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Original Research

Analysis of Potential Non-Canonical or Alternate STAT5 Functions in Immune Development and Growth

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Abstract

Background: Signal transducer and activator of transcription (STAT) proteins play key roles in development, growth, and homeostasis. These roles have principally been assigned to their "canonical" function as inducible transcriptional activators acting downstream of cytokines and other factors. However, variant "non-canonical" functions have also been identified. The potential *in vivo* role for non-canonical STAT functions was investigated in the zebrafish model. **Methods**: Two zebrafish Stat5.1 mutants were generated using CRISPR/Cas9 that should impact canonical functionality: one with a deleted transactivation domain (ΔTAD) and another with a disrupted tyrosine motif (ΔTM). Immune cell development, growth, and adiposity of these Stat5.1 mutants were assessed in comparison to a Stat5.1 knockout (KO) mutant in which both canonical and non-canonical functions were ablated. **Results**: Both the ΔTAD and ΔTM mutants showed significantly reduced embryonic T lymphopoiesis, similar to the KO mutant. Additionally, adult ΔTAD and ΔTM mutants displayed a decrease in T cell markers in the kidney, but not as severe as the KO, which also showed T cell disruption in the spleen. Severe growth deficiency and increased adiposity were observed in all mutants, but ΔTAD showed a more modest growth defect whereas ΔTM exhibited more profound impacts on both growth and adiposity, suggesting additional gain-of-function activity. **Conclusions**: These results indicate that canonical Stat5.1 plays a major role in T cell development and growth throughout the lifespan and non-canonical Stat5.1 functions also contribute to aspects of adult T lymphocyte development and growth, with alternate functions impacting growth and adiposity.

Keywords: cytokine; JAK-STAT; STAT5; non-canonical; immunity; growth

1. Introduction

Mammals possess seven signal transducer and activator of transcription (STAT) proteins: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6 [1]. The myriad roles of STATs across development, growth, and homeostasis have been largely attributed to their so-called "canonical" function as inducible transcription factors downstream of cytokines and other factors [2,3]. In this modality, ligation of cytokine or other receptors triggers the tyrosine phosphorylation of inactive unphosphorylated STAT (uSTAT) monomers in the cytoplasm via associated kinases such as JAKs, allowing dimerization of phosphorylated STAT (pSTAT) molecules through reciprocal interactions between a phosphotyrosine-containing motif on one STAT protein with the SH2 domain on another. The subsequent nuclear translocation of these activated STAT dimers enables them to activate the transcription of target genes, impacting key cell processes, notably including differentiation, survival, proliferation, and activation [4–6]. However, STATs have also been demonstrated to regulate critical cell processes through alternative "non-canonical" functions [7].

Mammalian STAT5A and STAT5B proteins share more than 90% amino acid similarity and have both unique as well as overlapping functions [8]. Both STAT5A and STAT5B contribute to immune regulation, although STAT5B appears to play the dominant role, presumably via canonical signaling downstream of various interleukin (IL) receptors [9]. STAT5A is a master regulator of mammary gland development and lactogenesis attributed to a canonical signaling function downstream of prolactin [10], whereas STAT5B facilitates the regulation of growth and metabolism assigned to canonical growth hormone (GH) signaling [11–13]. Non-canonical functions of STAT5 proteins have also been reported. These include gene repression by pSTAT5B during oncogenesis [14], uSTAT5B participating in the regulation of megakaryocyte differentiation [15], and non-nuclear roles of STAT5A/B in maintaining the structural integrity of sub-cellular organelles such as the endoplasmic reticulum and Golgi body [16]. Various STAT5B mutations have been associated with disrupted growth and immunity but with differential clinical severity depending on the site of mutation, further suggesting the potential for non-canonical functions [12,17–19].

Zebrafish possess two STAT5 proteins, Stat5.1 and Stat5.2 [20]. Stat5.1 displays the closest similarity with mammalian STAT5B and has been shown to contribute to lymphopoiesis throughout the life course [21], as well as the regulation of growth and sex-specific adiposity [21,22].

However, these publications used loss-of-function (LOF) knockout (KO) mutant models that would abrogate both canonical and non-canonical functions. In this study, two Stat5.1 mutants were generated using CRISPR/Cas9-based mutagenesis to examine potential non-canonical or alternate functions. The impact of these Stat5.1 mutants on lymphopoiesis, growth, and adiposity was examined in direct comparison to a Stat5.1 KO mutant. The results provided evidence of predominantly canonical functions but also suggested that non-canonical and alternate Stat5.1 functions also exist.

2. Materials and Methods

2.1 Zebrafish Husbandry

Zebrafish were maintained using standard husbandry practices [23] following national guidelines for their care and use. All studies were approved by the Deakin University Animal Ethics Committee.

2.2 Generation of Stat5.1 Mutants

A single guide RNA (sgRNA) targeting the zebrafish *stat5.1* gene was designed using ZiFiTargetor v4.2 [24] and CHOPCHOP v3 [25], which had a minimum of three mismatches with potential off-target sites, all on other chromosomes (**Supplementary Table 1**). The sgRNA template was cloned into pDR274 and sgRNA synthesized by *in vitro* transcription using a MEGAscript T7 Transcription Kit (#AM1354, Thermofisher Scientific, Australia), purified using a MegaClear Kit (#AM1908, Thermofisher Scientific, Australia), and quantified. One cell stage wild-type (WT) zebrafish embryos were injected with ~1 nL of 100 ng/µL sgRNA, 200 ng/µL TrueCut Cas9 protein v2 (#A36498, Thermofisher Scientific, Australia), and 0.4% (w/v) phenol red. Founder fish were raised to adulthood, out-crossed with WT fish, and screened for the presence of mutations. Confirmed heterozygous fish were further outcrossed to minimize potential off-target mutations, with heterozygous progeny in-crossed to obtain homozygous mutant lines.

2.3 Genomic DNA Analysis

Genomic DNA from adult fin clips and whole embryos was isolated with QuickExtract DNA Extraction Solution (#QE09050, Gene Target Solution, Australia) following the manufacturer's instructions. This was subjected to a polymerase chain reaction (PCR) for high-resolution melt (HRM) analysis with the *stat5.1*-specific primers 5′-GCGAGGGTGTGAAAAACAGT and 5′-CCCTTTCTGTCTTCCTGTCCA or gel electrophoresis with the alternate primers 5′-GTGGTAAAAATGTGTGGATGAACTCTG and 5′-GTCTCATGTATCCAAGGCAACTCG to identify potential mutants, which were confirmed by Sanger sequencing at the Australian Genome Research Facility.

2.4 Whole-Mount in Situ Hybridization

Embryos were collected and raised in egg water with 0.0003% (w/v) phenylthiourea (PTU) for 5 days and then fixed in 4% paraformaldehyde/phosphate-buffered saline (PFA/PBS) overnight at 4 °C, followed by 100% methanol at –20 °C. Whole-mount *in situ* hybridization (WISH) was performed on rehydrated embryos according to a published protocol [26]. Embryos and juvenile fish were imaged with a MVX10 monozoom microscope using a 1 × MVXPlan Apochromat lens (NA = 0.25) and DP74 camera (#N5667400, Olympus, Tokyo, Japan), with the images captured on CellSens Dimension 1.6 software (Olympus, Tokyo, Japan), and areas of WISH staining determined using ImageJ software (v1.x, National Institutes of Health (NIH), Bethesda, MD, USA).

2.5 Body Measurements

Embryos and juvenile fish were imaged as described above and the length was determined using ImageJ software. Adult fish were imaged using an iPhone 13 pro max, wide camera-26 mm f1.5 12 MP, 3024 × 4032, 1.5 µm pixel size (Apple Inc., Cupertino, CA, USA) next to a ruler, with standard length measured from snout to caudal peduncle [27]. Following removal of eggs adult female zebrafish were weighed and subjected to total lipid extraction using 8:4:3 (v/v) chloroform:methanol:NaCl (0.03%), incubating the organic phase at 65 °C in a fumehood until dry to determine total lipid as described [28], which was expressed as a percentage of body weight.

2.6 Gene Expression Analysis

Total RNA was extracted from whole juvenile fish using TRIsure™ (#BIO-38033, Meridian Biosciences, OH, USA, and from the kidney and spleen of adult male fish using an RNEasy Mini Kit (#74136, Qiagen, Australia) according to the manufacturer's instructions. RNA concentrations were determined using a NanoDrop 2000 Spectrophotometer (#ND-2000, Thermofisher Scientific, Australia), and cDNA was synthesized from 500 ng RNA using a QuantiTect cDNA Synthesis Kit (#205311, Qiagen, Australia). Samples were subjected to quantitative real-time reverse-transcription PCR (qRT^2-PCR) with primers for *actb* (5′-TGGCATCACACCTTCTAC, 5′-AGACCATCACCAGAGTCC), *cd8* (5′-ACTCTTCTTCGGAGAGGTGAC, 5′-ACAGGCTTCAGTGTTGTTTGAA), *cish.a* (5′-TCACCGAGACGCATTGACGAACC, 5′-AGACTGAAACGACATTGCCTG), *cish.b* (5′-CAGTCAGGAATGGTTACAAGGG, 5′-TATGCGGATGTTAGTAGGGC), *fasn* (5′-CATATTCTGGGTGTGCGTGAC, 5′-GCTTTACAGGAGACTCCTCTTTC), *gh1* (5′-TCTTATGCCTGAGGAACGC, 5′-AGGTCTGGCTGGGAAACTC), *ghrb* (5′-CAACACACAGGGTCAGACTTCAAC, 5′-CATTCATTCCTCTGGGAGTTG), *igf1a* (5′-CCGCATCTCATCCTCTTTCTC, 5′-CCTGTCTCCACACACAAACTGC), *igf2a* (5′-AGTGTCACAGGCTCTTCACAAG, 5′-GATGGGACTCC

TCTCCTTAACC), *ighm* (5′-CCGAATACAGTGCCACAAGC, 5′-TCTCCCTGCTATCTTTCCGC), *nkld* (5′-TGGTGAAATCCCAACAGAGCA, 5′-TTTCATCCTGAGTTGCACCA), *srebf1* (5′-GAAGCTAAGCTCAATAAGTCTGC, 5′-TCAGAGACTTGTTCTTCTGGATG) and *tcra* (5′-ACTGAAGTGAAGCCGAAT, 5′-CGTTAGCTCATCCACGCT) using Sensifast SYBR Green Lo-ROX Kit (#BIO-94050, Meridian Biosciences, USA) on an Aria Mx Real-time PCR System (Integrated Sciences, Australia). Data were normalized to *actb* and fold-change was calculated as described [29].

2.7 Western Blot Analysis

A synthetic gene encoding wild-type Stat5.1 with a C-terminal Flag-tag was obtained from GeneArt (Thermofisher Scientific, Australia) and cloned into pBK-CMV vector, with KO, TAD, and TM mutants generated using a Q5 Site-directed Mutagenesis Kit (#E0554S, New England Biolabs, Australia). The resulting plasmids were transfected into HEK293 along with pBK-CMV-Jak3-A573V [30] using Lipofectamine 2000 Transfection Reagent (#11668019, Thermofisher Scientific, Australia) and Western blot performed as described [31] using mouse monoclonal antibodies for Flag M2 (#F1804, Sigma-Aldrich, Australia), pSTAT5A(Y694) (#ab30648, Abcam, Australia) or beta-actin (#A5441, Sigma-Aldrich, Australia), followed by rabbit anti-mouse Ig conjugated to horseradish peroxidase.

2.8 Statistical Analysis

Statistical analysis was performed with GraphPad Prism v8.0.0 for Windows (GraphPad Software, San Diego, CA, USA) using One-way ANOVA with Tukey's multiple comparison test.

3. Results

3.1 Generation of Zebrafish Stat5.1 Mutants

Zebrafish Stat5.1 is highly homologous to human STAT5B, both at the level of protein domains as well as individual amino acid residues [20], including the region spanning the SH2 domain, the tyrosine motif (TM) phosphorylated in response to the external stimuli [32], and the transactivation domain (TAD) (Fig. 1A). The region adjacent to the TM was targeted using CRISPR-Cas9 genome editing [33,34] with a specific sgRNA (Fig. 1B). Two different mutant alleles were generated, which were out-crossed twice and bred to homozygosity and sequenced (Fig. 1C): *mdu033*, a 4 bp deletion causing a frameshift that results in the complete deletion of the TAD (ΔTAD) and *mdu032*, a 12 bp in-frame deletion resulting in the removal of 4 amino acids in the TM but with other sequences intact. These mutants were subsequently compared to wild-type zebrafish and a previously generated knockout Stat5.1 KO mutant [21] (Fig. 1D).

3.2 Impact of Stat5.1 Mutations on Lymphopoiesis

Mammalian STAT5 proteins play important roles in the development of lymphoid cells, particularly STAT5B [35]. We have recently shown that zebrafish Stat5.1 KO mutants exhibited severe disruption in T lymphopoiesis throughout the life course, as well as, other lymphoid defects [21]. Thus, we compared the impact of the other Stat5.1 mutants on this aspect of biology.

Embryonic lymphopoiesis was investigated using WISH on 5 dpf embryos using specific T cell markers: *rag1* for early T cells [36] and *lck* for mature T cells [37,38]. KO embryos showed a significant reduction in the area of *rag1* staining in comparison to WT, as described [21]. Both ΔTAD and ΔTM mutants showed a comparable significant reduction in the area of *rag1* staining, similar to the KO (Fig. 2A,C). Likewise, the area of *lck* staining was also reduced in both ΔTAD and ΔTM mutants compared to the WT to a similar extent as seen with the KO (Fig. 2B,D).

Adult lymphopoiesis was examined in the kidney marrow, a primary lymphoid organ that plays an equivalent role to mammalian bone marrow [39,40], and the spleen, a secondary lymphoid organ [41]. Expression of four different lymphocyte-specific markers was analyzed: *tcra* (T cells) [42], *cd8* (cytotoxic T cells) [43], *ighm* (B cells) [44], and *nkld* (NK cells) [45]. In the kidney, expression of *tcra* and *cd8* were both significantly reduced in the KO compared to the WT, as described [21]. Expression of *cd8* was also significantly reduced in the ΔTAD and ΔTM mutants (Fig. 2E,F), although the reduction was not statistically significant for *tcra* in ΔTM and was more subtle than observed in the KO. No significant changes in the expression of *ighm* or *nkld* were observed in any of the mutants (Fig. 2E,F). In the spleen, the KO, but not the other mutants, exhibited significantly reduced *cd8* expression compared to the WT, with the KO also significantly lower than the ΔTAD. The expression of *ighm* was upregulated in the KO compared to the WT, but remained unaltered in ΔTAD and displayed a significant reduction in ΔTM compared to the KO. The relative expression of *tcra* and *nkld* remained unchanged in all mutants compared to the WT (Fig. 2F).

3.3 Impact of Stat5.1 Mutations on Growth and Adiposity

Mammalian STAT5B/Stat5b plays a crucial role in the regulation of growth and adiposity [46]. Moreover, zebrafish Stat5.1 KO mutants have also been found to exhibit reduced growth but increased adiposity along with dysregulation of growth and lipid metabolism genes [21,22]. During all developmental stages and into adulthood for both sexes, the ΔTAD and ΔTM mutants displayed a significant reduction in length compared to the WT (Fig. 3A–D, F–H), as well as weight in adults (Fig. 3I,J). However, there were subtle differences. Both the ΔTAD and ΔTM mutants were slightly larger than the KO at the embryonic stage (Fig. 3A,C). The trend continued for the ΔTAD mutant, and although not statistically significant at the juvenile stage

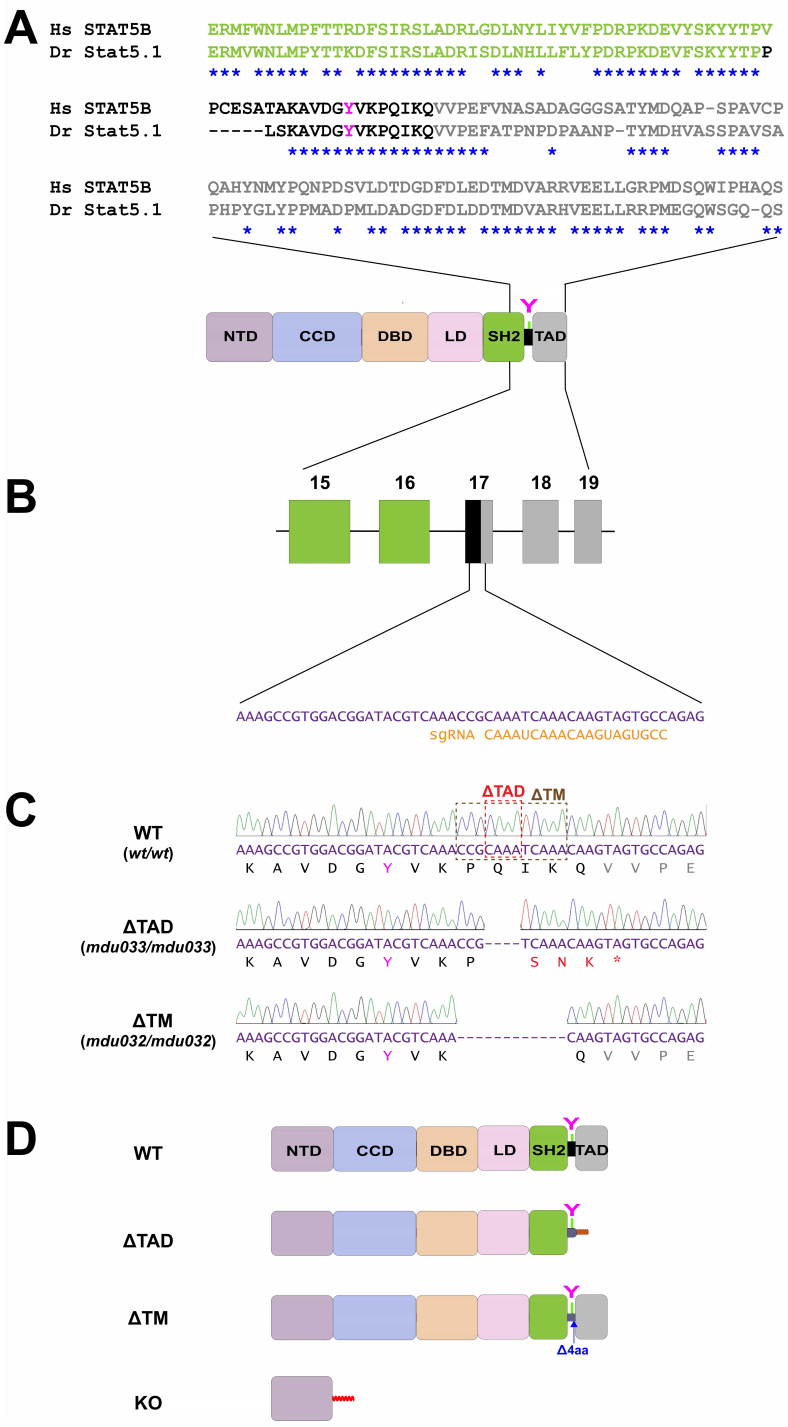

Fig. 1. Generation of zebrafish Stat5.1 mutants using CRISPR/Cas9. (A) Schematic diagram of STAT5B/Stat5.1 and its constituent N-terminal domain (NTD), coiled-coil domain (CCD), DNA-binding domain (DBD), linker domain (LD), Src-homology 2 domain (SH2), tyrosine residue (Y), and transactivation domain (TAD). An amino acid alignment for the indicated region of *Homo sapiens* (Hs) STAT5B and *Danio rerio* (Dr) Stat5.1 is shown above, with identical residues indicated by asterisks. (B) Part of the zebrafish *stat5.1* gene. Exons are shown as boxes in color matching the corresponding domain, along with the sequence targeted by the sgRNA. (C) Sequence traces, corresponding nucleotides, and encoded amino acids for homozygous wild-type (WT) (*wt/wt*), ΔTAD (*mdu033/mdu033*) and ΔTM (*mdu032/mdu032*) Stat5.1. The dotted boxes on the WT trace indicate the sequences deleted for the specified mutant. The *mdu033* allele represents a 4 bp deletion leading to an altered reading frame after P701 that results in 3 *de novo* residues followed by a stop codon (shown in red), whereas the *mdu032* allele denotes a 12 bp in-frame deletion that results in the removal of 4 residues after K700 with all remaining C-terminal sequences intact. (D) Schematic representation of Stat5.1 WT along with the ΔTAD, ΔTM, and KO (*mdu022/mdu022*) mutants.

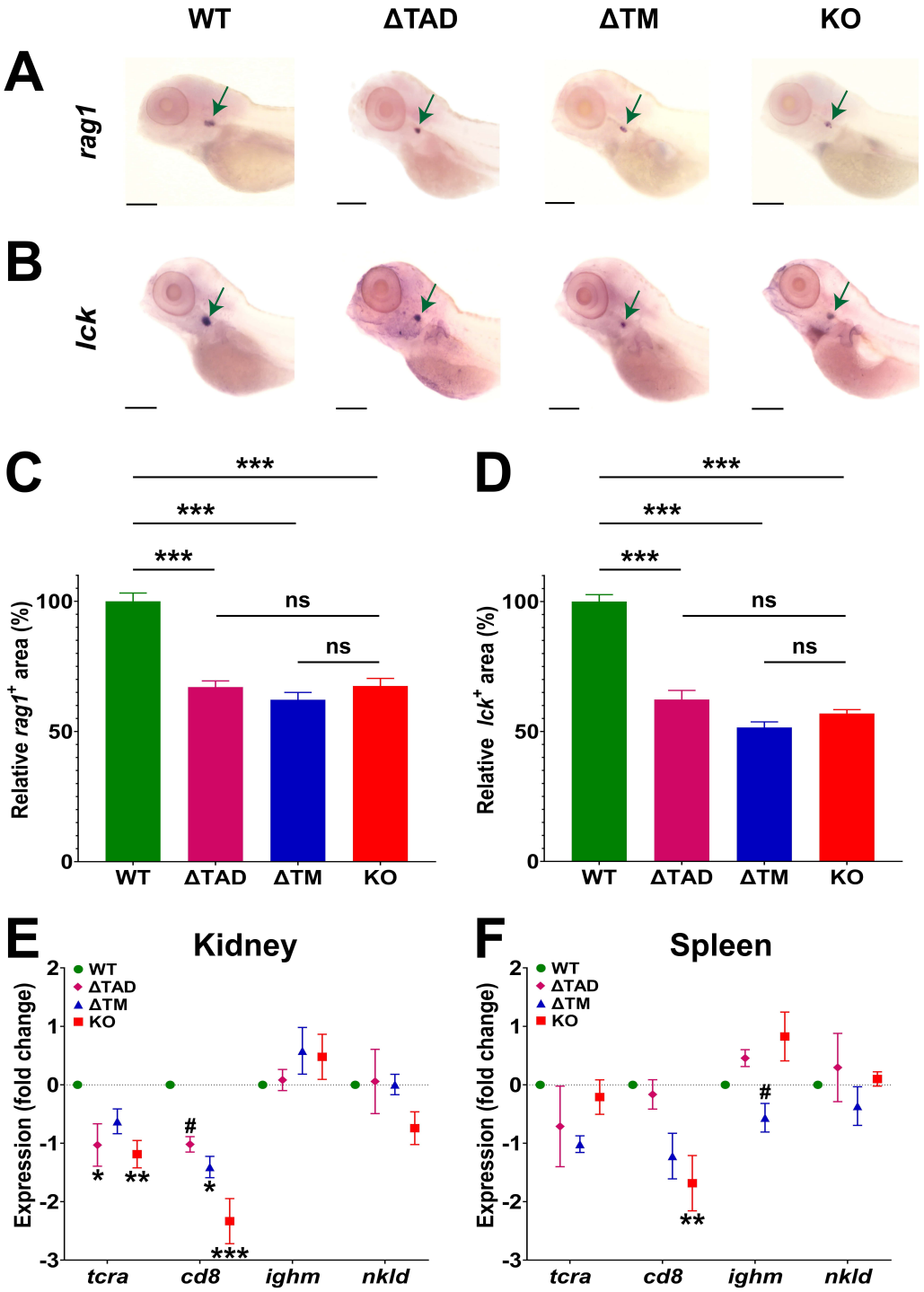

Fig. 2. Analysis of lymphopoiesis in Stat5.1 mutants. (A–D) Expression analysis of lymphoid markers using Whole-mount *in situ* hybridization (WISH) in 5 dpf WT (*stat5.1*$^{wt/wt}$), deleted transactivation domain (ΔTAD) (*stat5.1*$^{mdu033/mdu033}$), ΔTM (*stat5.1*$^{mdu032/mdu032}$) and knockout (KO) (*stat5.1*$^{mdu022/mdu022}$) embryos showing representative individuals for *rag1* (A) and *lck* (B). Green arrows indicate the thymic region and 0.5 mm scale bars are shown. The relative area of expression for *rag1* (C) and *lck* (D) was quantified comparative to the WT that was set at 100%, with mean and standard error of the mean (SEM) (n = 25–30) displayed. (E,F) Expression analysis of representative lymphoid marker genes by quantitative real-time reverse-transcription polymerase chain reaction (qRT^2-PCR) in the kidney (E) and spleen (F) from adult male fish of the indicated genotypes. Primary data were normalized relative to *actb* and represented as fold-change showing mean and SEM (n = 5). For panels (C–F), the level of statistical significance is indicated. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ compared to WT; #: $p < 0.05$ compared to KO; ns: not significant.

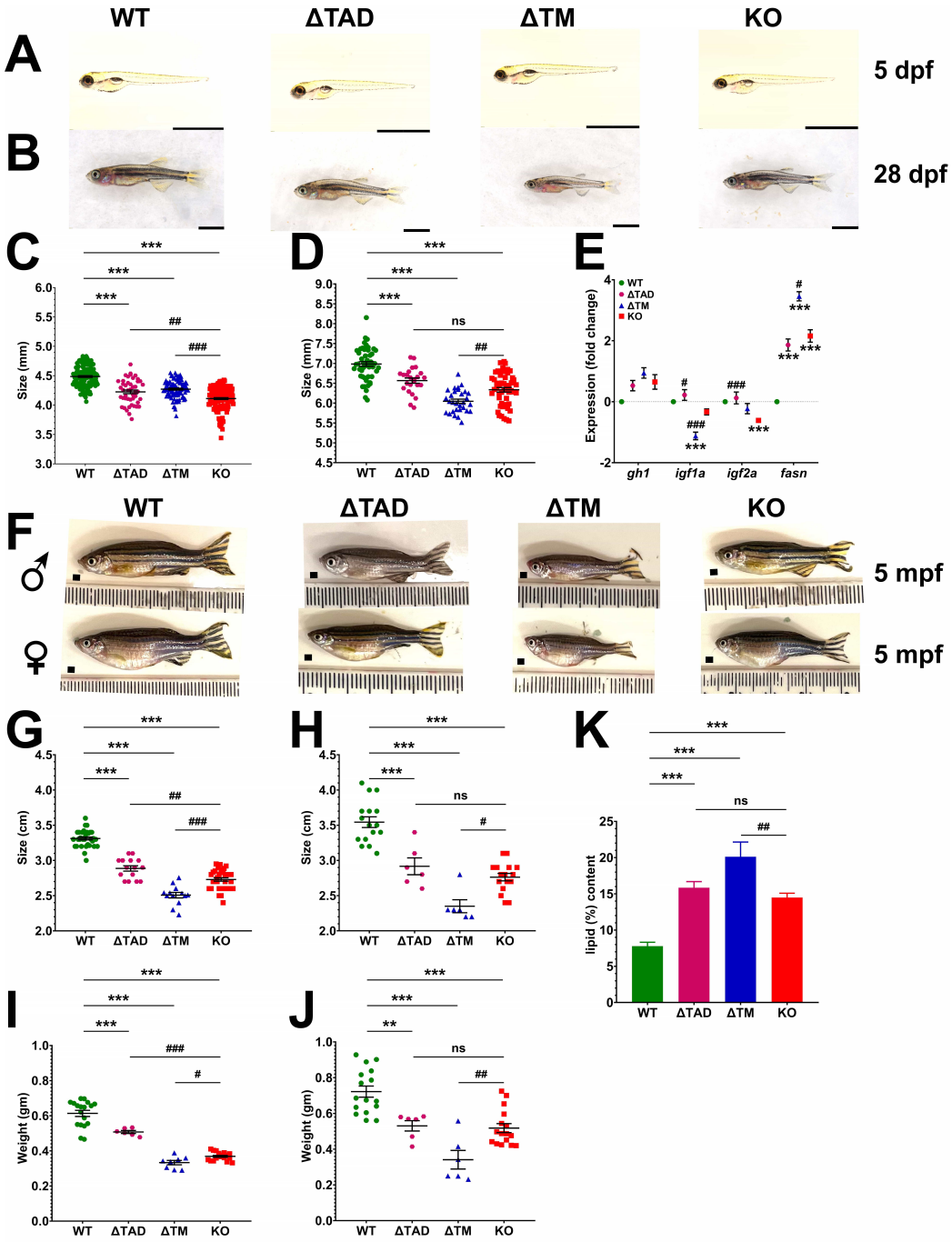

Fig. 3. Analysis of growth and adiposity in Stat5.1 mutants. (A–B, F) Images of representative WT (*stat5.1*$^{wt/wt}$), ΔTAD (*stat5.1*$^{mdu033/mdu033}$), ΔTM (*stat5.1*$^{mdu032/mdu032}$), and KO (*stat5.1*$^{mdu022/mdu022}$) individuals at 5 dpf (A), 28 dpf (B), and 5 mpf for male (♂) and female (♀) adults (F). Scale bars represent 2 mm in panels (A) and (B), with ruler increments of 1 mm in panel (F). (C,D,G–J) Analysis of size for fish with the indicated genotypes, showing quantitation of length at 5 dpf (C), 28 (D) and 5 mpf for male (G) and female (H) fish, and weight at 5 mpf for male (I) and female (J) fish of the indicated genotypes showing mean and SEM (5 dpf: n = 90–150; 28 dpf: n = 30–45; 5 mpf: n = 6–15) (J,K). (E) Expression analysis of the indicated genes involved in growth and lipid biosynthesis by qRT^2-PCR in 28 dpf WT, ΔTAD, ΔTM, and KO juveniles. Data were normalized relative to the *actb* and represented as relative fold-change showing mean and SEM (n = 5). (K) Quantitation of total lipid content for WT, ΔTAD, ΔTM, and KO of 5 mpf adult females each, represented as a percentage of wet weight showing mean and SEM (n = 6). For panels (C–E), (G,H), and (I,J) the level of statistical significance is indicated. **: $p < 0.01$, ***: $p < 0.001$ compared to WT; #: $p < 0.05$, ##: $p < 0.01$, ###: $p < 0.001$ compared to KO; ns: not significant.

(Fig. 3B,D), it was for adult males but not females (Fig. 3F–J). In contrast, the ΔTM mutant was significantly smaller compared to the KO at the juvenile stage (Fig. 3B,D), as well as in adulthood of both sexes (Fig. 3F–J). Total lipid content was significantly increased in all Stat5.1 mutants compared to the WT, with the ΔTM mutant significantly higher than the KO (Fig. 3K).

To investigate the molecular underpinnings of the growth deficiency and enhanced adiposity, 28 dpf juvenile fish were analyzed for the expression of genes specific for growth, *gh1* (growth hormone 1), *igf1a* and *igf2a* (insulin-like growth factors) [47], and lipid metabolism, *fasn* (fatty acid synthases) [48]. As described [21], KO juveniles showed reduced *igf2a* and elevated *fasn* expression, but no significant changes in *gh1* or *igf1a* compared to the WT (Fig. 3E). Expression of *gh1*, *igf1a* and *igf2a* in the ΔTAD mutant remained unchanged, but *fasn* was significantly upregulated. ΔTM showed significant downregulation of *igf1a* expression, whereas expression of *gh1* and *igf2a* remained unaltered, while expression of *fasn* was upregulated, becoming significantly elevated relative to the KO (Fig. 3E).

4. Discussion

The critical function of STAT proteins as inducible transcription factors downstream of cytokines is well documented [2,49]. However, there is growing evidence that in addition to such canonical roles, STATs also participate in non-canonical functions [7]. However, the *in vivo* impact of these non-canonical functions remains poorly understood since most studies are based on KO/LOF mutants that abrogate both canonical and non-canonical functions. Zebrafish Stat5.1 is structurally and functionally conserved with STAT5B, and ablation of either impacted growth, adiposity, and lymphopoiesis [11,21,22]. This study sought to investigate potential non-canonical functions of zebrafish Stat5.1.

Two Stat5.1 mutants were successfully generated using CRISPR-Cas9 genome editing. The first mutant, ΔTAD (p.P701fsX705*), truncated the entire TAD, thereby ablating its ability to act as a transcriptional activator. This is similar to the naturally-occurring splice variants encoding STAT5β proteins that also lack a TAD. These variants are able to bind to the promoter region of responsive genes but not initiate transcription, and potentially hinder the binding of full-length STAT5 proteins to these sites, thereby disrupting canonical functionality [50,51]. The second mutant, ΔTM (p.P701-K704del), deleted four amino acid residues at positions +3 to +6 downstream of the phosphotyrosine (pY) motif that includes three residues within the critical +1 to +5 region responsible for SH2 domain/pY recognition [52–54]. Loss of these amino acid residues may impact the specificity of SH2 domain interactions [55] and so perturb functionality. Western blot analysis confirmed the expression of proteins of appropriate sizes and demonstrated that both the ΔTAD and ΔTM mutants retained the ability to be tyrosine-phosphorylated (**Supplementary Fig. 1A**). The ΔTAD or ΔTM mutants were compared to a recently described Stat5.1 KO/LOF mutant [21] as well as wild-type (WT) fish. If the ΔTAD mutant exhibited a particular phenotype similar to the KO, this was likely due to loss of canonical functionality. Conversely, if it displayed a phenotype similar to the WT, then non-canonical functionality was potentially responsible.

As we have recently described, the Stat5.1 KO mutant exhibited disrupted growth at all developmental stages compared to WT fish with enhanced adiposity observed in adult females along with reduced *igf2a* and increased *fasn* [21]. This finding suggests that altered metabolism was responsible, consistent with studies on an alternate Stat5.1 KO mutant [56]. The Stat5.1 ΔTAD mutant also showed significant growth deficiency, increased female adiposity, and altered target gene expression compared to the WT, indicating that loss of canonical functionality was likely responsible for the metabolic perturbation. The zebrafish *vizzini* line, which harbors a LOF mutation in the zebrafish growth hormone gene paralogue *gh1*, displayed even more severe growth defects and increased adiposity [57]. This suggests canonical Stat5.1 functionality downstream of growth hormone regulates zebrafish growth and adiposity, and the more severe phenotype in the *vizzini* line was likely due to redundancy with other downstream pathways.

The Stat5.1 KO mutants further displayed decreased embryonic and adult T lymphopoiesis, but the B and NK cells of these mutants were largely unaffected, as described [21]. The Stat5.1 ΔTAD mutant exhibited a similar significant reduction in embryonic T lymphopoiesis compared to the WT. However, adult lymphopoiesis was impacted less uniformly by the mutants. In adult kidney marrow, T lymphopoiesis was significantly impacted in the ΔTAD mutant compared to the WT, but to a lesser extent than in the KO, whereas in the adult spleen only the KO displayed disrupted T lymphopoiesis. The ΔTAD mutant also showed no alterations in B and NK cell markers. This suggests that canonical functionality predominates in embryonic and some aspects of adult T lymphopoiesis, but that non-canonical functionality might contribute to other facets of adult T lymphopoiesis. LOF mutations of zebrafish *il2rg.c*, *il7r*, *jak1,* or *jak3* all impacted embryonic T lymphopoiesis in a similar, if not more extreme manner [31,58,59]. Loss of *il2rg.c* or *jak3* also caused a significant reduction in adult T lymphocyte populations, but B lymphocytes and NK cells were additionally impacted [59,60]. Collectively, this indicates that canonical Stat5.1 functionality downstream of IL-2R family members plays a major role in regulating T lymphopoiesis, but redundant signaling via Stat5.2 and/or other Stat proteins, and parallel signaling pathways such as PI3K and IRS also likely contributes to certain T cell lineages, as well as B and NK cells [61,62].

The mechanism by which non-canonical Stat5.1 functions regulate aspects of adult lymphopoiesis remains to be determined, although precedents exist for non-canonical STAT5 functions impacting mammalian immune cell development. For example, human *STAT5B* mutations that delete most of the protein—and so likely LOF—are associated with severe T and NK lymphopenia [12,17], but shorter truncations that would still ablate canonical functions result in milder effects, such as reduced $CD8^+$ T cells with normal levels of total T and NK cells [18,19]. Alternatively, suppression of genes mediating Th9 cell differentiation by IL-2-induced pSTAT5 was important for controlling the balance of Th9 versus Th17-like differentiation [63], while IL-7-induced pSTAT5 has been demonstrated to repress the transcription of *Igk* genes to maintain the proliferation of large pre-B cells and restrict transition to small pre-B cells [64]. Other STATs have also been found to exert their effects via non-canonical functions. For example, uSTAT1 can mediate transcriptional activation of IFN-induced genes independent of IFN stimulation [65–67], while uSTAT3 contributes to the expression of various NFκB-dependent genes, as well as oncogenes such as *RANTES*, *IL6*, *IL8*, *MET*, and *MRAS* [68,69]. Current therapeutic agents target canonical STAT function, being directed at either upstream signaling components such as JAKs or the SH2 domains of STATs [70]. However, these agents may not be effective against diseases in which non-canonical functions are involved. Elucidation of critical non-canonical functionalities will inform the development of more appropriate therapeutic agents that accurately target specific STAT functions.

The distinct phenotypes of the ΔTM mutant were intriguing. During embryonic lymphopoiesis and for some components of adult lymphopoiesis its phenotype mirrored that of the KO (and ΔTAD) mutant, indicative of LOF. However, in other aspects of adult lymphopoiesis it mirrored the WT (and ΔTAD mutant), suggesting normal function. In contrast, the ΔTM mutant disrupted growth and adiposity to an even greater extent than observed in the KO, and was associated with lower expression of *igf1a* rather than *igf2a*, and also higher *fasn* expression than the KO, revealing possible gain-of-function (GOF) properties. To gain further insights, ΔTM heterozygotes were in-crossed and the progeny were analyzed for *rag1* expression prior to genotyping (**Supplementary Fig. 1B**). This revealed that both homozygous and heterozygous ΔTM mutants showed significantly reduced embryonic T lymphopoiesis compared to the WT, indicating the mutant can act dominantly over the wild-type. Expression analysis was also performed on genes shown to be regulated by Stat5.1 in our recent NGS analysis [21], including the *ghrb*, *srebf1*, and *cish.a* genes, the latter possessing tetrameric STAT5 sites in its promoter able to be bound by Stat5.1, with its expression decreased following Stat5.1 ablation, unlike the related *cish.b* [71]. The *ghrb*, *srebf1*, and *cish.a* genes, but not *cish.b*, showed substantial down-regulation in the KO (**Supplementary Fig. 1C**). Interestingly, while *ghrb* expression was also significantly decreased in the ΔTM mutant, this was not the case for *srebf1* and *cish.a*, nor for *igf2a*, another STAT5 target, suggesting that the ΔTM mutant can still activate the transcription of these genes. This contrasts its effects on the *fasn* gene, also a STAT5 target [72], that displayed even more elevated expression than the KO and *igf1a* which was reduced compared to WT and KO. This indicates that ΔTM mutants can likely still interact with and regulate at least some direct target genes but differentially, and may also have the capability to interfere dominantly with WT functions on some of these genes. Additionally, it could potentially bind to activated Stat5.2, thereby interfering with its function.

5. Conclusions

This study explored the relative involvement of canonical versus non-canonical and other functions for zebrafish Stat5.1 *in vivo*. The results revealed strong impacts of Stat5.1 mutations on embryonic lymphopoiesis, growth, and adiposity that largely appear to be due to loss of canonical functions. However, there was also evidence of potential non-canonical Stat5.1 functions regulating adult T lymphopoiesis and growth, with growth and adiposity also impacted by alternate functions.

Availability of Data and Materials

All data generated or analyzed during this study are included in this article, or available upon request.

Author Contributions

ACW and CL designed the research study. NA and CL performed the research. NA, ACW and CL analyzed the data. NA and ACW wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

This study was approved by the Deakin University Animal Ethics Committee under projects 23-2019, 24-2019, 25-2019, 14-2022, 15-2022, and 16-2022.

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Conflict of Interest

The authors declare no conflict of interest. Given his role as Guest Editor, ACW had no involvement in the peer-

review of this article and has no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to Amedeo Amedei.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2808187.

References

[1] Santos ME, Athanasiadis A, Leitao AB, DuPasquier L, Sucena E. Alternative splicing and gene duplication in the evolution of the FoxP gene subfamily. Molecular Biology & Evolution. 2011; 28: 237–247.

[2] Morris R, Kershaw NJ, Babon JJ. The molecular details of cytokine signaling via the JAK/STAT pathway. Protein Science. 2018; 27: 1984–2009.

[3] Herrington J, Smit LS, Schwartz J, Carter-Su C. The role of STAT proteins in growth hormone signaling. Oncogene. 2000; 19: 2585–2597.

[4] Takeda K, Akira S. STAT family of transcription factors in cytokine-mediated biological responses. Cytokine & Growth Factor Reviews. 2000; 11: 199–207.

[5] Bromberg J, Darnell JE Jr. The role of STATs in transcriptional control and their impact on cellular function. Oncogene. 2000; 19: 2468–2473.

[6] O'Shea JJ, Gadina M, Schreiber RD. Cytokine signaling in 2002. Cell. 2002; 109: S121–S131.

[7] Awasthi N, Liongue C, Ward AC. STAT proteins: a kaleidoscope of canonical and non-canonical functions in immunity and cancer. Journal of Hematology and Oncology. 2021; 14: 198.

[8] Ambrosio R, Fimiani G, Monfregola J, Sanzari E, De Felice N, Salerno MC, *et al*. The structure of human STAT5A and B genes reveals two regions of nearly identical sequence and an alternative tissue specific STAT5B promoter. Gene. 2002; 285: 311–318.

[9] Maurer B, Kollmann S, Pickem J, Hoelbl-Kovacic A, Sexl V. STAT5A and STAT5B-twins with different personalities in hematopoiesis and leukemia. Cancers. 2019; 11: 1726.

[10] Liu X, Robinson GW, Wagner KU, Garrett L, Wynshaw-Boris A, Hennighausen L. Stat5a is mandatory for adult mammary gland development and lactogenesis. Genes & Development. 1997; 11: 179–186.

[11] Baik M, Yu JH, Hennighausen L. Growth hormone-STAT5 regulation of growth, hepatocellular carcinoma, and liver metabolism. Annals of the New York Academy of Sciences. 2011; 1229: 29–37.

[12] Pugliese-Pires PN, Tonelli CA, Dora JM, Silva PCA, Czepielewski M, Simoni G, *et al*. A novel STAT5B mutation causing GH insensitivity syndrome associated with hyperprolactinemia and immune dysfunction in two male siblings. European Journal of Endocrinology. 2010; 163: 349–355.

[13] Bennett C, Lawrence M, Guerrero JA, Stritt S, Waller AK, Yan Y, *et al.* CRLF3 plays a key role in the final stage of platelet genesis and is a potential therapeutic target for thrombocythemia. Blood. 2022; 139: 2227–2239.

[14] Yan S, Lim SJ, Shi S, Dutta P, Li WX. Unphosphorylated STAT and heterochromatin protect genome stability. The FASEB Journal. 2011; 25: 232–241.

[15] Park HJ, Li J, Hannah R, Biddie S, Leal-Cervantes AI, Kirschner K, *et al.* Cytokine-induced megakaryocytic differentiation is regulated by genome-wide loss of a uSTAT transcriptional program. The EMBO Journal. 2016; 35: 580–94.

[16] Lee JE, Yang Y, Liang F, Gough DJ, Levy DE, Sehgal PB. Nongenomic STAT5-dependent effects on Golgi apparatus and endoplasmic reticulum structure and function. American Journal of Physiology-Cell Physiology. 2012; 302: C804–C820.

[17] Bernasconi A, Marino R, Ribas A, Rossi J, Ciaccio M, Oleastro M, *et al.* Characterization of immunodeficiency in a patient with growth hormone insensitivity secondary to a novel STAT5b gene mutation. Pediatrics. 2006; 118: e1584–e1592.

[18] Foley CL, Al Remeithi SS, Towe CT, Dauber A, Backeljauw PF, Tyzinski L, *et al*. Developmental adaptive immune defects associated with STAT5B deficiency in three young siblings. Journal of Clinical Immunology. 2021; 41: 136–146.

[19] Vidarsdottir S, Walenkamp MJE, Pereira AM, Karperien M, van Doorn J, van Duyvenvoorde HA, *et al*. Clinical and biochemical characteristics of a male patient with a novel homozygous STAT5B mutation. The Journal of Clinical Endocrinology & Metabolism. 2006; 91: 3482–3485.

[20] Lewis RS, Ward AC. Conservation, duplication and divergence of the zebrafish stat5 genes. Gene. 2004; 338: 65–74.

[21] Heidary S, Awasthi N, Page N, Allnutt T, Lewis RS, Liongue C, *et al*. A zebrafish model of growth hormone insensitivity syndrome with immune dysregulation 1 (GHISID1) Cellular and Molecular Life Sciences. 2023; 80: 109.

[22] Xiong S, Mei J, Huang P, Jing J, Li Z, Kang J, *et al*. Essential roles of stat5.1 / stat5b in controlling fish somatic growth. Journal of Genetics and Genomics. 2017; 44: 577–585.

[23] Lawrence C. Advances in zebrafish husbandry and management. Methods in Cell Biology. 2011; 6: 429–451.

[24] Sander JD, Zaback P, Joung JK, Voytas DF, Dobbs D. Zinc Finger Targeter (ZiFiT): an engineered zinc finger/target site design tool. Nucleic Acids Research. 2007; 35: W599–W605.

[25] Labun K, Montague TG, Krause M, Torres Cleuren YN, Tjeldnes H, Valen E. CHOPCHOP v3: expanding the CRISPR web toolbox beyond genome editing. Nucleic Acids Research. 2019; 47: W171–W174.

[26] Thisse C, Thisse B. High-resolution *in situ* hybridization to whole-mount zebrafish embryos. Nature Protocols. 2008; 3: 59–69.

[27] Parichy DM, Elizondo MR, Mills MG, Gordon TN, Engeszer RE. Normal table of postembryonic zebrafish development: Staging by externally visible anatomy of the living fish. Developmental Dynamics. 2009; 238: 2975–3015.

[28] Folch J, Lees M, Stanley GHS. A simple method for the isolation and purification of total lipides from animal tissues. Journal of Biological Chemistry. 1957; 226: 497–509.

[29] Regier N, Frey B. Experimental comparison of relative RT-qPCR quantification approaches for gene expression studies in poplar. BMC Molecular Biology. 2010; 11: 57.

[30] Basheer F, Bulleeraz V, Ngo VQT, Liongue C, Ward AC. *In vivo* impact of JAK3 A573V mutation revealed using zebrafish. Cellular and Molecular Life Sciences. 2022; 79: 322.

[31] Sertori R, Liongue C, Basheer F, Lewis KL, Rasighaemi P, de Coninck D, *et al.* Conserved IL-2Rγc signaling mediates lymphopoiesis in zebrafish. Journal of Immunology. 2016; 196: 135–143.

[32] Leonard WJ. Cytokines and immunodeficiency diseases. Nature Reviews Immunology. 2001; 1: 200–208.

[33] Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. Nature Protocols. 2013; 8: 2281–2308.

[34] Sertori R, Trengove M, Basheer F, Ward AC, Liongue C. Genome editing in zebrafish: a practical overview. Briefings in Functional Genomics. 2016; 15: 322–330.

[35] Heltemes-Harris LM, Farrar MA. The role of STAT5 in lymphocyte development and transformation. Current Opinion in Immunology. 2012; 24: 146–152.

[36] Willett CE, Cherry JJ, Steiner LA. Characterization and expres-

sion of the recombination activating genes (rag1 and rag2) of zebrafish. Immunogenetics. 1997; 45: 394–404.
[37] Carmona SJ, Teichmann SA, Ferreira L, Macaulay IC, Stubbington MJT, Cvejic A, *et al*. Single-cell transcriptome analysis of fish immune cells provides insight into the evolution of vertebrate immune cell types. Genome Research. 2017; 27: 451–461.
[38] Langenau DM, Ferrando AA, Traver D, Kutok JL, Hezel JP, Kanki JP, *et al. In vivo* tracking of T cell development, ablation, and engraftment in transgenic zebrafish. Proceedings of the National Academy of Sciences of the United States of America. 2004; 101: 7369–7374.
[39] Amatruda JF, Zon LI. Dissecting hematopoiesis and disease using the zebrafish. Developmental Biology. 1999; 216: 1–15.
[40] Murayama E, Kissa K, Zapata A, Mordelet E, Briolat V, Lin H, *et al*. Tracing hematopoietic precursor migration to successive hematopoietic organs during zebrafish Development. Immunity. 2006; 25: 963–975.
[41] Boehm T, Hess I, Swann JB. Evolution of lymphoid tissues. Trends in Immunology. 2012; 33: 315–321.
[42] Haire RN, Rast JP, Litman RT, Litman GW. Characterization of three isotypes of immunoglobulin light chains and T-cell antigen receptor alpha in zebrafish. Immunogenetics. 2000; 51: 915–923.
[43] Takizawa F, Dijkstra JM, Kotterba P, Korytář T, Kock H, Köllner B, *et al*. The expression of CD8α discriminates distinct T cell subsets in teleost fish. Developmental & Comparative Immunology. 2011; 35: 752–763.
[44] Zapata A, Diez B, Cejalvo T, Gutiérrez-de Frías C, Cortés A. Ontogeny of the immune system of fish. Fish & Shellfish Immunology. 2006; 20: 126–136.
[45] Pereiro P, Varela M, Diaz-Rosales P, Romero A, Dios S, Figueras A, *et al*. Zebrafish Nk-lysins: first insights about their cellular and functional diversification. Developmental & Comparative Immunology. 2015; 51: 148–159.
[46] Barclay JL, Nelson CN, Ishikawa M, Murray LA, Kerr LM, McPhee TR, *et al*. GH (growth hormone)-dependent STAT5 signaling plays an important role in hepatic lipid metabolism. The Journal of Clinical Endocrinology & Metabolism. 2010; 95: 5454–5455.
[47] Lichanska AM, Waters MJ. How growth hormone controls growth, obesity and sexual dimorphism. Trends in Genetics. 2008; 24: 41–47.
[48] Latasa M, Griffin MJ, Moon YS, Kang C, Sul HS. Occupancy and function of the –150 sterol regulatory element and –65 E-box in nutritional regulation of the fatty acid synthase gene in living animals. Molecular & Cellular Biology. 2003; 23: 5896–5907.
[49] Akira S. Functional roles of STAT family proteins: lessons from knockout mice. Stem Cells. 1999; 17: 138–146.
[50] Moriggl R, Gouilleux-Gruart V, Jähne R, Berchtold S, Gartmann C, Liu X, *et al*. Deletion of the carboxyl-terminal transactivation domain of MGF-Stat5 results in sustained DNA binding and a dominant negative phenotype. Molecular & Cellular Biology. 1996; 16: 5691–5700.
[51] Mui AL, Wakao H, Kinoshita T, Kitamura T, Miyajima A. Suppression of interleukin-3-induced gene expression by a C-terminal truncated Stat5: role of Stat5 in proliferation. The EMBO Journal. 1996; 15: 2425–2433.
[52] Liu BA, Engelmann BW, Nash PD. The language of SH2 domain interactions defines phosphotyrosine-mediated signal transduction. FEBS Letters. 2012; 586: 2597–2605.
[53] Gianti E, Zauhar RJ. An SH2 domain model of STAT5 in complex with phospho-peptides define "STAT5 binding signatures" Journal of Computer-Aided Molecular Design. 2015; 29: 451–470.
[54] Zhou S. SH2 domains recognize specific phosphopeptide sequences. Cell. 1993; 72: 767–778.
[55] Lin J, Li P, Liu D, Jin H, He J, Rasheed M, *et al*. Critical role of STAT5 transcription factor tetramerization for cytokine responses and normal immune function. Immunity. 2012; 36: 586–599.
[56] Huang P, Xiong S, Kang J, Mei J, Gui JF. Stat5b regulates sexually dimorphic gene expression in zebrafish liver. Frontiers in Physiology. 2018; 9: 676.
[57] McMenamin SK, Minchin JEN, Gordon TN, Rawls JF, Parichy DM. Dwarfism and increased adiposity in the gh1 mutant zebrafish vizzini. Endocrinology. 2013; 154: 1476–1487.
[58] Iwanami N, Mateos F, Hess I, Riffel N, Soza-Ried C, Schorpp M, *et al*. Genetic evidence for an evolutionarily conserved role of IL-7 signaling in T cell development of zebrafish. The Journal of Immunology. 2011; 186: 7060–7066.
[59] Basheer F, Lee E, Liongue C, Ward AC. Zebrafish model of severe combined immunodeficiency (SCID) due to JAK3 mutation. Biomolecules. 2022; 12: 1521.
[60] Sertori R, Jones R, Basheer F, Rivera L, Dawson S, Loke S, *et al.* Generation and characterization of a zebrafish IL-2Rγc SCID model. International Journal of Molecular Sciences. 2022; 23: 2385.
[61] Liao W, Lin J, Leonard W. Interleukin-2 at the crossroads of effector responses, tolerance, and immunotherapy. Immunity. 2013; 38: 13–25.
[62] Leonard WJ, Lin JX, O'Shea JJ. The γ_c family of cytokines: basic biology to therapeutic ramifications. Immunity. 2019; 50: 832–850.
[63] Canaria DA, Yan B, Clare MG, Zhang Z, Taylor GA, Boone DL, *et al*. STAT5 represses a STAT3-independent Th17-like program during Th9 cell differentiation. The Journal of Immunology. 2021; 207: 1265–1274.
[64] Mandal M, Powers SE, Maienschein-Cline M, Bartom ET, Hamel KM, Kee BL, *et al*. Epigenetic repression of the Igk locus by STAT5-mediated recruitment of the histone methyltransferase Ezh2. Nature Immunology. 2011; 12: 1212–1220.
[65] Chatterjee-Kishore M, Wright KL, Ting JP-, Stark GR. How Stat1 mediates constitutive gene expression: a complex of unphosphorylated Stat1 and IRF1 supports transcription of the LMP2 gene. The EMBO Journal. 2000; 19: 4111–4122.
[66] Kumar A, Commane M, Flickinger TW, Horvath CM, Stark GR. Defective TNF-α-induced apoptosis in STAT1-null cells due to low constitutive levels of caspases. Science. 1997; 278: 1630–1632.
[67] Cheon H, Stark GR. Unphosphorylated STAT1 prolongs the expression of interferon-induced immune regulatory genes. Proceedings of the National Academy of Sciences of the United States of America. 2009; 106: 9373–9378.
[68] Yang J, Chatterjee-Kishore M, Staugaitis SM, Nguyen H, Schlessinger K, Levy DE, *et al*. Novel roles of unphosphorylated STAT3 in oncogenesis and transcriptional regulation. Cancer Research. 2005; 65: 939–947.
[69] Yang J, Liao X, Agarwal MK, Barnes L, Auron PE, Stark GR. Unphosphorylated STAT3 accumulates in response to IL-6 and activates transcription by binding to NFkappaB. Genes & Development. 2007; 21: 1396–1408.
[70] Brachet-Botineau M, Polomski M, Neubauer HA, Juen L, Hédou D, Viaud-Massuard MC, *et al.* Pharmacological inhibition of oncogenic STAT3 and STAT5 signaling in hematopoietic cancers. Cancers. 2020; 12: 240.
[71] Lewis RS, Noor SM, Fraser FW, Sertori R, Liongue C, Ward AC. Regulation of embryonic hematopoiesis by a cytokine-inducible SH2 domain homolog in zebrafish. The Journal of Immunology. 2014; 192: 5739–5748.
[72] Hogan JC, Stephens JM. The regulation of fatty acid synthase by STAT5A. Diabetes. 2005; 54: 1968–1975.