




## RESEARCH ARTICLE OPEN ACCESS

# SMC2 and Condensin II Subunits Are Essential for the Development of Hematopoietic Stem and Progenitor Cells in Zebrafish

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## ABSTRACT

Hematopoietic stem and progenitor cells (HSPCs) play a pivotal role in blood cell production, maintaining the health and homeostasis of individuals. Dysregulation of HSPC function can lead to blood-related diseases, including cancer. Despite its importance, our understanding of the genes and pathways underlying HSPC development and the associated pathological mechanisms remains limited. To elucidate these unknown mechanisms, we analyzed databases of patients with blood disorders and performed functional gene studies using zebrafish. We employed bioinformatics tools to explore three public databases focusing on patients with myelodysplastic syndrome (MDS) and related model studies. This analysis identified significant alterations in several genes, especially *SMC2* and other condensin-related genes, in patients with MDS. To further investigate the role of *Smc2* in hematopoiesis, we generated *smc2* loss-of-function zebrafish mutants using CRISPR mutagenesis. Further analyses of the mutants revealed that *smc2* depletion induced G2/M cell cycle arrest in HSPCs, leading to their maintenance and expansion failure. Notably, although the condensin II subunits (*ncaph2*, *ncapg2*, and *ncapd3*) were essential for HSPC maintenance, the condensin I subunits did not affect HSPC development. These findings emphasize the crucial role of condensin II in ensuring healthy hematopoiesis via promoting HSPC proliferation.

Chang-Kyu Oh, Man S. Kim, and Unbeom Shin contributed equally to this work.

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## 1 | Introduction

Hematopoietic stem and progenitor cells (HSPCs) are multipotent progenitor cells with the capacity for both self-renewal and differentiation (Seita and Weissman 2010). Located within the bone marrow, these cells continuously generate all types of blood cells, including the erythroid, myeloid, and lymphoid lineages (Copley and Eaves 2013; Sawai et al. 2016). Consequently, they play crucial roles in supporting immune responses and efficient oxygen transport throughout the body (Shevyrev et al. 2023). Furthermore, HSPCs dynamically maintain blood homeostasis by adjusting their proliferation or maturation according to the body's needs (Glauche et al. 2009). Proper HSPC development, maintenance, and function are thus essential for the overall homeostasis of the body and effective response to various challenges.

Defective HSPC development and function can lead to various blood diseases and disorders (Robertson et al. 2016; Risitano et al. 2007; Bao et al. 2019). Myelodysplastic syndrome (MDS) results from malfunctioning HSPCs, leading to inadequate or aberrant production of certain blood cell types (Zhan and Park 2021). Aplastic anemia, characterized by insufficient production of blood cells, often originates from dysfunctional HSPCs in the bone marrow (Medinger et al. 2018). Furthermore, leukemia, a cancer that affects both blood and bone marrow, can originate from mutations within HSPCs, causing uncontrolled production of atypical white blood cells (Passequé et al. 2003). These disorders highlight the vital role of HSPCs in maintaining blood health, emphasizing the need for comprehensive research into HSPC biology and related diseases.

Blood disorders primarily arise from hematopoietic homeostasis failure and the aberrant regulation of molecular mechanisms supervising hematopoiesis (Sachs 1996; Enciso et al. 2015). Therefore, to understand the roles of genes potentially associated with hematopoietic malignancies, it is crucial to functionally validate those genes related to regular hematopoiesis. Zebrafish are excellent vertebrate model systems for studying hematopoiesis and blood diseases owing to their unique experimental advantages and conserved hematopoietic system, similar to that of mammals (Han et al. 2017; Traver et al. 2003). For instance, our studies using zebrafish embryos revealed the association of FAM213A with both the prognosis of acute myeloid leukemia (AML) and the modulation of oxidative stress and myelopoiesis (Oh, Ha, et al. 2020). Additionally, we determined the association between TPD52, a poor prognostic indicator of AML, and HSPC maintenance, specifically its influence on cell proliferation during zebrafish embryogenesis (Kang et al. 2020). The condensin complex subunit, CAP-G2, is intricately linked to erythroid cell differentiation (Xu et al. 2006). Zebrafish research has provided diverse insight into the functions of genes associated with blood disorders.

Condensin is an essential protein complex involved in chromosomal condensation and segregation during mitosis and meiosis (Ono et al. 2003; Hagstrom et al. 2002). The structure of the condensin complex depends on the structural maintenance of chromosome (SMC) proteins and non-SMC condensin complex subunits. The heterodimers of SMC2 and SMC4 form the primary axis of the condensin complex, where both subunits

feature crucial ATP- and nucleotide-binding motifs (Ono et al. 2003; Hagstrom et al. 2002; Palou et al. 2018). By complementing these SMC proteins, non-SMC subunits complete the condensin complex. Based on their configuration of non-SMC subunits, condensins are categorized as condensin I (composed of CAP-H, CAP-G, and CAP-D2) and condensin II (composed of CAP-H2, CAP-G2, and CAP-D3) (Hirano 2012). These complexes, which are evolutionarily conserved among vertebrates, are instrumental in chromosomal assembly and segregation (Ono et al. 2003; Hirano 2012; Hirano et al. 1997).

Each condensin subunit plays a distinct role during mitosis, influencing the chromosome structure according to its cellular distribution ratio (Skibbens 2019). Condensin I facilitates cohesin dissociation from chromosome arms and chromosome shortening via prometaphase and metaphase, whereas condensin II primarily contributes to chromosome condensation in early prophase (Hirota et al. 2004). Individual non-SMC components affect the development and function of specific cells or organs. For example, the condensin I complex regulates the survival of proliferating cells in the neural retina of the zebrafish (Seipold et al. 2009). Condensin II subunits function as regulators of mammalian T-cell differentiation and cell fate determination in *Drosophila* (Gosling et al. 2007; Klebanow et al. 2016).

Despite these insights, our understanding of the specific functions of SMC2 and the associated non-SMC proteins in blood disorders and hematopoiesis remains incomplete. This study aims to identify candidate genes and pathways implicated in blood diseases and their associated hematopoietic processes. We analyzed genomic data from patients diagnosed with MDS and performed loss-of-function experiments using zebrafish to determine how the condensin complex contributes to HSPC development. Our findings will enhance our understanding of the condensin complex's role in hematopoiesis and its relevance to blood disorders.

## 2 | Methods

### 2.1 | Analysis of RNA-seq Datasets

Three MDS-associated transcriptome datasets, bulk RNA-seq datasets under the accession GSE173108 (Mian et al. 2023), GSE183325 (Berastegui et al. 2022), and GSE131581 (Ochi et al. 2020) were downloaded from Gene Expression Omnibus (GEO) and reanalyzed. At first, a typical differential analysis on four different bulk RNA-seq datasets was processed through DESeq. 2 (Love et al. 2014) ( $\log_2$  fold change  $> 0$  for upregulation,  $\log_2$  fold change  $< 0$  for downregulation, and  $p$  value  $< 0.05$ ) and visualized using EnhancedVolcano. From the differentially expressed genes (DEGs), protein-protein interaction (PPI) networks were generated through STRING (Szklarczyk et al. 2023), which is a protein interaction database containing known direct/indirect associations, where each interaction score was higher than 0.9. Those PPI networks narrowed down into three protein clusters from the DEGs, and one of the protein complexes from those clusters known to be irrelevant to MDS to the best of our knowledge was selected. Finally, one of

the protein families (condensin complex), *smc2*, was considered as a potential MDS-associated gene for further investigation.

## 2.2 | Zebrafish Maintenance and Chemical Treatment

Wild-type (WT) AB zebrafish were maintained in an automatic circulation system (Genomic-Design) at 28.5°C. All experiments were performed in accordance with the guidelines of the Ulsan National Institute of Science and Technology (UNIST) Institutional Animal Care and Use Committee (IACUC) (approval number: UNISTIACUC-20-09). Eggs and embryos were maintained with E3 solution in incubators at 28.5°C. Nocodazole (500 µg/mL [Sigma Aldrich, USA]) dissolved in dimethyl sulfoxide (DMSO) was prepared to induce G2/M cell cycle arrest. Twelve zebrafish embryos were incubated with nocodazole (500 ng/mL) from 24 h post fertilization (hpf) for 48 h, whereas the control group was treated with 0.1% DMSO.

## 3 | Results

### 3.1 | Alteration of Condensin Components in Samples From Patients With MDS and an Experimental MDS Model

Based on the hypothesis that there are genetic alterations related to general hematopoiesis in the development of blood diseases (Bao et al. 2019), we examined multiple databases of patients with such conditions. As MDS involves challenges in both normal blood cell development and HSPC genesis and function (Zhan and Park 2021), we conducted an analysis on MDS to understand the mechanisms related to hematopoiesis, including HSPC development.

To investigate the functional mechanisms of genes associated with MDS, we focused on identifying candidate genes using RNA-seq datasets from patients with MDS. Three bulk RNA-seq datasets were collected: (i) human CD34+ HSPCs from patients with MDS (GSE173108) (Mian et al. 2023), (ii) four human hematopoietic stem cells characterized as CD34+ CD38– CD90+ CD45RA– (GSE183325) (Berastegui et al. 2022), and (iii) cells with cohesin mutation linked to MDS (GSE131581) (Ochi et al. 2020). Equipped with these datasets, we conducted a comprehensive analysis to identify significantly DEGs and determine the overlapping gene lists across all three datasets. Furthermore, we performed protein-interaction profiling for these candidates to understand their potential interplays.

We first performed differential analyses of gene expression levels in three groups: (i) patients with MDS and healthy individuals (GSE173108), (ii) patients with MDS and healthy young adults (GSE183325), and (iii) STAG2 knock-out HL-60 cell lines presenting MDS-like phenotypes and WT controls (GSE131581). A significant number of DEGs were revealed in each comparison: 9329 in the first, 4107 in the second, and 2184 in the third (Figure 1A). Interestingly, one gene was upregulated, whereas the expression of 80 genes was consistently downregulated in all three groups (Figure 1B). One gene that

was found to be upregulated is SLC29A2, which, according to the results of previous studies, has been shown to have little impact on MDS (Follo et al. 2019; Kutyna et al. 2023). Thus, we focused on transcriptional profile changes in downregulation.

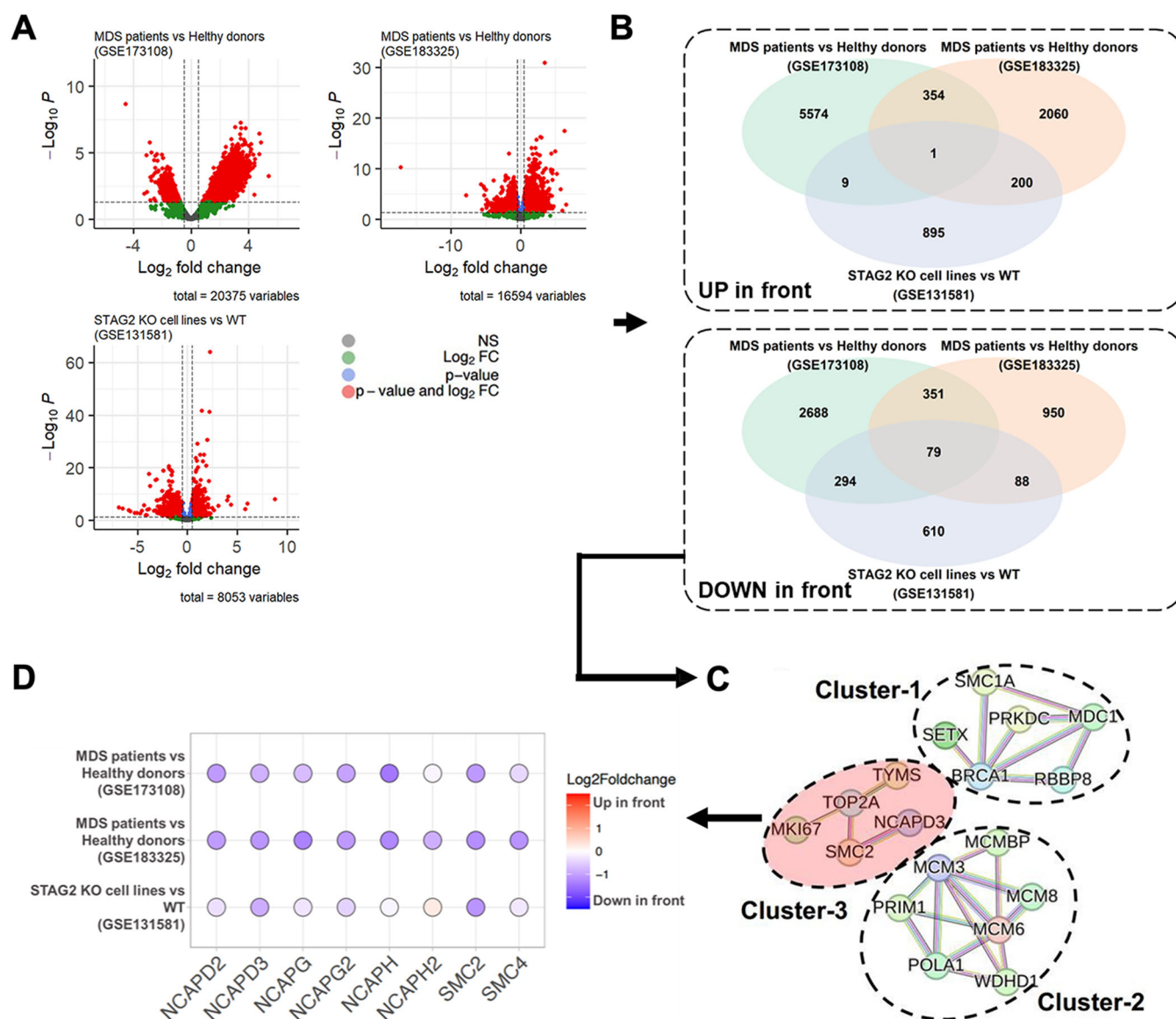
Next, we generated PPI networks from the overlapping DEG list of downregulated genes. Subsequently, we narrowed them down to three protein families that were significantly related to each other (Figure 1C). The first group of Cluster 1 includes genes such as *SMC1A*, *MDC1*, *SETX*, *BRCA1*, *PRKDC*, and *RBBP*, which are primarily involved in DNA repair processes (BRCA1-C complex [*BRCA1* and *RBBP*] and the DNA repair complex [*BRCA1* and *PRKDC*]). Cluster 2 contains genes such as *PRIM1*, *MCM3*, *MCMBP*, *POLA1*, *MCM6*, *MCM8*, and *WDHD1*, which are involved in DNA replication (regulation of DNA-directed DNA polymerase activity containing the MCM complex [*MCM3*, *MCM6*, and *MCM8*] and alpha DNA polymerase complex [*MCM3*, *POLA1*, and *PRIM1*]). Finally, the third group, Cluster 3, involves genes such as *TYMS*, *MKI67*, *TOP2A*, *SMC2*, and *NCAPD3*, which play essential roles in cell division processes like chromatid segregation and mitotic spindle checkpoint signaling.

Genes or pathways involved in DNA replication and repair are associated with MDS (Zhou et al. 2015; Borges et al. 2023; Ribeiro et al. 2016; Flach et al. 2021). However, the role of the condensin complex in MDS has not yet been thoroughly explored. Consequently, our study focused on Cluster 3 of the condensin complex, which includes genes such as *SMC2* and *NCAPD3*. We further examined the expression patterns of these genes and expanded our analysis to other genes in the complex—*SMC4*, *NCAPG*, *NCAPG2*, *NCAPD2*, *NCAPH*, and *NCAPH2*—, as shown in Figure 1D. Notably, all condensin complex genes were significantly downregulated. *SMC2*, in particular, exhibited the most consistent suppression across all three datasets, indicating a potentially strong link with MDS. Collectively, our findings suggested that the condensin complex may be involved in MDSs, with *SMC2* possibly playing a pivotal role in this association.

### 3.2 | Generation of Zebrafish *smc2* Null Mutants

Our bioinformatics study, using datasets of patients with MDS and cellular models, revealed an association between the condensin complex, including *SMC2*, and blood diseases. To elucidate its role in hematopoiesis, we used a zebrafish model, capitalizing on the considerable conservation of *SMC2* between zebrafish and humans (Supporting Information S1: Figure 1). This evolutionary conservation established the relevance of zebrafish in modeling human hematopoiesis in our study.

Focusing on the function of *smc2* in hematopoiesis, we created *smc2* zebrafish mutants via CRISPR/Cas9 mutagenesis using a single guide RNA (sgRNA) targeting exon 7 in *smc2*, resulting in mutants with three distinct allelic variants (Figure 2A). All three mutants possess small insertions/deletions (in/del) alleles that generate frameshift mutations, possibly leading to non-sense mRNA decay becoming functionally null (Shalem et al. 2014). To examine the stability of *smc2* mRNA in the mutants, we performed whole-mount in situ hybridization



**FIGURE 1** | Selection procedure of target proteins using RNA-seq datasets of MDS-associated samples via three different comparisons. (A) Volcano plots of significantly differentially expressed MDS-associated genes for the three comparisons: (i) MDS patients versus healthy donors (GSE173108), (ii) MDS patients versus healthy young adults (GSE183325), and (iii) STAG2 knock-out (KO) HL-60 cells versus WT HL-60 cells (GSE131581). (B) Venn diagrams comparing the three DEGs for up/downregulations. (C) Protein-protein interaction (PPI) networks connected using significant DEGs. (D) Transcriptional profiles of the condensin family across the three comparisons.

(WISH) using *smc2* probes and quantitative PCR (qPCR) analyses. Our observations revealed that *smc2* mRNA began to decrease from 24 hpf and was mostly abolished at 72 hpf, confirming that our mutants lacked normal Smc2 function (Figure 2B,C).

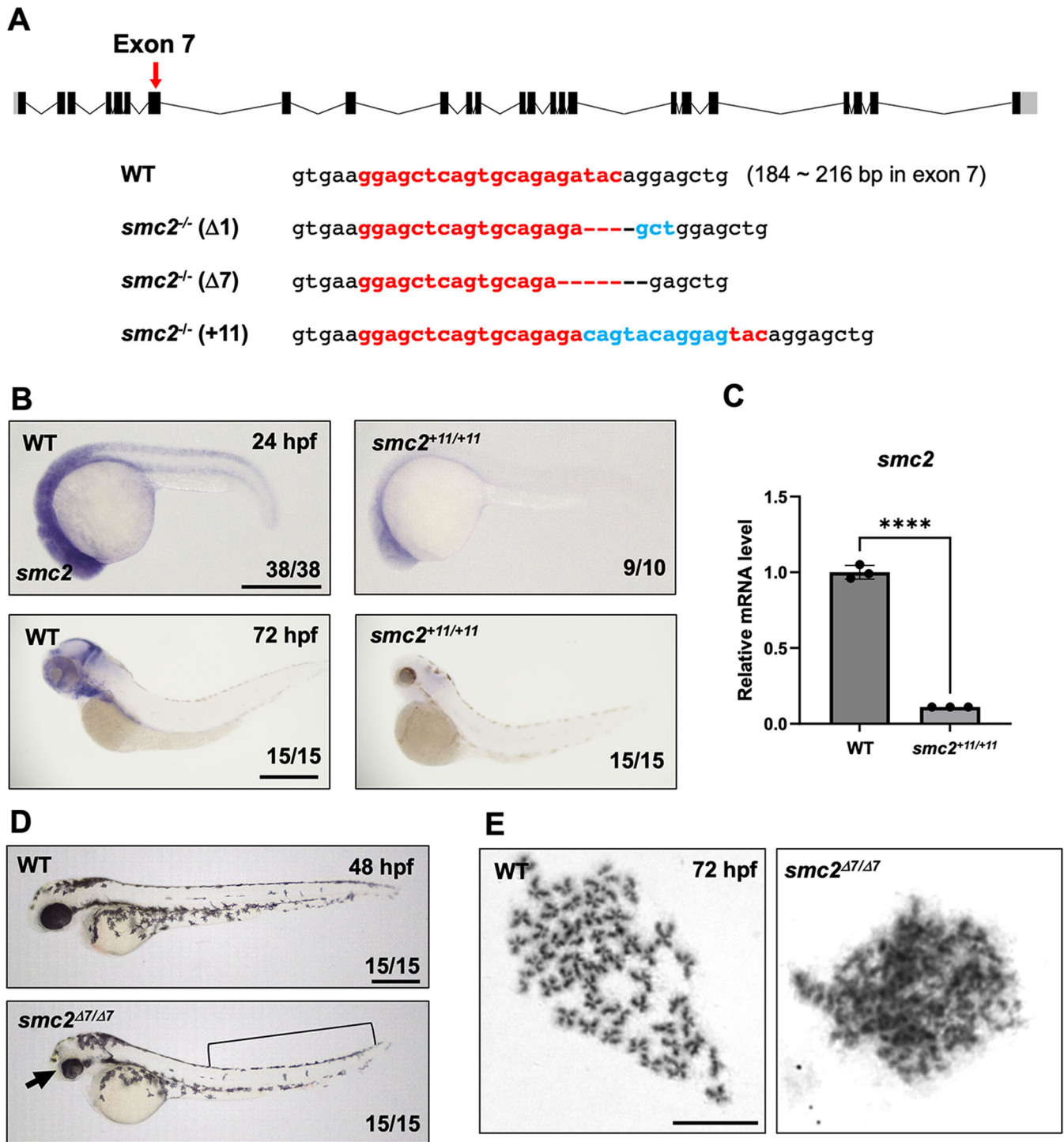
To characterize the effects of *smc2* depletion during embryogenesis, we monitored the morphology of *smc2* mutants. Homozygous mutant embryos displayed distinct morphological abnormalities from 48 hpf, including reduced head size, smaller eyes, and aberrant yolk extension, leading to lethality by 5-day post fertilization (dpf) (Figure 2D). To examine the effects of Smc2 on chromosomal condensation at the organismal level, we performed chromosome-spreading assays using zebrafish *smc2* mutants. WT zebrafish embryos exhibited normal condensation during mitosis, whereas *smc2* mutants failed to form normally

condensed chromosomes (Figure 2E). Collectively, our results demonstrated that *smc2* null mutants created using CRISPR/Cas9 gene editing successfully function as loss-of-function reagents.

### 3.3 | Smc2 Is Necessary for HSPC Maintenance

Zebrafish hematopoiesis proceeds sequentially with primitive and definitive waves of blood cells, similar to that of the mammalian system (Gore et al. 2018; Davidson and Zon 2004). Primitive hematopoiesis generates erythroid and myeloid populations from unipotent mesodermal precursors, whereas definitive multipotent HSPCs arise from the hemogenic endothelium (Espanola et al. 2020; Zhen et al. 2013). To examine whether any hematopoietic events are affected by the loss of





**FIGURE 2** | Generation of *smc2* mutants via CRISPR/Cas9 mutagenesis. (A) A diagram of *smc2* targeted by a sgRNA at exon 7 (a red arrow) and the sequences of three different *smc2* mutant alleles generated using CRISPR/Cas9 mutagenesis. Numbers in parentheses with WT sequences indicate the position in the open reading frame of the targeted exon 7 of *smc2*. The sgRNA sequence is shown in red bold letters. Deletions are illustrated as dashed lines and insertions as blue letters. (B) Whole-mount in situ hybridization (WISH) using a *smc2* probe of *smc2* mutant embryos and its WT sibling controls at 24 and 72 h post fertilization (hpf). The numbers shown at the bottom of the images represent the count of representative outcomes observed relative to the total number of embryos obtained. Data from two independent experiments. Scale bar = 400  $\mu$ m. (C) Quantitative PCR (qPCR) analysis assessing the relative expression levels of the *smc2* gene in WT and *smc2* null mutant zebrafish embryos at 72 hpf. Expression levels of *smc2* were normalized to  $\beta$ -actin, with results presented as relative fold changes. Data were quantified and expressed as the mean  $\pm$  standard error of the mean (SEM) from three independent biological replicate experiments. Statistical analysis was performed using a *t*-test to calculate *p*-values. (\*\*\*\**p* < 0.0001). (D) Lateral view of *smc2* mutant embryos and WT controls at 48 hpf. *smc2* mutants demonstrated morphological defects in the head (black arrow) and yolk extension (black square bracket). Data from two independent experiments. Scale bar = 400  $\mu$ m. (E) Chromosome-spreading assay of WT and *smc2* mutant embryos at 72 hpf from three independent experiments performed. The scale bar indicates the designated length in the images. Scale bar = 10  $\mu$ m.

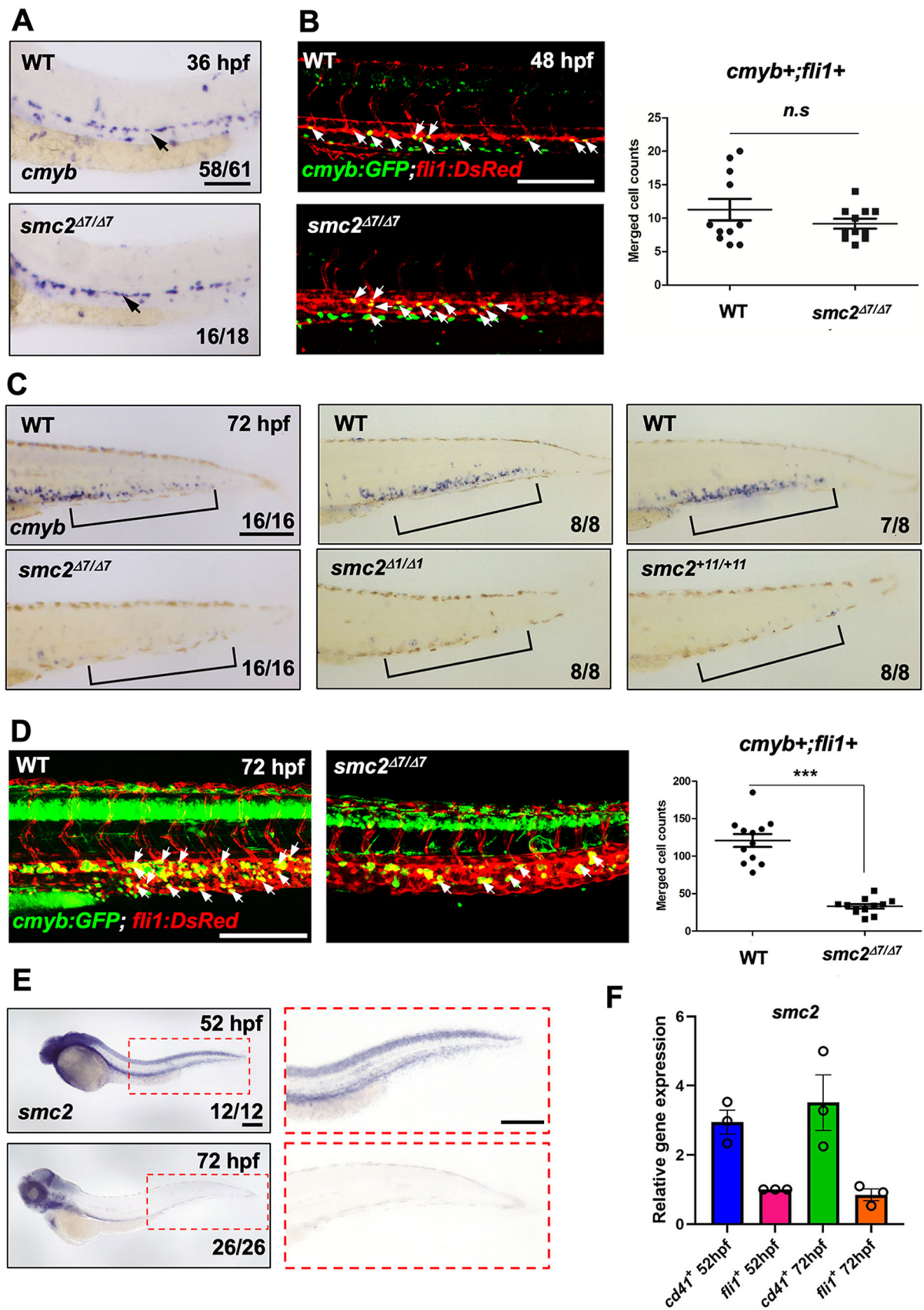


FIGURE 3 | Legend on next page.

Smc2, we performed WISH on *smc2* mutants using each blood marker. WISH using the erythroid-specific marker, *gata1*, and the myeloid marker, *mpx*, showed that primitive hematopoiesis commenced normally in the absence of *smc2* (Supporting Information S1: Figure 2A). Next, we examined definitive HSPC formation in the hemogenic endothelium of the dorsal aorta using a probe targeting *cmyb* at 36 hpf. Similar to WT sibling control embryos, *smc2* mutants showed normal *cmyb* expression in the dorsal aorta (Figure 3A). This was further corroborated via confocal microscopy of *cmyb:GFP;fli1:DsRed* transgenic reporter lines, which showed no differences in the initial formation of HSPCs in the dorsal aorta at 48 hpf between *smc2* mutants and controls (Figure 3B). Additionally, blood circulation in the aorta, essential for the specification and emergence of HSPCs in the dorsal aorta (North et al. 2009), was normal in the mutants as shown in Supporting Information S2: Movie 1.

After emergence from hemogenic endothelial cells in the dorsal aorta, HSPCs migrate to the caudal hematopoietic tissue (CHT) through the circulatory blood to expand and mature into functional blood stem cells (Mahony and Bertrand 2019; Tamplin et al. 2015). WISH analysis using a *cmyb* antisense probe and confocal imaging of *cmyb:GFP;fli1:DsRed* zebrafish embryos at 72 hpf showed that the HSPC population in the CHT was robustly reduced in *smc2* mutants compared to WT controls (Figure 3C,D). Additionally, the absence of lymphoid cells in the thymus, as indicated by a lymphoid-specific *rag1* probe at 4 dpf, suggested a failure in lymphoid generation from definitive HSPCs (Supporting Information S1: Figure 2B). Collectively, these findings implied that although Smc2 is not necessary for HSPC emergence in the dorsal aorta, it plays a crucial role in maintaining these progenitor cells within the CHT.

To further characterize the role of Smc2 in HSPC maintenance, we traced the spatial expression pattern of *smc2* during embryogenesis using WISH. *smc2* was broadly expressed in the anterior part of the embryo, including the head, and was prominently observed in the CHT at 52 hpf, although its expression declined by 72 hpf (Figure 3E), indicating its potential importance in HSPC maintenance from as early as 52 hpf. To closely investigate *smc2* expression within the CHT, we utilized *CD41:GFP* and *fli1:GFP* transgenic reporter lines to examine its expression levels in FACS-sorted *CD41*-positive HSPCs and

*fli1*-positive vascular endothelial cells (Supporting Information S1: Figure 3). Interestingly, at both 52 and 72 hpf, *smc2* expression was markedly higher in *CD41*-positive HSPCs than in *fli1*-positive endothelial cells (Figure 3F). The sustained high expression of *smc2* in HSPCs appears critical for their maintenance during this period, suggesting a possible cell-autonomous effect of Smc2 on HSPC maintenance in zebrafish embryos.

### 3.4 | Depletion of Smc2 Inhibited HSPC Proliferation in the CHT

To evaluate the phenotypes of *smc2* mutants with HSPC maintenance failure, we tracked HSPCs within the CHT during the homing and expansion stages which are critical for HSPC development (Mahony and Bertrand 2019; Xue et al. 2017). Using *CD41:GFP* transgenic animals in an *smc2* mutant background, we monitored *CD41*-positive HSPCs starting at 44 hpf, the onset of HSPC homing to the CHT. From 44 to 52 hpf, the number of HSPCs in the CHT showed a significantly larger difference between the control and mutant groups (Figure 4A). Although HSC emergence normally occurs during this period (Figure 3B), the noticeable difference starting from 44 hpf in mutants suggests possible impaired HSPC lodgment within the CHT. Furthermore, although HSPC numbers continuously increased in the WT group from 44 to 52 hpf, the mutant group showed little to no increase, indicating potential defects in both lodgment and subsequent expansion. Restoration of normal HSPC formation at 52 hpf in mutants through the injection of normal *smc2* mRNA confirms the role of *smc2* in these processes (Figure 4B). Additional experiments using morpholino (MO) to knock down *smc2* showed that although initial HSPC emergence was unaffected, their maintenance or expansion from 52 to 72 hpf was disrupted, mirroring findings in *smc2* mutants (Supporting Information S1: Figure 4). Confocal microscopy using *cmyb:GFP;fli1:DsRed* further confirmed a significant reduction in HSPCs at 52 hpf in *smc2* mutants (Figure 4C). These results highlight the crucial role of Smc2 in the maintenance and expansion of HSPCs in the CHT of zebrafish embryos from 52 hpf.

To investigate the cellular mechanisms underlying the observed HSPC maintenance defects in *smc2* mutants, we evaluated cellular apoptosis and proliferation rates. Acridine orange (AO)

**FIGURE 3** | SMC2 is required for hematopoietic stem and progenitor cell (HSPC) maintenance. (A) WISH of *smc2* mutant embryos and WT controls using HSPC marker *cmyb* in the dorsal aorta (black arrows). The numbers shown at the bottom of the images represent the count of representative outcomes observed relative to the total number of embryos obtained. Results from three independent experiments. (B) Confocal imaging of *smc2* mutant embryos and sibling controls with a *cmyb:GFP;fli1:DsRed* transgenic background at 48 hpf (white arrows indicate HSPCs). Right panel: quantification of *cmyb*+;*fli1*+ double positive cells in the dorsal aorta at 48 hpf (WT,  $n = 11$ ; mutants,  $n = 10$ ). Data were quantified and expressed as the mean  $\pm$  SEM. Statistical analysis was performed using a *t*-test to calculate *p*-values (*n.s.*, no significance at  $p > 0.05$ ). Data from a single experiment. (C) WISH using a HSPC-specific *cmyb* antisense probe of three distinct *smc2* mutant embryos and their WT controls in caudal hematopoietic tissue (CHT) at 72 hpf. Black square brackets indicate CHT. Data obtained from a single experiment. (D) Confocal imaging of WT embryos and *smc2* mutants at 72 hpf using *cmyb:GFP;fli1:DsRed* transgenic zebrafish. White arrows indicate HSPCs. Right panel: quantification of *cmyb*+;*fli1*+ double positive HSPCs in the CHT at 72 hpf (WT,  $n = 11$ ; mutants,  $n = 10$ ; \*\*\* $p < 0.001$ ). Results from two independent experiments. Statistical analysis was performed using a *t*-test to calculate *p*-values. (E) Transcriptional expression of *smc2* during embryogenesis with *smc2* WISH of WT embryos at 52 and 72 hpf. The red-dotted box is a magnified image of the CHT at 52 and 72 hpf. Combined data from two independent experiments. (F) qPCR analysis of *smc2* expression in *CD41*-positive HSPCs and *fli1*-positive endothelial cells at 52 and 72 hpf. The expression levels of *smc2* were normalized to the  $\beta$ -actin, with results presented as relative fold changes. Data were quantified and expressed as the mean  $\pm$  SEM of three independent biological replicates. Scale bar = 200  $\mu$ m.



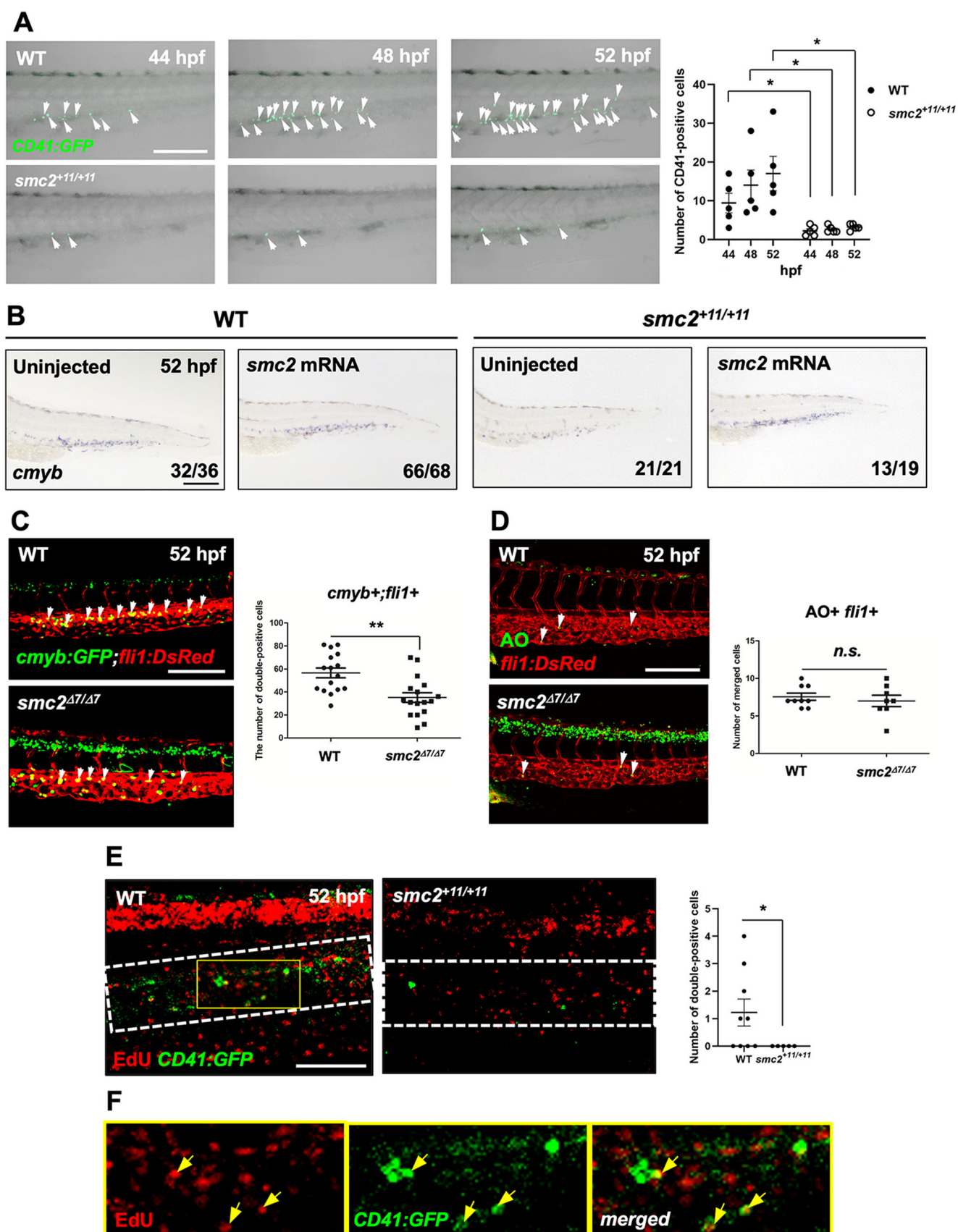


FIGURE 4 | Legend on next page.



staining of endothelium-specific *fli1:DsRed* transgenic embryos indicated no significant changes in apoptosis within the CHT of *smc2* mutants at 52 hpf, whereas a notable increase in apoptosis was observed within the neural tissues of the mutants (Figure 4D). Similarly, TUNEL staining confirmed no alteration in cell death under the *smc2* MO knockdown conditions (Supporting Information S1: Figure 5A). Moreover, vasculature formation in the CHT, including the appearance of vascular plexus—a critical HSPC niche for retention (Wattrus and Zon 2018)—appeared normal in the absence of *Smc2* (Figure 4C,D and Supporting Information S1: Figure 5B,C). In contrast, EdU staining for cell proliferation revealed a complete absence of detectable cell division in *smc2* mutants and morphants across all tissues, including the CHT, during a 10-min EdU exposure at 52 hpf (Supporting Information S1: Figure 5D,E). This suggests that *Smc2* plays a critical, systemic role in cell proliferation. Further EdU staining after 24-h exposure detected some proliferating cells in neural cells and the CHT of mutants (Figure 4E,F). However, these were significantly fewer compared to the WT controls, and particularly, the number of *CD41*- and EdU-double positive HSPCs in the CHT was completely absent in *smc2* mutants relative to the WT group. Collectively, these results demonstrate that *Smc2* is essential for the proliferation of HSPCs within the CHT, which is critical for the proper maintenance and expansion of the blood progenitor cell population.

### 3.5 | G2/M Arrest Disrupts HSPC Maintenance in the CHT of *smc2* Mutants

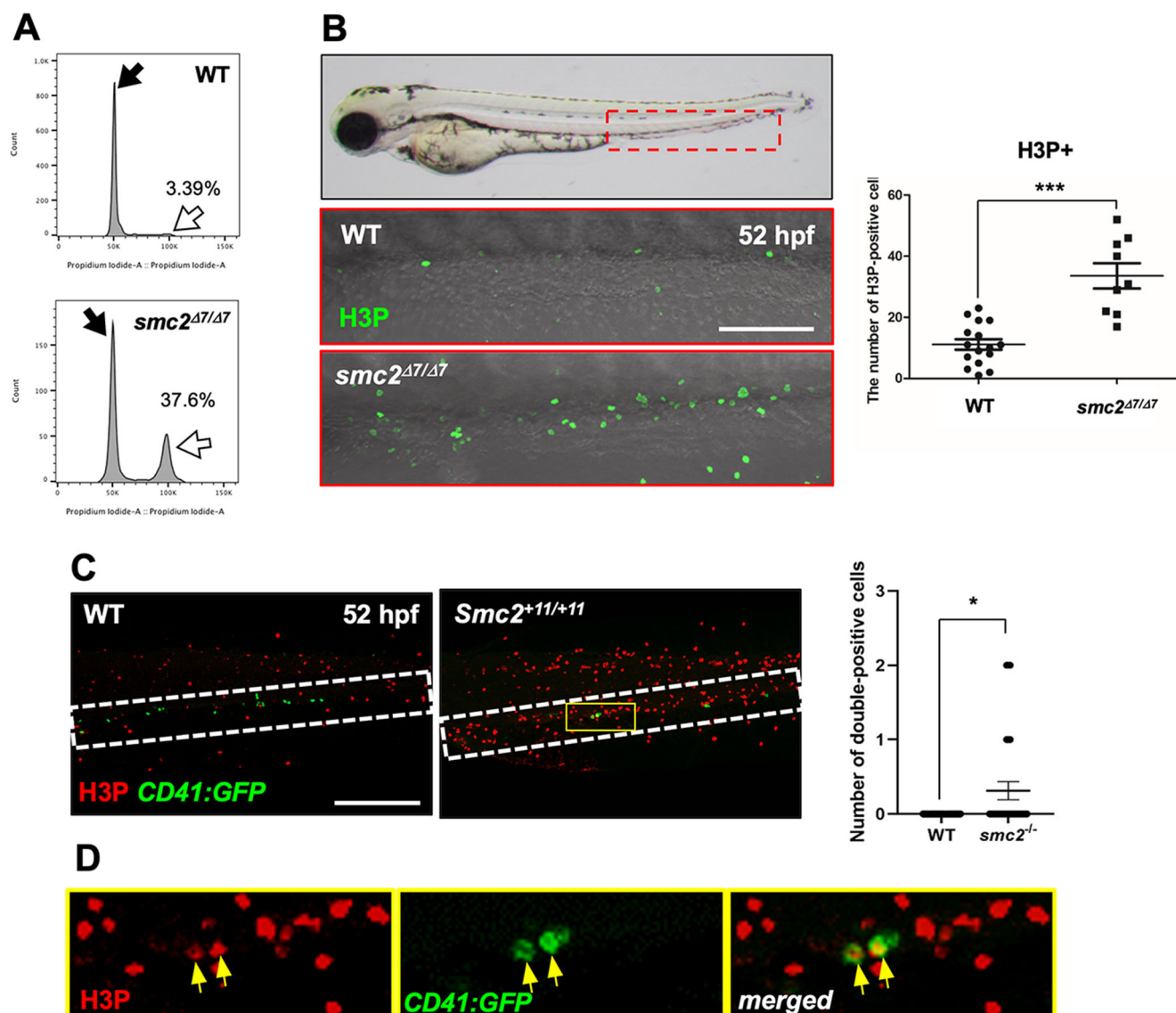
Previous studies have established the critical role of condensin subunits, including SMC proteins, in chromosome segregation and cell cycle progression (Jensen and Shapiro 1999; Graumann 2001). Specifically, non-SMC2 subunits such as *Ncap1* are essential for cell cycle progression, with their knockdown suppressing the proliferation of pancreatic cancer cell lines (Kim et al. 2019). To investigate the potential impact of the loss of *smc2* on cell cycle events and its consequent effect on HSPC proliferation, we performed DNA content analysis on *smc2* mutant and control embryos at 52 hpf using flow cytometry after staining the DNA with propidium iodide. The results revealed an increased occurrence of G2/M phase cell cycle arrest in *smc2* mutants compared to WT controls

(Figure 5A). To ascertain if this cell cycle arrest was induced in the HSPCs, we conducted immunohistochemistry staining with H3P, a marker for G2/M cell cycle arrest (Sun et al. 2014; Jin et al. 2017). The number of H3P-positive cells within the CHT was significantly higher in *smc2* mutants than that in WT controls (Figure 5B). Additionally, using *CD41:GFP* transgenic zebrafish embryos, we found that H3P+ and *CD41*+ double-positive cells were present in *smc2* mutants, whereas no double-positive cells were observed in WT controls (Figure 5C,D). This result indicated that cell cycle arrest occurred at the G2/M phase within HSPCs lacking *smc2*. Likewise, nocodazole treatment, which can induce cell cycle arrest at the G2/M phase as a microtubule polymerization inhibitor (Blajeski et al. 2002), blocked HSPC expansion and maintenance in the CHT, similar to that in *smc2* mutants (Supporting Information S1: Figure 6). Taken together, these findings suggested that G2/M cell cycle arrest in HSPCs, induced by the loss of *Smc2*, leads to failure in HSPC expansion and maintenance.

### 3.6 | Condensin II Subunits Are Crucial for HSPC Maintenance

To further understand the role of the condensin complex in hematopoiesis, we examined the function of other condensin subunits in hematopoiesis via knockdown approaches using splice-blocking MOs (Supporting Information S1: Figure 7). Notably, *smc4* knockdown led to the loss of HSPCs in the CHT, like the phenotype of *smc2* null mutants, indicating a consistent requirement of both *Smc2* and *Smc4* in HSPC development (Figure 6A). Furthermore, our functional assessment revealed a distinct role between condensin I and II subunits. Knockdown of condensin II components (*ncaph2*, *ncapg2*, and *ncapd3*) significantly reduced HSPCs in the CHT like *smc2* mutants, whereas knockdown of condensin I subunits (*ncaph1*, *ncapg1*, and *ncapd2*) showed no visible alteration of *cmyb* expression in the CHT (Figure 6B,C). To further validate the specific requirement of condensin II for HSPC proliferation, we performed EdU and H3P staining using morphants against *ncapg1* and *ncapg2*. The *ncapg2* knockdown markedly decreased the number of Edu+ cells and increased the number of H3P+ cells in the CHT, compared to controls (Figure 6D,E). In contrast, *ncapg1* knockdown did not alter the number of Edu+ or H3P+

**FIGURE 4** | *Smc2* is required for HSPC proliferation in the CHT. (A) Confocal imaging of *smc2* mutant embryos and sibling controls with a *CD41:GFP* transgenic background at 44, 48, and 52 hpf. Right panel: quantification of *CD41*-positive HSPCs in the CHT (WT at 44 hpf,  $n = 5$ ; mutants at 44 hpf,  $n = 5$ ; WT at 48 hpf,  $n = 5$ ; mutants at 48 hpf,  $n = 5$ ; WT at 52 hpf,  $n = 5$ ; mutants at 52 hpf,  $n = 5$ ;  $*p < 0.05$ ). White arrows indicate *CD41*-positive HSPCs. Data from a single experiment. (B) WISH of *smc2* mutant embryos and WT controls at 52 hpf following injection with *smc2* mRNA. Black square brackets indicate the *cmyb* signal in the CHT. The numbers shown at the bottom of the images represent the count of representative outcomes observed relative to the total number of embryos obtained. Data from two independent experiments. (C) Confocal microscopy images of *smc2* mutant embryos and their sibling controls in the *cmyb:GFP;fli1:DsRed* background. White arrows indicate HSPCs. Right panel: quantification of *cmyb*+/*fli1*+ cells in the CHT at 52 hpf (WT,  $n = 16$ ; mutants,  $n = 17$ ;  $**p < 0.01$ ). Data from a single experiment. (D) Confocal imaging of *smc2* mutant embryos and WT controls at 52 hpf with acridine orange (AO) staining using *fli1:DsRed* transgenic zebrafish embryos. White arrows indicate AO-positive apoptotic cells. Right panel: quantification of AO+/*fli1*+ cells in the CHT (WT,  $n = 8$ ; mutants,  $n = 8$ ; n.s., no significance at  $p > 0.05$ ). Data from a single experiment. (E) Confocal microscopy imaging of EdU-stained (incorporating 24 h) *smc2* mutant embryos and WT sibling controls in the background of *CD41:GFP* at 52 hpf. White-dotted box indicates CHT. Right panel: quantification of Edu- and *CD41*-double positive cells in the CHT (WT,  $n = 9$ ; mutants,  $n = 5$ ;  $*p < 0.05$ ). The results represent data from two independent experiments. (F) Higher magnification images of the yellow box in EdU-stained WT;*CD41:GFP* transgenic zebrafish embryos from (E). Yellow arrows indicate Edu- and *CD41*-double positive cells in the CHT. All quantification data were quantified and expressed as the mean  $\pm$  SEM. Statistical analysis was performed using a *t*-test to calculate *p*-values. Scale bar = 200  $\mu$ m.



**FIGURE 5** | G2/M cell cycle arrest disrupts HSPC maintenance in the CHT in *smc2* mutants. (A) Fluorescence-activated cell sorting (FACS) analysis of DNA content in WT and *smc2* mutant embryos at 52 hpf following propidium iodide (PI) staining. Black and white arrows indicate the G1 and G2/M phase peak, respectively. The indicated percentage represents the DNA content in the G2/M phase. Data from two independent biological replicate experiments. (B) Confocal microscopy images of *smc2* mutants ( $n = 9$ ) and WT controls ( $n = 16$ ) immunohistochemically stained with the H3P antibody in the CHT (red-dotted box) at 52 hpf. Red-dotted box indicates CHT. Right panel: quantification of H3P+ cells in the CHT ( $***p < 0.001$ ). Data combined from two independent experiments. (C) Confocal imaging of H3P-stained *smc2* mutant embryos ( $n = 29$ ) and WT sibling controls ( $n = 50$ ) in the CHT at 52 hpf using *CD41:GFP* transgenic zebrafish. White-dotted box indicates CHT. Right panel: quantification of H3P- and *CD41*-double positive cells in the CHT ( $*p < 0.05$ ). The results represent data from two independent experiments. (D) Higher magnification images of the white-dotted box in H3P-stained *smc2<sup>-/-</sup>;CD41:GFP* transgenic zebrafish embryos from (C). Yellow arrows indicate H3P- and *CD41*-double positive cells in the CHT of *smc2* mutants. All quantification data were quantified and expressed as the mean  $\pm$  SEM. Statistical analysis was performed using a *t*-test to calculate *p*-values. Scale bar = 200  $\mu$ m.

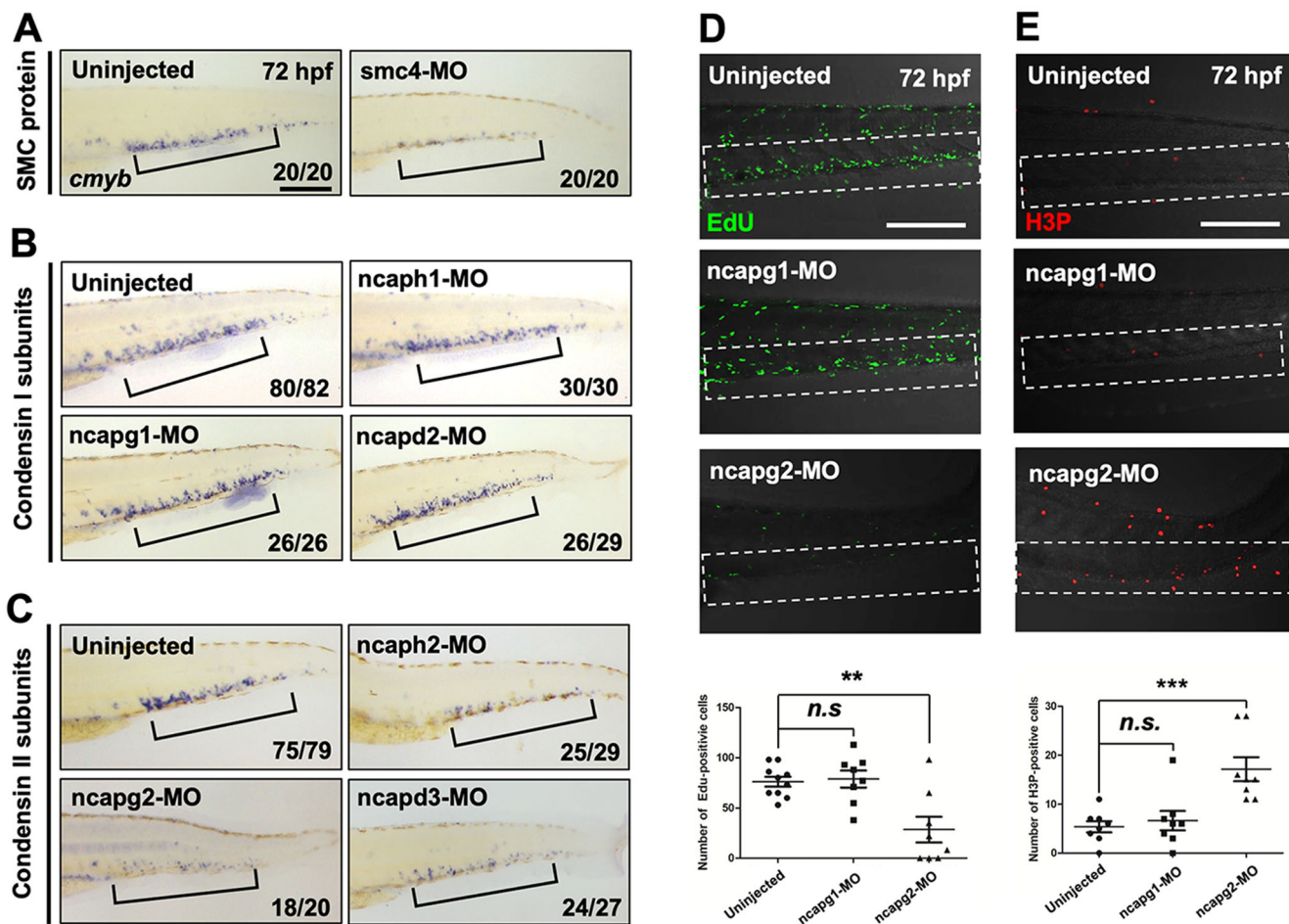
cells. Collectively, these findings suggested that condensin II plays an essential role in the regulation of HSPC proliferation and maintenance within the CHT.

#### 4 | Discussion

MDS includes a group of diverse bone marrow disorders characterized by insufficient production of one or more blood cell types, often leading to anemia, infection, or bleeding (Dotson and Lebowicz 2023). Central to these disorders is the

dysfunction of HSPCs, which play a critical role in the genesis of all the blood cell types (Zhan and Park 2021; Veiga et al. 2021; Li 2013). Various mutations or abnormalities can affect both HSPCs and the bone marrow microenvironment in MDS (Ganan-Gomez et al. 2022; Shastri et al. 2017). Our analysis using patient databases and cellular models revealed a significant correlation between the expression of condensin complex subunits, particularly SMC2, and MDS occurrence.

Research utilizing animal models, particularly mice and zebrafish, has been pivotal in elucidating MDS characteristics.



**FIGURE 6** | Condensin II subunits are required for HSPC maintenance. (A–C) WISH using the *cmyb* antisense probe at 72 hpf in WT embryos injected with morpholinos (MOs) targeting *smc4* (A), condensin I subunits (*ncaph1*, *ncapg1*, and *ncapd2*) (B), condensin II subunits (*ncaph2*, *ncapg2*, and *ncapd3*) (C), and their corresponding uninjected controls. Black square brackets indicate *cmyb*-positive HSPCs in the CHT. The numbers shown at the bottom of the images represent the count of representative outcomes observed relative to the total number of embryos obtained. WISH data from a single experiment. (D) Confocal microscopy images of EdU-stained morphants of *ncapg1* and *ncapg2* and their uninjected controls at 72 hpf. White-dotted box indicates CHT. Bottom panel: quantification of EdU+ cells in the CHT (uninjected,  $n = 10$ ; *ncapg1*-MO,  $n = 10$ ; *ncapg2*-MO,  $n = 10$ ; n.s., no significance at  $p > 0.05$ ;  $**p < 0.01$ ). Data from a single experiment. (E) Confocal microscopy images of H3P-stained morphants targeting *ncapg1* and *ncapg2* and their uninjected controls at 72 hpf. Bottom panel: quantification of H3P+ cells in the CHT (uninjected,  $n = 10$ ; *ncapg1*-MO,  $n = 10$ ; *ncapg2*-MO,  $n = 10$ ; n.s., no significance at  $p > 0.05$ ;  $***p < 0.001$ ). All quantification data were quantified and expressed as the mean  $\pm$  SEM. Statistical analysis was performed using a *t*-test to calculate *p*-values. Data from a single experiment. Scale bar = 200  $\mu$ m.

Diagnosis in these models typically relies on the morphology of blood cells, but the embryonic stage presents challenges, necessitating the use of specific blood cell markers. For example, mutations of TET2 and APC on chromosome 5q, known to be associated with MDS in mice, lead to enhanced self-renewal capacity of HSPCs and impair their differentiation. This resulted in MDS-related hematologic features including abnormal monocytosis or leukocytosis in mutants (Quivoron et al. 2011; Lane et al. 2010). In zebrafish, knockdown of TET2 resulted in defective erythroid development, an anemic phenotype, and a decrease in HSPC markers at 36 hpf (Ge et al. 2014). Additionally, the SF3B1 mutation, commonly observed in MDS patients, disrupt erythroid differentiation and HSPC development in zebrafish (De La Garza et al. 2016). Thus, various models of MDS demonstrate the disease's phenomena through defects in the differentiation of leukocytes, including erythroid, and the development of HSPCs.

Our investigation into *smc2*'s role revealed that although hemogenic endothelium specification and initial HSPC emergence are normal in *smc2* mutants, these mutants exhibit significant deficits in HSC expansion and maintenance. Specifically, a marked reduction in CD41-positive HSPCs from 44 hpf indicates potential abnormalities in HSPC lodgment in the CHT. Further analysis of HSPC counts between 44, 48, and 52 hpf showed diminished expansion in the mutants, supported by EdU staining, which highlighted proliferation defects. Moreover, an increase in H3P-positive cells points to potential cell cycle arrest at the G2/M phase in *smc2* mutants, suggesting a multifaceted impact on initial homing, cell proliferation, and cell cycle regulation of HSPCs. To fully elucidate these processes, continued investigation is necessary, including further tissue-specific functional studies to verify the potential cell-autonomous effects of *Smc2* on HSPCs.



These results align with our previous research using zebrafish models, which demonstrated a strong link between various blood disease-related genes and hematopoiesis, specifically supervising HSPC development (Oh, Ha, et al. 2020; Oh, Kang, et al. 2020; Kang et al. 2020). In particular, the effects of condensins, including SMC2, on HSPC development observed in MDS patients and cellular models suggest that SMC2 may be functionally related to the onset of MDS.

Furthermore, our studies distinguished between the roles of condensin I and II in HSPC development. Notably, the suppression of condensin II subunits led to HSPC maintenance failure, whereas condensin I knockdown had no evident effects on HSPC development. This indicated a distinct and crucial role of the condensin II complex in zebrafish hematopoiesis.

Condensins and their subunits play crucial roles in various developmental processes and cancer pathogenesis (Gosling et al. 2007; Klebanow et al. 2016). For example, the overexpression of SMC2 is associated with tumorigenesis, and the blockade of SMC2 with antibodies has been proposed as a novel cancer treatment (Montero et al. 2020). Furthermore, the ectopic expression of SMC2 was observed in colon cancer, and its suppression impaired tumor growth in a xenograft mouse model (Dávalos et al. 2012). SMC4 expression is markedly increased in human gliomas, and its activity is associated with the aggressiveness of the TGF- $\beta$  signaling pathway (Jiang et al. 2017). Moreover, the fusion protein NUP214-ABL1, associated with SMC4, has been identified in T-cell acute lymphoblastic leukemia as a proliferation and survival enhancer for T lymphoblasts (De Keersmaecker et al. 2014).

In addition to the major subunits, non-SMC condensin components contribute to tumorigenesis (Dávalos et al. 2012; Jiang et al. 2017). Condensin I subunits play vital roles in organogenesis and oncogenesis. For instance, NCAPG has been identified as a prognostic marker of hepatocellular carcinoma (Liu et al. 2017), and NCAPD2 was reported as a factor in the prognosis of triple-negative breast cancer (Zhang et al. 2020). Additionally, NCAPHs show high expression in pancreatic cancer (Kim et al. 2019), highlighting the integral role of condensin I in solid tumor growth.

In contrast, condensin II subunits play a more prominent role in hematopoiesis and blood disorders. CAP-G2 participates in erythropoiesis by interacting with the SCL protein, and a mutation in NCAPH2 leads to defects in T-cell development in mouse cell strains (Xu et al. 2006; Gosling et al. 2007). Mutations in the condensin II subunit, Caph2, have been linked to genome instability and T-cell lymphoma due to uncontrolled accumulation of mature T cells in the lymph nodes (Woodward et al. 2016). Likewise, our study extends this understanding by demonstrating the essential role of condensin II subunits in normal hematopoiesis and their potential correlation with MDS. This emphasizes the need for comprehensive research on the role of the condensin II complex in blood health and disease.

In summary, our findings reinforce the critical role of condensin complexes, particularly condensin II, in hematopoiesis and blood disorders. These results highlight the need for further

research into the specific mechanisms through which condensin II affects HSPC function and contributes to MDS development. However, it is important to recognize that our results and interpretation are based on specific experimental conditions and may not fully represent the complexity of in vivo conditions. Therefore, more detailed research on the tissue-specific effects of condensin complexes and distinct molecular mechanisms of condensin II subunits in hematopoiesis at the cellular level is needed. Future studies exploring the interplay between condensin complexes and HSPC functionality may provide new strategies for diagnosing and treating hematological diseases.

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#### Author Contributions

Y.L., C.-K.O., and K.M. designed the experiments. C.-K.O., M.S.K., U.S., J.W.K., Y.H.K., H.S.K., J.S.R., S.A., E.Y.C., S.Y., U.N., and T.C. performed the experiments. C.-K.O., M.S.K., U.S., K.M., and Y.L. analyzed the data and wrote the manuscript.

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#### Ethics Statement

Zebrafish were raised in accordance with Ulsan National Institute of Science and Technology and Use Committees (IACUC: UNISTIACUC-20-09).

#### Conflicts of Interest

The authors declare no conflicts of interest.

#### Data Availability Statement

All data generated or analyzed during this study are included in this published article and its supplementary information files.

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## Supporting Information

Additional supporting information can be found online in the Supporting Information section.