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Anti-viral divergent function of Atlantic Salmon *trim25* Paralogues in Infectious Salmon Anaemia Virus Infection

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Abstract

Aquaculture production of Atlantic salmon is a rapidly growing food sector, offering numerous advantages over terrestrial livestock production. Yet, despite these advantages, the production efficiency, in addition to the health and welfare of marine raised Atlantic salmon is highly impacted by infectious diseases. In order to develop better preventative and therapeutic tools for infectious diseases, a better understanding of the immune response is required. In model species, the E3 ubiquitin ligase TRIM25 is a well characterised component of the host antiviral response, acting as both a sensor and a signal transducer for the RIG-like receptor pathway. In Atlantic salmon (*Salmo salar*), *trim25* presents as two copies - ENSSSAG00000054152 and ENSSSAG00000046838 located on chromosome 2 (Ssa02) and 12 (Ssa12) respectively, and has been identified in multiple viral and viral mimic challenges as a virus-induced gene. To date, its role in the host response against viral pathogens remains uncharacterised. Viruses such as Infectious Salmon Anaemia Virus (ISAV) constantly threaten marine-raised Atlantic salmon, and novel strategies for prevention and treatment of viral outbreaks are fundamental for more efficient production. This research aims to characterise the function of the Atlantic salmon *trim25* to better understand the host response to viruses, leading to improvements in the management of viral diseases in salmon farming. Using Atlantic salmon head kidney 1 (SHK-1) cells and ISAV as a model infection, we show paralog-specific virus-inducibility and antiviral function of *trim25*. Whilst structural analyses showed that the two copies of *trim25* retain their functional domains, using CRISPR-

Cas genome editing in combination with qPCR and epigenetic (ATAC and ChIP) sequencing we have demonstrated that Ssa02 *trim25* exclusively has an impact on viral replication. Additionally, we demonstrate the conservation of the role of *trim25* in the RIG-like receptor pathway using RNA sequencing. Finally, we show that overexpression of *trim25* leads to protection from ISAV infection in a salmonid cell line. This research highlights the paralog specific antiviral role of *trim25* in fish, and paves the way for biotechnological applications to reduce the impact of viral outbreaks in salmon aquaculture.

Key Words: CRISPR, Genome editing, Aquaculture, Innate Immunity, Ubiquitin

1. Introduction

Tripartite Motif proteins (TRIM) are a large sub-family of RING E3 ubiquitin ligases, defined by their conserved protein structure (RBCC) - RING domain, B box domain(s), and Coiled Coil domain[1]. E3 ubiquitin ligases act as matchmakers in the ubiquitin ligase pathway, conferring specificity to this post-translational modification system. E3 ligases catalyse the addition of ubiquitin to a broad range of substrates, thereby altering their stability, activation state and localization[2,3]. TRIM E3 ligases show an incredible repertoire of functions across vertebrate species, regulating processes from immune response to cell division[4]. In teleost fish, this gene family has undergone large expansions, resulting in an expanded toolkit of TRIM genes, the vast majority of which still require characterisation[5]. Whilst humans have between 70-80 TRIM genes in the genome, zebrafish have upwards of 200[6], including novel, fish specific FinTRIMs and Blood-thirsty like genes[4,7,8]. Furthermore, in salmonids, TRIM diversity is further expanded by teleost (Ts3R) and salmonid specific additional whole genome duplication events (Ss4R) – two extras relative to mammals[4], leading to potentially multiple copies of each gene[9].

TRIM25 plays a fundamental role in innate immune responses, particularly against viruses, and is well-characterised in humans[10], rodents[11], birds[12], and some fish species[13-18]. However, its mode of action is still unclear and/or disputed, with multiple suggested mechanisms. Primarily, TRIM25 is predicted to activate interferon-mediated innate immune response via K63 linked ubiquitination of RIG-I, activating the Rig-like receptor interferon stimulated gene pathway[10]. However, in opposition, *Hayman et al.*[19] proposed that Riplet protein, not TRIM25, is responsible for RIG-I activation. Further to this hypothesis, *Choudry et al.*[20] conclude that TRIM25 destabilises viral RNA, yet is redundant for activating RIG-I. Whilst the precise mechanism of TRIM25 remains undeciphered, both mechanisms converge on the consensus that TRIM25 protects the host against viral infection.

In fish, TRIM25 has been confirmed as an antiviral E3 ligase, with most studies supporting the hypothesis that TRIM25 acts via the Rig-like receptor (RLR pathway), including rainbow trout (*Oncorhynchus mykiss*)[17], common carp (*Cyprinus carpio*)[18], grass carp (*Ctenopharyngodon*

Idella)[21], Japanese flounder (*Paralichthys olivaceus*)[16], yellow croaker (*Larimichthys crocea*)[22], grouper (*Epinephelus coioides)*[23] and zebrafish (*Danio rerio*)[24]. Currently, no literature in fish proposes an alternative mechanism to RLR activation, however, due to the challenging nature of knock-out/down studies in fish, the majority of studies rely on plasmid-derived overexpression of TRIM25. Overexpression of TRIM25, as discussed in Hayman *et al.*[19], may lead to protection via the induction of the RLR pathway as a side effect of the overexpression, rather than the direct activity of the TRIM25 protein. It is therefore necessary to develop additional, more refined methods for the characterisation and validation of gene function in hard-to-study species, by using novel technologies such as CRISPR-Cas gene editing.

In Atlantic salmon the molecular mechanism of TRIM25 has not yet been described, however, increasing evidence across taxa points towards the conservation of the antiviral mechanism in salmon as demonstrated in other vertebrates. Evidence exists for the virus-inducibility of *trim25* in Atlantic salmon both *in-vitro*[25-27] and *in-vivo*[26,28-30], but its antiviral activity mechanism is not yet confirmed.

Infectious salmon anaemia virus (ISAV), belonging to the family *Orthomyxoviridae*, poses a significant threat to Atlantic salmon aquaculture sustainability and is a good model-virus to study the function of TRIM25. Studies applying genomics techniques and RNA sequencing, have repeatedly identified a *trim25*-like gene being upregulated in response to ISAV[28]. Additionally, it has been shown that ISAV segments 7[31] and 8[32] antagonise the host interferon response, potentially through interacting with this TRIM25 protein. Whilst no detailed mechanism has been suggested, it may be the case that ISAV non-structural protein (segment s7ORF1) acts similarly to the NS1 protein of influenza A, a virus in the same family as ISAV. Influenza A NS1 antagonises the host immune response to infection by inhibiting TRIM25 mediated ubiquitination of RIG-I[11]. This interaction between NS1 and TRIM25 blocks the K63-linked ubiquitination and activation of a downstream signalling molecule, RIG-1, which in the absence of NS1 induces the production of pro-inflammatory type 1 and 3 interferons, leading to activation of innate and adaptive immune responses[11,33,34]. This mechanism exemplifies the interplay between the immune-regulatory post-translational modifications by E3 ligases, as well as the co-evolution of immune evasion mechanisms of viruses.

By developing our understanding of the Atlantic salmon cellular immune response to this virus, in addition to the life cycle and mechanisms of the virus, we may be able to develop better targets for gene editing, vaccine antigens and selective breeding programs. Using Salmon Head Kidney cells (SHK-1) and ISAV infection as a model, here we aim to elucidate the mechanism of TRIM25 in this species using a combination of molecular biology techniques, including CRISPR-Cas9 mediated knockdown and plasmid driven overexpression. This research presents the first evidence of TRIM25 knock-out and

characterisation in a salmonid cell line, and shows for the first-time sub-functioning of the *trim25* paralogs in the establishment of an effective antiviral response.

3. Materials and methods

Phylogenetic analysis

The gene tree for *trim25* was obtained from Ensembl (version 115), constructed based on the longest transcript of each gene. A subtree was then extracted in Wasabi online suite (http://wasabiapp.org/). The coding sequence of filtered genes were aligned using Multiple Alignment using Fast Fourier Transform (MAFFT) aligner[35] with default parameters. A neighbor joining phylogenetic tree was then constructed in the online suite of MAFFT (version 7), using conserved sites (1782 of total 2043 sites) and a jukes cantor substitution model. Bootstrap values were produced using 500 iterations. Finally, the tree was visualized using the iTOL platform[36].

Sequence analysis

The annotated TRIM proteins were extracted from the Atlantic salmon Ensembl proteome (Ssal_v3.1) using the following keywords: *'tripartite.motif'*, *'bloodthirsty'*, and *'finTRIM'*. The sequences were filtered to leave only one transcript per gene (the largest). To infer the phylogenetic tree, a multiple sequence alignment was performed using MAFFT with the L-INS-i iterative refinement method and a maximum of 1,000 cycles. This alignment was used to construct a Maximum-Likelihood tree with IQ-TREE[37] using ModelFinder to determine the best-fit substitution model (JTT+F+R5 according to BIC), and 10,000 ultrafast bootstraps to assess branch supports. The option "*-bnni*" was also used to reduce the risk of overestimating branch supports in case of severe model violations. Pairwise distance matrices for sequence similarity were calculated from the aligned protein sequences with the function dist.alignment() from the R package 'seqinr'. This function outputs a root distance matrix that can be converted to a similarity matrix as follows: $S = 1 - (\text{root distance})^2$. The tree was illustrated using the R package '*ggtree'*. Tip labels include Ensembl information: gene name, chromosome number, and accession code.

Cell culture

Atlantic salmon head kidney (SHK-1) cells (RRID:CVCL_4299) were grown as a monolayer on Nunc™ cell culture flasks (Thermo Fisher scientific, Massachusetts, USA) of varying sizes. Media for SHK-1 was L-15 media (Sigma Aldrich, Missouri, USA) supplemented with 5% heat-inactivated Foetal Bovine Serum (FBS) (Gibco™, Massachusets, USA) and 1% Pen-Strep (P/S) (Gibco™), 40mM β Mercaptoethanol (Gibco™), with 30% pre-conditioned media. Cells were routinely passaged at 80-90% confluency 1/3 split, reaching confluency in approx. 10

days. Cells were detached using 0.25% Trypsin-EDTA, phenol red (Gibco™) for approximately 5 mins with gentle agitation.

ISAV infection

Virus was thawed in palm of hand, briefly vortexed and diluted in L-15 media, 2% heat inactivated FBS, 500uM BME with 1% P/S. Virus was diluted to desired MOI for infection, with a concentration of virus (PFU/mL) calculated by TCID50 on SHK-1 cells. The plates were sealed with parafilm and incubated at 15°C for three hours. After the incubation, the inoculum was removed and fresh, virus free reduced serum media added. Plates were returned to the 15°C incubator until harvesting at designated timepoints.

RT-qPCR of *trim25* paralogs and ISAV

Samples were harvested as follows – supernatant was aliquoted and frozen at -80ºC immediately for TCID50 for viral infections. Remaining media was aspirated from cells, which were lysed with TRIzol™ (Thermo Fisher scientific) and stored at -80ºC. RNA was extracted from TRIzol™ using the Direct-Zol™ kit (Zymo Research, California, USA), with oncolumn DNAse I digestion of host genomic DNA. RNA was eluted in nuclease free water and quantified with nanodrop (Thermo Fisher scientific) and/or Qubit (Invitrogen, California, USA) and stored at -80ºC. Following RNA extraction, samples were amplified using Reverse transcription-polymerase chain reaction (RT-QPCR) using the Luna® Universal One-Step RT-qPCR Kit (New England Biosciences, Massachusetts, USA) and the CFX QPCR Machine (Bio-Ra, California, USA) according to manufacturer's instructions.

Each infection sample was amplified using ISAV Segment 8 (see Table 1) and Elf-1α primers in technical duplicates. The ISAV viral load was calculated as a fold change in the number of virus particles (ddCT) relative to housekeeping gene Elf-1α, with fold change represented vs control samples. Primer specificity was checked via sequencing of RT-qPCR products for *trim25* RT-qPCRs.

PCR for sequencing of paralogs

Sequencing primers were designed to span the start codon (ATG) of *trim25* paralogs in the Atlantic salmon genome (ENSSSAG00000054152 – Ssa02 and ENSSSAG0000004683 – Ssa12). Forward and reverse primers (Table 1) were designed to ensure CRISPR gRNAs were contained within the forward and reverse sequences with a large enough amplification window to account for large insertion/deletion events.

171 Table 1. PCR primers

	Sequence	Application
Ssa02 *trim25* RT-qPCR Fwd	TGTAGCATCTGCCTCTGTGC	RT-qPCR
Ssa02 *trim25* RT-qPCR Rev	GGAGGGCGCTTTTATTCGAC	RT-qPCR
Ssa12 *trim25* RT-qPCR Fwd	AAAGCGACCTCCCCAATGAG	RT-qPCR
Ssa12 *trim25* RT-qPCR Rev	AACGACGCCATACAGGTGAG	RT-qPCR
ISAV Segment 8 Fwd[38]	GAAGAGTCAGGATGCCAAGACG	RT-qPCR
ISAV Segment 8 Rev[38]	GAAGTCGATGATCTGCAGCGA	RT-qPCR
EF1α Fwd[39]	CACCACCGGCCATCTGATCTACAA	RT-qPCR
EF1α Rev[39]	TCAGCAGCCTCCTTCTCGAACTTC	RT-qPCR
Ssa02 *trim25* Fwd	GTAGCATCTGCCTCTGTGCT	Sequencing
Ssa02 *trim25* Rev	CAGATACGGTCGCGCAGGTC	Sequencing
Ssa12 *trim25* Fwd	TGTAGTTCCATCATGGCTGACA	Sequencing
Ssa12 *trim25* Rev	CAGATACGGTCTCGCAGGTC	Sequencing
TRIM25_Flag pcDNA 3.1 (+) Fwd	tgtggtggaattctgcagatGCCACCATGGCTGACAATATG	Cloning
TRIM25_Flag pcDNA 3.1 (+) Fwd	cggccgccactgtgctggatCTACTTATCGTCGTCATCTTTGTAGTC	Cloning

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173 CRISPR-cas9 gene editing

174 CRISPR-Cas9 gRNAs were designed to target the first exon of the catalytic RING domain of
175 the two *trim25* paralogs (genes ENSSSAG00000054152 and ENSSSAG00000046838) using
176 CRISPOR[40], with 20bp NGG Sp Cas9-HF1 PAM motif. Guide RNA and Tracer RNA were
177 combined in equimolar ratio to create duplex guide RNA. Duplex guide RNA was combined at
178 equimolar ratio with SpCas9-HF-1 to create a final concentration of 12uM Ribonucleoprotein.
179 Ribonucleoprotein was electroporated into SHK1 cells (cells at approx. 80% confluence)
180 suspended in Optimem (Thermo Fisher scientific) using Neon Transfection system (Thermo

Fisher scientific) at 1600V 10ms 3 pulses, at 1uM final concentration of Cas9 to approximately $1x10^5$ cells in Optimem media. Cell were dispensed into 24 well plates containing 1mL of antibiotic free media. Media was changed for antibiotic containing media at 48 hours post infection. Cells were passaged when confluency was reached, with whole genomic DNA harvested for sequencing using QuickExtract ™ (Sigma Aldrich), at day 10 post electroporation.

Table 2. gRNA sequences

	Sequence	Application
Ssa02 *trim25* gRNA	AGCGCCCTCCCCAGTGAGAT	CRISPR-Cas9 editing
Ssa12 *trim25* gRNA	AAGAGTGTCTCCTAGAAACA	CRISPR-Cas9 editing
Ssa02 + Ssa12 *trim25* gRNA	ACTACAGCTGTCCACAATGC	CRISPR-Cas9 editing

Gene cloning and plasmid construction

A *trim25* cDNA sequence (A0A1S3L7A9_SALSA, XP_013986861.1) representing the longest transcript of the chromosome 12 copy of *trim25* was codon optimised and synthesised by TWISTBIO. A Gibson cloning assembly kit (New England Biolabs) was used to subclone *trim25* with Flag tag (TRIM25_flag) out of pTWIST ssTRIM25 into pcDNA 3.1 (+) using primers in table 1, creating plasmid pcTRIM25_flag

Transfection of TRIM25 plasmids

CHSE-214 cells (CVCL_2780) were transfected with 100ng of pcDNA 3.1 (+) or pcDNA 3.1 TRIM25_flag at 1300V 30ms 1 pulse as per previously published optimisation[41]. Transfected cells were allowed to recover before selection for antibiotic resistance marker (NeoR) using G418. A G418 kill curve was generated to calculate sufficient selection pressure. 2 mg/mL G418 was used to select cells for a period of approximately 1 month, followed by maintenance media of 500 µg/mL.

Overexpression of plasmid derived trim25_flag mRNA was measured using RT-qPCR. Briefly, cellular RNA was harvested and purified as previously described. DNAse treatment was used to remove genomic DNA. cDNA was generated using the SuperScript™ VILO™ cDNA Synthesis Kit, with 60ng RNA added to each reaction. No reverse transcriptase controls were included to ensure amplification was from RNA not genomic DNA or plasmid DNA. SYBR™ Green Universal Master mix (Thermo Fisher Scientific) was used to perform RT-qPCR reactions, including no RT controls and no cDNA controls. RT-qPCR reactions were run in

duplicate for TRIM25_flag expression and EF1α house-keeping in order to calculate a ddCT and fold change value.

RNA sequencing

SHK-1 cells (Wild type = No gRNA, *trim25* KO = Ssa02 *trim25* KO + Ssa12 *trim25* KO) infected with 0.1 MOI ISAV were harvested at 24- hours and 48-hour post infection with TRIzol™. RNA was extracted from TRIzol™ using the Direct-Zol™ kit (Zymo Research), with on column DNAse I digestion of host genomic DNA. RNA was eluted in nuclease free water, and quality checked and quantified using EZ drop 1000 (Blue-Ray Biotech).

mRNA was isolated from total RNA using poly-T oligo–attached magnetic beads and fragmented prior to cDNA synthesis with random hexamer primers. Double-stranded cDNA libraries were prepared through end repair, A-tailing, adapter ligation, size selection, PCR amplification, and purification. Library quality and quantity were assessed by Qubit, real-time PCR, and Bioanalyzer. Qualified libraries were pooled according to effective concentration and sequenced on an Illumina NovaSeq platform by Novogene (Beijing, China).

Raw FASTQ reads were quality-filtered using fastp to remove adapters, poly-N reads, and low-quality sequences. Clean reads were evaluated for Q20, Q30, and GC content and used for all downstream analyses. Reads were aligned to the reference genome (Ssal_v3.1) using HISAT2 with splice-aware mapping based on gene annotation files. Gene-level read counts were generated with featureCounts, and expression levels were calculated as FPKM values. Differential expression analysis was performed using DESeq2 for datasets with biological replicates and edgeR for datasets without replicates, with P values adjusted using the Benjamini–Hochberg method. Genes with adjusted P values ≤ 0.05 and |log2 fold change| ≥ 1 were considered significantly differentially expressed, with thresholds adjusted when necessary to ensure sufficient gene numbers for downstream analyses. Functional enrichment analysis of differentially expressed genes was conducted using cluster. Profiler for Gene Ontology (GO) and KEGG pathway analyses, with enrichment significance defined as adjusted $P < 0.05$.

4. Results

Trim25 is present as two copies following the salmonid-specific whole-genome duplication

Considering the major expansion of the TRIM gene family in teleost fish as well as the occurrence of an additional, salmonid-specific, whole-genome duplication event (Ss4R), we first explored *trim25* gene abundance in the salmonid family. *Trim25* sequences were retrieved from the respective gene gain/loss tree in the Ensembl genome browser (release 115), resulting in the identification of ENSSSAG00000046838 and ENSSSAG00000054152. In total, gene sequences for six salmonid species (*Salmo salar*: Atlantic salmon, *Salmo trutta;* Brown trout, *Oncorhynchus mykiss;* Rainbow trout*, Oncorhynchus kisutch;* Coho salmon *and Oncorhynchus tshawytscha;* Chinook salmon) were visualized on ITOL[36] (Figure 1), together with Northern Pike (*Esox lucious*), a close ancestor of salmonids that did not undergo the lineage-specific whole genome duplication.

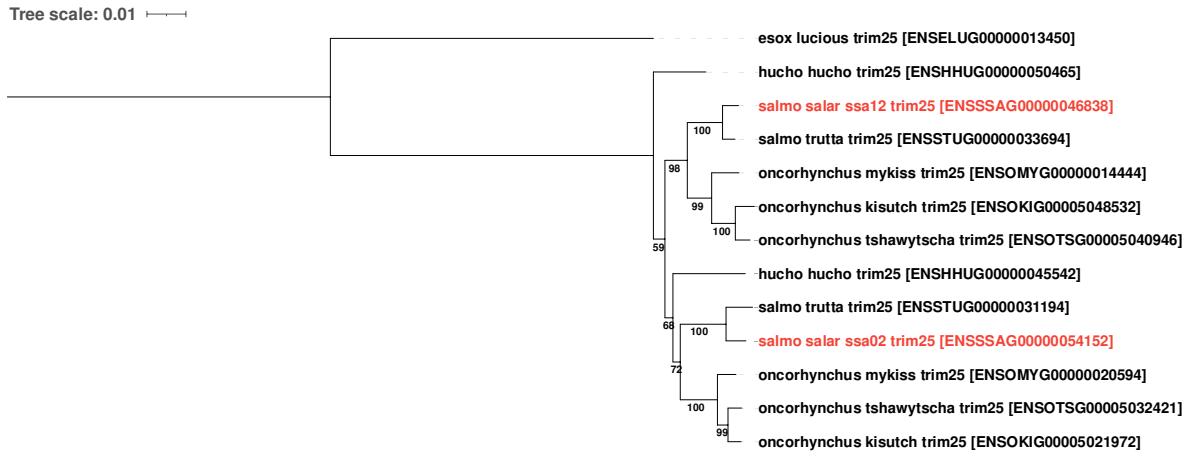


Figure 1: Gene tree of Atlantic salmon trim25*. Atlantic salmon trim25 Ssa02 ENSSSAG00000054152 and Ssa12 ENSSSAG00000046838 are denoted in red. Gene tree obtained from Ensembl[42] and visualized in iTOL[36]. Bootstrap values are shown on tree and were created using Jukes Cantor substitution model with 500 iterations.*

Accordingly, Northern pike (*Esox Lucius*) has only one copy of *trim25*, while all salmonid species obtain two gene paralogues, each located on separate phylogenetic clusters. Atlantic salmon *trim25* paralogues clustered closely with brown trout, while rainbow trout, coho salmon and Chinook salmon formed a separate cluster. Our findings therefore suggest that the expansion of the salmonid *trim25* gene is likely attributed to the salmonid-specific whole genome duplication (Ss4R) whereas retention and clustering of gene copies indicates that divergence of these duplicates likely occurred in the common salmonid ancestor.

Focussing on Atlantic salmon, homology analysis was performed with Orthofinder[43] salmon to identify paralogous copies of un-annotated *trim25* in the Atlantic salmon genome (Supplementary Figure 1). Orthofinder analysis identified only two *trim25* genes in the same orthogroup; the closest homolog being *trim65* (ENSSSAG00000059081) which shares 36.36% identity with ENSSSAG00000054152 and 33.77% identity with ENSSSAG00000046838. To further verify the number of annotated copies of *trim25*, in addition to comparing 'TRIM25-Like genes' in the Atlantic salmon genome (Ssal_v3.1), annotated TRIM genes in the Ensembl Atlantic salmon genome database were aligned using MAFFT and a Maximum-likelihood phylogenetic tree was constructed (Figure 2).

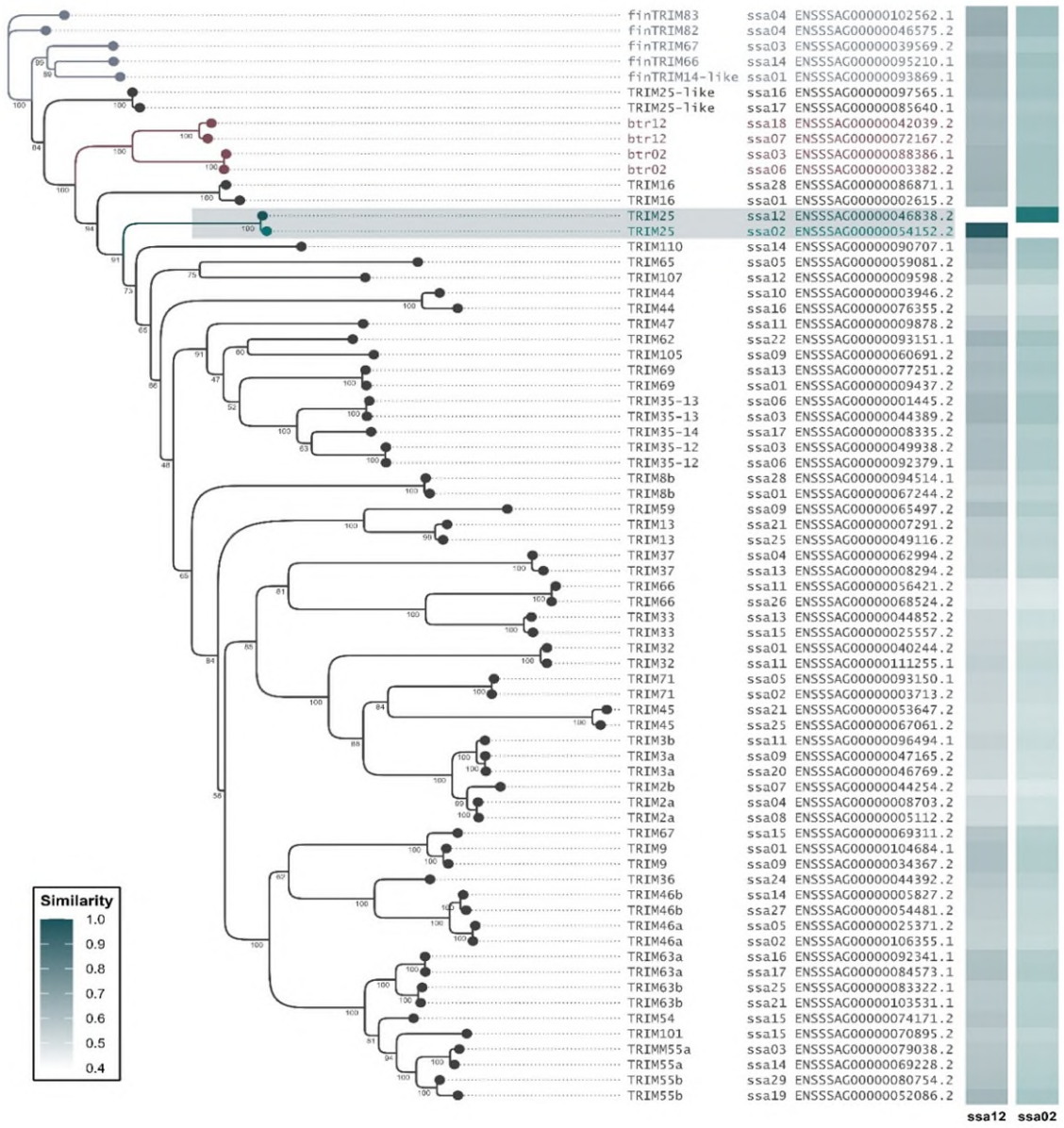


Figure 2: *Maximum likelihood tree for annotated TRIM genes in Atlantic salmon. Heatmap columns indicate the similarity between the two TRIM25 paralogs and each of the remaining TRIMs. Ultrafast Bootstrap values are indicated next to the corresponding node. TRIM25*

sequences used for alignment are Ssa02 ENSSSAG00000054152.2 and Ssa12 ENSSSAG00000046838.2.

The presence of only two *trim25* copies in the Atlantic salmon genome was confirmed, located on chromosome 02 (Ssa02) and chromosome 12 (Ssa12). The two copies share a high degree of homology, with 94.28% identity at the cDNA level (longest transcript), 97% at the RNA level (longest transcript), and 91.41% identity at the protein level (largest protein)[44]. Two additional TRIM25-like genes were identified in Ensembl, located on Ssa16 (ENSSSAG00000097565) and Ssa17 (ENSSSAG00000054152). However, both these genes shared only 28-31% similarity at the protein level with Ssa02 and Ssa12 *trim25* and do not form part of the same gene cluster.

Based on the observed sequence similarity, we investigated potential functional differences between the two *trim25* paralogs. Sequences from protein coding transcripts were aligned and functionally annotated based on the InterPro predicted annotations (Figure 3).

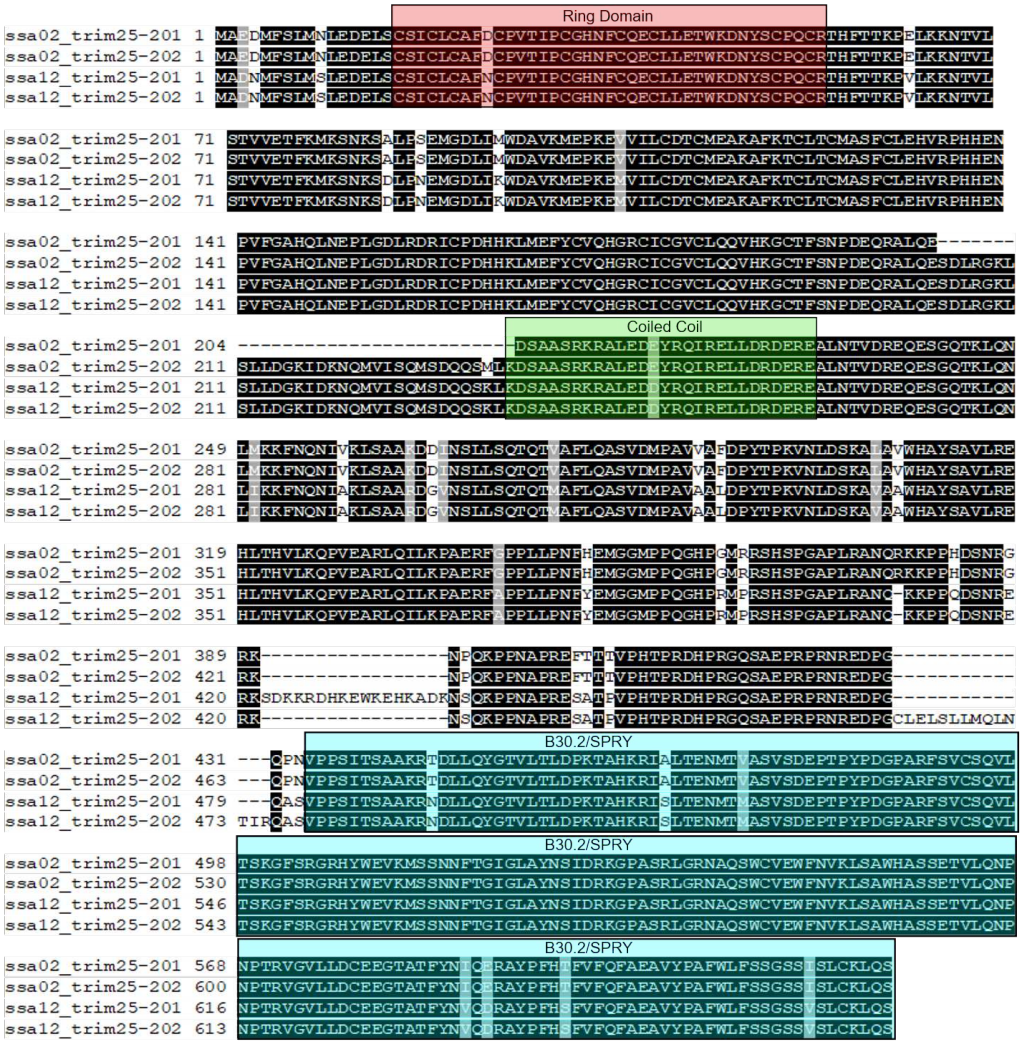


Figure 3: *Multiple sequence alignment of amino acid sequences of Ssa02 trim25 (ENSSSAG00000054152) and Ssa12 trim25 (ENSSSAG00000046838) associated protein*

coding transcripts from Ensembl. Domains are highlighted according to InterPro[45] representative domain as detailed in UniProt. Alignments created with T-Coffee[46].

Multiple sequence alignments of the protein-coding sequences of Ssa02 *trim25* and Ssa12 *trim25* show high levels of conservation (Figure 3), particularly in the catalytic E3 ligase RING domain. The RING domain alignment contains only one difference between peptide sequences, with an asparagine (N) replaced with aspartic acid (D) at position 25. The B30.2/SPRY domain is more variable, with 7 substitutions throughout the domain, perhaps reflecting the role of these domains as hypervariable regions which confer specificity of TRIM proteins with their substrate proteins.

Ssa02 trim25 is upregulated in response to Poly(I:C) stimulation in vitro and in vivo

To determine potential paralog-specific expression patterns and regulation, we used publicly available RNA, ChIP and ATAC-seq data of Atlantic salmon head kidney samples challenged *in vivo* and *in vitro* with the viral mimic Poly(I:C). This data was generated as part of the AQUA-FAANG project (Clark *et al.*[47]; Supplementary material 3; Table S4*)*. Using the data from this project, the expression of both paralogs was studied *in vivo* (head kidney tissue) and *in vitro* (primary cultures of head kidney leukocytes) upon Poly(I:C) stimulation – Fig. 4A. Ssa02 *trim25* showed significant upregulation in the RNA-seq dataset upon Poly(I:C) stimulation both *in vivo* (FC = 5.97) and *in vitro* (FC = 4.28), whilst Ssa12 *trim25* was not significantly upregulated – *in vivo* FC = 1.57, *in vitro* FC = 1.39 (Figure 4A).

In addition to transcriptomics information, a previously produced epigenetics dataset of histone modification (ChIP-seq) and chromatin accessibility (ATAC-seq) regulatory signals (peaks) was also analyzed, that was generated from the same [*in vivo/vitro*] viral stimulation challenge as the RNA-seq experiment [Naseer *et al.*[48], accession numbers *ATAC PRJEB50077, ChIP PRJEB56698 and RNA PRJEB50076*].

Chromatin immunoprecipitation (ChIP) and sequencing is widely used to determine the degree to which chromatin is associated with activating and/or deactivating epigenetic modifications and transcription factors. DNA is associated with acetylation of histone H3 (H3K27ac) – a marker of activate, open chromatin or methylation of histone H3 (H3k27me3) – a marker of closed, silenced chromatin. In combination with Assay for Transposase-Accessible Chromatin (ATAC) sequencing, these epigenetic sequencing technologies provide genome-wide identification of open chromatin regions, regulatory regions/elements and transcription factors.

ATAC sequencing revealed a higher degree of chromatin accessibility of Ssa02 *trim25* upon Poly(I:C) stimulation, whilst for Ssa12 *trim25* remained constant relative to PBS (Figure 4B). ChIP-seq data showed elevated H3K27ac (histone mark of gene activation) for both paralogs following Poly(I:C) stimulation, but the presence of the repressor mark H3K27me3 on the

promoter of Ssa12 *trim25* indicates that Ssa12 *trim25* remained silenced despite the increase observed in both ATAC and H3K27ac datasets. On the contrary, for Ssa02 *trim25* there was no presence of the repressor mark in the promoter regions following Poly(I:C) stimulation. Epigenetic sequencing revealed different regulation of the accessibility of the promoter regions for the *trim25* paralogs, suggesting that differences in expression are due to differences in genomic regulation of promoter activity.

Interferon-Sensitive Response Elements (ISRE) are DNA sequences found in the promoter regions of Interferon stimulated genes (ISGs). Following the autocrine or paracrine sensing of interferon, transcription factor Interferon Stimulated Gene factor 3 (ISGF3) binds to these regions, inducing the upregulation of hundreds of ISGs. Despite differences observed in ChIP sequencing of the promoter accessibility, ISRE motifs at promoter regions of the *trim25* genes are highly similar, with the sequence of Ssa02 *trim25* ISRE site (TTTCGTTC) only differing in one base to the Ssa12 *trim25* ISRE (TTTCGTTT**T**C) – Figure 4B ISRE track).

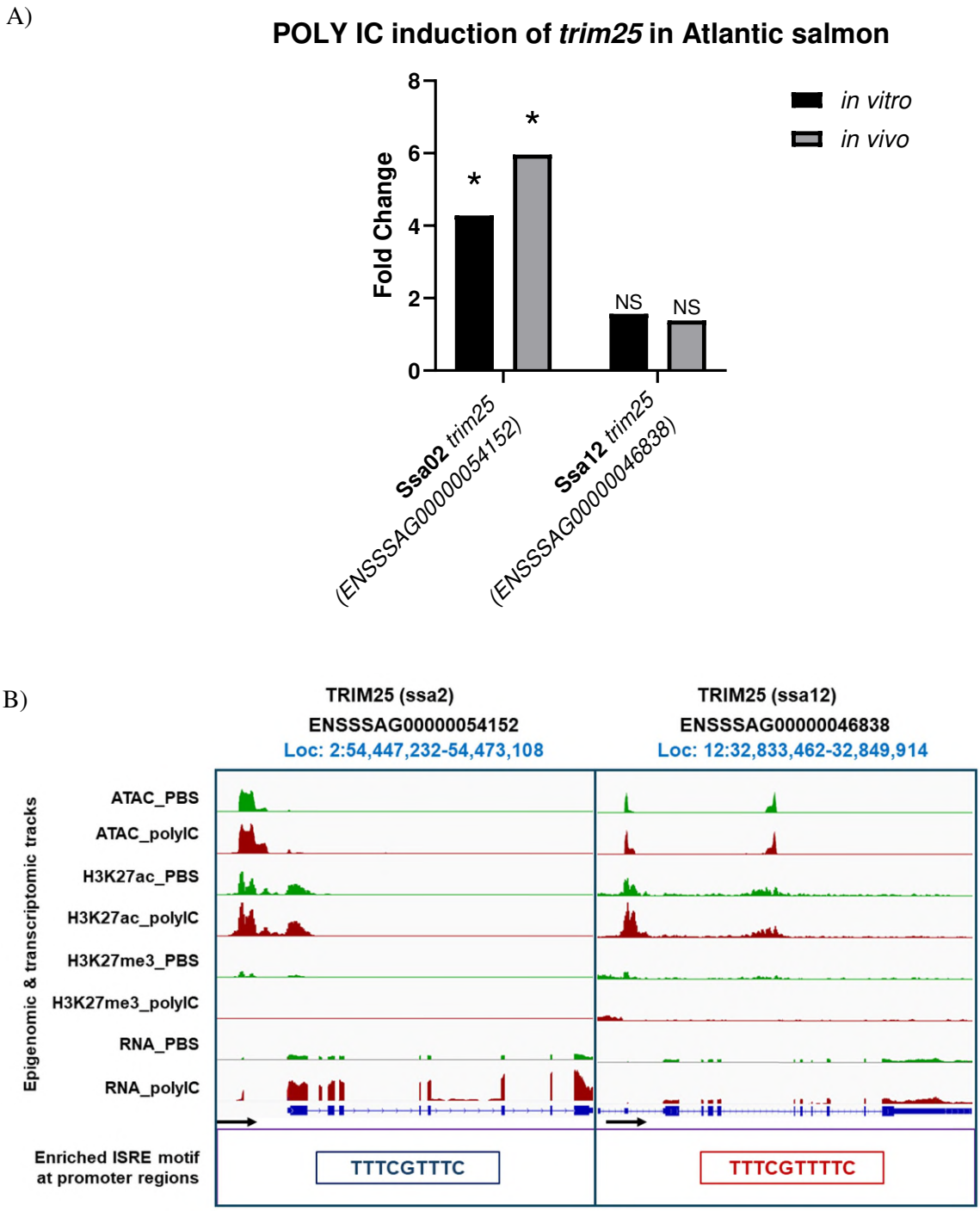


Figure 4: A) *Visual representation of RNA-seq fold change values for Atlantic salmon trim25 paralogs upon Poly(I:C) stimulation specific induction. In vivo data is from fish stimulated intraperitoneally with either PBS or Poly(I:C), whilst in vitro data is from primary leukocyte cultures derived from Atlantic salmon head kidney; RNA was sequenced and analysed as described by Clark et al.*[47] *as part of the AQUA-FAANG project.* ***B)*** *CHIP, ATAC and RNA sequencing functional annotation of PBS and Poly(I:C) stimulated Atlantic salmon in vivo data*

displayed using Integrative genomics viewer (IGV). Data generated by the AQUA-FAANG project[48]. ATAC and ChIP seq bigwig files were loaded into IGV for visualization of the tracks. For RNA-seq mapped bam files were loaded directly into IGV.

Ssa02 *trim25* but not Ssa12 *trim25* is virus-induced *in vitro*

To confirm the paralog-specific induction of *trim25* in response to viral pathogens, paralog-specific RT-qPCR primers on the divergent regions between the two copies of *trim25* were developed. The expression of each paralog was measured in ISAV infected SHK-1 cells from 0 to 48h post infection (Figure 5).

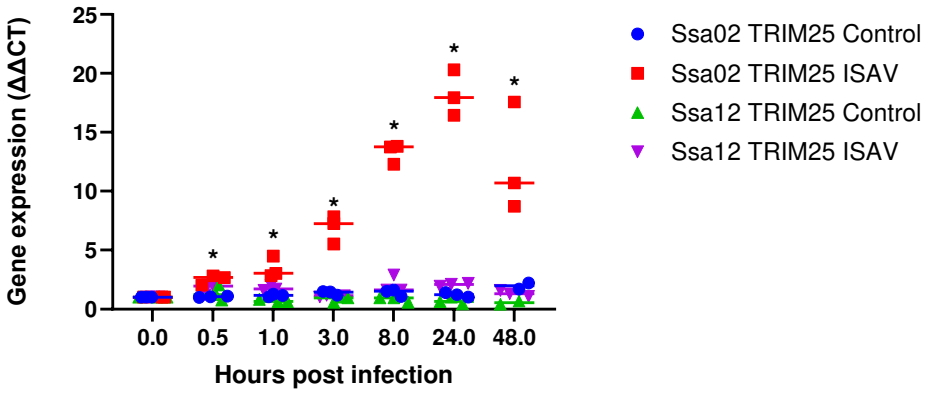


***Figure 5**: Trim25 paralog expression upon ISAV infection. Transcript levels of Ssa02 trim25 and Ssa12 trim25 were measured relative to the housekeeping gene (EF1α) using real-time qPCR. qPCR products (n = 3 per paralog) were sequenced with light-run sequencing to confirm specific amplification. Area under curve analysis was used to determine overall significant differences between expression – Supplementary Figure 2, and One-way ANOVA with Dunnett's multiple comparisons test used to determine significant differences at each time points, denoted by (*), * = p value <0.05.*

Ssa02 *trim25* is significantly upregulated from 0.5h post infection, reaching a peak at 24h post infection and remaining significantly upregulated until 48h, whilst Ssa12 *trim25* expression remains unchanged over the time course of infection. Having confirmed the virus inducibility of *trim25*, the next step was to determine whether *trim25* is functional in viral replication.

Sa02 *trim25* knock out, but not Ssa12 *trim25*, results in increased ISAV replication

Having confirmed that *trim25* is inducible by viral infection, the next step was to determine whether TRIM25 plays a functional role in viral replication. In addition, targeted editing of paralogous copies would provide further evidence for sub functionalization. Using CRISPR–Cas9, with guide RNAs (gRNAs) designed to target divergent regions of the *trim25* paralogs (Figure 6A), we efficiently edited both copies of *trim25*. Editing efficiencies and knockout

scores exceeded 75% (Figure 6B). Highly efficient and specific editing was achieved for Ssa02 *trim25*, Ssa12 *trim25*, as well as for both copies simultaneously. Sequencing traces confirming the edits are provided in the supplementary material (Supplementary figures 1–3).

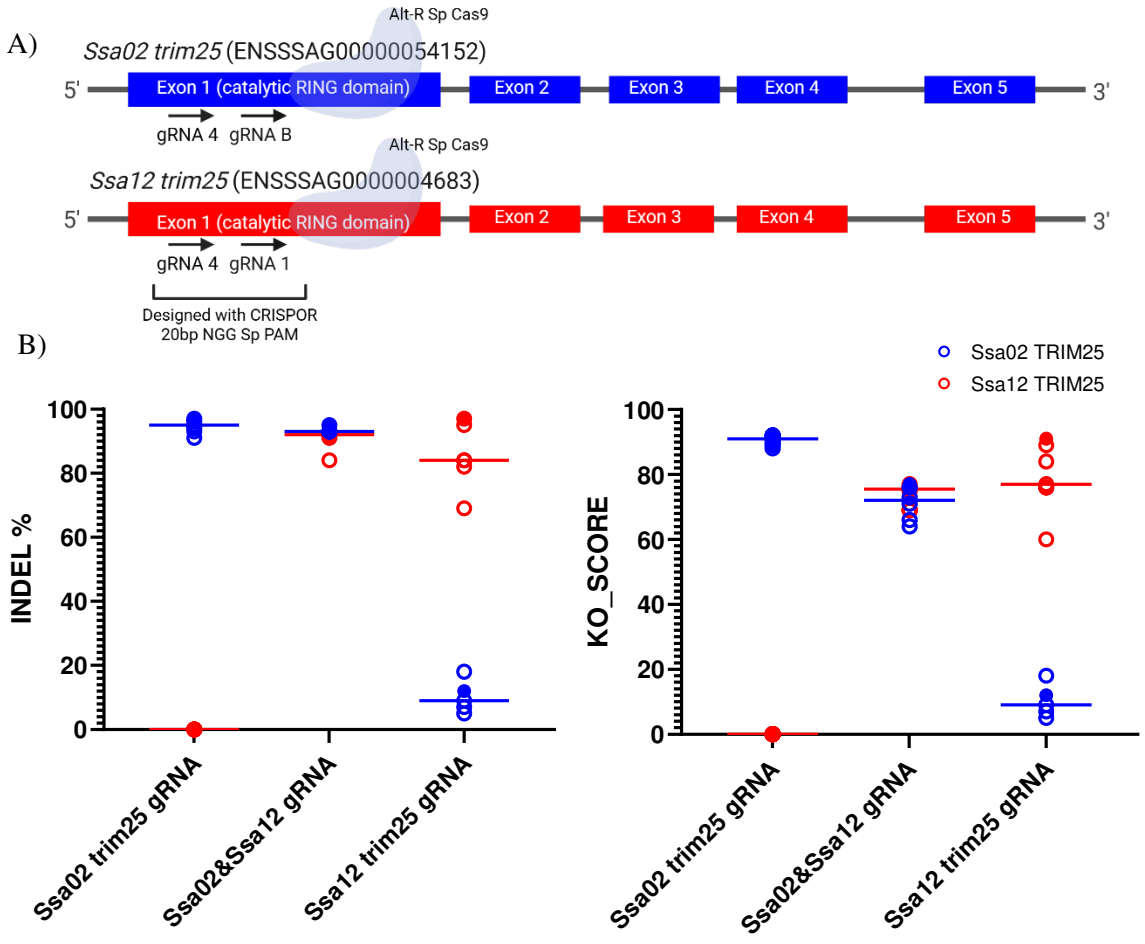


***Figure 6**: A) Schematic of gRNA and primer design for trim25 editing. All gRNAs target the first coding exon. gRNA 4 targets both paralogs of trim25, whilst gRNA B targets Ssa02 trim25 specifically and gRNA 1 targets Ssa12 trim25 specifically. B) Editing efficiencies (INsertion DELetion efficiency left, Knock Out score right) achieved with paralog-specific gRNAs. KO_Score is calculated by summing frequencies of frame shift mutations + deletions >21 base pairs. Analysis performed in ICE Synthego[49].*

To determine the impact of paralog-specific editing of Ssa02 *trim25* and Ssa12 *trim25*, polyclonal cell pools were infected with ISAV, and ISAV segment 8 RNA was quantified via RT-qPCR and TCID50. Editing and knockout percentages at the time of infection are shown in Figure 7A.

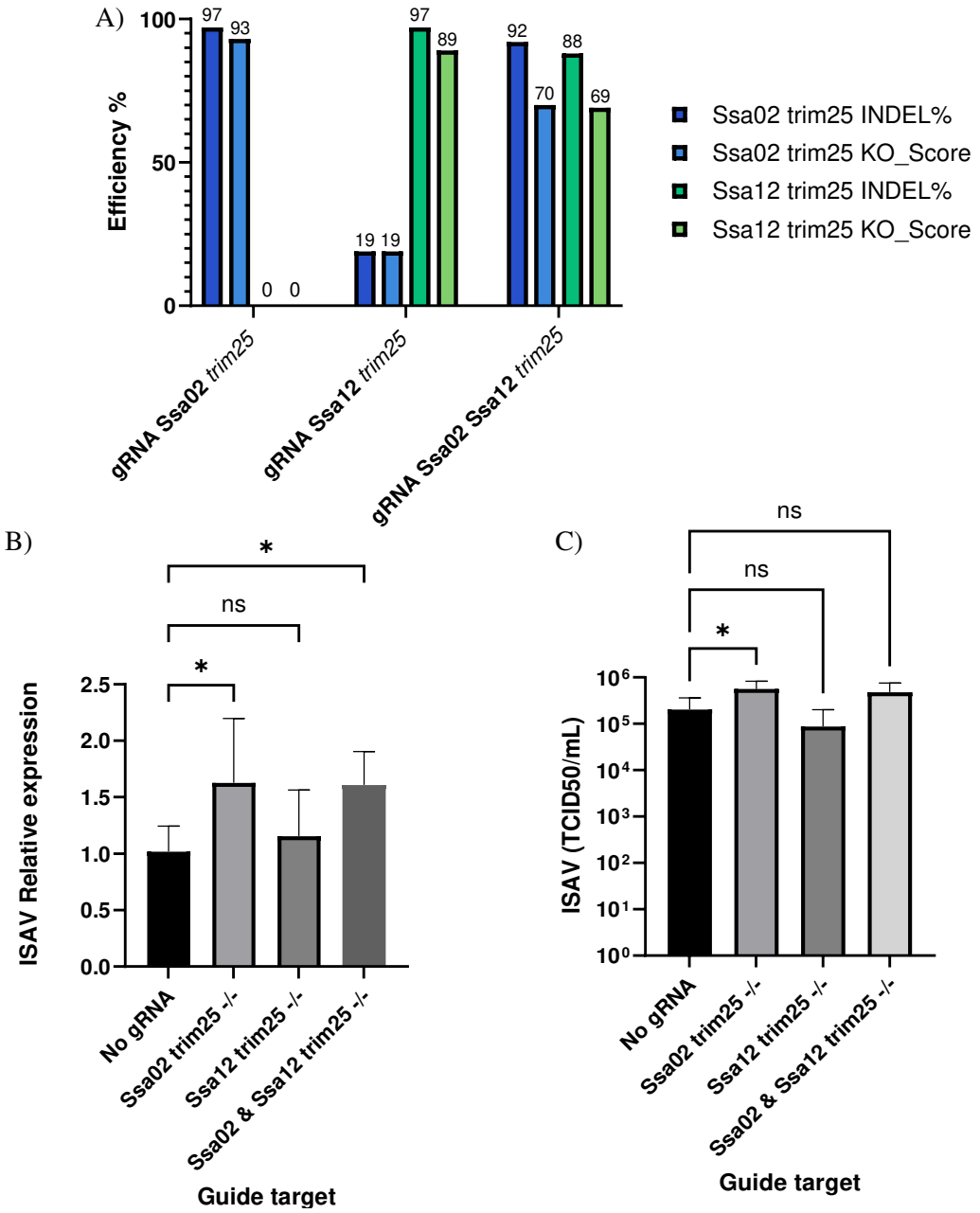


***Figure 7**: A) Editing efficiency of trim25 knock-out polyclonal SHK-1 cell pool at Day 0 of ISAV infection. B) Intracellular ISAV Viral segment 8 expression in TRIM25 knockouts assessed by RT-qPCR and C) by TCID50 (Extracellular virus). Viral load was assessed at 7dpi, MOI 0.01, n = 6. Sequencing traces from ICE Synthego analysis tool[49] are included in supplementary file Figure 2. Statistical significance was determined by One-way ANOVA, with multiple comparisons determined by Dunnett's multiple comparisons test. * = p value < 0.05.*

ISAV infection of the Ssa02 *trim25* knockout and the double knockout polyclonal cell pools resulted in a higher titre of intracellular ISAV than wild type SHK-1, whilst Ssa12 *trim25* knock out did not induce a higher titre of ISAV (Figure 7B). The titre of viable ISAV particles in the

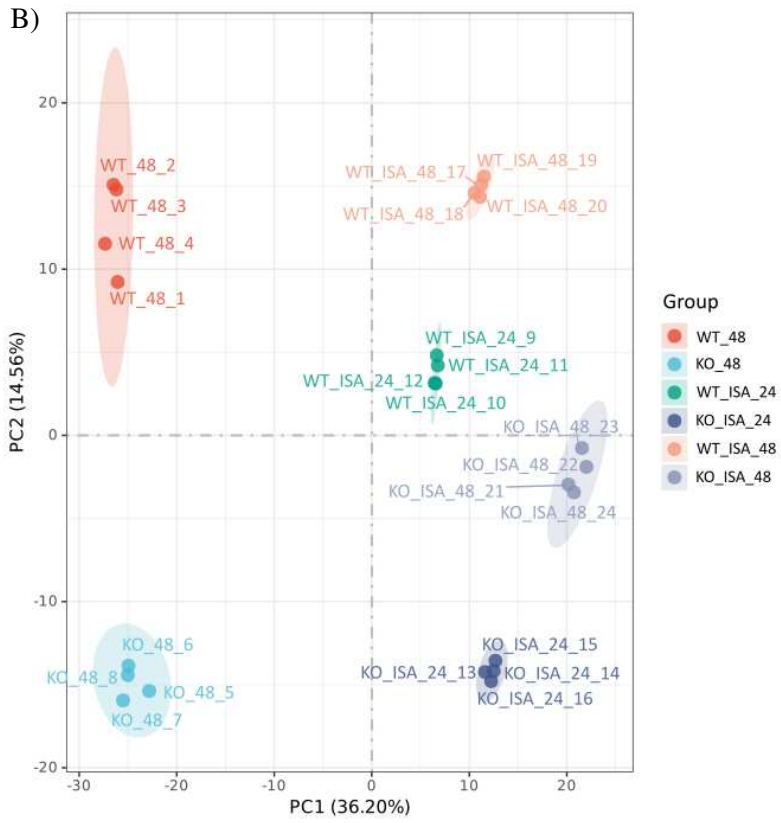
cellular supernatant was assessed by TCID50 and confirmed increased production of ISAV in Ssa02 *trim25*, yet not in the double knock-out (Figure 7C).

RNA sequencing of knock-out cells highlights the role of *trim25*

To determine the transcriptomic changes resulting from the knock-out of both paralogues of *trim25*, RNA sequencing was performed on uninfected and ISAV infected SHK-1 cells with polyclonal double-knock out (Ssa02 + Ssa12 *trim25* KO) and wild-type controls (non-targeting guide), 24 and 48 hours post infection. Editing percentages at time of infection are presented in Figure 8A. Sequencing resulted in an average of 47,623,243 per sample (range 42,287,294 – 54,340,954), with an average of 84% uniquely mapped reads (83.34%-86.87%) to the Atlantic salmon genome.

A)

Ssa02 *trim25*		Ssa12 *trim25*	
INDEL	**KO_Score**	**INDEL**	**KO_Score**
84%	62%	94%	75%



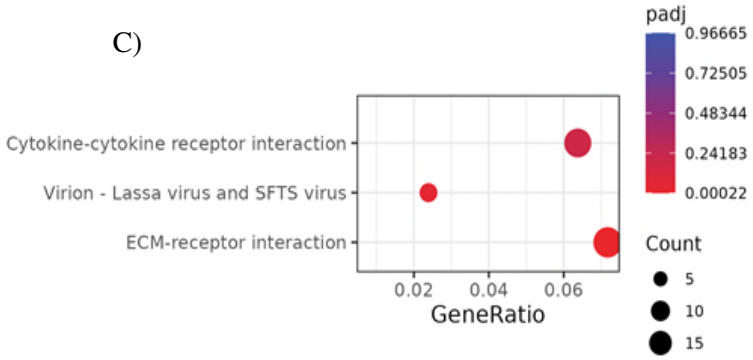


Figure 8: *A) Trim25 editing efficiency of polyclonal SHK-1 cell pools at the time of infection (Day = 0). B) Principal component analysis of ISAV infected trim25 polyclonal KO SHK-1 cells and wild types. WT = No Guide RNA (negative control), ISA_24 = 24h post ISAV infection, ISA_48 = 48h post ISAV infection, KO = gRNA targeting both copies of Atlantic salmon trim25. C) KEGG dot plot showing differentially expressed pathways in uninfected WT vs KO cells.*

Principal component analysis of WT (Non-targeting guide) and KO (Ssa02 & Ssa12 *trim25* KO) at 24 and 48 hours post infection demonstrates compact clustering of biological replicates, with the different experimental groups separated based on gene editing status (PC2) and infection status (PC1) (Figure 8B). In non-infected cells, differential gene expression resulted in the expression of 567 unique genes in KO cells, and 853 unique differentially expressed genes in WT cells. KEGG pathway enrichment analysis revealed that these unique genes down regulated in KO cells have roles in cytokine-cytokine receptor interaction, ECM-receptor interaction and viral (Lassa and SFTS) pathways (Figure 8C). Cytokine-cytokine receptor associated genes include Tumour Necrosis Factor Superfamily Member 11 (*tnfs11*), a tumour necrosis factor superfamily 14-like gene (ENSSSAG00000099494), immune regulators (*csf2rb*; Colony Stimulating Factor 2 Receptor, *CXCR3*; C-X-C motif chemokine receptor 3, ENSSSAG00000001430; interleukin-2 receptor subunit, *M17*; IL-6 subfamily cytokine, *il16*; Interleukin 16 and ENSSSAG00000006510; permeability factor 2) and regulators of JAK-STAT pathway (*prl2*; Prolactin 2)

Differences in transcriptomic responses between wild type and edited cells were detected by comparing gene expression levels of samples treated with ISAV virus and control conditions i.e. (WT vs WT + ISAV) v (G4 vs G4 + ISAV). Total read counts of *trim25* showed a measurable decrease in the abundance of trim25 reads across uninfected and infected cells (Figure 9A), additionally the larger decrease in Ssa12 *trim25* is consistent with the higher editing efficiency at time of infection (Figure 8A). Despite a reduction in abundance of *trim25* at all given conditions, significant differences when comparing WT vs G4 are only seen for Ssa12 *trim25*. In uninfected cells Ssa12 *trim25* abundance is significantly reduced (FC = 0.33), and interestingly upon ISAV infected this reduction is significant and consistent over both timepoints – 24 hours FC=0.45 and 48 hours FC=0.45, indicating that *trim25* expression levels does not respond to infection.

When comparing *trim25* expression in WT cells alone, the paralog previously discussed specific induction of Ssa02 *trim25* is confirmed by the significant upregulation of Ssa02 *trim25* – 5.0-fold change at 48 hours post infection (p adj. 1.93E-146) whilst Ssa12 *trim25* is not differentially expressed at 24 or 48 hours in WT SHK-1 cells upon infection.

Since in model species TRIM25 typically acts through the activation of the Rig-like receptor pathway, we analysed the genes in this pathway (KEGG pathway sasa04622) to determine the impact of the reduction of *trim25* abundance (Figure 9B). The genes down-regulated in the ISAV-infected KO compared to the ISAV-infected WT include MAVS (WT FC = 319, KO = 6.41), MDA5 (WT FC = 2.95, KO FC = NS), MITA (WT FC = 3.90, KO = 2.73), p38 (WT FC =2.27, KO = NS), IFN α (WT FC = 76.3, KO = 41.5) and CXCL10 (WT FC = 319, KO = 48.9). This indicates a disruption of the Rig-like receptor pathway as a result of *trim25* knock-out,

despite not seeing reductions in abundance of RIG-I itself (Figure 9C). It should be considered that the predicted activating mechanism of *trim25* is ubiquitination (K63) and stabilisation of RIG-I: MAVS interaction. Accordingly, *trim25* knock out may not alter the transcriptional levels of RIG-I transcripts, but rather act exclusively on the ubiquitination and activation state of the protein.

Genes upregulated in *trim25* knock-out immune response relative to wild type immune response include *DDX58* (RIG-I), *DHX58* (LGP2), *ZNFX1*, Ssa02 *trim25*, *ADAR*, *IRF3* and *IRF7.* This upregulation may be indicative of compensational upregulation of these genes in response to lack of anti-viral immune response due to knock-down of RLR signalling.

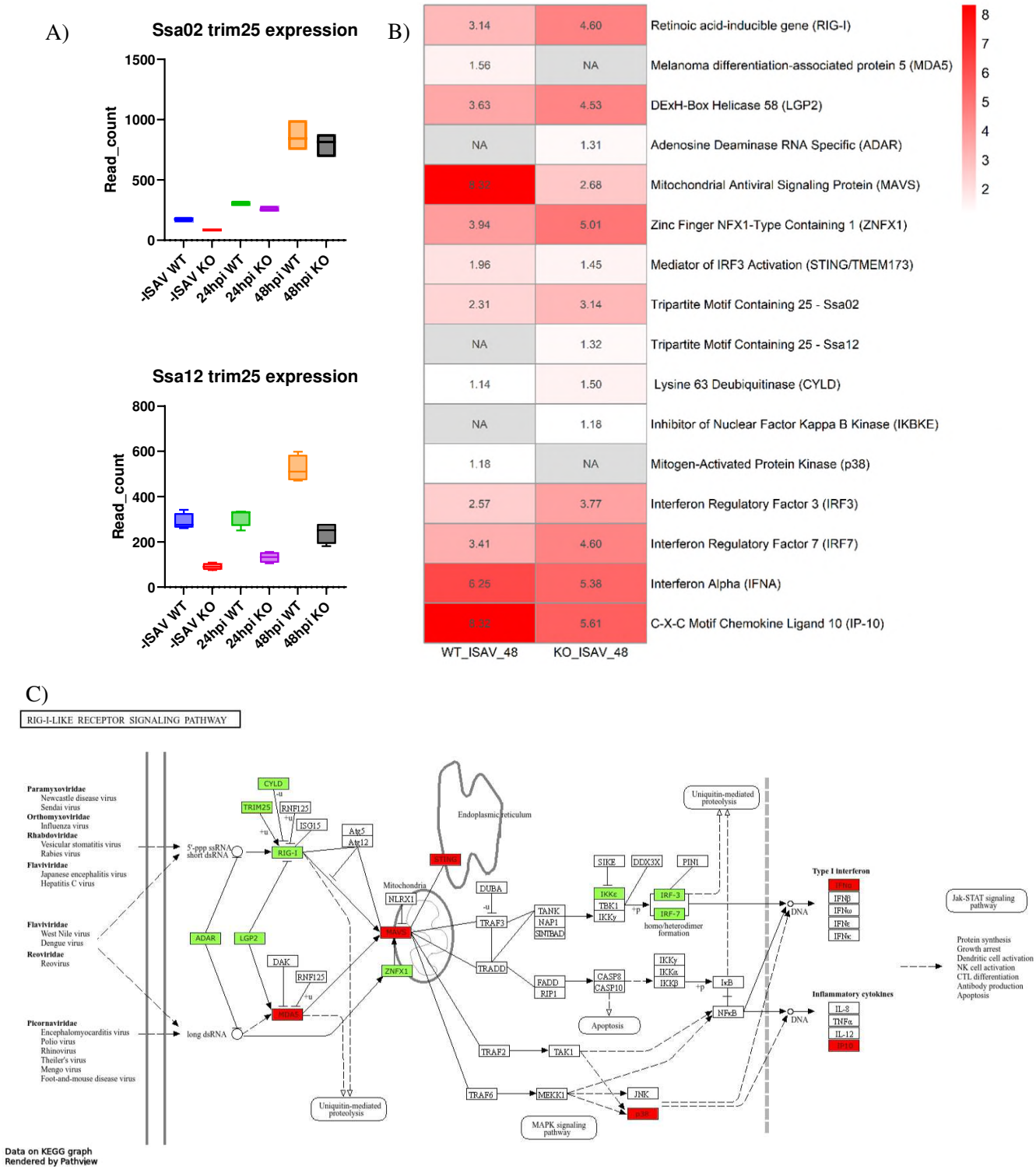


***Figure 9**: A) trim25 read count averages across 4 samples in WT and KO. -ISAV – non-infected controls. KO = Knock out WT = Wild type. B) Genes identified in KEGG pathway enrichment belonging to KEGG sasa04622. Log2FC is calculated relative to un-infected, time matched control of same genotype (i.e. WT vs WT ISAV 48hr, KO vs KO ISAV 48hr). Colour represents Log2FC. Genes possessing non-significant fold changes in either group are not displayed. Where multiple copies of genes are upregulated, an average FC is displayed. C) Annotated KEGG pathway sasa04622 red indicates genes with lower fold change following infection in edited vs wild type, green indicates genes with higher fold change following infection in edited vs wild type.*

trim25 over-expression protects cells from infection

To determine whether higher expression of *trim25* could lead to an enhanced immune response against ISAV, CHSE-214 cells were transfected by electroporation with either a *trim25* expressing plasmid (pcTRIM25_flag) or the empty plasmid backbone(pcDNA 3.1). Recombinant *trim25-flag* expression was confirmed using RT-qPCR (Figure 10A). Control (backbone) and *trim25* overexpressing cells were infected with ISAV, viral copies were quantified utilizing RT-qPCR for segment 8 as previously described (Figure 10B).

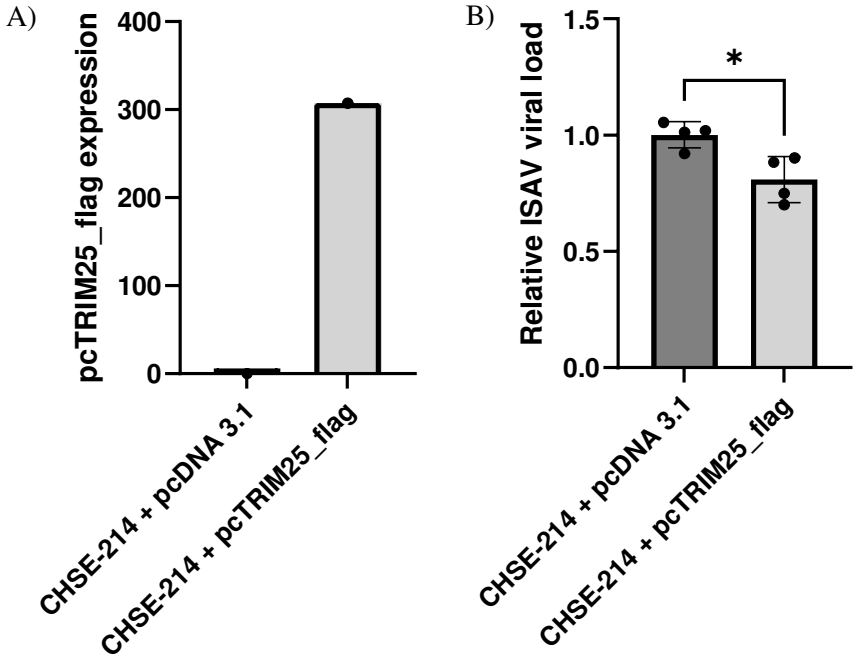


Figure 10: *A) Recombinant trim25 expression from polyclonal cell pools overexpressing either pcDNA 3.1 (+) or pcTRIM25_flag following long term antibiotic selection. Primers designed to recombinant trim25_flag plasmid. RT-qPCR performed with -Reverse transcriptase control. Gene expression values calculated relative to housekeeping gene EF1a. (n = 1) B) Relative viral copy number of pcDNA 3.1 and pcTRIM25_flag expressing CHSE-214 cells infected with 0.1 MOI ISAV at 7dpi. Viral copy number quantified by RT-qPCR and calculated relative to housekeeping gene EF1α. Statistical significance determined by unpaired T test (* = p value < 0.05, n = 4).*

5. Discussion

In the current study, the E3 ubiquitin ligase gene *trim25* was characterised and mechanistically interrogated in Atlantic salmon. Applying molecular biology, genome editing and RNA sequencing with bioinformatic tools, we investigated the role of the two *trim25* paralogs in the Atlantic salmon genome, and confirmed the role of the paralogs located in Ssa02 in anti-viral immune response. *Trim25* has to date been demonstrated to have anti-viral activity in zebrafish[24], orange spotted grouper[14], rainbow trout[17], Japanese flounder[16] and common carp[21], while only virus-inducibility has been described so far in Atlantic salmon[26-29], Nile tilapia[15] and Grass carp[21]. To date, no TRIM genes have been characterised in Atlantic salmon, and *trim25* represents a good starting point to characterize the complicated TRIM repertoire of this valuable aquaculture species.

Atlantic salmon TRIM25 has two copies, resulting from WGD

The Atlantic salmon shares three whole genome duplication (WGD) events with other teleost fish, two ancestral to vertebrates and another teleost specific (Ts3R), in addition to having undergone a fourth salmonid-specific (Ss4R) duplication around 80 million years ago[9]. This offers the opportunity to investigate genome evolution following WGD duplication events. In this study, the focus is the *trim25* gene, part of the E3 Tripartite Motif ubiquitin ligase family. There are multiple conserved TRIM genes across humans and fish, amongst these is the TRIM25-like family[6]. As shown in our study, *trim25* was duplicated in the Ss4R salmonid-specific duplication, leading to two paralogous copies of *trim25* in Ssa02 and Ssa12. The location of these *trim25* paralogues is consistent with Lien *et al.*[9], where regions of high homology are observed between Ssa02 and Ssa12, suggesting a common origin from a single chromosome present in the salmonid common ancestor. Whilst other genes in the Atlantic salmon genome are annotated as “TRIM25-LIKE” in Ensembl, they only share moderate homology with *trim25*.

Atlantic salmon *trim25* retains the canonical TRIM protein architecture, comprising a catalytic RING domain, B-box, and coiled-coil (R–B–CC) domains. Accordingly, it is expected to support the ubiquitin-conjugating activity characteristic of E3 ubiquitin ligases. Sequence alignment of the two *trim25* paralogues shows that only one residue differs in the RING domain. While specific changes in the RING domain of *trim25* have been shown to alter its antiviral function – R54P substitution abolishes the action of human *trim25* against alphavirus by preventing *trim25* mediated ubiquitination[50] - both *trim25* paralogues retain this catalytic arginine residue at this position, suggesting that structurally both could be functional. Additionally, both cysteine residues 50 and 53, required for antiviral activity in human *trim25*, are conserved in both copies of Atlantic salmon *trim25*[51]. Finally, lysine is required at position

65 in human *trim25*, and again is conserved in both copies of *trim25* in salmon[52].Taken together, the RING domain in both copies of Atlantic salmon TRIM25 retains the catalytic residues necessary for the anti-viral activity characterized in human TRIM25, therefore any functional differences between these two genes / proteins are most likely not the result of non-functional catalytic RING domains.

The B-box domains of both copies of *trim25* are identical except for one residue – M/V at residue 106. This is a conservative substitution, and as such should maintain the structure and function of the B-box. The B-box is associated with formation of higher order structures and self-association[53]. Therefore, it is unlikely that a lack of oligomerization ability due to differences in the B-box domain would explain any differences in the anti-viral capacity of the two Atlantic salmon TRIM25 proteins.

The largest differences between the two *trim25* paralogs are observed in the PRY/SPRY domain, a region which defines the RNA binding capacity of *trim25*[54]. Further, the PRY/SPRY region has been suggested to act as a scaffold for the recruitment of other RNA-binding proteins, in addition to allowing *trim25* to auto-ubiquitinate[55]. Therefore, the differences between the two Ssa02 and Ssa12 *trim25* paralogues may lead to binding to different substrates in different pathways. Further study of whether the differences observed in this domain have an effect on substrate affinity and antiviral properties would be possible via recombinant expression of these two proteins. This would shed light on the function of both copies of *trim25*, and derive whether the functional differences are due to protein structure or genomic regulation.

Utilizing publicly available data from the AQUA-FAANG project, it was possible to combine epigenetic and transcriptomic data to decipher the differential expression and regulation of both *trim25* paralogues. The data demonstrates that Poly(I:C) stimulation removes the repressor mark (H3K27me3) from the promoter region upstream of the Ssa02 *trim25* gene, facilitating its significant upregulation. In contrast, the repressive mark at the promoter region upstream of the Ssa12 copy remains unchanged following Poly(I:C) stimulation, which correlates with the non-significant upregulation of this gene copy observed in the transcriptomic data. The epigenetic changes observed in Ssa02 *trim25* translate into high inducibility from Poly(I:C) both *in vitro* and *in vivo*, whilst the epigenetic silencing of the promoter region of Ssa12 *trim25* results in a sustained repression upon Poly(I:C) stimulation.

Paralogue-specific RT-qPCR in Atlantic salmon cells (SHK-1) upon ISAV infection confirms that the chromosome 2 copy of *trim25* is the main copy of *trim25* upregulated in response to infection. Previously, the inducibility of *trim25* has been demonstrated in immortalized cell lines, primary cell cultures and in fish in response to viruses[25-28,56,57] and viral mimics[47,48].

However, the limitations of RNA sequencing; namely shotgun sequencing and read sharing make the interpretation of RNA sequencing complicated for duplicated genomes; hence the validation of unbalanced paralogous expression by RT-qPCR in this project. It remains to be seen whether the imbalanced induction is unique to types of viral genome classes (e.g. RNA vs DNA viruses), or certain pathogens (viruses vs bacterial pathogens). As more high-quality RNA sequencing data – particularly high quality, long read sequencing - becomes available for a broader range of pathogens, it will be possible to determine the *trim25* response of a broad range of bacterial and viral pathogens, which would add a deeper understanding to the divergent roles of these paralogs.

Enriched interferon-stimulated response elements (ISRE) motifs at promoter regions are highly similar, with the sequence of their ISRE sites only differing in one base. The impact of this difference is unknown as ISRE sites are understudied in Atlantic salmon, however it has been demonstrated in humans that single nucleotide polymorphisms (SNPs) in ISRE promoter sites can dramatically alter the expression of a gene[58], and in rainbow trout that ISRE site number and location can drastically alter gene expression[59]. The impact of SNPs on gene expression is not currently understood in salmon, despite evidence that they can be highly impactful in humans, and therefore warrant further studies on a broader range of paralogous genes.

Only Ssa02 trim25 retains the ancestral antiviral function

Using CRISPR-Cas9 genome editing, we specifically and efficiently edited the two paralogs of *trim25* both independently and together. Often the presence of multiple gene copies makes CRISPR editing challenging, however our study demonstrated that by developing guide RNAs targeted to unique regions of the gene it is possible to specifically target highly similar gene copies, which has allowed us to demonstrate the sub-functionalization of *trim25* gene copies. Knock-down of Ssa02 *trim25* or Ssa02 and Ssa12 simultaneously led to an increase in ISAV viral load, whilst Ssa12 *trim25* knockdown did not lead to a significant increase. TCID50 analysis of viral titre confirmed the significant increase of infective viral particles in Ssa02 *trim25* KO cells, however the double KO (Ssa02 and Ssa12 *trim25* KO) did not produce significant differences. This may in part be explained by the differing sensitivities of TCID50 relative to RT-qPCR, or perhaps the intracellular and the extracellular (supernatant) virus amounts did not correlate at this timepoint. Samples were taken at 7 days post infection which is typically prior to cytopathic effect, therefore viral content of supernatant is likely a result of budding and/or exocytosis[60]. Additionally, whilst qPCR controls for cell number by calculating viral genomes relative to the expression of a housekeeping gene, TCID50 does not normalise and may be highly influenced by cell number and evaporation / concentration of supernatant.

Lastly, whilst these results show a significant difference in viral genome copies and viral titre, the polyclonal cell pool nature of this work dilutes the effect of editing. Further refinements and validation of these results should include the establishment of a single cell clone using a suitable cell line (SHK-1 has not been used for single cell cloning to date), and alternatively technologies such as base editing could be utilized to create an edited cell pool with only two genotypes instead of the many varied edits typically observed when utilizing the NHEJ pathway for Cas9 editing. Current research into Atlantic salmon gene function using CRISPR editing often relies on cells from different salmonid species; normally CHSE-214 (Chinook)[61], which are single cell cloneable and therefore allow the isolation of a uniform cell pool. Whilst this is a valid technique, it requires switching the species in the interest of an improved methodology.

Atlantic salmon trim25 knockout leads to key changes in the antiviral response

The effect of knocking out both copies of *trim25* in SHK-1 cells following infection with ISAV was determined by RNA sequencing. By comparing the transcription profiles of infection between wild type and knock-out we can see that numerous elements of the Rig-like receptor pathway are disrupted; most notably MAVS and IFNα. These changes suggest that *trim25* is acting through the canonical RLR pathway in Atlantic salmon. IFN signalling in teleost fish is highly redundant, complex and operates as a positive feedback loop. Therefore, given the polyclonal nature of the knock-out cells it is entirely feasible that the autocrine and paracrine signalling capacity of IFNα through the INAR1 and IFNAR2 in the JAK-STAT pathway confers protection to even cells that lack the initial capacity to activate the RLR pathway due to the lack of TRIM25-RIG-I activation.

Trim25 is a ubiquitin ligase involved in a wide range of cellular, immune and homeostatic pathways. It has continuously been demonstrated it has a key role in the cellular death pathway[62] where high expression of *trim25* in cancer patients is associated with poor prognosis[63,64]. *Trim25* is a global transcriptional regulator in the centre of metastasis-related transcriptional networks, highlighting the multifaceted role of this gene[50,65]. Therefore, it is unsurprisingly that when comparing WT to knock out cells that many metabolic, cell cycle and immune pathways are likely dysregulated in RNA sequencing. This further confounds the interpretation of *trim25* knock out on the RLR signalling pathway.

Trim25 overexpression leads to partial protection against ISAV in Atlantic salmon cells

Finally, stable transfection of CHSE-214 cells with *trim25* showed that over expression of TRIM25 partially protects these cells from ISAV infection. Although transient over-expression of plasmid was considered for this experiment, transfection efficiency is typically below 10%

for fish cells[66] and therefore a stable integration and long-term selection technique was utilized.

Over expression is a typical way of studying the role of a gene, however it is not without limitations. Introducing foreign DNA, antibiotic selection and electroporation all disrupt cellular processes. Nonetheless, we showed that *trim25* overexpression increases the protection against ISAV relative to cells that had undergone the same procedures but with an empty vector. Further work is ongoing to determine whether the nature of this protection is due to viral destabilisation as described by Choudhury *et al*.[20] or increased activation of the RLR pathway[67].

Our research provides valuable insight into the involvement of *trim25* in antiviral immunity and RLR signalling. Previous research has identified *trim25* as being upregulated in fish with higher resistance to ISAV[28]. By combining the insight provided in our research, with this information it may be able to determine the genetic basis for this resistance, and selectively breed for higher ISAV resistant lines. Further validation of the substrates and pathways of *trim25* will be beneficial to validate the substrates and mechanism, perhaps by the harnessing of synthetic biology tools such as substrate trapping strategies[50,68].

6. Conclusion (200 words)

This study characterises the Atlantic salmon *trim25* gene, an E3 ubiquitin ligase involved in antiviral defence. Following the salmonid specific Ss4R genome duplication, two *trim25* paralogues were retained and are conserved across salmonids. Although both paralogs maintain key structural domains associated with antiviral function, our results show evidence of functional divergence, with only Ssa02 *trim25* actively involved in the antiviral response to ISAV infection. Functional experiments demonstrate that disruption of Atlantic salmon *trim25* increases viral load, whilst its overexpression confers protection, with transcriptomics highlighting its role in the RIG-I–like receptor pathway. Overall, these findings provide the first functional evidence of *trim25*-mediated antiviral defence in Atlantic salmon and demonstrate sub-functionalisation of duplicated *trim25* genes following the salmonid genome duplication. By characterising genes and determining how they regulated the immune system we can deepen our understanding of the anti-viral immune response in fish. In high value aquaculture species such as Atlantic salmon, the impact of this research could be applied through the genomic selection and breeding of fish with more robust, stronger immune responses, which may contribute to the reduction in the burden of infectious disease in aquaculture.

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