Application of CRISPR-based gene editing tools in Atlantic salmon

Mari Torsdotter Raudstein

Thesis for the degree of Philosophiae Doctor (PhD)
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Contents

Scientific environment

The work presented in this thesis was conducted at the Institute of Marine Research (IMR), Bergen and Matre, spanning from November 2020 to January 2024. The studies were part of the TUNESAL project (grant number 301602) funded by the Research Council of Norway.

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Abstract

Atlantic salmon is the most important species in the Norwegian aquaculture industry. The industry suffers from several sustainability challenges, such as genetic introgression of escapees and spreading of infectious diseases, which impact the surrounding environment and the welfare and health of the fish. Evidently, there is an overarching need to protect the farmed fish from infectious diseases. Changing the genotype of the fish by gene editing is a viable approach, and one powerful method to achieve this is by using CRISPR/Cas technology. However, effective application of this technology requires the ability to perform the edits with accuracy. Additionally, we need more species-specific knowledge of which genes to edit. Such knowledge can be obtained using CRISPR/Cas-based loss-of-function and gain-of-function approaches. Put differently, CRISPR/Cas has valuable applications for both basic fish research and the aquaculture industry.

The primary aim of this thesis was to implement new CRISPR-based gene editing tools in Atlantic salmon. This aim was the focus of Paper I and Paper II, where we tested CRISPR/LbCas12a technology and base editing. The secondary aim included using CRISPR-based tools to make *in vivo* models that can increase our understanding of biological processes related to the Atlantic salmon immune system. This aim is covered in the final manuscript, Paper III, where CRISPR/Cas9 was applied to knockout the immune genes Immunoglobulin (Ig) M and Interferon gamma (IFNg) in Atlantic salmon.

The experiments in this thesis have been performed by microinjecting fertilized salmon eggs, followed by high-throughput sequencing to reveal the editing outcomes. In Paper I, CRISPR/LbCas12a was used to knock out the pigmentation gene *solute carrier family 45 member 2* (*slc45a2*) gene, resulting in an albino or albino mosaic pigmentation phenotype. We also conducted experiments where templates having a target or non-target strand orientation were added to evaluate the knock-in possibilities of LbCas12a, achieving up to 54% integration efficiency. Finally, LbCas12a and SpCas9 CRISPR complexes were injected in the same individual to

compare the two nucleases. Interestingly, they both produced similar mutation rates, but more perfect integration occurred at the LbCas12a cleavage site compared to the SpCas9 cleavage site. In Paper II, the cytidine base editor AncBE4max was used to introduce a premature stop codon in *slc45a2*. We achieved highly efficient C-to-T conversion of up to 89% of our target base. Some undesired effects such as indels, bystander edits, and conversion of C-to-non-Ts were also observed but in small amounts. In Paper II we also further developed an alternative to base editing, single nucleotide replacement, using conventional CRISPR/Cas9. Here, templates featuring point mutations at various positions from the cleavage site were added, revealing that the insertion efficiency was affected by the mutation's distance from the cleavage site.

In Paper III, we employed CRISPR/Cas9 to successfully knock out the two IgM heavy chain loci in Atlantic salmon, achieving more than 95% mutagenesis at both target sites. Mutagenesis on the IFNg target site(s) was found to be approximately 57%, although with some uncertainty. The investigations regarding IFNg were therefore postponed but may be interesting for future studies. The effect of the IgM knockout on protein level was assessed by flow cytometry analysis and revealed a mean average reduction in IgM positive (IgM^+) B cells of 91% in peripheral blood of the gene-edited salmon.

In conclusion, we have implemented two new methods for making genetic changes in Atlantic salmon, CRISPR/LbCas12a and base editing, expanding the toolkit for gene editing in this species. We also employed CRISPR/Cas9 to generate a F0 IgM^+ B cell-deficient Atlantic salmon, which may serve as a model to elucidate the role of this key gene and our understanding of the Atlantic salmon immune system in general.

Samandrag

Atlantisk laks er den viktigaste arten i norsk havbruksnæring. Næringa i Noreg er i dag ramma av fleire berekraftsufordringar, til dømes genetisk innblanding av rømt laks, og spreiing av smittsame sjukdommar. Dette påverkar både miljøet rundt, og fører til redusert velferd og helse hos fisken. Det er difor eit openbart behov for å beskytte oppdrettsfisken mot smittsame sjukdommar. Ei mogleg løysing på dette kan vere å endre fisken sin genotype ved bruk av genredigering, noko som kan bli gjort ved bruk av CRISPR/Cas genteknologi. Effektiv bruk av denne teknologien krev imidlertid evna til å uføre genredigering med høg nøyaktigheit. I tillegg trenger vi meir artsspesifikk kunnskap om kva gen som kan redigerast. Slik kunnskap kan vi få ved hjelp av CRISPR/Cas-baserte «loss-of-function» og «gain-of-function» eksperiment. Sagt på ein annan måte har CRISPR/Cas verdifulle bruksområde for både fiskeforsking og for havbruksnæringa.

Hovudmålet med denne avhandlinga var å implementere nye CRISPR-baserte verktøy i atlantisk laks. Dette målet er fokuset i Paper I og Paper II, kor vi testa CRISPR/LbCas12a genteknologi og base-redigering. Det sekundære målet var å bruke CRISPR-baserte verktøy til å lage *in vivo* modellar som kan bidra til å auke forståinga vår rundt biologiske prosessar knytt til laksen sitt immunsystem. Dette målet vart dekka i det siste manuskriptet, Paper III, kor CRISPR/Cas9 vart brukt til å slå ut dei to immungena Immunoglobulin (Ig) M og Interferon gamma (IFNg) i atlantisk laks.

Eksperimenta i denne avhandlinga har blitt utført ved å mikroinjisere fertiliserte lakseegg, etterfølgt av høgkapasitets-sekvensering for å undersøke resultata av genredigeringa. I Paper I vart CRISPR/LbCas12a brukt til å slå ut genet som kodar for pigmentering i laks, solute carrier family 45 member 2 (*slc45a2*), noko som resulterte i ein albino- eller albinomosaikk-pigmenteringsfenotype. Vi utførte òg eksperiment der donor templat med anten target- eller non-target DNA-tråd-orientering vart inkludert for å vurdere moglegheitene for å setje inn genetisk materiale med LbCas12a nukleasen. Her oppnådde vi opp til 54% integrerings-effektivitet. Til slutt injiserte vi både LbCas12a og SpCas9 CRISPR-kompleks i same individ for å samanlikne dei to

nukleasane. Dei to produserte liknande mutasjonseffektivitet, men det var meir perfekt integrasjon av templatet på LbCas12a kuttstaden samanlikna med SpCas9 kuttstaden.

I Paper II brukte vi cytidin base-editoren AncBE4max for å introdusere eit stoppkodon i *slc45a2*. Vi oppnådde høg konverteringseffektivitet, opp til 89% C-til-T konvertering av den ønska mål-basen. I tillegg vart nokre uønska effektar oppdaga, som insersjonar og delesjonar, bystander-redigering, og konvertering av C-til-ikkje-T, men i liten skala. I Paper II vidareutvikla vi også eit alternativ til base-redigering, enkelt-base utbytting, ved bruk av konvensjonell CRISPR/Cas9. Her inkluderte vi templat med mutasjonar i ulike posisjonar frå kuttstaden. Her såg vi at integrerings-effektiviteten var påverka av mutasjonen sin avstand frå kuttstaden.

I Paper III vart CRISPR/Cas9 brukt til å slå ut IgM hos atlantisk laks, med meir enn 95% mutagenesis på begge IgM loci. Mutagenesis på IFNg mål-staden vart funne til å gjennomsnittleg vere ca. 57%, men med litt usikkerheit rundt dette. Utforskinga av effekten av å slå ut IFNg vart difor utsett i dette arbeidet, men er interessant for framtidige studiar. Effekten av å slå ut IgM på proteinnivå vart undersøkt ved hjelp av flow cytometry. I den genredigerte fisken var det gjennomsnittlege nivået av IgM positive (IgM+) B celler redusert med 91%.

For å konkludere har vi implementert to nye metodar for å utføre genetiske endringar i atlantisk laks, CRISPR/LbCas12 og base-redigering, og har dermed utvida verktøykassen for genredigering i denne arten. I tillegg brukte vi CRISPR/Cas9 til å lage ein F0 atlantisk laks med reduserte IgM^+ B celle-nivå, som i framtida kan fungere som ein modell for å auke forståinga vår rundt dette genet, i tillegg til forståinga vår av den atlantiske laksen sitt immunforsvar generelt.

List of papers

Paper I

Mari Raudstein, Erik Kjærner-Semb, Morten Barvik, Silje Broll, Anne Hege Straume, Rolf Brudvik Edvardsen. In vivo CRISPR/LbCas12a-mediated knock-in and knock-out in Atlantic salmon (*Salmo salar* L.). Transgenic Res 32, 513-521 (2023)

Paper II

Mari Raudstein, Anne Hege Straume, Erik Kjærner-Semb, Morten Barvik, Rolf Brudvik Edvardsen. Highly efficient *in vivo* C-to-T base editing in Atlantic salmon (*Salmo salar*) – A step towards aquaculture precision breeding. Aquaculture 581 (2024)

Paper III

Mari Raudstein, Michelle Peñaranda, Søren Grove, Erik Kjærner-Semb, H. Craig Morton, Rolf Brudvik Edvardsen. Generation of IgM^+ B cell-deficient Atlantic salmon (*Salmo salar)* by CRISPR/Cas9-mediated IgM knockout. Manuscript

Abbreviations

ABE	Adenine base editor
Cas	CRISPR-associated
CBE	Cytidine base editor
CD	Cluster of differentiation
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
crRNA	CRISPR RNA
CTL	Cytotoxic T cell
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DSB	Double-strand break
dsDNA	Double-stranded DNA
EPA	Eicosapentaenoic acid
GCF	Germ cell free
gRNA	Guide RNA
HDR	Homology-directed repair
IFNg	Interferon gamma
IgM	Immunoglobulin M
Indels	Insertions and deletions
IPNV	Infectious pancreatic necrosis virus
LC-PUFA	Long-chain polyunsaturated fatty acid
MHC	Major histocompatibility complex
Mstn	Myostatin

NHEJ	Non-homologous end joining
NUC	Nuclease lobe
ODN	Oligodeoxynucleotide
PAM	Protospacer adjacent motif
pegRNA	Prime editing guide RNA
Pol	Polymerase
QTL	Quantitative trait locus
REC	Recognition lobe
RNA	Ribonucleic acid
RNP	Ribonucleoprotein complex
Slc45a2	Solute carrier family 45 member 2
ssDNA	Single stranded DNA
TALEN	Transcription activator-like effector nuclease
Th	Helper T cell
tracrRNA	Trans-activating CRISPR RNA
ZFN	Zinc-finger nuclease

PART ONE | SYNOPSIS

Introduction

1. CRISPR/Cas gene editing

Gene editing (sometimes called genome editing) is an approach for making specific changes to the genome of a cell or an organism. This could be to insert, remove, or alter DNA. Advances in biotechnology have given rise to gene editing techniques that rely on programmable nucleases. Zinc finger nucleases (ZFNs) (Kim et al., 1996) and transcription activator-like effector nucleases (TALENs) (Christian et al., 2010) had leading roles in gene editing but were limited by their time-consuming processes, as well as low efficiency and specificity. More recently, the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) technology has emerged as the primary tool for gene editing, overcoming the shortcomings of its predecessors. The CRISPR system was first identified in prokaryotes, described as a family of repetitive DNA sequences characterized by direct, short repeated sequences, interspaced by similarly sized non-repetitive sequences (Jansen et al., 2002). Additionally, CRISPR-associated (Cas) genes were identified adjacent to the CRISPR loci. Interestingly, researchers discovered that the CRISPR/Cas system provided the prokaryotes with an adaptive-like immune system (Barrangou et al., 2007). When infected with viruses, the bacteria insert small fragments of the viral DNA in between the repeated sequences of the CRISPR loci. Accordingly, viral DNA constitutes the non-repetitive sequences, also termed spacers. Upon subsequent viral infection, the bacteria transcribe precursor CRISPR RNA (pre-crRNA) molecules from the CRISPR loci. These are then processed by Cas proteins and accessory factors into mature crRNAs capable of recognizing and destroying the invading virus together with the Cas proteins (Rath et al., 2015). In 2012, two groups of scientists almost in parallel demonstrated the possibility of adapting the bacterial CRISPR/Cas system as a tool for targeted gene editing (Jinek et al., 2012, Gasiunas et al., 2012).

1.1. Components

There are currently two major classes of CRISPR/Cas systems (Class 1 and Class 2), encompassing six types and 33 subtypes (Makarova et al., 2011, Makarova et al., 2015, Makarova et al., 2020). The main difference between the two classes is that the Class 1 systems possess multi-subunit crRNA-effector complexes, whereas the Class 2 systems are defined by the presence of a single crRNA-effector complex (Makarova et al., 2015). Belonging to Class 2 type I, the Cas9 nuclease derived from *Streptococcus* sp. (Sp) was the first nuclease to be utilized experimentally for gene editing. Similar to the bacterial defense mechanism, employing the CRISPR/Cas9 system for gene editing requires a guide RNA (gRNA) molecule and a Cas9 nuclease, which together form the effector complex (Figure 1).

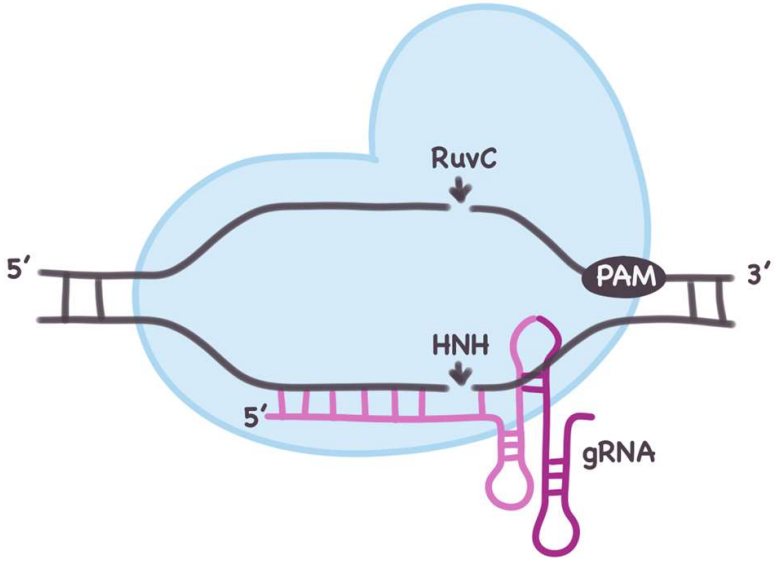

Figure 1 Cas9-gRNA effector complex. The PAM is located on the 3' end of the DNA strand non-complementary to the gRNA sequence. The gRNA consists of crRNA (light pink) and tracrRNA (dark pink). The HNH domain cleaves the DNA strand complementary to the gRNA, whereas the RuvC domain cleaves the non-complementary strand, producing a blunt end double-strand break.

The Cas9 protein is often thought of as a genetic scissor, as it is capable of cutting DNA. The protein has a bilobed architecture, composed of regions termed the target recognition (REC) lobe and the nuclease (NUC) lobe (Nishimasu et al., 2014). The REC lobe, consisting of bridge helix, REC1, and REC2 domains, binds the gRNA molecule. The gRNA is made of a crRNA sequence and a trans-activating crRNA (tracrRNA) sequence. The crRNA sequence encompasses an (approximately) 20 nt-long seed sequence complementary to the target DNA which is comparable to the spacers of the CRISPR/Cas system in bacteria – this sequence is often also termed the protospacer. The tracrRNA is a small noncoding RNA required for Cas9 cleavage (Jinek et al., 2012). The NUC lobe is composed of RuvC and HNH domains, which each cleave one DNA strand. A protospacer adjacent motif (PAM), a short DNA sequence downstream of the protospacer sequence, is necessary for Cas9 cleavage. The PAM is nuclease-specific and dependent on the bacteria the nuclease is derived from. For Cas9 derived from *Streptococcus* sp. the PAM sequence is 5'-NGG-3'. The NUC lobe also contains the PAM-interacting domain that enables interaction with the PAM and initiates binding to the target DNA (Nishimasu et al., 2014).

1.2. Mechanisms

The mechanisms of CRISPR/Cas gene editing can be divided into three steps: target site recognition, DNA cleavage, and repair. The gRNA directs the Cas9 nuclease to the target sequence of interest through the protospacer component. Once the effector complex has recognized a target site with an appropriate PAM, DNA melting is triggered followed by the formation of an RNA-DNA heteroduplex (Asmamaw and Zawdie, 2021). CRISPR/Cas gene editing relies on the generation of a double-strand break (DSB) and subsequent repair. Approximately 3-4 bases upstream of the PAM, the HNH domain cleaves the DNA strand complementary to the protospacer, while the RuvC domain cleaves the non-complementary strand (Jinek et al., 2012). The DSB is then repaired by the host cellular machinery mainly through two mechanisms, the non-homologous end joining (NHEJ) or homology-directed repair (HDR) pathway. The outcome of the gene editing depends on the repair pathway taking place. NHEJ facilitates the DSB repair by directly ligating the DNA fragments. It is the

predominant and most efficient repair mechanism, occurring in all cell cycle phases except in mitosis. However, NHEJ is error-prone and can result in random insertions and deletions (indels) at the cleavage site. The indels may lead to frameshift mutations or premature stop codons, rendering the protein not expressed or non-functional. While NHEJ seals DSBs with little or no homology, HDR utilizes a template for repair. HDR allows the use of an exogenous DNA template to generate almost any desired DNA change. However, the frequency of HDR is lower compared to NHEJ. Put differently, the two repair mechanisms can be exploited to knockout genes through NHEJ, or to knock in genetic material through HDR.

Non-homolgous end joining

NHEJ utilizes a nuclease to trim broken DNA, DNA polymerases to fill in new DNA, and a ligase to seal the DSB (Figure 2) (Lieber, 2010). The key factors of (vertebrate) NHEJ are Ku70/80, Artemis, DNA-PKcs, Polymerase (pol) X members, and a ligase complex, consisting of XLF, XRCC4, and DNA ligase IV. Ku70/80 first binds to the DNA ends and can be thought of as a "docking station" for the nuclease, polymerases, and ligase complex (Lieber, 2010). Artemis and DNA-PKcs form a complex that is responsible for end processing and has a diverse array of nuclease activities, including 5' and 3' endonuclease activities (Lieber, 2010). Pol X members (mu and lamda) bind to the Ku70/80 dock and recruit nucleotides for DSB repair. The ligase complex can ligate across gaps as well as incompatible DNA ends. During the activities of the nuclease and polymerases, nucleotides are randomly added and removed, constituting the basis of the formation of indels (Yang et al., 2020). Furthermore, the NHEJ pathway exhibits great flexibility in terms of the order of events (Lieber, 2010), explaining why the outcome can vary although the starting point is identical (i.e., cleavage 3-4 bp upstream of the PAM).

Homology-directed repair

When the cell is in the S (DNA synthesis) or G2 (cell growth before mitosis) phase and a donor template is available, HDR may occur (Figure 2). The first step to HDR is initiated by the MRN complex, which together with the nuclease CtlP, process the DNA ends to form single-stranded (ss) 3' overhangs (Yang et al., 2020, Symington, 2016). As free ssDNA is unstable, the overhangs are quicky bound and shielded by replication protein A (RPA). Assisted by recombination "mediators", RPA is replaced by DNA repair proteins, RAD51, which forms protein filaments on the ssDNA (Yang et al., 2020). The 3' protein filament mediates homology searches and strand invasion of homologous DNA templates, forming a displacement loop (D-loop). Nucleotides are added by multiple polymerases, including the replicative polymerases Pol delta and Pol epsilon, until homology with the other ssDNA 3' overhang is achieved (Wright et al., 2018). Finally, the nucleotides of the opposite strand are filled in, using the sequence information obtained from the sister chromatid or the exogenous DNA donor template.

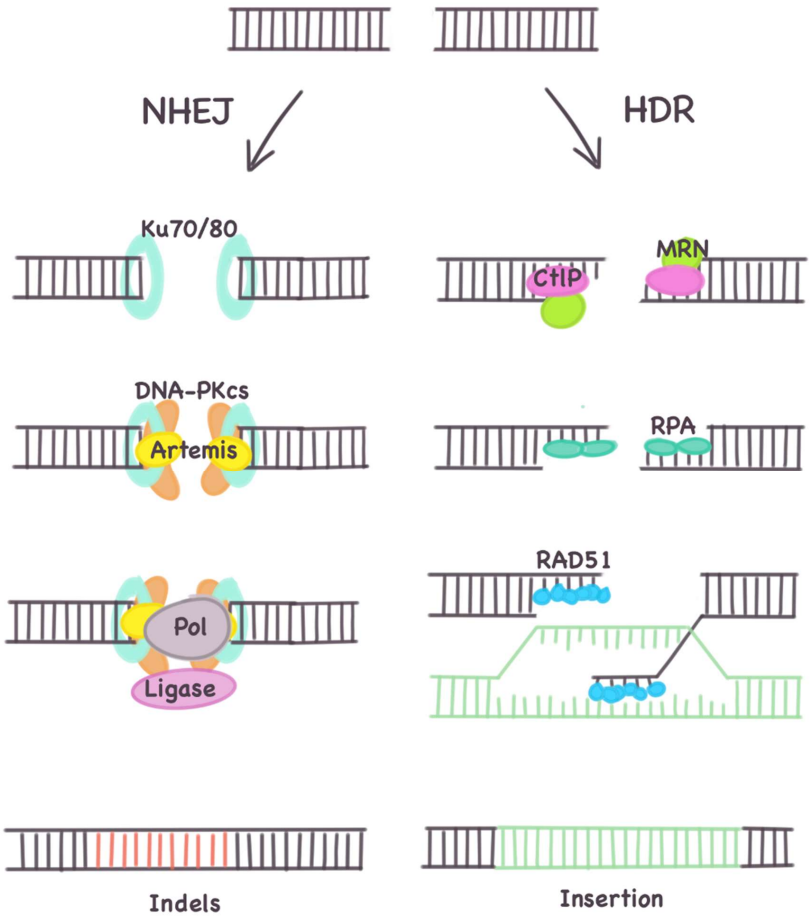

Figure 2 The non-homologous end joining (NHEJ) and homology-directed repair (HDR) pathways. The double-strand break produced by the CRISPR/Cas effector complex is repaired mainly through two pathways: NHEJ or HDR. The outcome of the gene editing is dependent on which pathway occurs. NHEJ may result in the generation of insertions and deletions (indels), rendering the protein non-functional, whereas HDR may be utilized to insert any desired genetic change to the DNA by the use of a template.

1.3. Other CRISPR-based gene editing tools

Because the CRISPR/Cas9 system was the first to be adopted for gene editing, this technology remains the most characterized. However, other methods utilizing the specificity of the gRNA-Cas complex have been developed to add versatility to the CRISPR/Cas gene editing toolkit, including novel or modified nucleases.

Cas12a nuclease

Compared to Cas9, the Cas12a nuclease (Figure 3) was more recently discovered as a tool for gene editing (Zetsche et al., 2015). While the two nucleases share some similarities, they also exhibit major differences. They both belong to the Class 2 CRISPR system, and thus exert their gene editing functionality using a single crRNA-Cas effector complex. However, Cas9 recognizes the crRNA through a duplex structure between crRNA and tracrRNA, as well as the secondary structure of the tracrRNA, whereas only the crRNA is required for Cas12a cleavage (Zetsche et al., 2015). For target site recognition and binding, the unique PAM sequence for Cas12a is 5'-TTTV-3', in which V can be either A, C, or G. Furthermore, although Cas12a is comprised of a REC lobe and a NUC lobe, the NUC lobe consists of a single nuclease domain, RuvC, in contrast to the RuvC and HNH domains of Cas9 (Yamano et al., 2016, Nishimasu et al., 2014). The RuvC domain cleaves both DNA strands distantly from the PAM: 18 bp downstream of the PAM on the strand non-complementary to the protospacer, and 23 bp on the complementary strand, producing staggered ends with a 5' overhang (Zetsche et al., 2015). As with Cas9, the endogenous repair mechanisms predominated by NHEJ and HDR are initiated to seal the DSB and thus facilitate the alterations to the DNA.

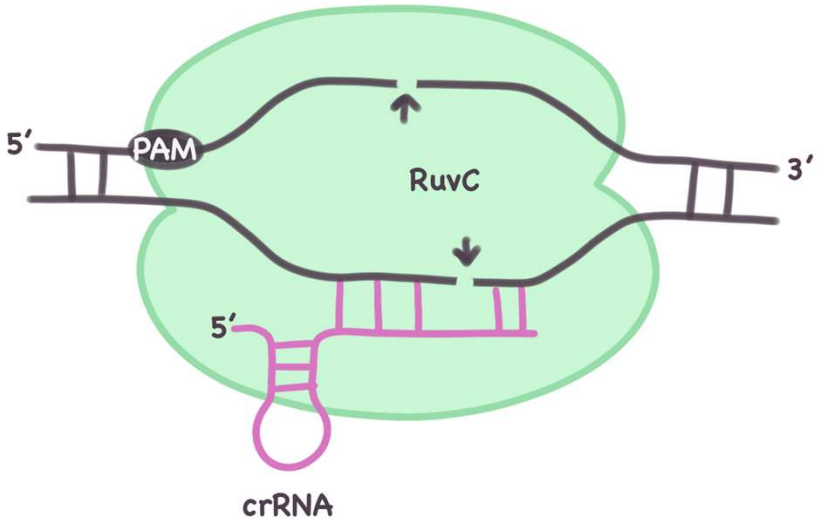

Figure 3 Cas12a-crRNA effector complex. The PAM is located on the 5' end of the DNA strand non-complementary to the crRNA sequence. The RuvC domain cleaves both the non-complementary and complementary strand, producing a staggered double-strand break with approximately 5 bp overhang.

Base editors

The nucleases Cas9 and Cas12a both induce DSBs to the DNA. An alternative approach, termed base editing, has been developed to facilitate the conversion of a single DNA base into another without the need for a DSB, a donor template or relying on HDR (Komor et al., 2016). Base editing also mitigates the frequent and somewhat unpredictable indels associated with NHEJ. The base editors are composed of a catalytically disabled nuclease fused to a nucleobase deaminase, and in some cases, a DNA glycosylase inhibitor (Rees and Liu, 2018). The two primary classes of base editors are cytosine base editors (CBEs) and adenine base editors (ABE), converting C-to-T and A-to-G, respectively. (Komor et al., 2016, Gaudelli et al., 2017).

Current CBEs consist of cytidine deaminases fused to a Cas9 nickase (nCas9) and uracil DNA glycosylase inhibitor (UGI) domains (Figure 4) (Koblan et al., 2018). Only a limited number of cytidine deaminases are known to work on DNA, and these enzymes require ssDNA (Harris et al., 2002, Komor et al., 2016). The nCas9, harboring mutations in the RuvC domain, induces a nick rather than a DSB in the complementary DNA strand, resulting in the generation of ssDNA. The deaminase then converts cytosine to uracil within a specific editing window on the non-complementary target DNA strand. The UGIs are included to prevent the removal of uracil catalyzed by UGs and initiation of the base excision repair pathway, which changes the U:G base pair back to a C:G base pair. The thymine base is permanently incorporated when the cell utilizes the uracil-containing strand as a template during DNA replication or repair. The ABEs function similarly to the CBEs, but because there are no enzymes known to deaminate adenine in DNA, a novel deaminase able to process DNA was created from a TadA protein (Gaudelli et al., 2017). TadA is in its original form an adenine deaminase that converts adenine to inosine in tRNA (Kim et al., 2006). Current ABEs consist of a modified TadA protein fused with nCas9, and as with CBEs, the nCas9 nicks the complementary strand (Gaudelli et al., 2017, Richter et al., 2020). The deaminase converts adenine to inosine, which is read as a guanine base by polymerases, and following DNA repair or replication, the original A:T base pair is replaced with a G:C base pair (Gaudelli et al., 2017). CBEs and ABEs enable the four possible transition mutations (C-to-T, A-to-G, T-to-C, and G-to-A). More recently, the development of novel base editors allowing the transversion mutations C-to-G and A-to-C have further expanded the scope of base editing (Kurt et al., 2021, Chen et al., 2023).

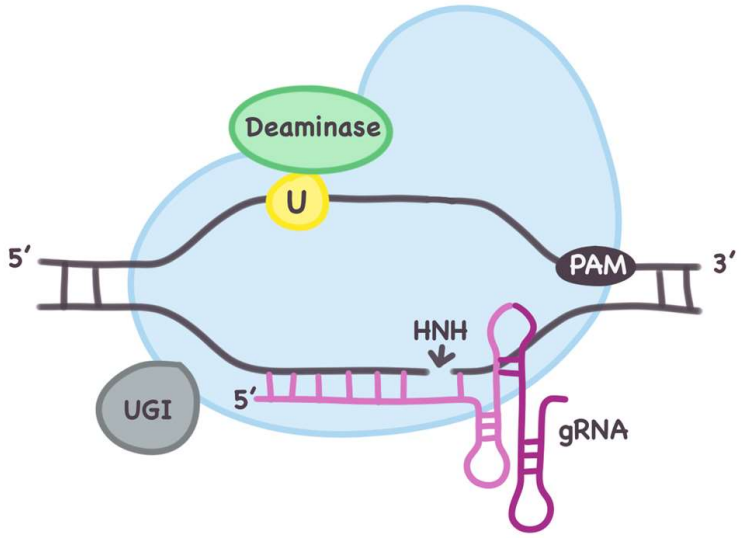

Figure 4 Generation four cytidine base editor comprised of a Cas9 nickase (nCas9), uracil DNA glycosylase inhibitor (UGI) and deaminase. The nCas9 does not induce a double-strand break but rather nicks the complementary DNA strand. The deaminase converts the target base cytidine into uracil (U).

Prime editing

Prime editing, another CRISPR-based technology developed by the same researchers who pioneered base editing, also relies on nicking of the DNA rather than DSBs. Unlike base editing, prime editing allows for all possible base conversions as well as the insertion of short genetic sequences (Anzalone et al., 2019). The prime editing complex is comprised of an nCas9 fused to an engineered reverse transcriptase. The system is directed by a prime editing gRNA (pegRNA), which in addition to the conventional gRNA part, encompasses a primer binding site and a reverse transcriptase template at its 3’ end. After nCas9 nicks the non-complementary strand, a 3’ ssDNA flap is generated. The primer binding site hybridizes with the 3’ flap, initiating reverse transcription of the new genetic material using the template of the pegRNA. The 3’ flap is extended and eventually displaces the unedited 5’ flap. During DNA repair, the desired edit is incorporated in the opposite DNA strand.

1.4. Challenges and limitations

CRISPR/Cas-based gene editing has great advantages compared to its predecessors, but there are still challenges to this technology. A primary concern is unintended changes beyond the gene of interest, termed off-target effects. To mitigate off-target effects, it is necessary to optimize the gRNA design, ensuring that the protospacer sequence is specific to the target gene (i.e., it does not have any matching sequence at other sites in the genome). However, even with careful design, studies have revealed the possibility for gRNA binding even with up to five bp mismatches between the target sequence and the protospacer (Zhang et al., 2015). The consequences of off-target effects can potentially lead to detrimental phenotypes, especially if affecting essential genes. Assessing potential off-target sites is therefore important when applying this technology. A second issue related to the gRNAs is their varying ability to produce efficient mutagenesis at the target site. Multiple programs have been developed to consider putative target sites with some also predicting the gRNA efficiency, although with varying accuracy (Konstantakos et al., 2022).

While achieving a gene knockout is a relatively straightforward process, the insertion of genetic material remains challenging. As mentioned, the NHEJ pathway tends to predominate, occurring more frequently than HDR. Various strategies are employed to shift this balance, such as the use of inhibitors targeting components of the NHEJ pathway (Aksoy et al., 2019) or the optimization of the donor DNA by considering factors like template polarity, length of the homology arms, use of ssDNA or dsDNA, and the decision to linearize or maintain the plasmid in its native state (Fernandez et al., 2018, Richardson et al., 2016). Furthermore, the base editing and prime editing technologies were developed as an effort to correct point mutations and introduce small genetic changes without the need for a DSB or relying on the low-efficiency HDR pathway. Still, efficient insertion of larger elements such as whole genes is yet to be solved.

Finally, the availability of suitable target sites poses another challenge in CRISPR/Cas gene editing, as it necessitates the presence of a specific PAM depending on the nuclease. Efforts to address this include the discovery and application of novel Cas

enzymes exhibiting distinct PAM requirements, such as Cas12a, as well as the exploration of engineering PAM-free nucleases (Collias and Beisel, 2021, Walton et al., 2020). Base editing and prime editing add another layer to the target site availability by requiring not only a PAM but also have a restricted targeting window. However, the expanding gene editing toolkit provides an array of techniques, allowing researchers to choose from various options if a particular one is not suitable for the desired target site. Although not all the limitations and restrictions of CRISPR/Cas have been solved yet, the technology's versatility and efficiency has spouted its use as the main tool for gene editing and revolutionized many areas of science, medicine, and industry.

2. Application of CRISPR in aquaculture

The aquaculture industry represents one of the fastest growing food production sectors and is expected to grow due to the high demand for seafood. Aquaculture may be defined as the breeding, rearing, and harvesting of organisms in all types of water environment, encompassing a variety of species including fish, shellfish, and algae (NOAA). The largest region for aquaculture is Asia, accounting for nearly 90% of the world's total production, followed by America and Europe, which account for 5% and 3.7%, respectively (FAO, 2023). Marine and freshwater fish are by far the largest sector of aquaculture, followed by crustaceans and mollusks (FAO, 2023). The Norwegian aquaculture industry mainly rears Atlantic salmon (*Salmo salar*), producing more than 1 billion tons of fish annually. Despite steadily increasing over the past decades, further expansion of the industry is hindered by sustainability challenges, many of which are related to environmental impact or animal welfare. Possible genetic introgression of escapees, spreading of infectious disease, and sea lice infestations represent some of the major challenges that need to be addressed (Karlsson et al., 2016, Taranger et al., 2014, Sommerset et al., 2023). CRISPR/Cas presents a promising approach to help solve these challenges in different ways.

2.1. Hindering genetic introgression

Despite some uncertainty in the numbers, the Norwegian Directorate of Fisheries reports escape of approximately 50.000 - 300.000 salmon from the sea pens in Norway each year (Fiskeridirektoratet, 2021). A major concern associated with these escapees is the potential for the farmed fish to migrate upriver and interbreed with wild populations (Taranger et al., 2014). The use of sterile fish in aquaculture has therefore been proposed as an effort to eliminate genetic introgression and minimize negative ecological effects of farmed salmon (Cotter et al., 2000). The most efficient way of creating sterile salmon has been to apply high pressure to induce triploidy in newly fertilized eggs (Johnstone et al., 1989). However, the use of triploid salmon was recently discontinued in Norway due to the fish having lower standards for health and welfare compared to its diploid counterparts (Stien et al., 2023, Rimstad et al., 2023).

As an alternative approach, gene editing can be used to create a genetically sterile salmon. CRISPR/Cas9 technology has successfully been used to produce germ cell free (GCF) Atlantic salmon by knockout of the *dead end* gene, a gene important for germ cell development (Wargelius et al., 2016). Following the GCF fish for a full production cycle, they were found to perform similarly to non-edited fish (Kleppe et al., 2022). The body growth rate, condition factor, and relative liver size were slightly smaller, likely due to no onset of maturation. This further illustrates the benefits of using GCF fish in production as early maturation in males may be associated with poor growth and reduced disease resistance (Taranger et al., 2010).

2.2. Introducing favorable traits

Breeding programs were applied early on in the salmon industry to obtain fish with traits favorable for production, including body weight, age of sexual maturation, and disease resistance (Gjedrem, 2012). Over the years of salmon farming, the fish now reach market size sooner, need less feed, have improved survival rate, and reduced frequency of early maturation (Gjedrem, 2012, Thodesen and Gjedrem, 2006). However, due to lack of documentation there is some uncertainty whether these phenotypic traits are attributed to genetic improvement (Gjedrem, 2012). The variable production environment, long production time, and sea lice and infectious diseases makes it difficult to document the benefits of genetic selection (Næve et al., 2022). Nevertheless, the salmon industry is a relatively young industry with considerable potential for genetic gain, especially with the application of more accurate and efficient breeding methods (Næve et al., 2022). For example, CRISPR/Cas been suggested as a way to enhance current breeding programs (Robinson, 2022, Gratacap et al., 2019, Lu et al., 2021, Yang et al., 2021). Research is now being conducted on using CRISPR/Cas gene editing in various aquaculture species like channel catfish (*Ictalurus punctatus*) and blue catfish (*Ictalurus furcatus*) (Coogan et al., 2022, Simora et al., 2020, Xing et al., 2022, Wang et al., 2023), common carp (*Cyprinus carpio*) (Zhong et al., 2016), Atlantic salmon (Datsomor et al., 2019b, Datsomor et al., 2019a, Wargelius et al., 2016, Güralp et al., 2020), and Nile tilapia (*Oreochromis niloticus*) (Wu et al., 2023, Li et al., 2020), focusing on traits related to enhanced

growth or fatty acid metabolism, disease resistance, and as already mentioned, sterility.

Enhanced growth

Body weight has been a key trait of interest since the beginning of salmon aquaculture. Myostatin (mstn) is a suppressor of muscle growth, and a natural occurring deletion in the *mstn* gene of the Belgian blue cattle has been found to cause the characteristic double-muscled phenotype (Grobet et al., 1997). Several studies to enhance growth of aquaculture fish have therefore focused on targeting this gene. Improved growth by disrupting *mstn* has been achieved in channel catfish (Coogan et al., 2022) and Nile tilapia (Wu et al., 2023) resulting in a higher body weight of the gene-edited fish. Furthermore, there are currently two CRISPR gene-edited fish approved for sale on the Japanese food market: a red sea bream (*Pagrus major*) mutated in the *mstn* gene, resulting in fish that grow larger but with the same amount of food, and a tiger puffer (*Takifugu rubripes*), in which the leptin receptor gene controlling appetite was disrupted, causing the fish to eat more and increase the rate of which they gain weight (JapanEmbracesCRISPR-editedFish, 2022).

Higher omega-3 content

Omega-3 long-chain polyunsaturated fatty acids (LC-PUFAs), particularly EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid), have been shown to be beneficial for human health. Farmed salmon are these days fed a diet containing mostly vegetable oils due to a lack of fish oil and fish meal resources, resulting in lower levels of EPA and DHA (Datsomor et al., 2019b). Salmon can synthesize omega-3 LC-PUFAs and using CRISPR/Cas to understand the mechanisms of salmon omega-3 synthesis and possibly increase the fish' intrinsic production is an interesting and potentially beneficial approach (Datsomor et al., 2019a, Datsomor et al., 2019b). For example, Datsomor et al. demonstrated that *elovl2* has a key role in omega-3 synthesis in salmon, suggesting that selective breeding for *elovl2* may increase conversion of vegetable oils into EPA and DHA. Furthermore, the *elovl2* gene from masu salmon (*Oncorhynchus masou*) was inserted in channel catfish, which have lower levels of EPA and DHA compared to salmon (Xing et al., 2022). The insertion

resulted in higher DHA content in muscle from transgenic fish compared to non-transgenic fish. Alternatively, CRISPR/Cas9 has been applied to create a gene-edited rapeseed, containing eight genes enabling the production of LC-PUFAs. The oil derived from this plant was recently approved for use in fish feed by the Norwegian authorities.

Disease resistance

One of the most promising applications of CRISPR/Cas technology in salmon is its potential to introduce traits that may confer disease resistance (Gratacap et al., 2019, Robinson, 2022). The annual Fish Health report by the Norwegian Veterinary institute assesses the mortality rates in Norwegian salmon farms. Last year, approximately 15% of the salmon died during production due to diseases attributable to pathogens like viruses, bacteria, and parasites (Sommerset et al., 2023). This figure translates to nearly 50 million fish, emphasizing that one of the most critical challenges facing the industry is infectious and parasitic diseases. Various strategies are currently employed to help control disease, including vaccination, thermal or mechanical sea lice removal, and selective breeding. Compared to other traits like growth and reproduction, using CRISPR/Cas to study immune-related genes and disease resistance is still in its early stages. Most of the work has been performed in catfish, where researchers successfully integrated an exogenous antimicrobial peptide using a CRISPR-based HDR approach. The gene encoding the antimicrobial peptide cathelicidin derived from alligators was inserted into the channel catfish genome, resulting in a transgenic fish expressing cathelicidin (Simora et al., 2020). Cathelicidin was later inserted into blue catfish in which the transgenic fish showed higher survival rate compared to wild type counterparts following infection with *Flavobacterium covae*, a pathogen causing columnaris disease affecting mainly the gills, skin, and fins (Wang et al., 2023). CRISPR/Cas9 has also been used in channel catfish to disrupt the immune genes toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1) and rhamnose binding lectin (RBL) (Elaswad et al., 2018), and in rohu carp (*Labeo rohita*) to disrupt the toll-like receptor 22 (TLR22) gene involved in innate immunity, the latter resulting in a fish lacking TLR22 mRNA expression (Chakrapani et al., 2016).

Model animals lacking specific target genes are often used to study immune-related pathways, but first attempting knockout in cell lines may also provide insight into what genes are involved in disease resistance. A few studies report immune gene-related knockout in cell lines of salmon (Pavelin et al., 2021, Dehler et al., 2019), olive flounder (*Paralichthys olivaceus*) (Kim et al., 2021), and grass carp (*Ctenopharyngodon idella*) (Ma et al., 2018). For example, knockout of the NEDD-8 activating enzyme 1 (*nae1*) gene in Atlantic salmon SHK-1 cells led to reduced replication of the infectious pancreatic necrosis virus (IPNV) (Pavelin et al., 2021). Although the use of CRISPR/Cas on immune-related genes is still limited, this research field is expected to further advance to better understand the mechanisms underlying disease resistance and the fish immune system.

3. The teleost immune system

The immune system is essential for the survival of an organism and its main task is to recognize and eliminate infectious pathogens like bacteria, viruses, fungi, or parasites. Additionally, the immune system recognizes and destroys damaged host cells and tissues. As in mammals, the teleost immune system encompasses both innate (non-specific) and adaptive (specific) components (Uribe et al., 2011, Zhu et al., 2013). The innate defenses are quickly initiated following infection and recognizes a vast array of pathogens based on molecular structures commonly found on pathogen cell surfaces. The adaptive immune system discriminates between different pathogens based on small differences in their structures and thus has the ability to target specific pathogens. Together with its high specificity, the adaptive immune system is also characterized by its memory. When first exposed to a pathogen, the primary infection, the adaptive immune responses develop a memory of that infection. Upon re-exposure to same pathogen, the secondary infection, this immunological memory will enable the adaptive immune responses to assemble more rapidly, and the pathogen to be eliminated at a faster rate. In other words, immunological memory is acquired through active infection. Importantly, however, it can also be acquired through vaccination. Fish vaccination may in some cases provide the organism with long-term protection (e.g., (Tobar et al., 2011)), although it must be noted that the mechanisms of the protection elicited by the vaccine are not fully understood (Yamaguchi et al., 2019). The components involved in adaptive immunity are therefore of particular interest for aquaculture research for example to develop more efficient vaccines.

3.1. The adaptive immune system

Generally speaking, the adaptive immune system can be divided into humoral immunity, involving B cells and their antibody production, and cellular immunity, involving T cells and their array of effector functions. Figure 5 shows an overview of key cells and molecules involved in the adaptive immune system. Activation of both T and B cells is necessary for the organism to elicit an effective immune response and is achieved when the cells recognize a pathogen through a process of antigen presenting.

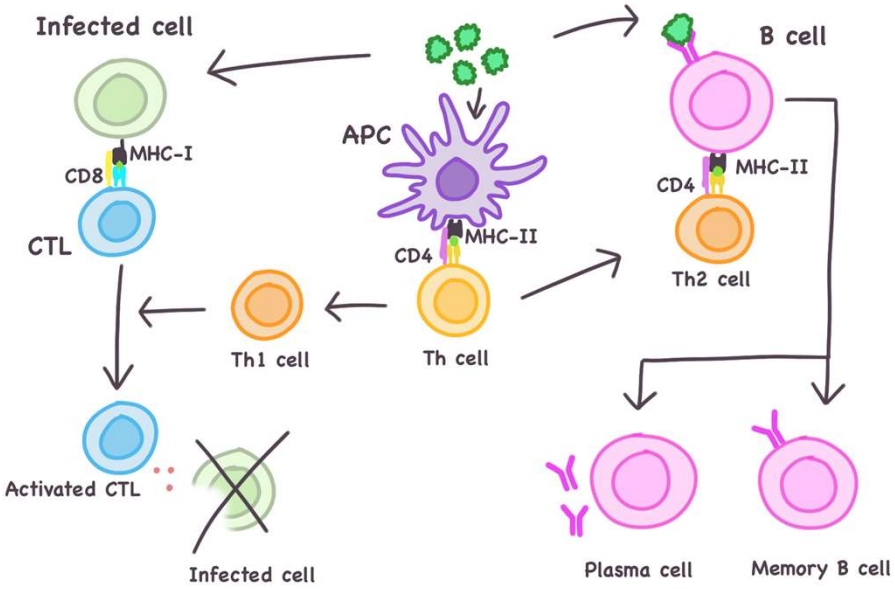

Figure 5 Key components of the adaptive immune system. The humoral immunity comprises the B cells and associated antibodies, whereas the cell-mediated immunity comprises the T cells and their effector functions.

Briefly described, pathogens are internalized by a group of specialized antigen-presenting cells (APCs) (e.g., macrophages) through phagocytosis, or by B cells through their B cell receptors, which have affinity for epitopes on pathogenic microorganisms. Then, the pathogens are degraded into smaller peptide fragments. The peptides are presented on major histocompatibility complex (MHC) molecules, which are cell surface proteins with very high specificity in their binding site. The MHC molecules can be divided into two classes, MHC class I and MHC class II. MHC-I is expressed by nearly all cells of the organism, whereas MHC-II is expressed only by the APCs, at least in mammals. Whether the antigen is presented on MHC-I or MHC-II largely depends on the type of pathogen: intracellular pathogens are mainly presented on MHC-I by infected cells, whereas extracellular pathogens may be taken up by the APCs and presented on MHC-II. Although scarce, the evidence available suggests that the functions of fish MHC-I and MHC-II are similar to those of mammals (Yamaguchi and Dijkstra, 2019).

Once activated, the B cells may differentiate into two types of cells, plasma cells and memory B cells. The plasma cells produce large quantities of antibodies with affinity to the pathogen. Antibodies may bind to and neutralize the pathogen before they can invade host cells and are therefore primarily associated with fighting extracellular pathogens. Antibodies, or immunoglobulins (Ig), are glycoproteins built up of two heavy chains and two light chains. The Igs can bind to specific epitopes found on pathogens (in the same manner as the B cell receptors – in fact, the B cell receptors are membrane-bound Igs), enabling them to recognize a vast array of microorganisms in a highly specific manner. There are multiple Ig isotypes, classified by their heavy chain. Three isotypes have been reported in teleosts: IgM, IgD, and IgT (Bilal et al., 2021). IgM is the predominant serum Ig in salmonids, whereas IgT is mainly associated with mucosal immunity (Zhang et al., 2010, Hordvik, 2015). Antibodies also play important roles in the innate mechanisms like opsonization, which enhances phagocytosis performed by phagocytic cells, and the activation of the complement system. Some activated B cells differentiate into memory B cells that can survive in the organism in a latent state long after being exposed to the pathogen. During a secondary infection, memory B cells that express a B cell receptor specific to that pathogen, will elicit an enhanced and improved adaptive immune response.

Intracellular pathogens will largely be able to avoid the antibodies and are mainly targeted by the cellular immunity comprising the T cells. There are two classes of T cells in fish, helper T cells (Th cells) and cytotoxic T cells (CTLs) (Nakanishi et al., 2015). The Th cells produce cytokines and regulate the immune response to the pathogen, for example by activation of CTLs and also B cells. The CTLs recognize and eliminate infected host cells. The T cells are classified based on certain cell surface proteins called cluster of differentiation (CD) molecules as well as their effector function once activated. All T cells express various types of CD molecules, but the ones most important for identification are CD4 found on Th cells, and CD8 found on CTLs.

3.2. Species-specific variation

The teleosts are comprised of nearly 30,000 species classified into approximately 70 orders and 500 families (Ravi and Venkatesh, 2018). Although teleosts exhibit both innate and adaptive immune factors, fundamental differences exist between species, partly because key genes are lacking from certain lineages. For example, the Atlantic cod (*Gadhus morhua*) and related fish lack MHC-II molecules entirely but have multiple MHC-I molecules (Star et al., 2011). This suggests that they may have evolved alternative mechanisms for adaptive immunity perhaps through an expanded number of MHC-I genes (Star et al., 2011). Furthermore, even though there are three Ig isotypes (IgM, IgD, and IgT) characterized in teleosts, not all species possess each isotype: IgT has not been detected in medaka (*Oryzias latipes*) (Magadán-Mompó et al., 2011) or channel catfish (Bengtén et al., 2006). On the other hand, due to a whole-genome duplication event occurring in salmonids, rainbow trout and Atlantic salmon exhibit three subtypes of IgT (Zhang et al., 2017, Hordvik, 2015), as well as two subtypes each of IgM and IgD (Hordvik, 2015). This gene expansion might influence the salmon's immune capabilities, but its precise impact on the response to pathogens is still unknown. Species-specific investigations of the Atlantic salmon immune system are therefore necessary to gain knowledge of traits related to disease resistance.

4. Aims and research questions

The sustainability challenges affecting the Norwegian salmon aquaculture industry impacts the surrounding environment and the welfare and health of the fish. Solving these challenges is necessary to protect the fish from harm. One solution may be to use CRISPR/Cas gene editing technology to change the genotype of the fish and make it more robust. To do so, we need to be able to perform the gene editing with accuracy, but also knowledge of which genes to edit. Such knowledge can be obtained using CRISPR/Cas-based loss-of-function and gain-of-function approaches. In other words, CRISPR/Cas can be used for both basic fish research purposes, and for application in the aquaculture industry.

The primary aim of this thesis was to implement new CRISPR-based gene editing tools in Atlantic salmon. The secondary aim included using such tools to make *in vivo* models that can increase our understanding of biological processes related to the Atlantic salmon immune system.

The first aim is the focus of Paper I and Paper II, where we tested new CRISPR-based tools in Atlantic salmon: In Paper I, we conducted experiments to assess both the knockout and knock-in possibilities of the LbCas12a nuclease (derived from *Lachnospiraceae bacterium*). In Paper II, we investigated two methods for making single nucleotide changes to the genome: cytidine base editing, and conventional CRISPR/Cas9 using DNA templates featuring point mutations at various positions in the template. The second aim is covered in the final paper, Paper III, in which we applied CRISPR/Cas9 to knockout the immune genes IgM and Interferon gamma (IFNg) in Atlantic salmon.

Summary of the papers

Paper I presents the *in vivo* application of CRISPR/LbCas12a gene editing in Atlantic salmon. Microinjections of LbCas12a ribonuclease protein (RNP) complexes targeting the *solute carrier family 45 member 2* (*slc45a2*) gene were performed to assess both the knockout and knock-in possibilities of the LbCas12a nuclease. Knockout of the *slc45a2* gene results in pigmentation loss and the fish exhibit an albino or mosaic pigmentation phenotype, enabling easy visual recognition of successfully gene edited individuals. In the initial experiment, three crRNAs were tested, in which two were multiplexed (i.e., injected simultaneously in the same individual) and one was injected singly. High-throughput sequencing (HTS) was used to assess the editing efficiency of the crRNAs. The crRNA injected singly failed to produce mutagenesis at the target site, whereas the two other crRNAs showed very different mutation efficiencies. The most efficient of the two displayed an average mutation rate of 77%. In a subsequent experiment, we used the most efficient crRNA and included ssDNA templates with either a target or non-target DNA strand orientation to assess the insertion efficiency. Individual integration rates of up to 34% and 55% using the target and non-target strand template, respectively, were observed. We achieved a significantly higher rate of perfect integration (i.e., no indels up- or downstream of the insert) using the non-target strand template. Finally, SpCas9 and LbCas12a RNPs were co-injected with ssDNA templates corresponding to the respective cut sites of the nucleases. While both nucleases produced similar mutation rates, an average of 55% and 58% by LbCas12a and SpCas9, respectively, LbCas12a showed a higher occurrence of perfect integration at its cleavage site. Furthermore, LbCas12a produced larger deletions compared to SpCas9.

In Paper II, we report two methods for making precise edits to the Atlantic salmon genome, including the first application of base editing in any fish aquaculture species. By microinjecting cytidine base editor AncBE4max mRNA together with gRNA targeting *slc45a2*, we successfully converted our target C to T. This edit introduced a premature stop codon, resulting in fish displaying an albino or mosaic pigmentation phenotype. HTS revealed highly efficient correct conversion of our target base in the

sampled individuals, with an average ranging from 50% to 66%, depending on the concentration of the base editor mRNA. Moreover, up to 89% correct conversion was achieved in one individual. Although at a low rate, sequence reads containing errors such as indels, conversion from C to non-Ts, and bystander edits were also observed. Additionally, we performed an experiment using conventional CRISPR/Cas9 combined with ssDNA templates featuring point mutations at various positions upstream and downstream of the Cas9 cleavage site. Here, we demonstrated that the insertion efficiency was dependent on where the mutation was located: the further away from the cleavage site, the lower insertion efficiency.

In the final paper, CRISPR/Cas9 was applied to knockout the immune genes IgM and IFNg in Atlantic salmon. We tested two gRNAs for IgM, and two gRNAs for IFNg. These gRNAs were multiplexed, and we created two groups of CRISPR fish: Immune-1 injected with gRNAs IgM-1 and IFNg-1, and Immune-2 injected with gRNAs IgM-2 and IFNg-2. gRNA targeting *slc45a2* was also included for visual recognition. HTS was used to assess the editing efficiency of the gRNAs. The IgM-2 gRNA resulted in an impressive mutation rate of more than 95% at both IgM loci known in salmon, whereas the IgM-1 produced less than 10% mutagenesis. The IFNg-2 gRNA produced an average mutation rate of 57%, and the IFNg-1 was inefficient. We decided to focus on functional assays for IgM because of the high gRNA efficiency of IgM-2. In addition, a second IFNg gene was annotated in Atlantic salmon during the work with this study, causing some uncertainty to whether both IFNg genes were mutated. To assess the phenotypical outcomes of the gene editing, flow cytometry analysis was conducted on peripheral blood extracted from ~500 g fish. The results revealed a striking 91% reduction in the number of IgM positive (IgM^+) B cells in the mutated fish in the Immune-2 group compared to their wild type siblings. These findings suggest that we were able to disrupt the DNA resulting in a functional protein knockout. To our surprise, the fish survived being reared in a non-sterile environment.

Discussion

Implementation of new CRISPR-based tools

A decade since the discovery of CRISPR/Cas9 gene editing (Jinek et al., 2012, Gasiunas et al., 2012), novel or improved CRISPR-based tools are under continuous development. These tools are most often developed and tested in human cell lines, or model species such as rats, mice, pigs, or zebrafish. To improve and extend the possibilities for gene editing in aquaculture, there is a need to implement and test new tools in target species like Atlantic salmon. In Paper I and Paper II, two CRISPR-based tools were tested for the first time in Atlantic salmon: CRISPR/LbCas12a gene editing and base editing using the cytidine base editor AncBE4max. What works in model organisms will not necessarily translate to Atlantic salmon, and we did in fact try other techniques than those reported herein. For example, we added the NHEJ inhibitor NU7441 to the injection mix as this was found to increase HDR in zebrafish (Aksoy et al., 2019). However, these experiments did not yield the anticipated effect observed in the models and were consequently omitted (data not shown). Differences in egg size and composition, injection protocols, and incubation temperature are some of the factors that vary between species and may affect the outcome.

Paper I and Paper II present proof-of-principle studies, and we targeted the pigmentation gene *slc45a2*. Disruption of *slc45a2* results in fish displaying an albino or mosaic pigmentation phenotype (Edvardsen et al., 2014), allowing for easy visual confirmation of successful gene editing. We could therefore conclude that both the LbCas12a nuclease and base editor AncBE4max works in salmon based on the phenotype alone. For a more precise assessment of the gene editing efficiency, we employed HTS. Recent advancements in sequencing technologies have significantly increased the sequence throughput while reducing costs and time. Using HTS, thousands of sequence reads are generated from the genomic DNA obtained from each fish. This approach enables us to accurately determine the amount of wild type (i.e., unedited DNA) and edited DNA in individual fish.

In Paper I, we demonstrate the applicability of CRISPR/LbCas12a in Atlantic salmon. The use of this nuclease enables gene editing in AT-rich regions which are inaccessible to Cas9, meaning we now have more opportunities to edit genes of interest. We also found that perfect insertion of the template FLAG sequence was achievable using both a non-target and target strand orientation of the template. However, a significantly higher insertion efficiency was observed using the non-target strand template, agreeing with findings in zebrafish (Moreno-Mateos et al., 2017). This is an interesting observation as it could influence the template design. In some cases, the FLAG sequence was integrated but with undesired indels up- or downstream of the insert (i.e., not perfect insertion). Previously, a HDR strategy using Cas9 mRNA and gRNA targeting *slc45a2* indicated a correlation between the template orientation and indel location (Straume et al., 2020). For LbCas12a, the influence of template polarity on indel location is still uncertain due to few reads containing both the FLAG insert and indels, but this aspect could be interesting to investigate in future work.

Comparing the LbCas12a and SpCas9 nucleases, we found that they both produced a similar percentage of mutated reads (indel-containing reads and HDR reads). However, the integration efficiency of the FLAG template was higher at the LbCas12a cleavage site than the SpCas9 cleavage site, consistent with observations in zebrafish (Moreno-Mateos et al., 2017). This is a most intriguing finding, given that efficient insertion poses a challenge in CRISPR/Cas gene editing. Another finding was the larger deletions produced by LbCas12a than those produced by SpCas9. An observation also reported in zebrafish (Meshalkina et al., 2020) and rice (Hu et al., 2017). Larger deletions may be advantageous when aiming to create knockout models, as they increase the chance of protein function loss, even if the deletion leads to in-frame mutations. One limitation to the comparison between the nucleases was different target sites in *slc45a2*: the LbCas12-associated crRNA targeted exon 1 and the Cas9-associated gRNA targeted exon 6. Additionally, the findings are based on only these two target sites in a single gene. Further research is warranted to explore the full knock-in possibilities of LbCas12a.

Paper II presents two methods for single nucleotide replacement in Atlantic salmon, including the first application of base editing. Using the fourth-generation base editor AncBE4max, we achieved a very high C-to-T conversion of the target base in the F0 generation, introducing a premature stop codon. In three individuals, more than 80% correct conversion was observed. This was an unexpected high efficiency, both because previous experiments in our lab using an earlier version of the base editor did not work, and also because the efficiency observed in this study is higher than reported by others using the same base editor (Carrington et al., 2020, Yuan et al., 2020, Zhao et al., 2020). Notably, differences in data analysis approaches in these studies may contribute to varying reported efficiencies. In our study, we used two concentrations of the base editor mRNA, 150 ng/µL or 300 ng/µL. The higher concentration group resulted in a seemingly higher average correct conversion rate than in the lower concentration group. However, variations in efficiency between experiments are common, and a definitive conclusion whether one concentration over the other is advantageous is uncertain. Our sequencing data also revealed some undesired effects of the base editor, such as indels, bystander edits, and incorrect conversion of C to non-Ts. While these errors were present in only a small percentage of the sequencing reads, they still need to be considered when employing base editing in future applications.

Base editing has some restrictions regarding potential target sites, as the target base needs to fall within the editing window of the deaminase component. Additionally, base editors can only perform four out of the twelve possible transition mutations. As a solution to this, we also employed conventional CRISPR/Cas9 in combination with ssDNA templates to edit a single nucleotide. Although this has been done in salmon previously (Straume et al., 2021), our focus this time was to explore the possibility of editing a base further away from the cleavage site than before, and to investigate whether the editing efficiency was influenced by the mutation's location in the template. Injecting six templates featuring point mutations at various positions upstream and downstream from the cleavage site revealed a correlation between the position of the mutation and editing efficiency: the further away upstream of the cleavage site the mutation was positioned, the lower efficiency. With this

understanding, we now know that if a single nucleotide polymorphism (SNP) falls within the appropriate range of the cleavage site, CRISPR/Cas9 in combination with ssDNA templates can be used to implement the desired change.

Taken together, the implementation of LbCas12a, base editing, and single nucleotide replacement in Atlantic salmon, expands the toolkit for gene editing in this species. A versatile toolkit is advantageous both for research purposes and for potential future applications to introduce traits that enhance disease resistance.

Generation of a B cell-deficient salmon model

A deeper understanding of the salmon immune system is needed to address the challenge of infectious disease, whether the solution involves gene editing, improved treatments, or alternative approaches. Animal models featuring specific gene knockouts are useful for understanding gene functions or disease mechanisms. In Paper III, our objective was to create fish with a targeted knockout of the two genes IgM and IFNg, chosen for their key roles in the immune system. IgM is primarily associated with humoral immunity and immunological memory, while also participating in various innate responses such as complement activation, neutralization, and opsonization. IFNg is a cytokine produced primarily by T cells upon viral infection and regulates several biological pathways, including promotion of cellular immunity and differentiation of other T cells (Schroder et al., 2003). The choice of these genes was also influenced by the necessity to confirm potential effects of the knockouts on protein level – for IgM and IFNg we had antibodies available, which is not always guaranteed for Atlantic salmon.

We designed two gRNAs for each gene, intending this experiment to serve as a pilot to assess gRNA efficiencies. During the microinjections, the gRNAs were multiplexed, resulting in two groups of CRISPR fish injected with gRNAs targeting both IgM and IFNg. We also included gRNA targeting *slc45a2*, as mutagenesis of this gene is correlated with mutagenesis of the gene of interest (Wargelius et al., 2016), allowing for visual confirmation of successful gene editing (i.e., lack of pigmentation indicates that the CRISPR injection mix has been delivered properly). This study

differs somewhat from the proof-of-principle approach in Paper I and Paper II, as our primary goal was to achieve high mutagenesis of the target sites to ensure a phenotypic effect. The two gRNAs targeting IgM displayed very different efficiencies. Variations in efficiency were also observed for the gRNAs targeting IFNg, although the overall mutation efficiency was lower compared to the IgM gRNAs. Such variance in gRNA efficiency is common in CRISPR experiments (and was also observed when testing crRNAs in Paper I), although the specific reasons for these differences are unclear. Proposed factors causing such variation include genome availability, gRNA secondary structures, and GC content (Konstantakos et al., 2022) Interestingly, the Geneious software used for visualizing DNA sequences suggested similar efficiencies for the two IgM gRNAs according to its built-in gRNA prediction tool (Doench et al., 2016). These findings emphasize the importance of designing multiple gRNAs to ensure the selection of one that exhibits sufficient editing efficiency *in vivo*.

Despite the initial intent for this experiment to be a pilot study, we opted to make use of the fish available and evaluate the knockout effect on protein level. As briefly mentioned in the summary section, the investigations regarding IFNg were put on hold. Performing the functional assay for IFNg required working in a laminar flow cabinet to maintain a sterile environment for the blood cells. Unfortunately, due to limited resources we were unable to conduct this assay concurrently to functional analysis of IgM. Because preliminary data indicated higher efficiency of the IgM gRNA than the IFNg gRNA, we decided to focus on IgM in this study and not prioritize the IFNg assay. We also realized during the work with the study that a second IFNg gene was annotated with the release of the third version of the salmon genome assembly. The newly annotated IFNg gene, located on chromosome 17, exhibited high similarity to the other IFNg, located on chromosome 7. The primers used for amplification of the IFNg target site during library preparation for HTS were an exact match for both genes, indicating that both genes were likely amplified. Consequently, it is challenging to conclusively determine whether the observed average mutation efficiency of 57% represents the mutation rate across both target sites, or if it resulted from one gene being extensively mutated while the other

remained unaffected. To be certain of the mutation rates at chromosome 7 and 17, designing new primers that are specific to each gene is necessary.

To assess the phenotypic effect of the CRISPR-induced IgM mutagenesis, we conducted flow cytometry analysis on leukocytes isolated from peripheral blood. The monoclonal anti-salmon IgM antibody F1-18, recognizing both IgM subclasses A and B (Hedfors et al., 2012), was used to stain IgM^+ B cells. In the knockout fish in the Immune-2 group (i.e. injected with the high-efficiency gRNA IgM-2), a significant reduction in the IgM^+ B cells was observed compared to the non-injected wild type controls. The Immune-1 group, in which the IgM-1 gRNA did not produce efficient mutagenesis, displayed similar IgM^+ B levels as the wild type controls. Although some IgM^+ B cells persisted, this may be attributed to the retention of some wild type DNA in the knockout fish, or the presence of in-frame mutation variants that did not produce a phenotypic effect. This may also partly explain why the fish was able to survive despite being reared in a non-sterile environment. A study characterizing IgM knockout rats revealed normal IgM levels in heterozygous mutants and undetectable levels in homozygous mutants (Ménoret et al., 2010). We speculate that the remaining IgM^+ B cells are capable of normal proliferation and differentiation, and that the reduced levels of IgM^+ B cells are not detrimental to the fish. It is also possible that IgT^+ B cells and antibodies can compensate for the IgM KO. Potential challenges may arise in the next generation if obtaining homozygous fish. Breeding the fish to the next generation would be of interest as homozygous fish may be favorable for future experiments, given that retained wild type DNA potentially rescues the phenotype.

The IgM^+ B cell-deficient salmon represent the first application CRISPR-based tools to knockout an immune-related gene *in vivo* Atlantic salmon. The knockout fish may help us further elucidate the role of IgM in Atlantic salmon. Conducting challenge experiments can help us evaluate the disease mechanisms of various pathogens and determine whether they trigger the humoral immune response comprised of B cells and antibodies. The humoral immune response is necessary for establishing immunological memory achieved through either infection or vaccination. The knockout fish, lacking IgM^+ B cells, could therefore provide valuable insight into

vaccine development. Vaccination is a major strategy to prevent disease outbreaks in aquaculture, but this strategy is limited by the inability to produce efficient vaccines for all types of pathogens.

Since our understanding of the teleost immune responses lags behind that of mammals, creating salmon models featuring knockout of other key genes to study their functional role in the immune system is also of interest. Additionally, knockout of genes that may be more directly involved in resistance to specific pathogens holds significant interest. This approach not only expands our understanding of the teleost immune system but may also uncover potential targets for gene editing to enhance disease resistance in the fish.

Gene editing for disease resistance

There are multiple gene editing strategies to improve disease resistance in Atlantic salmon. Three major approaches include 1) removing whole or parts of specific genes, 2) introducing gene variants from the same species, and 3) introducing genes from different species.

The removal of entire genes or specific gene segments linked to disease susceptibility represents a straightforward strategy. For instance, CRISPR technology has been successfully applied in pigs to disrupt CD163, a protein expressed on the cell surface of alveolar macrophages, serving as an entry receptor for the porcine reproductive and respiratory syndrome virus (PRRSV) (Whitworth et al., 2016). The resulting gene-edited pigs exhibited resistance to infection from both PRRSV subtypes. However, the complete removal of genes may lead to unintended effects. Subsequent studies revealed that selectively removing a portion of the gene had no impact on CD163 expression on the cell surface, while still granting resistance to PRRSV (Burkard et al., 2017, Salgado et al., 2024). Targeting viral entry receptors or proteins facilitating replication of fish viruses appears to be a possible approach. One candidate gene may be the aforementioned *nae1*, since knockout and chemical inhibition of *nae1* in salmon cell lines resulted in a significant reduction in IPNV replication (Pavelin et al., 2021).

In some instances, a population may have evolved advantageous gene variants in response to varying disease pressures in different habitats (Kjærner-Semb et al., 2016). Changing the genetic sequence of farmed fish to incorporate such gene variants can be a valid strategy and is often a goal of breeding programs. A classic example is the identification of a quantitative trait locus (QTL) – in which *nae1* is located – associated with IPNV resistance (Houston et al., 2008, Moen et al., 2009). Subsequent selective breeding for this QTL significantly reduced outbreaks with IPNV compared to previous years (Sommerset et al., 2023). It is worth noting that many disease resistance traits are likely polygenic, meaning that they are controlled by multiple genes rather than a single one. While identifying and targeting multiple genes concurrently may require more effort, the precision of CRISPR technology allows bypassing potential negative side effects associated with breeding, such as the inheritance of undesirable traits.

Finally, the introduction of genes that may confer disease resistance from other species is an enticing opportunity. The coho salmon (*Oncorhynchus kisutch*) and pink salmon (*Oncorhynchus gorbuscha*) are able to elicit effective immune responses against sea lice and therefore display natural tolerance to the parasite (Jones et al., 2007, Fast et al., 2002). Although it is unclear whether the resistance in coho and pink salmon stems from species-specific gene regulation or from gene variants, replicating this type of immune response in Atlantic salmon could have substantial impact on the overall welfare of the fish. As mentioned in the introduction, some progress has already been made in introducing exogenous genes into aquaculture fish: researchers have integrated the antimicrobial peptide cathelicidin, derived from alligators, into the catfish genome, resulting in enhanced resistance against bacterial disease (Wang et al., 2023).

These three approaches exhibit varying levels of “invasiveness” into the original genome of the fish, a factor that may influence public perception of gene editing. Both the first and second approaches are changes within species and fall under the category of precision breeding. Precision breeding involves using techniques such as gene editing to accurately change the DNA of animals and plants in a way so that the

changes are equal to those achievable using conventional breeding methods (FSA, 2023). The third approach involves the insertion of genes from other species and is often referred to as genetic modification or transgenesis. This is different from precision breeding in that DNA from one plant or animal species is inserted into another (un)related species in a way that could not have occurred using traditional methods (FSA, 2023). Nevertheless, due to the advancements in gene editing technology, particularly with the discovery of CRISPR, genetic modification can be performed with much greater efficiency than before. These advancements have also led to debate regarding gene-edited animals. England has recently passed the Genetic Technology (Precision Breeding) Act, allowing the development and marketing of precision bred plants and animals. Moreover, Japan and Argentina have already granted approval for the sale of CRISPR fish (JapanEmbracesCRISPR-editedFish, 2022, Fishfarmingexpert, 2018).

An important aspect to consider when applying CRISPR technology is to avoid off-target effects, the unintended editing of regions similar to the target of interest, as these could impact the phenotype. Thorough investigation of potential off-target sites is necessary, and the fish must be monitored to detect any potential negative effects. The overarching objective is, of course, to mitigate welfare problems rather than introduce them.

Concluding remarks

The work presented in this thesis expands the toolkit for performing gene editing in Atlantic salmon by implementing CRISPR/LbCas12a and base editing. Additionally, it demonstrates that using conventional CRISPR/Cas9 and ssDNA templates, the insertion of point mutations at various positions from the cleavage site is possible. Furthermore, CRISPR/Cas9 technology was used to create an IgM^+ B cell-deficient salmon that may serve as a model to elucidate this key protein's function in the immune system. Although there are still challenges to CRISPR-based gene editing, the technology holds great potential in contributing to improve the Atlantic salmon's welfare and health in the future – either through increased knowledge or by actually changing the genome of the farmed fish to improve disease resistance.

Future work

Since we only targeted one gene, *slc45a2*, as proof-of-principle in Paper I, we suggest there is a need for further exploration of the knock-in possibilities of LbCas12a in salmon. Therefore, targeting additional genes, as well as optimizing factors such as arm length or concentration of donor templates should be considered. While not covered in this thesis, efforts were made to enhance HDR using CRISPR/Cas9. We have some preliminary data indicating insertion of green fluorescent protein in salmon, but with unknown efficiency (data not shown). Further investigations to achieve gene insertion, either using LbCas12a or Cas9, is of high interest.

Similarly, in Paper II, *slc45a2* was the only target gene when using the base editor. Applying the base editor AncBE4max to edit cytidine bases in other genes would therefore be interesting. However, given the base editor restrictions, it may prove challenging to find suitable candidate genes, i.e., genes that have a C within the editing window that will lead to a stop codon or an amino acid change when edited. Testing and implementing the other types of base editors, or even prime editing, is therefore also a possibility.

The conclusion of the final paper states the possibility of using the IgM knockout fish as a model for subsequent experiments. The remaining fish from this experiment are housed in our facilities in Matre and are earmarked for breeding to produce a homozygous F1 generation. Additionally, we performed new microinjections using the most efficient gRNAs (i.e., IgM-2 and IFNg-2), creating two groups of CRISPR fish with either IgM or IFNg knockout. A priority is to conduct the IFNg assay to determine whether the IFNg knockout results in a phenotypic effect as we were unable to perform this assay in Paper III. Both IFNg and IgM knockout fish will be subjected to disease trials in future experiments.

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PART TWO | PAPERS

PAPER I

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RESEARCH

In vivo CRISPR/LbCas12a-mediated knock-in and knock-out in Atlantic salmon (*Salmo salar* L.)

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Abstract Genome editing using the CRISPR/Cas system offers the potential to enhance current breeding programs and introduce desirable genetic traits, including disease resistance, in salmon aquaculture. Several nucleases are available using this system, displaying differences regarding structure, cleavage, and PAM requirement. Cas9 is well established in Atlantic salmon, but Cas12a has yet to be tested in vivo in this species. In the present work, we microinjected salmon embryos with LbCas12a ribonucleoprotein complexes targeting the pigmentation gene *solute carrier family 45 member 2* (*slc45a2*). Using CRISPR/LbCas12a, we were able to knock-out *slc45a2* and knock-in a FLAG sequence element by providing single-stranded DNA templates. High-throughput sequencing revealed perfect HDR rates up to 34.3% and 54.9% in individual larvae using either target or non-target strand template design, respectively. In this work, we demonstrate the in vivo application of CRISPR/LbCas12a in Atlantic salmon, expanding the toolbox for editing the genome of this important aquaculture species.

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Introduction

Norway is the world's largest producer of farmed Atlantic salmon (*Salmo salar*) and exported over 1 million tons of fish in 2022. The salmon industry has steadily increased over the years, but further expansion is currently hindered due to several sustainability issues. One of the problems is infectious diseases attributable to viruses, bacteria, or parasites (Sommerset et al. 2023). To combat this problem, breeding programs have been applied to develop fish with more robust performance in the sea pens (Thodesen and Gjedrem 2006; Kjøglum et al. 2008). However, selective breeding is time-consuming, especially in species with a long generation time like the Atlantic salmon. New breeding technologies such as genome editing (GE) using the CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated) system may facilitate current breeding programs and further introduce favorable genetic traits including disease resistance (Gratacap et al. 2019) without the need for breeding the fish for many generations.

CRISPR/Cas9 was first utilized as a tool for GE in 2012 by Jennifer Doudna and Emmanuelle Charpentier (Jinek et al. 2012). Since then, it has become widely used owing to its efficiency and versatility. The

system consists of two components, the Cas nuclease and a single guide (sg) RNA molecule, together forming the nuclease effector complex. The sgRNA molecule is comprised of both a crisprRNA (crRNA) and a trans-activating crRNA (tracrRNA). The tracrRNA-part enables the recruitment of the nuclease, while the crRNA is programmable and can be designed to target a specific region within the genome. In this manner, the nuclease effector complex is guided to the target site of interest by the pre-programmed crRNA. Prior to binding the DNA, the nuclease effector complex requires the recognition of a short sequence termed the protospacer adjacent motif (PAM). Once bound, the nuclease initiates a double-strand break (DSB) to the DNA, and the endogenous repair mechanisms that follow are exploited to do GE. Repair usually occurs by two main mechanisms: non-homologous end joining (NHEJ) or homology-directed repair (HDR). During NHEJ, the cut ends are trimmed, nucleotides are recruited, and the strands are re-ligated. However, this process often results in erroneous repair with random insertions and deletions (indels), and may lead to a gene knock-out (KO) if produced in a coding exon. This approach allows us to study KO phenotypes and has previously been applied in salmon (Wargelius et al. 2016; Datsomor et al. 2019) as well as other aquaculture species such as common carp (*Cyprinus carpio)* (Zhong et al. 2016), Nile tilapia (*Oreochromis niloticus*) (Jiang et al. 2017), and rainbow trout (*Oncorhynchus mykiss)* (Cleveland et al. 2018). On the other hand, the HDR mechanism can be utilized to knock-in (KI) genetic material by providing a donor template together with the nuclease effector complex. KI by HDR has been done in salmon using oligodeoxynucleotides (ODNs) to perform single nucleotide replacement or insert FLAG sequence elements (Straume et al. 2020, 2021). Inserting larger genetic material remains challenging but has been achieved in other aquaculture species such as channel catfish (*Ictalurus punctatus)* (Simora et al. 2020; Xing et al. 2022).

Cas9 derived from *Streptococcus pyogenes* was among the first nucleases to be established for GE and is still widely used today. However, the restriction for a particular PAM site narrows down the scope of target sites available for GE. More recently, novel nucleases derived from other bacterial species, e.g., Cas12a (previously Cpf1) from *Acidaminococcus* sp. (AsCas12a) or Lachnospiraceae bacterium (LbCas12a), have also been shown effective for GE (Zetsche et al. 2015). The nucleases Cas9 and Cas12a represent different types of Cas enzymes, type II and V, respectively. The two types differ in characteristics such as structure, cleavage, and importantly, PAM requirement. Cas9 forms a complex with the sgRNA, recognizes PAM 5′-NGG-3′ downstream of the protospacer, and usually produces a blunt-end cleavage 3 bases upstream of the PAM (Jinek et al. 2012). In contrast, Cas12a forms a complex with the crRNA only, recognizes PAM 5′-TTTV-3′ (V represents A, C, or G) upstream of the protospacer, and cleaves about 18 bp downstream of the PAM on the non-target strand, and 23 bp downstream on the target strand, producing staggered ends (Zetsche et al. 2015).

Its unique characteristics make Cas12a a promising tool for GE for several reasons. Firstly, the T-rich PAM requirement enables GE in AT-rich regions not accessible to Cas9. Secondly, the shorter crRNA utilized by Cas12a is easier and cheaper to synthesize compared to the crRNA and tracrRNA molecules needed by Cas9 (Zetsche et al. 2015). Finally, Cas12a has been shown to induce higher HDR efficiency than Cas9 in zebrafish (Moreno-Mateos et al. 2017). While Cas12a has been established in model organisms such as zebrafish (Meshalkina et al. 2020; Fernandez et al. 2018; Han et al. 2022) and silkworm (Dong et al. 2020), as well as salmonid cell lines (Gratacap et al. 2020), the in vivo application has yet to be reported in any aquaculture species. Therefore, this study aimed to investigate if the CRISPR/Cas12a system could be established as a GE tool in Atlantic salmon. To address this question, we targeted the pigmentation gene *solute carrier family 45 member 2* (*slc45a2*) and microinjected salmon embryos with LbCas12a ribonucleoprotein complexes (RNPs). We also injected RNPs in combination with a ODN FLAG template to assess the HDR efficiency of LbCas12a versus Cas9. We performed high-throughput sequencing (HTS) of individual larvae to determine the efficiency and accuracy of the integrations. To our knowledge, this is the first report of GE using the CRISPR/LbCas12a system in vivo Atlantic salmon. By implementing the use of LbCas12a, we expand the toolbox for editing the genome of this important aquaculture species.

Materials and methods

Target site selection

Target site selection for Cas9 GE of *slc45a2* is described in previous work (Edvardsen et al. 2014). Target site selection for LbCas12a GE was done as follows: the gene sequence of *slc45a2* was obtained from the Atlantic salmon reference genome assembly v2 on the National Center for Biotechnology Information (NCBI) website. (GenBank: GCF_000233375.1, NCBI) (https://www.ncbi.nlm.nih.gov/gene/106563596/: NC_027300.1 (117874712..117899795, complement)). Target sites containing the PAM site 5′-TTTV-3′ for CRISPR/Cas12a cleavage were identified using Geneious Prime software (v. 11.0.12). Candidate target sites were selected from the first exons and BLASTN (Altschul et al. 1990), available on the NCBI website, was used to screen for crRNA sequences with limited chance for off-target cleavage in the Atlantic salmon genome (ICSASG_v2, GCF_000233375.1).

crRNA and sgRNA preparation

Alt-R L.b. Cas12a crRNAs targeting *slc45a2* exon 1 and exon 2 were ordered from Integrated DNA Technologies (IDT) (Coralville, USA). Cas9 sgRNA targeting *slc45a2* exon 6 was synthesized as described in Gagnon et al. (2014) with the following exceptions: the QIAquick PCR Purification Kit (Qiagen) was used to purify the sgDNA templates, the HiScribe T7 Quick High Yield RNA synthesis kit (NEB) was used for in vitro transcription, and the RNeasy Mini Kit (Qiagen) was used to purify the synthesized sgRNA. An overview of crRNA and sgRNA sequences can be found in Supplementary File 1.

ODN design

ODN templates for KI using LbCas12a were designed based on previous studies (Straume et al. 2021; Moreno-Mateos et al. 2017; Richardson et al. 2016). One target and one non-target strand template were designed asymmetrically by copying 90/36 nt from each side of the cut sites, with a 27–29 nt insert comprised of the (CG-)FLAG-TAA sequence. The CG addition was included when needed to keep the open reading frame of FLAG, whereas the stop codon TAA was included to ensure an albino phenotype. Finally, the PAM sites were mutated to avoid repeated cutting. ODN design for KI using Cas9 is described previously (Straume et al. 2021). ODN template sequences can be found in Supplementary File 1. ODNs were ordered from IDT (Coralville, USA).

Cas nucleases

Alt-R L.b. Cas12a (Cpf1) Ultra and Alt-R S.p. Cas9 Nuclease V3 were ordered from IDT (Leuven, Belgium).

Fertilization

Salmon eggs and sperm were obtained from Mowi (Askøy, Norway). The eggs were fertilized in 0.5 mM reduced glutathione (Sigma-Aldrich) solution (pH 10) to prevent chorion hardening. The embryos were incubated for 3 + hours at 6–8 ℃ until the first cell was visible.

Ribonucleoprotein complex assembly

Ribonucleoprotein (RNP) complexes were assembled by mixing the appropriate Cas nuclease with crRNA or sgRNA to a final concentration of 100 ng/µL of both components. The RNP complexes were incubated at room temperature for 20 min.

Microinjection

Glass capillaries (O.D 1.0 mm, I.D 0.50 mm, 10 cm) (Sutter Instrument) were pulled using a PC-100 needle puller (Narishige, Japan). Fertilized eggs were injected using a FemtoJet® 4i injector (Eppendorf). The injection mix contained the pre-formed RNP complex (100 ng/µL), and for the KI experiments, the appropriate ODN template (1.5 µM). Following injection, the eggs were incubated at 6 ℃ until sampling.

Sampling

The larvae were killed with an overdose of buffered MS-222: Tricaine Methane Sulfonate and sampled after 600–700 day-degrees. Individuals showing albino and mosaic pigmentation phenotypes were selected. A non-injected control was included. DNA was isolated from fin clips using the Agencourt

DNAdvance Kit (Beckman Coulter) according to the manufacturer's instructions.

Library preparation

Libraries were prepared for Illumina sequencing using a two-step PCR protocol based on Gagnon et al. (2014) to assess mutation rates, and HDR efficiency and accuracy. The first PCR was performed using Q5 High-Fidelity DNA Polymerase (NEB) on genomic DNA to amplify a fragment that covered the targeted mutagenesis site. Successful amplification was verified by 1% agarose gel. The PCR products were diluted 1:4 and used as templates for a second PCR to barcode individual samples using primers containing adapters with indexes. Primers used for amplification of the target sites can be found in Supplementary File 1. Equal volumes of barcoded fragments were pooled to form a library, which was subsequently purified using the QiaQuick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The library was sequenced on the MiSeq platform (Illumina) using MiSeq Kit v.3 with 300 bp paired-end reads.

Mutation analysis

Preprocessing of the sequence data was done as previously described (Straume et al. 2021). Reads retained after filtering were mapped to the respective reference amplicon sequences using Muscle (v. 3.8.1551) (Edgar 2004). The processed sequence data was analyzed with custom Python scripts and visualized with Geneious Prime software (v. 11.0.12). Only variants with more than 100 reads were included. For deletion size analysis, only variants $\geq 1\%$ were included. Total read counts and wild-type (WT) read counts were calculated for all samples. The WT counts were subtracted from the total read counts to find the total number of reads containing mutations. The numbers of different mutation-containing reads were subsequently used for calculating the integration rates.

Statistics

The D'Agostino & Pearson Normality test was used to assess Gaussian distribution of the data. Since some groups did not follow a normal distribution, non-parametric tests were used. For the comparison between groups, we used the non-parametric Mann–Whitney or Wilcoxon paired tests. The tests were carried out using GraphPad Prism (v. 9.5.1).

Results and discussion

CRISPR/LbCas12a knock-out of *slc45a2*

Atlantic salmon incubate at low temperatures and we therefore chose to use the LbCas12a nuclease due to AsCas12a showing less activity at 25 ℃ compared to LbCas12a in zebrafish (Moreno-Mateos et al. 2017), and no activity in rice (Hu et al. 2017). To test LbCas12a activity, we designed three different crRNAs, one targeting exon 1 and two targeting exon 2 of *slc45a2*. Two of the crRNAs (slc45a2-ex1 and slc45a2-ex2) were multiplexed, and one (slc45a2-ex2-2) was injected singly. In the group injected with a single RNP complex, no individuals exhibited an albino or mosaic pigmentation phenotype at the time of sampling, and we concluded that crRNA slc45a2-ex2-2 had no or very low efficiency. From the group injected with two RNP complexes, we sampled 19 larvae showing albino or mosaic pigmentation phenotypes (Fig. 1A; Supplementary File 2, Fig. S1). A non-injected control was included. Experimental data regarding the number of individuals injected, sampled, and exhibiting albino or mosaic pigmentation phenotypes can be found in Supplementary File 1.

HTS was used to assess the mutation rates in the individual samples and revealed varying efficiency of the two crRNAs (Fig. 1B; Supplementary File 3, Table S1). crRNA slc45a2-ex1 generated an average mutation rate of 77.3% (48.3–99.1%) whereas slc45a2-ex2 resulted in an average mutation rate of 16.8% (2.0–48.1%). In comparison, previous experiments using Cas9 mRNA and sgRNA produced similar mutation rates to the crRNA slc45a2-ex1 (Straume et al. 2020, 2021). However, editing efficiencies vary greatly between Cas9-experiments, also when using the same sgRNA. Various factors such as the Cas9 mRNA and sgRNA quality can affect the outcome. Furthermore, the egg quality, including survivability, and microinjection procedure may contribute to creating variation between experiments. The microinjection procedure involves aiming directly at the developing cell, which can be difficult due to the opaque nature of the eggs. The injection volume will also

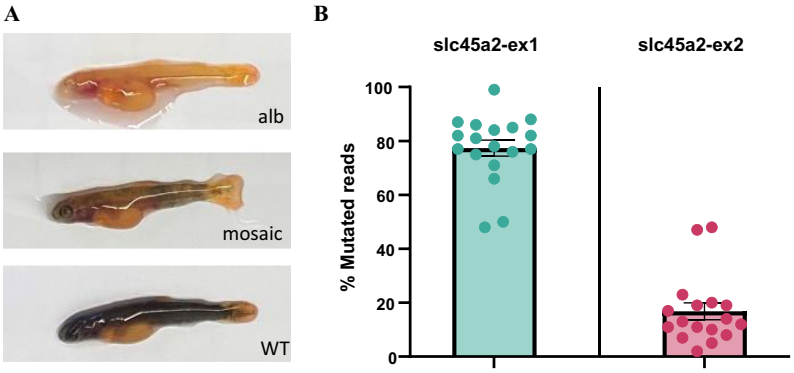

Fig. 1 *slc45a2* knock-out using LbCas12a nuclease. **A** Albino (alb), mosaic, and wild-type (WT) phenotypes in salmon larvae injected with a LbCas12a RNP complex targeting the pigmentation gene *slc45a2*. **B** The degree of mutation in larvae was assessed using amplicon sequencing (MiSeq). The percentage (%) of reads supporting mutations is reported for crRNAs slc45a2-ex1 (n = 18) and slc45a2-ex2 (n = 17). Error bars indicate SEM

differ between individual embryos due to the opening of the needle. Taken together, our results show that LbCas12a is applicable as a tool for gene KO in Atlantic salmon, although with varying efficiency depending on the crRNA sequence, both within the same gene and even the same exon. Therefore, designing several crRNAs is recommended to ensure a high mutation rate and KO effect. This is especially important for salmon, where crossing out to F1 generation is impractical due to the long generation time.

CRISPR/LbCas12a-mediated HDR knock-in of FLAG

We used the slc45a2-ex1 crRNA in combination with a target or non-target strand FLAG ODN template (Fig. 2A) to investigate the possibilities of HDR-mediated KI using the LbCas12a nuclease. We sampled 27 individuals from the group injected with the target strand template and 20 individuals from the group injected with the non-target strand template (Supplementary File 2, Fig. S2 and S3).

The rate of perfect HDR occurring in individual larvae was assessed using amplicon sequencing data (Fig. 2B; Supplementary File 3, Table S2). Perfect HDR was defined as the perfect integration of the FLAG sequence, without indels in the insert itself, nor up- or downstream of the insert. When calculating the perfect HDR rate in each larva, we removed WT reads from the total reads obtained, ending up with the mutated reads. This allowed us to look at the amount of HDR events out of the total CRISPR events and removed potential variation between the two groups due to crRNA efficiency. Individual differences were observed; 9 out of 27 individuals in the group injected with the target strand template (T), and 14 out of 20 individuals in the non-target strand template (NT) group had sequence reads showing perfect HDR. Within these fish, the average percentage of perfect HDR was 9.4% (SEM 4.4%) for the T group and 9.7% (SEM 4.0%) for the NT group. Three fish from the T group and four fish from the NT group had perfect HDR above 10%: 17.4, 26.6, and 34.3% for T, and 12.7, 14.7, 27.8, and 54.9% for NT. Taken together, we achieved perfect HDR efficiencies similar to what we have previously seen with Cas9 mRNA in Atlantic salmon (Straume et al. 2021). We achieved high rates of perfect HDR using both target and non-target strand designs of the FLAG template. This contrasts with Moreno-Mateos et al. (2017) who observed almost no HDR using a target strand-oriented template, although with a small number of samples (Moreno-Mateos et al. 2017). On the other hand, and in agreement with Moreno-Mateos et al. (2017), our NT group gave significantly more perfect HDR than the T group.

For some sequence reads, the FLAG element had been correctly inserted but with indels up- or downstream of the insert (Fig. 2B; Supplementary File 3, Table S2). This occurred in 6 out of 27, and 11 out

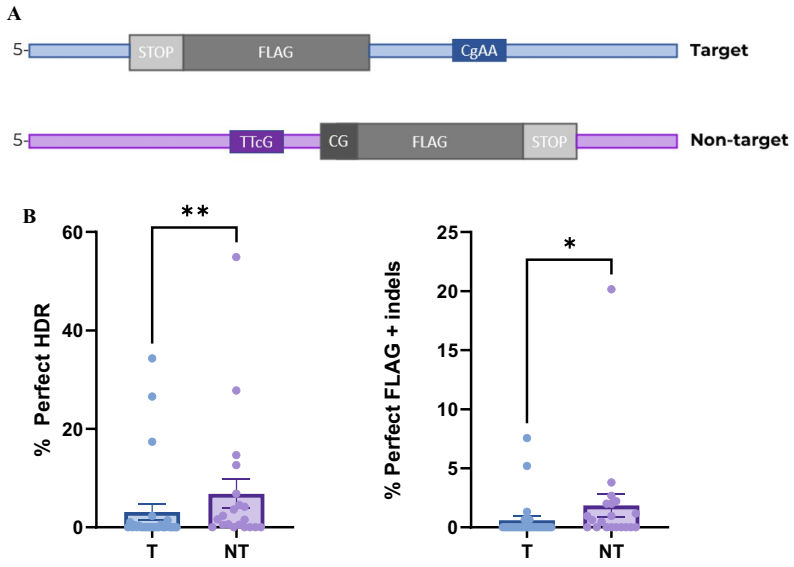

Fig. 2 *slc45a2* FLAG knock-in using LbCas12a nuclease**. A** Asymmetrical ODNs containing 90/36 nt from each side of the LbCas12a cut sites, with a 27–29 nt insert comprised of the FLAG sequence followed by a stop codon (TAA). Additional nucleotides (CG) were added to keep the open reading frame for FLAG in the non-target strand ODN. PAM sites were mutated to avoid repeated cutting. **B** Salmon embryos injected with a LbCas12a RNP complex and either target (T, n = 27) or non-target (NT, n = 20) strand ODN template design. Integration rates were assessed by amplicon sequencing (MiSeq). Perfect HDR: sequence reads with perfect match to the template sequence. Perfect FLAG + indels: reads showing integration of FLAG but with indels on either side of the insert. Error bars indicate SEM. *indicate $P < 0.05$, **indicate $P < 0.01$

of 20 individuals in the T and NT group, respectively. The average percentage of reads displaying perfect FLAG + indels was 2.7% (SEM 1.2%) in the T group, and 3.4% (SEM 1.7%) in the NT group. We have previously demonstrated that template polarity determines the location of indels when doing KI with Cas9 and symmetrical ODNs in salmon (Straume et al. 2020). Later, we found that the indel location was determined by template polarity also when using asymmetrical ODNs for the *dnd* gene, but not for *slc45a2* (Straume et al. 2021). In the current study, we had few samples displaying perfect FLAG + indels, but observed a trend in ODN polarity-driven indel positioning also for LbCas12a (Supplementary File 3, Table S2).

Furthermore, we observed sequence reads where HDR had occurred with several errors, such as partially inserted FLAG or FLAG containing substitutions and insertions. Imperfect HDR reads were found in 19 out of 27 individuals in the T group, and in 15 out of 20 individuals in the NT group. The average of imperfect HDR reads in these fish were 2.9% (SEM 0.9%) and 6.0% (SEM 1.5%) in the T and NT group, respectively (Supplementary File 3, Table S2).

Combining LbCas12a and Cas9

The possibility of using Cas12a and Cas9 at the same time could be practical both for KO and KI approaches. We microinjected salmon embryos with LbCas12a/slc45a2-ex1 RNP and non-target strand FLAG template in combination with Cas9/slc45a2-ex6 RNP and target strand FLAG template. The FLAG sequences were in the same orientation to prevent hybridization between the two different ODNs. A total of 29 individuals were sampled (Supplementary File 2, Fig. S4).

In addition to testing LbCas12a RNP for the first time in Atlantic salmon, this is also the first time we report using Cas9 RNP. Because the two RNPs were combined, we assume equivalent amounts of the RNPs were injected in each embryo, removing

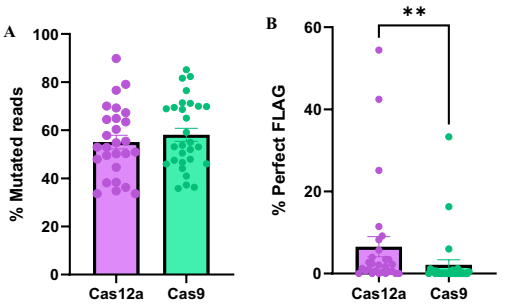

Fig. 3 slc45a2 knock-out (**A**) and FLAG knock-in (**B**) using LbCas12a and Cas9 nucleases. Salmon embryos were co-injected with LbCas12a and Cas9 RNPstargeting exons 1 and 6 of the pigment gene *slc45a2*, respectively, and FLAG templates. **A** The mutation rates were assessed by sequencing of the target sites. The percentage (%) of reads supporting mutations is reported for LbCas12a (n = 27) and Cas9 (n = 29) nucleases. **B** The rates of perfect FLAG integration (%) calculated by dividing the perfect FLAG reads by the total number of mutated reads. Error bars indicate SEM. **indicate $P < 0.01$

uncertainty regarding the delivery. When comparing LbCas12a and Cas9, we saw that the two nucleases produced similar mutation rates for the crRNA and sgRNA tested in this study, with an average of 55.1% for LbCas12a/slc45a2-ex1, and 58.1% for Cas9/slc45a2-ex6 (Fig. 3A; Supplementary File 3, Table S3). Resembling mutation efficiencies agrees with what has been reported previously in zebrafish (Meshalkina et al. 2020). Furthermore, the number of indel variants generated by the nucleases was also found to be similar (Supplementary File 2, Fig. S5). GE is commonly used to generate KO animals in order to study gene function. Here, achieving a large deletion is preferred as it increases the chance of loss-of-function even if the indels lead to in-frame mutations. Previous studies in rice and zebrafish have reported that LbCas12a generates larger deletions compared to Cas9 (Hu et al. 2017; Meshalkina et al. 2020). As the target sites for our crRNA and sgRNA are located in different exons of *slc45a2*, we cannot compare the two nucleases directly. However, in our data we observed that an average of 63.4% of the LbCas12a/slc45a2-ex1-generated deletions were 10 bp or more, whereas an average of 29.4% of the Cas9/slc45a2-ex6-induced deletions were 10 bp or more (Supplementary File 3, Table S4). Nevertheless, as we have only acquired deletion size data from one target site for LbCas12a and one for Cas9, we cannot conclude whether this outcome is attributed to the editing features of LbCas12a, or other factors such as the specific target sites or initiation of the microhomology-mediated end joining repair pathway. It is also worth noting that since the RNPs were multiplexed, they might influence each other. For example, we observed complete removal of the region between the cut sites of the two nucleases. To examine the extent of this large deletion, we performed PCR using primers targeting upstream of the LbCas12a cut site and downstream of the Cas9 cut site. In 8 out of 28 samples, we observed a gel band indicating that the entire region of 30 kb was removed for an unknown proportion of the CRISPR events (Supplementary File 2, Fig. S6). While certain GE approaches support the generation of large deletions, the target sites should be carefully considered when multiplexing crRNAs and sgRNAs.

As for the HDR events in the co-injected individuals, the sequencing revealed that perfect integration of FLAG (regardless of indels up- or downstream of the insert) occurred significantly more often at the LbCas12a cut site compared to the Cas9 cut site (Fig. 3B; Supplementary File 3, Table S3). At the LbCas12a cut site, 22 out of 28 samples had perfect FLAG integration. Within these samples, the average was 8.3% (SEM 3.0%) and the highest individual displayed 54.4% perfect FLAG. In contrast, we observed perfect FLAG integration in 11 out of 29 samples at the Cas9 cut site, with an average of 5.6% (SEM 3.1%) and the highest individual having 33.3% perfect FLAG reads. Higher HDR efficiency using LbCas12a compared to Cas9 has previously been demonstrated in zebrafish, where LbCas12a in combination with the optimal DNA donor was found to improve HDR in two of four loci tested when compared to SpCas9 (Moreno-Mateos et al. 2017). However, our data includes FLAG integration only at a single locus using crRNA and sgRNA targeting different exons. Further studies are necessary to corroborate whether LbCas12a exhibits improved HDR efficiency compared to Cas9 in Atlantic salmon.

Surprisingly, exon 1 fragments were found at the cut site of Cas9 in exon 6, and vice versa, exon 6 fragments at the cut site of LbCas12a in exon 1. Exon 1 fragments, likely originating from the homology arms of the LbCas12a-associated template, had been inserted into the Cas9 cut site to a greater extent than the other way around. In 20 out of 29 fish, an average

of 4.7% (SEM 1.5%) of the reads showed that exon 1 fragments had been inserted into the Cas9 cut site. On the other hand, in 3 out of 27 fish, an average of 2.3% (SEM 1.0%) of the reads showed exon 6 fragments at the LbCas12a cut site (Supplementary File 3, Table S5).

Conclusion

In the present work, we successfully applied the LbCas12a nuclease to KO the *slc45a2* gene in Atlantic salmon. Application of this nuclease brings important advantages such as additional target opportunities, especially in AT-rich regions of the genome, owing to the 5′-TTTV-3′ PAM requirement. Furthermore, the deletions created by LbCas12a were larger than the Cas9-mediated deletions for the crRNA and sgRNA tested in this study, which is likely to be an advantage for gene KO studies. We also performed KI of a FLAG sequence element by using ODN templates together with the LbCas12a RNP, achieving perfect HDR rates of up to 54.8% in individual larva. Finally, when comparing larvae injected simultaneously with both LbCas12a and Cas9 RNPs, we observed similar mutation rates, but more FLAG integration at the LbCas12a cut site, suggesting improved KI using the LbCas12a nuclease. However, as we only targeted a single gene using one crRNA and one sgRNA, further studies are necessary to generate more data on the KI possibilities of LbCas12a in salmon. Establishing the use of CRISPR/LbCas12a expands the toolbox for GE in salmon and may also inspire the use of this nuclease in other non-model species.

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Author contributions RBE and MR designed the study. MR designed the crRNAs and ODNs for LbCas12a GE. AHS designed the ODN and prepared the gRNA for Cas9 KI. MR, RBE, MB, and SB performed the microinjections. MR, SB, and RBE collected the tissue samples. MR purified the DNA and prepared the Illumina MiSeq libraries. EKS performed the bioinformatic analysis of the sequence data. MR and RBE analyzed the results and wrote the paper. All authors have read and approved the final manuscript.

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Data availability All data used for calculating the different rates can be found in Supplementary File 3.

Declarations

Competing interests The authors declare no competing interests.

Conflict of interest The authors declare no conflict of interest.

Ethical approval All animal experiments within the study were conducted in accordance with the Norwegian Animal Welfare Act.

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Supplementary information

Supplementary File 1: crRNA/sgRNA-, ODN- and primer sequences, and experimental data

Supplementary File 2: Supplementary Figures

Supplementary Fig. S1: Salmon larvae injected with LbCas12a RNP targeting *slc45a2*

Supplementary Fig. S2: Salmon larvae injected with LbCas12a RNP targeting *slc45a2* and target strand ODN template

Supplementary Fig. S3: Salmon larvae injected with LbCas12a RNP targeting *slc45a2* and non-target strand ODN template

Supplementary Fig. S4: Salmon larvae injected with LbCas12a and Cas9 RNPs targeting *slc45a2* and corresponding templates

Supplementary Fig. S5: Indel variants in larvae injected with LbCas12a and Cas9 RNPs targeting *slc45a2* and corresponding templates

Supplementary File 3: MiSeq data used for calculating the different rates

Table S1: Cas12a KO

Table S2: Cas12a KI (target and non-target)

Table S3: Cas12a vs. Cas9

Table S4: Deletion size

Table S5: Homology arms

Guide name	Guide type	Target site	Nuclease	Sequence (5'-3')
slc45a2-ex1	crRNA	exon 1, slc45a2	Cas12a	GAAGGGAATTTTGCTATGCG
slc45a2-ex2	crRNA	exon 2, slc45a2	Cas12a	CAGCAGACTTCATCGATGGG
slc45a2-ex2-2	crRNA	exon 2, slc45a2	Cas12a	ACGTCTGCTCGTACACAGAC
sgRNA-ex6	sgRNA	exon 6, slc45a2	Cas9	GTCTTATCGGCCTGTTCCCC

ODN template	Reference	Sequence (5'-3')
Cas12a_alb_ex1_targetODN		GACACTAAGAAGCACTGGCGTGACGAAGGCTGCCTCTTACTTGTCGTCATCGTCTTTGTAGTCCACCGCATAGCAAAATTCCCTTCCGAACATCACCA TGCCATGCAGCAGCAACCTCCCCCGAGACCGCCTGGGAAGCTCCACCACCCCAAA
Cas12a_alb_ex1_non-targetODN		TTGTGTTTGGGGTGGTGGAGCTTCCCAGGCGGTCTCGGGGGAGGTTGCTGCTGCATGGCATGGTGATGTTCGGAAGGGAATTTTGCTATGCGGACT ACAAAGACGATGACGACAAGTAACGGTGGAGGCAGCCTTCGTCACGCCAGTGCTTCTTA
Cas9_alb_ex6_targetODN	Straume et al. 2021	CTGCAGATGTCCAGAGGCTGCTGCTGCCTTACATCGGTCTGAAGGGGCTCTACTTCGTAGGATACTTTGTGTTTGGTCTGGGCACAAGTCTTGACTAC AAAGACGATGACGACAAGTAATTATCGGCCTGTTCCCCAACATTATCACCACCCTCA

Primer name	Target site	Nuclease	Sequence (5'-3')
Cas12_alb_ex1_F_miseq	exon 1, slc45a2	Cas12a	tctttccctacacgacgctcttccgatctGCTATGTCCCTCCTCACAGG
Cas12_alb_ex1_R_miseq	exon 1, slc45a2	Cas12a	tggagttcagacgtgtgctcttccgatctGCTGATCAGCCACACAAGGC
Cas12_alb_ex2_F_miseq	exon 2, slc45a2	Cas12a	tctttccctacacgacgctcttccgatctCGGACAGACTGAATCTGTAACTTG
Cas12_alb_ex2_R_miseq	exon 2, slc45a2	Cas12a	tggagttcagacgtgtgctcttccgatctCCACAAGCATAACAGTAAGAGCC
Alb2_ex6_F_miseq	exon 6, slc45a2	Cas9	tctttccctacacgacgctcttccgatctCAGATGTCCAGAGGCTGCTGCT
Alb2_ex6_R_miseq	exon 6, slc45a2	Cas9	tggagttcagacgtgtgctcttccgatctTGCCACAGCCTCAGAATGTACA
			lowercase letters indicate miseq adapters

Injection group	No. embryos injected	No. survivals	No. alb	No. mosaic
Cas12a/slc45a2-ex1*	395	158	2	18
Cas12a/slc45a2-ex2*	395	158	2	18
Cas12a slc45a2-ex2-2	347	N/A	0	0
Cas12a/slc45a2-ex1 + T strand template	568	393	4	23
Cas12a/slc45a2-ex1 + NT strand template	754	490	1	19
Cas12a/slc45a2-ex1 + NT strand template*, Cas9/slc45a2-ex6 + T strand template*	673	388	18	11

*multiplexed

In vivo CRISPR/LbCas12a-mediated knock-in and knock-out in Atlantic salmon (*Salmo salar* L.)

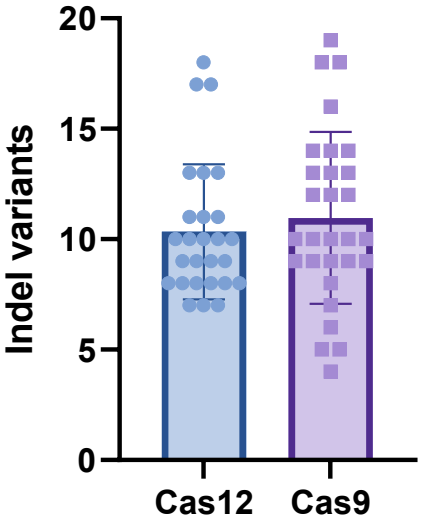
Mari Raudstein[1], Erik Kjærner-Semb[1], Morten Barvik[1], Silje Broll[1], Anne Hege Straume[1], Rolf B. Edvardsen[1]

Supplementary Fig. S1 Sampling of salmon larvae injected with LbCas12a RNP targeting exon 1 and exon 2 of *slc45a2*. A total of 19 larvae showing albino or mosaic pigmentation phenotype were sampled (one additional larvae was sampled after the photo was taken). One pigmented (labelled WT) larvae for comparison

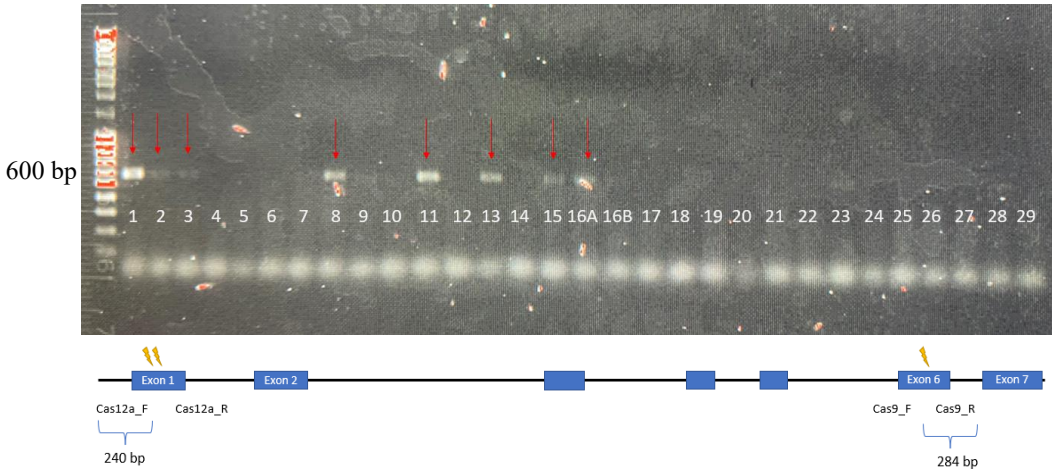
Supplementary Fig. S2 Sampling of salmon larvae injected with LbCas12a RNP targeting exon 1 of *slc45a2* in combination with target strand ODN template. A total of 27 individuals showing albino or mosaic pigmentation phenotypes were sampled. One wild-type (labelled WT) larvae for comparison

Supplementary Fig. S3 Sampling of larvae injected with LbCas12a RNP targeting exon 1 of slc45a2 in combination with non-target template. A total of 20 individuals showing albino or mosaic pigmentation phenotypes were sampled. One wild-type (labelled WT) larvae for comparison

Supplementary Fig. S4 Sampling of larvae injected with LbCas12a RNP targeting exon 1 of slc45a2 and non-target template in combination with Cas9 RNP targeting exon 6 of slc45a2 and target template. A total of 29 individuals showing albino or mosaic pigmentation phenotypes were sampled. One wild-type (labelled WT) larvae for comparison



Supplementary Fig. S5 Number of indel variants in each individual larvae injected with LbCas12a RNP and non-target strand template, in combination with Cas9 RNP and target strand template



Supplementary Fig. S6 Knock-out (KO) of a 30 kb region in *slc45a2* using LbCas12a and Cas9. Salmon embryos were injected with two RNPs: LbCas12a and Cas9 targeting exon 1 and exon 6 of slc45a2, respectively. Gel electrophoresis of fragments amplified using Cas12a F primer and Cas9 R primer was performed to determine excision of the whole region between the cut sites of the two nucleases. The red arrows indicate samples where the whole region has been KO. The 240 and 284 bp labels illustrate the number of bases from the primer binding to the cut site, meaning that the resulting band should be 524 bp. Both primers have adapters of 30 bp length, so the total band size is 584 bp

SampleID	crRNA target exon	WT reads	Mutated reads	Total reads	%WT reads	%mutated reads
4201 (WT pigmentation)	exon 1	35820	9072	44887	79.80	20.21
4202	exon 1			1303		
4203	exon 1	642	68408	69050	0.93	99.07
4204	exon 1	11253	49764	61023	18.44	81.55
4205	exon 1	7086	39261	46347	15.29	84.71
4206	exon 1	10356	53892	64241	16.12	83.89
4207	exon 1	7859	48354	56213	13.98	86.02
4208	exon 1	11892	39060	50952	23.34	76.66
4209	exon 1	6970	46075	53045	13.14	86.86
4210	exon 1	3944	30056	34000	11.60	88.40
4211	exon 1	10969	35825	46794	23.44	76.56
4212	exon 1	10848	47915	58763	18.46	81.54
4213	exon 1	10028	42724	52752	19.01	80.99
4214	exon 1	12924	40479	53403	24.20	75.80
4215	exon 1	11626	42296	53922	21.56	78.44
4216	exon 1	16541	41054	57595	28.72	71.28
4217	exon 1	27007	25221	52228	51.71	48.29
4218	exon 1	10920	32829	43749	24.96	75.04
4219	exon 1	13893	26932	40825	34.03	65.97
4220	exon 1	21879	22086	43969	49.76	50.23
Non-injected control	exon 1	45593	130	45723	99.72	0.28
4201 (WT)	exon 2	28202	256	28458	99.10	0.90
4202	exon 2			NA		
4203	exon 2	27269	8311	35580	76.64	23.36
4204	exon 2	13357	12394	25751	51.87	48.13
4205	exon 2	25902	3084	28986	89.36	10.64
4206	exon 2	22785	5544	28329	80.43	19.57
4207	exon 2	21755	5096	26851	81.02	18.98
4208	exon 2	31257	2711	33968	92.02	7.98
4209	exon 2	21532	3381	24913	86.43	13.57
4210	exon 2			NA		
4211	exon 2	42832	852	43684	98.05	1.95

4212	exon 2	23867	5007	28874	82.66	17.34
4213	exon 2	18910	4450	23360	80.95	19.05
4214	exon 2	24439	2697	27136	90.06	9.94
4215	exon 2	31920	1543	33463	95.39	4.61
4216	exon 2	28516	2176	30695	92.90	7.09
4217	exon 2	17969	16041	34013	52.83	47.16
4218	exon 2	28144	3820	31964	88.05	11.95
4219	exon 2	35996	4627	40623	88.61	11.39
4220	exon 2	23617	3680	27297	86.52	13.48
Non-injected control	exon 2	32193	0	32193	100.00	0.00

SampleID	perfect_HDR reads > 100	FLAG_Indels reads > 100	Imperfect_HDR reads > 100	Total reads > 100	WT reads > 100	%mutated reads	%Perfect_HDR	%FLAG_Indels	%Imperfect_HDR
7201	0	0	187	39053	16758	57.09	0.00	0.00	0.84
7202	0	0	1747	35276	7683	78.22	0.00	0.00	6.33
7203	0	0	0	36900	5925	83.94	0.00	0.00	0.00
7204	0	1905	1184	35275	10060	71.48	0.00	7.56	4.70
7205	490	164	751	24440	3313	86.44	2.32	0.78	3.55
7206	0	0	0	39944	5151	87.10	0.00	0.00	0.00
7207	162	0	147	46895	4411	90.59	0.38	0.00	0.35
7208	0	143	128	25140	2859	88.63	0.00	0.64	0.57
7209	0	0	0	35314	5826	83.50	0.00	0.00	0.00
7210	0	0	697	30497	4157	86.37	0.00	0.00	2.65
7211	0	0	219	33726	6453	80.87	0.00	0.00	0.80
7212	0	0	154	37587	15297	59.30	0.00	0.00	0.69
7213	0	0	0	42859	19645	54.16	0.00	0.00	0.00
7214	0	0	2851	30807	9202	70.13	0.00	0.00	13.20
7215	174	0	408	51091	10624	79.21	0.43	0.00	1.01
7216	0	0	196	37854	11808	68.81	0.00	0.00	0.75
7217	0	0	254	45451	11035	75.72	0.00	0.00	0.74
7218	0	395	3788	39525	9444	76.11	0.00	1.31	12.59
7219	3736	0	0	38036	16542	56.51	17.38	0.00	0.00
7220	6113	1196	0	36428	13428	63.14	26.58	5.20	0.00
7221	147	172	289	36730	9832	73.23	0.55	0.64	1.07
7222	0	0	0	41188	14623	64.50	0.00	0.00	0.00
7223	256	0	101	48005	22856	52.39	1.02	0.00	0.40
7224	0	0	159	40240	13083	67.49	0.00	0.00	0.59
7225	318	0	207	36027	13391	62.83	1.40	0.00	0.91
7226	0	0	756	44967	19661	56.28	0.00	0.00	2.99
7227	6176	0	0	28504	10501	63.16	34.31	0.00	0.00
TAR_AVG_ALL							3.12	0.60	2.03
TAR_AVG_POS							9.37	2.69	2.88

7301	1025	494	1149	37839	13135	65.29	4.15	2.00	4.65
7302	10395	0	182	41948	4554	89.14	27.80	0.00	0.49
7303	199	194	605	50166	8043	83.97	0.47	0.46	1.44
7304	2410	0	336	23824	7414	68.88	14.69	0.00	2.05
7305	111	209	1105	47436	25996	45.20	0.52	0.97	5.15
7306	798	214	298	44270	22193	49.87	3.61	0.97	1.35
7307	0	135	894	36558	15204	58.41	0.00	0.63	4.19
7308	0	0	3464	39227	18294	53.36	0.00	0.00	16.55
7309	0	0	3315	32775	10437	68.16	0.00	0.00	14.84
7310	0	0	0	30229	11603	61.62	0.00	0.00	0.00
7311	12148	593	333	33883	11747	65.33	54.88	2.68	1.50
7312	255	2164	0	28206	17468	38.07	2.37	20.15	0.00
7313	162	0	1419	49691	39620	20.27	1.61	0.00	14.09
7314	0	0	834	41784	14586	65.09	0.00	0.00	3.07
7315	0	581	703	39528	24223	38.72	0.00	3.80	4.59
7316	1688	295	373	40729	27394	32.74	12.66	2.21	2.80
7317	1196	347	2287	36296	18790	48.23	6.83	1.98	13.06
7318	299	230	0	49561	30029	39.41	1.53	1.18	0.00
7319	639	0	0	36328	22267	38.71	4.54	0.00	0.00
7320	208	0	0	56681	23763	58.08	0.63	0.00	0.00
NONT_AVG_ALL							6.81	1.85	4.49
\ONT_AVG_POS							9.74	3.37	5.99

SampleID	Perfect 5' reads	Perfect 3' reads	%Perfect 5' reads	% Perfect 3' reads
7201	0.00	0.00	0.00	0.00
7202	0.00	0.00	0.00	0.00
7203	0	0	0.00	0.00
7204	1905	1905	7.56	7.56
7205	164	0	0.78	0.00
7206	0	0	0.00	0.00
7207	0	0	0.00	0.00
7208	143	0	0.64	0.00
7209	0	0	0.00	0.00
7210	0	0	0.00	0.00
7211	0	0	0.00	0.00
7212	0	0	0.00	0.00
7213	0	0	0.00	0.00
7214	0	0	0.00	0.00
7215	0	0	0.00	0.00
7216	0	0	0.00	0.00
7217	0	0	0.00	0.00
7218	395	0	1.31	0.00
7219	0	0	0.00	0.00
7220	1196	0	5.20	0.00
7221	172	172	0.64	0.64
7222	0	0	0.00	0.00
7223	0	0	0.00	0.00
7224	0	0	0.00	0.00
7225	0	0	0.00	0.00
7226	0	0	0.00	0.00
7227	0	0	0.00	0.00
TAR_AVG_ALL				
TAR_AVG_POS				

7301	0	494	0.00	2.00
7302	0	0	0.00	0.00
7303	194	0	0.46	0.00
7304	0	0	0.00	0.00
7305	209	209	0.97	0.97
7306	0	214	0.00	0.97
7307	0	135	0.00	0.63
7308	0	0	0.00	0.00
7309	0	0	0.00	0.00
7310	0	0	0.00	0.00
7311	593	0	2.68	0.00
7312	489	1675	4.55	15.60
7313	0	0	0.00	0.00
7314	0	0	0.00	0.00
7315	463	118	3.03	0.77
7316	0	295	0.00	2.21
7317	347	0	1.98	0.00
7318	0	230	0.00	1.18
7319	0	0	0.00	0.00
7320	0	0	0.00	0.00
NONT_AVG_ALL				
NONT_AVG_POS				

SampleID	Cas12a_Perfect_FLAG	Cas12a total reads > 100	Cas12a WT reads > 100	Cas9_Perfect_FLAG	Cas9 total reads > 100	Cas9 WT reads > 100	%Cas12a_mut	%Cas9_mut	%Cas12_FLAG	%Cas9_FLAG
7101	0	42416	4319	0	19928	2966	89.82	85.12	0.00	0.00
7102	998	38669	13564	0	21125	6626	64.92	68.63	3.98	0.00
7103	106	39319	11781	0	22853	4039	70.04	82.33	0.38	0.00
7104	0	39451	12913	185	24720	4539	67.27	81.64	0.00	0.92
7105	656	33992	15157	0	37029	11165	55.41	69.85	3.48	0.00
7106	675	28956	8889	321	33363	7857	69.30	76.45	3.36	1.26
7107	589	44353	16187	0	31351	14734	63.50	53.00	2.09	0.00
7108	1563	31165	14091	0	31991	9944	54.79	68.92	9.15	0.00
7109	0	37749	7888	224	29840	10443	79.10	65.00	0.00	1.15
7110	129	34578	8092	6728	28883	8680	76.60	69.95	0.49	33.30
7111	0	446	337	0	20855	9651		53.72		0.00
7112	8723	24870	8844	0	34330	20263	64.44	40.98	54.43	0.00
7113	0	45146	25013	0	37866	17755	44.60	53.11	0.00	0.00
7114	10748	41983	16649	0	32772	13397	60.34	59.12	42.43	0.00
7115	0	0	0	0	27553	7891		71.36		0.00
7116A	5047	37927	17831	151	34908	10070	52.99	71.15	25.11	0.61
7116B	210	40885	20333	0	34303	18524	50.27	46.00	1.02	0.00
7117	270	36334	24114	102	32151	9803	33.63	69.51	2.21	0.46
7118	114	44727	27568	0	25613	13821	38.36	46.04	0.66	0.00
7119	1010	35419	17638	1000	37797	21151	50.20	44.04	5.68	6.01
7120	277	43723	27877	122	37273	19406	36.24	47.94	1.75	0.68
7121	423	36495	19008	0	31970	17068	47.92	46.61	2.42	0.00
7122	125	44825	21103	0	29892	15690	52.92	47.51	0.53	0.00
7123	233	35960	17641	1881	31079	19515	50.94	37.21	1.27	16.27
7124	1074	38869	25804	0	27216	13072	33.61	51.97	8.22	0.00
7125	104	42996	26612	0	45807	29411	38.11	35.79	0.63	0.00
7126	0	44793	22609	0	36472	23217	49.53	36.34	0.00	0.00
7127	1706	42840	27937	107	49450	24479	34.79	50.50	11.45	0.43
7128	674	42770	18032	107	30806	13869	57.84	54.98	2.72	0.63
Non-injected control	0	45723	45593	0	31707	31570	0.28	0.43	0.00	0.00
AVG							55.09	58.10	6.55	2.06
AVG_FLAG_positive_only							NA	NA	8.34	5.61

SampleID	Cas12a del size < 10 bp	Cas12a del size >= 10 bp	%deletions >= 10 bp	Cas9 del size < 10 bp	Cas9 del size >= 10 bp	%deletions >= 10 bp
7101	5164	36013	87.46	4912	3275	40.00
7102	14757	6895	31.84	7193	3327	31.63
7103	4153	19752	82.63	7255	4806	39.85
7104	7651	17765	69.90	5394	1171	17.84
7105	782	16920	95.58	10719	8860	45.25
7106	3783	13249	77.79	13742	861	5.90
7107	6799	21198	75.72	6067	3823	38.66
7108	3773	6812	64.36	11745	2607	18.16
7109	8877	16477	64.99	8112	4745	36.91
7110	0	11976	100.00	1493	1790	54.52
7111				6656	1297	16.31
7112	5710	1402	19.71	4270	1186	21.74
7113	1761	14685	89.29	6158	3198	34.18
7114	2653	6773	71.85	4586	7155	60.94
7115				12808	532	3.99
7116A	2967	6411	68.36	6176	8894	59.02
7116B	11645	8055	40.89	5586	3052	35.33
7117	3465	3397	49.50	6752	9630	58.78
7118	7242	3204	30.67	5518	1865	25.26
7119	4440	5973	57.36	3588	0	0.00
7120	0	11066	100.00	4796	7657	61.49
7121	4038	6741	62.54	4573	789	14.71
7122	9673	11436	54.18	6363	2587	28.91
7123	4729	12253	72.15	4126	1693	29.09
7124	2936	5385	64.72	7490	1110	12.91
7125	10579	4231	28.57	6866	682	9.04
7126	13861	8023	36.66	6104	438	6.70
7127	3102	6136	66.42	9471	2240	19.13
7128	11126	10933	49.56	6906	2610	27.43
ɔn-injected cont	0	0		0	0	
Average			63.43			29.44

SampleID	ex6 fragments inserted in Cas12a cut site	Total reads > 100 (cas12a target site)	WT reads > 100 (cas12a target site)	ex1 fragments inserted in Cas9 cut site	Total reads > 100 (cas9 target site)	WT reads > 100 (cas9 target site)	%ex6 fragments in Cas12a cut site	%ex1 fragments in Cas9 cut site
7101	0	42416	4319	1278	19928	2966	0.00	7.53
7102	978	38669	13564	630	21125	6626	3.90	4.35
7103	0	39319	11781	387	22853	4039	0.00	2.06
7104	0	39451	12913	1882	24720	4539	0.00	9.33
7105	0	33992	15281	331	37029	11165	0.00	1.28
7106	0	28956	8889	757	33363	7857	0.00	2.97
7107	122	44353	16187	342	31351	14865	0.43	2.07
7108	447	31165	14091	0	31991	9944	2.62	0.00
7109	0	37749	7888	575	29840	10443	0.00	2.96
7110	0	34578	8092	144	28883	8680	0.00	0.71
7111	0	446	337	0	20855	9651		0.00
7112	0	24870	9195	160	34330	20450	0.00	1.15
7113	0	45146	25013	1012	37866	17918	0.00	5.07
7114	0	41983	16649	655	32772	13500	0.00	3.40
7115	0	0	0	725	27553	7891		3.69
7116A	0	37927	17831	684	34908	10070	0.00	2.75
7116B	0	40885	20333	0	34303	18707	0.00	0.00
7117	0	36334	24114	1259	32151	9803	0.00	5.63
7118	0	44727	27568	0	25613	13946	0.00	0.00
7119	0	35419	17638	5292	37797	21355	0.00	32.19
7120	0	43723	27877	499	37273	19554	0.00	2.82
7121	0	36495	19008	106	31970	17219	0.00	0.72
7122	0	44825	21103	0	29892	15847	0.00	0.00
7123	0	35960	17641	107	31079	19691	0.00	0.94
7124	0	38869	25979	0	27216	13196	0.00	0.00
7125	0	42996	26612	0	45807	29789	0.00	0.00
7126	0	44793	22609	0	36472	23425	0.00	0.00
7127	0	42840	27937	804	49450	24822	0.00	3.26
7128	0	42770	18032	0	30806	13988	0.00	0.00

PAPER II

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Highly efficient *in vivo* C-to-T base editing in Atlantic salmon (*Salmo salar*) – A step towards aquaculture precision breeding

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ABSTRACT

The salmon industry has the potential to provide nutritious food to the expanding world population. However, the industry faces several sustainability challenges related to environmental impact and fish welfare. Genome editing presents a promising approach for precision breeding, where small, targeted changes to the fish genome may facilitate the introduction of advantageous traits like disease resistance, delayed maturation, or enhanced growth. This study presents the first application of base editing in an aquaculture fish species. By co-injecting cytidine base editor AncBE4max mRNA and gRNA targeting the pigmentation gene *solute carrier family 45 member 2* in Atlantic salmon embryos, we were able to induce a specific C-to-T conversion, thereby introducing a premature stop codon and achieving an albino phenotype. High-throughput sequencing revealed an average of 50–66% correct conversion of the target base depending on the base editor mRNA concentration and up to 89% in a single individual. Our results suggest that AncBE4max provides a simple and efficient approach to making precise single nucleotide edits in the Atlantic salmon genome. Additionally, we used conventional CRISPR/Cas9 combined with oligonucleotide templates to insert point mutations at various positions up- and downstream of the Cas9 cleavage site, demonstrating that the insertion efficiency was affected by the position of the mutation. The application of genome editing tools that allow for precise changes in this species is a step towards aquaculture precision breeding and the introduction of traits promoting a more sustainable industry.

1. Introduction

The salmon aquaculture industry holds the potential to meet the dietary needs of our ever-growing global population by offering nutritious and protein-rich food. Norway is the world's largest Atlantic salmon (*Salmo salar*) producer, but the industry faces several sustainability challenges related to environmental impact and fish welfare, such as genetic introgression of escaped salmon into wild populations (Karlsson et al., 2016; Taranger et al., 2014), precocious maturation, infectious diseases, and sea lice infestations (Sommerset et al., 2023). Traditional breeding programs have been implemented in the industry to select fish with traits that are advantageous for production. Emerging breeding techniques, such as genome editing (GE) using CRISPR/Cas, present a promising approach for precision breeding – where precise, targeted changes enable the introduction of genetic traits that would be both time-consuming and difficult to achieve through conventional breeding. In other words, GE can be used to design robust aquaculture fish that are more resistant to diseases, mature at a later age, have improved growth, and a higher nutritional status. Progress has already been made using GE in aquaculture species. For instance, CRISPR/Cas9 has been used to increase fatty acid production in channel catfish (*Ictalurus punctatus*) (Xing et al., 2022), induce sterility in Atlantic salmon (Wargelius et al., 2016), and enhance disease resistance in blue catfish (*Ictalurus furcatus*) (Wang et al., 2023).

The CRISPR/Cas system consists of a pre-designed RNA molecule that guides a CRISPR-associated endonuclease to the target site of interest. The nuclease initiates a double-strand break (DSB) to the DNA, which is subsequently repaired by the cell's endogenous repair mechanisms. Most frequently, the DSB is repaired by the non-homologous end-joining (NHEJ) pathway. The NHEJ mechanism usually entails random insertions and deletions (indels), which in some cases may lead to gene knockout (KO). However, if a template is available to the cell, the homology-directed repair (HDR) pathway may occur. HDR allows the change or insertion of DNA but is frequently accompanied by the indels

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formed by the competing NHEJ pathway. More recently, base editors, comprising a modified Cas9 fused with a deaminase, have emerged as a tool for precise single nucleotide editing without inducing a DSB or requiring a template. The two major classes of base editors, cytidine and adenine base editors (CBEs and ABEs), facilitate the conversion of target C•G base pairs to T•A (Komor et al., 2016) and A•T base pairs to G•C (Gaudelli et al., 2017), respectively, enabling all possible transition mutations. Because they do not induce a DSB, the base editors are considered to reduce the frequency of undesired indels compared to regular CRISPR/Cas. Base editing has been successfully used to edit target bases in a variety of organisms, including plants (Zong et al., 2017; Kang et al., 2018), mice (Kim et al., 2017), and zebrafish (Zhang et al., 2017; Qin et al., 2018) but has to our knowledge not been established in Atlantic salmon or any other fish aquaculture species.

In the present paper, base editing was, for the first time, applied to Atlantic salmon. We used the 4th generation CBE AncBE4max (Koblan et al., 2018) to convert C to T in the pigmentation gene *solute carrier family 45 member 2* (*slc45a2*), thereby introducing a premature stop codon. We employed high-throughput sequencing (HTS) to evaluate the base editor's efficiency and detect any undesired effects. Although base editors are promising, they require the target base to fall within the editing window. This, together with the inability to perform transversion mutations, imposes some limitations. When base editing is not feasible, an alternative approach utilizing HDR for single nucleotide replacement (SNR) can be employed. Previously, we demonstrated the applicability of using oligonucleotide (ODN) templates to introduce point mutations near the Cas9 cleavage site in salmon (Straume et al., 2021). To further develop this methodology, we combined CRISPR/Cas9 with ODN templates containing point mutations at various positions upstream or downstream of the cleavage site. HTS was used to identify whether the location of the mutation affected the HDR efficiency. This paper demonstrates two distinct methods for SNR in Atlantic salmon, which we believe will serve as invaluable tools for advancing precision breeding in the aquaculture industry.

2. Methods

2.1. gRNA design and synthesis

We targeted the *slc45a2* gene due to the albino phenotype achieved when disrupted. The *slc45a2* gene sequence was obtained from the Atlantic salmon reference genome assembly v2 on the National Center for Biotechnology Information (NCBI) website ((GenBank: GCF_000233375.1, NCBI), https://www.ncbi.nlm.nih.gov/gene/106563596/:). The base editing window of AncBE4max CBE is approximately 12–18 bp upstream of the protospacer adjacent motif (PAM) (Koblan et al., 2018), and a target C needs to be within this window. We designed a gRNA targeting exon 7 of the *slc45a2* gene, ensuring the base editor requirements were met. Successful conversion of the target C to T introduces a stop codon (TAG). For SNR using ODN templates, we used a gRNA targeting exon 6 of *slc45a2* previously described in Edvardsen et al., 2014. The gRNA sequences were screened against the Atlantic salmon reference genome using BLASTN (Altschul et al., 1990) available from NCBI to limit unwanted off-target effects. The gRNA used for BE had no off-target sites, and the gRNA used for SNR had only one potential off-target site, with a gap adjacent to the PAM site (Supplementary Fig. S1). gRNA was *in vitro* transcribed (IVT) from DNA templates based on Gagnon et al., 2014 with the following exceptions: The QIAquick PCR purification kit (Qiagen) was used to purify the DNA templates, the HiScribe T7 Quick High Yield RNA synthesis kit (NEB) was used for IVT, and the RNeasy Mini Kit (Qiagen) was used to purify the synthesized gRNA. Oligos for gRNA synthesis were ordered from Eurofins Genomics (Germany). The gRNA sequences are found in Supplementary Table S1.

2.2. Base editor mRNA and Cas9 mRNA synthesis

The base editing construct pCMV_AncBE4max was a gift from David Liu (Addgene plasmid #112094; https://www.addgene.org/112094/; RRID:Addgene 112094) (Koblan et al., 2018). The AncBE4max plasmid was obtained as an *Escherichia coli* bacterial stab and isolated using the Qiagen MiniPrep kit, linearized with *Sap*I restriction enzyme (NEB), and purified using the MinElute Reaction Cleanup kit (Qiagen). AncBE4max base editor mRNA was *in vitro* transcribed using mMESSAGE mMACHINE T7 Transcription Kit (ThermoFisher) and purified using the RNeasy Mini Kit (Qiagen). Cas9 mRNA for SNR using ODNs was synthesized as previously described in Edvardsen et al., 2014.

2.3. ODN template design

The template design was based on Richardson et al., 2016 and previous work (Straume et al., 2021). Six ODNs were designed: four containing a mutation at distinct positions upstream of the Cas9 cleavage site (−12, −24, −49, or −59), one featuring a combination of these, and one containing a mutation downstream of the cleavage site (+12). The PAM site was mutated to introduce a stop codon and avoid repeated cutting. ODNs were ordered from Eurofins Genomics (Germany). The template sequences are found in Supplementary Table S2.

2.4. Microinjections

Salmon eggs and sperm were obtained from Aquagen (Steigen, Norway) and Mowi (Askøy, Norway). The eggs were fertilized in 0.5 mM reduced glutathione (Sigma-Aldrich) solution (pH 10) to prevent chorion hardening. The embryos were incubated for 3–4 h at 6–8 °C until the first cell was visible. Fertilized eggs were injected using glass capillaries (O·D 1.0 mm, I·D 0.50 mm, 10 cm) (Sutter Instrument) pulled by a PC-100 needle puller (Narishige, Japan), and a FemtoJet® 4i injector (Eppendorf). The base editing injection mix had a final concentration of either 150 ng/μL or 300 ng/μL AncBE4max mRNA and 50 ng/μL or 100 ng/μL gRNA. The injection mix for SNR using ODNs had a final concentration of 150 ng/μL Cas9 mRNA, 50 ng/μL gRNA, and 1.5 μM ODN template (+12, −12, −24, −49, −59, or combination) based on previous results (Straume et al., 2021). The injected embryos were incubated at 6 °C until sampling.

2.5. Sampling, DNA isolation and sequencing

After approximately 650 day-degrees, the larvae were euthanized with buffered MS-222: Tricaine Methane Sulfonate. Individuals exhibiting an albino or mosaic pigmentation phenotype were sampled. DNA was isolated from fin clips using the Agencourt DNAdvance Kit (Beckman Coulter). Libraries were prepared for Illumina sequencing based on Gagnon et al., 2014 with the following exceptions: Q5 High-Fidelity DNA Polymerase (NEB) was used to amplify the target site, and barcoded fragments were purified using the QiaQuick Gel Extraction kit (Qiagen). Primers used for target site amplification are listed in Supplementary Table S3. The library was sequenced on the MiSeq platform using MiSeq Kit v.3 (Illumina) with 300 bp paired-end reads.

2.6. Mutation efficiency analysis

The sequence data was processed as described in Straume et al., 2021. Reads retained after filtering were mapped to the *slc45a2* reference amplicon sequences using Muscle (v. 3.8.1551) (Edgar, 2004). The processed sequence data was subsequently analyzed using custom Python scripts and visualized with Geneious Prime software (v. 11.0.12). Sequence variants accounting for less than 0.1% of the total number of reads in a given sample were not included.

3. Results

3.1. AncBE4max base editor induces highly efficient C-to-T conversion in *slc45a2*

To explore the potential of base editing as a GE tool in Atlantic salmon, we injected salmon embryos with AncBE4max base editor mRNA (150 ng/μL or 300 ng/μL) and gRNA targeting *slc45a2*. We chose to target the *slc45a2* gene as proof of principle due to the albino phenotype achieved when disrupted. Data regarding the numbers of individuals injected, surviving until the sampling point, and that were sampled, is available in Supplementary Table S4. This type of data varies between experiments due to different gRNA efficiencies and other variables, as previously reported (Raudstein et al., 2023). We sampled 6 and 8 individuals exhibiting a complete or mosaic albino pigmentation phenotype from the 150 and 300 ng/μL groups, respectively (Supplementary Fig. S2 and S3). The albino phenotypes are clear indications of successful base editing, as the target base C-to-T conversion results in a premature stop codon (TAG) and gene KO (Fig. 1A). HTS was performed to assess the outcome of the base editing and revealed highly efficient conversion of the target base (Fig. 1B, Supplementary File 2). In the group injected with 150 ng/μL mRNA, an average of 50.8% of the sequence reads displayed the correct C-to-T conversion. Individual mutation rates within this group ranged from 17.6 to 75.2%. Similarly, in the 300 ng/μL mRNA injection group, we achieved an average conversion rate of 66.3%, with individual rates ranging between 36.0 and 88.9%. Furthermore, three of the eight sampled larvae in the latter group exhibited more than 80% correct conversion reads.

Despite the high rates of correct conversion, the HTS also revealed undesired effects such as indels, bystander edits, and incorrect conversions (Fig. 1B, Supplementary File 2). In the 150 ng/μL group, we observed an average of 6.0% indel-containing reads, whereas the 300 ng/μL group displayed an average of 8.2% indel reads. Both small and large deletions were observed. The deletions variants usually appeared near what would be the cleavage site of a conventional Cas9 nuclease, centered around the target base, or extended from near the target base to the Cas9 cleavage site (Supplementary Fig. S4). Instances of bystander edits, defined as the conversion of non-target C bases within the protospacer region, were observed at positions 3, 10, and 13 of the protospacer (the target C being in position 5) (Fig. 2). In the 150 ng/μL group, an average of 4.9% sequence reads exhibited bystander edits, distributed as 1.0, 3.1, and 0.8% for positions 3, 10, and 13, respectively. In the 300 ng/μL group, the average bystander edit rate was 4.4%, distributed as 0.9, 2.3, and 1.2% for said positions. Finally, incorrect conversions, in which the target C was converted to either A or G, occurred at low rates. C-to-A conversion occurred on average in 0.4% of the reads in the 150 ng/μL group and in 0.2% of the reads in the 300 ng/μL group, whereas C-to-G conversion occurred on average in 2.9% of the reads in the 150 ng/μL group, and 2.5% of the reads in the 300 ng/μL group.

3.2. ODN templates enable knock-in of point mutations at various positions

In cases where the desired target site is unsuitable for base editing,

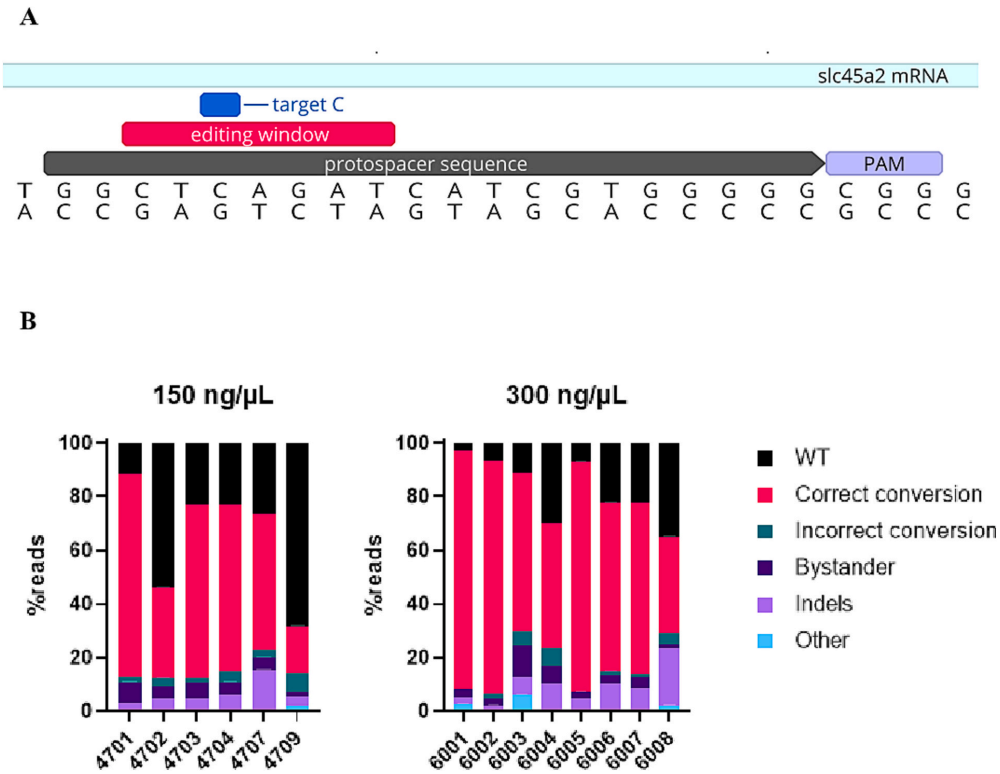

Fig. 1. Base editing in Atlantic salmon. A Target site for C-to-T base editing in *slc45a2* exon 7. The base editing window of AncBE4max is from nucleotide position 3 to 9 in the protospacer sequence. Image from Geneious. B Different base editing outcomes in individual salmon larvae injected with AncBE4max base editor mRNA and *slc45a2* gRNA, revealed by sequencing of the target site.

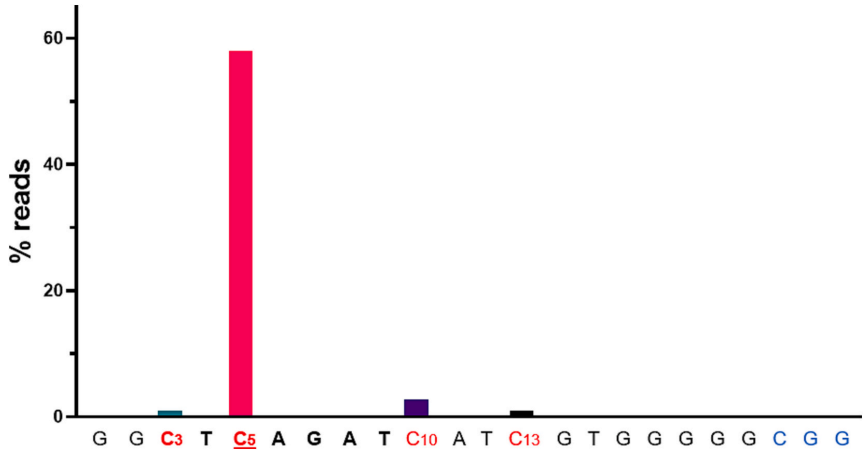

Fig. 2. Salmon embryos injected with AncBE4max base editor mRNA and gRNA targeting *slc45a2*. The average percentage of reads containing bystander edits in the two concentration groups combined. The optimal editing window for the cytidine deaminase ranges between nucleotide position 3 to 9 (indicated in bold). The PAM site is indicated in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

other approaches for SNR may be utilized. To test SNR efficiencies at different distances from the Cas9 cleavage site, we injected salmon embryos with Cas9 mRNA, gRNA targeting *slc45a2* and one ODN template featuring a single point mutation at a distinct position upstream of the cleavage site (positions −12, −24, −49, or −59, as well as one template containing all four mutations) (Fig. 3A). We also injected one group of embryos with a template containing a mutation +12 downstream of the cleavage site. Data on individuals injected, surviving, and sampled is found in Supplementary Table 5. The PAM site was mutated in all templates to prevent repeated editing and induce a stop codon. We sampled and performed HTS of 15 to 16 albino and mosaic individuals from each of the six groups to examine the HDR efficiency. Integration of the complete template sequence without any indels or mismatches outside the targeted positions was defined as "perfect HDR".

The average perfect HDR rate decreased as the mutation site moved further upstream from the cleavage site (Fig. 3B, Supplementary File 2). The downstream ODN mutation (+12) displayed the lowest perfect HDR percentage of all groups. When looking at the combination template, containing the four point mutations located upstream of the PAM, we observed the same decreasing efficiency based on the mutation position. Here, sequence reads containing one (−12), two (−12 and −24), three (−12, −24, and −49), or all four mutations simultaneously, accounted for an average of 7.9, 7.1, 3.7, and 2.6% of the total amount of reads, respectively (Supplementary Fig. S4, Supplementary File 2).

4. Discussion

To our knowledge, this study demonstrates the first application of base editing in any fish aquaculture species. By microinjecting Atlantic salmon embryos with base editor AncBE4max mRNA and gRNA targeting *slc45a2*, we precisely introduced a premature stop codon, leading to visual albino or mosaic phenotypes. Our deep sequencing data revealed up to 88.9% correct conversion of the target C to T in a single individual. The average conversion rates were 50.8 and 66.3% using different concentrations of the base editor mRNA. We normally experience slight differences in efficiency between experiments, and we cannot definitively conclude whether a higher mRNA concentration is advantageous. As we have performed HTS to determine the mutation rate in single individuals, it is difficult to compare our data directly to other studies using different approaches for analyzing their data. However, our results agree with what others have reported using AncBE4max, in that the base editor efficiently induces the correct conversion (Carrington et al., 2020; Yuan et al., 2020; Zuo et al., 2023; Koblan et al., 2018). For example, a study involving six target sites detected an average base conversion rate of 42.7% in pooled zebrafish embryos (Zhao et al., 2020).

Despite the impressive C-to-T conversion rate, various undesired effects such as indels, bystander edits, and incorrect conversions were notable in our data. Indel-containing reads accounted for an average of ~7.1% of the reads across both concentration groups, making indels the most common undesired effect. AncBE4max mRNA encodes nCas9 (Koblan et al., 2018), a modified Cas9 protein mutated in the RuvC-like cleavage domain, rendering it incapable of inducing DSBs (Jinek et al., 2012). During C-to-T base editing, the cytidine deaminase enzyme converts the target C to U, and the nCas9 protein creates a single-strand break (nick) in the opposite strand. Occasionally, the U base may be excised by uracil-DNA glycosylase following deamination, forming an apurinic or apyrimidinic (AP) site. This can lead to nicking of the deaminated strand by DNA AP lyase, which, if occurring simultaneously with nCas9 nicking the opposite strand, can result in a DSB as suggested by Rees and Liu (Rees and Liu, 2018). In such cases, the NHEJ repair mechanism may introduce indels. While this can explain some of the indel variants observed in our data, some variants resembled indels generated by the conventional Cas9, appearing near the Cas9 cleavage site.

The optimal AncBE4max editing window in which the deaminase shows the greatest efficiency ranges from nucleotide position 3 to 9 in the protospacer sequence (Koblan et al., 2018). In our experiments, three Cs were in proximity to our target base, specifically at positions 3, 10, and 13 in the protospacer. An average of ~4.7% bystander edits were detected across both concentration groups. The C10 was edited to a greater extent (approximately 2–3%) than C3 and C13 (approximately 1% each). Almost all the sequence reads displaying bystander edits also had correct conversion of the target C. Bystander edits are undesirable when the goal is to do precise edits like single amino acid substitutions. Lastly, we observed a low occurrence of sequence reads containing conversion of C to non-Ts. Taken together, our results indicate that AncBE4max offers a simple and efficient way to precisely edit targeted bases in Atlantic salmon.

Base editing is a valuable tool for precision breeding, but certain limitations remain when using this technology. For example, the target base needs to fall within the editing window, while non-target bases

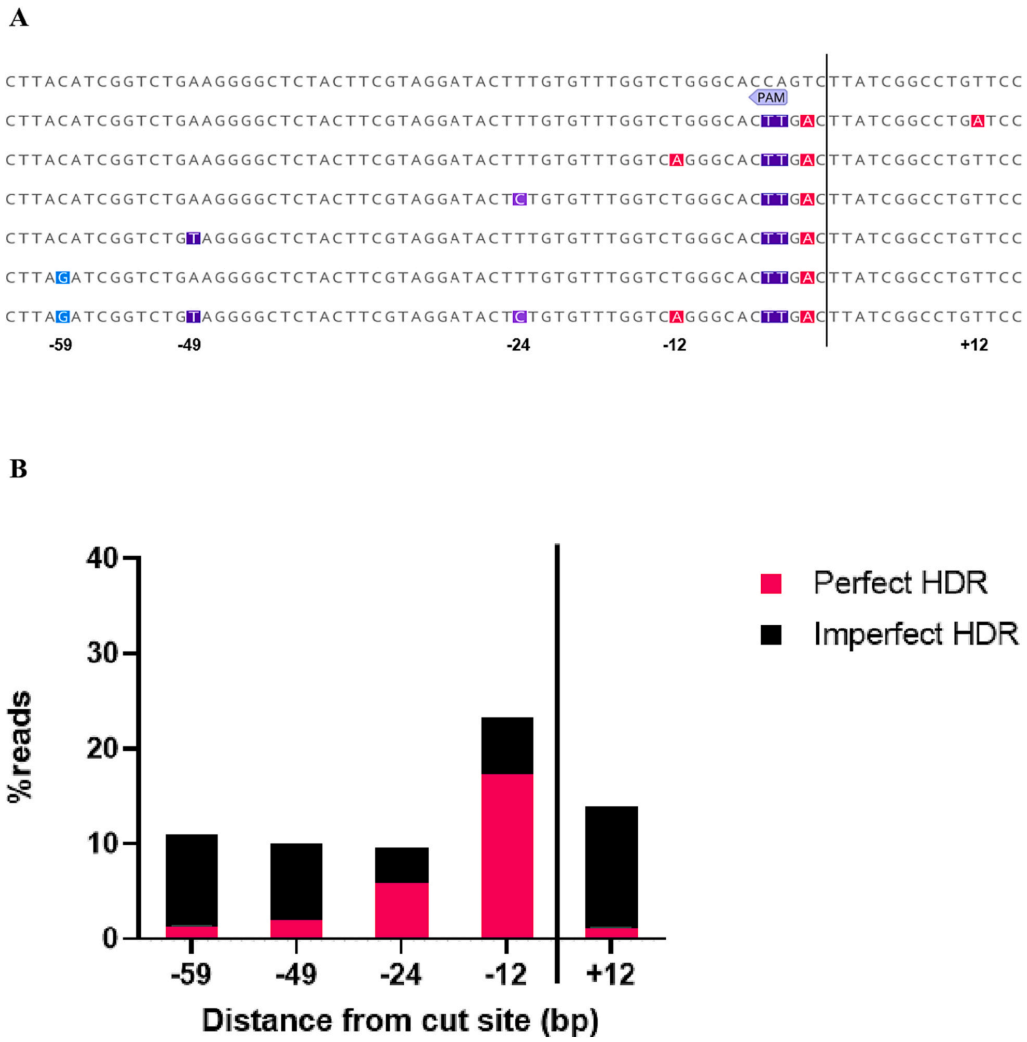

Fig. 3. Single nucleotide replacement in Atlantic salmon using conventional CRISPR/Cas9 and ODN templates. A ODN templates featuring point mutations at distinct positions upstream or downstream of the Cas9 cleavage site (indicated by a black line). The PAM site is also mutated to prevent repeated cutting and induce a TGA stop codon. B Average HDR efficiencies revealed by sequencing of larvae injected with Cas9 mRNA, gRNA targeting *slc45a2* exon 6 and the respective templates. Pink bars indicate perfect HDR, defined as sequence reads with a perfect match to the template sequence, including both the point mutation and PAM mutation. Black bars indicate imperfect HDR, defined as sequence reads where the PAM site is mutated but without nucleotide replacement at the targeted positions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

should be avoided in this window to prevent bystander editing. Additionally, the two primary classes of base editors, CBEs and ABEs, can achieve only 4 out of 12 possible single base substitutions. To overcome these limitations, we further developed our previously used method for SNR, where we inserted a point mutation near the Cas9 cleavage site using ODN templates (Straume et al., 2021). In the present work, we provided templates with point mutations at various positions to investigate whether the distance from the cleavage site would affect the insertion efficiency. Our findings revealed that it was possible to introduce mutations at positions up to −59 bases upstream of the cleavage site but with decreased efficiency compared to the mutations introduced closer to the cleavage site. Furthermore, because of the competitive relationship between the HDR and NHEJ pathways, this approach may exhibit lower efficiency and result in more indel generation compared to base editing. There are also well-known concerns regarding unexpected or unwanted off-target effects when employing GE technologies. In this work, we carefully designed the gRNAs to ensure specific target sites and limit potential editing at other sites. The gRNA used for base editing had no off-target sites, whereas the gRNA used for SNR with ODNs had one potential off-target site, although in a non-coding region and with a 1-base gap adjacent to the PAM site. Because the focus of the current study was to assess the efficiency of base editing and SNR using the *slc45a2* gene as proof of concept, we did not investigate off-target effects outside the *slc45a2* locus. However, for future applications in

aquaculture where other genes are relevant, sequencing of potential off-target sites should be performed.

In addition to CBE and SNR using ODNs explored in the current study, other alternatives for precise GE exist. Recent studies have reported the development of novel base editors that enable transversion mutations, such as C-to-G (Kurt et al., 2021) and A-to-C (Chen et al., 2023), expanding the possibilities of base editing. Furthermore, the versatile prime editing technology allows for all possible base conversions, as well as the insertion of short genetic sequences (Anzalone et al., 2019). The rapid advancements in GE technology have sparked discussions about its application as a new breeding technique. Recently, the United Kingdom passed into law the Genetic Technology (Precision Breeding) Act, facilitating the development and marketing of GE plants and animals. This legislative progress may promote the application of precision breeding in aquaculture, with its potential to accelerate the introduction of traits favorable for production. For instance, male fish with delayed maturation are favored due to potential negative effects associated with early maturation, such as increased disease susceptibility and reduced growth (Taranger et al., 2010). The maturation age is strongly linked to the *vestigial-like protein 3* (*vgll3*) locus, with certain single nucleotide polymorphisms (SNPs), including two missense mutations, explaining approximately 33–36% of the phenotypic variation in the fish (Ayllon et al., 2015). Although the specific SNPs governing the maturation age are not functionally demonstrated, it is conceivable that fine-tuned GE could be applied to precisely edit such SNPs and investigate the impact on the phenotype. Furthermore, as our understanding of the salmon genome advances, also putative causative SNPs for other valuable traits, like disease resistance or enhanced nutritional status, can be functionally validated and potentially introduced to the farmed fish.

5. Conclusion

In summary, we present the first application of base editing in an aquaculture fish species, achieving highly efficient conversion of the targeted C to T in multiple Atlantic salmon individuals. Despite some undesired effects, our results suggest that CBE AncBE4max offers a simple and efficient way of converting single bases in this species. When base editing is not feasible, an HDR-mediated approach using conventional CRISPR/Cas9 and ODN templates can be employed to insert point mutations across a wider range than previously described in salmon. Moreover, the growing collection of CRISPR-based GE tools now available encourages the implementation of precision breeding in aquaculture. Precision breeding may facilitate the introduction of genetic traits that promote fish health, such as delayed maturation or disease resistance, ultimately contributing to a more sustainable aquaculture industry.

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Ethical statement

All animal experiments within the study were conducted in accordance with the Norwegian Animal Welfare Act.

CRediT authorship contribution statement

Mari Raudstein: Formal analysis, Investigation, Resources, Visualization, Writing – original draft, Writing – review & editing. **Anne Hege Straume:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Erik Kjærner-Semb:** Formal analysis, Software, Writing – review & editing. **Morten Barvik:** Investigation, Writing – review & editing. **Ståle Ellingsen:** Funding acquisition, Supervision, Writing – review & editing. **Rolf Brudvik Edvardsen:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare no competing interests.

Data availability

Raw data will be made available upon request. MiSeq read counts used for data analysis are found in Supplementary File 2.

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Appendix A. Supplementary data

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Supplementary information

Supplementary Material 1: Supplementary tables and figures

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Supplementary Fig. S2: Salmon larvae injected with base editor (150 ng/µL)

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Supplementary Material 2: Excel sheet containing MiSeq read counts used for data analysis

Supplementary File

Highly efficient *in vivo* C-to-T base editing in Atlantic salmon (*Salmo salar*) – a step towards aquaculture precision breeding

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Supplementary Table S1. gRNA sequences used for base editing and single nucleotide replacement (SNR) using ODN templates. The PAM is underlined. Red letters indicate the editing window of the base editor, and the bold letter indicates the target cytidine base.

gRNA	Description	Sequence (5'-3')
Slc45a2_exon7	gRNA targeting exon 7 of slc45a2 for base editing	GGCTCAGATCATCGTGGGGGCGG
Slc45a2_exon6	gRNA targeting exon 6 of slc45a2 for ODN SNR	CCAGTCTTATCGGCCTGTTCCCC

Supplementary Table S2. ODN template sequences used for single nucleotide replacement. Red letters indicate the point mutation in the template. The PAM site was mutated and is underlined.

Template	Sequence (5'-3')
+12	CTGCAGATGTCCAGAGGCTGCTGCTGCCTTACATCGGTCTGAAGGGG CTCTACTTCGTAGGATACTTTGTGTTTGGTCTGGGCACTTGACTTATCG GCCTGATCCCCAACATTATCACCACCCTCA
-12	CTGCAGATGTCCAGAGGCTGCTGCTGCCTTACATCGGTCTGAAGGGG CTCTACTTCGTAGGATACTTTGTGTTTGGTCAGGGCACTTGACTTATCG GCCTGTTCCCCAACATTATCACCACCCTCA
-24	CTGCAGATGTCCAGAGGCTGCTGCTGCCTTACATCGGTCTGAAGGGG CTCTACTTCGTAGGATACTCTGTGTTTGGTCTGGGCACTTGACTTATCG GCCTGTTCCCCAACATTATCACCACCCTCA
-49	CTGCAGATGTCCAGAGGCTGCTGCTGCCTTACATCGGTCTGTAGGGG CTCTACTTCGTAGGATACTTTGTGTTTGGTCTGGGCACTTGACTTATCG GCCTGTTCCCCAACATTATCACCACCCTCA
-59	CTGCAGATGTCCAGAGGCTGCTGCTGCCTTAGATCGGTCTGAAGGGG CTCTACTTCGTAGGATACTTTGTGTTTGGTCTGGGCACTTGACTTATCG GCCTGTTCCCCAACATTATCACCACCCTCA
Combination -12, -24, -49, -59	CTGCAGATGTCCAGAGGCTGCTGCTGCCTTAGATCGGTCTGTAGGGG CTCTACTTCGTAGGATACTCTGTGTTTGGTCAGGGCACTTGACTTATCG GCCTGTTCCCCAACATTATCACCACCCTCA

Supplementary Table S3. Primer sequences for amplification of the *slc45a2* target sites. Uppercase letters indicate target-specific primers. Lowercase letters indicate adapter overhang.

Primer	Description	Sequence (5'-3')
Slc45a2_exon7_F_miseq	Primer for amplification of base editing target site	tctttccctacacgacgctcttccgatctGGATTCTTCCTGTTGTGACACC
Slc45a2_exon7_R_miseq	Primer for amplification of base editing target site	tggagttcagacgtgtgctcttccgatctGAGGTTATTCCACGTATCTGATG
Slc45a2_exon6_F_miseq	Primer for amplification of SNR using ssODNs target site	tctttccctacacgacgctcttccgatctCAGATGTCCAGAGGCTGCTGCT
Slc45a2_exon6_R_miseq	Primer for amplification of SNR using ssODNs target site	tggagttcagacgtgtgctcttccgatctTGCCACAGCCTCAGAATGTACA

Supplementary Table S4. Number of salmon embryos injected, larvae that survived until the sampling point, and larvae sampled in the base editing experiment.

Experimental group	No. embryos injected	No. surviving larvae	No. alb/mosaic not sampled	No. alb/mosaic sampled
150 ng/µL	559	298	0	6
300 ng/µL	471	209	0	8

Supplementary Table S5. Number of salmon embryos injected, larvae that survived until the sampling point, and larvae sampled in the single nucleotide replacement experiment.

Experimental group	No. embryos injected	No. surviving larvae	No. alb/mosaic not sampled	No. alb/mosaic sampled
+12	321	218	~5	19
-12	312	195	~20	19
-24	300	177	0	19
-49	303	194	~5	19
-59	298	184	0	16
Combi	277	186	0	19

A

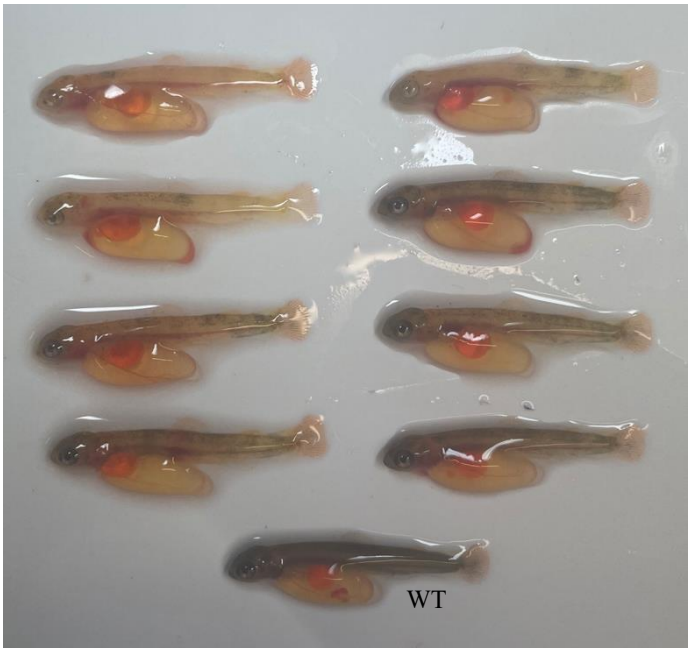
```
> gRNA_BE on NC_059442.1 Salmo salar chromosome ssa01, Ssal_v3.1, whole
genome shotgun sequence
Length=174498729

 Score = 42.8 bits (46),  Expect = 0.001
 Identities = 23/23 (100%), Gaps = 0/23 (0%)
 Strand=Plus/Minus

gRNA   1          GGCTCAGATCATCGTGGGGGCGG  23
                  |||||||||||||||||||||||
Sbjct  131398776  GGCTCAGATCATCGTGGGGGCGG  131398754
```

B

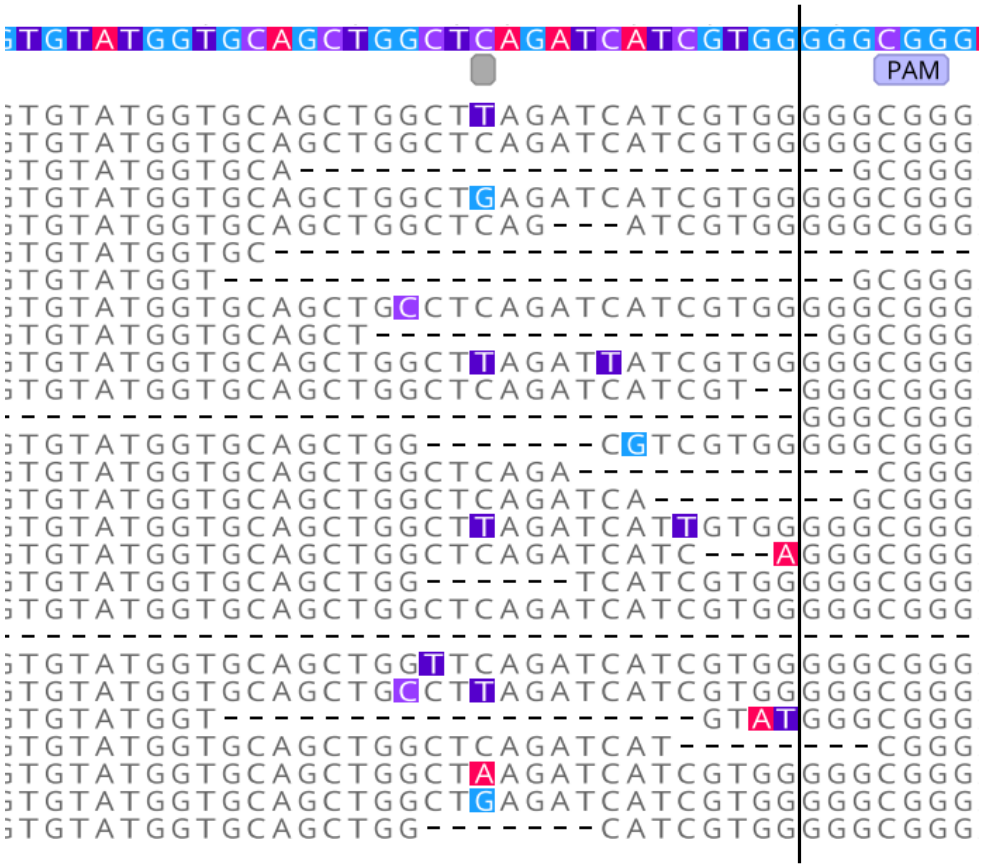
```
> gRNA_SNR on NC_059442.1 Salmo salar chromosome ssa01, Ssal_v3.1, whole
genome shotgun sequence
Length=174498729

 Score = 42.8 bits (46),  Expect = 0.001
 Identities = 23/23 (100%), Gaps = 0/23 (0%)
 Strand=Plus/Minus

gRNA   1          CCAGTCTTATCGGCCTGTTCCCC  23
                  |||||||||||||||||||||||
Sbjct  131400916  CCAGTCTTATCGGCCTGTTCCCC  131400894



> gRNA_SNR on NC_059458.1 Salmo salar chromosome ssa17, Ssal_v3.1, whole
genome shotgun sequence
Length=87489397

Score = 33.7 bits (36),  Expect = 0.57
Identities = 22/23 (96%), Gaps = 1/23 (4%)
Strand=Plus/Minus

gRNA   1          CCAGTCTTATCGGCCTGTTCCCC  23
                  ||||| ||||||||||||||||
Sbjct  68371385  CCAGT-TTATCGGCCTGTTCCCC  68371364
```

Supplementary Fig. S1. gRNA BLAST search against the salmon genome for potential off-target sites. The PAM site is underlined. **A** The gRNA used for base editing (BE) showed one target site (on target). **B** The gRNA used for single nucleotide replacement (SNR) showed two target sites. The top is on target, and the bottom is off target, with a 1-base gap near the PAM site.

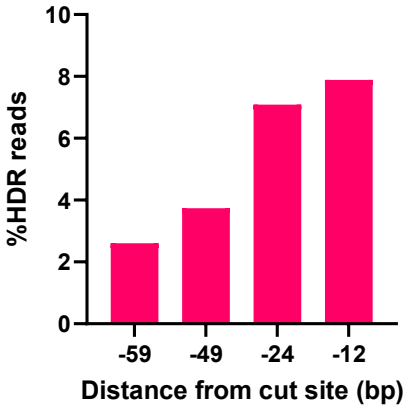
Supplementary Fig. S2. Salmon larvae injected with 150 ng/µL AncBE4max mRNA and gRNA targeting *slc45a2* exon 6.



Supplementary Fig. S3. Salmon larvae injected with 300 ng/µL AncBE4max mRNA and gRNA targeting *slc45a2*. One wild-type (WT) larvae for comparison.



Supplementary Fig. S4. Example of indel variants in an individual larvae injected with base editor AncBE4max mRNA and gRNA targeting *slc45a2*. The target base C is indicated by the grey box. The cleavage site of a conventional Cas9 nuclease is indicated by a black line. Image from Geneious.



Supplementary Fig. 5. HDR efficiencies of the combination template featuring four distinct point mutations in positions -59, -49, -24 and -12 upstream of the PAM. The bars represent the % of HDR reads containing that particular mutation.

SampleID	Total reads > 0.1%	WT reads	correct_conversion	wrong_A	wrong_G	Deletions	Insertions	p[3]	p[10]	p[13]
4701	6558	767	4966	0	116	167	0	114	388	25
4702	7836	4222	2641	38	223	310	32	66	241	45
4703	8341	1918	5424	0	106	339	0	125	285	98
4704	9457	2198	5906	52	285	508	63	67	376	19
4707	7926	2110	4007	33	183	1162	18	41	164	165
4709	8377	5705	1473	83	514	275	44	73	28	25
AVG 150 ng/µL										
6001	127806	3740	113666	0	201	3013	103	0	3575	155
6002	119029	7563	103556	104	2427	2540	0	166	2673	0
6003	100885	11470	59750	0	5125	5934	293	531	3787	7654
6004	113843	34239	53092	535	7144	6780	4889	5143	2077	105
6005	103170	7355	88611	0	368	4243	339	234	1444	915
6006	124775	27626	78580	558	1450	12204	233	201	3948	0
6007	126924	28461	81359	290	1053	9240	1703	1857	2865	171
6008	139907	48853	50366	196	5520	29437	246	428	1365	528
AVG 300 ng/µL										
Non-injected control	9853	9727	15	0	0	0	0	0	12	0

SampleID	%WT	%Correct conversion	%Wrong conversion (C->A)	%Wrong conversion (C->G)	%Deletions	%Insertions	%bystander p[3]	%bystander p[10]	%bystander p[13]	Other
4701	11.70	75.72	0.00	1.77	2.55	0.00	1.74	5.92	0.38	0.23
4702	53.88	33.70	0.48	2.85	3.96	0.41	0.84	3.08	0.57	0.23
4703	22.99	65.03	0.00	1.27	4.06	0.00	1.50	3.42	1.17	0.55
4704	23.24	62.45	0.55	3.01	5.37	0.67	0.71	3.98	0.20	0.00
4707	26.62	50.56	0.42	2.31	14.66	0.23	0.52	2.07	2.08	0.54
4709	68.10	17.58	0.99	6.14	3.28	0.53	0.87	0.33	0.30	1.87
AVG 150 ng/µL	34.42	50.84	0.41	2.89	5.65	0.30	1.03	3.13	0.79	0.54
6001	2.93	88.94	0.00	0.16	2.36	0.08	0.00	2.80	0.12	2.62
6002	6.35	87.00	0.09	2.04	2.13	0.00	0.14	2.25	0.00	0.00
6003	11.37	59.23	0.00	5.08	5.88	0.29	0.53	3.75	7.59	6.29
6004	30.08	46.64	0.47	6.28	5.96	4.29	4.52	1.82	0.09	0.00
6005	7.13	85.89	0.00	0.36	4.11	0.33	0.23	1.40	0.89	0.00
6006	22.14	62.98	0.45	1.16	9.78	0.19	0.16	3.16	0.00	0.00
6007	22.42	64.10	0.23	0.83	7.28	1.34	1.46	2.26	0.13	0.00
6008	34.92	36.00	0.14	3.95	21.04	0.18	0.31	0.98	0.38	2.12
AVG 300 ng/µL	17.17	66.35	0.17	2.48	7.32	0.84	0.92	2.30	1.15	1.31
Non-injected control	98.72	0.15	0.00	0.00	0.00	0.00	0.00	0.12	0.00	1.00

SampleID	Total_reads > 20 (~0.1%)	WT_reads > 20	Total HDR reads (PAM mutated, regardless of indels or not)	Perfect HDR (SNR + PAM mutated)	Perfect HDR PAM (no SNR and no indels)	KO reads (Tot - (WT + HDR))	%Total HDR reads	%Perfect HDR	%Perfect HDR PAM only	%KO
8001	19137	0	0	0	0	19137	0.00	0.00	0.00	100.00
8002	18127	0	1544	0	1510	16583	8.52	0.00	8.33	91.48
8003	13896	729	1235	404	589	11932	8.89	2.91	4.24	85.87
8004	11580	64	394	22	372	11122	3.40	0.19	3.21	96.04
8005	16065	71	2324	112	2099	13670	14.47	0.70	13.07	85.09
8006	15111	277	2226	1155	685	12608	14.73	7.64	4.53	83.44
8007	13103	727	288	0	205	12088	2.20	0.00	1.56	92.25
8008	15548	559	1366	106	1038	13623	8.79	0.68	6.68	87.62
8009	13894	777	3498	122	2813	9619	25.18	0.88	20.25	69.23
8010	9022	129	1036	24	926	7857	11.48	0.27	10.26	87.09
8011	13064	483	3139	257	2521	9442	24.03	1.97	19.30	72.27
8012	14425	685	5488	29	5252	8252	38.05	0.20	36.41	57.21
8013	15393	509	1271	43	1060	13613	8.26	0.28	6.89	88.44
8014	16798	361	3517	24	3325	12920	20.94	0.14	19.79	76.91
8015	11353	984	2746	351	2329	7623	24.19	3.09	20.51	67.15
8016	20004	853	1650	138	1124	17501	8.25	0.69	5.62	87.49
AVG (+12)							13.83	1.23	11.29	82.97
8101	15549	0	4503	3917	535	11046	28.96	25.19	3.44	71.04
8102	13132	298	3181	2713	274	9653	24.22	20.66	2.09	73.51
8103	19035	0	3043	1813	1191	15992	15.99	9.52	6.26	84.01
8104	15758	0	4886	310	73	10872	31.01	1.97	0.46	68.99
8105	15577	34	8682	7096	33	6861	55.74	45.55	0.21	44.05
8106	16532	0	6713	5473	1061	9819	40.61	33.11	6.42	59.39
8107	16691	0	1469	1469	0	15222	8.80	8.80	0.00	91.20
8109	20502	314	2999	2306	624	17189	14.63	11.25	3.04	83.84
8110	17793	0	818	230	27	16975	4.60	1.29	0.15	95.40
8111	8255	108	1708	989	636	6439	20.69	11.98	7.70	78.00
8112	18506	222	2727	1680	968	15557	14.74	9.08	5.23	84.06
8113	33908	49	233	212	21	33626	0.69	0.63	0.06	99.17
8114	16569	51	8853	8445	161	7665	53.43	50.97	0.97	46.26

8115	15232	686	3052	2783	216	11494	20.04	18.27	1.42	75.46
8116	13544	25	2152	1572	210	11367	15.89	11.61	1.55	83.93
AVG (-12)							23.33	17.32	2.60	75.89
8201	17078	128	813	283	113	16137	4.76	1.66	0.66	94.49
8203	18400	1036	5303	3855	384	12061	28.82	20.95	2.09	65.55
8204	17702	2177	690	397	189	14835	3.90	2.24	1.07	83.80
8205	16382	796	470	402	68	15116	2.87	2.45	0.42	92.27
8206	14578	112	1663	377	1286	12803	11.41	2.59	8.82	87.82
8207	10449	910	1963	1546	343	7576	18.79	14.80	3.28	72.50
8208	11637	380	1728	1097	293	9529	14.85	9.43	2.52	81.89
8209	27386	138	643	196	447	26605	2.35	0.72	1.63	97.15
8210	14598	338	391	270	38	13869	2.68	1.85	0.26	95.01
8211	17401	1691	3421	2859	513	12289	19.66	16.43	2.95	70.62
8212	11495	2623	224	178	46	8648	1.95	1.55	0.40	75.23
8213	15586	2986	1045	485	300	11555	6.70	3.11	1.92	74.14
8214	15655	3403	825	403	310	11427	5.27	2.57	1.98	72.99
8215	18534	1513	2286	646	136	14735	12.33	3.49	0.73	79.50
8216	11869	2680	783	389	394	8406	6.60	3.28	3.32	70.82
AVG (-24)							9.53	5.81	2.14	80.92
8301	19209	142	183	51	132	18884	0.95	0.27	0.69	98.31
8302	24122	34	430	110	147	23658	1.78	0.46	0.61	98.08
8303	18485	338	1146	49	1076	17001	6.20	0.27	5.82	91.97
8304	19048	506	387	42	220	18155	2.03	0.22	1.15	95.31
8305	11150	838	726	139	375	9586	6.51	1.25	3.36	85.97
8306	18373	2104	1056	181	803	15213	5.75	0.99	4.37	82.80
8307	14341	960	5266	340	4127	8115	36.72	2.37	28.78	56.59
8308	19296	4810	3170	62	515	11316	16.43	0.32	2.67	58.64
8309	19762	1609	1335	504	798	16818	6.76	2.55	4.04	85.10
8310	18679	1555	3202	224	2501	13922	17.14	1.20	13.39	74.53
8311	24847	1093	991	487	474	22763	3.99	1.96	1.91	91.61
8312	37298	0	0	0	0	37298	0.00	0.00	0.00	100.00
8313	15461	1997	2194	196	1929	11270	14.19	1.27	12.48	72.89
8314	15701	1769	2399	1687	658	11533	15.28	10.74	4.19	73.45

8315	15211	1961	2512	895	1173	10738	16.51	5.88	7.71	70.59
8316	13113	2170	1398	160	1007	9545	10.66	1.22	7.68	72.79
AVG (-49)							10.06	1.93	6.18	81.79
8401	19094	33	38	0	38	19023	0.20	0.00	0.20	99.63
8402	22270	77	12386	1271	10811	9807	55.62	5.71	48.55	44.04
8403	18314	1219	1755	315	1207	15340	9.58	1.72	6.59	83.76
8404	23253	893	2673	1603	976	19687	11.50	6.89	4.20	84.66
8405	15860	2234	1305	321	956	12321	8.23	2.02	6.03	77.69
8406	19418	2892	4651	85	4029	11875	23.95	0.44	20.75	61.15
8407	18676	2435	754	175	579	15487	4.04	0.94	3.10	82.92
8408	14512	1870	1873	48	432	10769	12.91	0.33	2.98	74.21
8409	16577	2897	3824	138	3494	9856	23.07	0.83	21.08	59.46
8410	19830	1973	129	21	108	17728	0.65	0.11	0.54	89.40
8411	23146	4801	767	23	604	17578	3.31	0.10	2.61	75.94
8412	15311	3249	1256	202	992	10806	8.20	1.32	6.48	70.58
8413	13886	3847	640	104	513	9399	4.61	0.75	3.69	67.69
8414	18982	5426	637	29	608	12919	3.36	0.15	3.20	68.06
8415	23185	5299	893	94	799	16993	3.85	0.41	3.45	73.29
8416	16179	7293	350	22	328	8536	2.16	0.14	2.03	52.76
AVG (-59)							10.95	1.37	8.47	72.83

SampleID	Total_reads > 20 (~0.1%)	WT_reads > 20	Total HDR (PAM mutated, regardless of indels)	12+24+49+59+PAM	12+24+49+PAM	12+24+PAM	12+PAM	59+PAM	49+PAM	24+PAM	Perfect PAM (NO SNR)
8501	14768	171	5237	2101	172	534	163	0	0	46	298
8502	17720	534	3831	537	42	742	96	0	0	0	239
8503	20194	1549	761	151	0	193	130	0	0	0	287
8504	14568	1582	3460	477	1227	844	142	87	0	0	447
8505	20714	449	1214	212	37	451	85	0	0	0	232
8506	23443	333	1135	78	126	362	80	0	0	0	132
8507	9839	1142	492	98	32	101	31	0	0	0	55
8508	15361	2516	3640	1299	746	547	166	0	0	0	136
8509	26155	1905	2189	271	413	582	197	0	0	0	28
8510	9915	1000	2218	164	31	1783	109	0	0	0	86
8511	18932	2869	396	27	0	60	31	0	0	0	126
8512	17784	4035	573	0	0	86	315	0	0	0	74
8513	18954	3675	1426	500	84	244	318	0	0	0	82
8514	31423	3573	1627	44	0	1246	154	0	0	0	62
8515	23426	4812	1905	681	105	583	133	0	0	0	80
8516	16724	5453	790	70	50	287	204	0	0	0	112
AVG (combi)											

SampleID	%Total HDR	%(12+24+49+59)	%(12+24+49)	%(12+24)	%(12 only)	%(59 only)	% (49 only)	% (24 only)	% PAM only	%total(-12)	%total(-24)	%total(-49)	%total(-59)
8501	35.46	14.23	1.16	3.62	1.10	0.00	0.00	0.31	2.02	20.11	19.32	15.39	14.23
8502	21.62	3.03	0.24	4.19	0.54	0.00	0.00	0.00	1.35	8.00	7.45	3.27	3.03
8503	3.77	0.75	0.00	0.96	0.64	0.00	0.00	0.00	1.42	2.35	1.70	0.75	0.75
8504	23.75	3.27	8.42	5.79	0.97	0.60	0.00	0.00	3.07	18.47	17.49	11.70	3.87
8505	5.86	1.02	0.18	2.18	0.41	0.00	0.00	0.00	1.12	3.79	3.38	1.20	1.02
8506	4.84	0.33	0.54	1.54	0.34	0.00	0.00	0.00	0.56	2.76	2.41	0.87	0.33
8507	5.00	1.00	0.33	1.03	0.32	0.00	0.00	0.00	0.56	2.66	2.35	1.32	1.00
8508	23.70	8.46	4.86	3.56	1.08	0.00	0.00	0.00	0.89	17.95	16.87	13.31	8.46
8509	8.37	1.04	1.58	2.23	0.75	0.00	0.00	0.00	0.11	5.59	4.84	2.62	1.04
8510	22.37	1.65	0.31	17.98	1.10	0.00	0.00	0.00	0.87	21.05	19.95	1.97	1.65
8511	2.09	0.14	0.00	0.32	0.16	0.00	0.00	0.00	0.67	0.62	0.46	0.14	0.14
8512	3.22	0.00	0.00	0.48	1.77	0.00	0.00	0.00	0.42	2.25	0.48	0.00	0.00
8513	7.52	2.64	0.44	1.29	1.68	0.00	0.00	0.00	0.43	6.05	4.37	3.08	2.64
8514	5.18	0.14	0.00	3.97	0.49	0.00	0.00	0.00	0.20	4.60	4.11	0.14	0.14
8515	8.13	2.91	0.45	2.49	0.57	0.00	0.00	0.00	0.34	6.41	5.84	3.36	2.91
8516	4.72	0.42	0.30	1.72	1.22	0.00	0.00	0.00	0.67	3.65	2.43	0.72	0.42
AVG (combi)	11.60	2.56	1.18	3.33	0.82	0.04	0.00	0.02	0.92	7.89	7.09	3.74	2.60

PAPER III

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