenótipo

Abstract

Model organisms are non-human species, that due to similarities with the human organism, are studied in the expectation of discovering gene functions, cure for diseases, improvements in healthcare and welfare. *Danio rerio*, *Drosophila melanogaster* and *Mus musculus* are examples of model organisms widely used in all biomedical research fields. To study gene function, production of knockout animals is an important approach. The CRISPR/Cas9 targeted mutagenesis technology offers the possibility of targeting any gene of interest as long as there is a proto-spacer adjacent motif (PAM) in that region, a gRNA and a Cas9 protein. Cas9 protein makes a DSB in the DNA that the cell tries to fix through the NHEJ mechanism. This mechanism is not always efficient and small base deletions or insertions may arise, causing a frameshift that leads to the production of a deficient protein or null protein, causing a knockout of the gene. A common protocol for gRNA production and knockout generation that fits all three model organisms above referred, is not yet available. In this project, we first did a proof of principle with a pre-existing protocol for gRNA production and knockout zebrafish production. When establishing the zebrafish protocol, the main objective was to use the same protocol structure to produce knockout animals in both fruit fly and mouse, making the necessary optimizations regarding differences in embryonic development. To do this, genes that would cause a phenotypic readout were chosen: *tyrosinase* in zebrafish and mouse, and *yellow* in fruit fly. The *tyrosinase* gene in zebrafish was successfully mutated and mosaic phenotypic and genotypic disruption was observed. Co-microinjection of gRNA for the *yellow* gene in fruit fly with Cas9 protein didn't produce a positive result, since Cas9 protein is required in a much higher concentration in the cell. For this animal model, we concluded it was best to micro-inject the gRNA in embryos already producing the Cas9 protein. In mouse, injection of Cas9 protein and gRNA targeting the *tyrosinase* gene resulted in the successful generation of 41 animals, but we fail to observe a clear *tyrosinase* mutant.

Keywords: CRISPR, mutation, Knockout, mosaic, phenotype

1. Introduction

1.1 Model organisms and their manipulation to produce knockouts

Naturalist Charles Darwin (1809-1882) was on a voyage around the world on board of "Beagle" for 5 years. His observations and studies of specimens during those 5 years, led him to present his evolutionary theory and in 1859, he published "On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life". Charles Darwin's evolutionary theory (Darwin, 1869) says that all living beings have a common ancestor. Based on that theory, all organisms share a common ancestor and biology. This argument follows naturally to the conclusion that non-human organisms can be studied to pursue the ultimate goal of medicine development. Using animals in such studies instead of humans brings obvious advantages both at the experimental and ethical planes. Experiments in non-human organisms can bring benefits not just to humans, but also to the animals themselves. Work of Mendel in pea plants and Morgan in fruit flies, that identified Mendel's determinants as the chromosomes, are a clear example. Model organisms are all non-human species that are biologically studied in the expectation of discovering gene functions, cure for diseases, improvements in healthcare and welfare that can then be applied in other organisms. Models are chosen according to the experimental manipulation that is planned. Characteristics such as life cycle, genetic manipulation tools, housing requirements (cage, vials, type of feed, etc.…) and genetic similarity are important when choosing a model organism. According to Nature Glossary the definition for model organism is "An organism suitable for studying a specific trait, disease, or phenomenon, due to its short generation time, characterized genome, or similarity to humans; examples are a fly, fish, rodent or pig, whose biology is well known and accessible for laboratory studies" (Nature Education, 2017).

Drosophila melanogaster as model organism and transgenesis techniques used

Drosophila melanogaster was probably the first animal being used for genetic studies by Thomas Morgan, who received the Nobel Prize in 1933 of Physiology or Medicine for discovering the role of chromosomes in heredity (Nobel Prize in Physiology or Medicine, 1933). Drosophila is a model organism widely used by researchers for several reasons: flies are easy and inexpensive to maintain in laboratory; there are almost non-ethical issues in using Drosophila, allowing almost any genetic modification; females can lay about 100 eggs per day (Shapiro, 1932); at 25 ºC it takes 10 days to have adult flies so many flies can be generated very fast; it has only 4 pairs of chromosomes, being the 4th so small that is usually discarded; females show meiotic recombination but males don't

(Morgan, 1912); balancer chromosomes (modified chromosomes used to prevent crossing over between homologous chromosomes during meiosis) exist carrying genetic markers that are easily visible. These chromosomes allow for an homozygous lethal mutation to be maintained heterozygous in the population (Bloomington Drosophila Stock Center, 2013); and the full genome is already sequenced and fully annotated. The National Human Genome Research Institute made a comparison between the fruit fly and human genomes estimating that 60% of genes are conserved in both species and about 75% of human disease genes have a recognizable match in the fruit fly genome.

Transgenesis in the fruit fly was detected in 1982 through the works of Rubin and Spradling with transposable elements, a technique now called P-element Transgenesis, that is based on a transposon called P element, a highly mobile element present in the DNA (Rubin & Spradling, 1982). P-elements encode a functional transposase that enables them to "jump" inside a genome (Hummel, 2008). For production of transgenic flies according to this technique, two constructs need to be microinjected into the embryo, one that contains the gene of interest and a marker gene (mini-white) and another one, called Helper plasmid, that contains the transposase that will catalyse the "jump" of the DNA of interest into the fly genome (Fig.1). *Mini-white* rescues the red colour of the fly eye, since microinjected flies have a *white* background. In P-element Transgenesis, the transgene is randomly incorporated in the fly genome making it a useful tool to produce transgenic animals, because the transgene of interest is expressed either way. It is possible to produce mutants using P element Transgenesis since an incomplete excision can occur. Fly embryos are microinjected in the syncytial stage, when the embryo is a multinucleated cell with no cytoplasmic membranes involving the nuclei. Microinjection in this stage increases the possibility of targeting every nucleus. Also, microinjection is made in the posterior end of the embryo where the pole cells will appear (pole cells give origin of the germline cells), increasing the chances of germ line transgene incorporation and subsequent transmission to progeny.

In P-element Transgenesis, integration of DNA is random but transgenesis in fly evolved and new techniques arise where DNA is integrated in known sites of the fly genome. One of those techniques is based on Integrase φC31, where an integrase isolated from a phage induces recombination between two non-identical sequences, one called attP (from phage) and the other called attB (from bacteria). This mechanism was translated into the production of transgenic flies, microinjecting a plasmid that contains an attB region into the fly embryo that already possesses an attP site similar to the attP site of the phage (Fig 2). The microinjected plasmid also contains a *mini-white* as marker gene (Groth *et al*., 2004). Integrase φC31 can be used in another approach using stocks originated from integration of a specific cassette randomly into the genome, that cassette stands for Minos Mediated Integration Cassette (MiMIC). MiMIC makes use of the transposon Minos, containing a DNA cassette flanked by 2 inverted attP regions. Replacement of this DNA cassette by a functionally relevant DNA element (enhancer, gene trap, etc) is achieved by φC31-mediated

integration. In this case, original flies have *yellow* background and the cassette has a *yellow*$^{+}$ marker, so the flies are phenotypically wildtype, but when microinjection is successful and the cassette is replaced, flies loose the *yellow*$^{+}$ marker and become yellow (Venken *et al*., 2011). The latter 2 techniques are not random like P-element Transgenesis but attP/MiMiC regions are in known regions of the genome and are useful to produce transgenic animals, not to study gene function (unless the gene of interest has an attP/MiMiC region and in that case, some strategies can be employed to produce a knockout, like the Gal4-UAS: system) (Ou & Lei, 2013).

Mus musculus as model organism and transgenesis techniques used

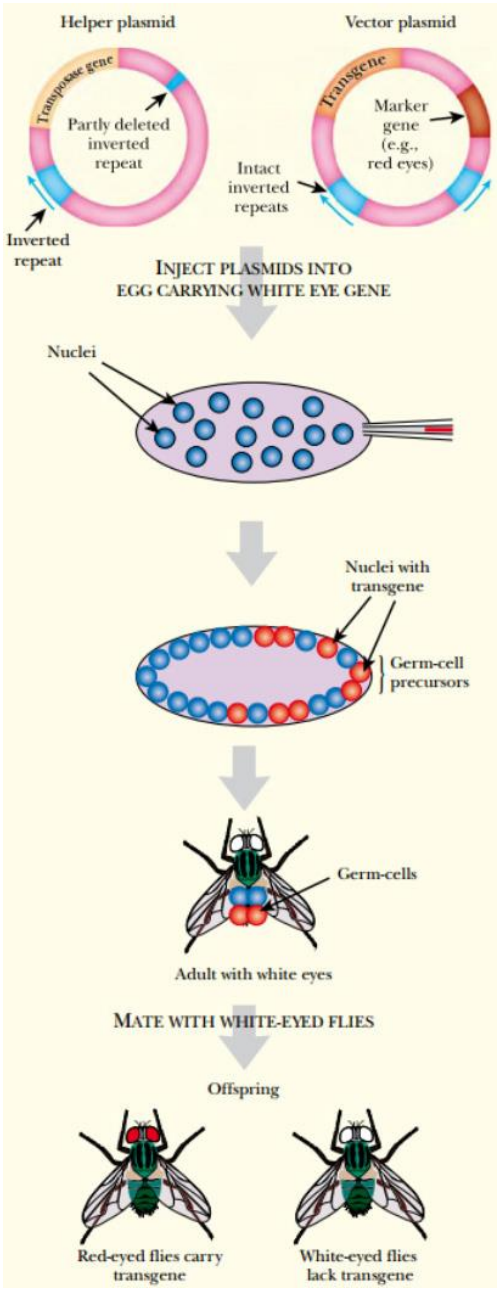


Figure 1 – *Drosophila Random P Transgenesis* (Abdul Razzaq, n.d.)

Mice were first used by Mendel in 1860 but he was forbidden to breed mice within the monastery so he started his work in sweet peas (The Jackson Laboratory). Lucien Cuénot, in 1902, was then the first person to use mice and he demonstrated the Mendelian inheritance in mammals, using the coat colours in mice (Cuenot, 1905). Mice are biologically very similar to humans and suffer from the same diseases for the same genetic reasons making them one of the most used model organism. On average, 85% of mouse coding regions are identical to human. Some genes are 99% similar but others are just 60% (National Human Genome Research Institute). Besides genetic similarity, there are other reasons that make mouse a good model organism, such as: one year in the mouse equals to 30 human years (Dutta & Sengupta, 2016), this accelerated lifespan allows the study of an entire life cycle; their maintenance is cost-effective, they are small to handle, reproduce fast; and can be genetically manipulated to mimic any human disease or condition.

Jon Gordon, in 1980, was able to produce the first transgenic mouse by microinjecting purified DNA directly into the pronuclei of fertilized mouse oocytes (Gordon *et al*., 1980). This became a widely used technique for mouse transgenic production. But the integration of this DNA seems to be random (Lacy *et al*., 1983) making it impossible to replace, for example, a gene that causes a certain disease. Other technique used for transgenic and mutant mice production consists in the manipulation of mouse embryonic stem cells (ES cells). Using this approach it is possible to manipulate a desired locus by introducing a loss or gain of function in vitro (Bradley *et al*., 1984; Thomas & Capecchi, 1987). ES cells are present in 3.5 day blastocysts and are pluripotent, meaning that are able to contribute to different cell lineages (Martin, 1981). When in a petri dish, these ES cells may be transfected with the desired DNA that is introduced into the cell's genome by homologous recombination between the donor DNA and the target genomic locus of ES cell's DNA. Transformed ES cells that contain the desired alteration are then injected into blastocysts that are in turn transferred to a surrogate mother. Typically, surrogate mothers and ES cell donor animals have different colour coats. This way, the born pups that will have incorporated the altered ES cells will display a quimeric colour coat (Bradley *et al*., 1984; Koller & Smithies, 1992). The ES cells technique made it possible to knockout a gene through the homologous recombination mechanism but it is still a long and expensive process (Hall *et al*., 2009).

Danio rerio as model organism and transgenesis techniques used

In 1981, George Streisinger was the first to clone a vertebrate and it was the zebrafish *Danio rerio*. George was the father of zebrafish as a research model and has turned it into a very useful scientific model organism to study development and gene function (Streisinger *et al*., 1981). There's an online resource, the Zebrafish Information Network (ZFIN) where genetic, genomic and developmental information can be found. The reasons why zebrafish is such a good model organism are: its genome is already sequenced; has a rapid embryonic development attaining sexual maturity in 60-90 days; adults are small and are housed in large groups, requiring few space and lowering the maintenance costs; adult zebrafish breed very fast and can produce until 300 embryos at a time; fertilization in zebrafish is external, allowing the easy manipulation; embryos also have the advantage of being large and transparent (Burke, 2016). Zebrafish has similar behaviour as compared to mammalian models concerning toxicity testing and diurnal sleep cycle (Jones, 2007). Even existing 70% of gene similarity between human and zebrafish (Howe *et al*., 2013), limitations in using zebrafish as an organism model exists, for example as a human disease model. Some human diseases are caused by genes that do not exist in zebrafish, making impossible to use this organism as a human disease model for a variety of human diseases. Zebrafish is also not a good organism model for human diseases that take place in a body part that zebrafish don't have, like mammary glands or prostate (Burke, 2016).

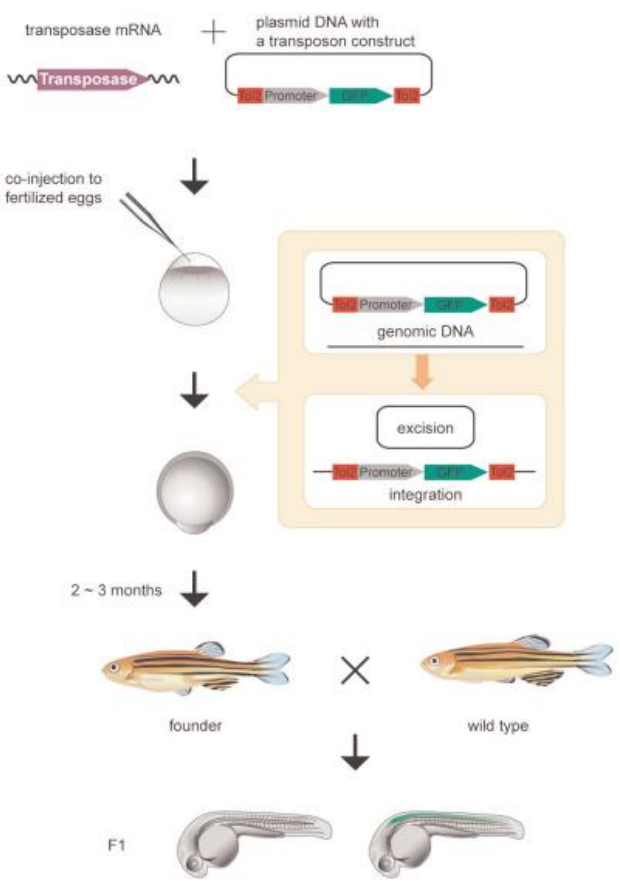


Figure 3 *– Zebrafish Tol2 transgenesis system* (Kawakami, 2007)

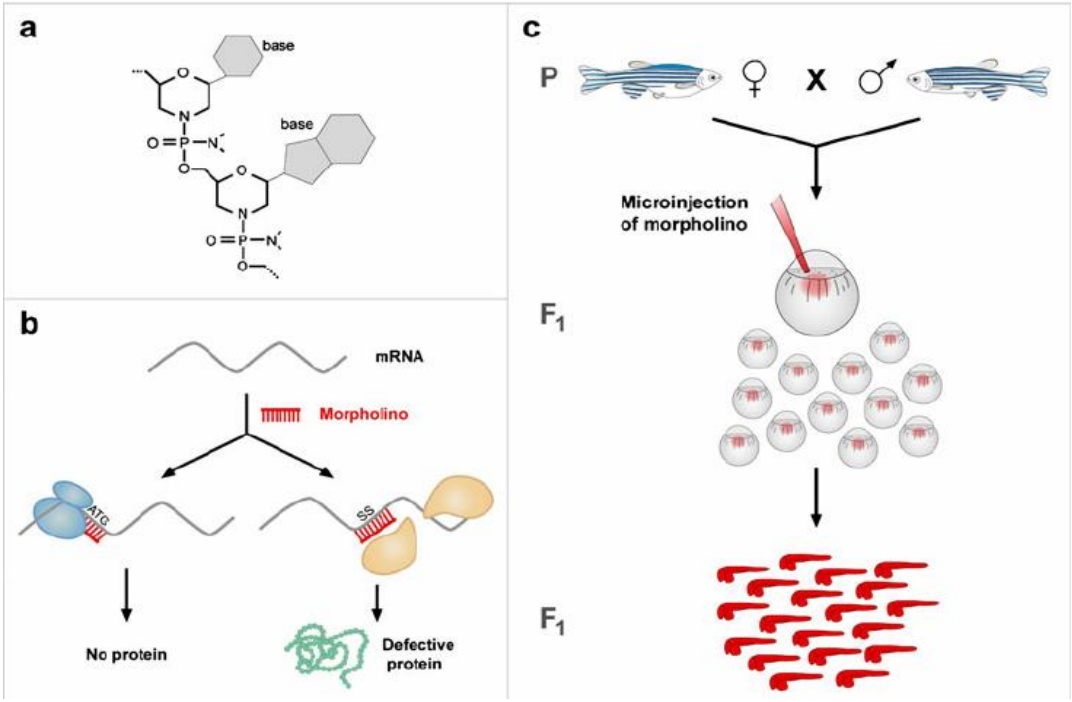


Figure 4 – *Zebrafish Morpholinos* (Codarin *et al*., 2009)

Stuart and colleagues, in 1988, after publication of the first transgenic mice production (Gordon *et al*., 1980), applied successfully the same technique in zebrafish, with their group being the first to produce a transgenic zebrafish (Stuart *et al*., 1988). However, those results had a very low efficiency rate , and although this was being increased over time, a new technique using transposons has been developed in zebrafish, called the Tol2 transposon system (Kawakami & Shima, 1999; Kawakami *et al*., 2000). Evidence of this active transposon was first reported in Medaka fish in 1996 (Koga *et al*., 1996). A couple of years later, Tol2 was isolated from a mutational insertion in the Medaka *tyrosinase* locus and showed to have autonomous mobility (Kawakami *et al*., 1998). The Tol2 system consists on a construct containing 2 cis-regulatory sequences (CREs) from the Tol2 element positioned 5´and 3´ of a promoter sequence followed by a fluorescent protein. This construct was named Tol2 vector. The Tol2 vector is co-injected with mRNA encoding for the Tol2 transposase into a one-cell stage embryo. Once translated, the Tol2 protein will catalyse the excision of the region of the Tol2 vector between the CREs and its integration in the genomic DNA (Fig. 3) (Kawakami & Shima, 1999; K Kawakami *et al*., 2000; Kawakami, 2007). There's another approach capable of blocking a gene in initial stages of embryo development, allowing the study of its function, the morpholinos (Nasevicius & Ekker, 2000; Summerton, 1999). Morpholinos are synthetic molecules and exist in two types: the ATG morpholinos, that block the initiation of translation of proteins, and the Splice morpholinos that bind and interfere with the RNA splicing machinery resulting on a truncated protein (Fig. 4). However, this mechanism is transient because morpholinos are degraded through time (Bill *et al*., 2009; Morcos, 2007).

Neither the Tol2 system nor the Morpholinos are able to induce targeted mutagenesis in zebrafish and, in 2008, zinc finger nucleases (ZNFs) were adapted to create targeted double strand breaks in the zebrafish genome (Doyon *et al*., 2008; Meng *et al*., 2008). ZNFs were produced to cleave DNA (Kim *et al*., 1996) and are a fusion between a restriction enzyme, FokI, and a DNA recognition domain containing 3 (or more) zinc finger motifs. ZNF heterodimerization in a position of the DNA leads to a double-strand break (DSB).

DNA double strand break repairs

Cells fix double strand breaks in the DNA by two different mechanisms, homologous recombination (HR) or Non-Homologous End Joining (NHEJ). Homologous recombination only occurs if a donor sequence with homology arms is present (Filippo *et al*., 2008), a technique used to produce transgenic animals. Otherwise, NHEJ will occur and for that mechanism a nuclease to reconstruct the damaged DNA, a polymerase to fill in the gaps and a ligase to restore the strand integrity are required (Ma *et al*., 2004). It seems obvious to think that these enzymes work by this order to reconstruct the DNA cut but these enzymes have a functional flexibility big enough to allow the NHEJ mechanism to occur in many ways (Fig. 5).

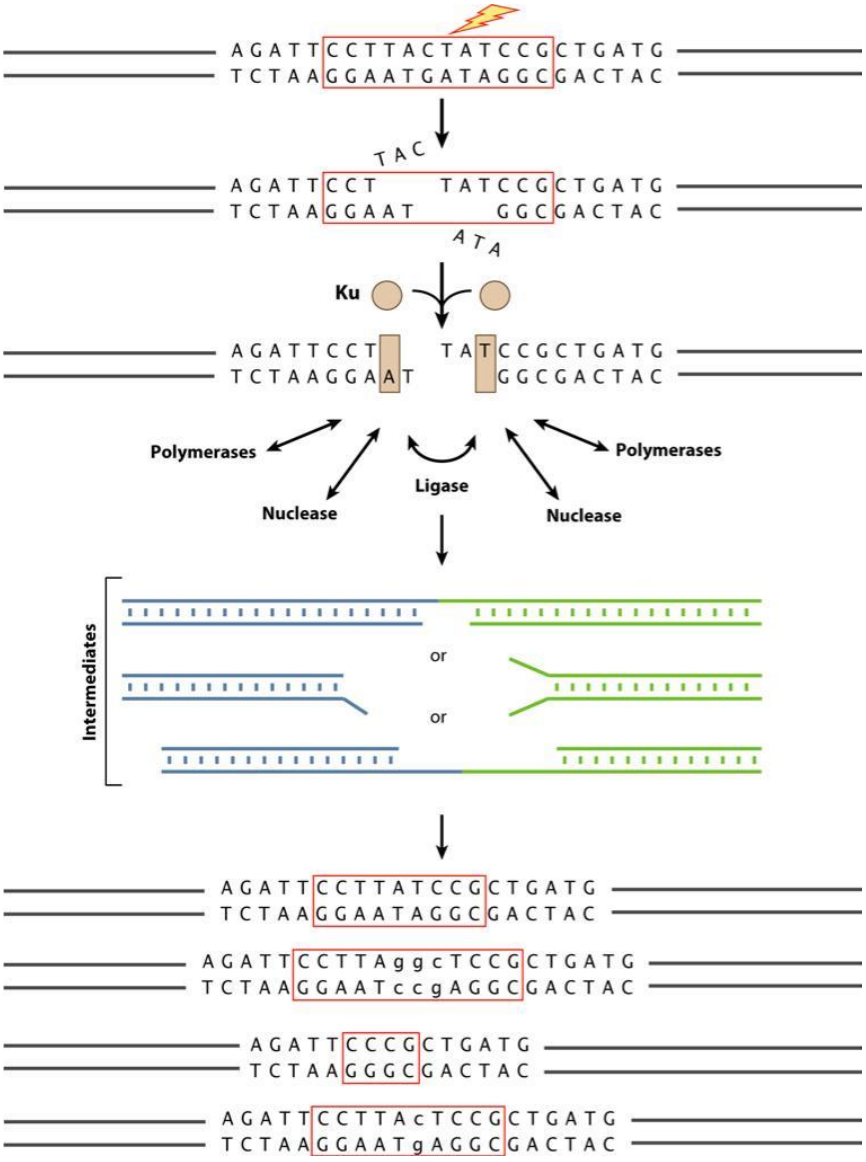


Figure 5 *– DSB repair: flexibility of enzyme functions lead to different repairs in a DSB break* (Lieber, 2010).

This flexibility can result in loss of nucleotides or junctions with nucleotide addition (Lieber, 2007, 2010), causing a frameshift that can result on a different protein translation or the complete gene knockout (Puchta *et al*., 2015).

An animal that has a gene knockout is an organism in which a particular gene or genes have been made inoperative. And knocking out genes is important for research purposes. If we remove a piece from a machine it's possible to know how it works and what's the importance of that piece and its function, for genes is the same logic, by knockin out a gene it's possible to understand what is its function. Nowadays, despite several animal genomes being sequenced, many genes still have an unknown function, and by knocking out a gene it is possible to study its function. Knockins (insertion of a gene) and knockouts are also widely used to produce and create disease models (Hall *et al*., 2009).

1.2 Bacteria CRISPR adaptive immunity system

Viruses are the biggest predators of bacteria, infecting prokaryotic cells with its DNA or RNA and making the bacteria machinery transcribe and translate its genetic material. Bacteria have both an innate immune system, that recognizes certain infection characteristics, and an adaptive immune system that can recognize specific pathogen characteristics (Rath *et al*., 2015). In 1987, in *Escherichia coli*, five homologous sequences of 29 nucleotides arranged in direct repeats with 32 nucleotides interspacing were found (Fig. 6). Those sequences were called REP (from repeats) sequences and were thought to act as mRNA stabilizers (Ishino *et al*, 1987).

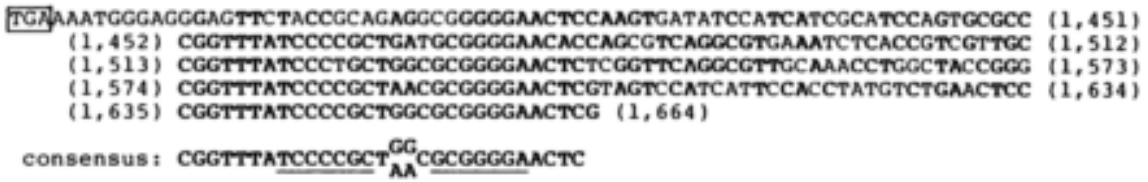


Figure 6 *- Direct repeated sequences of iap gene of E. coli. There are 29 highly conserved nucleotides, 14 of which (underlined in the bottom) contain a dyad symmetry. In brackets are the nucleotide numbers in the gene* (Ishino *et al*., 1987).

Later in 2000, Mojica's group identified that those short-repeated elements, generally in clusters, had one peculiarity: sequences were always regularly spaced by a unique sequence of constant length (Mojica *et al*., 2000). They called those clusters SRSRs (Short Regularly Spaced Repeats). Another feature present in those clusters is the presence of a conserved sequence, called leader, that is located upstream of every cluster locus. This leader directs transcription (Rath *et al*., 2015). Searching these SRSRs in all available microbial genomes, resulted in hits in 20 microbial species widespread among physiological and phylogenetic groups (Mojica *et al*., 2000). In 2002, those sequences were named CRISPR (Clustered Regularly Interspaced Palindromic Repeats) (Jansen *et al*., 2002), name that is used nowadays to refer to this molecular system. Alongside with CRISPR, three Cas (CRISPR-associated) genes were also identified. Cas genes are present in prokaryotes that contain CRISPR, absent in non-CRISPR-containing prokaryotes and are found to be located invariably adjacent to the CRISPR locus, suggesting that Cas genes and CRISPR have a functional relationship. Cas genes showed characteristic motifs of helicases and exonucleases (Jansen *et al*., 2002).

In 2005, work in *S. pyogenes* showed that CRISPRs could acquire phage DNA by discovering that seven out of the nine spacers included in *S. pyogenes* CRISPRs corresponded to a phage sequence (Pourcel *et al*., 2005). Another work, in this case in *S. thermophilus*, showed that about 75% of CRISPR spacers from this bacterium corresponded to *S. thermophilus* phages and 20% corresponded to *S. thermophilus* and *Lactococcus lactis* plasmids (Bolotin *et al*., 2005). Both these works pointed that CRISPR spacers have phage DNA and extra chromosomal origin, but it was Mojica's group that proposed a role for CRISPRs in microbial immunity showing that those extra chromosomal elements,

included in the spacers, fail to infect the cells (Mojica *et al*., 2005). In 2007 this hypothesis was further reinforced by experimental work in *S. thermophilus* by Barrangou and colleagues. They showed that resistance against a bacteriophage could be acquired by integrating a genome fragment of that phage into the CRISPR locus (Barrangou *et al*., 2007). Each spacer integration promotes a duplication of a new repeat, creating a new spacer-repeat unit. *S. thermophilus* also allowed the discovery of plasmid cleavage in this system. Cleavage of DNA was performed 3 nucleotides upstream of a proto-spacer adjacent motif (PAM) by an endonuclease. When an invading DNA appears, selection of which spacer precursors (proto-spacers) will integrate the CRISPR locus, is determined by the recognition of PAM. PAMs are usually 3 nucleotides long and differ between CRISPR types (Barrangou *et al*., 2007; Deveau *et al*., 2008; Horvath *et al*., 2008). In 2008, Brouns and colleagues demonstrated how those acquired spacers are used. CRISPRs are transcribed and a complex of Cas proteins cleaves the CRISPR RNA (crRNA) in each repeat, and retains the cleavage product that corresponds to a certain phage (Fig. 7). CrRNAs serve as guide RNAs that allow the Cas protein complex to interfere with the (Brouns *et al*., 2008).

Three types of CRISPR systems were identified, but type II is the system currently used to manipulate eukaryotic cells. In Type II CRISPR system, phage or plasmid DNA that tries to infect a cell is cut into small fragments halting the infection. In addition, those small fragments are

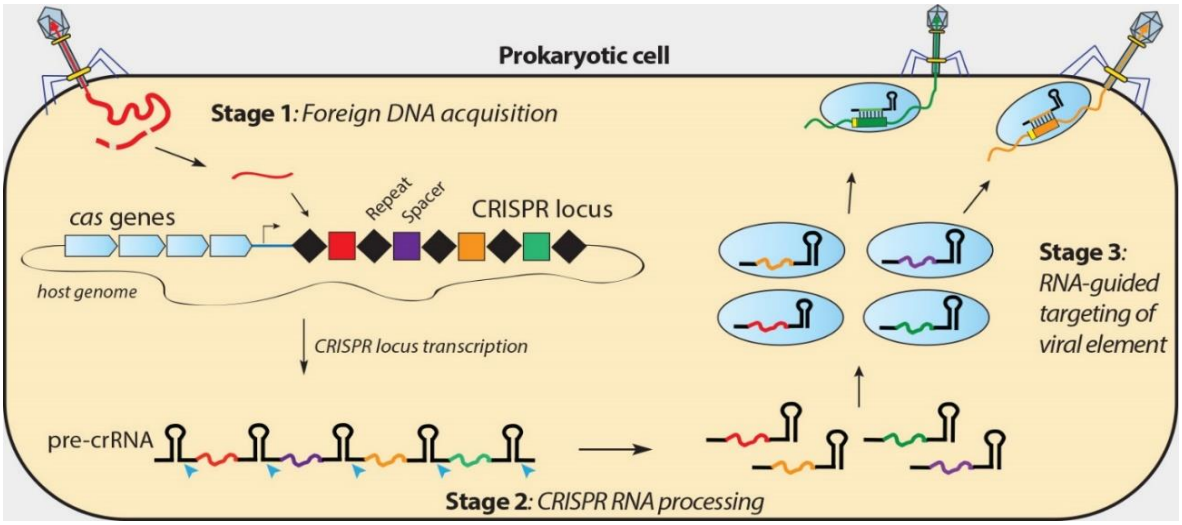


Figure 7 *– Bacteria CRISPR/Cas immunity system (Doudna lab http://rna.berkeley.edu/crispr.html)*

incorporated into the CRISPR locus in short repeats (about 20 bp each). When new infection occurs, those loci are transcribed and those transcripts are processed into small RNAs (called CRISPR RNA – crRNA) that will guide Cas proteins to the target invading DNA based on sequence complementarity of crRNA and invading DNA (Fig. 8). In this system, only one protein, Cas9, is required to inactivate a gene (Jinek *et al*., 2012). The Cas9 protein, discovered in Streptococcus species, has a key role in Type II CRISPR system, participating in processing crRNA and destroying target DNA. Cas9 contains

two nuclease domains, a RuvC-like nuclease domain and a HNH-like nuclease domain, that cut the upstream strand and the downstream strand, respectively (Sapranauskas *et al*., 2011).

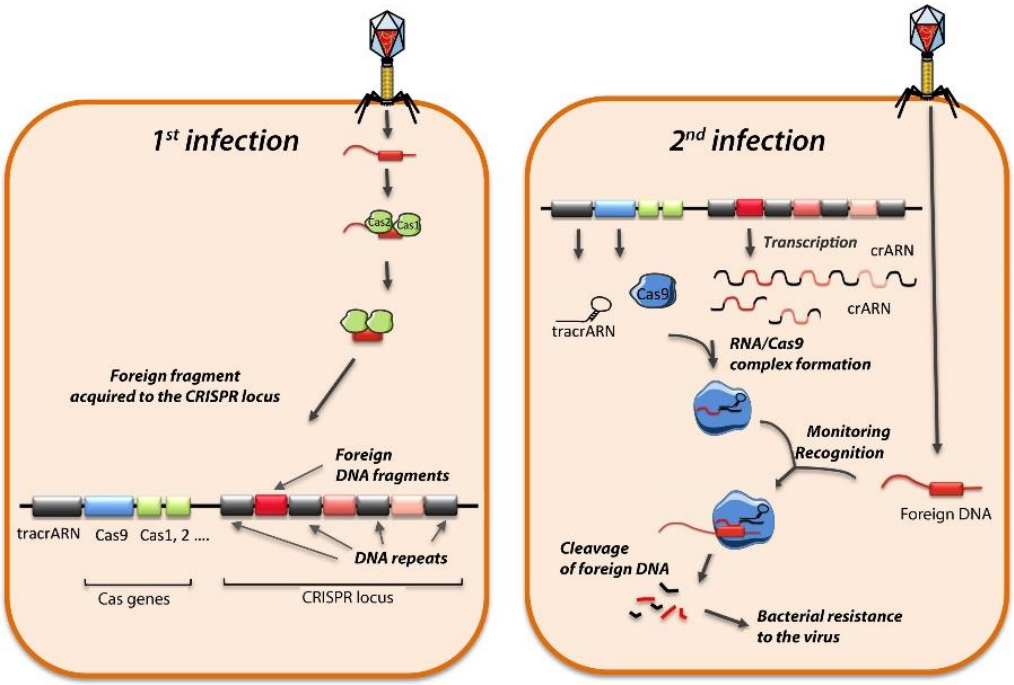


Figure 8 *– CRISPR/Cas9 mechanism after 1st phage infection and 2nd infection by the same phage* (Charpentier & Barrangou, 2017).

The CRISPR immunity system is divided into three stages: adaptation/acquisition, biogenesis/expression and interference. In the adaptation stage, a unique sequence from invading DNA, the protospacer, is incorporated into the CRISPR locus becoming a new spacer. This stage gives bacteria a genetic memory of invading DNA. Cas1 and Cas2 are two nucleases that are the key factors for the spacer integration into the CRISPR locus, but the mechanism through which these nucleases effect that integration is not fully understood. In Type II CRISPR, Cas9 is essential to identify the sequence that will be the protospacer by recognizing PAM sequences, and it is assumed that after that recognition, Cas9 recruits Cas1 and Cas2 to deliver the new protospacer into the CRISPR locus (Hille & Charpentier, 2016; Rath *et al*., 2015). The second stage, expression/biogenesis, refers to the transcription of the CRISPR locus to produce a CRISPR ribonucleoprotein complex. Primarily, the CRISPR locus is transcribed into pre-crRNA that is later processed into guide crRNAs, each containing memorized sequences of previous invaders. In type II system, it is known that a separate trans-activating RNA (tracrRNA) is required for the maturation of crRNA, but its mechanism is still unknown (Hille & Charpentier, 2016). In the third and final stage, interference, crRNAs binds to Cas9 protein and the complex locates the corresponding targets to be degraded. For interference to occur, the presence of PAM and complementarity between crRNA and invader DNA are necessary (Hille & Charpentier, 2016; Rath *et al*., 2015).

To target mutagenesis in vitro, Cas9 is complexed with crRNA and tracrRNA (Deltcheva *et al*., 2011). Both Cas9 nuclease domains cut the target DNA, with double strand breaks, 3 nucleotides

upstream of the PAM sequence, which in the case of Cas9 protein is NGG. Doudna and Charpentier showed that Cas9 protein required a base-paired structure between crRNA and tracrRNA to cleave DNA, so they developed a simpler system which combined crRNA and tracrRNA into a single guide RNA (sgRNA). Cas9 is effective with separate tracrRNA and crRNA as it is with sgRNA (Jinek *et al.*, 2012).

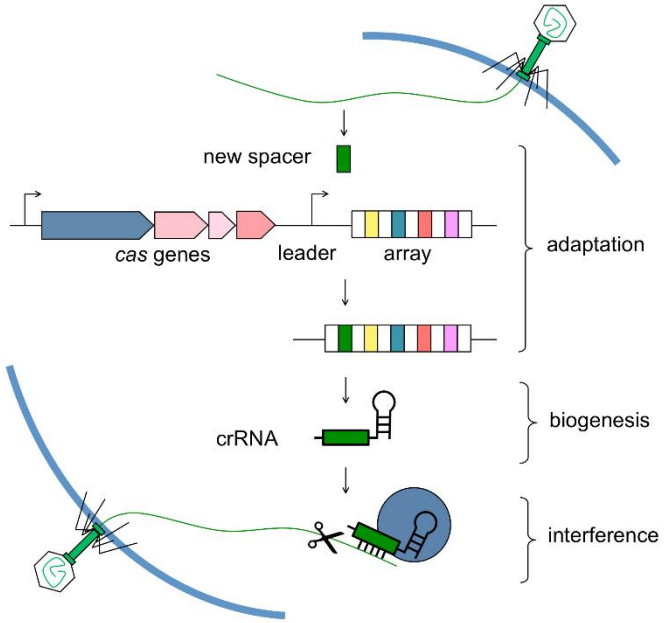


Figure 9 *– Stages in CRISPR/Cas immunity system: adaptation, biogenesis and interference* (Marraffini Laboratory - *http://marraffini.rockefeller.edu/research.html*)

To produce mutant animals using the CRISPR/Cas9 technology, only microinjection of Cas9 (in protein or in mRNA) and a sgRNA complementary to the chosen gene target is required. This technology offers many advantages over all techniques referred above: it is easier and cheaper to design and produce, since only Cas9 protein (or Cas9 mRNA) and sgRNA are necessary; sgRNA and Cas9 protein (or mRNA) can be directly injected into embryos; it is possible to make more than one mutation at once by co-injecting 2 or more gRNAs; the possibilities to target the mutation are bigger than ever since you can almost target any gene as long as a PAM sequence exists; it is also possible to make knock-ins with this technique by co-injecting oligonucleotides that will be incorporated into the genome by homologous recombination. However, limitations also exist: one of the major limitations of the CRISPR/Cas9 technology is off-targets: the mutation can occur in a non-specific region with similar homology to the real target site (even tough, off targets of CRISPR technology are fewer than other techniques); even with microinjection in 1-cell stage embryos, it does not mean that the mutation will occur in all cells nor that it happens in both alleles, creating mosaic animals; and the generations of multiple different mutated alleles. When DSB happens, the repair process of NEHJ is different in every animal producing different mutations from the same cut (The Jackson Laboratory b). CRISPR/Cas9 has been successful in many animals, invertebrates and vertebrates. Indels have been

introduced at about 90% efficiency in *C. elegans*, Drosophila, rabbit, chicken, mouse, zebrafish and human cells (Bortesi *et al*., 2016).

1.3 The *tyrosinase* and the *yellow* gene

Tyrosinase is an enzyme responsible for the conversion of tyrosine into melanin in melanocytes. Melanin gives colour to skin, hair and eyes and is also found in the retina were has it a role in vision (Genetics Home Reference - U.S. National Library of Medicine, 2017). Mutations in the *tyrosinase* gene cause oculocutaneos (OCA1) in humans and identical phenotypes are found in mice (King *et al*., 2003). In mice, a single nucleotide exchange in the coding region of *tyrosinase* causes the classical albino mutation (Jackson & Bennett, 1990). In zebrafish, the *tyrosinase* gene is expressed first in the retinal pigment epithelium and then in the neural crest (Camp & Lardelli, 2001). The *tyrosinase* gene was chosen in this project exactly because it is expected that after knocking out the *tyrosinase* gene a lack of pigmentation phenotype would be easily observed.

The *yellow* gene (y) is located in the drosophila X chromosome and controls the pigmentation pattern of the adult fly cuticle and larval mouth parts. When the *yellow* gene is mutated adult flies have a phenotypically distinct yellow pigmentation on its cuticle (Biessmann & Alberts, 1985). Just as for the *tyrosinase* gene, the *yellow* gene was chosen for convenient phenotype scoring: *yellow* knockout mutants will have yellow cuticles and can be easily differentiated from wildtype flies with normal brownish cuticle (Fig. 10).

Figure 10 *– Different body color between a Drosophila wildtype female (1) and a Drosophila yellow female (2)* (Rampasso & Vilela, 2017)

2. Aims

Despite the fact that the CRISPR/Cas9 technique is already well established in these three organisms, there is a lack of a general common protocol suitable for all 3 animals. In this project, our focus was to establish a single protocol for guide RNA production and CRISPR mediated knockout generation that would fit all 3 model organisms: *Danio rerio*, *Drosophila melanogaster* and *Mus musculus*.

The main objective of this MSc project was to optimize a general protocol of guideRNA production that would fit three model organisms, *Danio rerio*, *Drosophila melanogaster* and *Mus musculus*, commonly used at CF and produce mutant animals using CRISPR technology. To achieve this goal, we:

- Chose a gene that would be responsible for an observable phenotypic characteristic. The knockout of that gene was expected to generate a different phenotype;
- Used a guideRNA production protocol that had been already shown to be successful in *Danio rerio*;
- Started with the validation of such a protocol in *Danio rerio* using embryo microinjection of guideRNA and Cas9 protein;
- Moved to *Drosophila melanogaster* and adjusted microinjection concentrations of guideRNA and Cas9 protein to produce mutant individuals;
- Performed embryo microinjection in *Mus musculus* also adjusting the microinjection concentrations to produce mutant individuals.

A second objective of this project, after producing mutant animals, was to establish a mutant stable line. To achieve this goal, we:

- Crossed *Danio rerio* mutants between themselves and screened the progeny for non-pigmented individuals
- To establish a line *tyrosinase* knockout, injected animals need to be crossed with wildtype individuals
- Crossed *Drosophila melanogaster* mutants with *yellow* flies for two generations and screened the progeny for yellow cuticle colour to establish a mutant stock for *yellow* knockout;
- Crossed *Mus musculus* mutants with albino animals and screened the progeny for non-pigmented animals, then crossed between themselves to establish a stock for *tyrosinase* knockout.

Due to the organism diversity, every step after the production of the guideRNA is different so, the next chapters, Material and Methods and Results, will be divided by organism.

3. Material and Methods

3.1 Production of guideRNA

To produce guideRNA, a modified protocol from Gagnon et al (2014) was used (Gagnon *et al*., 2014).

Template for guideRNA production

To produce guideRNA, a first template is generated, consisting on two oligos annealed. The first is a variable gene-specific oligo, comprising a suitable promoter, the target site (without the PAM region) and an overlap region. This overlap region will anneal with the second oligo that is constant and contains tracrRNA that will bind to the Cas9 protein.

A suitable promoter can be T7 or SP6. If the guideRNA sequence starts with GG, a suitable promoter is T7, if it starts with GA then is SP6. If there is no GG or GA, a GGG upstream to the guideRNA and the T7 promoter are added. The sequence for the T7 promoter is TAATACGACTCACTATA and for the SP6 promoter is ATTTAGGTGACACTATA. The overlap region is GTTTTAGAGCTAGAAATAGCAAG. In the end, there are two template possibilities:

T7: TAATACGACTCACTATA -**N20**-GTTTTAGAGCTAGAAATAGCAAG
SP6: ATTTAGGTGACACTATA-**N20**-GTTTTAGAGCTAGAAATAGCAAG

where N20 is the target site sequence chosen to the target gene.

The constant oligonucleotide, regardless of the choice of the promoter or target gene, is:

5’AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC 3’

These oligonucleotides were synthesized by Sigma.

The first step of the guideRNA production protocol was the annealing of both oligonucleotides:

Table 1 *– Solution for annealing of oligonucleotides*

Oligonucleotide	µl
Gene-specific (100 µM)	1
Constant (100 µM)	1
Water	8
	10 µl total

Followed by a temperature cycle using a thermocycler:

Table 2 *– Thermocycler conditions for annealing oligonucleotides*

95ºC	5 minutes
95ºC ramp. Rate to 85ºC	´-2ºC/second
85ºC ramp. Rate to 25ºC	´-0.1ºC/second
4ºC	Hold

The next step was the Fill in with T4 polymerase (NEB) to produce a double strand oligonucleotide:

Table 3 – Fill in with T4 polymerase

Reagent	µl
dNTPs (10nM)	2.5
10x NEB buffer	2.2
100x NEB BSA	0.2
T4 NEB DNA Polymeras	0.5
Water	4.8
	10 µl total

Samples were incubated for 20 minutes at 12ºC. After incubation 80 µl of water was added to the template followed by purification using a PCR cleanup column, eluting in 30 µl of water. Expected DNA yield should be between 100-200 ng/µl. After measuring the DNA, a 1% agarose gel or QIAxcel ScreenGel®, was performed to verify that the product had the correct size of ~120bp.

Transcription of template to produce guideRNA

After purification, the template was in vitro transcribed with Ambion Megashortscript T7 or Megascript SP6 kit, depending on the promoter, to produce guideRNA. From this point onward all procedures were made in RNase-free conditions. To maximize the transcription, the incubation step was prolonged to an overnight incubation when using T7 kit and 4 hours when using SP6 kit.

Extraction and Purification of guideRNA

Recovery of guideRNA was performed with Qiagen micro-RNA purification Kit or with a Phenol/chloroform extraction: to 20 µl of transcription product, 115 µl of nuclease-free water and 15 µl of Sodium Acetate Stop (Ambion kit) were added. Next, 150 µl of phenol/chloroform pH 4.5 (or pH 8) was added, mixed well and centrifuged for 5 minutes at 4ºC. Upper layer (lower layer if using phenol/chloroform pH 8) was transferred to another tube with 350 µl 100% EtOH, incubated for 15 min at -80ºC or with dry ice to precipitate RNA. RNA was centrifuged 20 minutes at 4ºC to form a RNA pellet. The supernatant was discarded and the pellet washed with 500 µl 70% EtOH spinning 15 minutes at 4ºC. The supernatant was discarded again and the pellet was left to dry and resuspended in 20 µl of nuclease-free water. RNA was aliquoted according to the concentration needed for microinjection.

3.2 Cas9 Protein

Cas9 Protein was batch-produced at 1 mg/ml in 20mM Tris Ph 8, 10mM $MgCl_2$ and 0.2M KCl buffer, at the Weizmann Institute of Science, Israel.

4. Danio rerio (zebrafish)

4.1 Methods

Targeting strategy

For zebrafish, the *tyrosinase* gene was chosen for mutation. *Tyrosinase* is responsible for the black pigmentation of the body and eyes of the animal. So, a knockout of this gene should produce a visible phenotype with lack of pigmentation.

Choosing guideRNA

In zebrafish, the CRISPRz database was used to look for guides already validated for the zebrafish *tyrosinase* gene. We chose the one used by Jao *et al*. (2013): GGACTGGAGGACTTCTGGGGAGG (PAM site underlined). Since *tyrosinase* guideRNA (without the PAM site) started with GG, a suitable promoter was T7, thus being the *tyrosinase* gene-specific oligonucleotide (ordered from Sigma):

TAATACGACTCACTATA**GGACTGGAGGACTTCTGGGG**TTTTAGAGCTAGAAATAGCAA
G

The guideRNA production protocol was performed and guideRNA was ready for microinjection.

Danio rerio breeding

Zebrafish were housed at the CF Fish Facility. Wildtype TU adults (around 6 males and 12 females) were crossed for each microinjection trial, setting 6 crosses for each trial (1 male to 2 females). Those crosses were made between 4 and 6 p.m. with fish housed in spawning tanks. Spawning tanks contain an insert reservoir, that have holes in the bottom, and a spacer that fits the tank, separating males and females from physical contact, but sharing the water. Animals stay overnight in these tanks being close to each other but not being able to breed, so when the spacer is removed early in the morning (when the lights turn on), fish spawn and eggs are fertilized. Spacers are taken one at a time, meaning that each cross produced embryos for a single microinjection. When the first laying of the first cross was injected, then the spacer from the second cross was removed, and so on. Eggs fall through the holes in the insert reservoir, preventing the cannibalization of the embryos

by the parents. Fish were used to breed once a week. Eggs were collected into petri dishes with the help of a tea strainer and blue water (Methylene blue) and were ready to be aligned for microinjection. After laying, animals were housed back into the housing tanks (Martins *et al*., 2016). All animal procedures were made under rigorous standards of animal welfare and complied with the 2010/63/EU (European Parliament and the Councli pf the European Union, 2010).

Microinjection needles and microinjection set-up

Agarose petri dishes with trenches (Fig. 11) were used to align and microinject embryos. Alignment and microinjection of embryos were performed under a Zeiss Discovery V8 scope and microinjection with a PV820 Pneumatic Picopump (WPI). Microinjection needles were bought from Biomedical Instruments and were loaded with Eppendorf Microloader™ tips.

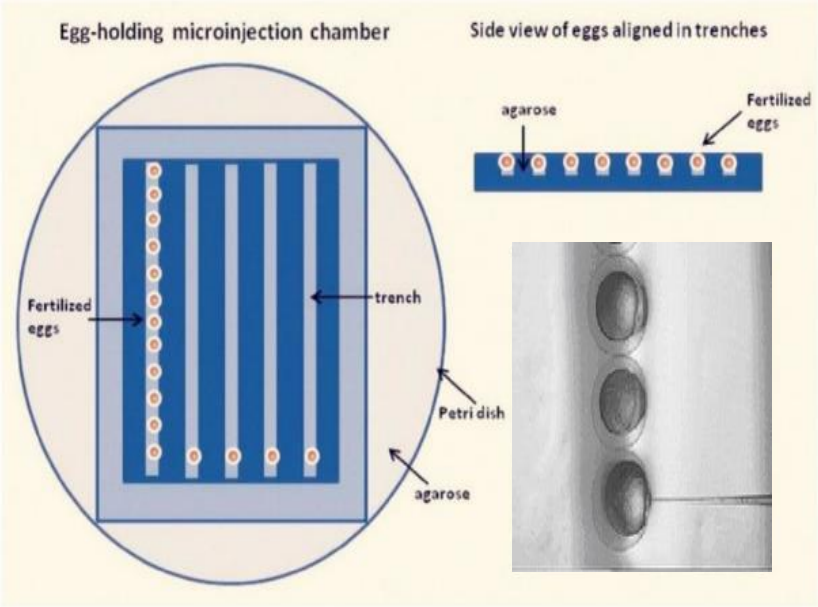


Figure 11 *– Agarose plates and zebrafish embryo alignment. Image modified from Wang et al. and Lu Zhe* (Wang *et al*., 2013; Zhe, n.d.)

Embryo Microinjection

Collected embryos were aligned with the cell positioned to the right side so it can be directly injected (Fig. 11) Only one-cell stage embryos were microinjected, embryos in other stages were discarded. Uninjected embryos were also kept as controls for each laying/cross.

Different concentrations of guideRNA and Cas9 protein were tested (Table 4). Phenol red was added to the mix to serve as a visible marker for the injection into the embryo. After injection,

embryos were incubated and bleached (to disinfect embryo surface) at 24hpf. Survival rates were recorded 24 hours after injection (See Appendix 1).

Table 4 *– Microinjection concentrations of gRNA and Cas9 protein into zebrafish embryos*

Cas9 Protein (ng/µl)	sgRNA (ng/µl)
210	526
260	174
260	438
315	526
500	438
600	200

Screening for mutations

Forty-eight hours after injection it was possible to evaluate the result from targeting the *tyrosinase* gene because *tyrosinase* expression already started (Camp & Lardelli, 2001). Individuals with visible phenotypes were incubated until 5 days old, at which age larvae entered the nursery. For non-phenotype targeting there is a possibility to screen for the mutation in early stages by DNA extraction from 24hpf embryos, following HotSHOT protocol (Meeker *et al.*, 2007).

The HotSHOT protocol consists in collecting pools of two embryos into a PCR strip (without blue water; if not possible, blue water can be removed with a micropipette) with 50 µl of 50mM NaOH to cause cell lysis during a 15 minutes incubation at 95ºC, followed by a cooling step at 4ºC. If using a thermomixer for incubation, ice can be used to cool down the samples; if using a thermocycler, an additional step of 4ºC can be added. For buffering, 5 µl of 1mM Tris-HCL pH 7.5 was added to the samples. After embryo DNA extraction, a 25 µl PCR reaction mix was prepared using 5 µl of extracted embryo DNA (See Appendix 5 for primers used). Uninjected embryos were always used as controls. After PCR reaction (Table 5), samples were loaded in a 3% agarose gel for 1 hour at 80V. Uninjected eggs should have a single band while injected positive eggs should have a smear or more than one single band. This method can and should be used to test guide efficiency, according to which a respective number of fish are raise to adulthood. The PCR product was purified and sent for sequencing. If the CRISPR process is successful and there is integration or deletion of nucleotides in the target region, this can be easily seen in the sequence chromatogram that will be a mix of different alleles present in the sample.

Primers

For PCR from embryo DNA extraction, amplicons should be around 100bp, 50bp from the cut site to each side, the smaller the better to search for indels, but for the *tyrosinase* gene the primers used were the same as Joa *et al*. (2013) and amplicons were around 315bp (Appendix 5).

Genotyping the adults

By two months old, fish are big enough to be fin clipped to sample tissue for genotyping. DNA extraction was performed with proteinase K: Tissue was sampled into 200 µl of lysis Buffer (50 Nm Tris-HCL pH 8.5, 1 mM EDTA and 0.5% Tween-20) and proteinase K was added to a final concentration of 200 µg/µl immediately before DNA extraction (samples can be frozen before extraction), samples were incubated for 2h at 55ºC in a thermomixer followed by a denaturation step of 10 minutes at 95ºC. After incubation, samples were centrifuged 10 minutes at 13.200rpm at 4ºC. The supernatant was collected to a new tube and stored at 4ºC for up to 3 months or -20ºC for longer periods.

After DNA extraction, a 25 µl PCR reaction (Table 5) was performed and samples loaded on QIAxcel ScreenGel®. Different band sizes (or a smear – QIAxcel ScreenGel® has an "smear analysis" option") should appear.

Table 5 *PCR cycle for tyrosinase gene*

	Temperature	Time
Initial Denaturation	95°C	30 seconds
34 Cycles	95°C	30 seconds
	60°C	30 seconds
	68°C	2 minute
Final Extension	72°C	5 minutes
Hold	12ºC	

Sequencing

For sequencing the mutation, positive fish should be outcrossed and the F1 genotyped. The PCR product sent for sequencing and screened for jammed chromatogram near the PAM site. For the *tyrosinase* gene in particular, incrosses between F0 mutant individuals were made and non-pigmented progeny was grown to adulthood for genotyping.

4.2 Results

GuideRNA production

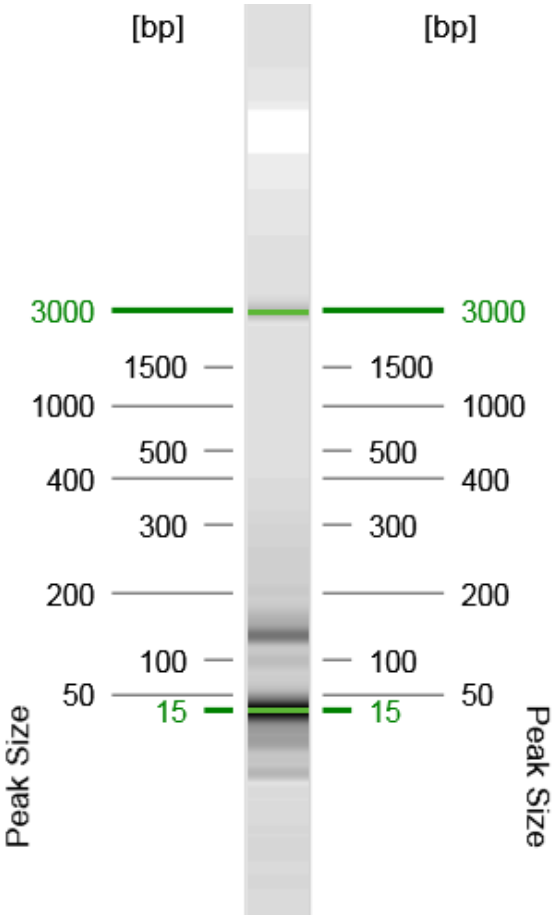


Figure 12 *– QIAxcel ScreenGel®, for tyrosinase gRNA template*

After annealing the oligos and fill-in with T4 polymerase, the template was purified using a PCR column (QIAquick PCR Purification Kit) and eluted in 30 µl of water. DNA concentration was expected to be 100-200ng/µl, tyrosinase template was 145 ng/µl. Template was then loaded in QIAxcel ScreenGel®, and a band of ~120bp was observed (smaller fragments are primer dimer) (Fig. 12) (as described in protocol).

RNA extraction with Qiagen micro-RNA purification kit showed that very few RNA was extracted, 54 ng/µl in our first attempt and 10 ng/µl in our second attempt, which was not sufficient to microinject. Based on these results, we did a comparison between RNA extraction with the Qiagen kit and Phenol/chloroform extraction. After in vitro transcription, the sample was divided into two tubes and we tested the 2 protocols. With phenol/chloroform we could extract 3278 ng/µl while with Qiagen kit we were only able to extract 20,8 ng/µl. According to the ratios of the absorbance parameters and by applying the Beer-Lambert Law, guideRNA extracted with Phenol/chloroform was contaminated with protein (A^{260nm}/A^{280nm} = 1.66) but free from organic contaminants (A^{260nm}/A^{230nm} = 2.26), so we therefore chose to follow the phenol/chloroform protocol for RNA extraction.

Screening for mutations

DNA embryo extraction

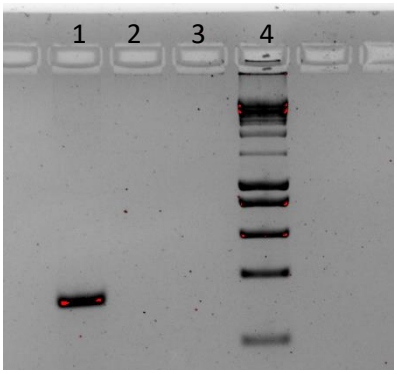


Figure 13 *– PCR amplification of 314bp fragment of the tyrosinase zebrafish gene run in 1% Agarose gel: Amplification from fin sample genomic DNA preparation (1) Amplification from a 24hpi embryos genomic DNA preparations (2 and 3), geneRuler 200bp (Thermo Scientific) (4).*

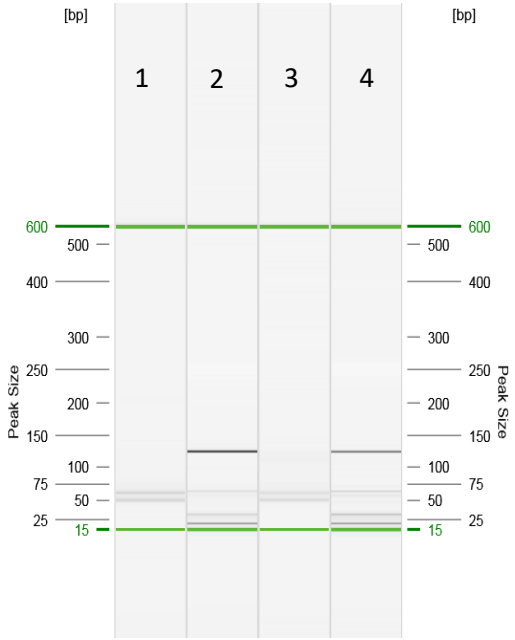
At 24hpi embryo DNA was extracted with 10 pools of 2 injected embryos each and 2 pools of two non-injected embryos as control but nothing was amplified in the PCR reaction. A new pair of primers and different enzymes were tested but still nothing was amplified. Next, extraction from DNA embryos and fin samples were compared. Only by using DNA extracted from fin samples was it possible to amplify the correctly sized band (Fig. 13).

In an effort to understand and troubleshoot lack of amplification of the *tyrosinase* fragment from 24hpi genomic DNA template, we tested different amplification protocols:

Protocol 1 – protocol from HotSHOT using Thermocycler for incubation and 5 µl of template DNA for PCR reaction

Protocol 2 – protocol from HotSHOT but using Thermomixer for incubation and an additional final step of 5 minutes centrifugation at 13550 rpm and 1,5 µl template DNA for PCR reaction

Different genes regions were amplified, in the *tyrosinase* gene and *DIA1R* gene, as a control. The primers used for amplification of *tyrosinase* gene were the same ones that worked for the fin sample and that were used by Jao *et al.* (2013). For amplification of the *DIA1R* gene another pair of primers that had also worked for the fin sample, were used. Results from the above PCR reactions are in Figure 14.



Overall Result Table							
AI35_Embryo DNA PCR_2017-01-20							
B1 cc1		B2 cc2		B3 jm1		B4 jm2	
Size [bp]	Mol. [nmol/l]	Size [bp]	Mol. [nmol/l]	Size [bp]	Mol. [nmol/l]	Size [bp]	Mol. [nmol/l]
600	n/a	63	9.66	600	n/a	57	9.66
		124	36.62			63	12.61
		600	n/a			124	22.64
						600	n/a

***Figure** 14 - Left panel: QIAxcel ScreenGel® analysis (1) protocol 1 for tyrosinase gene; (2) protocol 1 for DIA1R gene; (3) protocol 2 for tyrosinase gene and (4) protocol 2 for DIA1R gene. On the right, there's an overall result table with DNA concentration measured in the samples*

As we can see from figure 14, we suceeded in amplifyng *DIA1R* gene with template DNA extracted from 24h embryos but fail to amplify *tyrosinase* gene., in both embryo DNA extraction protocols. With these results and since extraction using the HotSHOT protocol clearly worked, we decided to extract genomic DNA from different ages and use genomic DNA extracted as DNA template for PCR reaction to see when was the *tyrosinase* gene amplified. For genomic DNA extracted from 72hpf larvae, no amplification was detected but with genomic DNA extracted from 5-day-old larvae a very faint band starts to appear (Fig. 15).

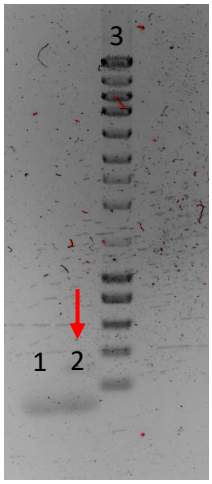


***Figure 15** – PCR amplification of 314bp fragment of the tyrosinase zebrafish run in 1% Agarose gel: Amplification from 72h larvae genomic DNA preparation (1); Amplification from 5-day-old larvae genomic DNA preparation (2); GeneRuler 200bp (Thermo Scientific) (3)*

In another approach, we tested the addition of DMSO to the PCR reaction mix of 24hpf and 72hpf embryo DNA extraction (Fig.16). These results were not pursued since animals were already growing and for *tyrosinase* in specific (that causes a phenotypic result when mutated), DNA embryo extraction was not essential.

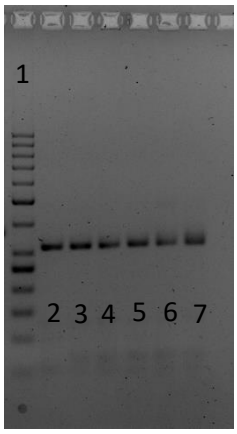


Figure 16 *– PCR amplification of 341bp fragment of the tyrosinase zebrafish run in 1% Agarose gel: GeneRuler 50 bp (Thermo Scientific) (1); Amplification from 24 h embryos genomic DNA preparation (in pools of two embryos) (2, 3, 4, 5, 6 and 7).*

Phenotype screening

At 48hpf, injected embryos were screened for lack of pigmentation and the number of individuals with mosaicism was scored. Although in our first microinjection trial we did not see a lack of pigmentation in larvae, we still decided to grow some fish and some adult individuals did grow with non-pigmented cells. So, despite the fact that this mutation causes lack of pigmentation, it is possible that sometimes it could not have a larval phenotype. See Appendix 2 for mosaic individuals that were identified and respective microinjection mix concentrations. Eighteen larvae were identified with mosaicism, but in total we had 40 fish that showed pigmented mosaicism as adults (see Figure 17 for mosaicism example in larvae). This phenotype was achieved with different concentration mixes (Appendix 2). Mosaic animals that were raised but that didn't show a lack of pigmentation as larvae, were obtained from our first injection with 260 ng/µl of Cas9 protein and 174 ng/µl of sgRNA.

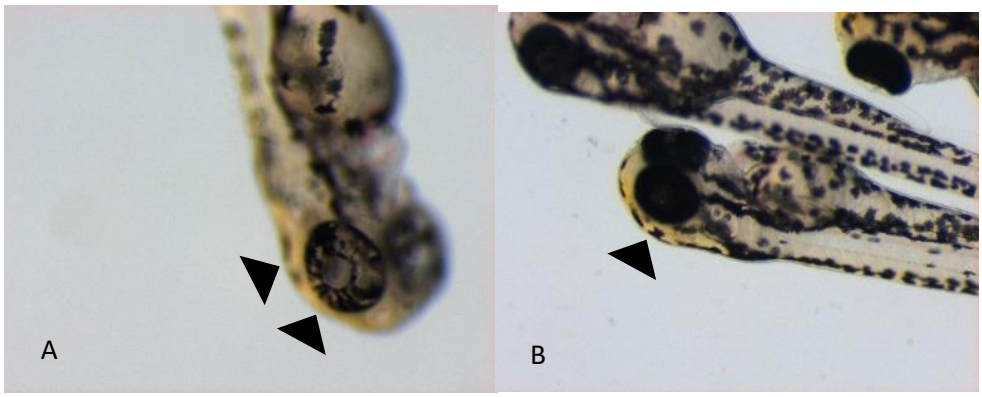
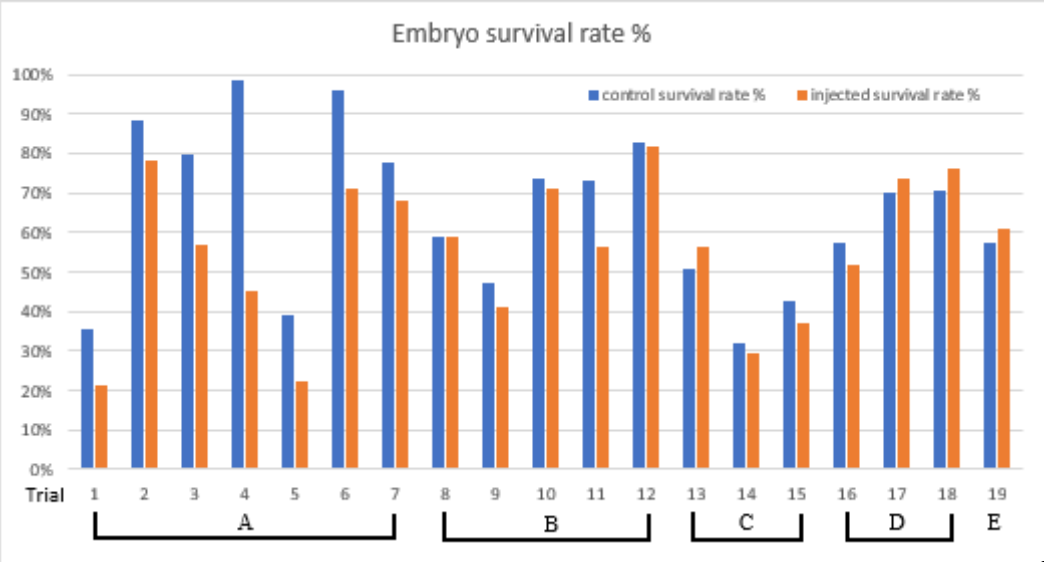


Figure 17 *– Injected 72h larvae for tyrosinase knockout: Lack of pigmentation in some cells of the eye (A) and wild type phenotype (B).*

Table 6 Mosaic animals with lack of pigmentation phenotype found according to concentrations mixes

Cas9 Protein (ng/ul)	guideRNA (ng/ul)	Larvae with mosaic pigmentation
210	526	0
260	174	0
260	438	1
315	526	17
500	438	0
600	200	0

Embryo survival rates were different from uninjected embryos and injected embryos, being lower in the injected ones. Despite results from Condition A (see Graphic 1) that are probably due to the lack of experience, that was optimized trough trials, injected embryo survival is close to control embryo survival rate in all other conditions (B, C, D and E). Also, it seems that toxicity does not affect embryo survival, since that the lowest concentration mix condition (B) showed very similar survival rate to the most concentration mix conditions (D and E).



Graphic 1: Embryo survival rate: Control embryo survival rate vs injected embryo survival rate in different microinjection concentrations of Cas9 protein and guideRNA (A, B, C, D and E). A – 260 ng/µl Cas9 protein + 174 ng/µl guideRNA, B – 260 ng/µl Cas9 protein + 438 ng/µl guideRNA, C – 500 ng/µl Cas9 protein + 438 ng/µl guideRNA, D – 315 ng/µl Cas9 protein + 526 ng/µl guide RNA and E – 600 ng/µl Cas9 protein + 200 ng/µl guideRNA

Genotyping

Genotyping F0 injected animals

Fourteen adult fish (F0 injected animals) were genotyped for screening indels. TU wildtype DNA was used as a control. All samples amplified the wildtype band, but also amplified smaller or bigger fragments that indicate indel occurrence (Fig. 18). Overall table of results of measured band sizes can be found in Appendix 3.

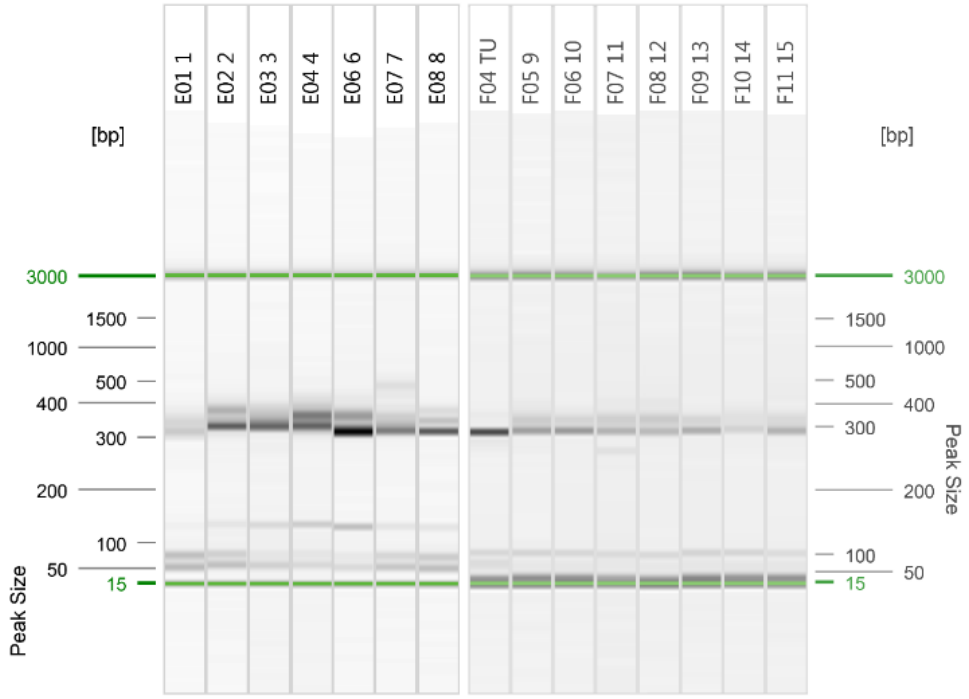


Figure 18 *– Screening for indels: Wildtype sample (F04 TU) amplified DNA with ~315 bp, all other samples were from mosaic individuals that in addition to wildtype band, also showed smaller or bigger fragments proving that guideRNA cut the DNA and NEHJ events have occurred.*

Sequencing

PCR products from these samples were sent for sequencing, but it was a very jammed chromatogram making it impossible to draw any conclusion. To really get conclusions of which indels have really occurred it may be best to run a high concentration agarose gel and extract each amplicon and send it for sequencing, which was not done due to time constrains.

Genotyping F1 fish

Non-pigmented larvae (Fig. 19) from incrosses of mosaic animals, were grown and fins sampled at 2-month-old fish. From figure 19, it's possible to see that all non-pigmented individuals (D01 – D08) lack the wildtype amplicon (D09), and instead, are composed of different F0 mutations.

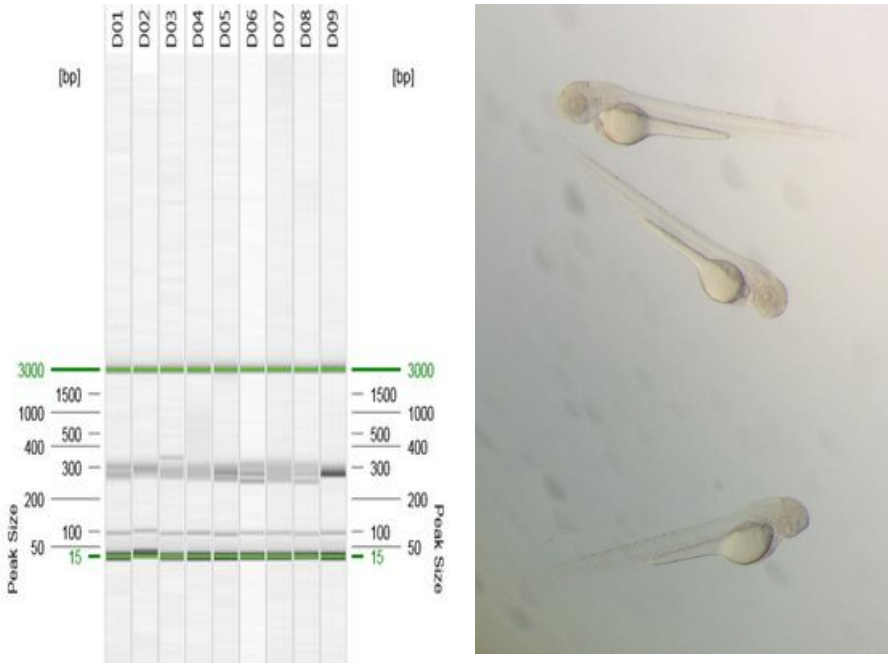


Figure 19 *– Genotyping of Non-pigmented incrossed animals (left side); Non-pigmented 72h larvae (right side)*

5. Drosophila melanogaster (fruit fly)

5.1. Methods

Targeting strategy

For fruit fly, *yellow* was the targeted gene to be knockout. *Yellow* is a spontaneous recessive mutation that gives a yellow colour to the body of the fly (Biessmann & Alberts, 1985). *Yellow* mutation already exists and it's been part of fly crossings strategies. In this case we tried to mimic the existing spontaneous mutation of yellow body colour in wildtype flies and vasa_Cas9 flies. Vas_cas9 (Bl #51324) are flies that express Cas9 protein under the germ-line promotor *vasa* and were used for microinjection of only guideRNA instead of Cas9 protein and guideRNA co-injection in non-expressing cas9 flies.

Choosing guideRNA

In Drosophila, guideRNA was found in BreakingCas site (Oliveros *et al*., 2016) using the *yellow* gene sequence (NM_143655.4) as template for search fit guides. The guide chosen had a 99.9 score: GGTTTTGGACACTGGAACCGTGG (PAM site underlined). This guide was also used in Basset et al. (2013) experiments. Since yellow guideRNA (without the PAM site) started with GG the suitable promotor was T7, being the *yellow* gene-specific oligonucleotide (ordered from Sigma):

TAATACGACTCACTATAGGG**GGTTTTGGACACTGGAACCGG**TTTTAGAGCTAGAAATA
GCAAG

Production of guideRNA was performed according to the protocol described in "Production guideRNA" methods section, page 16 and it was ready to microinject.

Microinjection needles and microinjection set-up

Embryos were aligned under a Leica MZ6 scope and microinjected under a Zeiss Primovert microscope adapted to microinjection, with a Narishige micromanipulator connected to a PV820 Pneumatic Picopump. Capillaries from WPI (Thin wall single- barrel Standard Borosilicate 1mm with filament) were pulled on a Sutter P-2000 needle puller to produce microinjection needles. Needles were loaded with Eppendorf Microloader™ tips.

Embryo Microinjection

Flies (Canton S and Bl#51324) were maintained in laying pots with petri dishes containing apple juice and yeast. Embryos were collected between 40min-1h after dish change and injected as soon as possible while still in a syncytial stage. For microinjection, embryos were dechorionated first with 50% bleach and aligned (around 50 per slide) all to the same side. Embryos were covered with oil 10s (VWR chemicals) to prevent dehydration but still allow gas exchanges since embryos were dechorionated (Al-Dosary *et al*., 2010). Microinjection was performed in the posterior side of the embryo, where pole cells, which will later give rise to the fly gonads, are located, increasing the chances of the mutation to occur in the germ-line and being transmitted to the progeny. First, injections of Cas9 protein with guideRNA into Canton S (wildtype) flies were performed. Different concentrations of Cas9 protein, to a maximum concentration of 800 ng/ µl, were tested. Next, I performed a series of injections into vas_cas9 flies that already express the Cas9 Protein, of only guideRNA at 500 ng/ µl or 1000 ng/ µl. Twenty-four hours after injection, larvae were collected into a vial with food and yeast and were left 10 days at 25ºC until adult eclosion.

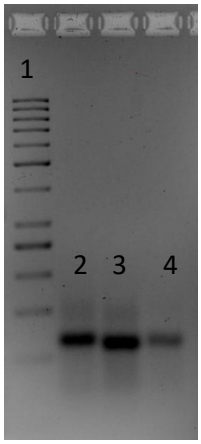
Screening for mutation

In flies injected with Cas9 protein and guideRNA, one should see body colour mosaicism in the F0 injected flies, as for the zebrafish *tyrosinase* injections. To obtain a whole-body yellow fly, the mosaic F0 males should be individually crossed to virgins from *yellow* stock. If the mosaicism of the F0 male extends to the germ-line, a subset of the F1 females should now be all yellow. For vas_Cas9 flies, that only express Cas9 protein under a germ-line promotor, F0 generation should not have any phenotype for yellow. To follow the mutation in this case, injected males should be crossed with *yellow* virgins, and F1 progeny virgin females should be crossed with *yellow* males. If the strategy is successful, a subset of F2 males will carry the *yellow* mutation and have a yellow body phenotype. In this case, F0 animals will only be mosaic in the germ-line that is the site of Cas9 production.

5.2 Results

GuideRNA production

After annealing the oligos and fill-in with T4 polymerase, template was purified using a PCR column (QIAquick PCR Purification Kit) and eluted in 30 µl of water. Concentration of DNA was expected to be between 100-200ng/ µl, yellow template was 150 ng/ µl. Template was then loaded into a 1% agarose gel, a band of ~120bp was observed (Fig. 20) (as described in protocol).



RNA extracted with Phenol/chloroform was 1930 ng/ µl. According to the ratios of the absorbance parameters and by applying the Beer-Lambert Law, guideRNA was contaminated with protein (A^{260nm}/A^{280nm} = 1.51) but free from organic contaminants (A^{260nm}/A^{230nm} = 1.99).

Figure 20 *– Annealed and filled-in templates on 1% agarose gel, for yellow (2) tyrosinase mouse guide 1 (3) and tyrosinase mouse guide 2 (4). GeneRuler 50 bp (Thermo Scientific) (1)*

Screening for mutation

When microinjecting Cas9 protein and guideRNA into drosophila embryos, it was expected that mosaic flies would appear, but no phenotype was present and all F0 injected flies had homogeneous wildtype colour cuticle. These results are consisting with Lee *et al*. (2014) findings where his group only achieved mutations, when injecting Cas9 protein, at a 4000 ng/ µl final concentration (Lee *et al.*, 2014). Unfortunately, our Cas9 protein stock was at a 1000 ng/ µl and, bearing in mind that the guideRNA was co-injected further diluting our Cas9 protein solution, the maximum Cas9 protein concentration we could achieve for microinjection in this project was 800 ng/µl. Microinjection concentrations can be found in Appendix 4. To further proceed with this strategy, we should next try to inject Cas9 coding plasmid instead of protein (Screening strategy in Diagram 1).

There is yet another strategy possible in this model system that is the use of *Drosophila* stocks already expressing the Cas9 protein. In this case, only the guideRNA should be injected (see Diagram 2). If the Cas9 is being expressed under the control of a germ-line promoter (eg. Vasa-Cas9), no mosaics will be seen in F0 injected flies. However, if the strategy is successful, some of the F1 females, resulting from F0 injected males crossed to *yellow* virgins, will carry the induced *yellow* mutation and have a yellow phenotype. Unfortunately, we did not have available the correct Cas9 stock to follow the second strategy. Instead, we had a Cas9 stocks with the Cas9 transgene positively marked with a *yellow* rescue gene (*y*+). In this case, the crossing scheme to recover possible successful *yellow* induced mutations further complicates and an extra generation is necessary (see Diagram 3).

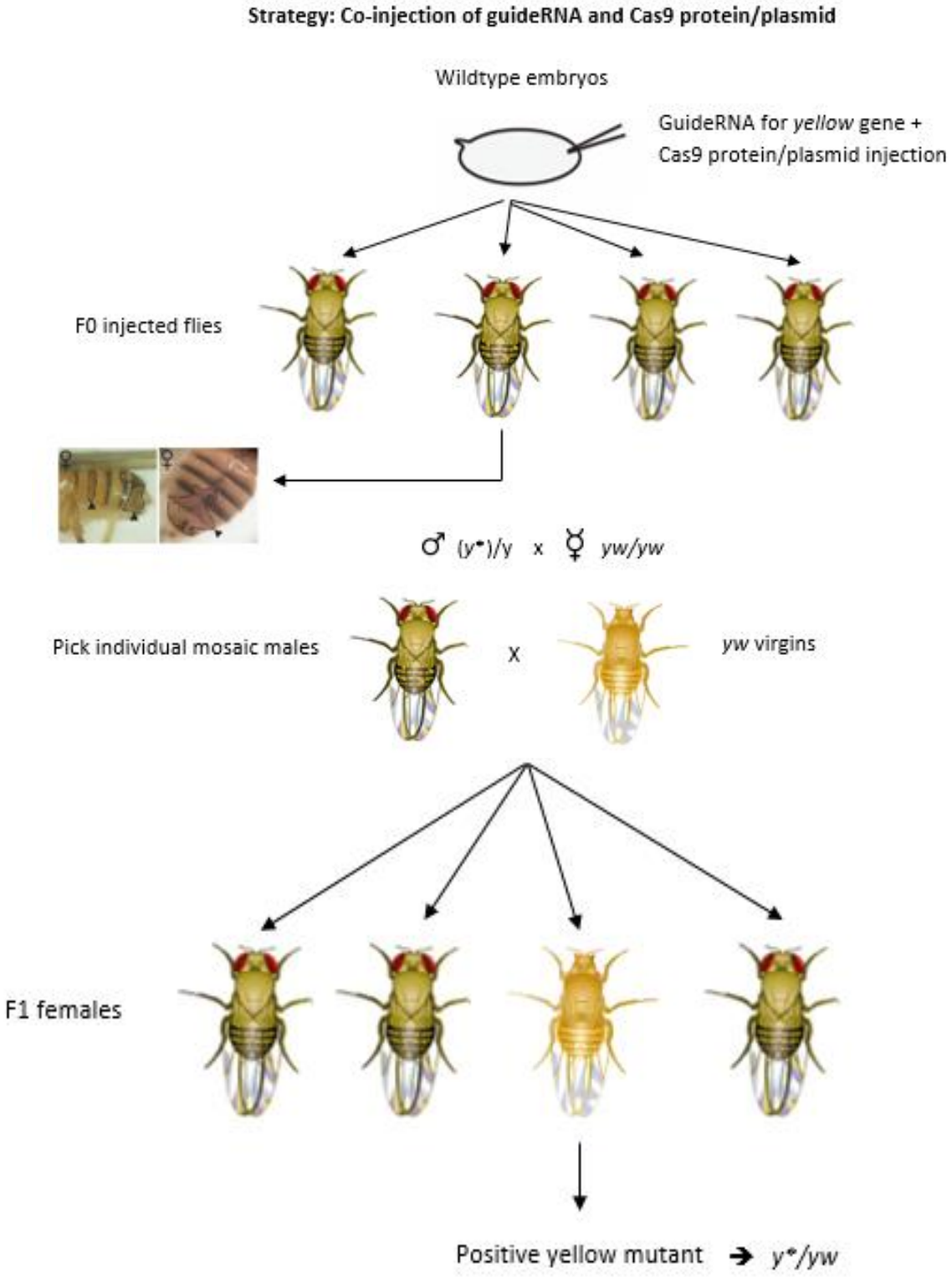


Diagram 1 *– GuideRNA and Cas9 protein/plasmid microinjection scheme for mutation screening*

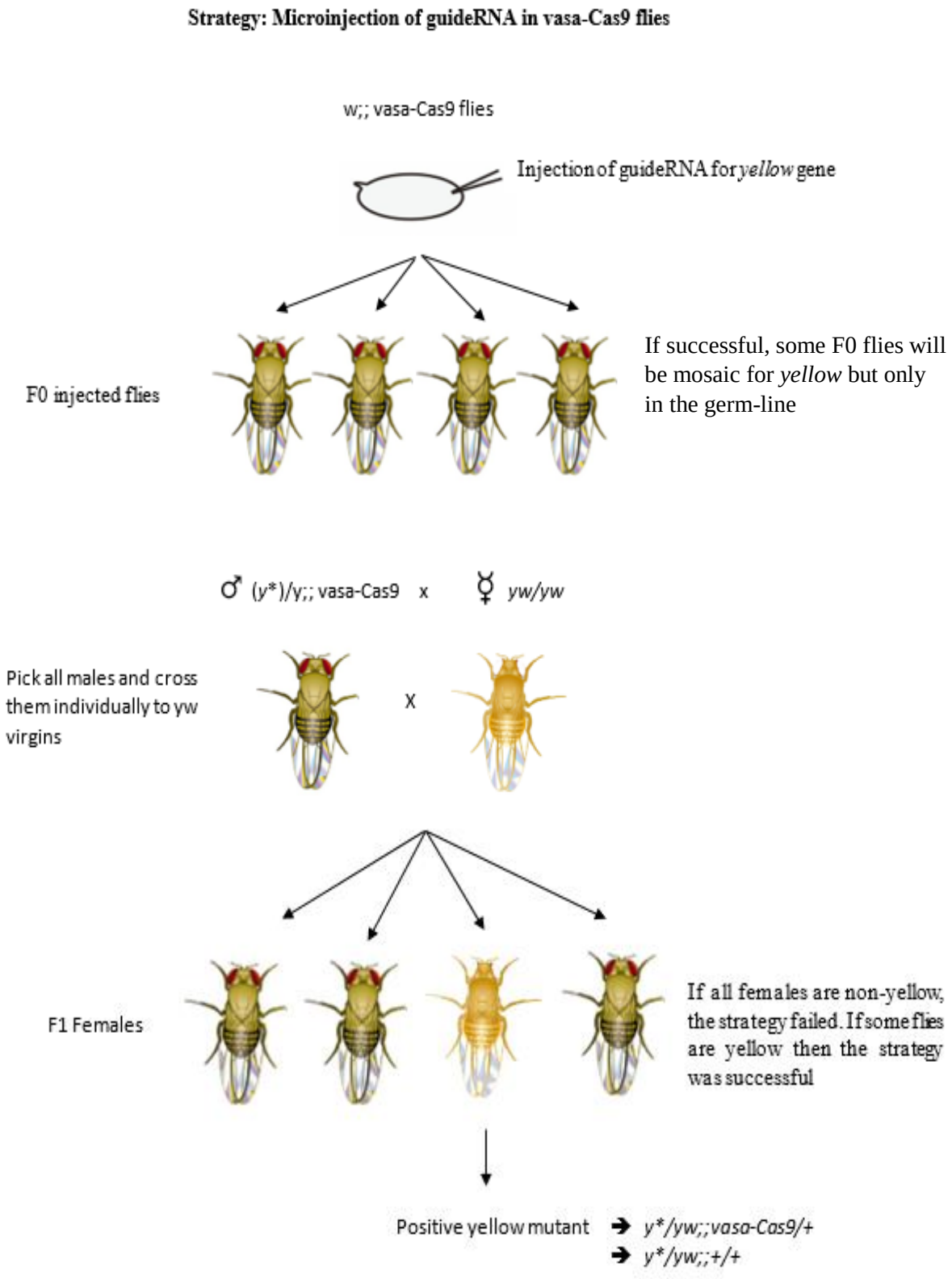


Diagram 2 - *Injection of guideRNA for yellow gene in vasa-Cas9 flies and screening for mutation*

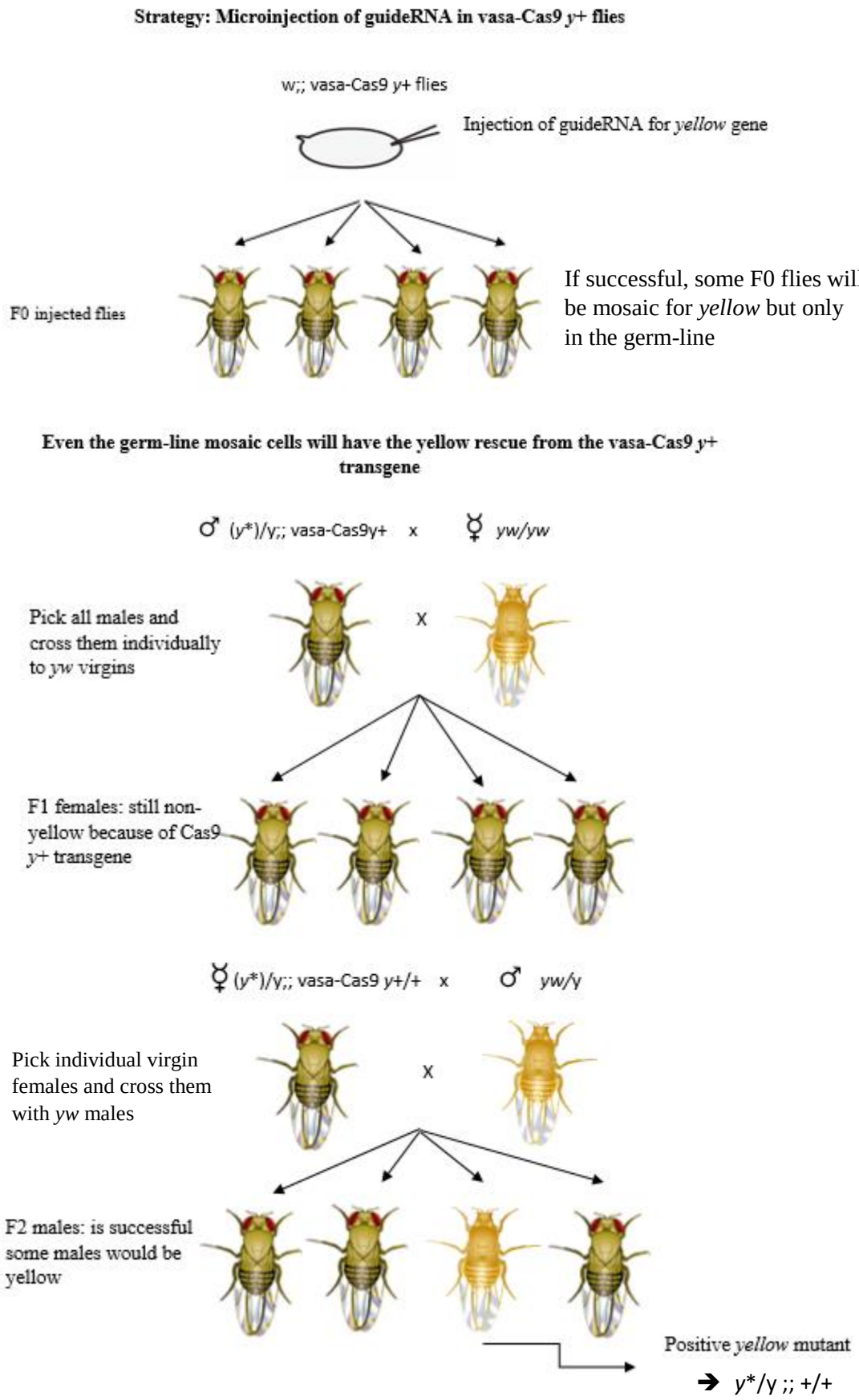


Diagram 3 – Injection of guideRNA for yellow gene in vasa-Cas9 y+ flies and screening for mutation

6. *Mus musculus* (mouse)

6.1 Methods

Targeting strategy

For mouse, the gene chosen to be knockout was, like *Danio rerio*, the *tyrosinase* gene. The *tyrosinase* gene has the same function as in *Danio rerio*, giving black pigmentation to the body of the mouse. So, a knockout of this gene should produce a visible phenotype by lacking colour in body and eyes.

Choosing guideRNA

In *Mus musculus*, guideRNA was also found in the BreakingCas site (Oliveros *et al.*, 2016) using the *tyrosinase* gene (ENSMUSG00000004651) sequence as a template in the search for fit guides. In this case, there were two chosen guides. One near the first ATG: GGTCATCCACCCCTTTGAAGGG (PAM site underlined) with 86.7 score. And another one, that was the best-scored guide for this gene, with 98.8 score: GGACCACTATTACGTAATCCTGG (PAM site underlined). Since *tyrosinase* guideRNAs (without the PAM site) started with GG the suitable promoter was T7, being the *tyrosinase* gene-specific oligonucleotides (ordered from Sigma):

Tyr_grna1: TAATACGACTCACTATAGGG**GGTCATCCACCCCTTTGAA**GGTTTTAGAGCTA GAAATAGCAAG

Tyr_grna2: TAATACGACTCACTATAGGG**GGACCACTATTACGTAATCC**GTTTTAGAGCTA GAAATAGCAAG

Production of guideRNA was performed according to the protocol described in "Production guideRNA" methods section, page 16 and it was ready to microinject.

Mus musculus animals

Animals were housed in the CF Vivarium. For this project, 25 C57Bl/6J female mice were used for embryo collection, and 12 C57Bl/6J males were used as studs (only copulated with females to fertilize the eggs). Nine NMRI females were used as surrogate mothers. All animal procedures were made under rigorous standards of animal welfare and complied with the Directive 2010/63/EU (European Parliament and the Councli pf the European Union, 2010).

C57BL/6J females were superovulated with 5IU PMSG (Sigma) at 2p.m. and 46 hours after with 5IU hCG (Sigma) and mated with C57BL/6J males right after hCG administration via intra-peritoneal injection. 16-18 hours after, females were sacrificed and oocytes collected into a hyaluronidase medium (Sigma), to degrade the cumulus cells. After cumulus cells degradation, embryos were washed in M2 and M16 medium (Sigma) and left incubate at 37ºC, 5%CO2 for 3h.

Microinjection needles and microinjection set-up

Fertilized oocytes, checked by the presence of pronuclei, were chosen under a Zeiss Discovery V8 scope. Microinjection of embryos was performed with a Zeiss Observer Z1 microscope adapted to microinjection with Eppendorf Transfer Man NK2 micromanipulator and Eppendorf FemtoJet Microinjector. Capillaries from WPI (Thin wall single- barrel Standard Borosilicate 1mm with filament) were pulled on a Sutter P-2000 needle puller, to produce microinjection needles. Holding needles were bought from Eppendorf (VacuTip). Microinjection needles were loaded with Eppendorf Microloader™ tips.

Embryo Microinjection

After 3h, fertilized embryos were transferred into a microinjection chamber (Fig. 21), consisting on a drop of M16 medium covered with paraffin oil (Sigma). Embryos were microinjected in the pronuclei with a continuous flow from Eppendorf FemtoJet microinjector. Different concentrations were tested, first only the *tyrosinase* guide 1 was co-injected, at a concentration of 20ng/µl, with Cas9 protein at 100ng/µl. Next, the 2 guides were co-injected, 20 ng/µl each, with 100ng/µl Cas9 protein. Lastly, the 2 guides were co-injected, 50 ng/µl each, with 60 ng/µl Cas9 protein.

Right after injection, embryos were transferred into a surrogated mother, if primed surrogated mothers were not available for the day of embryo microinjection, embryos were incubated until 2-cell stage and then transferred to a surrogated mother or frozen. NMRI females were used as surrogated mothers and a maximum of 40 embryos were transferred to a single female. Twenty-one days after pups were born.

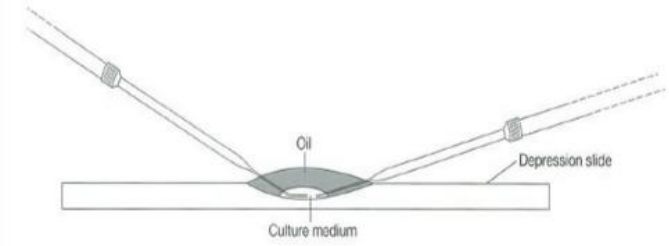


Figure 21 *– Mice embryo Microinjection Chamber*

Screening for mutations

This case is identical to the zebrafish *tyrosinase* mutation. Animals were expected to have a lack of pigmentation in the coat colour, this phenotype should be visible in 5-day-old pups, when pigmentation starts to appear. About 21 days after pups were born, they can be weaned and an ear sample can be taken to genotype and search for indels.

Primers

Primer design was done using NCBI primer Blast and OligoPerfect™ Designer (Thermo Fisher Scientific). Primers can be found in Appendix 5. For *tyrosinase* guide 2 a first design pair of primers (amplification of ~340bp) was not functional and a new pair was designed but was not possible to amplify a smaller fragment, being the new amplicon of ~544 bp, not a perfect size to found indels.

DNA extraction

All DNA extractions of ear samples were performed with REDExtract-N-Amp™ Tissue PCR Kit. PCR reaction mix for the *tyrosinase* guide 1 indels was also performed using REDExtract-N-Amp™ Tissue PCR Kit in a 25 µl reaction mix. But for *tyrosinase* guide 2, REDextraction reagents to PCR didn't work. This amplification was performed with Dream taq PCR Master Mix and a small modification in the PCR cycle. After PCR reactions, samples were loaded into a 3% agarose gel or in QIAxcel ScreenGel®.

***Table 7**: PCR cycle for tyrosinase guide 1 indels (A) and PCR cycle for tyrosinase guide 2 indels (B)*

A	Temperature	Time
Initial Denaturation	95°C	30 seconds
34 Cycles	95°C	30 seconds
	58°C	30 seconds
	68°C	2 minute
Final Extension	72°C	5 minutes
Hold	12ºC	

B	Temperature	Time
Initial Denaturation	95°C	30 seconds
38 Cycles	95°C	30 seconds
	58°C	30 seconds
	68°C	2 minute
Final Extension	72°C	5 minutes
Hold	12ºC	

6.2 Results

Production of guide RNA

After annealing the oligos and fill-in with T4 polymerase, template was purified using a PCR column (QIAquick PCR Purification Kit) and eluted in 30 µl of water. Concentration of DNA was expected to be between 100-200ng/µl, tyrosinase guide 1 template was 190 ng/µl and tyrosinase guide 2 was 165 ng/µl. Template was then loaded into a 1% agarose gel, a band of ~120bp was observed (Fig. 20) (as described in protocol).

RNA extracted from *tyrosinase* guide 1 with Phenol/chloroform was 1700 ng/ µl. According to the ratios of the absorbance parameters and by applying the Beer-Lambert Law, guideRNA was contaminated with protein (A^{260nm}/A^{280nm} = 1.59) but free from organic contaminants (A^{260nm}/A^{230nm} = 2.44). RNA extracted from *tyrosinase* guide 2 with Phenol/chloroform was 1683 ng/ µl. According to the ratios of the absorbance parameters and by applying the Beer-Lambert Law, guideRNA was free from protein contaminants (A^{260nm}/A^{280nm} = 2.03) and free from organic contaminants (A^{260nm}/A^{230nm} = 2.03).

Screening for mutations

In total, 41 animals were born from microinjection (See Appendix 6 for mix concentrations and survival rates). None of these animals showed lack of pigmentation, being identical to wildtype animals. Some lack of pigmentation in the tail was found, but a similar pattern was also found in wildtype mice, so that phenotypic result was discarded.

Table 8 Mouse microinjection concentrations of guideRNA and Cas9 protein; embryo survival rates and number of born pups

Cas9 protein (ng/µl)	guideRNA (ng/µl)	Embryo survival %	Born pups
100	20 (tyr 1 guideRNA)	71%	11
100	20 (2 guideRNAs)	73%	18
100	50 (2 guideRNAs)	72,50%	12

<u>Genotyping</u>

Two different PCR reactions were performed (Table 6), one for *tyrosinase* guide 1 injection indels screening (Fig. 23), expected size of ~200bp, and another one for *tyrosinase* guide 2, expected size of ~544 bp. All samples from guide 1 injected pups amplified a single band of ~200 bp around the tyrosinase guide 1 target site, just like the wildtype control sample. These results point to a lack of

efficiency of *tyrosinase* guide 1. Likewise, all samples from *tyrosinase* guide 2 injected pups amplified a single band of ~544 bp around the guide 2 target site, just like the wildtype control sample (Fig. 22). Again, these results point to a lack of efficiency of *tyrosinase* guide 2.

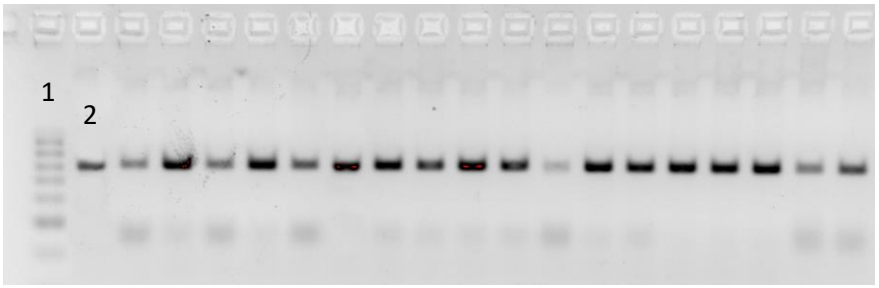


Figure 22 *- Amplification of genomic DNA from tyrosinase guide 2 injected animals for screening tyrosinase guide 2 indels. Ladder: GeneRuler 200bp (1) WT control sample (2).*

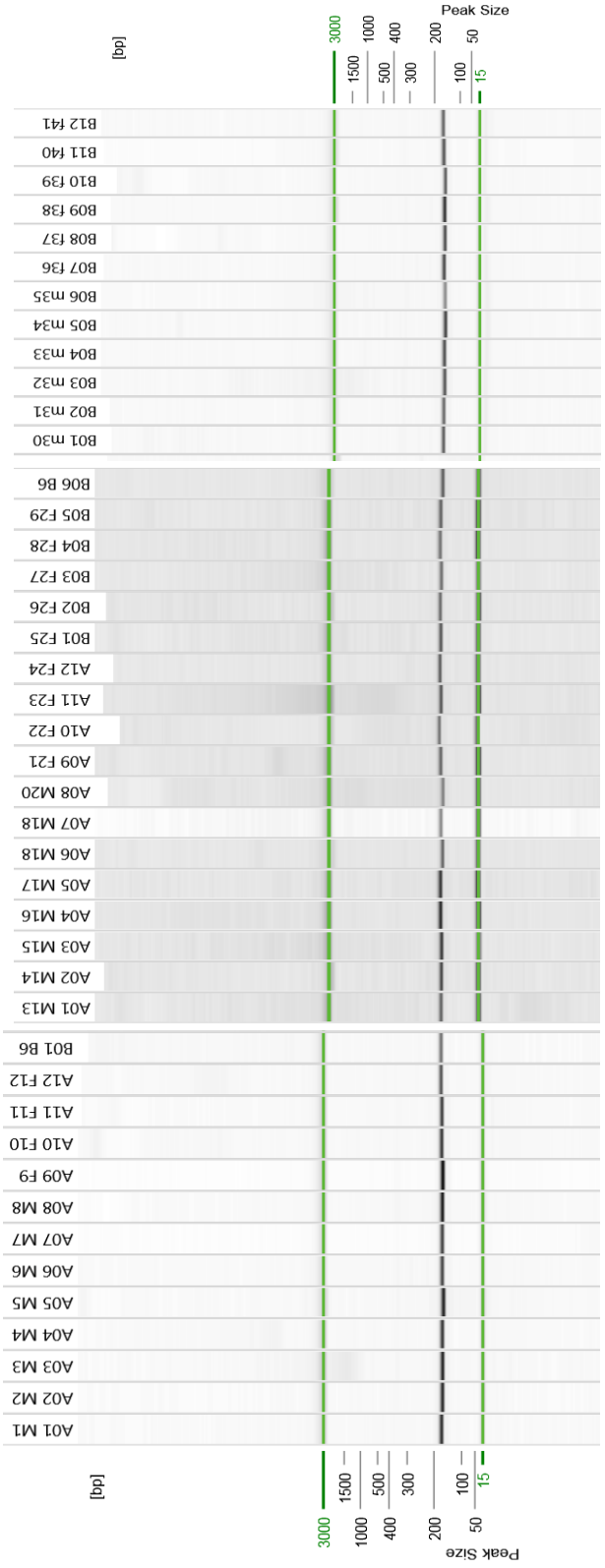


***Figure 23** – QIAxcel ScreenGel® analysis of PCR amplification for screening tyrosinase guide 1 indels. WT control sample (B01 B6)*

7. General Discussion

In this project, our main objective was to establish a unique protocol for guideRNA production and CRISPR knockout generation that would fit all three organisms currently used in CF: *Danio rerio*, *Drosophila melanogaster* and *Mus musculus*. Our starting point was the reproduction of the protocol of Gagnon *et. al* (2014), that is a well-established for mutant production using CRISPR/Cas9, in zebrafish followed by its adaptation to the two other model species. In order to achieve this goal, production of *tyrosinase* mutant zebrafish through Gagnon *et.al* (2014) protocol was achieved. After establishment of this technique in zebrafish, production of *tyrosinase* knockout in mice and *yellow* knockout in fruit fly was tested.

In zebrafish, we were able to reproduce the protocol and produce a *tyrosinase* knockout animal. But, some steps of the protocol were not reproducible. First, RNA extraction with Qiagen micro-RNA purification Kit had a very low yield. Concentrations extracted were, most of the times, not enough for embryo microinjection, probably due do column saturation. The chosen alternative method was RNA extraction with Phenol/chloroform. The guideRNA extracted this way, was effective in knocking out *tyrosinase* gene although the absorbance parameters showed a slight protein contamination (A^{260nm}/A^{280nm} = 1.66). We have failed to amplify a *tyrosinase* fragment for genotyping when using genomic DNA extracted from embryos. However, we showed that the extraction protocol worked for amplification of other amplicons and that the *tyrosinase* primer pair also worked when the template was extracted from later stage larvae or adults. When added DMSO, amplification was observed and since *tyrosinase* gene starts to be transcribed 16.5 hpf (Camp et al., 2001), this problem that we encountered must be due to a technical problem of little genomic DNA and PCR optimization. Another aspect, probably due to fast embryo development, is the mosaicism encountered. It was expected that knocking out *tyrosinase* would lead to lack of pigmentation. However, a mosaic lack of pigmentation occurs. This means that some cells were *tyrosinase* knockout but others were not, the bigger the number of targeted cells, the bigger the number of non-pigmented areas in the animal. In some extreme cases, we found some individuals with almost all cells pigmented except for a stripe in the eye. These individuals were probably injected in a slightly later stage in comparison to the others. This mosaic phenotype is in accordance with Ablain *et al* (2015), where a mosaic gene disruption also occurred (Ablain *et al*., 2015). For mutation to occur, the CRISPR machinery (guideRNA and Cas9 protein), have to be in the cell nucleus. We would only have a clonal mutant animal if the errors were induced right before the first mitosis. However, as initial stages of development are very fast, that developmental windows are very narrow. To add to the speed of the initial cell divisions, we also have to consider the dynamics of the CRISPR machinery itself. It seems thus possible that CRISPR-induced mutations will only occur at a later developmental stage, when mitosis has slowed down, and only in cells that still have the machinery available. For this last point we have to consider, not only the half-

life of the machinery components, but also the dilution and potentially asymmetrical distribution of this components from cell to cell. What follows from these arguments is that the later the injection, the less likely it is that all cells receive the CRISPR components necessary for DSBs and mutant generation, and less mutant cells the animal will have. Additionally, as cell division continues, the more diluted the CRISPR components will be in each cell and the less likely the DSBs and induced mutations. This mosaic phenotype is represented in our genotyping results, where each individual shows different mutations. In F1 animals, this phenotypic mosaicism disappears because they are derived from the gametes and contribution of only 1 allele from each mosaic parent, but not the gene disruption mosaicism, as seen in genotyping of F1 results, where the wildtype band disappears but different band sizes are still amplified, meaning that continues to be a mosaic. To get rid of this mosaicism outcross matings with wildtype animals need to be performed until a stock is established. However, the protocol that we followed showed to be efficient in zebrafish where a *tyrosinase* gene knockout occurred.

In fruit fly we were not able to reproduce the Gagnon *et.al* (2014) CRISPR components delivery protocol, namely co-injection of guideRNA and protein Cas9. In particular, for the delivery of Cas9 protein, the injection concentration of this component had to be higher than the concentration of our stock Cas9 protein solution (Lee *et al*., 2014). As we did not have any reliable protocol for protein concentration at our disposal, this limitation could only be circumvented by *de novo* protein synthesis, which was not an option within the scope of this thesis. There are alternative CRISPR components delivery methods in the fly system, which should be explored in future work. It has been shown that microinjection of *in vitro* synthetize guideRNA is more efficient than injection of a guideRNA encoding plasmid (Bassett *et al*., 2013; Gratz *et al*., 2014) but, these efficiency is accompanied with injection of a Cas9mRNA encoding plasmid and of Cas9 protein. An alternative source of Cas9 delivery is the use of Cas9-transgenic flies. In this case, the fly is expressing Cas9 protein and only *in vitro* synthetized guideRNA is microinjected into the fly embryo. The two main disadvantages of using Cas9 flies are: the fact that the possibility of targeting any fly line is removed and that after production of mutant flies it is needed to remove the Cas9 transgene. Both disadvantages can be overcome by prior or after additional crosses. In the first case, one should first cross the fly line of interest to transgenic Cas9 flies establishing a stable stock and only after inject guideRNA to target mutation. In the latter case, additional crosses should be made after to eliminate the Cas9 transgene in the final mutated stock. Using Cas9 transgenic flies increases the efficiency of CRISPR/Cas9 technique (Port *et al.*, 2015). Different transgenic Cas9 flies are already available and stocks differ in expression patterns, activity and chromosomes carrying the transgene. Using vas-cas9 (Bl #51324) flies, as an alternative to Cas9 protein injection, solves the Cas9 protein stock low concentration problem. However, we have to bear in mind that the Cas9 transgene in this particular stock is positively marked with a *yellow*$^{+}$ gene, turning the screening of *yellow* mutants more complicated, only

possible in F2 animals and beyond the temporal scale of this thesis. Alternatively, and for future work, we can also make use of other Cas9-expressing stocks that are neither in a *yellow* background nor bearing a *yellow*$^{+}$ marked Cas9 transgene. Such stocks exists but are not available at common stock centres for purchasing and can only be acquired by establishment of collaborations.

Regarding mouse *tyrosinase* gene knockout, we injected 2 guideRNAs, targeting different regions of the gene: one near the ATG site and a second one that was further down the coding region but with a higher score in the software tool used. No phenotypic results were visible and genotyping F0 results were in accordance with this observation. One possibility to explain the failure of *tyrosinase* knockout mice generation can be the protocol that we were following, that it was optimized for fish CRISPR/Cas9 target mutagenesis. To address that, a protocol directed to mice CRISPR/Cas9 target mutagenesis should be followed with the same guideRNA sequence. For example, in Henao-Meija *et al.* (2017) protocol, guideRNA production consists also in the annealing of two oligos, one with the guideRNA desired and another one with T7 promotor sequence. Another possibility is that the target mutagenesis occurred, but the induced mutations did not produce a visible phenotype and indels were just 1-2bp long and beyond the separation limit of the Qiaxcell gel. This possibility could be tested be using and alternative genotyping method, in particular, the T7 endonuclease assay (NEB). T7 endonuclease recognizes and cleaves non-perfectly matched DNA or heteroduplexes. The T7 cleaved sample can be easily run in an agarose gel and would show 2 bands if a mutation had occurred (Dad *et al*., 2014).

Challa *et.al* (2016) used the same targeting strategy as us in this project, actually one of the used guide was just 2 nucleotides different from ours *tyrosinase* guide 1. In their experiments, the injected embryos were C57Bl/6J bred to albino Tyr C57Bl/6J. The *tyrosinase* mutation is recessive, meaning that both alleles have to be mutated to result in a phenotypically albino animal. By doing injections in heterozygous embryos, they could easily identify animals in which the DSBs and loss-of-function mutations only occurred in one allele. They had two black animals out of thirteen positive born animals that had indel mutations. One way of increasing knockout efficiency is augmenting the deletion size and that can be achieved by co-injecting two guideRNAs (Lin *et al*., 2014). However, in Challa *et al.* (2016) work this proved to be a very rare event. We don't have any reason to doubt that the protocol used in this thesis does not work - microinjection of *in vitro* synthetized guideRNA in and Cas9 protein is a technique already established for the mouse model. (Harms *et al*., 2014; Henao-Mejia *et al.*, 2016; Ma *et al*., 2017; Thermo Fisher Scientific, n.d.). Animals born from this master thesis need to be further tested to conclude if they bear no mutations.

Overall, further work needs to be done, in particular regarding Drosophila and mouse mutant screening. Addressing if a mutation occurs but it's being overcome by wildtype cells. Either way, establishing an easier and more efficient protocol for CRISPR/Cas9 targeted mutagenesis in zebrafish

was one of the main objectives of this project and it was accomplished being used for different target genes now at the CF Fish Facility.

8. General Conclusions

With this project, we made a proof of principle of an existing protocol for guideRNA production with consequent production of mutant zebrafish. We can conclude that the protocol is easy to follow and less time consuming than other CRISPR guideRNA production protocols, which are two key features. Also, it is cheaper since there is no need to clone vectors. Regarding RNA extraction after transcription, we conclude that phenol/chloroform is more efficient in RNA extraction and cheaper that Qiagen micro-RNA extraction kit. Even though guideRNA was slightly contaminated with protein it was still efficient in producing DSB in tyrosinase gene in zebrafish.

We conclude that DNA extraction from zebrafish embryos works but for *tyrosinase* gene in specific, more extraction and PCR optimization are needed.

We can also conclude that a single guideRNA is sufficient to knockout a gene through NHEJ cell repair. Also, NHEJ can produce different mutations with the exact same DSB break, producing different knockout animals. Also, even in the individual itself, there is mosaic gene disruption.

In the work done with fruit fly we can conclude that microinjection of Cas9 protein requires a huge concentration, confirming other results already in literature.

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Appendix

Appendix 1 - *Danio rerio* embryo microinjection survival rates and concentration injected of guideRNA and Cas9 protein

	Name	Cas9 Protein	sgRNA	Dead after injection	Alive (24h after)	Alive (48h after)	% survival (24h after)
Microinjection 1	Laying 1	260 ng/ul	173.9 ng/ul	102	28		21,54%
	Control laying 1			27	15		35,71%
	Laying 2	260 ng/ul	173.9 ng/ul	13	46		77,97%
	Control laying 2			4	30		88,24%
	Laying 3	260 ng/ul	173.9 ng/ul	72	95		56,89%
	Control laying 3			14	55		79,71%
	Laying 4	260 ng/ul	173.9 ng/ul	34	28		45,16%
	Control laying 4			1	79		98,75%
	Laying 5	260 ng/ul	173.9 ng/ul	60	17		22,08%
	Control laying 5			14	9		39,13%
	Laying 6	260 ng/ul	173.9 ng/ul	33	82		71,30%
	Control laying 6			3	72		96,00%
	Laying 7	260 ng/ul	173.9 ng/ul	27	58		68,24%
	Control laying 7			16	55		77,46%
Microinjection 2	Laying 1	260 ng/ul	437,7 ng/ul	90	129		58,90%
	Control laying 1			95	137		59,05%
	Laying 2	260 ng/ul	437,7 ng/ul	140	98		41,18%
	Control laying 2			84	75		47,17%
	Laying 3	260 ng/ul	437,7 ng/ul	26	64		71,11%
	Control laying 3			50	141		73,82%
	Laying 4	260 ng/ul	437,7 ng/ul	61	79		56,43%
	Control laying 4			34	92		73,02%
	Laying 5	260 ng/ul	437,7 ng/ul	20	91		81,98%
	Control laying 5			23	110		82,71%
Microinjection 3	Laying 1	500 ng/ul	437,7 ng/ul	99	128	32	56,39%
	Control laying 1			109	112		50,68%
	Laying 2	500 ng/ul	437,7 ng/ul	76	32	37	29,63%
	Control laying 2			137	65		32,18%
	Laying 3	500 ng/ul	437,7 ng/ul	102	60	69	37,04%
	Control laying 3			43	32		42,67%
Microinjection 4	Laying 1.1	315 ng/ul	526 ng/ul	39	43		52,44%
	Laying 1.2			62	73		54,07%
	Laying 1.3			35	38		52,05%
	Control laying 1 (all)			20	27		57,45%
	Laying 2	600 ng/ul	200 ng/ul	93	145		60,92%
	Control Laying 2			56	75		57,25%
Microinjection 5	Laying 1	315 ng/ul	526 ng/ul	75	208		73,50%
	Control laying 1			34	79		69,91%
	Laying 2	315 ng/ul	526 ng/ul	90	287		76,13%
	Control laying 2			41	99		70,71%

Appendix 2 – Overall table results QIAxcel ScreenGel®, from indels screening of F0 injected zebrafish animals for *tyrosinase* knockout

Overall Result Table											
C160628120_2017-04-13_15-30-36											
E1 1	E2 2	E3 3	E4 4	E5 5	E6 6	E7 7	E8 8	E9 C	E10 2	E11 1	E12 1
Size [bp]	Size [bp]	Size [bp]	Size [bp]	Size [bp]	Size [bp]	Size [bp]	Size [bp]	Size [bp]	Size [bp]	Size [bp]	Size [bp]
15	15	15	15	15	15	15	15	15	15	15	15
54	58	57	58	51	53	53	51	52	52	51	52
77	79	77	79	73	131	76	72	74	74	74	75
133	135	136	135	324	316	132	130	3000	3000	3000	3000
318	308	332	332	3000	359	318	317				
340	331	3000	361		3000	433	348				
3000	376		3000			479	377				
	3000					3000	3000				

Overall Result Table							
C160727134_2017-06-28_14-58-28							
F4 TU	F5 9	F6 10	F7 11	F8 12	F9 13	F10 14	F11 15
Size [bp]	Size [bp]	Size [bp]	Size [bp]	Size [bp]	Size [bp]	Size [bp]	Size [bp]
15	15	15	15	15	15	15	15
74	29	65	76	64	70	30	29
107	67	106	101	100	106	65	69
261	105	290	234	289	291	108	105
287	290	331	290	335	329	296	290
3000	328	3000	334	400	3000	334	331
	3000		3000	3000		3000	3000

Appendix 3 – Drosophila embryos microinjection mixes concentrations

Cas9 protein (ng/ul)	sgRNA (ng/ul)
300	250
500	250
800	250
800	500

Appendix 4 – Primers table

Tyr_Fwd	GCGTCTCACTCTCCTCGACTCTTC
Tyr_ Rv	GTAGTTTCCGGCGCACTGGCAG
Yellow_Fwd	ATACAGCTGGAGATTGCGCCA
Yellow_Rv	CCAGGTAGCTCGTATCTCCGAATT
Tyr1mouse_Fwd	TGGCAAAAGAATGCTGCCC
Tyr1mouse_Rv	AACCCATGAAGTTGCCTGAG
Tyr2mouse_Fwd	ATGAAGCACCAGGGTTTCTG
Tyr2mouse_Rv	GAGCGGTATGAAAGGAACCA