Cohesin components Stag1 and Stag2 differentially influence haematopoietic mesoderm development in zebrafish embryos

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Abstract

Cohesin is a multiprotein complex made up of core subunits Smc1, Smc3 and Rad21, and either Stag1 or Stag2. Normal haematopoietic development relies on crucial functions of cohesin in cell division and regulation of gene expression via three-dimensional chromatin organisation. Cohesin subunit STAG2 is frequently mutated in myeloid malignancies, but the individual contributions of Stag variants to haematopoiesis or malignancy are not fully understood. Zebrafish have four Stag paralogues (Stag1a, Stag1b, Stag2a and Stag2b), allowing detailed genetic dissection of the contribution of Stag1-cohesin and Stag2-cohesin to development. Here we characterize for the first time the expression patterns and functions of zebrafish stag genes during embryogenesis. Using loss-of-function CRISPR-Cas9 zebrafish mutants, we show that stag1a and stag2b contribute to primitive embryonic haematopoiesis. Both stag1a and stag2b mutants present with erythropenia by 24 hours post-fertilisation. Homozygous loss of either paralog alters the number of haematopoietic/vascular progenitors in the lateral plate mesoderm. The lateral plate mesoderm zone of scl-positive cells is expanded in stag1a mutants with concomitant loss of kidney progenitors, and the number of spi1-positive cells are increased, consistent with skewing toward primitive myelopoiesis. In contrast, stag2b mutants have reduced haematopoietic/vascular mesoderm and downregulation of primitive erythropoiesis. Our results suggest that Stag1 and Stag2 proteins cooperate to balance the production of primitive haematopoietic/vascular progenitors from mesoderm.
INTRODUCTION

Cohesin is a large multi-subunit protein complex that was originally characterised for its role in sister chromatid cohesion during mitosis (Losada, 2008; Onn et al., 2008; Nasmyth and Haering, 2009). Cohesin subunits Smc1A, Smc3 and Rad21 form a large ring-shaped structure that entrap and holds together DNA strands (Nasmyth, 2011). A fourth subunit of either Stag1 or Stag2 binds to cohesin by contacting Rad21 and Smc subunits (Shi et al., 2020), and is required for the association of cohesin with DNA.

Additional roles for cohesin include DNA damage repair and the control of gene expression (Dorsett and Strom, 2012). The gene expression function of cohesin is thought to derive from cohesin’s role in three-dimensional genome organisation (Bonora et al., 2014; Rowley and Corces, 2018). Together with the zinc finger protein, CCCTC-binding factor (CTCF), cohesin organizes the genome into large loops known as topologically-associating domains (TADs) (Vietri Rudan and Hadjur, 2015; Hnisz et al., 2016; Rowley and Corces, 2018). The current theory is that cohesin forms loops by extrusion of DNA through the cohesin ring, and CTCF bound in convergent orientation limits extrusion to delineate loop size (Fudenberg et al., 2018; Hansen, 2020).

Inside TADs, cohesin can mediate smaller loops that link genes to their regulatory elements (Merkenschlager and Odom, 2013). Differential formation of sub-TAD gene regulatory loops is thought to be key to cell type specification during development (Hnisz et al., 2016). Several previous studies have linked mutations in cohesin subunits with tissue-specific changes in gene expression (Dorsett, 2009; Merkenschlager, 2010; Horsfield et al., 2012; Kawauchi et al., 2016). Therefore, via its role in genome organization, cohesin plays a crucial role in developmental gene expression.

Germline mutations in genes encoding the cohesin loader NIPBL, or in cohesin subunits, cause a spectrum of human developmental disorders, the best known of which is Cornelia de Lange Syndrome (CdLS). These disorders, known as “cohesinopathies”, are characterized by multifactorial developmental anomalies, intellectual disability and growth delay (Liu and Krantz, 2009). On the other hand, somatic mutations in cohesin subunits contribute to the development of several types of cancer, including bladder cancer (15-40%), endometrial cancer (19%), glioblastoma (7%), Ewing’s sarcoma (16-22%) and myeloid leukemias (5-53%) (De Koninck and Losada, 2016; Hill et al., 2016; Waldman, 2020). How pathogenicity arises from cohesin mutation is poorly understood, but for both cohesinopathies and cancers, causality is thought to derive primarily from the gene expression function of cohesin rather than its cell division role (Hill et al., 2016; Waldman, 2020).

Notably, there is a particularly high frequency of cohesin gene mutations in myeloid malignancies (Kon et al., 2013; Yoshida et al., 2013; Leeke et al., 2014; Thol et al., 2014; Thota et al., 2014; Papaemmanuil et al., 2016). The high frequency of cohesin mutations in myeloid cancers likely reflects cohesin’s role in determining haematopoietic lineage identity and controlling the differentiation of haematopoietic stem cells (Mazumdar et al., 2015; Mullenders et al., 2015; Viny et al., 2015; Galeev et al., 2016; Viny et al., 2019).

Several previous studies have investigated the role of cohesin in animal development. In Drosophila, Nipped-B and cohesin control cut gene expression in the wing margin (Dorsett et al., 2005) and mutations in Nipped-B or cohesin genes have dosage-dependent effects on the expression of developmental genes (Dorsett, 2009; Gause et al., 2010). In mice, deficiency in Nipbl or cohesin subunits results in multifactorial developmental abnormalities that mimic CdLS (Kawauchi et al., 2009; Remeseiro et al., 2012b; Smith et al., 2014; Newkirk et al., 2017). Zebrafish models show that...
Nipbl and cohesin are important for tissue-specific gene regulation (Monnich et al., 2009; Rhodes et al., 2010; Muto et al., 2011), including expression of hox genes (Muto et al., 2014) and runx genes (Horsfield et al., 2007).

Although animal models have been crucial to understanding the developmental origins of both cohesinopathies (Kawauchi et al., 2016) and haematological malignancies (Viny and Levine, 2018), much remains to be discovered. It is still unclear how cohesin contributes to cell type specification in early development and cell lineage specification. Furthermore, whether all the protein components of cohesin operate equivalently in development is undetermined.

In zebrafish, a forward genetic screen determined that mutation in cohesin subunit rad21 led to loss of runx1 expression in the posterior lateral plate mesoderm of zebrafish embryos during early somitogenesis. Knock down of the Smc3 subunit of cohesin also eliminated mesoderm runx1 expression (Horsfield et al., 2007). Runx1 is essential for definitive haematopoiesis, and is itself affected by mutations and translocations in myeloid malignancies (Downing et al., 2000; Speck, 2001). Previous research shows that runx1 is directly regulated by Rad21-cohesin in zebrafish (Horsfield et al., 2007; Marsman et al., 2014) and leukaemia cell lines (Antony et al., 2020). Loss of mesoderm-expressed runx1 at the very earliest time of blood development in rad21 mutants suggests that the onset of haematopoietic differentiation from the mesoderm might require functional cohesin.

The Stag subunits differ from core cohesin subunits Rad21, Smc1 and Smc3 in that they have redundant roles in cell division, such that a complete loss of Stag2 is tolerated due to partial compensation by Stag1. In addition, Stag1 preferentially associates with CTCF to organise TADs whereas Stag2 mediates short-range cell-specific interactions (van der Lelij et al., 2017; Liu et al., 2018; Cuadrado and Losada, 2020). In mice, homozygous loss of Stag1 is lethal at embryonic day 11.5 (E11.5) (Remeseiro et al., 2012a). While adult loss of Stag2 is tolerated, homozygous Stag2-null mouse embryos die by mid-gestation with developmental delay and defective heart morphogenesis (De Koninck et al., 2020). When Stag2 is ablated somatically in adults, increased self-renewal of HSCs accompanied by myeloid skewing is observed (Viny et al., 2019; De Koninck et al., 2020). However, early lethality of Stag mutations makes investigating the embryonic function of Stag1/Stag2 cohesin difficult in mammalian models.

In this study, we characterised the expression of Stag paralogues in early zebrafish development, and investigated whether, like Rad21, cohesin Stag subunits affect haematopoietic differentiation from mesoderm in zebrafish embryos.

RESULTS

Evolution and embryonic expression of zebrafish Stag paralogues

Zebrafish have four gene paralogues encoding Stag proteins: stag1a, stag1b, stag2a and stag2b. To determine if these paralogues are likely to be functional, we characterised their evolutionary conservation and expression in zebrafish embryos.

Phylogenetic analysis of Stag protein sequences using the PhyML algorithm segregated Stag1 and Stag2 into distinct clusters. Stag2b clustered more closely with other vertebrate Stags than Stag2a, while the two Stag1 paralogues had similar levels of divergence (Figure 1A). Whole-mount in situ
hybridisation (WISH) (Figure 1B) and quantitative RT-PCR (qPCR) (Figure 1C,D) was then used to analyse expression of the four stag paralogues in zebrafish embryogenesis.

At early gastrula stages, all four stag paralogues showed ubiquitous expression although stag2a expression was noticeably reduced compared with stag1a/b and stag2b. By 24 hours post-fertilisation (hpf), expression of stag1a/b and stag2b was robust in anterior regions with high cellular density.
Characterisation of zebrafish Stag paralogues

similar to that observed for genes encoding other cohesin subunits (Monnich et al., 2009), while
stag2a was barely expressed above background (Figure 1B).

We used qPCR to quantify mRNA expression of the stag paralogues at different embryonic
timepoints. All four paralogues were both maternally deposited and zygotically expressed with
stag1b and stag2b being the most expressed throughout embryogenesis. Notably, stag1a was
predominantly zygotically expressed whereas stag2a showed maternal deposition that was
downregulated post-midblastula transition (Figure 1C,D).

In summary, all four Stag paralogues are expressed during development, indicating that they have
total potential to be functional.

Generation of stag1 and stag2 mutant zebrafish lines

To determine the physiological roles of the four paralogues, we generated loss-of-function germline
zebrafish mutants in individual stag genes. CRISPR guide RNAs (Supplementary Table 2) were
designed to truncate the Stag paralogues upstream of the STAG domain, which spans exons 6 and 7
in all paralogues. We recovered the following germline mutations: 38 bp insertion in exon 3 of
stag1a, 13 bp deletion in exon 3 of stag1b and 7 bp deletion in exon 3 of stag2b (Figure 2A and
Supplementary Figure 2). No germline mutations could be recovered in stag1b despite evaluating
multiple guide RNAs. The three zebrafish stag mutant alleles we successfully generated were named
stag1a
agt204, stag1b
agt205, and stag2b
agt207.

To confirm knockdown and to evaluate paralogue compensation, we measured the mRNA levels of
the four paralogues at 48 hpf using qPCR (Figure 2B). In stag1a
agt204 mutants, stag1a mRNA was
significantly reduced and was accompanied by significant downregulation of stag1b and stag2b
mRNA. In stag1b
agt205 mutants, stag1b mRNA was significantly reduced and was accompanied by a
significant upregulation of stag1a and stag2a mRNA levels, indicating potential transcriptional
compensation. In stag2b
agt207 mutants, stag2b mRNA was modestly but significantly reduced with no
changes in the other paralogues.

The stag1a
agt204, stag1b
agt205, and stag2b
agt207 zebrafish mutants were all homozygous viable to
adulthood, and fertile. While stag1a
agt204 mutant had no apparent larval phenotype, both stag1b
agt205
and stag2b
agt207 mutants exhibited mild developmental delay. In addition, stag2b
agt207 mutants had
displaced pigment cells in the tail fin by 54 hpf with a penetrance of ~80-85% (Figure 2C). Injection
of 200 pg functional stag2b mRNA in stag2b
agt207 mutants rescued the displaced pigment cells
(Supplementary Figure 3A).

Despite the presence of a 7 bp deletion in the stag2b gene in stag2b
agt207 mutants, downregulation of
the stag2b transcript was rather modest (Figure 2B). Therefore, we sought to confirm loss of function
in stag2b
agt207 mutants by determining if a morpholino oligonucleotide targeting stag2b phenocopies
the stag2b
agt207 mutation. Injection of 0.5 mM stag2b morpholino generated the same pigment cell
displacement phenotype that was observed in the stag2b
agt207 mutant with no observable toxicity.
Furthermore, injection of 0.5 mM stag2b morpholino into stag2b
agt207 embryos caused no additional
abnormalities (Supplementary Figure 3B). These observations indicate that the stag2b
agt207 allele is
likely to be a true loss of function.

Overall, it appears that three of the Stag paralogues, Stag1a, Stag1b and Stag2b, are individually
dispensable for zebrafish development and reproduction. We were not able to recover zebrafish
Characterisation of zebrafish Stag paralogues

[Diagram A]

-8:38I  stag1a  GRCz10/danRer10

-9:13D  stag1b

-5:7D  stag2b

[Diagram B]

**A**

stag1a mRNA at 48 hpf

stag1b mRNA at 48 hpf

stag2a mRNA at 48 hpf

stag2b mRNA at 48 hpf

**B**

relative mRNA

WT  stag1a+  stag1b+  stag2b+

WT  stag1a+  stag1b+  stag2b+



**C**

6 dpf  wildtype

6 dpf  stag2b+
Characterisation of zebrafish Stag paralogues

Figure 2. Generation of zebrafish stag germline mutants. CRISPR-Cas9 genome editing was used to generate germline mutations in stag1a, stag1b and stag2b. (A) Exon diagrams of the respective paralogues showing details of the editing strategy. sgRNA binding sites are marked by red arrowheads with the type of mutation generated indicated above. (B) mRNA levels of the four paralogues in each of the mutant lines indicated on the x-axis. Each data point represents mRNA isolated from a pool of 30 embryos. All graphs are mean +/- one standard deviation. * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001; one-way ANOVA. Expression was normalised to the reference gene, b-actin (Supplementary Figure 1C) (C) stag2b<sup>a207</sup> mutants have displaced pigment cells in the tail fin, zoom-ins are shown in insets. Mutants also show mild developmental delay with late swim bladder inflation as indicated by the black arrow. Scale bars are 200 μm.

mutant for stag2a; its early maternal expression supports the idea that this subunit may be essential in the germline.

Stag mutations reduce primitive erythroid cells in 24 hpf zebrafish embryos

Previously, we found that a nonsense mutation in cohesin subunit rad21 inhibits primitive erythropoiesis and blocks the emergence of differentiated myeloid cells (Horsfield et al., 2007). Therefore, we were interested to determine if cohesin stag subunit mutations also affect haematopoiesis in zebrafish embryos. Whole mount in situ hybridisation (WISH) was used to determine if expression of markers of primitive and definitive haematopoiesis is affected in zebrafish stag mutants at 24 hpf and 36 hpf. We found that stag1b<sup>nz205</sup> mutants had no haematopoietic phenotype (data not shown), but that the stag1a<sup>a204</sup> and stag2b<sup>a207</sup> mutations both had modest effects on embryonic haematopoiesis.

Expression of gata1 marks primitive erythroid cells, and expression of spi1 (also known as pu.1), primitive myelopoiesis. Expression of gata1 at 24 hpf was downregulated in stag1a<sup>a204</sup> and stag2b<sup>a207</sup> homozygous and heterozygous mutants, indicating loss of primitive erythroid cells. gata1 expression was rescued by injection of functional stag1a or stag2b mRNA (Figure 3A). In contrast, we found that stag1a and stag2b mutation had divergent effects on primitive myelopoiesis: stag1a<sup>a204</sup> increased spi1 expression, while stag2b<sup>a207</sup> had no effect (Figure 3B). The results suggest that Stag1a and Stag2b promote gata1-mediated primitive erythropoiesis and in addition, Stag1a restricts spi1-mediated primitive myelopoiesis.

Definitive haematopoietic stem cells (HSCs) in the ventral wall of the dorsal aorta are marked by runx1 and cmyb expression at 36 hpf. HSC expression of runx1 was moderately reduced in stag1a<sup>a204</sup> mutants and unchanged in stag2b<sup>a207</sup> mutants (Supplementary Figure 4). Quantitative PCR of RNA isolated from 48 hpf stag1a<sup>a204</sup> and stag2b<sup>a207</sup> embryos showed that transcript levels of cmyb, mpx and lyz mRNA were similar between mutants and wild type, indicating that definitive myelopoiesis is intact in the mutants. In contrast, gata1 expression remained reduced in both stag1a<sup>a204</sup> and stag2b<sup>a207</sup> mutants at 48 hpf (Figure 3C), indicating that the deficiency in erythropoiesis is sustained from early development. Therefore, Stag1a and Stag2b appear to promote erythropoiesis during embryonic haematopoiesis, but are dispensable for myelopoiesis.

Stag1a and Stag2b are important for specification of scl-positive cells in the haematopoietic mesoderm

A null cohesin rad21 mutation causes a striking, complete loss of runx1 expression in the posterior lateral mesoderm (PLM) of zebrafish embryos at 4-15 somite stages (Horsfield et al., 2007). This
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**Figure 3** legend on following page
observation prompted us to investigate whether stag mutations also affect expression of runx1 and other lineage-defining genes in the intermediate mesoderm.

WISH with a riboprobe detecting runx1 expression in the PLM on 15 hpf embryos (14 somites) revealed that stag1a<sup>ne204</sup> and stag2b<sup>ne207</sup> mutants had relatively normal PLM runx1 expression (Figure 4A). We observed minor expansion in the PLM domain of runx1 in stag1a<sup>ne204</sup> mutants, and minor localised reduction of runx1 expression in stag2b<sup>ne207</sup> mutants; however, qPCR revealed that total runx1 transcript levels are not significantly different between mutants and wild type (Figure 4C). Therefore, unlike rad21 mutation, stag1a or stag2b mutations are by themselves not sufficient to cause dramatic changes to runx1 expression.

Expression of the scl (tal-1) gene marks a subset of cells in the PLM that will later go on to assume either vascular or haematopoietic identity. Surprisingly, we observed significant differences in the expression pattern of scl in the PLM of stag1a<sup>ne204</sup> and stag2b<sup>ne207</sup> mutants at 15 hpf (14 somites) (Figure 4B). An expanded lateral domain of scl expression appeared in the PLM of stag1a<sup>ne204</sup> mutants, and was rescued by injection of stag1a mRNA (Figure 4B). In contrast, scl expression was reduced in the anterior PLM of stag2b<sup>ne207</sup> mutants, and this was rescued by injection of stag2b mRNA (Figure 4B). The observed changes in scl expression were reinforced by qPCR analysis (Figure 4C), which showed an increase of scl transcript in stag1a<sup>ne204</sup> and decrease in stag2b<sup>ne207</sup> mutants, respectively. In contrast to observations in 24 hpf embryos, gata1 transcript levels were increased in stag1a<sup>ne204</sup> mutants along with a slight increase in spi1 mRNA. Expression of the vascular marker, fli1, was not significantly altered (Figure 4C).

The results suggest that during early somitogenesis in stag1a<sup>ne204</sup> mutants, scl-positive cell numbers are expanded and accompanied by the upregulation of primitive haematopoietic markers. In contrast, both scl and gata1 are downregulated in stag2b<sup>ne207</sup> mutants suggesting a reduction in scl-positive haematopoietic/vascular progenitors.

Loss of Stag1a, but not Stag2b, alters gene expression domains in the posterior lateral mesoderm

During early somitogenesis, the PLM contains non-overlapping stripes of pax2a-expressing pronephric progenitors adjacent to the scl-expressing cells. We were curious to know whether
A

WT  stag1a−/−  stag2b−/−

runx1 at 15 hpf

16/16  31/31

B

WT  stag1a−/− + stag1a mRNA

stag2b−/− + stag2b mRNA

scl at 15 hpf

33/33  5/11

22/23  38/38

C

mRNA levels at 15 hpf

WT  stag1a−/−  stag2b−/−

gata1  spi1  scl  runx1  flt1

Relative mRNA

0  1  2  3  4

**  **  *  **

Changes in the scl-positive population in the stag mutants influenced adjacent cell populations, such as pronephric progenitors, in the mesoderm.

At 12 hpf (10 somites), scl expression was expanded in stag1a<sup>nz204</sup> mutants, while in stag2b<sup>nz207</sup> mutants, scl expression was slightly reduced (Figure 5A). This finding is consistent with observations of 15 hpf embryos (Figure 4B,C; Supplementary Figure 5A). Notably, the PLM zone of pax2a expression was reduced concomitant with expansion of scl-expressing cells in the PLM of stag1a<sup>nz204</sup> mutants (Figure 5B; Supplementary Figure 5B). These results suggest that scl-positive haematopoietic/endothelial progenitors are expanded at the expense of pronephric progenitors in stag1a<sup>nz204</sup> mutants. In contrast, in stag2b<sup>nz207</sup> mutants with reduced scl transcript, expression of...
pax2a was maintained in the PLM but reduced in the optic stalk compared with wild type (Figure 5B, C; Supplementary Figure 5B).

Figure 5. stag1a and stag2b mutations affect cell identity in the posterior lateral mesoderm at 12 hpf. (A) scl expression in whole-mount embryos at 12 hpf, posterior views of the PLM; dorsal to the top. In stag1an204 homozygous mutant embryos scl expression is expanded. In stag2bn207 homozygous mutant embryos, scl expression is reduced. Changes in expression are marked by arrows and the number of embryos is indicated below each panel. (B) pax2 expression in whole-mount embryos at 12 hpf; posterior views of the PLM; dorsal to the top. In stag1an204 homozygous mutant embryos, pax2 expression is markedly reduced. In stag2bn207 homozygous mutant embryos, pax2 expression is comparable to wild type. Changes in expression are marked by arrows and the number of embryos is indicated below each panel. (C) pax2 expression in whole-mount embryos at 12 hpf, lateral views of the head region; anterior to the left. Anterior pax2 expression is specifically reduced in the optic stalk of stag2bn207 homozygous mutant embryos. (D) Multiplexed in situ HCR of scl (Alexa Fluor 488, false colour yellow), gata1 (Alexa Fluor 594, false colour red) and fli1 (Alexa Fluor 647, false colour blue) expression at 15 hpf. High magnification maximum intensity projections of a single PLM stripe; posterior views with anterior to the left. Expression domains of scl broadly overlap gata1 and fli1 in all embryos. Ectopic scl expression, indicated by white arrow, in stag1an204 homozygous mutant embryos does not overlap gata1 or fli1 expression domains. In stag2bn207 homozygous mutant embryos, expression of all three markers is reduced. Scale bars are 10 µm. The number of embryos analysed is indicated below the respective panels.
A subset of scl-positive cells also express gata1 and acquire a haematopoietic fate while the remaining cells express fli1 acquiring an endothelial fate. We next wanted to determine whether scl-positive cells are skewed towards a haematopoietic or vascular fate in the stag mutants. Multiplex in situ hybridisation using HCR revealed that the expression of gata1 and fli1 largely overlap that of scl in the PLM (Figure 5D; Supplementary Figure 5A). Ectopic scl expression seen in stag1a\textsuperscript{nz204} mutants did not overlap gata1 or fli1 expression, but gata1 expression appeared more intense than wild type, consistent with qPCR results (Figure 5D; Figure 4C; Supplementary Figure 5A). We detected no differences in the relative composition of scl\textsuperscript{+}/gata1\textsuperscript{+} and scl\textsuperscript{+}/fli1\textsuperscript{+} cells in the PLM of Figure 6. stag1a and stag2b mutations differentially alter the production of primitive myeloid cells in the anterior lateral mesoderm at 12 hpf. (A) scl expression in whole-mount embryos at 12 hpf. Ventral views of ALM are shown; dorsal to the top. scl expression is comparable to wildtype in stag1a\textsuperscript{nz204} homozygous mutant embryos and reduced in stag2b\textsuperscript{nz207} homozygous mutant embryos. (B) scl expression in whole-mount embryos at 15 hpf. Top panels show lateral views and bottom panels show ventral views of the ALM. Expanded scl expression in the ALM of stag1a\textsuperscript{nz204} mutants is rescued upon injection of functional stag1a mRNA. The reduced scl expression in the ALM of stag2b\textsuperscript{nz207} mutants is rescued upon injection of functional stag2b mRNA. Changes in expression are marked by arrows and the number of embryos is indicated below each panel. (C) Multiplexed in situ HCR of runx1 and spi1 at 15 hpf. Maximum intensity projections of a single ALM stripe; lateral views with dorsal to the top. Expression domains of runx1 (Alexa Fluor 647) and spi1 (Alexa Fluor 514, false colour blue) broadly overlap in all embryos. Both runx1 and spi1 are expanded in stag1a\textsuperscript{nz204} embryos but reduced in stag2b\textsuperscript{nz207} embryos. Changes in expression are indicated by white arrows. Scale bars are 10 µm. The number of embryos analysed is indicated below the respective panels.
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...mutations (Supplementary Figure 5A). The results suggest that in stag1a
expression does not appear to skew cell fate in the PLM, but transiently increases gata1 expression.

In stag2b
 mutants, the expression domains of scl, gata1 and fli1 was reduced in the PLM (Figure 5D; Supplementary Figure 5A). Cell composition of the PLM was unchanged in stag2b
 mutants (Supplementary Figure 5A), suggesting that reduced scl, gata1 and fli1 does not influence PLM cell fate.

Stag1a or Stag2b loss differentially affects the production of primitive myeloid cells in the anterior lateral mesoderm

We next asked if stag mutants also affect haematopoietic cell specification in the anterior lateral mesoderm (ALM), a site of primitive myelopoiesis (Berman et al., 2005). At 12 hpf, scl expression in the rostral blood island marks a population of cells fated to become spi1-positive myeloid cells or fli1-positive endothelial cells.

scl expression was normal in the ALM of stag1a
 mutants at 12 hpf (Figure 6A) but by 15 hpf scl expression was markedly increased in stag1a
 (Figure 6B, Supplementary Figure 5C). Increased scl expression in the ALM of stag1a
 mutants was reversed by injection of functional stag1a mRNA, which reduced scl expression to below normal. In stag2b
 mutants, scl expression was reduced in the ALM at both 12 and 15 hpf, and was robustly rescued upon injection of stag2b mRNA (Figure 5B).

Multiplex HCR expression analysis showed that the population of ALM cells that co-express runx1 and spi1 are expanded in the ALM of stag1a
 mutants (Figure 6C). In contrast, the same spi1/runx1-positive ALM population was reduced in stag2b
 mutants. Since there was also a modest increase in spi1-positive cells in stag1a
 mutants at 24 hpf (Figure 3B), these results are consistent with the idea that excess scl in stag1a
 mutants promotes myelopoiesis in the anterior blood island.

Taken together, the results suggest that in early somitogenesis, Stag1a normally restricts scl expression in the ALM and PLM, such that its loss in stag1a
 mutants results in a modest expansion of primitive erythroid and myeloid cells at the expense of pronephros specification. In contrast, Stag2b positively regulates the number of scl-expressing cells and its loss in stag2b
 mutants leads to a reduction of scl-derived lineages. However, by 24 hpf gata1-positive cells are reduced in both stag1a
 and stag2b
 mutants, suggesting that erythropaenia is a common consequence of an imbalance in scl-positive cells. Because both stag1a
 and stag2b
 mutants are homozygous viable, there must be sufficient redundancies and plasticity to overcome these stag mutations in later development.

DISCUSSION

All four Stag paralogues are expressed in early embryogenesis, suggesting that they are likely to have a function in early development. The maternally and zygotically expressed stag1b and stag2b are the most abundant of the zebrafish Stags. While zebrafish Stag1a and Stag1b are more or less equally related to mammalian Stag1, the higher zygotic expression of stag1b suggests that it is the most predominant isoform in zebrafish. Of the two Stag2 isoforms, stag2b is the most abundantly


expressed and is also most closely related to mammalian Stag2, suggesting that Stag2b is likely to be the predominant Stag2 in zebrafish.

The *stag2a* parologue mRNA is present in early embryos up until the mid-blastula transition and then is rapidly downregulated. Interestingly, we detected robust *stag2a* expression in the ovaries of adult zebrafish (Supplementary Figure 6), and little expression elsewhere in adults. It is possible that *stag2a* is required in oocytes for development pre-zygotic genome activation, but is dispensable at later stages. Significantly, we were not able to isolate a CRISPR mutant for *stag2a*, raising the possibility that Stag2a is essential in oocytes its loss does not allow for transmission of a mutation.

All three germline mutations successfully isolated for the Stag paralogues are homozygous viable and fertile, indicating that there is likely to be functional redundancy among Stag proteins throughout development and reproduction. Compensation could be partly transcription based, for example, *stag1b* mutant embryos upregulated expression of *stag1a* and *stag2a*. Fish that were mutant for the most abundant Stags, *stag1b* and *stag2b*, exhibited a slight developmental delay as larvae, and had displaced pigment cells in the tail fin. However, only the *stag1a* mutant (which had no morphological phenotype) and *stag2b* mutants produced haematopoietic phenotypes in embryos younger than 48 hpf. The sharp increase of *stag1a* expression and the abrupt downregulation of *stag2a* at the mid-blastula transition (leaving *stag2b* as virtually the sole zygotic Stag2) might explain why these two particular mutations caused phenotypes in embryos.

Analysis of primitive haematopoiesis from 24-48 hpf showed that both the *stag1a* and *stag2b* mutants had a profound decrease in erythroid cells. These findings are in partial agreement with data from mice. Somatic removal of Stag2 in mice resulted in increased myeloid progenitors and decreased megakaryocyte-erythrocyte progenitors, with consequential myeloid skewing (Viny et al., 2019; De Koninck et al., 2020). However, there is no haematopoietic phenotype in *Stag1*-mutant mice (Viny et al., 2019), contrasting with the erythropaenia we observed in zebrafish *stag1a* mutants at 24 and 48 hpf.

Although *stag1a* and *stag2b* mutants both had erythroid deficiency, unexpectedly, only the *stag1a* mutant presented with additional early haematopoietic alterations. These included a reduction in *runx1*-positive definitive HSCs at 36 hpf in *stag1a* mutants, and striking changes to expression of *scl* in the PLM at 12 and 15 hpf.

The basic helix-loop-helix protein Scl/Tal-1 is expressed in mesoderm and marks both vascular and haematopoietic lineages. Scl is thought to program ventral mesoderm to a haematopoietic fate (Orkin, 1995; Davidson and Zon, 2000; Prummel et al., 2020). Overexpression of zebrafish *scl* leads to an overproduction of blood from mesoderm at the expense of other non-axial mesoderm fates (Gering et al., 1998). Consistent with this, we observed a reduction in expression of *pax2a* in the pronephric mesoderm in *stag1a* mutants that had expanded expression of *scl*. However, a concomitant increase in expression of downstream haematopoietic markers *gata1* and *spil* was only transitory in *stag1a* mutants. Expression of *gata1* and *spil* is increased in 15 hpf *stag1a* mutants but by 24 hpf, *spil* expression was normal and *gata1* expression was reduced.

Stag2 depletion in mice induces both an increase in self-renewal and reduced differentiation capacity in HSCs (Viny et al., 2019). Stag2-deficient mice had downregulation of *spil* target genes that promote myeloid differentiation. ChIP-sequencing experiments in mice showed that recruitment of *Spil* to genomic binding sites is reduced in the absence of Stag2 (Viny et al., 2019). In zebrafish, loss
of Stag2b had little effect on spi1 expression, but did lead to reduced primitive haematopoiesis overall.

The phenotypes of stag1a<sup>nz204</sup> and stag2b<sup>nz207</sup> mutants have opposite effects on scl expression in early somitogenesis (12 and 15 hpf), but a similar reduction in gata1-positive cells by 24 hpf. We suggest that loss of Stag2b leading to reduced scl expression limits the pool of progenitors that can contribute to primitive haematopoiesis. Conversely, we propose that increased scl expression caused by loss of Stag1a increases haematopoietic progenitors that are subsequently exhausted by early differentiation. These scenarios would explain the erythropenia observed in both stag1a<sup>nz204</sup> and stag2b<sup>nz207</sup> mutants by 24 hpf (Figure 7).

![Figure 7. Hypothetical model explaining the effects of Stag1a and Stag2b loss on primitive erythropoiesis.](image)

A remaining question is the mechanism by which stag1a<sup>nz204</sup> and stag2b<sup>nz207</sup> mutants differentially affect scl expression. High levels of Bmp signalling induce lateral plate mesoderm and specify haematopoietic fate (Davidson and Zon, 2000; Prummel et al., 2020). Bmp signalling cooperates with Wnt signalling to promote blood fate through activation of homeobox transcription factors Cdx1 and Cdx4 (Lengerke et al., 2008). Previous studies show that mutations in cohesin subunits interfere with canonical Wnt signalling (Avagliano et al., 2017; Chin et al., 2020), so it is possible that loss of Stag1a or Stag2b differentially affect the balance of Bmp and Wnt signalling that directs the production of scl-positive cells. Further experimentation will be needed to determine whether this is the case.

In summary, we have characterised the expression and function of zebrafish Stag paralogues in early development and haematopoiesis. We found a surprising role for the Stag1a orthologue in restricting primitive vascular/haematopoietic cell numbers. In contrast, Stag2b loss-of-function reduced progenitor numbers. Subfunctionalisation and homozygous viability of the zebrafish stag mutants...
offer a unique opportunity to dissect cohesin’s developmental functions in the absence of interference from cell cycle phenotypes.

**MATERIALS AND METHODS**

**Zebrafish maintenance**

Wild type (WIK) and mutant fish lines were maintained according to established protocols (Westerfield, 1995). Zebrafish procedures were carried out in accordance with the Otago Zebrafish Facility Standard Operating Procedures. Zebrafish mutant lines were developed under AUP-19-17 approved by the University of Otago Animal Ethics Committee. For all experiments, embryos were incubated at 22 °C or 28 °C.

**CRISPR-Cas9 editing**

At least three sgRNAs were designed for each *stag* gene using the publicly available CHOPCHOP CRISPR design tool (Montague et al., 2014). sgRNAs were synthesised using a cloning-free approach as previously described (Varshney et al., 2016). Recombinant Cas9 protein was obtained commercially (PNA Bio Inc., Newbury Park, California, USA). Ribonucleoprotein complexes (RNPs) were assembled by mixing sgRNA and Cas9 protein at concentrations of 100 pg/embryo and 300 pg/embryo, respectively in 300 mM KCl. RNPs were incubated for 5 minutes at 37 °C before injection into 1-cell stage WIK embryos. Editing efficiencies were evaluated by genotyping eight embryos from each injection clutch using high resolution melt analysis (HRMA). The most efficient sgRNAs were used to generate germline mutant lines (Supplementary Table 2). Primers used for genotyping are listed in Supplementary Table 3.

**Morpholino and mRNA rescue injections**

Morpholinos were purchased from GeneTools LLC (Philomath, Oregon, USA) for the *stag* genes (Supplementary Table 4). 1-cell stage zebrafish embryos were injected with 0.5 mM of morpholino. Full-length mRNA constructs in pcDNA3.1+/C-(K)DYK vectors were obtained from GenScript Biotech (Piscataway, New Jersey, USA) for each *stag* gene. mRNA was synthesised using the mMessage mMachine transcription kit (Ambion, Austin, Texas, USA) and 200 pg was injected into *stag* mutant embryos at the 1-cell stage.

**Whole-mount in situ hybridisation (WISH)**

WISH was performed as previously described (Thisse and Thisse, 2008). Digoxigenin-labelled riboprobes for the four *stag* genes were synthesized from PCR clones inserted into pGEM®-T Easy vectors (Promega, Madison, Wisconsin, USA) using T7/Sp6 RNA polymerase (Roche Diagnostics, Basel, Switzerland). Anti-DIG alkaline phosphatase antibody (Roche Diagnostics, Basel, Switzerland) was used for detection, followed by visualization with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) (Roche Diagnostics, Basel, Switzerland). Embryos were imaged using a Leica M205 FA epifluorescence microscope (Leica, Wetzlar, Germany Applications Suite). Primers used for the amplification of *stag* riboprobes are listed in Supplementary Table 3.
**Quantitative PCR (qPCR)**

Total mRNA was extracted from pools of 30 embryos using NucleoSpin RNA kit (Macherey-Nagel, Bethlehem, PA, USA). Complementary DNA (cDNA) was synthesized with qScript cDNA SuperMix (Quanta Biosciences, Beverly, MA, USA). Expression levels of the *stag* paralogues (primer sequences in Supplementary Table S3) and haematopoietic markers were measured using SYBR Premix Ex Taq II (Takara Bio Inc., Kusatsu, Japan) on a Roche LightCycler400. Reference genes were *b-actin* and *rpl13a*.

**Hybridisation chain reaction (HCR)**

HCR probe sets for *pax2*, *scl*, *runx1*, *gata1*, *spi1* and *fli1* were obtained from Molecular Instruments, Inc. (California, USA). HCR was performed as per the manufacturer’s protocol for zebrafish embryos. Embryos were mounted in 1% agarose and imaged on Nikon C2 confocal microscope (Nikon Corp, Tokyo, Japan NIS-Elements). Image analysis was performed using ImageJ. For embryos shown in figures, maximum intensity projections were generated and brightness/contrast was adjusted with no further processing. For quantitative analysis, individual channels were background-subtracted, auto-thresholded using the RenyiEntropy algorithm (Kapur et al., 1985) and fluorescence intensities were measured. Colocalization analysis was performed using the JACoP plugin (Bolte and Cordelières, 2006) in ImageJ.

**Statistical Analysis**

GraphPad PRISM 7 was used for performing all statistical analysis. One-way ANOVAs (Tukey’s multiple comparisons tests) were used for estimating the statistical significance of qPCR and HCR data.

**CONFLICTS OF INTEREST**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**AUTHOR CONTRIBUTIONS**

SK and JAH designed experiments. SK and AL performed experiments. SK, AL and JAH analyzed data. SK and JAH wrote the paper. All authors read and approved the final manuscript.

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Supplementary Figure 1. Stability of reference genes used for quantitative RT-PCR (qPCR) normalisation. (A) Ct values of b-actin and rpl13a in wildtype embryos at 48 hpf. (B) Ct values of b-actin and rpl13a in wildtype and mutant embryos at 15 hpf. (C) Ct values of b-actin and rpl13a in wildtype and mutant embryos at 48 hpf. Expression of rpl13a is altered in mutants. **P≤0.01; one-way ANOVA.
Supplementary Figure 2. Genomic DNA sequence detail of zebrafish *stag* gene germ-line CRISPR mutants. Nucleotide alignments of wildtype and mutant homozygous sequences are shown. The sgRNA sites are annotated in blue.
Supplementary Figure 3. The \textit{stag2b} mutation: confirmation of loss of function and mutant phenotype. (A) The ectopic pigment cells seen in the tail fin of \textit{stag2b-/-} embryos (red arrows) are rescued upon injection of \textit{stag2b} mRNA. Lateral views of tail fin zoom-ins are shown, anterior to the left. (B) Injection of 0.5 mM \textit{stag2b} morpholino does not induce any additional phenotypes in \textit{stag2b-/-} embryos, and confirms that Stag2 loss causes displaced pigment cells (red arrows) and tail fin folds (blue arrow). Lateral views of full-length embryos and tail fin zoom-ins are shown, anterior to the left. Numbers of embryos are indicated below the respective panels.
Supplementary Figure 4. Whole-mount in situ hybridization analysis of expression of runx1 and cmyb at 36 hpf. Lateral views are shown, anterior to the left. Insets show zoom-ins of the dorsal aorta region. Reduced runx1 expression in stag1a-/- embryos is indicated by an arrow. Number of embryos is indicated below the respective panels. Scale bars are 100 µm.
Supplementary Figure 5. Stag mutations affect gene expression in the posterior lateral mesoderm (PLM) and anterior lateral mesoderm (ALM). (A) Multiplexed in situ HCR of scl (yellow), gata1 (red), and fli1 (blue) expression at 15 hpf. Dorsal PLM views are shown, anterior to the top. Extra scl expression in stag1a-/- embryos and reduction of PLM expression in stag2b-/- embryos is marked by white arrows. Quantitative analysis of fluorescence integrated densities indicates scl, gata1 and fli1 trend to non-significant upregulation in stag1a-/- embryos in the PLM. Composition of the PLM is equivalent in all embryos. (B) Multiplexed in situ HCR of scl (yellow) and pax2a (cyan) expression at 15 hpf. Posterior views of a single PLM stripe are shown, dorsal to the left. Arrows mark lateral expansion of scl into the pax2a domain in the middle stag1a-/- panels and reduced scl expression in the lower stag2b-/- panel. (C) Quantitative analysis of fluorescence integrated densities of ALM markers shows an increase of runx1 and spi1 expression in stag1a-/- embryos. **** P ≤ 0.0001; one-way ANOVA.
Supplementary Figure 6. *in situ* hybridization of *stag2a* in adult zebrafish ovary. Expression of *stag2a* as detected by *in situ* hybridization (blue/purple) is clearly visible in Stage I, II and III oocytes in this transverse section through wild type adult zebrafish ovary.
### Supplementary Table 1. List of accession identifiers for proteins used for phylogenetic analysis.

| Protein | Accession ID |
|---------|-------------|
| Hs STAG1 | NP_005853.2 |
| Hs STAG2 | NP_001036214.1 |
| Gg STAG1 | XP_015146838.1 |
| Gg STAG2 | XP_004940885.1 |
| Mm Stag1 | NP_001344193.1 |
| Mm Stag2 | NP_001071180.1 |
| Xt stag1 | NP_001121432.1 |
| Xt stag2 | XP_002931833.2 |
| Dr stag1a | NP_001349269.1 |
| Dr stag1b | XP_692120.3 |
| Dr stag2a | NP_001093498.1 |
| Dr stag2b | XP_005173250.1 |

### Supplementary Table 2. sgRNA sequences used to generate CRISPR mutants. PAM sequences are marked in blue.

| Target gene | Sequence 5’-3’ | CHOPCHOP in silico efficiency |
|-------------|----------------|------------------------------|
| stag1a      | GGGCTTTATGGCAGTCCAGA | 49.5 |
| stag1b      | CGGGAGGAGGCCGAATGGAG | 54.11 |
| stag2b      | GCCCCTGGAGAGAAGGGAAAAGG | 45.19 |

### Supplementary Table 3. Primer sequences used in this study.

| Target gene | Forward primer | Reverse primer |
|-------------|----------------|----------------|
| stag1a      | CTGGACCTTACATGACCGGC | TATCCAGCGTCATGGACACG |
| stag1b      | CCAGGTTGATGGCAGAAAGGTG | GGCGTCCAGATGGCTTTTCCAT |
| stag2a      | AGCCGCTCTCAAGGATCGAAT | CAGCGTCAGCGCTTTAATGG |
| stag2b      | CAATAGCAGAGATCCGCGGA | GACACTTCAGACCGACCTCA |
| gata1a      | TTACTGCCACCCGTGGATGT | TTGGCGAACTGGAGCCTTGT |

**Primers used for qPCR**

| Target gene | Forward primer | Reverse primer |
|-------------|----------------|----------------|
| stag1a      | CTTTGCGCCAACACTGTT | GAGTTCTGCTCTTGCCC |
| stag1b      | GTCTGAAAGCATTTCTGGGCT | GGCAATCTCAGGTAACCGT |
| stag2a      | AAGGGCGAACATGGCCAAATC | GACGACGACCTACCCCTT |
| stag2b      | CACTTCACCTGGCTTGT | GCACCTTCAGACCGACTT |

**Primers used for in situ hybridisation**

| Target gene | Forward primer | Reverse primer |
|-------------|----------------|----------------|
| stag1a      | CTGGACCTTACATGACCGGC | TATCCAGCGTCATGGACACG |
| stag1b      | CCAGGTTGATGGCAGAAAGGTG | GGCGTCCAGATGGCTTTTCCAT |
| stag2a      | AGCCGCTCTCAAGGATCGAAT | CAGCGTCAGCGCTTTAATGG |
| stag2b      | CAATAGCAGAGATCCGCGGA | GACACTTCAGACCGACCTCA |

**Primers used for genotyping**

| Target gene | Forward primer | Reverse primer |
|-------------|----------------|----------------|
| stag1a CRISPR | GCCTCGGAAGATCTCCCATCAG | GCACACCTGCATAGCAGCTT |
| stag1b CRISPR | GCCCGCTAAATAGGCAAGGCCCA | AAGCAGCACCAACACTC |
| stag2b CRISPR | GTCCTCGGCTTCCAGGCGAA | TGACCTGCATGGCCTT |

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Supplementary Table 4. Morpholino sequences used in this study.

| Name | Sequence 5'-3' | Binding site |
|------|----------------|--------------|
| stag1a | GGTTAGATTTGTTACAGGTCT | 5'UTR |
| stag2b | GTAATCCGGTGCGCAGATATTTC | ATG |