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Original Research

Socs3a is Dispensable for Zebrafish Hematopoiesis and is Required for Neuromast Formation

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Abstract

Background: Suppressor of cytokine signaling (SOCS)3 is a regulatory protein that participates in an important negative feedback loop downstream of several critical cytokines, especially members of the interleukin-6 (IL-6) family. As a result, SOCS3 has been shown to impact the development and function of blood and immune cells. Zebrafish harbor duplicates of SOCS3, Socs3a and Socs3b, both of which possess conserved functional domains. **Methods**: This study explored the role of zebrafish Socs3a by creating a whole genome knockout using CRISPR/Cas9, with a focus on hematopoiesis and neuromast formation. **Results**: A zebrafish Socs3a knockout mutant was successfully generated. Characterization of this mutant revealed that normal hematopoiesis was not impacted nor was neutrophils lacking Socs3a displayed normal responses to injury or their production during emergency granulopoiesis. Neuromast formation was severely impacted in Socs3a knockout zebrafish. **Conclusions**: Zebrafish Socs3a mutants display normal hematopoiesis and myeloid function, but the formation of the lateral line neuromast was affected by the absence of Socs3a.

Keywords: cytokine; suppressor of cytokine signaling 3 protein; myelopoiesis; zebrafish

1. Introduction

The suppressor of cytokine signaling (SOCS) family of regulatory proteins participates in a negative feedback pathway downstream of cytokines and other factors. Among the SOCS family of proteins, SOCS3 plays a number of roles in the regulation of hematopoiesis as well as the function of immune cells, specifically the myeloid lineage [1], shown to be mediated by its SOCS box domain [2]. SOCS3 is induced principally by cytokines that activate signal transducer and activator of transcription 3 (STAT3), which includes interleukin 6 (IL-6), leukemia inhibitory factor (LIF) and granulocyte colony-stimulating factor (G-CSF) [3,4], Critical roles have been identified for SOCS3 in the generation and regulation of neutrophils and other myeloid cells, with critical roles in controlling inflammation and infection [1]. It also participates in diverse other roles, such as in placental trophoblasts [5].

Elevated expression of SOCS3 has been found to correlate with the development of chronic inflammatory diseases including inflammatory bowel disease and rheumatoid arthritis [6,7]. Conversely, decreased expression or mutations of SOCS3 have also been implicated in the etiology of other disorders like Crohn's disease as a byproduct of uncontrolled inflammation [8]. Furthermore, as STAT3 is an oncogene, SOCS3 is considered a tumor suppressor protein [9]. Suppression of SOCS3, typically through methylation, often results in enhanced proliferation and tumor development due to loss of negative regulation within various pathways [10,11]. As a result, a number of SOCS3 mutations, both loss of function and activating are implicated in various hematological malignancies [12,13].

The zebrafish has been increasingly used as a model to study hematopoiesis and immune cell function [14,15]. Intriguingly, zebrafish contain two SOCS3 molecules, termed Socs3a and Socs3b, with both displaying conserved functional domains [16] and induction downstream of STAT3 proteins [17]. Moreover, zebrafish knockouts of Stat3 [18] and Socs3b [19] indicated the presence of a conserved STAT3/SOCS3 module in the control granulopoiesis and myeloid function, particularly inflammation. In contrast, Socs3a has been implicated in regeneration [17] and the development of the fish-specific neuromast organ [20], suggesting it may have divergent functions.

To better understand the functional split between the zebrafish SOCS3 duplicates, we generated a Socs3a knockout line using Clustered Regularly Interspaced Short Palindromic Repeats / CRISPR-associated protein 9 (CRISPR/Cas9) for further characterization. In contrast to Socs3b knockouts, the Socs3a knockouts showed normal granulopoiesis and neutrophil function, with no signs of inflammation. However, the Socs3a knockouts displayed disrupted lateral line neuromast formation that was absent in Socs3b knockouts.

2. Materials and Methods

2.1 Zebrafish Husbandry

Wildtype, Socs3b knockout [18], Stat3 knockout [19] and Tg(*mpx*:GFP) [21] zebrafish were maintained in a purpose-built Tecniplast aquarium according to national guidelines and standard husbandry practices [22]. Embryos required for experiments were kept in E3 water for 24 h, after which 1-phenyl-2-thio-urea (PTU) (Sigma-Aldrich Technologies, Melbourne, Australia) was added to 0.003% (w/v) to increase transparency by inhibiting pigmentation.

2.2 Line Generation

Nucleotide sequences of the *socs3a* gene were obtained from the National Center for Biotechnology Information (NCBI) data bank and the corresponding genomic DNA (gDNA) and mRNA sequences were aligned to identify exons and introns. A specific guide RNA (sgRNA) was designed using the online software package ZiFiT Targeter v4.2). Potential off-targeting was assessed using an online tool at Integrated DNA Technologies (IDT DNA, https://www.idtdna.com/page), which indicated a low chance of off-targeting (overall score = 95) and with only one potential gene on the same chromosome as *socs3a* (*srebf*, chr3) having 4 mismatches, including 3 immediately adjacent to the protospacer adjacent motif (PAM). Appropriate oligonucleotides were generated with attached T7 promoter sites and overhanging ends for ligation into the DR274 expression vector. Subsequently, sgRNAs were generated through *in vitro* transcription using a MegaShortScript™ T7 Transcription Kit (Thermo-Fisher Scientific Pty Ltd, Scoresby, VIC, Australia) followed by purification with a MegaClear™ Kit (Thermo-Fisher Scientific).

Mutants were created by injecting ~1 nL of 100 ng/µL sgRNAs and ~500 ng Cas9 mRNA (Sigma-Aldrich) into zebrafish embryos at the one-cell stage. Screening of founder (F0) zebrafish for mutations employed high-resolution melt (HRM) analysis on a CXF96 Thermal Cycle (Bio-Rad, South Granvlle, Australa) with primers flanking the target site (5′-TGAATCAGGCACCAAGAAC and 5′-GTTCTTGGTGCCTGATTCA), with potential mutants characterized by Sanger sequence analysis (Australian Genome Research Facility). Zebrafish harboring mutant alleles were outcrossed for two generations to wildtype zebrafish to reduce the possibility for off-target effects. Third-generation zebrafish carrying the mutant allele were incrossed to generate homozygous zebrafish for the desired allele. These were then outcrossed to the Tg(*mpx*:GFP) [21] transgenic line.

2.3 Whole-Mount in situ Hybridization

Embryos at appropriate developmental stages were fixed with 4% (w/v) paraformaldehyde (PFA) (Sigma-Aldrich) at 4 °C overnight. Embryos were then subjected to whole-mount *in situ* hybridization (WISH) using anti-sense RNA probes labelled with with digoxygenin (DIG) (Sigma-Aldrich), as published [23].

2.4 Mitotracker Staining

Embryos were stained with 200 nM Mitotracker Red CMX Ros (Molecular Probes, Invitrogen Australia, Mount Waverley, Australia) for 2 h at 28 °C in the dark. Following this, excess staining was removed with two subsequent 10 min washes in 1 × E3 media. Embryos were anesthetized with 5 µg/mL benzocaine (Sigma-Aldrich) in 1 × E3 media prior to imaging.

2.5 In Vivo Analysis

Transgenic zebrafish embryos at 3 days post fertilization (dpf) were anesthetized with 5 µg/mL benzocaine and subjected to tail fin wounding assays as previously described [19] to assess the response to injury, or injected with 100 ng/µL lipopolysaccharide (LPS) (Sigma-Aldrich) into the venous return as previously described [24] with embryos imaged prior to injections and 8 h following injection to quantify emergency granulopoiesis.

2.6 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was obtained from individual zebrafish embryos at 3 dpf with an RNeasy Mini Kit (Qiagen Pty Ltd, Clayton, Australia) using the manufacturer's protocol optimized for RNA isolation from small sample volumes. The extracted RNA was subjected to reverse transcription-polymerase chain reaction (RT-PCR) using specific primers (Table 1), with products analyzed via gel electrophoresis.

2.7 Imaging and Image Analysis

Embryos were imaged using a DP74 camera attached to an Olympus MVX10 microscope with CellSens Dimension v1.6 software (Olympus, Shinjuku, Japan) and UV excitation with either a green fluorescent protein (GFP) or red fluorescent protein (RFP) filter when required. Image quantification was performed using ImageJ v1.52k software (https://imagej.net/).

2.8 Experimental Setup and Statistics

Experiments were conducted on zebrafish obtained from an in-cross of the desired mutant allele. Experiments utilized a minimum of 20 embryos for each analysis group and genotype, with three independent repeats of each experiment performed. All statistical analysis was performed on the GraphPad Prism v8.4.3 software (GraphPad Software, Inc., San Diego, CA, USA). All data were tested for normality using a D'Agostino–Pearson omnibus normality test. Significant differences between data sets were tested using unpaired Students *t*-tests with Welch's correction if the data had variable standard deviations.

Table 1. Primers used for reverse transcription-polymerase chain reaction (RT-PCR) in this study.

Gene	Forward Primer	Reverse Primer
actb	TGGCATCACACCTTCTAC	AGACCATCACCAGAGTCC
cish.a	TCACCGAGACGCATTGACGAACC	AGACTGAAACGACATTGCCTG
cish.b	CAGTCAGGAATGGTTACAAGGG	TATGCGGATGTTAGTAGGGC
socs3a	CCACTTCAAGACCTTCAGC	TTGTCCGAACTGTCTCTG
socs3b	GCCGTTTTCACACTGAGCGT	GGATGTGGAAGAAGTGTCAGAGCT

3. Results

3.1 Generation of Socs3a Knockout Zebrafish

For the generation of a *Socs3a* knockout (KO) allele, the second exon of the *Socs3a* gene was targeted using a single-guide RNA (sgRNA) directed at sequences encoding the central SH2 domain (Fig. 1A). The sgRNA was injected along with Cas9 mRNA into embryos wildtype (WT) at the one-cell stage, which were subsequently raised with potential adult founder (F0) fish, and then outcrossed with WT zebrafish. The F1 progeny were screened using HRM, which identified a potential mutant allele in the *socs3a* gene. This mutant was out-crossed once more with WT fish before being in-crossed to produce zebrafish that were homozygous for the mutant allele. Sequence analysis of these identified a 4 base-pair (bp) deletion at the gRNA site (Fig. 1B). This caused a frameshift with an early stop codon in the alternate frame, resulting in a truncated Socs3a protein without functional SH2 and SOCS box domains (Fig. 1C). While the kinase inhibitory region (KIR) of the protein remained, SOCS3 has been found to require its SH2 domain for functionality [25]. Together this suggested the mutant represented a suitable knockout allele. Fish homozygous for this allele were designated Socs3a KO and compared with Socs3a WT zebrafish in subsequent experiments.

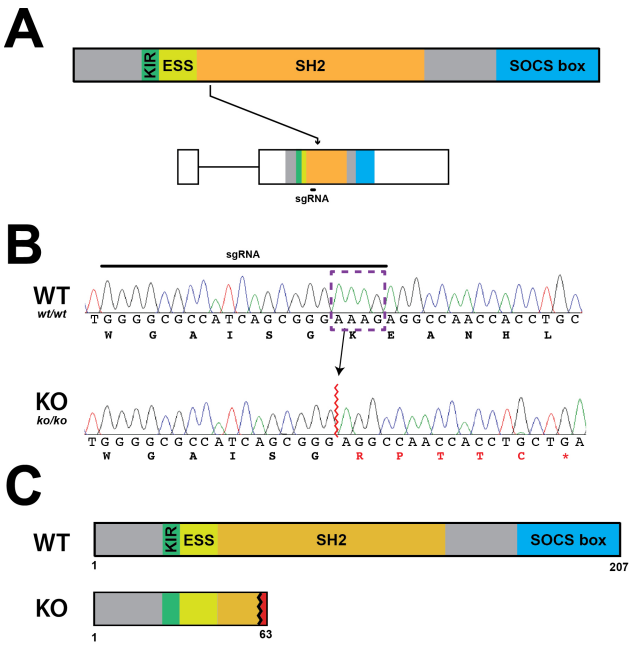

Fig. 1. Establishment of a Socs3a KO line. (A) Schematic diagram of the Socs3a protein indicating constituent domains (KIR, kinase inhibitory region; ESS, extended SH2 sub-domain; SH2, Src homology 2 domain; SOCS box, suppressor of cytokine signaling box) as well as *socs3a* gene showing the intron (thin line) and exons (boxes) and sequences coding each domain and targeted with the sgRNAs. (B) Sequence traces of representative homozygous wildtype (WT, *socs3a*$^{wt/wt}$) and knockout (KO, *socs3a*$^{ko/ko}$) fish at the genomic region targeted by the sgRNA showing nucleotides and encoded amino acids. Nucleotide sequences that were deleted in the mutant are boxed purple, with *de novo* protein sequences shown in red. (C) Schematic of Socs3a protein forms in WT and KO fish.

3.2 Socs3a is Dispensable for Primitive and Definitive Hematopoiesis

The impact of Socs3a ablation on early hematopoiesis was assessed via WISH using specific markers for relevant hematopoietic lineages. Neutrophils marked with *mpx* [21] were not affected in Socs3a KO mutants during the primitive wave of hematopoiesis as evaluated at 22 hours post fertilization (hpf) (Fig. 2A–C) or definitive hematopoiesis at 5 dpf (Fig. 2D–F). Furthermore, lymphocytes marked with *rag1* [26] (Fig. 2G–I) and erythrocytes marked with *hbbe1.1* [27] (Fig. 2J–L) at 5 dpf were also unaffected in Socs3a KO mutants.

3.3 Socs3a KO Shows Unaltered Emergency Granulopoiesis and Neutrophil Functionality

Neutrophils were further assessed in Socs3a WT and KO embryos crossed onto the Tg(*mpx*:GFP) genetic background [21]. Emergency granulopoiesis was analyzed following injection with LPS at 3 dpf. Neutrophil numbers significantly increased in both WT and Socs3a KO embryos, but there were no significant differences were observed between genotypes (Fig. 3A–E). Neutrophil response to injury was examined following caudal fin wounding. The number of neutrophils that underwent migration to the site of injury also followed a similar time course, with no significant difference in neutrophil migration in Socs3a KO in comparison to WT embryos (Fig. 3F,G).

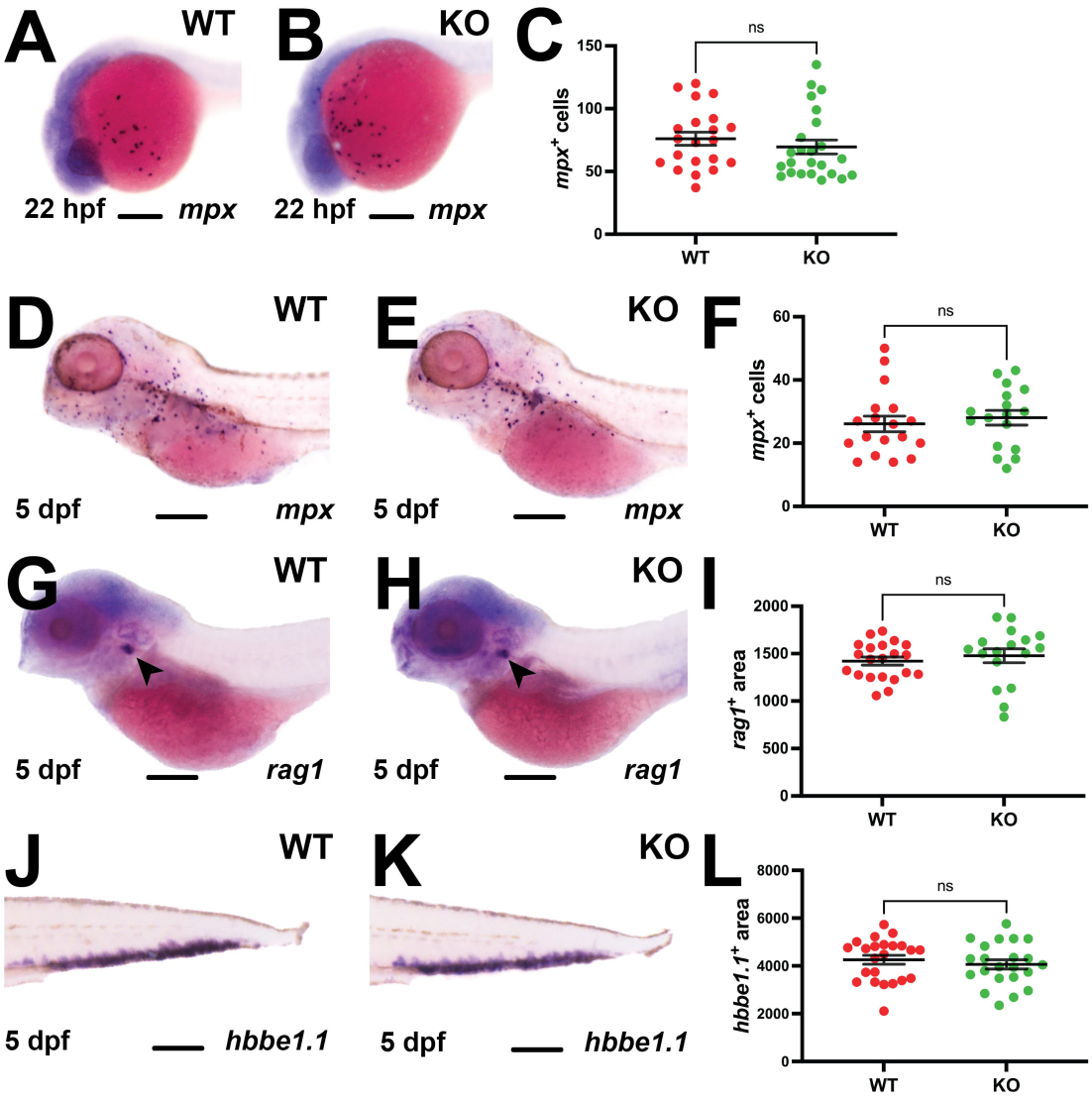

Fig. 2. Effect of Socs3a ablation on the early stages of hematopoiesis. (A,B,D,E,G,H,J,K) Representative images of wildtype (WT) and Socs3a knockout (KO) embryos analyzed using WISH with *mpx* at 22 hpf (A,B) and 5 dpf (D,E), as well as *rag1* (G,H) and *hbbe1*.1 (J,K) at 5 dpf, focusing on the main areas of staining for each, including 200 µm scale bars. (C,F,I,L) Quantitation of *mpx*+ cells at 22 hpf (C) and 5 dpf (F) and area of staining for *rag1* for (I) and (L) *hbbe1.1* at 5 dpf displaying individual embryos and mean ± SEM, with level of statistical significance indicated (ns, not significant; n = 17–24).

3.4 Socs3a is Required for the Generation of the Posterior Lateral Line Neuromasts

During the formation of the posterior lateral line (PLL), the migration of the PLL primordia (PLLp), is exclusively controlled by the chemokine *sdf1a* and its cognate receptor *cxcr4b*, with the chemokine expressed along the entire length of the migration path and receptor expressed in the leading zone of the migrating PLLp [28]. In addition to this, expression of *eya1* is observed in mature neuromasts as well as their migrating PLLp [29], while *atoh1a* is expressed solely in mature hair cells [30]. To identify any potential roles of Socs3a in these cells, WT and KO embryos were subjected to WISH with these markers (Fig. 4, with staining in the wild-type equivalent to that published [31]). Expression of *sdf1a* was comparable within the head of Socs3a WT and KO embryos, but staining within the trunk and tail was significantly reduced in KO embryos in comparison to WT embryos (Fig. 4A–C). Expression of *cxcr4b* was observed in the migrating PLLp, in the trunk of both Socs3a WT and KO embryos at 30 hpf (Fig. 4D,E). However, its relative posterior migration (Fig. 4F) and area of staining (Fig. 4G) were significantly lower in Socs3a KO embryos compared to WT counterparts. The number of mature neuromasts was significantly decreased in Socs3a KO compared to WT as assessed with *eya1* (Fig. 4H–J) and *atoh1a* (Fig. 4K–M), which was then confirmed using Mitotracker red staining (Fig. 4N–S).

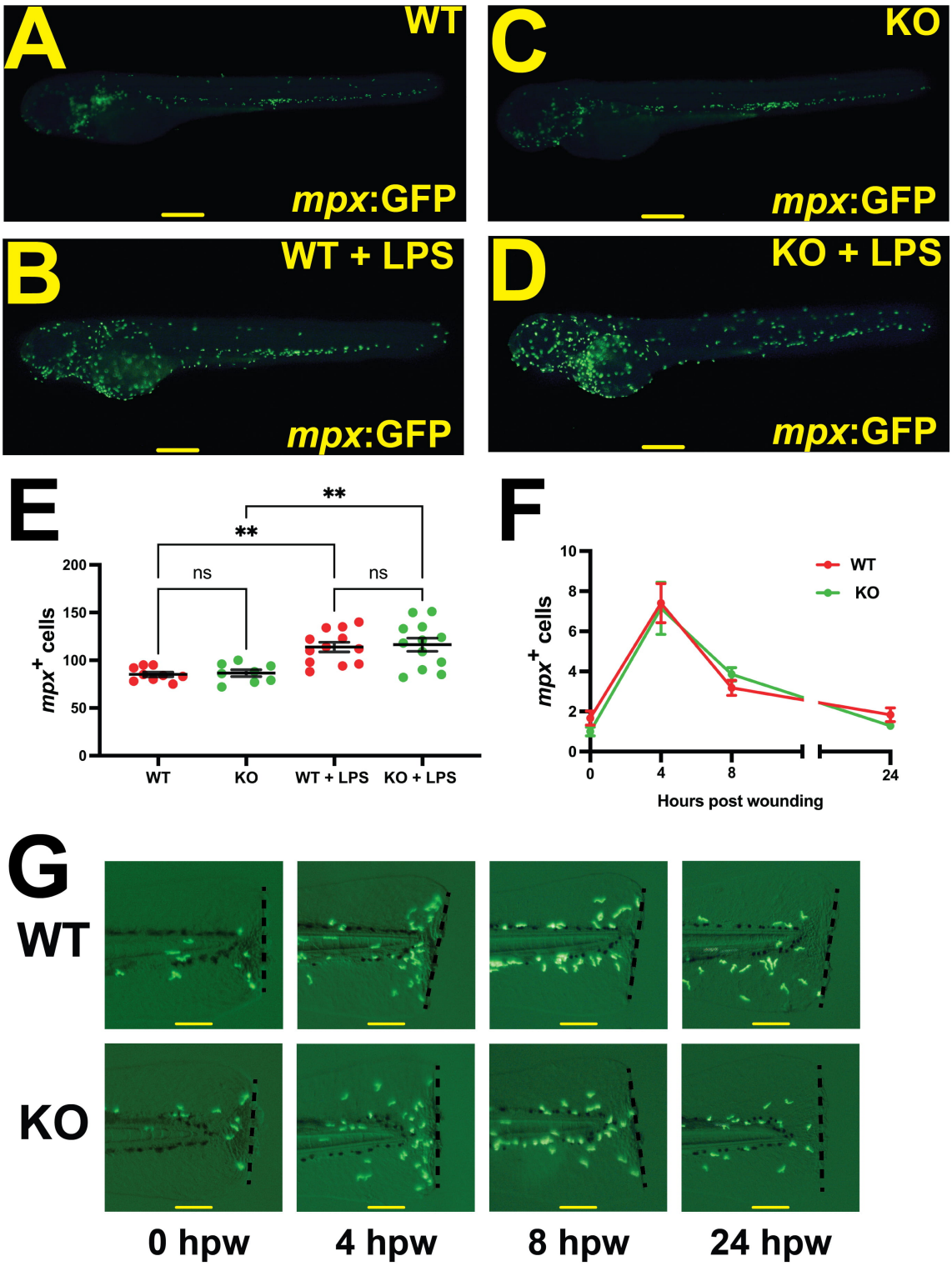

Fig. 3. Impact of Socs3a ablation on emergency granulopoiesis and neutrophil function. (A–E) Socs3a wildtype (WT) and knock-out (KO) embryos on a Tg(*mpx:GFP*) background were injected with LPS (+LPS) at 3 dpf and imaged 8 hours later showing (A–D) representative images and (E) quantitation of total *mpx*+ cells. (F,G) Embryos were separately inflicted with a tailfin wound at 3 dpf, with the caudal fin imaged at 4 h time points from 0 to 24 hours post wounding, with representative images (G) and quantification of (F) the *mpx*+ cells present at the site of wounding shown. Scale bars of 100 μm are shown for (A–D,G), with the mean ± SEM and statistical significance shown for (E,F) (** $p < 0.01$; ns, not significant; n = 8–12).

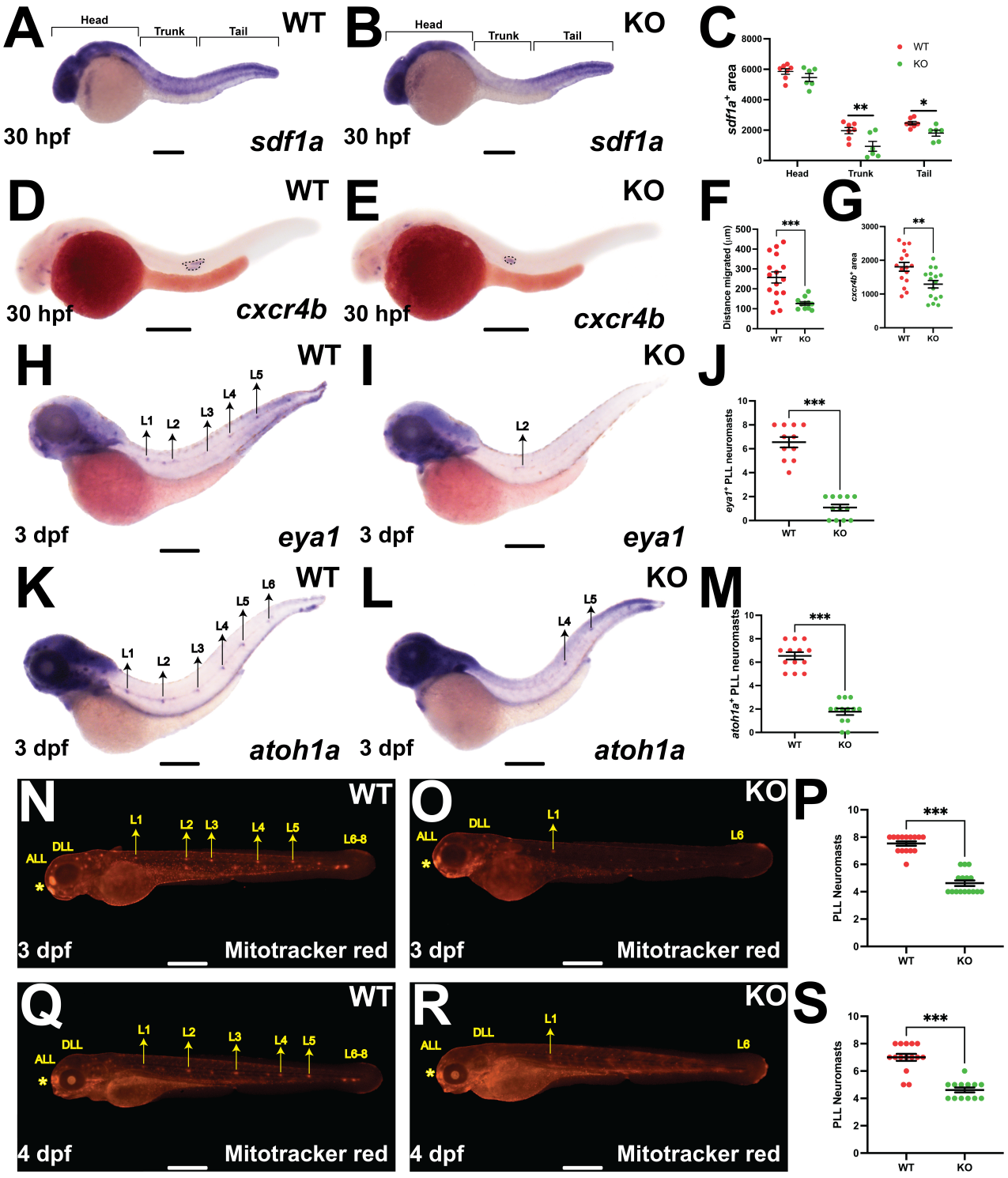

Fig. 4. Effect of Socs3a ablation on posterior lateral line neuromast formation. (A,B,D,E,H,I,K,L,N,O,Q,R) Representative images of Socs3a wildtype (WT) and knockout (KO) embryos analyzed using WISH with *sdf1a* (A,B) and *cxcr4b* (D,E) at 30 hpf, and *eya1* (H,I) and *atoh1a* (K,L) at 3 dpf or stained with Mitotracker red, allowing visualization of posterior lateral line (PLL) neuromasts L1-8 at 3 dpf (N,O) and 4 dpf (Q,R), with scale bars of 100 μm shown. (C,F,G,J,M,P,S) Quantification of the area of *sdf1a* staining in the head, trunk and tail as described [31] (C), distance traveled by *cxcr4b*+ primordia (F), area of *cxcr4b*+ primordia (G), and number of *eya1*+ (J), *atoh1a*+ (M) or Mitotracker red stained (P,S) neuromasts, showing individual points along with the mean ± SEM and statistical significance (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$; n = 10–15).

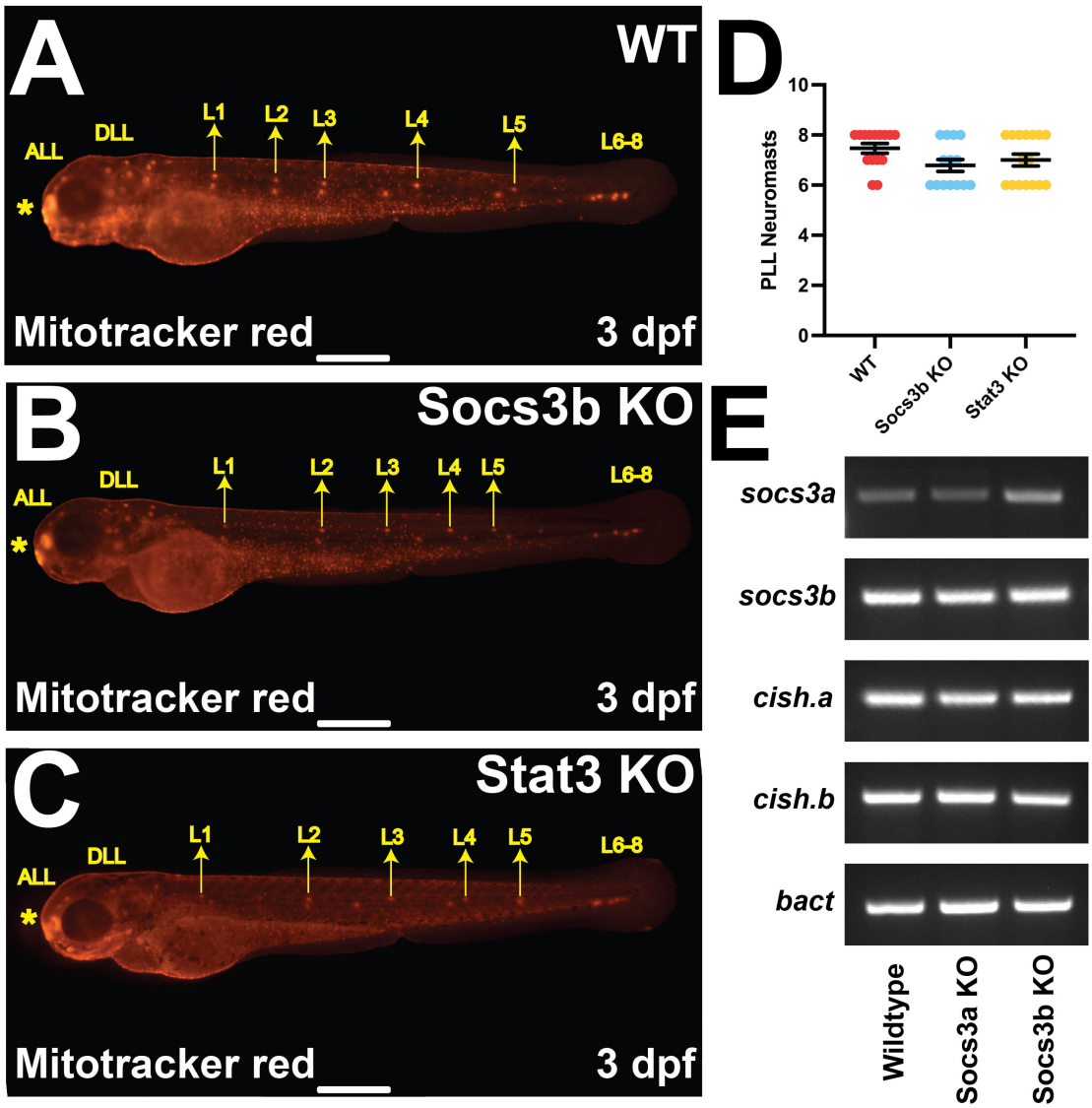

Fig. 5. Analysis of potential pathway components. (A–C) Representative images of wildtype (WT), Socs3b knockout (Socs3b KO) and Stat3 knockout (Stat3 KO) embryos analyzed with Mitotracker red at 3 dpf, with scale bars of 100 µm shown. (D) Quantitation of PLL neuromasts, showing individual points along with the mean ± SEM. No statistically significant differences were observed between groups (n = 14–16, repeated twice). (E) RT-PCR analysis of the indicated genes in WT, Socs3a KO and Socs3b KO embryos at 3 dpf.

3.5 Analysis of Other Potential Pathway Components

Given the significant impact on PLL neuromast development in Socs3a KO mutants, these cells were also examined using Mitotracker red staining in Socs3b and Stat3 KO [19] mutants. Both mutants showed normal formation of all PLL neuromasts, with no differences observed between them and WT (Fig. 5A–C). Finally, to assess potential compensation, expression levels of *socs3* and *cish* gene paralogs [32] were assessed in both Socs3a KO and Socs3b KO mutants by RT-PCR at 3 dpf (Fig. 5D,E). Expression of *socs3a* was found to be higher in Socs3b KO embryos in comparison to Socs3a KO and WT embryos, while *socs3b, cish.a* and *cish.b* expressions were unchanged.

4. Discussion

Zebrafish have duplicate SOCS3 proteins, Socs3a and Socs3b. Ablation of Socs3b in zebrafish revealed significant conservation of function with mammalian SOCS3 with respect to regulating neutrophils and macrophages both developmentally and functionally [18]. Socs3a has been shown to be expressed in PLL neuromasts [20], and implicated in the regeneration of hair cells [20], nerve cells [33] and liver [17], but a full understanding of its relationship with Socs3b remained lacking. To address this, CRISPR/Cas9 technology was used to create a Socs3a knockout allele, followed by the analysis of the homozygote mutants.

Sequencing of the Socs3a mutant revealed a 4 bp deletion that would severely truncate the protein, including

deletion of the SH2 and SOCS box domains that are crucial for SOCS3 function [34]. Unfortunately, no Socs3a-specific antibodies were available to confirm this at the protein level. However, the consistency between the key phenotypes observed in the Socs3a mutants with those observed following morpholino-mediated knockdown supports the assumption that this represents a loss-of-function mutation. The Socs3a mutant did not show any decrease in the level of *socs3a* transcripts. However, this was consistent with studies on other zebrafish SOCS family members: with Socs3b mutants [18] and Cish.a morphants [32] showing an increase in expression—which was also observed in Socs3a morphants [20]. This is likely due to SOCS proteins negatively regulating their upstream regulators [35]. Finally, the sgRNA used to generate the mutant was predicted to have no significant off-targets. Despite this, multiple independent outcrosses and comparisons of homozygous mutant and WT siblings generated from the same cross were employed to further minimize the likelihood of any such impacts.

We showed for the first time that Socs3a ablation did not have any significant impact on hematopoiesis, consistent with preliminary work using morpholino-mediated knockdown. This included no effect on early definitive lymphopoiesis or erythropoiesis, as we also recently reported for Socs3b ablation [18]. These results were collectively consistent with Socs3 KO mice that also lacked major impacts on the differentiation of lymphoid or erythroid cells [35]. However, Socs3 KO mice did show alterations in the function of both T and B lymphocytes in mammals [36,37], a phenotype that has yet to be explored in zebrafish. We additionally demonstrated that Socs3a ablation failed to impact normal granulopoiesis, emergency granulopoiesis induced by LPS, or the migration of neutrophils in response to wounding. This was in stark contrast with Socs3b KO zebrafish that exhibited dysregulated primitive and definitive granulopoiesis, altered LPS-mediated emergency granulopoiesis and decreased relative neutrophil migration [18]. Similarly, Socs3 ablation in mice caused elevated basal [38] and emergency [39] granulopoiesis, although neutrophil migration was not affected [40]. Together, this suggests that Socs3a does not share the critical negative regulatory role on neutrophil production and function of Socs3b, which is largely conserved with mammalian SOCS3. Analysis with additional myeloid markers would be of interest to understand whether Socs3a impacts monocytes/macrophages [41].

We also showed that Socs3a ablation perturbed the formation of PLL neuromasts. The formation of the PLL neuromasts is mediated by the chemokine Sdf1a (also known as Cxcl12a) and its receptors Cxcr4b/Cxcr7b that direct the deposition of the primordia along the lateral line [28]. In addition, Eya1 [42] and Atoh1a [43] are required for the proper function and maintenance of hair cells following neuromast formation. Socs3a KO embryos showed diminished expression of *sdf1a* along the length of the embryos and decreased expression of *cxcr4b* in the PLLp. Moreover, the distance traveled by the PLLp was significantly lower in time-matched Socs3a KO compared to WT embryos, which indicates a severe impact on primordia migration. Analysis of PLL neuromasts deposited along the lateral line with *eya1* and *atoh1a* markers indicated a significant decrease in Socs3a KO compared to WT embryos. This was separately confirmed with Mitotracker red staining that revealed a large reduction in the mature PLL neuromasts that formed. These results were consistent with a previous independent study employing morpholino-mediated knockdown of Socs3a that also resulted in a reduction in PLL neuromasts [20], reinforcing the critical role played. In contrast, no PLL neuromast defects were found in Socs3b KO zebrafish, showing this to be a unique Socs3a function. Stat3 KO embryos also possessed normal numbers of PLL neuromasts, which differed from what was observed with Stat3 morphants [20]—the reason for which remains unclear.

The presence of two *socs3* genes in zebrafish is a likely by-product of a whole genome duplication event, following which approximately 3–4% of duplicate genes were retained, with around a quarter developing divergent functions due to the addition of novel protein domains or altered spatiotemporal profiles [44]. However, such duplicates often also exhibit overlapping functions, complementing and compensating for the loss of the other [45,46]. For example, in the case of the zebrafish Csf1r duplicates, both retained their ancestral functions in macrophage cells, but Csf1ra developed an additional novel function in pigment cells that was not present in mammals [47]. Similarly, the zebrafish Cxcl8a and Cxcl8b duplicates were both required for neutrophil migration; however, forward migration and reverse migration were divided between the two duplicates, unlike mammals where the single CXCL8 performed both functions [48]. In the case of Socs3a and Socs3b, there was a high degree of conservation with mammalian SOCS3 proteins [16,49], while both Socs3a [50] and Socs3b [51] were shown to be regulated by Stat3, suggesting conserved function. Despite this, the Socs3a KO zebrafish did not exhibit the negative regulatory functions in myeloid cell production and neutrophil function conserved between Socs3b and mouse Socs3 [39,52], but instead displayed an alternative function in neuromast formation, a fish-specific mechanosensory organ [53]. The acquisition of this *de novo* function for Socs3a parallels that of SOCS3 in the placenta, an organ-specific to placental mammals [5]. However, the studies presented here do not rule out the potential of conserved mammalian functions as other studies have implicated Socs3a in other potentially relevant roles [33,54]. There is also the possibility of redundancy with Socs3b that can only be uncovered through analysis of double knockouts.

5. Conclusions

A zebrafish Socs3a knockout was generated, with its characterization showing Socs3a to be required for the formation of the fish-specific neuromast organ but dispensable for myeloid cell production and function. This contrasted with the zebrafish Socs3b knockout that showed normal neuromast development, but with a number of myeloid phenotypes largely conserved in mouse Socs3 knockouts. This indicates functional divergence of Socs3a in fish neuromast development that parallels that seen with mammalian Socs3 in placental development.

Availability of Data and Materials

All data reported in this paper will be shared by the corresponding author upon reasonable request.

Author Contributions

MLS, CL and ACW designed the research study. MLS performed the research. MLS analyzed the data. MLS wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Animal experiments were performed under the aegis of the 'Australian code for the care and use of animals for scientific purposes', 8th Edition 2013 (updated 2021) as part of projects approved by the Animal Ethics Committee of Deakin University (Ethics approvals: G23/2019, G24/2019, G25/2019).

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Conflict of Interest

Alister C. Ward is a Guest Editor and Editorial Board member but had no involvement in the peer-review of this article or access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to Natascia Tiso. The other authors declare no conflict of interest.

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