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PRINCIPAL INVESTIGATOR: Teresa Bowman

CONTRACTING ORGANIZATION: Albert Einstein College of Medicine

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14. ABSTRACT Myelodysplastic syndromes (MDS) are a spectrum of disorders arising from hematopoietic stem and progenitor cell (HSPC) dysfunction resulting in ineffective hematopoiesis and cytopenias. MDS in adults is largely thought to be an acquired disorder, but the recent identification of an inherited form of adult-onset MDS linked to germline mutations in the <i>DEAD-box Helicase 41</i> gene (<i>DDX41</i>) challenges this dogma. Germline mutations in <i>DDX41</i> primarily occur in the N-terminal domain leading to an early stop codon, suggesting that diminished function of DDX41 contributes to MDS initiation. DDX41 is a nucleic acid-activated ATPase linked to immunity, splicing, and R-loop regulation. All three of these processes have been implicated in MDS, thus the underlying mechanism for how DDX41 mutations contribute to hematologic malfunctioning is unclear. We demonstrated that Ddx41 regulation of R-loops is critical to restrain innate immune signaling that can trigger aberrant HSPC expansion. Elevated R-loops and innate immune signaling were also observed in human cells with lower levels of DDX41, suggesting conservation. We also defined how Ddx41 levels impact erythropoiesis. We determined that <i>ddx41</i> mutants develop anemia due to decreased expansion of erythroid progenitors and ineffective differentiation. Mechanistically, we discovered that elevated DNA damage triggered a cell cycle arrest that limited erythroid expansion in <i>ddx41</i> mutants. RNA-sequencing analysis revealed numerous expression and splicing changes in cell cycle and DNA repair factors that could trigger genomic instability and activate the DNA damage response. These findings have implications for understanding the etiology of MDS/AML as well as the development of novel approaches to target R-loops, DNA damage, and innate immune signaling to treat MDS/AML patients.					
15. SUBJECT TERMS Myelodysplastic syndrome, DDX41, hematopoiesis, inflammation, DNA damage					
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TABLE OF CONTENTS

	<u>Page</u>
1. Introduction	4
2. Keywords	4
3. Accomplishments	4-7
4. Impact	7
5. Changes/Problems	7
6. Products	7-8
7. Participants & Other Collaborating Organizations	8-9
8. Special Reporting Requirements	9
9. Appendices	10-90

1. INTRODUCTION:

Myelodysplastic syndromes (MDS) are a spectrum of disorders arising from hematopoietic stem and progenitor cell (HSPC) dysfunction resulting in ineffective hematopoiesis and cytopenias. MDS in adults is largely thought to be an acquired disorder, but the recent identification of an inherited form of adult-onset MDS linked to germline mutations in the *DEAD-box Helicase 41* gene (*DDX41*) challenges this dogma. Germline mutations in *DDX41* primarily occur in the N-terminal domain leading to an early stop codon, suggesting that diminished function of *DDX41* contributes to MDS initiation. *DDX41* is a nucleic acid-activated ATPase linked to immunity, splicing, and R-loop regulation. All three of these processes have been implicated in MDS, thus the underlying mechanism for how *DDX41* mutations contribute to hematologic malfunctioning is unclear. We demonstrated that *Ddx41* regulation of R-loops is critical to restrain innate immune signaling that can trigger aberrant HSPC expansion. Elevated R-loops and innate immune signaling were also observed in human cells with lower levels of *DDX41*, suggesting conservation. We also defined how *Ddx41* levels impact erythropoiesis. We determined that *ddx41* mutants develop anemia due to decreased expansion of erythroid progenitors and ineffective differentiation. Mechanistically, we discovered that elevated DNA damage triggered a cell cycle arrest that limited erythroid expansion in *ddx41* mutants. RNA-sequencing analysis revealed numerous expression and splicing changes in cell cycle and DNA repair factors that could trigger genomic instability and activate the DNA damage response. These findings have implications for understanding the etiology of MDS/AML as well as the development of novel approaches to target R-loops, DNA damage, and innate immune signaling to treat MDS/AML patients.

2. KEYWORDS:

Myelodysplastic syndrome, *DDX41*, hematopoiesis, inflammation, DNA damage

3. ACCOMPLISHMENTS:

Major goals of the project:

Goals as outlined in the approved Statement of Work:	Timeline Months	Site 1
Task: Seek regulatory approval for the use of animals	1-3	Local IACUC and DoD ACURO
Aim 1: To determine R-loop involvement in <i>Ddx41</i> regulation of hematopoiesis formation		
1.1 To determine if <i>DDX41</i> regulates R-loop levels.	1-6	Bowman lab
1.2 To determine if R-loops contribute to hematopoietic defects in <i>ddx41</i> mutants.	1-18	Bowman lab
1.3 To determine how R-loops regulate hematopoietic gene expression.	1-24	Bowman lab
Milestone Achieved: Delineate how <i>DDX41</i> -mediated R-loop regulation contributes to blood defects.	24	
Aim 2: To Determine R-loop-triggered DNA damage responses during development.		
2.1 To determine if inflammatory signaling is perturbed in <i>ddx41</i> mutants.	1-12	Bowman lab
2.2 To determine if STING-mediated inflammatory signaling plays a role in hematopoietic defects in <i>ddx41</i> mutants.	6-24	Bowman lab
2.3 To determine if elevated levels of R-loops alter inflammatory signaling in <i>ddx41</i> mutants.	12-18	Bowman lab
Milestone Achieved: Define how <i>DDX41</i> insufficiency alters the inflammatory milieu and the impact of this on hematopoiesis	24	

Accomplishments:

Task: Seek regulatory approval for the use of animals: The project was approved by both the IACUC at Albert Einstein College of Medicine (12/18/19) and the DOD ACURO (2/24/20).

Aim 1: To determine R-loop involvement in *Ddx41* regulation of hematopoiesis formation

Aim 1.1: To determine if DDX41 regulates R-loop levels (Outcomes from this sub-aim are published in Weinreb *et al. Dev Cell* 2021). To decipher if Ddx41 plays a role in R-loop regulation *in vivo*, we examined R-loop levels in zebrafish *ddx41* mutants by using the monoclonal antibody S9.6 that specifically recognizes RNA:DNA hybrid structures. RNA:DNA hybrid immunofluorescence signal in purified *cd41:gfp*⁺ hematopoietic stem and progenitor cells (HSPCs) was significantly higher in *ddx41* mutants compared to siblings (**Fig. 1**). To validate that the structures examined are indeed RNA:DNA hybrids, we demonstrated that the S9.6 levels were sensitive to RNASEH1, an enzyme that specifically cleaves the RNA in an RNA:DNA hybrid. We employed a transgenic zebrafish system for inducible expression of *RNASEH1-GFP*. These studies strongly support a role for Ddx41 in preventing deleterious R-loop accumulation *in vivo*.

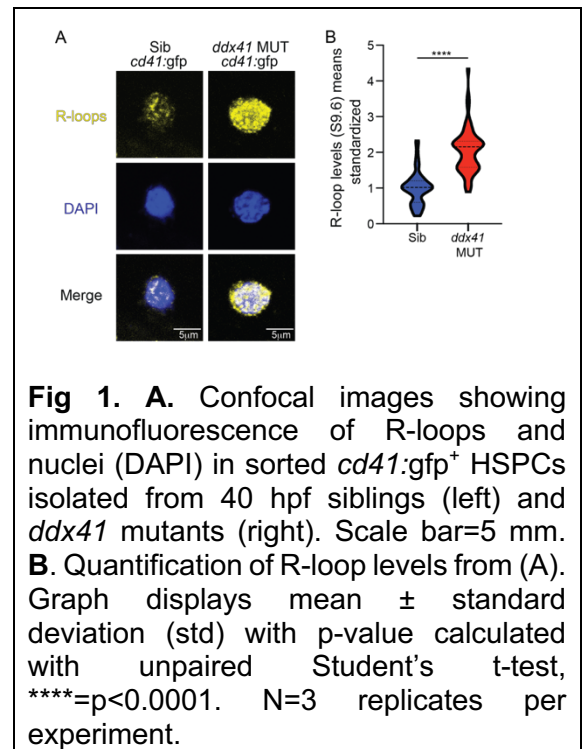
Aim 1.2: To determine if R-loops contribute to hematopoietic defects in *ddx41* mutants (Outcomes from this sub-aim are published in Weinreb *et al. Dev Cell* 2021 & Weinreb *et al. Haematological* 2022). Using the inducible *RNASEH1-GFP* transgenic zebrafish, we explored the impact of excess R-loops on hematopoietic phenotypes in *ddx41* mutants. We demonstrated that *ddx41* mutants have excessive HSPCs. As quantified by assessment of both absolute number of *runx1:mcherry*⁺ HSPCs per embryo and *runx1* *in situ* hybridization staining at 40 hpf, HSPC levels were significantly reduced in *ddx41* mutants after transient global RNASEH1-GFP expression (**Fig. 2A-E**). To elucidate if the impact of excess R-loops on HSPC expansion was cell-intrinsic, we injected *ddx41* mutants with either *ubi:gfp* (control) or *fli1:M27RNASEH1-GFP* which would deplete R-loops only in *fli1*⁺ endothelial cells including those that give rise to HSPCs. As quantified by assessment of *runx1:mcherry*⁺ HSPCs per embryo at 40 hpf, HSPC levels were significantly reduced in *ddx41* mutants injected with *fli1:M27RNASEH1-GFP* compared to control (**Fig. 2 F-G**). These data show that aberrant R-loop accumulation can intrinsically promote HSPC expansion *in vivo*.

Paradoxically, when we transiently overexpressed *RNASEH1-GFP*, rather than suppressing the erythrocyte defect in *ddx41* mutants it exacerbated the anemia as measured by a decrease in the amount of hemoglobinized erythrocytes. Through these studies, we uncovered that *ddx41* mutants display a strong DNA damage response that induces a cell cycle arrest in erythrocyte precursors and suppresses maturation. These findings are now published (Weinreb *et al. Haematologica*, 2021).

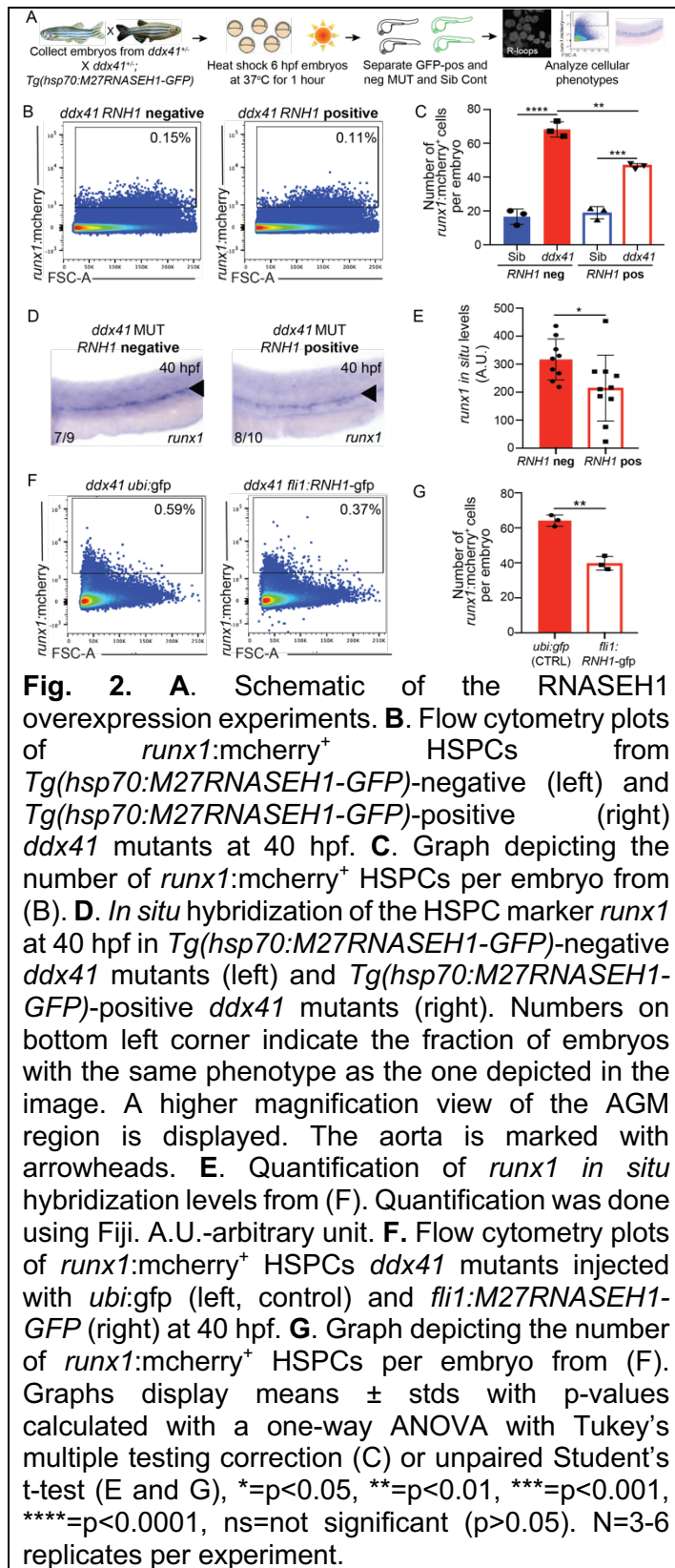
These data demonstrated that proper R-loop balance is critical for hematopoietic homeostasis.

Aim 1.3: To determine how R-loops regulate hematopoietic gene expression. R-loops are known to regulate gene expression via modulation of transcriptional elongation and epigenetic modifiers. Based on this, we hypothesized that DDX41-mediated R-loop regulation controls expression of critical genes for hematopoiesis, and that disruption of this regulation contributes to hematopoietic dysfunction in *ddx41* mutants. As our initial results regarding R-loop regulation of hematopoiesis were in erythrocytes, we first explored R-loop regulation of erythroid gene expression. This hypothesis was supported by a recent study that mapped the genomic locations of R-loops in human K562 erythroleukemia cells and identified an R-loop near the promoter of the *GATA1* gene. To identify R-loop-regulated gene expression in *ddx41* mutant erythrocytes, we performed RNA-seq on cells isolated from *ddx41* mutants and siblings either with or without R-loop depletion via RNASEH1-GFP overexpression. We observed few changes in transcript expression levels, but significant alterations in splicing. There were more than 600 RNASEH1-sensitive alternative splicing events in *ddx41* mutant cells.

We next tried to perform the same experiment in HSPCs. At this time point in development there are few HSPCs in wildtype animals, thus we experienced technical challenges getting high quality RNA for bulk RNA-seq. Moreover, we were interested in examining if R-loop accumulation might alter chromatin accessibility in HSPCs. To overcome these two hurdles, we undertook a single cell multiome approach. Using 10X Genomics chromium single cell ATAC + gene expression kit, we sequencing open chromatin and RNA from *runx1*⁺ HSPCs from *ddx41*



mutants and siblings with or without RNASEH1-GFP overexpression. The sequencing for these samples was received in December 2022 and we are currently analyzing them to determine the impact of excessive R-loops on chromatin configuration and gene expression. This work will part of a publication in the future.



Aim 2.1: To determine if inflammatory signaling is perturbed in *ddx41* mutants (Outcomes from this sub-aim are published in Weinreb *et al. Dev Cell* 2021). Growing evidence suggests that perturbed inflammatory signaling is a common feature of MDS that contributes to disease pathophysiology. In addition to a role in RNA:DNA hybrid resolution, DDX41 is implicated in inflammatory signaling. DDX41 acts as an intracellular DNA sensor that activates the STING (Stimulator of Interferon Genes) pathway in both mammals and zebrafish. When activated, STING triggers a signaling cascade leading to activation of TBK1 (Tank Binding Kinase 1), the transcription factors NF κ B (Nuclear Factor κ -B) and IRF3 (Interferon Response Factor 3), and production of Type I Interferon. Activation of the STING pathway in mice can induce HSPC cell cycle entry and mobilization, and thus could be involved in HSPC expansion. STING is activated by a number of cytosolic sensors in addition to DDX41. A recent study found that RNA:DNA hybrids can activate cGAS (cyclic GMP-AMP synthase), another cytosolic nucleic-acid sensor that triggers STING activation. To assess pathway activation in *ddx41* mutants and sibling controls, we measured phosphorylation of TBK1-Ser172 and NF κ B transcriptional activity. We showed elevated phospho-TBK1 and NF κ B transcriptional reporter activity in *ddx41* mutants compared to siblings. Pathway activation was diminished upon cGAS/STING knockdown and R-loop depletion. RNA-seq analysis revealed elevated Type I IFN gene expression in *ddx41* mutants compared to siblings. Using RT-qPCR, we demonstrated a decrease in Type I IFN responsive genes in *ddx41* mutants upon R-loop depletion. To assess the cell types with elevated inflammation, we examined *nfkb:gfp* levels in HSPCs and found that levels are higher in *ddx41* mutant HSPCs compared to sibling controls. *The data strongly indicate that excessive R-loops resulting from ddx41 loss trigger a cGAS/STING-mediated inflammatory response.* These findings are included in our published study (Weinreb *et al. Developmental Cell*, 2021).

Aim 2.2: To determine if STING-mediated inflammatory signaling plays a role in hematopoietic defects in *ddx41* mutants (Outcomes from this sub-aim are published in Weinreb *et al. Dev Cell* 2021). We tested the role of the cGAS/STING signaling components on the HSPC expansion in *ddx41* mutants. Lowering cGAS or STING

activation using numerous genetic approaches significantly reduced HSPC numbers in *ddx41* mutants, as measured by quantification of *cd41:gfp*⁺ HSPC numbers per embryo using flow cytometry and *runx1 in situ*

hybridization. *These results indicate that the cGAS-STING inflammatory cascade mediates HSPC accumulation in ddx41 mutants.* These findings are included in our published study (Weinreb *et al.* Developmental Cell, 2021).

Aim 2.3: To determine if elevated levels of R-loops alter inflammatory signaling in *ddx41* mutants (Outcomes from this sub-aim are published in Weinreb *et al.* Dev Cell 2021). We deciphered that the elevated phospho-TBK1 levels were dampened following cGAS morpholino-mediated knockdown and following RNA:DNA hybrid depletion via RNASEH1-GFP overexpression. Additionally, we demonstrated a decrease in some interferon-stimulated genes (ISGs) following R-loop depletion strengthening the model that R-loops activate a cGAS/STING inflammatory cascade leading to aberrant HSPC expansion. These findings are included in our published study (Weinreb *et al.* Developmental Cell, 2021).

Professional development:

As part of my professional development, I attended several conferences including the 2019 American Society of Hematology (ASH) Conference in Orlando, 2020 International Society of Experimental Hematology (ISEH) Conference (Virtual), the 2020 MDS Summit (Virtual), 2020 ASH Conference (Virtual), 2021 Keystone Hematopoiesis Symposium (Virtual), and 2021 ISEH Conference (Virtual). My student, Joshua Weinreb, who was the lead author on the publications related to this work, attended and presented his work at 2019 ASH and 2020 ISEH.

Dissemination of results:

We have presented the data from this project at National and International Conferences (ASH and ISEH) as well invited seminar series at US-based research institutes.

Plans for next reporting period:

This is the final report, so there is Nothing to Report.

4. IMPACT: Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

Impact on the principal discipline: Germline mutations in *DDX41* predispose individuals to developing blood cancers, especially MDS and AML. How defects in this factor might contribute to hematopoietic dysfunction remains unknown. Our studies have revealed that *DDX41* deficiency can cause expansion of blood-forming stem cells due to elevated inflammatory signaling via the cGAS/STING pathway. These mechanistic discoveries pave the way towards future biochemical studies deciphering how *DDX41* elicits these effects and the function of the cGAS/STING signaling in hematopoietic homeostasis. Moreover, these findings strongly suggest that inhibitors of the cGAS/STING pathway could be novel therapeutics for the treatment of MDS. Indeed, several STING antagonist are currently in pre-clinical and clinical develop for other diseases which could allow for more streamlined testing of these molecules for MDS.

Impact on other disciplines: Nothing to Report

Impact on technology transfer: Nothing to Report

Impact on society beyond science and technology: Nothing to Report

5. CHANGES/PROBLEMS:

Nothing to Report

6. PRODUCTS:

Journal publications:

Weinreb JT, Ghazale N, Pradhan K, Gupta V, Potts KS, Tricomi B, Daniels NJ, Padgett RA, De Oliveira S, Verma AK, and **Bowman TV**. Excessive R-loops Trigger an Inflammatory Cascade Leading to Aberrant HSPC Expansion. Developmental Cell, 2021; 56(5):627-640 e5. PMID: 33651979. PMCID: PMC8258699

Acknowledgement of federal support: Yes

Weinreb JT, Gupta V, Sharvit E, Weil R, and **Bowman TV**. Ddx41 inhibition of DNA damage signaling permits erythroid progenitor expansion in zebrafish. *Haematologica*, 2021; doi:10.3324/haematol.2020.257246. PMID: 33763998; PMCID: PMC8883538.

Acknowledgement of federal support: Yes

Other publications:

Bowman TV and Trompouki E. Sensing Stemness. *Current Stem Cell Reports*, 2021; doi: 10.1007/s40778-021-00201-w. PMID: 34868827. PMCID: PMC8639566

Acknowledgement of federal support: Yes

Weinreb JT and **Bowman TV**. Clinical and mechanistic insights into the roles of DDX41 in haematological malignancies. *FEBS Lett*, 2022; 596(21):2736-2745. doi: 10.1002/1873-3468.14487. PMID: 36036093. PMCID: PMC9669125 (available on 2023-11-01).

Acknowledgement of federal support: Yes

Makishima H, **Bowman TV**, Godley LA. DDX41-associated susceptibility to myeloid neoplasms. *Blood*, 2022; blood.2022017715. doi: 10.1182/blood.2022017715. Online ahead of print. PMID: 36455200.

Acknowledgement of federal support: Yes

Conference papers and presentations:

Nov 2019 Five Points Seminar, New York Genome Center, New York, NY
June 2020 Blood and Bone Seminar, Virtual Seminar Series
Dec 2019 2019 American Society of Hematology Annual Meeting, Abstract Achievement Award and Oral Presentation (awarded to JTW), Orlando, FL
Aug 2020 2020 International Society of Experimental Hematology Meeting, Oral Presentation, Virtual Conference
Nov 2020 Department of Genetics, Cell Biology, and Anatomy, University of Nebraska Medical Center, Omaha, NE (Virtual)
Dec 2020 ASH Myeloid Workshop, 2020 ASH Annual Meeting (Virtual)
Nov 2021 NIH Workshop: Biological Functions of DEAD/H-box Helicase in Health and Diseases
Dec 2021 Fred Hutchinson/University of Washington Heme Seminar Series (Virtual)
Jan 2022 Department of Genetic, Development, and Cell Biology Seminar Series, Iowa State University, Ames, IA
Jan 2022 Broad Institute, Cancer Biology Program Seminar Series, Cambridge, MA (Virtual)
Mar 2022 Cancer Epigenetics Program, Sylvester Comprehensive Cancer Center, University of Miami, Miami, FL (Virtual)
Sept 2022 2022 Zebrafish Disease Modeling 15 Conference, Sheffield, UK
Oct 2022 2022 Zebrafish Global Education Network Webinar Series
Oct 2022 2022 Edward P. Evans MDS Summit, Nashville, TN

Website or other Internet sites:

Nothing to report

Technologies or techniques:

Nothing to report

Inventions, patent applications, and/or licenses:

Nothing to report

Other Products:

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	Teresa V. Bowman, PhD
Project Role:	Principal Investigator
Research Identifier (e.g. ORCID ID):	

Nearest person month worked:	3
Contribution to project:	Dr. Bowman oversaw all of the work performed for the project, mentored and managed all participants, helped with data analysis, and wrote/edited all manuscripts.
Funding Support:	
Name:	Kathryn Potts, PhD
Project Role:	Postdoctoral Researcher
Research Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to project:	Dr. Potts performed the experiments in the project along with Joshua Weinreb.
Funding Support:	
Name:	Joshua Weinreb
Project Role:	Graduate Student
Research Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to project:	Joshua Weinreb performed all the experiments in the project along with Dr. Potts.
Funding Support:	Mr. Weinreb is supported by an F30 NIH predoctoral fellowship (1F30HL142161)
Name:	Varun Gupta
Project Role:	Bioinformatician
Research Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.6
Contribution to project:	Varun Gupta performed all the computational analysis of the RNA-sequencing data.
Funding Support:	

Changes in active other support to Principal Investigator:

Active Grants:

NIH/NIDDK: 1R01DK121738-01 2020-2024
 “Crosstalk of Splicing and Signaling during HSPC Fate Choices”
 Principal Investigator: Teresa Bowman

DOD/DTRA: 2020-2024
 “Long non-coding RNAs in Venezuelan Equine Encephalitis Virus infection”
 Principal Investigator: Steven Bradfute, University of New Mexico
 Collaborator/Consultant: Teresa Bowman (salary only)

Edward P. Evans Foundation MDS Discovery Research Grant: 2021-2024
 “Delineating mechanisms of DDX41 insufficiency in MDS”
 Principal Investigator: Teresa Bowman

NIH/NIDDK: 1R01DK131445: 2021-2024
 “Identification of novel regulators of HSC specification and maturation”
 Principal Investigator: Teresa Bowman

Other organizations:

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS:

Nothing to Report

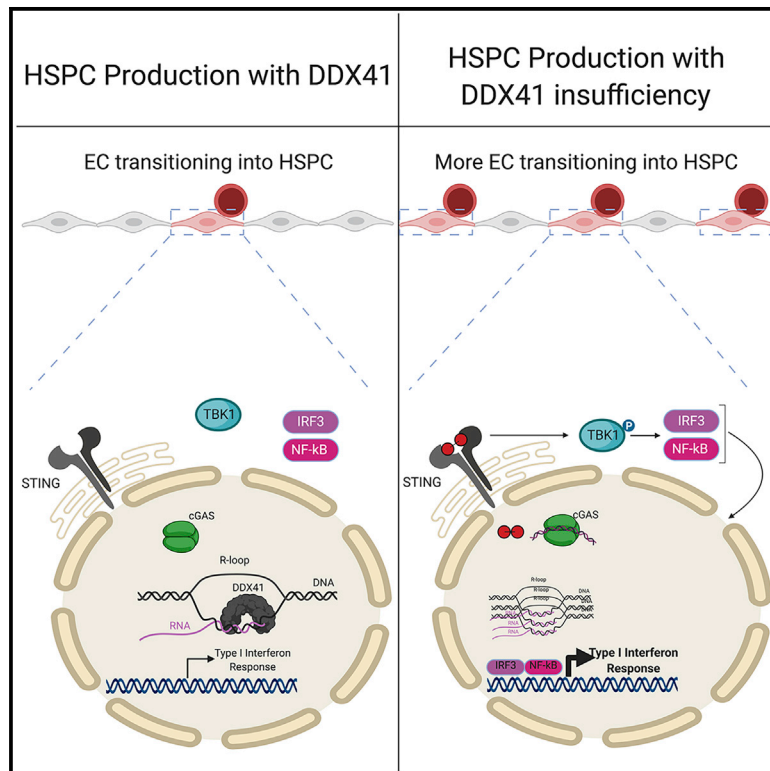
9. APPENDICES:

Publications produced as a result of this work.

Developmental Cell

Excessive R-loops trigger an inflammatory cascade leading to increased HSPC production

Graphical abstract



Authors

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In Brief

Weinreb et al. show a link between R-loops, inflammation, and the developing hematopoietic system. Ddx41 acts as a gatekeeper of HSPC production by suppressing R-loop accumulation and the cGAS-STING inflammatory pathway. Elevated R-loops and inflammatory signaling were also observed in human cells with decreased DDX41, suggesting possible conservation of mechanism.

Highlights

- Ddx41 regulates HSPC number *in vivo*
- Ddx41 constrains R-loop levels *in vivo*
- Elevated R-loop levels increase HSPC production via cGAS-STING inflammatory signaling
- DDX41 suppression of R-loop levels and inflammatory signaling is observed in human cells



Article

Excessive R-loops trigger an inflammatory cascade leading to increased HSPC production

Joshua T. Weinreb,^{1,2} Noura Ghazale,^{1,2} Kith Pradhan,^{1,3} Varun Gupta,⁴ Kathryn S. Potts,^{1,2} Brad Tricomi,^{2,4} Noah J. Daniels,⁵ Richard A. Padgett,⁵ Sofia De Oliveira,^{1,6} Amit Verma,^{1,2,7} and Teresa V. Bowman^{1,2,7,8,*}

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SUMMARY

Hematopoietic stem and progenitor cells (HSPCs) arise during embryonic development and are essential for sustaining the blood and immune systems throughout life. Tight regulation of HSPC numbers is critical for hematopoietic homeostasis. Here, we identified DEAD-box helicase 41 (*Ddx41*) as a gatekeeper of HSPC production. Using zebrafish *ddx41* mutants, we unveiled a critical role for this helicase in regulating HSPC production at the endothelial-to-hematopoietic transition. We determined that *Ddx41* suppresses the accumulation of R-loops, nucleic acid structures consisting of RNA:DNA hybrids and ssDNAs whose equilibrium is essential for cellular fitness. Excess R-loop levels in *ddx41* mutants triggered the cGAS-STING inflammatory pathway leading to increased numbers of hemogenic endothelium and HSPCs. Elevated R-loop accumulation and inflammatory signaling were observed in human cells with decreased *DDX41*, suggesting possible conservation of mechanism. These findings delineate that precise regulation of R-loop levels during development is critical for limiting cGAS-STING activity and HSPC numbers.

INTRODUCTION

The hematopoietic system is maintained by multipotent hematopoietic stem and progenitor cells (HSPCs) that sustain the stem cell pool via self-renewal divisions and generate all mature blood cells through multilineage differentiation. Precise maintenance of the HSPC pool is critical for healthy hematopoiesis throughout life. A paucity of functional HSPCs can result in bone marrow failure, and aberrant expansion of HSPCs is a common feature in clonal hematologic malignancies (Kurre, 2018; Sperling et al., 2017). Functional hematopoietic transplantation studies indicate that the frequency of HSPCs across vertebrates is astoundingly conserved (Fraint et al., 2020; Hess et al., 2013; Szilvassy et al., 2003). Shifts in HSPC frequency during embryogenesis are linked to inherited bone marrow failure syndromes, underlying the importance of identifying factors that ensure robustness in HSPC development (Kurre, 2018).

Inflammatory signaling plays a physiological role in regulating HSPC biology from the earliest stages of stem cell formation in the embryo and throughout the aging process (Espin-Palazon et al., 2018; Pietras, 2017). During development, nuclear factor

kappa-light-chain enhancer of activated B cells (NFκB) and interferon (IFN) signaling are critical for the initial establishment of definitive HSPCs (He et al., 2015; Espín-Palazón et al., 2014; Li et al., 2014; Sawamiphak et al., 2014). Prior work suggested that sterile inflammation, a non-pathogen-mediated activation of immunomodulatory signaling, is at the heart of the inflammatory signals required for HSPC formation. Our knowledge of the non-infectious stimuli and the downstream signaling cascades relevant for the impact of sterile inflammation on HSPC production in embryogenesis is incomplete.

Many of the transcription factors widely studied in HSPC development, such as AML/RUNX1, TAL1/SCL, and TEL1/ETV6, were first uncovered due to their aberrant function in hematologic malignancies. In humans, *DEAD-box helicase 41* (*DDX41*) was recently shown to be mutated in both germline and acquired forms of myelodysplastic syndrome (MDS), a clonal HSPC disorder (Sperling et al., 2017; Polprasert et al., 2015). The clinical link is suggestive, but the precise role for *DDX41* in HSPC biology has yet to be demonstrated. *DDX41* has pleiotropic cellular functions associated with inflammation, splicing, and, more recently, R-loops (Wang et al., 2018;



Polprasert et al., 2015; Zhang et al., 2013; Parvatiyar et al., 2012; Zhang et al., 2011).

R-loops are three-stranded nucleic acid structures consisting of an RNA:DNA hybrid and ssDNA. They are natural consequences of transcription that play roles in numerous cellular functions, including immunoglobulin class-switch recombination, mitochondrial replication, and transcriptional and epigenetic regulation (Crossley et al., 2019). Recent work demonstrated that the genome-wide R-loop landscape is dynamic during differentiation of human pluripotent stem cells into numerous lineages suggesting that the regulation of R-loop levels could be important during developmental fate transitions (Yan et al., 2020). Additionally, mutations in R-loop-regulating factors are found in humans with autoimmune disorders, suggesting that R-loop imbalance could affect inflammatory signaling (Günther et al., 2015; Lim et al., 2015).

Here, we show that DDX41 sits at a crossroad of R-loop metabolism and inflammation to regulate HSPC homeostasis. To uncover if DDX41 plays a role in HSPC development, we established a zebrafish *ddx41* loss-of-function mutant to study the *in vivo* requirements of Ddx41. We demonstrated that Ddx41 regulates HSPC number by increasing hemogenic endothelium. Mechanistically, loss of *ddx41* leads to R-loop accumulation triggering NF- κ B-mediated inflammation via activation of the cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) signaling pathway. This sterile inflammatory cascade in the mutants underlies their increased HSPC production. Elevated R-loop accumulation and inflammatory signaling were also observed in human cells with decreased DDX41, suggesting possible conservation of mechanism in humans. Together, our data establish that Ddx41 constrains HSPC production during development by maintaining R-loop homeostasis and preventing aberrant inflammatory signaling.

RESULTS

Ddx41 regulates HSPC number

As DDX41 mutations are linked to hematologic diseases driven by HSPC dysfunction, we hypothesized that this helicase might have a role in HSPC homeostasis. We explored this question with a zebrafish *ddx41* loss-of-function mutant (*ddx41*^{sa14887}) (Kettleborough et al., 2013). DDX41 is highly conserved between humans and zebrafish with an overall 89% identity and 94% similarity between the two proteins with almost identical DEAD box and helicase domains (Figure S1A), suggesting that the lessons learned about the *in vivo* role of zebrafish Ddx41 function in hematopoiesis will be relevant to human DDX41.

In zebrafish, definitive hematopoiesis begins ~24 h post fertilization (hpf), creating HSPCs that can self-renew and reconstitute all adult blood lineages (Nik et al., 2017). During development, HSPCs derive directly from the aortic hemogenic endothelium—which is located in the ventral floor of the dorsal aorta in a region known as the aorta gonad mesonephros (AGM)—via a process termed endothelial-to-hematopoietic transition (EHT) (Ottersbach, 2019; Bertrand et al., 2010; Boisset et al., 2010; Kissa and Herbomel, 2010). This process involves the transition of a subset of endothelial cells into HSPCs. We examined HSPC formation at the time of this transition via *in situ* hybridization for expression of *runx1*, a marker of hemogenic endothelium

and nascent HSPCs (Burns et al., 2002). At 40 hpf, levels of *runx1*⁺ cells were elevated in *ddx41* mutants compared with sibling controls (Figures 1A and 1B). We also demonstrated this increase by measuring the absolute number of HSPCs per embryo using flow cytometry quantification with the HSPC fluorescent reporters *runx1*:23:mcherry (hereafter referred to as *runx1*:mcherry) (Tamplin et al., 2015) and *cd41*:gfp (Lin et al., 2005). In agreement with the *runx1* *in situ* results, we observed a significant increase in the number of both *runx1*:mcherry⁺ and *cd41*:gfp⁺ HSPCs in *ddx41* mutants (Figures 1C–1F). Taken together, our results indicate that decreased *ddx41* levels lead to a significant increase in HSPCs.

We next wanted to elucidate if HSPC expansion in *ddx41* mutants is occurring via an increase in proliferation and/or production. To examine proliferation, we analyzed cell cycle status of *ddx41* mutant *cd41*:gfp⁺ HSPCs by flow cytometry quantification of DNA synthesis via 5-Ethynyl-2'-deoxyuridine (EdU) incorporation and DNA content via DAPI incorporation. At 40 hpf, *ddx41* mutant *cd41*:gfp⁺ HSPCs showed a reduction of cells in S phase and an accumulation in the G2/M phase compared with sibling controls (Figures S1C and S1D). These results suggest a decrease in proliferation rather than an increase, which indicates that HSPC expansion in *ddx41* mutants is not a result of increased cell division.

A second mechanism we tested was increased HSPC production. Hemogenic endothelial cells undergoing EHT express features of both endothelial cells and HSPCs (Bertrand et al., 2010). First, we examined the vasculature via *in situ* hybridization for *flt4* (vein) and *notch1b* (aorta) and observed no difference between siblings and *ddx41* mutants, suggesting that the artery-vein specification was unaffected (Figures S1E and S1F). Next, we performed flow cytometry to quantify endothelial cells (*kdr1*:dsred⁺), hemogenic endothelial cells (*cd41*:gfp⁺; *kdr1*:dsred⁺), and HSPCs (*cd41*:gfp⁺) in sibling controls versus *ddx41* mutants. At 40 hpf, we observed no significant difference in the number of *kdr1*:dsred⁺ endothelial cells, but a significant increase in the number of both *cd41*:gfp⁺ HSPCs and *cd41*:gfp⁺; *kdr1*:dsred⁺ double positive hemogenic endothelial cells in *ddx41* mutants (Figures 1G, 1H, S1G, and S1H). To visualize EHT changes, we performed whole mount time-lapse confocal imaging of *cd41*:gfp⁺; *kdr1*:dsred⁺ sibling controls and *ddx41* mutants between ~36–44 hpf (Videos S1 and S2). Consistent with the flow cytometry data, we observed that *ddx41* mutants had an increased number of *cd41*:gfp⁺; *kdr1*:dsred⁺ cells undergoing EHT morphological changes earlier in development than sibling controls. Taken together, our results indicate that the expansion of HSPCs in *ddx41* mutants is via an increased rate of EHT.

Ddx41 constraint of R-loop levels is critical for HSPC homeostasis

Cells possess numerous safeguards to tightly regulate R-loop levels, such as: formation inhibition by splicing factors, destruction by RNA:DNA hybrid ribonucleases, and removal by RNA:DNA hybrid helicases (Crossley et al., 2019). Based on the evidence that DDX41 binds to RNA:DNA hybrids and that its activity is stimulated by this interaction (Stavrou et al., 2018; Wang et al., 2018; Yoneyama-Hirozane et al., 2017), we hypothesized that Ddx41 might prevent R-loop accumulation *in vivo*. To address this question, we measured R-loop levels in *ddx41*

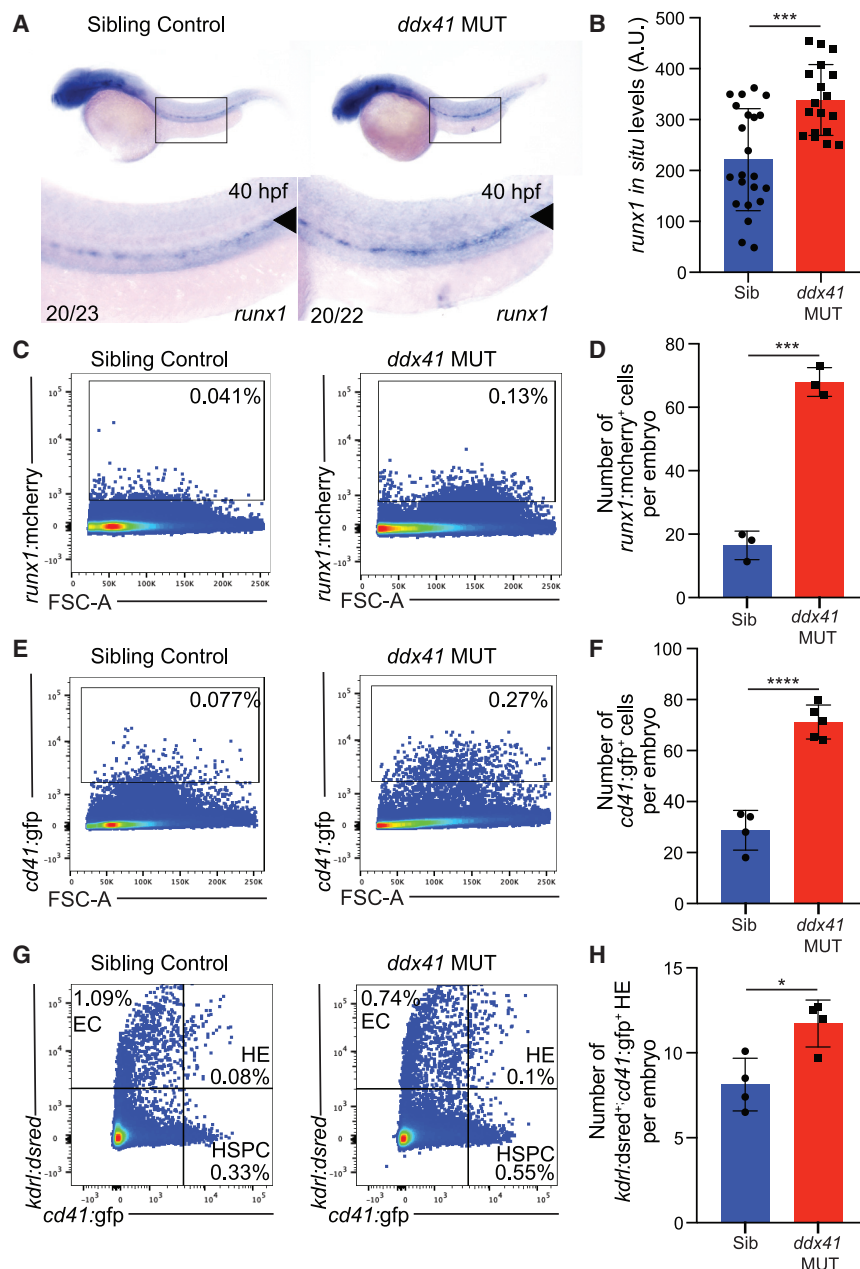


Figure 1. Ddx41 regulates HSPC number

(A) *In situ* hybridization of the HSPC marker *runx1* at 40 hpf in sibling controls (left) and *ddx41* mutants (right). Numbers on bottom left corner indicate the fraction of embryos with the same phenotype as the one depicted in the image. Inset underneath shows a higher magnification (8x) view of the boxed AGM region above. The aorta is marked with arrowheads.

(B) Quantification of *runx1* *in situ* hybridization levels from (A). Quantification was done using Fiji. a.u., arbitrary unit.

(C and E) Flow cytometry plots of *runx1*:mcherry⁺ (C) and *cd41*:gfp⁺ (E) HSPCs from sibling controls (left) and *ddx41* mutants (right) at 40 hpf.

(D and F) Graphs depicting the absolute number of *runx1*:mcherry⁺ (D) and *cd41*:gfp⁺ (F) per embryo at 40 hpf.

(G) Flow cytometry plots of *kdr*:dsred⁺, *cd41*:gfp⁺, and *kdr*:dsred⁺; *cd41*:gfp⁺ double positive cells from sibling controls (left) and *ddx41* mutants (right) at 40 hpf.

(H) Graphs depicting the absolute number of *kdr*:dsred⁺; *cd41*:gfp⁺ double positive hemogenic endothelial cells per embryo at 40 hpf. Graphs display means $\pm$ standard deviations (stds) with p values calculated with unpaired Student's t test, *p < 0.05, ***p < 0.001, ****p < 0.0001. N = 3–5 replicates per experiment.

at 6 hpf, separated transgenic and non-transgenic siblings according to GFP expression, and then assessed R-loops levels (Figure 2A). Our results showed that R-loop levels were increased nearly 2-fold in *ddx41* mutants compared with sibling cells and that the effect was suppressed by RNASEH1-GFP expression (Figures 2B and 2C). These data demonstrate that Ddx41 regulates R-loop levels *in vivo*.

We ascertained the effect of R-loop levels on hematopoiesis in *ddx41* mutants by measuring HSPC numbers in mutants and siblings following R-loop removal. As quantified by assessment of both absolute number of *runx1*:mcherry⁺ HSPCs per embryo and *runx1* *in situ* hybridization

mutants and siblings. We performed immunofluorescence with the S9.6 antibody, which is a monoclonal antibody that specifically binds RNA:DNA hybrids, and is a mainstay in the field for R-loop identification (Boguslawski et al., 1986). To confirm the S9.6 signal in zebrafish *ddx41* mutants was specific to RNA:DNA hybrids, we used Tg(*hsp:M27RNASEH1-GFP*) zebrafish that express human RNASEH1 fused to GFP under a heat shock-inducible promoter (Sorrells et al., 2018). RNase H1 is an enzyme which removes R-loops through the selective degradation of RNA in RNA:DNA hybrids and is the most rigorous confirmation to demonstrate R-loop dependency of a given phenotype (Suzuki et al., 2010; Paulsen et al., 2009). We induced RNASEH1-GFP expression by heat shocking *ddx41* mutants and siblings

staining at 40 hpf, HSPC levels were significantly reduced in *ddx41* mutants after RNASEH1-GFP expression (Figures 2D–2G). Next, we assessed if this effect was via a cell-intrinsic or cell-extrinsic mechanism. To address this, we isolated *cd41*:gfp⁺ HSPCs from 40 hpf sibling controls and *ddx41* mutants using fluorescent-activated cell sorting (FACS) and performed immunofluorescence with the S9.6 antibody to assess R-loop levels. Similar to the results in the whole embryo, R-loop levels were increased nearly 2-fold in *ddx41* mutant *cd41*:gfp⁺ HSPCs compared with siblings (Figure S2). To elucidate if the impact of excess R-loops on HSPC expansion was cell intrinsic, we injected *ddx41* mutants with either *ubi*:gfp (control) or *fli1*:M27RNASEH1-GFP, which would deplete R-loops only in *fli1*⁺

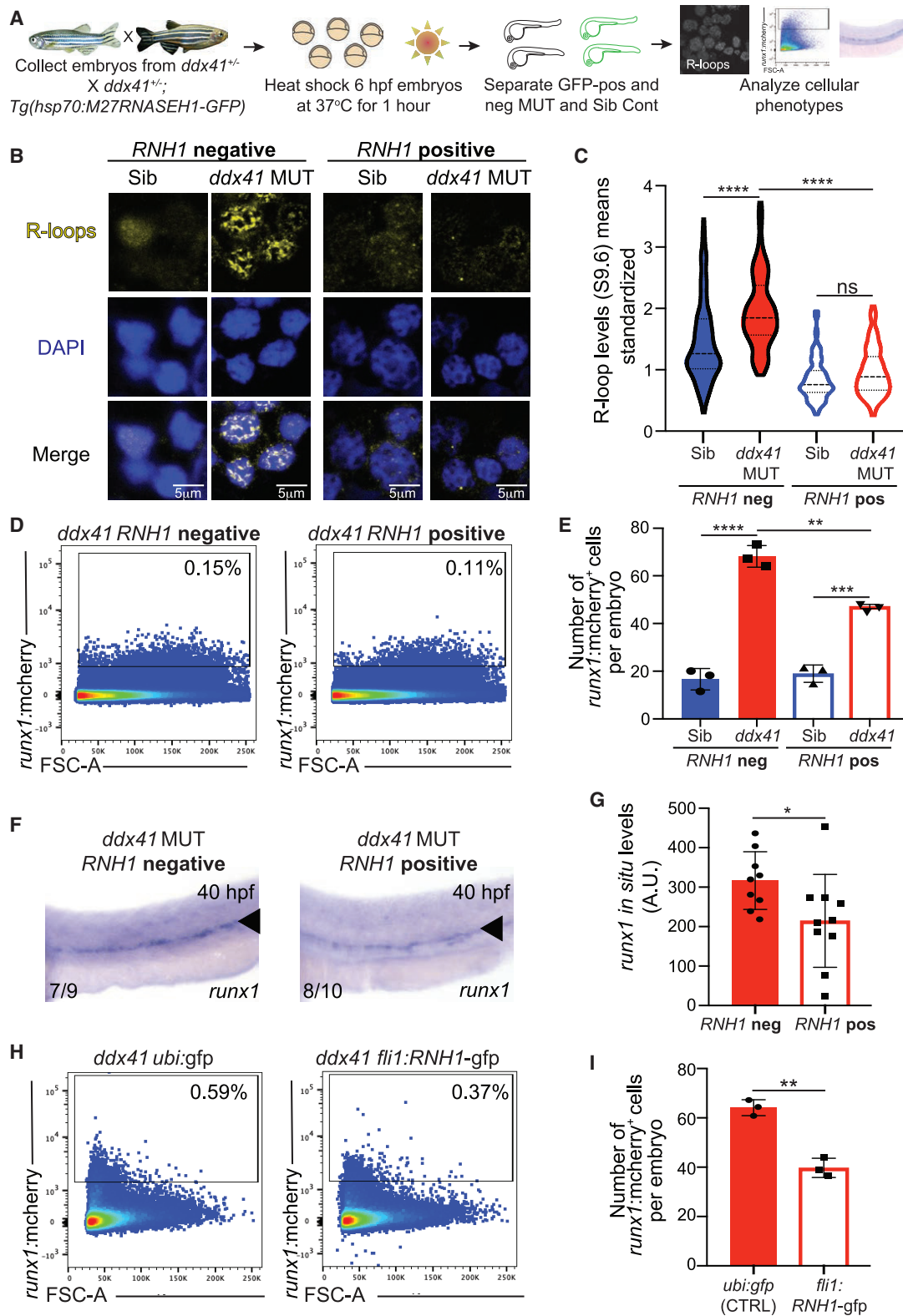


Figure 2. Ddx41 constraint of R-loop levels is critical for HSPC homeostasis

(A) Schematic of the RNASEH1 overexpression experiments.

(B) Confocal images showing immunofluorescence of R-loops and nuclei (DAPI) in cells isolated from 28 hpf siblings (left) and *ddx41* mutants (right) that are either *Tg(hsp70:M27RNASEH1-GFP)* negative (left) or *Tg(hsp70:M27RNASEH1-GFP)* positive (right). Scale bar, 5 μ m.

(legend continued on next page)

endothelial cells, including those that give rise to HSPCs (Lawson and Weinstein, 2002). As quantified by assessment of *runx1:mcherry*⁺ HSPCs per embryo at 40 hpf, HSPC levels were significantly reduced in *ddx41* mutants injected with *fli1:M27RNASEH1-GFP* compared with their control counterparts (Figures 2H and 2I). These data show that aberrant R-loop accumulation can intrinsically promote HSPC production from endothelial cells.

R-loops promote inflammatory gene expression in *ddx41* mutants

To delve into the mechanism underlying the HSPC defect in *ddx41* mutants, we conducted RNA-sequencing (RNA-seq) on *cd41:gfp*⁺ HSPCs isolated from *ddx41* mutants and siblings at 40 hpf. Of the nearly 19,000 genes analyzed, 1,173 were downregulated and 1,327 were upregulated in *ddx41* mutants compared with siblings (Figure 3A; Table S1).

To gain an insight into the affected pathways, we performed gene-set analysis on the upregulated and downregulated gene lists by comparing each to the molecular signature database (MSigDB), a platform that computes overlaps between experimentally derived gene lists and lists of genes in known pathways (Liberzon et al., 2015; Subramanian et al., 2005). In the downregulated list, genes associated with MTORC1 signaling, epithelial-mesenchymal transition, coagulation, and apical junctions were enriched (Figure 3B; Table S2). In the upregulated gene list, spliceosome was the top gene set with heme metabolism, DNA repair, and IFN- α response also enriched (Figure 3C; Table S3).

Ddx41 interacts with components of the spliceosome and is implicated in pre-mRNA splicing (Polprasert et al., 2015); thus, we also examined how *ddx41* loss affected mRNA splicing in HSPCs. A total of 2,009 alternative splicing events were observed between *ddx41* mutants and siblings, with exon skipping being the most frequently altered splicing event followed by intron retention (Figures S3A and S3B; Table S4). These splicing defects are similar to those found in *DDX41*-depleted and *DDX41*-mutated human cells (Polprasert et al., 2015). We were also able to detect other splicing defects not reported previously, including alternative 5'-splice site usage, alternative 3'-splice site usage, and changes in mutually exclusive exon usage in *ddx41* mutants as compared with siblings. To understand if particular pathways were enriched in the alternatively spliced gene list, we compared it with the MSigDB and determined that immune system pathways were among the top-enriched pathways (Figure S3C). Among the alternatively spliced factors were several NF- κ B pathway components, including *map3k7*,

nfk2, *nfkbiab*, and *irak3*. These results depict that *Ddx41* influences inflammatory gene expression in HSPCs at multiple levels.

Type I interferons and their target genes were significantly elevated in *ddx41* mutant HSPCs (Figure 3C; Table S3). We posited that excess R-loops might contribute to inflammatory signaling. The cytosolic nucleic acid sensor cGAS binds to—and can be activated by—RNA:DNA hybrids (Mankan et al., 2014). cGAS activity can induce a STING signaling cascade resulting in increased type I IFN-responsive gene expression (Ablasser et al., 2013; Li et al., 2013). To determine if R-loops contributed to inflammatory gene expression, we measured the expression of *ifn-phi1*, which is a zebrafish type I IFN, and several type I IFN-responsive genes identified in our RNA-seq analysis using RT-qPCR in embryos $\pm$ RNASEH1-GFP expression. Depletion of R-loops significantly reduced expression of many of these genes in *ddx41* mutants, demonstrating that excessive R-loops promote inflammatory signaling (Figure 3D).

Ddx41 regulates inflammatory signaling via the cGAS-STING pathway

Our model is that excess R-loops promote inflammation by activating the cGAS-STING pathway. We first assessed if cGAS and R-loops were in the same cellular compartment, and thus capable of activating cGAS-STING signaling as previously characterized (Mankan et al., 2014). Prior studies show that in addition to being in the cytoplasm, cGAS can also be found tightly tethered to chromatin in the nucleus, suggesting that it can bind to nuclear RNA:DNA hybrids (Gentili et al., 2019; Jiang et al., 2019; Volkman et al., 2019; Liu et al., 2018). Consistent with these prior reports, we found cGAS to be predominantly in the nucleus of zebrafish cells by immunofluorescence (Figures S4A–S4E). Additionally, R-loops were present in both cellular compartments of *ddx41* mutant embryos (Figures 2B, 2C, S4F, and S4G). In combination, these results demonstrate that interactions between R-loops and cGAS are possible in multiple cellular compartments.

Next, we ascertained if the loss of *ddx41* led to activation of the cGAS-STING pathway. Phosphorylation of Ser172 in Tank Binding Kinase 1 (TBK1) is induced downstream of cGAS-STING activation; thus, we measured phospho-TBK1 levels in sibling controls and *ddx41* mutant cells by immunofluorescence and western blots (Bai et al., 2017). Levels of phospho-TBK1 were significantly elevated in *ddx41* mutants and dependent on cGAS and elevated R-loop levels (Figures 4A–4C, S4H, and S4I). These data indicate that the cGAS-STING pathway is elevated when *ddx41* levels are diminished.

(C) Quantification of R-loop levels from (B).

(D) Flow cytometry plots of *runx1:mcherry*⁺ HSPCs from *Tg(hsp70:M27RNASEH1-GFP)*-negative (left) and *Tg(hsp70:M27RNASEH1-GFP)*-positive (right) *ddx41* mutants at 40 hpf.

(E) Graph depicting the number of *runx1:mcherry*⁺ HSPCs per embryo from (D).

(F) *In situ* hybridization of the HSPC marker *runx1* at 40 hpf in *Tg(hsp70:M27RNASEH1-GFP)*-negative *ddx41* mutants (left) and *Tg(hsp70:M27RNASEH1-GFP)*-positive *ddx41* mutants (right). Numbers on bottom left corner indicate the fraction of embryos with the same phenotype as the one depicted in the image. A higher magnification view of the AGM region is displayed. The aorta is marked with arrowheads.

(G) Quantification of *runx1 in situ* hybridization levels from (F). Quantification was done using Fiji. a.u., arbitrary unit.

(H) Flow cytometry plots of *runx1:mcherry*⁺ HSPCs *ddx41* mutants injected with *ubi:gfp* (left, control) and *fli1:M27RNASEH1-GFP* (right) at 40 hpf.

(I) Graph depicting the number of *runx1:mcherry*⁺ HSPCs per embryo from (H). Graphs display means $\pm$ stds with p values calculated with a one-way ANOVA with Tukey's multiple testing correction (C and E) or unpaired Student's t test (G and I), *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns, not significant (p > 0.05). N = 3–6 replicates per experiment.

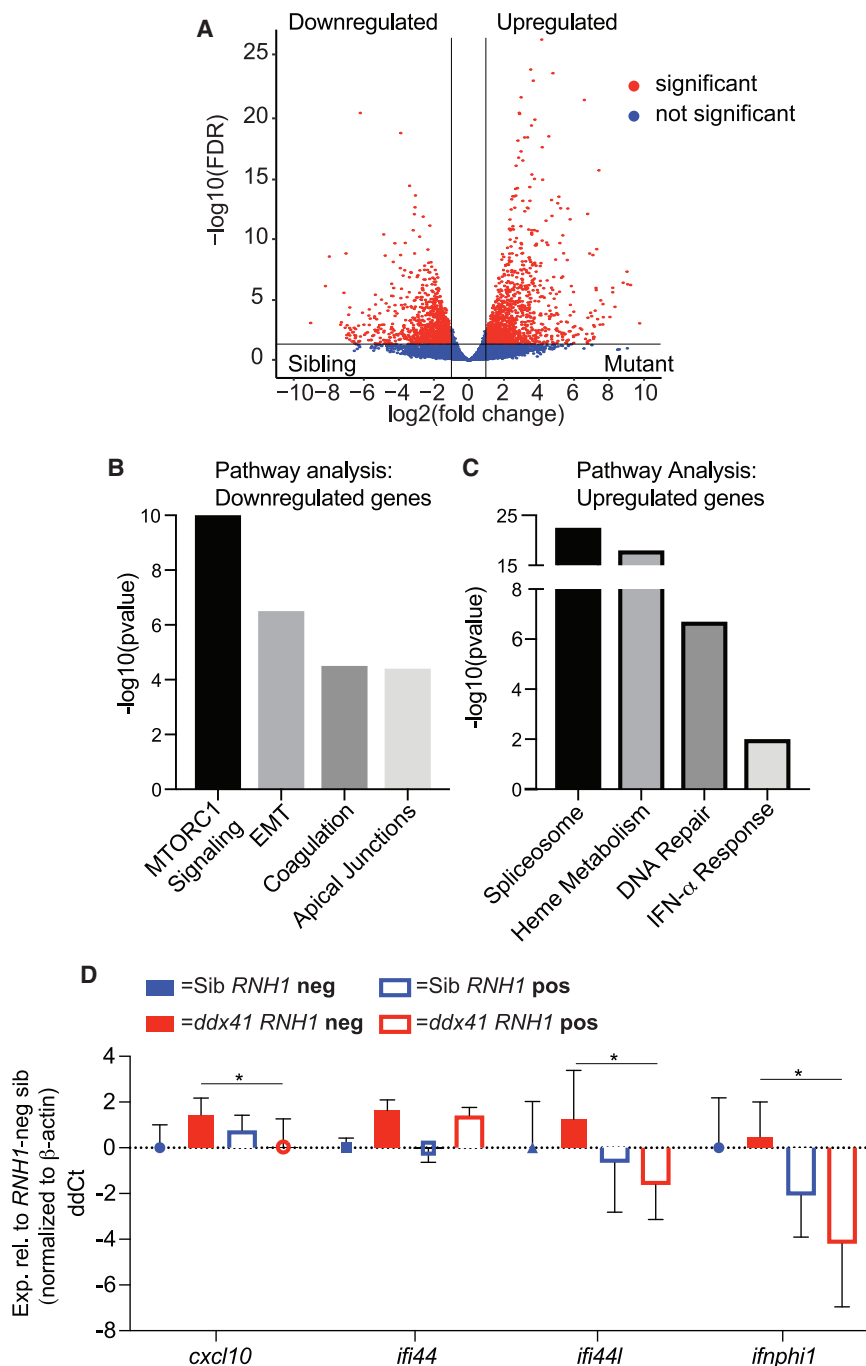


Figure 3. R-loops promote inflammatory gene expression in *ddx41* mutants

(A) Volcano plot displaying differentially expressed genes between *cd41:gfp*⁺ HSPCs from *ddx41* mutants and siblings. Significant differences are defined as FDR < 0.05 and $\log_2$ fold change > 1. Black vertical lines denote the fold-change threshold and the black horizontal line denotes the FDR threshold. Three biological replicates for both *ddx41* mutants and siblings were used to generate RNA-sequencing data.

(B and C) Representative charts of pathways significantly enriched in genes downregulated (B) or upregulated (C) in *ddx41* mutant HSPCs compared with sibling controls as determined by MSigDB analysis.

(D) Graph of RT-qPCR analysis of the expression of type I IFN-responsive genes between sibling controls and *ddx41* mutants that are *Tg(hsp70:M27RNASEH1-GFP)*-negative versus *Tg(hsp70:M27RNASEH1-GFP)*-positive. Expression levels were normalized to β -actin levels. All levels are relative to the RNASEH1-GFP (RNH1) negative sibling controls expressed as ddCt values, with positive values reflecting higher expression and negative values reflecting lower expression. Graph displays means $\pm$ standard error mean with p values calculated with an unpaired t test, *p < 0.05. N = 4 replicates per experiment.

flow cytometry and demonstrated a significant increase in *ddx41* mutants (Figures 4D and 4E). We also determined that NF κ B activity was increased in *runx1:mcherry*⁺ HSPCs and *kdr1:dsred*⁺ endothelial cells in *ddx41* mutants (Figures S4J–S4M). These data support that Ddx41 regulates NF κ B signaling in numerous cell types, including HSPC and endothelial cells.

Next, we determined if inhibiting components of the cGAS-STING pathway would dampen inflammatory signaling in *ddx41* mutants by measuring *nfkb:gfp* reporter activity (Figure 4F). Morpholino knockdown of either STING or cGAS lowered *nfkb:gfp* reporter activity in *ddx41* mutants (Figures 4G, 4H, S4E, and S4N–S4P). Taken together, these data indicate that cGAS-STING pathway activation is functionally important for the inflammatory response in *ddx41* mutants.

Ddx41 regulates HSPC number via the cGAS-STING inflammatory pathway

Based on our finding that Ddx41 regulates NF κ B levels via the cGAS-STING axis, we tested the role of these signaling components on the HSPC expansion in *ddx41* mutants (Figure 5A). Lowering cGAS and STING levels using numerous genetic approaches significantly reduced HSPC numbers in *ddx41*

Stimulation of the cGAS-STING pathway ultimately results in activation of the transcription factors IRF3/7 (interferon response factors 3 and 7) and NF κ B. As NF κ B is well established as a key inflammatory-stimulated transcription factor in regulating embryonic HSPC formation (Espin-Palazon et al., 2018), we examined the activation of this pathway. We measured activity using an NF κ B activity fluorescent reporter zebrafish that expresses GFP under the transcriptional control of a NF κ B-responsive promoter (*nfkb:gfp*) (Kanthar et al., 2011). We quantified the absolute number of cells with high *nfkb:gfp* reporter activity using

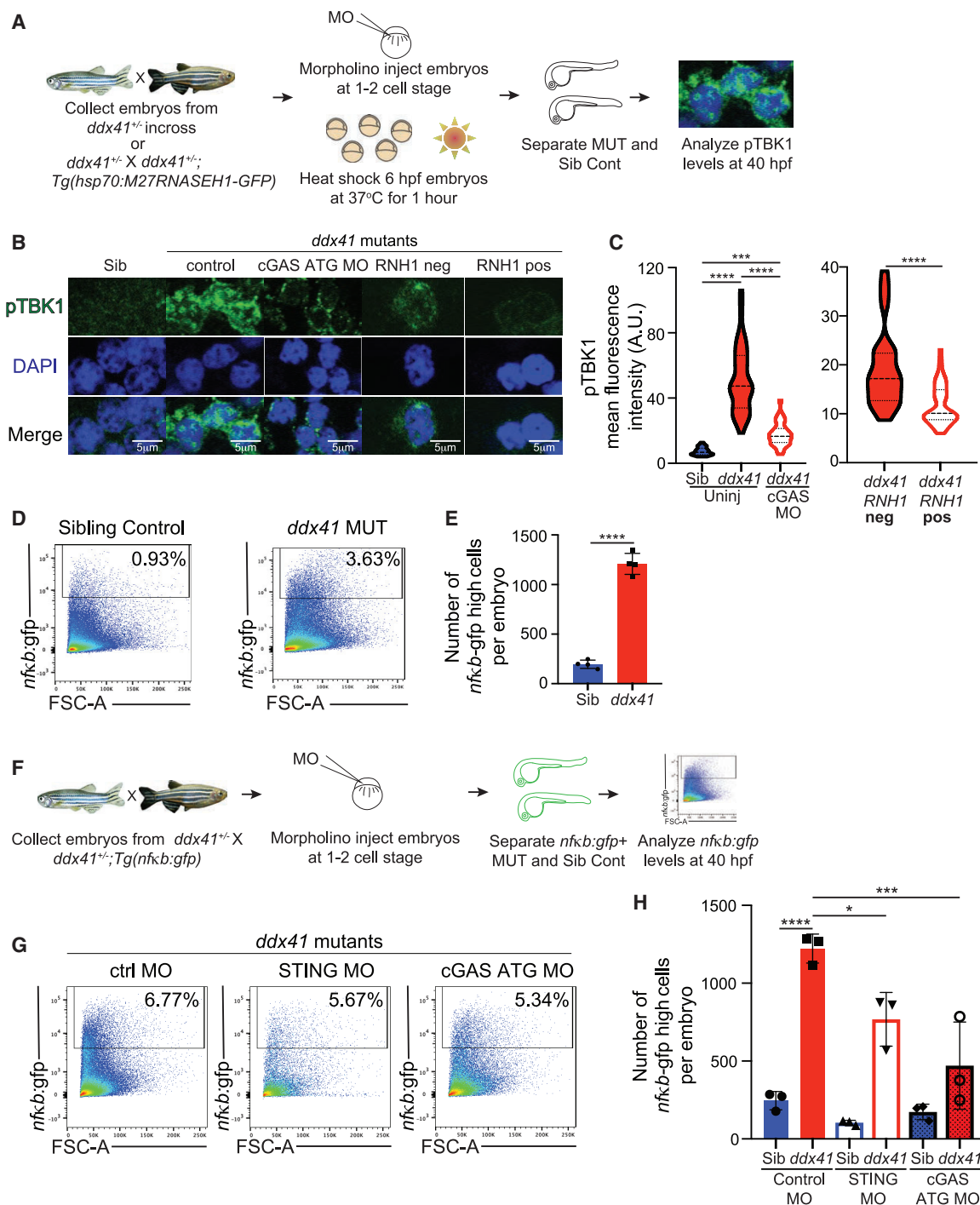


Figure 4. Ddx41 regulates inflammatory signaling via the cGAS-STING pathway

(A) Schematic of the Ser172-phosphorylated TBK1 (pTBK1) immunofluorescence experiments.

(B) Confocal images showing immunofluorescence of pTBK1 and nuclei (DAPI) in cells isolated from 40 hpf siblings, *ddx41* mutants (control), *ddx41* mutants injected with cGAS translation-blocking (ATG) MO, RNH1-negative *ddx41* mutants and RNH1-positive *ddx41* mutants. Scale bar, 5 μ m.

(C) Quantification of pTBK1 levels from (B).

(D) Flow cytometry plots of *NFκB:gfp*⁺ cells from sibling controls (left) and *ddx41* mutants (right) at 40 hpf.

(E) Graph depicting the absolute number of *NFκB:gfp*-high cells per embryo shown in (D).

(F) Schematic of the cGAS-STING pathway knockdown experiments with *NFκB:gfp* quantification.

(G) Flow cytometry plots of *NFκB:gfp*⁺ cells from *ddx41* mutants injected with control morpholino (left), splice-blocking *sting* morpholino (middle), or *cgas* ATG morpholino (right) at 40 hpf.

(H) Graph depicting the absolute number of *NFκB:gfp*-high cells per embryo shown in (G). Graphs display means $\pm$ stds with p values calculated with unpaired Student's t test (E) or a one-way ANOVA with Tukey's multiple testing correction (C and H), *p < 0.05, ***p < 0.001, ****p < 0.0001. N = 3–5 replicates per experiment.

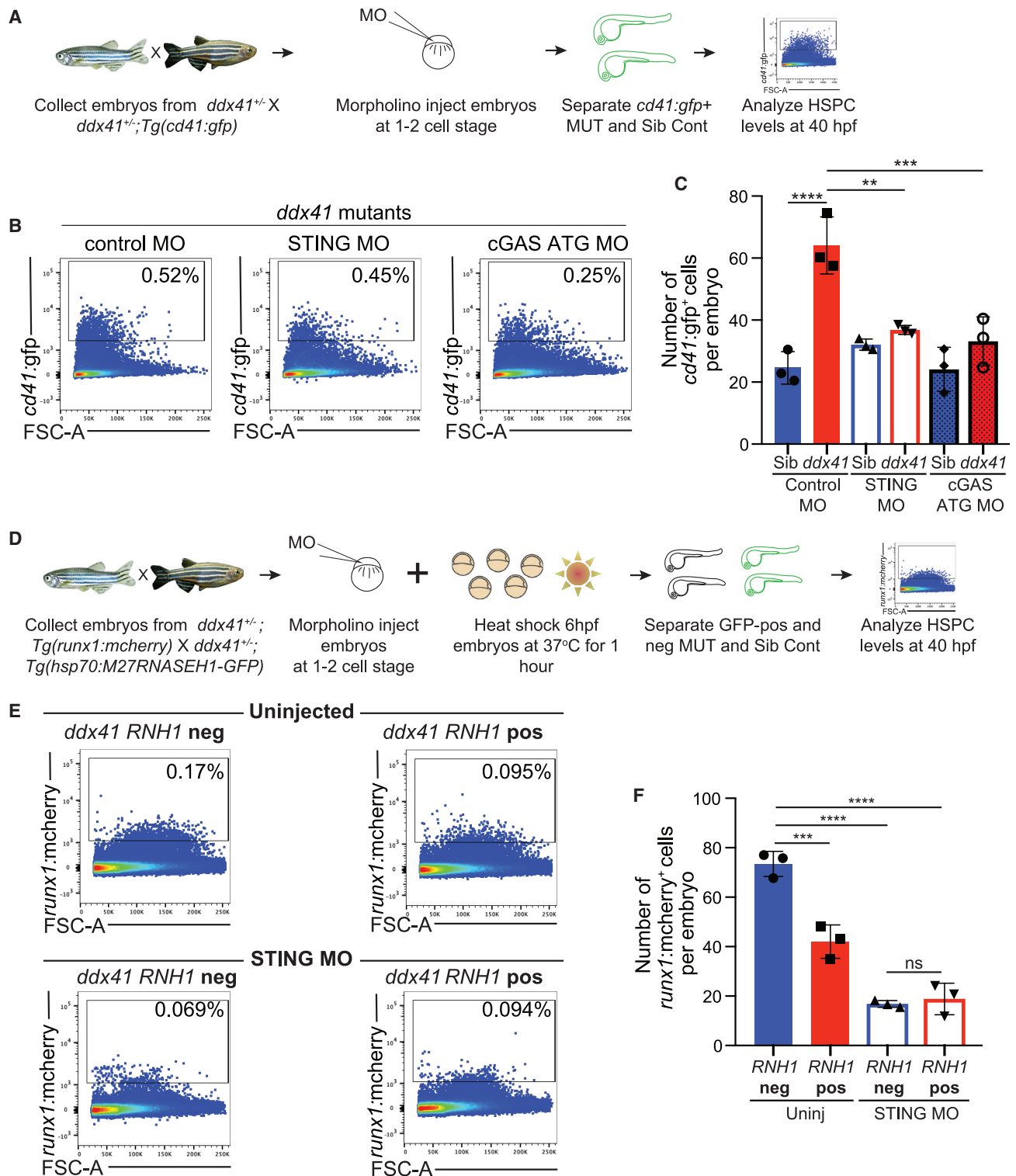


Figure 5. Ddx41 regulates HSPC number via the cGAS-STING inflammatory pathway

(A) Schematic of the cGAS-STING pathway knockdown experiments with HSPC quantification.

(B) Flow cytometry plots of *cd41:gfp*⁺ HSPCs from *ddx41* mutants injected with control morpholino (left), splice-blocking *sting* morpholino (middle), or translation-blocking (ATG) *cgas* morpholino (right) at 40 hpf.

(C) Graph depicting the absolute number of *cd41:gfp*⁺ cells per embryo shown in (B).

(D) Schematic of the STING knockdown and RNASEH1 overexpression experiment with HSPC quantification.

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mutants, as measured by quantification of *cd41:gfp*⁺ HSPC numbers per embryo using flow cytometry and *runx1* *in situ* hybridization (Figures 5B, 5C, and S5). These results indicate that the cGAS-STING inflammatory cascade promotes HSPC production in *ddx41* mutants.

Next, we determined if the increased HSPC formation from excessive R-loop levels was dependent or independent of the cGAS-STING signaling pathway. To do so, we assessed the impact of combined RNASEH1-GFP overexpression and STING knockdown on HSPC numbers in *ddx41* mutants (Figure 5D). If excess R-loops and STING are working via independent or parallel pathways, then we would expect an additive effect with further HSPC reduction compared with each manipulation alone. If they are working via a single pathway, then we would not see an additive suppression on HSPC expansion. We observed no difference between HSPC number in 40 hpf *ddx41* mutants with STING knockdown alone or in combination with RNASEH1-GFP overexpression (Figures 5E and 5F). These data support a single pathway with R-loops impacting HSPCs via a STING-mediated pathway.

Human cells with low *DDX41* have elevated inflammatory gene expression and R-loop levels

To test if the molecular features identified in zebrafish developmental HSPCs could be conserved in human cells, we examined clinical symptoms, gene expression, and R-loop levels in low versus high *DDX41* cells. We determined whether different *DDX41* expression levels were associated with different clinical outcomes. We compared blood counts and bone marrow blast levels in a cohort of 183 MDS patients (Pellagatti et al., 2010). The patients were stratified based on *DDX41* expression in their CD34⁺ HSPC bone marrow cells, comparing hematopoietic parameters in low- (bottom 20th percentile) versus high- (top 20th percentile) expressing patients (Figure S6A). Reduced white blood cell, neutrophil, and platelet counts were seen in MDS cases with lower levels of *DDX41* (Figures S6B–S6D). Significantly higher bone marrow blast counts were found in patients with lower *DDX41* levels (Figure 6A). Patients with low *DDX41* expression had inferior overall survival rates compared with patients with high levels of *DDX41* (Figure 6B). These data are suggestive that lower *DDX41* expression in HSPCs could contribute to hematologic changes in MDS.

Using this same patient dataset, we determined if lower *DDX41* levels in human CD34⁺ HSPCs are concordant with elevated inflammatory gene expression. We defined the genes with elevated expression in *DDX41* low versus high samples (Table S5), and then performed gene-set comparison with REACTOME pathways. Similar to our findings in *ddx41* mutant zebrafish, we observed that immune system pathways were also enriched (Figure 6C). We then took the genes that were highly expressed in *DDX41* low MDS samples and within the “Innate immune system” pathway and assessed how their expression correlated with blast counts in the cohort of 183 MDS patients

(Figure 6D). We observed a strong correlation between expression of these genes and high bone marrow blast counts (Figure 6E). These data support that low expression of *DDX41* in human CD34⁺ HSPCs leads to increased inflammatory signaling that correlates with increased blast levels.

Finally, we assessed if *DDX41* hypomorphic mutations could modulate R-loop levels. We focused on the *DDX41*^{R525H} mutant, which is a common hotspot somatic mutation that is thought to result in diminished helicase activity (Yoneyama-Hirozane et al., 2017; Polprasert et al., 2015). We analyzed R-loop levels in HEK-293 cells overexpressing wild-type *DDX41* (control) or mutant *DDX41*^{R525H}. We showed that R-loop levels were significantly increased in *DDX41*^{R525H}-overexpressing cells compared with *DDX41*^{wildtype}-overexpressing cells (Figures 6F and 6G). These data demonstrate that similar to our finding in zebrafish, human *DDX41* suppresses R-loop accumulation.

DISCUSSION

Here, we unveil a critical role for *Ddx41* as a key regulator of HSPC homeostasis via its role as a gatekeeper of inflammatory signaling. We demonstrated that R-loop dysregulation triggered by *ddx41* deficiency stimulates the cGAS-STING inflammatory cascade. This, in turn, increased the HSPC production during development by promoting EHT events. These results indicate that tight regulation of R-loop levels is critical for limiting HSPC numbers.

Inflammatory signaling, mainly via NFκB, is well established to play a critical role in HSPC emergence (Espin-Palazon et al., 2018). We determined that cGAS-STING is a new signaling pathway that feeds into NFκB to control HSPC production during embryonic hematopoiesis. Prior work showed that sterile inflammation drives NFκB-mediated inflammatory effects on HSPC formation (He et al., 2015; Espin-Palazón et al., 2014; Li et al., 2014; Sawamiphak et al., 2014). However, the upstream triggers of such signaling remain unclear. Our work shows that endogenous signals, such as excessive R-loops, can function as innate damage-associated molecular patterns (DAMPs) during development to alter HSPC formation. Repetitive elements and metabolic activity were recently identified as additional endogenous cues that regulate HSPC formation via sterile inflammatory signaling (Frame et al., 2020; Lefkopoulos et al., 2020). These findings open new avenues for considering the role for other naturally occurring internal inflammatory triggers as native constituents of the HSPC formation microenvironment.

Previous work demonstrated that *DDX41* can act as a sensor for cytoplasmic DNA and bacterial cyclic di-GMP/AMP activating a type I IFN immune response (Zhang et al., 2013; Parvatiyar et al., 2012; Zhang et al., 2011). Out of context, these data are in apparent contradiction to our findings that deficiency of *ddx41* results in increased type I IFN signaling. Previous studies were mainly performed in myeloid dendritic cells or monocytes, which may reflect how *DDX41* functions in immune effector cells.

(E) Flow cytometry plots of *runx1:mcherry*⁺ HSPCs from *Tg(hsp70:M27RNASEH1-GFP)*-negative (top left) and *Tg(hsp70:M27RNASEH1-GFP)*-positive (top right) uninjected *ddx41* mutants versus *Tg(hsp70:M27RNASEH1-GFP)*-negative (bottom left) and *Tg(hsp70:M27RNASEH1-GFP)*-positive (bottom right) STING morpholino-injected *ddx41* mutants at 40 hpf.

(F) Graph depicting the number of *runx1:mcherry*⁺ HSPCs per embryo from (E). Graphs display means ± stds with p values calculated with a one-way ANOVA with Tukey's multiple testing correction, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns, not significant (p > 0.05). N = 3–6 replicates per experiment.

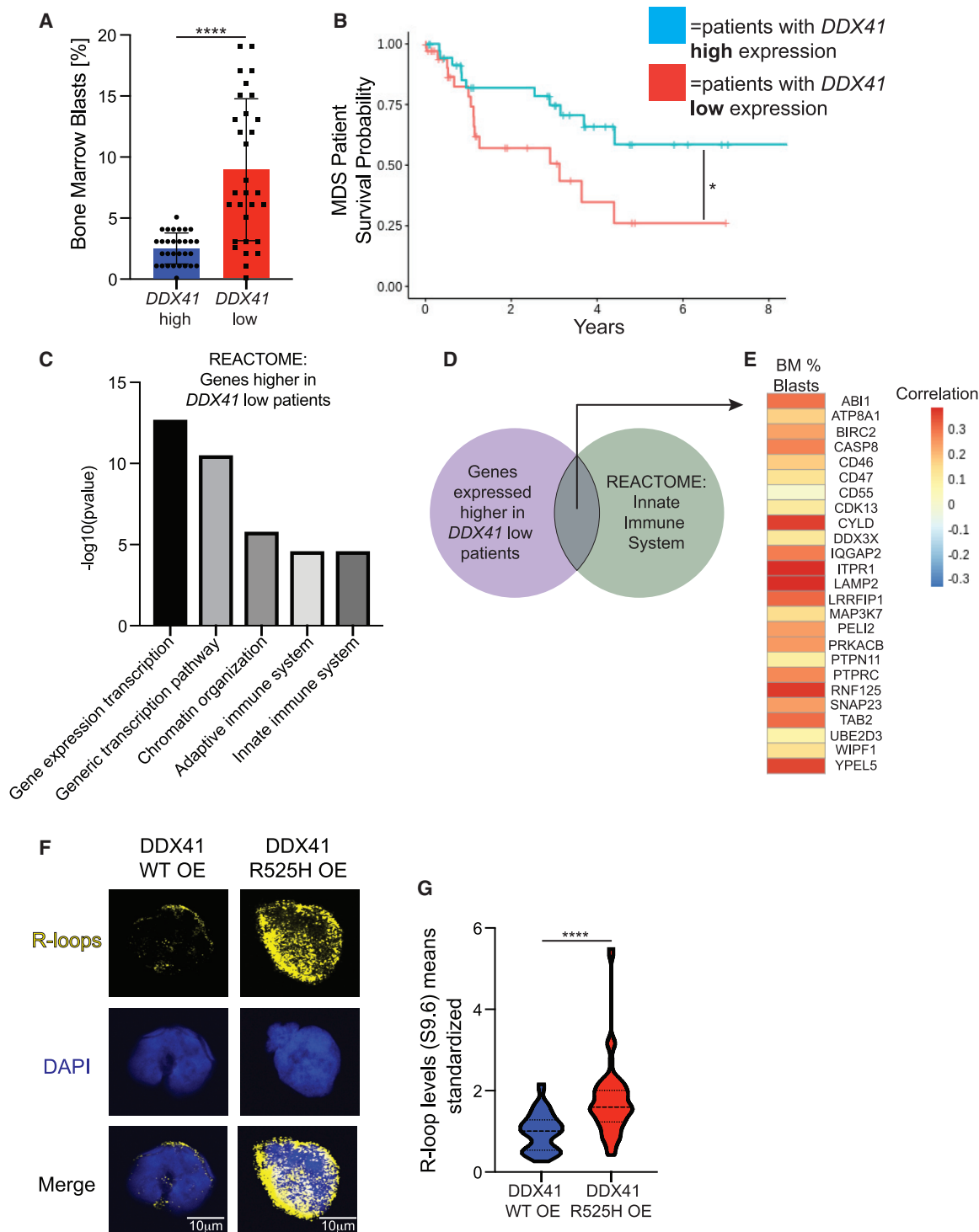


Figure 6. Conserved properties in *DDX41* low and mutated human cells

(A) Graph depicting bone marrow blast percentages of MDS patients with high (top 20%) or low (bottom 20%) *DDX41* expression.

(B) Kaplan-Meier survival curve comparing MDS patients with high or low *DDX41* expression.

(C) Representative charts of REACTOME pathways significantly enriched in *DDX41* low patients' CD34⁺ HSPCs compared with *DDX41* high patients' CD34⁺ HSPCs.

(D) Venn diagram showing overlap between genes that were highly expressed in *DDX41* low MDS samples and within the "Innate immune system" pathway and assessed for how their expression correlated with blast counts in the cohort of 183 MDS patients in (E).

(legend continued on next page)

DDX41 is ubiquitously expressed (Fagerberg et al., 2014). Zebrafish *ddx41* mutants have the loss-of-function mutation in all cells, opening the possibility of a different role for DDX41 in non-immune cell types, such as HSPCs. Another contributing factor to the differences between the prior immune signaling studies and our work is the pleiotropic functions of DDX41. Although numerous cellular roles of DDX41 are described, understanding how these processes interact and influence one another is still in its infancy. Our data indicate at least one example of how DDX41 regulation of R-loops and inflammatory signaling are intertwined.

Similarly, the function of cGAS-STING signaling has been studied mostly in immune effector cells where it induces anti-pathogen or anti-tumor immunity (Hoong et al., 2020; Devaux et al., 2018). More recently, activation of cGAS-STING has been associated with promoting stemness in CD8⁺ T cells, indicating the involvement of this pathway in cell-fate decisions (Li et al., 2020). In adult murine HSPCs, bacterial bis-(3′5′)-cyclic dimeric guanosine monophosphate-stimulated STING activation can promote proliferation of multipotent progenitors, while transiently suppressing long-term HSC function (Kobayashi et al., 2015). Of note, stimulation with cGAMP, the second messenger generated by cGAS, led to a different response. Our data suggest a stemness-promoting effect of intrinsic cGAS activation during HSPC production. Taken together, these findings indicate that there are contextual and cell-type-specific responses to STING pathway activation, implying greater complexity in STING-mediated effects than was previously appreciated.

Several groups showed that excessive R-loops promote DNA damage (Crossley et al., 2019). Due to this effect, R-loop imbalance is often associated with genomic instability in cancers. Mutations in R-loop-regulating factors are also found in inherited autoimmune disorders (Günther et al., 2015; Lim et al., 2015). Though this clinical linkage suggests R-loop imbalance could derail normal inflammatory signaling, our work provides *in vivo* and mechanistic evidence of this occurring. That said, our findings do not preclude genomic instability as another contributor to the effects of R-loops on HSPC production. Further exploration of the connections of DNA damage and inflammatory signaling in HSPC biology is needed.

DDX41 also interacts with multiple components of the spliceosome (Polprasert et al., 2015). Our work aligns with prior studies showing that *DDX41* insufficiency is associated with numerous deleterious splicing outcomes. If, and how, these splicing events contribute to hematopoietic defects is uncertain. We show that immune signaling components are commonly mis-spliced in *ddx41* mutants. Therefore, although we demonstrated a clear impact of R-loops on inflammatory signaling in *ddx41* mutants, our data do not preclude the possibility that splicing alterations could also contribute to perturbed inflammatory signaling and HSPC expansion. Mutations or deficiency of splicing factors,

similar to our results with *ddx41* deficits, cause R-loop imbalance in addition to splicing alterations (Sollier and Cimprich, 2015). The crosstalk of pre-mRNA processing and R-loop regulation is a major outstanding question in the field that remains to be addressed.

In summary, our study uncovers an important link between R-loops, inflammation, and the developing hematopoietic system. We demonstrate that the loss of *ddx41* leads to elevated R-loop levels, which trigger an inflammatory response contributing to increased HSPC production. This mechanistic connection between R-loops and inflammation gives us a greater understanding of HSPC development. Additionally, the conservation of mechanism observed in human cells with diminished DDX41 suggests our findings could have potential implications for HSPC-driven hematologic malignancies, such as MDS.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
 - Lead contact
 - Materials availability
 - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
 - Zebrafish
 - Cell lines
- METHOD DETAILS
 - Zebrafish embryo morpholino and construct injections
 - Whole-mount *in situ* hybridization
 - Flow cytometry
 - Cell cycle analysis
 - Single-cell immunofluorescence
 - Human cells
 - Fluorescent microscopy and time-lapse imaging
 - Image acquisition and processing
 - RNA-sequencing and splicing analysis
 - Reverse transcription-PCR
 - Western analysis
 - Patient database and survival data
- QUANTIFICATION AND STATISTICAL ANALYSIS
 - Statistics

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.devcel.2021.02.006>.

(E) Heatmap correlating clinical hematological variables with the genes in the REACTOME “innate immune signature” pathway from *DDX41* low patients’ CD34⁺ HSPCs (D). Each cell represents the correlation between the 183 values of the clinical variable (column), and the 183 values of the gene probe (row) matched by patient.

(F) Confocal images showing immunofluorescence of R-loops and nuclei (DAPI) in HEK-293 cells that are overexpressing DDX41 wildtype (left) or a DDX41 R525H mutant (right). Scale bar, 10 μ m.

(G) Quantification of R-loop levels from (F). Graphs display means $\pm$ stds with p values calculated with unpaired Student’s t test, *p < 0.05, ****p < 0.0001.

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AUTHOR CONTRIBUTIONS

J.T.W., A.V., and T.V.B. designed the project experimental approach. J.T.W., N.G., K.S.P., B.T., and S.D.O. performed the experiments. K.P. and V.G. performed bioinformatics analysis. N.J.D. and R.A.P. shared critical reagents. J.T.W. and T.V.B. analyzed the data. J.T.W. and T.V.B. wrote the manuscript; all authors reviewed and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L) for IF	Fisher	Cat# A-10667; RRID: AB_2534057
goat anti-mouse AlexaFluor-488 IgG2a secondary antibody for IF	Thermo Fisher Scientific	Cat# A-21135, RRID: AB_2535774
monoclonal S9.6 primary antibody for IF	This paper	N/A
Phospho-TBK1/NAK (Ser172) (D52C2) XP®Rabbit mAb (Alexa Fluor®647Conjugate) for western and IF	Cell Signaling Technology	Cat# 14590, RRID: AB_2798527
TBK1/NAK (D1B4) Rabbit mAb for western	Cell Signaling Technology	Cat# 3504T
Anti-GAPDH antibody [6C5] (ab8245), 100ug for western	Abcam	Cat# ab8245, RRID: AB_2107448
goat anti-mouse IgG-HRP for western	santa-cruz	Cat# sc-2005, RRID: AB_631736
goat anti-rabbit IgG-HRP	santa-cruz	Cat# sc-2004, RRID: AB_631746
rabbit anti-cGAS for IF and western	abcam	Cat# 224144
Critical commercial assays		
Click-IT EdU Flow Cytometry Assay Kit	Life Technologies/ThermoFisher	C10424
Deposited data		
Raw and analyzed data RNA-seq	This paper	GEO: GSE146995
Experimental models: cell lines		
Human HEK-293 cells overexpressing DDX41 constructs	Gift from Richard Padgett	N/A
Experimental models: organisms/strains		
Zebrafish: <i>ddx41</i> sa14887	Zebrafish International Resource Center (ZIRC) as part of the Sanger Institute Zebrafish Mutation Project	ZFIN: ZDB-ALT-130411-3515
Zebrafish: <i>sting</i> mk30	Rajshekar et al., 2018	ZFIN: ZDB-ALT-190912-7
Zebrafish: <i>cd41:gfp</i>	Lin et al., 2005	ZFIN: ZDB-TGCONSTRUCT-190821-1
Zebrafish: <i>kdrl:dsred</i>	Nakayama et al., 2012	ZFIN: ZDB-ALT-130522-1
Zebrafish: <i>runx1:mcherry</i>	Tamplin et al., 2015	ZFIN: ZDB-TGCONSTRUCT-150512-2
Zebrafish: <i>nfkB:egfp</i>	Kanter et al., 2011	ZFIN: ZDB-TGCONSTRUCT-120409-6
Zebrafish: <i>Tg(hsp70:M27RNASEH1-GFP)</i>	Sorrells et al., 2018	ZFIN: ZDB-ALT-150518-2
Oligonucleotides		
Primer for genotyping zebrafish <i>ddx41</i> _WT_Forward: GAGGCCAAGATGGTTTAT	This paper	N/A
Primer for genotyping zebrafish <i>ddx41</i> _WT_Reverse: GCCTCCACACCTTTTAGCAG	This paper	N/A
Primer for genotyping zebrafish <i>ddx41</i> _MUT_Forward: GCCTCCACACCTTTTAGCAG	This paper	N/A
Primer for genotyping zebrafish <i>ddx41</i> _MUT_Reverse: GAGGCCAAGATGGTTTAA	This paper	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Primer for <i>cxc10</i> _qPCR1: TGCAGAAAGAGCAGCAGAAA	This paper	N/A
Primer for <i>cxc10</i> _qPCR1: GGCTGGAAGAAGCTACAACG	This paper	N/A
Primer for <i>ifi44</i> _qpcrF1: TCTCTGAAGAAGCTGGCACA	This paper	N/A
Primer for <i>ifi44</i> _qpcrR1: ACTGGTCCTCCACCAGTTTG	This paper	N/A
Primer for <i>ifi44l</i> _qpcrF1: CGGCAGAAGGAAACAAAGTC	This paper	N/A
Primer for <i>ifi44l</i> _qpcrR1: GCGGATTTTACCGTCACACT	This paper	N/A
Primer for <i>ifn-phi1</i> -F: GTGGCAAGATACGCAAAGCC	This paper	N/A
Primer for <i>ifn-phi1</i> -R: ATTGACCCTTGCGTTGCTTG	This paper	N/A
Primer for <i>bactin</i> -F: GCTGTTTTCCCTCCATTGTT	Burns et al. G&D 2005	N/A
Primer for <i>bactin</i> -R: TCCCATGCCAACCATCACT	Burns et al. G&D 2005	N/A
Primer for <i>sting</i> _RTPCR_Ex1_F: TCTTACTCTGGCCGTTGCTT	This paper	N/A
Primer for <i>sting</i> _RTPCR_Ex3_R: ACCATGAGCCACGTTTCATCT	This paper	N/A
Primer for <i>cgas</i> _RTPCR_Ex2_F: TGCTGACTGTTCCCTGTGGAG	This paper	N/A
Primer for <i>cgas</i> _RTPCR_Ex4_R: GTGTGGCCATGTGATTCAG	This paper	N/A
Primer for <i>tubulin</i> _RTPCR_F: ATGCCTGCTGGGAAGTGTAT	This paper	N/A
Primer for <i>tubulin</i> _RTPCR_R: GGTGGAACAGCTGACGGTAT	This paper	N/A
Morpholino: splice-blocking <i>sting</i> GCCATGATACCTGGA	Gene tools	ZFIN: ZDB-MRPHLNO-140826-4
Morpholino: translation-blocking <i>cgas</i> CTGGTCTCCTGTGGCTGCTCATGAT	Gene tools	ZFIN: ZDB-MRPHLNO-171109-2
Morpholino: splicing-blocking <i>cgas</i> CAGATTGTGGGCATAGTATACCTCT	This paper	N/A
Morpholino: control CCTCTTACCTCAGTTACAATTATA	Gene tools	N/A
Recombinant DNA		
<i>ubi:gfp</i> plasmid	This paper	N/A
<i>fli1:M27RNASEH1-GFP</i> plasmid	This paper	N/A
Software and Algorithms		
FIJI/ImageJ	Dobrzycki et al., 2018	https://imagej.nih.gov/ij/
DE-App	Li and Andrade, 2017	https://yanli.shinyapps.io/DEApp/
MSigDB	Liberzon et al., 2015	https://www.gsea-msigdb.org/gsea/msigdb/index.jsp
rMATS	Shen et al., 2014	http://rnaseq-mats.sourceforge.net/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Teresa Bowman (teresa.bowman@einsteinmed.org).

Materials availability

The *fli1:naseh1-gfp* plasmid generated for this work is availability by request.

Data and code availability

The accession numbers for the RNA-Seq data reported in this paper are under Gene Expression Omnibus (GEO) GSE146995.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Zebrafish

Zebrafish were maintained as described (Lawrence, 2011). All fish were maintained according to IACUC-approved protocols in accordance with Albert Einstein College of Medicine research guidelines. We utilized several established zebrafish lines. Genotyping and phenotyping to confirm their identity was performed for each new generation and for embryos. Mutants for *ddx41* (*ddx41^{sa14887}*) were acquired from the Zebrafish International Resource Center (ZIRC) as part of the Sanger Institute Zebrafish Mutation Project (Kettleborough et al., 2013). The mutation results in a premature stop codon at Tyrosine 410. As early as 24 hpf, there appears to be slight brain death and abnormal curvature of the tail in *ddx41* mutants, allowing for phenotypic identification of mutants compared to siblings based on morphology (Figure S1B). Homozygous mutants are embryonic lethal, dying between 2–3dpf. This allows for the assessment of definitive hematopoietic development in zebrafish up to 3 dpf but precludes the assessment of hematopoiesis beyond this point of development. Mutants for *sting* (*sting^{mk30}*) were acquired from Mary Goll (Rajshekar et al., 2018). The following strains were used: *cd41:gfp* (Lin et al., 2005), *runx1:mcherry* (Tamplin et al., 2015), *kdr:dsred* (Nakayama et al., 2012), *nfkβ:gfp* (Kanter et al., 2011), and *Tg(hsp70:M27RNASEH1-GFP)* (Sorrells et al., 2018). *ddx41* genotyping was determined by mutant-specific PCR, which was initially verified by Sanger sequencing. Additionally, the genotype of *ddx41^{sa14887/+}* carriers were validated by paired mating of heterozygous adult animals followed by examination of embryos for the expected homozygous recessive phenotype. All transgenic strains used in this study were scored for their distinctive fluorescence pattern during embryogenesis. Genomic DNA for genotyping was isolated by alkaline lysis (Meeker et al., 2007). The *ddx41^{sa14887}* mutant and wildtype-specific PCR products were analyzed by gel electrophoresis. Genotyping primers are in the Key resources table.

Cell lines

The parental human embryonic kidney 293 cells (HEK-293) cell line was acquired from ATCC (CRL-1573). HEK-293 cells were grown on 100 mm plates in Dulbecco's Modified Eagle's Medium with 4.5 g/L glucose, l-glutamine, and sodium pyruvate (DMEM, Corning Cellgro #10-013-CV) supplemented with 10% (v/v) fetal bovine serum (Atlanta Biologicals) and 100 U/mL penicillin–streptomycin (Gibco) at 37°C and 5% (v/v) CO₂. The DDX41-V5 lentiviral expression vector (pLX304, clone ID: HsCD00442077) was obtained from DNASU Plasmid Repository. The DDX41^{R525H}-V5 plasmid was generated by introducing a point mutation in the DDX41-V5 vector using QuikChange site directed mutagenesis kit with the following primers (DDX41-R525H-F: CGCACCGGGCACTCGGGAAC and DDX41-R525H-R: GCCAATCCGGTGACATAGTTCTC). DDX41-V5 and DDX41^{R525H}-V5 vectors were used to generate lentiviral supernatants in HEK-293T cells. HEK-293 cells were subjected to 8ug/ul polybrene and viral supernatants for 24hrs followed by antibiotic selection using 10ug/ml of blasticidin to generate stable HEK-293 wildtype DDX41-V5 or mutant DDX41^{R525H}-V5 overexpression cell lines. DDX41 protein levels were validated using V5 (A190-120A, Bethyl) and DDX41 (NB100-57488, Novus Biosciences) antibodies. These constructs are published in (Polprasert et al., 2015).

METHOD DETAILS

Zebrafish embryo morpholino and construct injections

Embryos derived from *ddx41* heterozygous incrosses were injected with 1.5 ng morpholino (MO) and compared with control morpholino injected sibling controls. Morpholino sequences used: control morpholino (5'- CCTCTTACCTCAGTTACAATTATA-3'), splice-blocking *sting* (5'-GCCATGATACCTGGA-3') (van der Vaart et al., 2014), translation-blocking *cgas* (5'-CTGGTCTCCTGTGGCTGCTCATGAT-3') (Ge et al., 2015), and splice-blocking *cgas* (5'- CAGATTGTGGGCATAGTATACCTCT-3'). Translation-blocking morpholino was validated by western blot, and splice-blocking morpholinos were validated by RT-PCR. For construct injections, *tol2* RNA was injected with either 100μg of *ubi:gfp* or 200 μg of *fli1:M27RNASEH1-GFP*. Embryos were scored at ~40 hpf for presence of the transgene according to GFP expression using a Zeiss Stereo Discovery. V8 fluorescent dissecting scope.

Whole-mount *in situ* hybridization

In situ hybridization steps were performed as described previously by Thisse et al. (Thisse and Thisse, 2008), with minor modifications: before proteinase K permeabilization, embryos older than 28 hpf were bleached after re-hydration to remove pigmentation.

The bleaching was done for 5–10 minutes using a bleaching solution of 0.8% KOH, 0.9% H₂O₂ and 0.1% Tween 20. Embryos were then scored manually, imaged and genotyped. The *runx1* (Burns et al., 2002), *flt4* (Lawson et al., 2001), and *notch1b* (Lawson et al., 2001) probes were used, and *runx1* levels were quantified using FIJI in a similar manner to previously described methods (Dobrzycki et al., 2018).

Flow cytometry

At 40 hpf, mutant and sibling embryos were binned based on morphological differences. For generation of single-cell suspensions, 10–20 embryos were first removed from their chorions using pronase (Roche), and then homogenized by manual dissociation using a sterile razor blade followed by digestion with Liberase (Roche). For the digestion, dissociated embryos were resuspended in 600 μ l 1 $\times$ Dulbeccos-PBS (D-PBS) (Life Technologies) supplemented with a 1:65 dilution (9.23 μ l) of 5 mg/ml Liberase and then incubated at 37°C for 7 minutes. The reaction was stopped with the addition of 5% (30 μ l) fetal bovine serum (FBS) (Life Technologies). The cells were then filtered through a 40- μ m cell strainer (Falcon) and pelleted by centrifugation at 3000 rpm for 5 minutes. Cell pellets were resuspended in 400–700 μ l FACS buffer (0.9 $\times$ D-PBS, 5% FBS, 1% Penn/Strep (Life Technologies)). DAPI (4',6-diamidino-2-phenylindole) was added to a final concentration of 1 μ g/ml to facilitate exclusion of dead cells from the analysis. Samples were analyzed with a LSRII flow cytometer (BD Biosciences) and FlowJo software. Cells from non-fluorescent embryos were used to set gates above background. Quantification for the absolute number of cells was performed by acquiring all events in a tube on the flow cytometer to determine the total number of target cells. This number was then divided by the total number of embryos analyzed to calculate the number of target cells per embryo.

Cell cycle analysis

For EdU incorporation experiments, 38 hpf embryos were binned into 6 well plates, with $\sim$ 30 embryos per well. Embryos were incubated with 20mM EdU for 2 hours using the Click-IT EdU Flow Cytometry Assay Kit according to the manufacturer's instructions (Invitrogen C10424). Generation of single-cell suspensions of 40 hpf embryos was accomplished as described above. Samples were analyzed with a LSRII flow cytometer (BD Biosciences) and FlowJo software.

Single-cell immunofluorescence

Zebrafish

Single-cell suspensions were prepared as described above, and cell staining was performed as described in Sorrells & Nik et al. (Sorrells et al., 2018). The mouse monoclonal S9.6 (Boguslawski et al., 1986) primary antibody was used at a dilution of 1:50, the pTBK1 primary antibody (Cell Signaling Technology) was used at a dilution of 1:100, and the cGAS antibody (Abcam) was used at 1:200. All antibodies were diluted in blocking buffer (5% bovine serum albumin (BSA)/0.2% milk/PBS) and incubated for 3 h at room temperature followed by 3 washes in 0.1% Tween 20/PBS. The goat anti-mouse AlexaFluor-488 IgG2a (Thermo Fisher Scientific) and goat anti-mouse AlexaFluor-488 IgG (Fisher) secondary antibody were used at a dilution of 1:1000, while the mouse anti-rabbit AlexaFluor-488 (Thermo Fisher Scientific) secondary antibody was used at a dilution of 1:500; all secondary antibodies were incubated for 1 h at room temperature followed by 3 washes in 0.1% Tween 20/PBS. After final washes, cells were mounted with DAPI-Fluoromount-G (Southern Biotech), covered with a 25 $\times$ 25 mm glass coverslip and sealed with clear nail polish. Fluorescence intensity measurements of S9.6 were performed using FIJI. To quantify nuclear R-loop and cGAS levels, DAPI staining was used to define the nuclear border, and only R-loops or cGAS within the nucleus were measured using Fiji. To quantify non-nuclear R-loop levels, we measured R-loop levels using Fiji that were adjacent to, but not within the DAPI stained regions.

Human cells

HEK-293 cells grown on 25 $\times$ 25 mm glass coverslips were fixed in 4% paraformaldehyde (PFA) for 10 minutes followed by two 5-minute washes in D-PBS. Cells were permeabilized with 0.1% Triton-X in D-PBS for 10 minutes followed by two 10-minute washes in D-PBS. Cells were then blocked in 1% FBS/D-PBS for 1 h. The mouse monoclonal S9.6 (Boguslawski et al., 1986) primary antibody was used at a dilution of 1:100 in blocking buffer for 2 h at room temperature followed by 5 washes in D-PBS for 5 minutes each. The goat anti-mouse AlexaFluor-488 IgG2a was used at a dilution of 1:500 and incubated for 1 h at room temperature followed by 5 washes in D-PBS for 5 minutes each. After final washes, cells were mounted with DAPI-Fluoromount-G, placed on a glass slide and sealed with clear nail polish. Fluorescence intensity measurements of S9.6 were performed using FIJI.

Fluorescent microscopy and time-lapse imaging

Embryos were imaged using a Leica SP5 inverted confocal microscope (Leica). For time-lapse imaging, embryos were anesthetized with tricaine and then embedded in agarose (1% in E3 medium) containing tricaine in a zWEDGI device (Huemer et al., 2017). z-stacks were taken every 20 minutes over a six-hour period. Movies were created using Fiji software.

Image acquisition and processing

Brightfield and fluorescent images were acquired using a Zeiss Stereo Discovery.V8 with an AxioCam HRC camera. Confocal images for zebrafish single-cell immunofluorescence were acquired using a Leica SP5 AOBs Inverted DMI6000 microscope with a 63X oil objective and zoomed in fields were taken at 6.5X zoom. FIJI/ImageJ was used for quantification of images (Collins, 2007). FlowJo,

GraphPad Prism 8, Adobe Photoshop and Adobe Illustrator were used to generate figures. Graphical abstract was created with BioRender.com.

RNA-sequencing and splicing analysis

cd41:gfp⁺ HSPCs from *ddx41* mutants and siblings at 40 hpf were isolated by FACS on a Mo-Flo cell sorter (Beckman Coulter). RNA from these cells was subsequently isolated using the Zymo Quick-RNA microprep kit (R2080) according to the manufacturer specification. The TURBO DNA-free kit (Life Technologies) was used for DNA removal following RNA extraction. RNA and library quality was assessed using a bioanalyzer Pico Chip (Agilent) by the Einstein Genomics core facility. Libraries for RNA samples with RIN ≥ 9 were prepared by BGI using the Nugene low-input RNA sequencing kit. Three independent biological replicates were analyzed. On average, approximately 30 million paired-end 150 base pair sequencing reads were acquired per sample using the Illumina NovoSeq platform. Zebrafish RNA sequencing reads were first trimmed with TRIM GALORE software to check for quality of 150bp reads and then mapped to the *D. rerio* GRCz10 genome build using STAR Aligner (Dobin et al., 2013) (version 2.4.2a). The GRCz10.85 gtf annotation file was used. DE-Seq2 (Love et al., 2014) was used via DEApp (Li and Andrade, 2017) to determine differential gene expression (defined as 2-fold change, FDR $p < 0.05$) between *ddx41* mutant and sibling control HSPCs. Pathway analysis was analyzed using the Molecular Signatures Database (MSigDB) (Liberzon et al., 2015; Subramanian et al., 2005). Differential inclusion rates across 17,040 splicing events between *ddx41* mutants and siblings were calculated using rMATS (Shen et al., 2014) (replicate Multivariate Analysis of Transcript Splicing). Splicing events with inclusion differences $\geq 10\%$ and FDR ≤ 0.01 were considered significantly altered. Pathway analysis for alternatively spliced genes was also performed using MSigDB. RNA-seq data have been deposited at Gene Expression Omnibus (GEO) under GSE146995.

Reverse transcription-PCR

Mutant and sibling embryos were pooled based on morphology. Zebrafish embryo RNA was isolated from pools of 20–40 embryos at the 40 hpf stage using the TRIzol reagent (Thermo Fisher; 15596026) according to the manufacturer's protocol. Embryos were dissociated in TRIzol using a pestle. The High Capacity cDNA reverse transcriptase kit (Thermo Fisher; 4368814) was used to generate cDNA from 300ng input RNA. The cDNA was diluted 1:50 in nuclease-free water. Data were averaged from at least three independent replicates per sample. qPCR primers are in Key resources table. For RT-PCR analysis for morpholino functionality, wild-type embryos were first injected with control, splice-blocking *sting*, or splice-blocking *cgas* morpholinos. At 24 hpf, zebrafish embryo RNA was isolated and used to generate cDNA similar to the method described above. The cDNA was diluted 1:5 in nuclease-free water. The cDNA was then amplified using the RT-PCR primers in Key resources table, and the amplicons examined on an agarose gel to confirm proper exon-skipping induced by the splice-blocking morpholinos.

Western analysis

Embryos were pooled for protein collection on the basis of phenotype. Cell lysates of deyolked embryos were prepared with RIPA buffer (Boston BioProducts). Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad), followed by immunoblotting with primary and secondary antibodies. Primary antibodies were: mouse anti-TBK1 (Cell Signaling Technology, 1:1000), mouse anti-pTBK1 (Cell Signaling Technology, 1:1000), rabbit anti-cGAS (Abcam, 1:700) and rabbit anti-GAPDH (Abcam, 1:10000). Secondary antibodies were goat anti-mouse IgG-HRP (Santa Cruz, 1:2000) and mouse anti-rabbit IgG-HRP (Santa Cruz, 1:2000 for rabbit anti-cGAS, and 1:5000 for rabbit anti-GAPDH). Catalog numbers for all antibodies are listed in the Key resources table.

Patient database and survival data

Gene expression data from 183 MDS CD34⁺ samples and 17 controls were obtained from Gene Expression Omnibus (GEO) (GSE19429) (Pellagatti et al., 2010). Clinical annotations and overall survival were compared to gene expression as performed previously (Schinke et al., 2015). Pathway analysis between *DDX41* low and *DDX41* high patient CD34⁺ cells was performed using MSigDB, with clinical annotation being performed as described above. Each cell represents the correlation between the 183 values of the clinical variable (column), and the 183 values of the gene probe (row) matched by patient.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistics

Experiments were performed with a minimum of three replicates. Statistical analyses were performed as indicated in each figure using unpaired Student's t-test or a one-way ANOVA with Tukey's multiple testing correction as appropriate; error bars indicate the standard deviation of mean, unless otherwise indicated.



Ddx41 inhibition of DNA damage signaling permits erythroid progenitor expansion in zebrafish

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ABSTRACT

DEAD-box Helicase 41 (DDX41) is a recently identified factor mutated in hematologic malignancies whose function in hematopoiesis is unknown. Using an *in vivo* model of Ddx41 deficiency, we unveiled a critical role for this helicase in regulating erythropoiesis. We demonstrated that loss of *ddx41* leads to anemia caused by diminished proliferation and defective differentiation of erythroid progenitors. Mis-expression and alternative splicing of cell cycle genes is rampant in *ddx41* mutant erythroid progenitors. We delineated that the DNA damage response is activated in mutant cells resulting in an Ataxia-telangiectasia mutated (ATM) and Ataxia-telangiectasia and Rad3-related (ATR)-triggered cell cycle arrest. Inhibition of these kinases partially suppressed *ddx41* mutant anemia. These findings establish a critical function for Ddx41 in promoting healthy erythropoiesis via protection from genomic stress and delineate a mechanistic framework to explore a role for ATM and ATR signaling in DDX41-mutant hematopoietic pathologies.

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Introduction

Mutations in *DEAD-box Helicase 41* (DDX41) were identified recently in hematologic malignancies including myelodysplastic syndrome (MDS), acute myeloid leukemia (AML), and acute erythroid leukemia (AEL).^{1,2} Germline *DDX41* frameshift mutations are loss-of-function and somatic missense mutations are thought to result in hypomorphic helicase activity.^{1,3} The human genetics thus suggest that diminished function of this helicase is detrimental to hematopoiesis, but this has yet to be demonstrated in an animal model.

In particular, a significant number of *DDX41*-mutated MDS patients experience mild cytopenia in the years preceding diagnosis, indicating that anemia may be one of the first warning signs of disease.⁴ Anemia in MDS is attributed to numerous cellular mechanisms including erythroid precursor apoptosis, defective progenitor expansion, and ineffective erythrocytic maturation.⁵⁻⁷ The clinical findings suggest DDX41 could be important in erythropoiesis, but the cellular and molecular underpinnings remain unclear. Roles for DDX41 have been implicated in genomic stability, inflammation, and splicing, all processes linked to hematopoietic health, but the current lack of DDX41 mutant animal models has slowed exploration of its function in the blood system.⁸⁻¹¹

In order to uncover the *in vivo* role of DDX41 in erythropoiesis, we established a zebrafish *ddx41* loss-of-function mutant. We demonstrated that *ddx41* mutants develop anemia due to a decrease in erythroid progenitor expansion and defective differentiation. Mechanistically, the erythroid proliferative defect is due in part to ATM- and ATR-mediated cell cycle arrest induced by elevated DNA damage as well as mis-expression and alternative splicing of cell cycle regulators. Our data demonstrate that Ddx41 plays a critical role in hematopoiesis and provide a possible mechanism by which anemia may arise in *DDX41*-mutated hematopoietic pathologies.

Methods

Zebrafish

Zebrafish were maintained as described¹² and according to Institutional Animal Care and Use Committee-approved protocols in accordance with the Albert Einstein College of Medicine research guidelines. Genotyping was performed to confirm animal identity. Mutants for *ddx41* (*ddx41^{sa14887}*) were acquired from the Zebrafish International Resource Center.¹³ The mutation results in a premature stop codon at tyrosine 410. For all experiments, sibling controls are a mix of heterozygotes and wild types. Tg(*gata1:dsred*)¹⁴ transgenics were used. Genotyping details are in the *Online Supplementary Appendix* and the *Online Supplementary Table S7*.

Drug treatments

All drugs were dissolved in dimethyl sulfoxide (DMSO). Dilutions were made in E3 embryo water. KU60019 (ATM inhibitor) and AZ20 (ATR inhibitor) were used with DMSO as the vehicle control.

Whole-mount *in situ* hybridization and o-dianisidine staining

In situ hybridization was performed as previously described.^{15,16} After *in situ*, embryos were scored manually, imaged and genotyped. The *βe3-globin*,¹⁷ *cmyb*,¹⁸ and *gata1*¹⁹ probes were used, and *in situ* levels were quantified using FIJI.²⁰ O-dianisidine staining was performed as previously described.²¹

Flow cytometry

Mutant and sibling embryos were binned based on morphological differences. For generation of single-cell suspensions, 10–20 embryos were processed as previously described¹⁵ (also see the *Online Supplementary Appendix*). Quantification for the absolute number of cells was performed by acquiring all events in a tube on the flow cytometer to determine the total number of target cells. This number was then divided by the total number of embryos analyzed to calculate the number of target cells per embryo.

Cell cycle and apoptosis analyses

For 5-ethynyl-2'-deoxyuridine (EdU) incorporation experiments, embryos were incubated with 20 mM EdU for 2 hours. Single-cell suspensions of embryos were generated. Click-IT EdU Flow Cytometry Assay Kit was used according to the manufacturer's instructions. Flow cytometry analysis for active caspase-3 was performed as previously described.²² Samples were analyzed with a LSRII flow cytometer (BD Biosciences) and FlowJo software.

RNA sequencing and splicing analysis

Erythroid progenitors from *ddx41* mutants and siblings were isolated by fluorescently-activated cell sorting (FACS). RNA from these cells was subsequently isolated, DNase-digested and library prepared for sequencing. Details on library preparation, sequencing and bioinformatic analyses can be found in the *Online Supplementary Appendix*. All data are deposited under GEO accession number GSE160979.

Reverse transcription quantitative polymerase chain reaction

In order to validate the RNA sequencing (RNA-seq) data, we performed reverse transcription quantitative polymerase chain reaction (RT-qPCR). RNA was isolated from 40 hpf embryos. Details are listed in the *Online Supplementary Appendix* and *Online Supplementary Table S7*.

Single-cell immunofluorescence of zebrafish embryonic cells

Single-cell suspensions were prepared, and cell staining was performed as described in Sorrells & Nik *et al.*²² and detailed in the *Online Supplementary Appendix*. Fluorescence intensity measurements of γH2AX were performed using FIJI.

May-Grunwald Giemsa staining of primitive erythroid cells

May-Grunwald Giemsa staining was performed as previously described¹⁵ and as detailed in the *Online Supplementary Appendix*.

Statistics

Experiments were performed with a minimum of three replicates. Statistical analyses were performed as indicated in each figure using unpaired Student's *t*-test or a one-way ANOVA with Tukey's multiple testing correction as appropriate; error bars indicate the standard deviation of mean, unless otherwise indicated.

Results

Ddx41 regulates erythroid development

In order to explore a function for Ddx41 in hematopoiesis, we examined erythrocyte formation and differentiation in zebrafish *ddx41* homozygous loss-of-function mutants (*ddx41^{sa14887}*). Maternally-deposited Ddx41 (*data not shown*) helps the mutants develop and survive until 3 days post fertilization (dpf) (*Online Supplementary Figure S1A and B*). Based on this, we consider the mutants to be functionally hypomorphic with greatly diminished but not completely absent Ddx41 levels. DDX41 is highly conserved between humans and zebrafish suggesting that lessons learned about the *in vivo* role of zebrafish Ddx41 function in hematopoiesis will be relevant to human DDX41. In zebrafish, primitive hematopoiesis begins ~12–24 hours post fertilization (hpf), producing embryonic erythrocytes and myeloid cells that constitute the hematopoietic system early on in development.^{23,24} Cells of the erythrocytic lineage first arise from the intermediate cell mass (ICM) within the posterior lateral mesoderm (PLM) (Figure 1A). These erythrocytes express factors such as the progenitor transcription factor *c-myb* and the erythroid-specific transcription factor *gata1* starting during somitogenesis.^{19,25} Using *in situ* hybridization, we determined that these erythroid progenitor markers were expressed similarly in *ddx41* mutants compared to siblings (mix of *ddx41* heterozygotes + wild types) at 22 hpf, indicating initial erythroid specification is unaffected (Figures 1B to E). Oxygenated hemoglobinized erythrocytes are detectable beginning around 36 hpf using o-dianisidine staining.²⁶ In *ddx41* mutants, we observed little o-dianisidine-positive erythroid cells at 40 hpf (Figure 1F). We sorted *gata1:dsRed⁺* erythrocytes at 40 hpf and found that the *ddx41* mutant cells were larger than those from sibling controls (Figure 1G). This size difference could be indicative of delayed erythroid differentiation. As mutants display some developmental delay that becomes more severe as the embryos get older, it is possible that the erythroid delay is a side effect of the general developmental delay. In order to distinguish between these possibilities, we examined erythrocytes in *ddx41* mutants and siblings at 48 hpf. Oxygenated hemoglobin levels remained low in mutants at 48 hpf (Figures 1H to I). In order to assess maturation, we also bled *ddx41* mutants and sibling control embryos at 48 hpf and analyzed the morphology of isolated

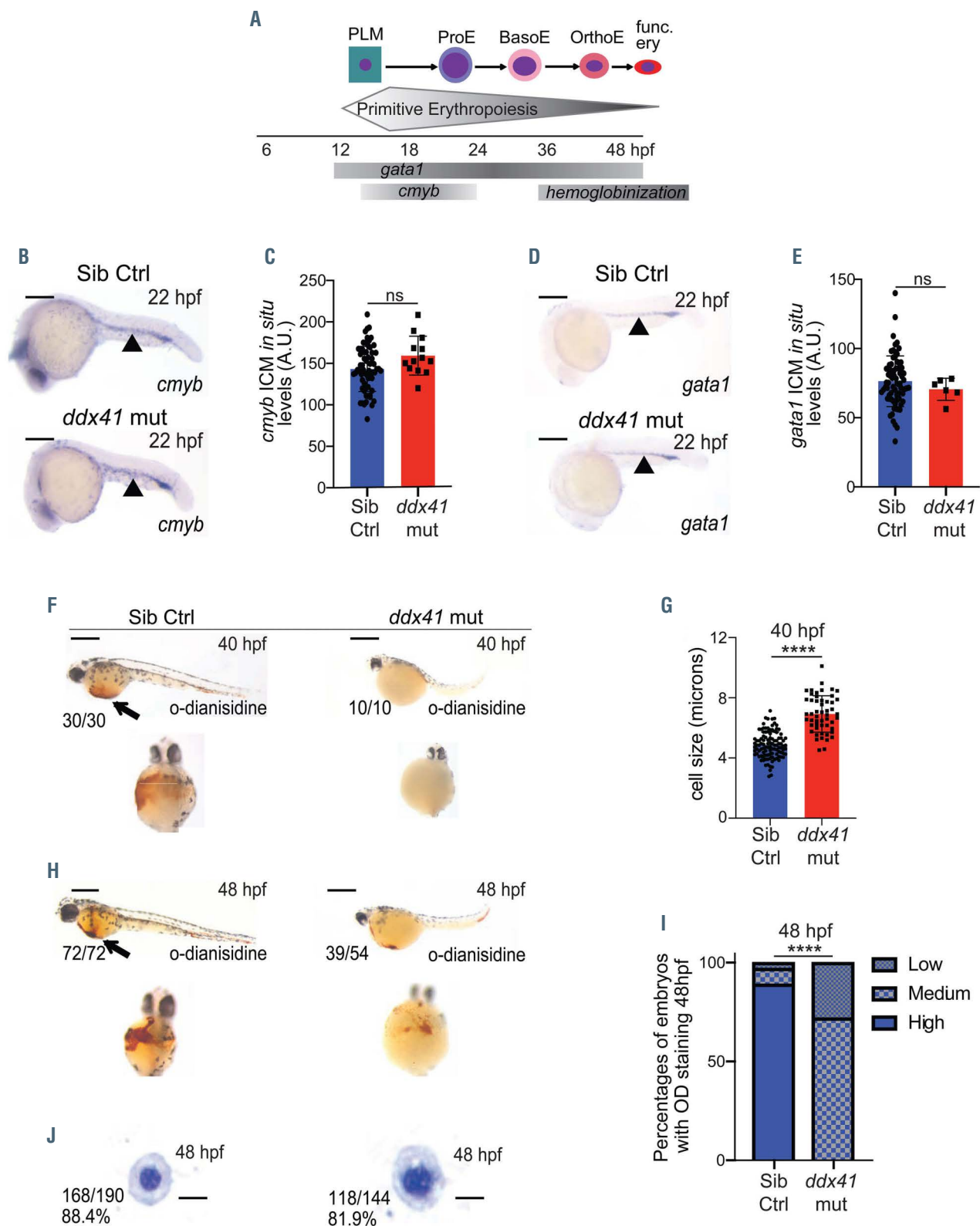


Figure 1. Loss of *ddx41* causes anemia. (A) Schema of primitive erythroid development, PLM: posterior lateral mesoderm; ProE: proerythroblasts; BasoE: basophilic erythroblasts; OrthoE: orthochromatophilic erythroblasts; func. Ery: functional erythrocytes. (B and D) *In situ* hybridization of the erythroid markers *cmyb* (B) (scale bars = 200 μ m) and *gata1* (D) (scale bars = 250 μ m) at 22 hours post fertilization (hpf) in sibling controls (top) and *ddx41* mutants (bottom). Arrowheads highlight the intermediate cell mass (ICM) region in the embryos. (C and E) Quantification of *cmyb* (C) and *gata1* (E) *in situ* hybridization levels from (B) and (D), respectively. Quantification was done using Fiji. (F and H) Staining for o-dianisidine, marking functional hemoglobin in mature primitive erythrocytes, in sibling controls (left) and *ddx41* mutants (right) at 40 hpf (F) (scale bars = 350 μ m) and 48 hpf (H) (scale bars = 400 μ m). Numbers on bottom left corner indicate the fraction of embryos with the same phenotype as the one depicted in the image. (G) Graph depicting size of erythrocytes in sibling controls and *ddx41* mutants at 40 hpf. (I) Graph depicting frequency of designated o-dianisidine staining levels in sibling controls and *ddx41* mutants at 48 hpf. (J) Representative images of orthochromatophilic erythroblasts stained with May-Grunwald-Giemsa from sibling controls (left) and *ddx41* mutants (right) at 48 hpf (scale bars = 5 μ m). Graphs display means $\pm$ standard deviations (stds) with *P*-values calculated with unpaired Student's *t*-test, ns=not significant ($P > 0.05$), **** $P < 0.0001$. For *in situs* and o-dianisidine staining $n=6-72$ embryos per experiment.

erythrocytes using May-Grunwald-Giemsa staining (Figure 1J). The *ddx41* mutant erythrocytes displayed a megaloblastoid-like phenotype, suggesting some abnormalities in erythrocyte maturation.

In order to acquire enough erythrocytes to perform the morphological assessment, we needed to bleed four times as many *ddx41* mutant embryos as compared to sibling control embryos, suggesting mutants had fewer erythrocytes than siblings. In order to test this hypothesis, we assessed the number of *gata1:dsred*⁺ erythroid progenitors in *ddx41* mutants and siblings using flow cytometry quantification. We determined that the absolute number of *gata1:dsred*⁺ erythrocytes per embryo was significantly reduced in *ddx41* mutants compared to siblings at both 28 and 40 hpf (Figures 2A to D). These data indicate that decreased erythrocyte number contributes to the development of anemia in *ddx41* mutants.

Erythroid progenitors arising from both primitive and definitive erythroid-myeloid progenitor (EMP)-derived waves are present during the developmental time points analyzed. The gene programs for the specification and differentiation of primitive and EMP-derived erythropoiesis are highly similar, but the developmental timings are distinct (Figure 2E). EMP specification begins around 26 hpf.²⁷ In order to determine whether there were defects in EMP-derived erythropoiesis, we performed *in situ* hybridization for the progenitor marker *c-myb* at 26 hpf and 36 hpf and *gata1* at 26 hpf in siblings and *ddx41* mutants (Figures 2F and G; *Online Supplementary Figure S1C to F*). Expression of both *c-myb* and *gata1* within the posterior blood island (PBI) region where EMP form were not decreased and in fact were increased in *ddx41* mutants as compared to siblings. As the gene programs are highly similar between these two waves of erythroid development, these data indicate that the reduction in erythrocytes in *ddx41* mutants is occurring at an erythroid progenitor stage after *c-myb* and *gata1* are both expressed, which is shortly after erythroid lineage specification.

In order to further characterize the maturation state of the erythrocytes at 40 hpf in *ddx41* mutants and siblings, we performed RT-qPCR for embryonic and larval globins. Expression of the embryonic globins $\alpha\epsilon 1$, $\alpha\epsilon 3$, $\beta\epsilon 1$, and $\beta\epsilon 3$ begins during somitogenesis with expression of all of these globins except $\beta\epsilon 3$ persisting in primitive and EMP-derived erythrocytes throughout larval development.²⁸ In contrast, levels of $\beta\epsilon 3$ globin diminish dramatically from 24–48 hpf, somewhat concomitant with the increasing expression of the larval $\beta\epsilon 2$ globin. The other larval globin $\alpha\epsilon 5$ is not expressed significantly until 14 dpf. In *ddx41* mutants, we determined that while the levels of the embryonic/larval globins $\alpha\epsilon 1$ and $\beta\epsilon 1$ were diminished, the levels of the embryonic-restricted $\beta\epsilon 3$ globin remained high, consistent with a maturational defect in primitive erythrocytes. Additionally, expression of the larval $\beta\epsilon 2$ globin was lower in mutants compared to sibling controls. Although *ddx41* mutants die before there are expansive numbers of maturing erythrocytes derived from EMP, these data indicate that mutants have fewer definitive erythrocytes compared to siblings. This finding suggests that similar to primitive erythroid progenitors, EMP are specified normally, but there is a later stage defect, although the underlying cause (e.g., diminished expansion, maturation or differentiation) cannot be deciphered. Together, our findings establish that Ddx41 is critical for erythrocyte expansion and maturation.

Cell cycle genes are mis-expressed and alternatively spliced in *ddx41* mutant erythroid progenitors

In order to mechanistically assess the underlying cause of the erythrocytic defect in *ddx41* mutants, we conducted RNA-seq on *gata1:dsred*⁺ erythrocytes isolated from *ddx41* mutants and siblings at 40 hpf. Over 1,800 genes were downregulated and more than 1,900 were upregulated in *ddx41* mutants compared to siblings (Figure 3A; *Online Supplementary Table S1*, log₂ fold-change ≥ 1 , adjusted *P*-value < 0.05). In order to understand if particular pathways were enriched in the differentially expressed genes, we performed gene set level analysis on the upregulated and downregulated gene lists by comparing each to the Molecular Signature Database (MSigDb), a platform that computes overlaps between classes of genes that are over- or underrepresented in lists of genes in known pathways.^{29,30} In the downregulated gene list, mRNA splicing was the top gene set with DNA replication, cell cycle, and DNA repair also enriched (Figure 3B; *Online Supplementary Table S2*). In the upregulated gene list, genes associated with adaptive immunity, posttranslational modifications, innate immune system, and cell cycle were enriched (Figure 3C; *Online Supplementary Table S3*). We validated the expression changes in several cell cycle and DNA-damage-associated genes using RT-qPCR (Figure 3D).

Ddx41 interacts with components of the spliceosome.¹ Additionally, the top downregulated pathway in our gene set was pre-mRNA splicing, thus we examined how *ddx41* loss affected mRNA splicing in erythrocytes. When comparing splicing between *ddx41* mutants and siblings, a total of 370 alternative splicing events were observed (Figures 3E; *Online Supplementary Table S4*). The specific splicing defects detected included exon skipping (SE), which was the most frequently altered splicing event, intron retention (RI), alternative 5'-splice site usage, alternative 3'-splice site usage, and changes in mutually exclusive exon usage. Alternative splicing within protein coding regions of a transcript can result in the introduction of premature termination codon (PTC) or generation of a novel peptide. For all SE and RI events (comprising nearly 85% of all splicing changes), we determined how the alternative splicing event might alter the protein sequence (Figure 3F; *Online Supplementary Table S5*). More than 50% of SE events altered the protein sequence and are predicted to generate novel peptides. Approximately 43% of SE and 90% of RI events are predicted to target the alternatively spliced transcript for nonsense-mediated decay (NMD) due to the introduction of a PTC. For example, the retained intron variant for homologous repair-associated factor structural maintenance of chromosome 5 (*smc5*) identified in *ddx41* mutants is predicted to result in NMD that could result in elevated DNA damage (Figure 3G). Another example of an NMD isoform expressed in *ddx41* mutant is the exon 3 skipped isoform of signal transducer and activator of transcription 1a (*stat1a*) that would diminish signaling by numerous cytokine pathways. Pathway analysis of these alternatively spliced factors revealed that those resulting in novel peptide sequences are enriched in mRNA metabolism, morphogenesis, and cell cycle, and those predicted to result in NMD are enriched for mRNA processing, DNA replication, and gene expression (Figure 3H; *Online Supplementary Table S6*). These results depict that Ddx41 influences the expression and splicing of cell cycle, DNA repair, and mRNA processing genes in erythrocytes.

Ddx41 deficiency triggers cell cycle arrest in erythroid progenitors

The diminished number of erythroid progenitors and dysregulated expression of cell cycle genes suggest that defects in erythrocyte proliferation could contribute to

the anemia in *ddx41* mutants. In order to examine proliferation, we analyzed cell cycle status of 30 hpf *gata1:dsred*⁺ erythroid progenitors by flow cytometry quantification of DNA synthesis via EdU incorporation and DNA content via DAPI incorporation. The *ddx41*

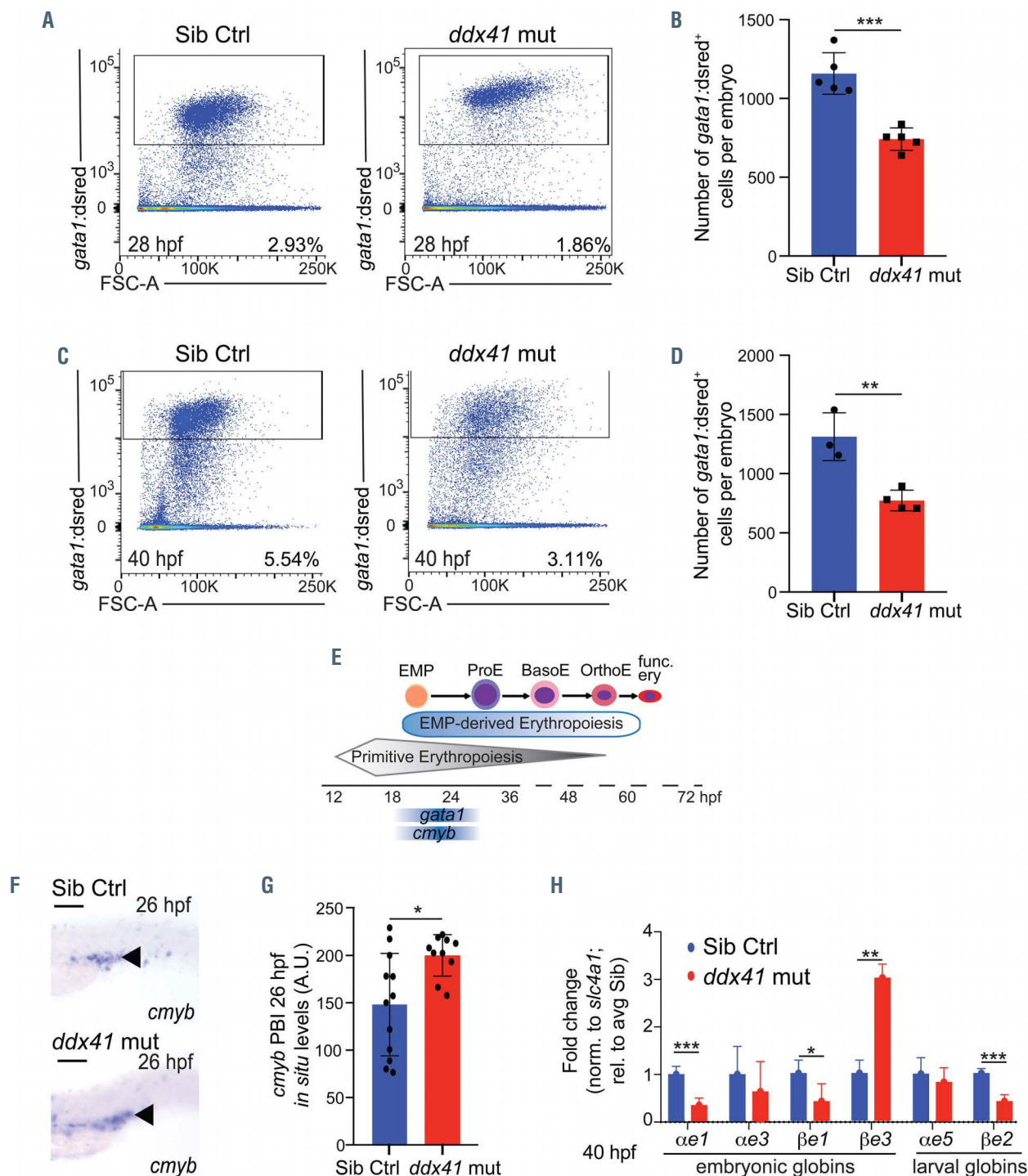


Figure 2. Ddx41 regulates erythroid progenitor numbers. (A and C) Flow cytometry plots of *gata1:dsred*⁺ erythroid cells from sibling controls (left) and *ddx41* mutants (right) at 28 days post fertilization (dpf) (A) and 40 hpf (C). (B and D) Graphs depicting the absolute number of *gata1:dsred*⁺ erythroid cells per embryo from (A) and (C), respectively. n=5 pools of ~5-20 embryos per pool. (E) Schema of erythroid-myeloid progenitor (EMP) development. ProE: proerythroblasts; BasoE: basophilic erythroblasts; OrthoE: orthochromatophilic erythroblasts; func. Ery: functional erythrocytes. (F) *In situ* hybridization of *cmyb* at 26 hpf in sibling controls (left) and *ddx41* mutants (right) (scale bars=150 μm). (G) Quantification of *cmyb* PBI *in situ* hybridization levels from (F). Quantification was done using Fiji; n=10-12 embryos. Graphs display means ± standard deviations (stds). (H) Graph of reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis of the expression of globin genes between sibling controls and *ddx41* mutants. Expression levels were normalized to *slc4a1* levels. Graph displays means ± standard error mean. The P-values were calculated with an unpaired t-test, *P<0.05, **P≤0.01, ***P≤0.001; n=3 replicates per genotype.

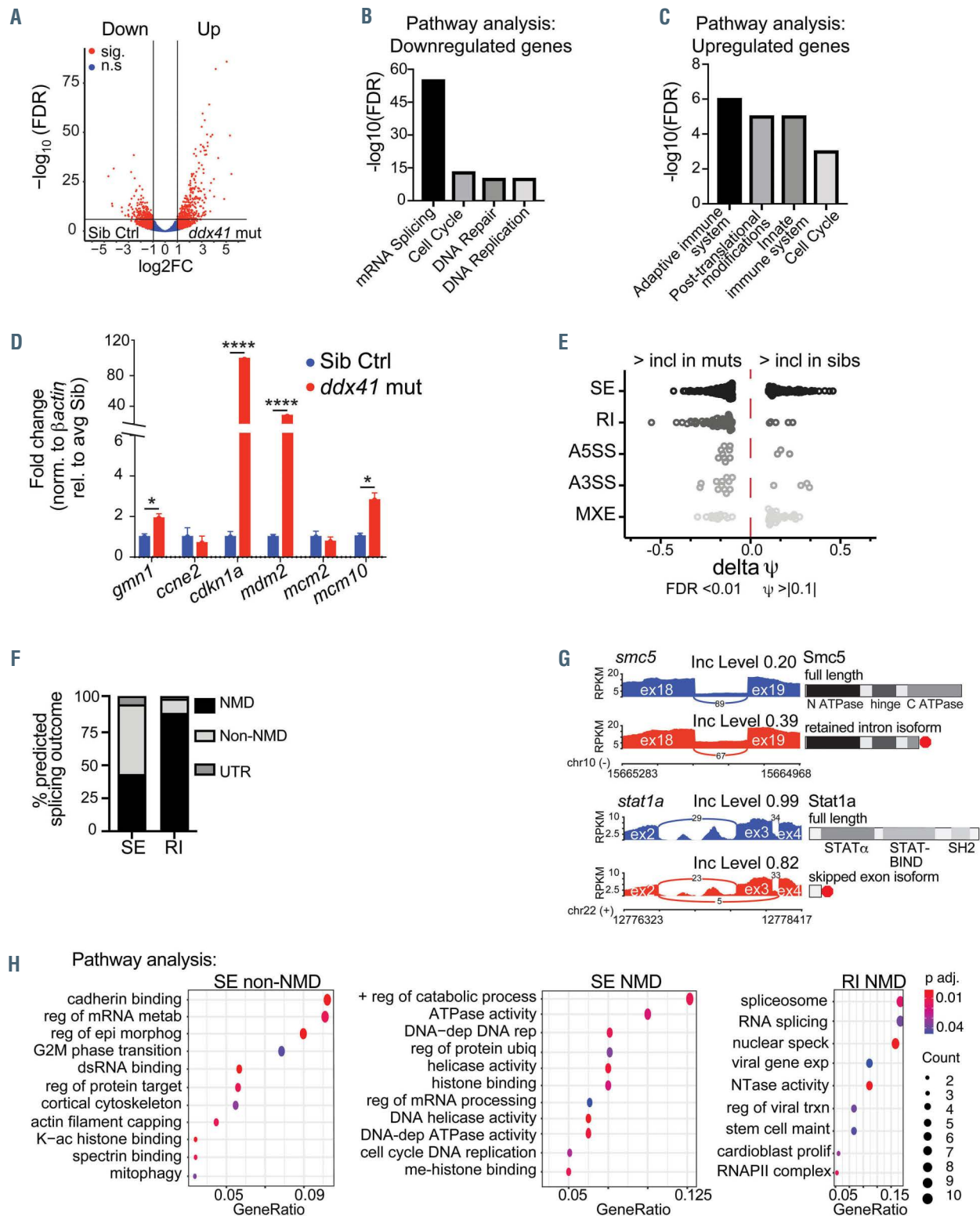


Figure 3. Cell cycle genes are mis-expressed and alternatively spliced in *ddx41* mutant erythroid progenitors. (A) Volcano plot displaying differentially expressed genes between *gata1:dsred⁺* erythrocytes from *ddx41* mutants and siblings. Significant differences are defined as false discovery rate (FDR) < 0.05 and log fold-change ≥ 1 . Black vertical lines denote the fold-change threshold and the black horizontal line denotes the FDR threshold. Five biological replicates for both *ddx41* mutants and siblings were used to generate RNA sequencing data. (B and C). Representative charts of pathways significantly enriched in genes downregulated (B) or upregulated (C) in *ddx41* mutant erythroid progenitors compared to sibling controls as determined by MSigDB analysis. (D) Graph of reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis of the expression of cell cycle and DNA damage-associated genes between sibling controls and *ddx41* mutants. Expression levels were normalized to β -actin levels. Graph displays means $\pm$ standard error mean. The *P*-values were calculated with an unpaired *t*-test, **P* < 0.05, *****P* $\leq$ 0.0001; *n* = 3 replicates per experiment. (E) Graph depicting the $\Delta\psi$ of individual splicing events between sibling controls and *ddx41* mutants as detected by analysis with rMATS. Significant differences are defined as FDR $\leq$ 0.01 and $\Delta\psi \geq 0.1$. SE: skipped exons; RI: retained introns; A5SS: alternative 5' splice site; A3SS: alternative 3' splice site; MXE: mutually exclusive exons. (F) Graph depicting the frequency of alternatively spliced isoforms in *ddx41* mutants that are predicted to result in nonsense-mediated (NMD) decay, protein sequence alterations (non-NMD), or changes in untranslated regions (UTR). (G) Sashimi plot for *smc5* (exons 18-19) and *stat1a* (exons 2-4) in *ddx41* mutant erythrocytes compared to sibling controls. RPKM: reads per kilobase of transcript per million mapped reads; Inc: inclusion. (H) Representative charts of pathways significantly enriched in alternatively spliced genes in *ddx41* mutant erythrocytes compared to sibling controls as determined by pathway analysis.

mutant *gata1:dsred*⁺ progenitors showed a reduction of cells in S phase and an accumulation of cells in the G0/G1 and G2/M phases compared to sibling controls (Figures 4A and B). These results are in-line with a decrease in proliferation in *ddx41* mutant erythrocytes caused by cell cycle arrests at the G0/G1-to-S phase and G2-to-M transitions. Prolonged cell cycle arrest can lead to apoptosis,³¹ thus we also assessed apoptosis in *ddx41* mutants. We measured levels of active caspase-3, an essential executor of apoptosis, in *ddx41* mutant and sibling *gata1:dsred*⁺ erythrocytes by flow cytometry. We observed a significant increase in active caspase-3 in *ddx41* mutant *gata1:dsred*⁺ erythrocytes at 30 hpf (Figures 4C-D). These data indicate that both cell cycle

arrest and elevated apoptosis in *ddx41* mutant erythrocytes may drive anemia.

Ddx41 regulation of ATM and ATR signaling contributes to proper erythropoiesis

These molecular and cellular phenotypes in *ddx41* mutants imply that loss of *ddx41* could promote DNA damage. In order to address this question, we analyzed the DNA damage response (DDR) by performing immunofluorescence (IF) for γ H2AX in *ddx41* mutants and siblings. We showed that γ H2AX levels were increased nearly two-fold in *ddx41* mutants compared to sibling cells (Figures 4E and F). These data demonstrate that *ddx41* deficiency triggers DDR *in vivo*.

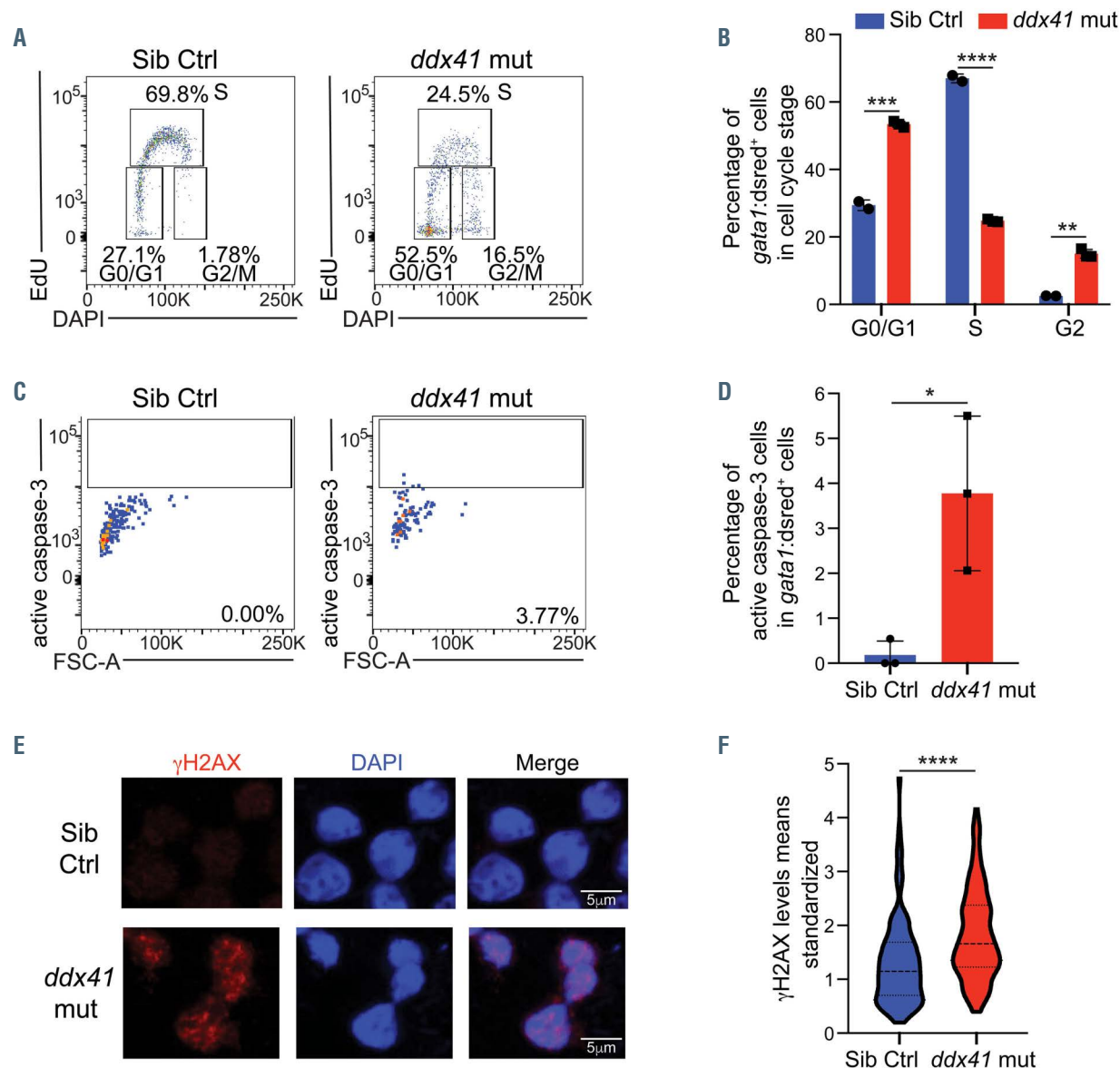


Figure 4. Ddx41 deficiency triggers cell cycle arrest and DNA damage response in erythroid progenitors. (A) Cell cycle analysis of *gata1:dsred*⁺ erythroid cells from sibling controls (left) and *ddx41* mutants (right) after a 2-hour pulse of 5-ethynyl-2'-deoxyuridine (EdU) at 28 hpf. EdU incorporation (y-axis) and DAPI content (x-axis) were measured by flow cytometry at 30 hours post fertilization (hpf). (B) Quantification of the percentage of cells in each cell cycle phase from (A). (C) Flow cytometry analysis of active-caspase 3 in *gata1:dsred*⁺ erythroid cells from sibling controls (left) and *ddx41* mutants (right). (D) Quantification of the percentage of *gata1:dsred*⁺ erythroid cells that are active caspase-3-positive from (C). (E) Confocal images showing immunofluorescence of nuclei (DAPI) and γ H2AX in cells isolated from 28 hpf siblings (top) and *ddx41* mutants (bottom). scale bars = 5 μ m. (F) Quantification of γ H2AX levels from (E). Graphs display means $\pm$ standard deviations (stds) with P-values calculated with unpaired Student's t-test, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. For flow cytometry, n=3-5 pools of ~5-20 embryos per pool. For immunofluorescence imaging, n=100-300 cells per genotype.

Our model is that loss of Ddx41 contributes to excessive DDR signaling and subsequent cell cycle arrest in erythrocytes, leading to anemia in *ddx41* mutants. If correct, then inhibiting components of the DDR pathway would i) reverse cell cycle defects and ii) increase erythrocyte levels. In order to test this model, we examined how the two primary mediators of DDR, Ataxia-telangiectasia-mutated (ATM) and Ataxia-telangiectasia and Rad3-related (ATR), affected erythrocytic cell cycle kinetics in *ddx41* mutants. We assessed cell cycle status of 30 hpf *ddx41*-mutant *gata1:dsred⁺* erythroid progenitors in embryos treated with DMSO vehicle control, the ATM inhibitor KU60019, or the ATR inhibitor AZ20. There was a significant increase of *gata1:dsred⁺* *ddx41*-mutant cells in S phase when treated with either ATM or ATR inhibitors as compared to DMSO vehicle control (Figures 5A and B). Additionally, pharmacological inhibition of ATM or ATR increased erythropoietic output in *ddx41* mutants, as measured by quantification of *gata1:dsred⁺* erythrocyte numbers per embryo using flow cytometry (Figures 5C to F). Although there was a trend towards an increase in erythrocyte numbers in control siblings treated with ATM or ATR inhibitors these changes were not statistically significant. Taken together, these data indicate that DDR signaling triggers a G0/G1 cell cycle arrest in *ddx41*-mutant erythrocytes that results in a reduction of erythroid progenitor cell number.

Finally, we wanted to assess if increasing the number of erythroid progenitors via ATM or ATR inhibition would increase the number of oxygenated erythrocytes in *ddx41* mutants. Surprisingly, we only observed a significant increase in o-dianisidine-positive erythrocytes in *ddx41* mutants treated with ATM inhibitor, but not ATR inhibitor (Figures 6A and B). These data indicate that Ddx41 regulation of ATM might have a broader impact on erythropoiesis than ATR signaling.

Discussion

Although DDX41 mutations are found in numerous human hematologic diseases, its function in hematopoiesis is unknown. Our work is the first to establish Ddx41 as a critical mediator of erythropoiesis with *ddx41* loss suppressing the expansion and maturation of erythrocytes. We showed a profound effect on the expression of cell cycle and DNA damage-associated genes in *ddx41* mutant erythroid progenitors consistent with the observed cell cycle arrest. The DNA damage response is elevated in *ddx41* mutant cells and triggers an ATM and ATR-triggered cell cycle arrest. Inhibition of ATM and ATR partially suppressed anemia in *ddx41* mutants. These findings establish Ddx41 as a positive regulator of erythropoiesis in part by preventing genomic stress and promoting proper erythroid progenitor expansion.

Patients with germline mutations in *DDX41* do not develop hematologic symptoms until later in life,¹ yet zebrafish *ddx41* mutants show anemia within 40 hpf. We posit that the difference has to do with the extent of Ddx41 deficiency. Zebrafish homozygous mutants have maternally deposited Ddx41 that is naturally depleted over the first few days of life. When the levels drop below a certain threshold, the mutants die, demonstrating it is an essential factor. In contrast, zebrafish *ddx41* heterozygous animals are phenotypically indistinguishable from wild-type animals during embryogenesis and in adulthood, sug-

gesting a 50% decrease of Ddx41 alone is insufficient to alter hematopoiesis. This is in agreement with the clinical observation that patients with germline *DDX41* mutations who develop hematologic malignancies often acquire somatic missense mutations in the second allele that are thought to diminish DDX41 ATPase activity.¹ Combined, the data indicate that when DDX41 levels decrease to less than 50%, this leads to hematologic defects, but when critically too low, it leads to lethality.

DDX41 was previously identified as a mediator of genomic stability in a cell line-based genome-wide siRNA screen.¹¹ However, a role for DDX41 in genomic integrity as well as the downstream consequences of its loss were never demonstrated *in vivo*. Our current work revealed that *Ddx41* regulates genomic integrity *in vivo*, and that loss of *ddx41* leads to both cell cycle arrest and apoptosis in erythrocytes that contributes to anemia in *ddx41* mutants. We established that ATM and ATR signaling contribute to these attributes, but only ATM inhibition significantly increased o-dianisidine-positive erythrocytes in *ddx41* mutants. The differential impact on oxygenated erythrocyte output by inhibition of ATM and ATR might indicate that Ddx41-regulated ATM signaling is more critical for proper erythropoiesis. ATM has an additional role in apoptosis, especially during development that might explain some of the phenotypic differences when comparing ATM and ATR inhibition effects on erythropoiesis. However, it should be noted that although the ATM and ATR kinases respond uniquely, there exists an extensive 'cross-talk' between them, which can make determining which precise pathway is involved in a phenotype confusing.³² Further dissection of the role of DDX41 in ATM and ATR pathway regulation will need to be investigated.

Splicing mutations are commonly found in hematologic malignancies.^{33,34} DDX41 interacts with multiple components of the spliceosome.¹ Our work aligns with prior studies showing *DDX41* insufficiency associates with numerous deleterious splicing outcomes. If and how these splicing events contribute to hematopoietic pathogenesis is unclear. We showed that components related to cell cycle and DNA repair are commonly mis-spliced in *ddx41* mutants. Therefore, it is possible that loss of *ddx41* may be mediating cell cycle arrest and activation of DDR via mis-splicing of crucial regulators of these pathways. The contribution of DDR pathway component mis-splicing in human cytopenias remains to be addressed.

In addition to the effect on cell cycle, we delineated maturation defects in *ddx41* erythrocytes marked by aberrant globin expression and a megaloblastoid-like morphology of mutant erythrocytes. Although we could not perform a complete analysis of definitive erythropoiesis as *ddx41* mutants die before EMP-derived or HSC-derived erythrocytes fully mature, the diminished expression of the larval $\beta e2$ globin suggests a decrease in EMP-derived definitive erythrocytes. This finding combined with the elevated *cmyb* and *gata1* levels in EMP cells suggests that this defect could be caused in part by maturation defects. As the treatment with the ATM inhibitor KU60019 only partially increased hemoglobinized erythrocytes in *ddx41* mutants, it suggests that deregulation of another pathway underlies additional maturational defects in *ddx41* mutants.

In summary, our study unveils a critical role for Ddx41 as a key gatekeeper to maintain cell cycle progression, a necessary component for erythrocytic development. We

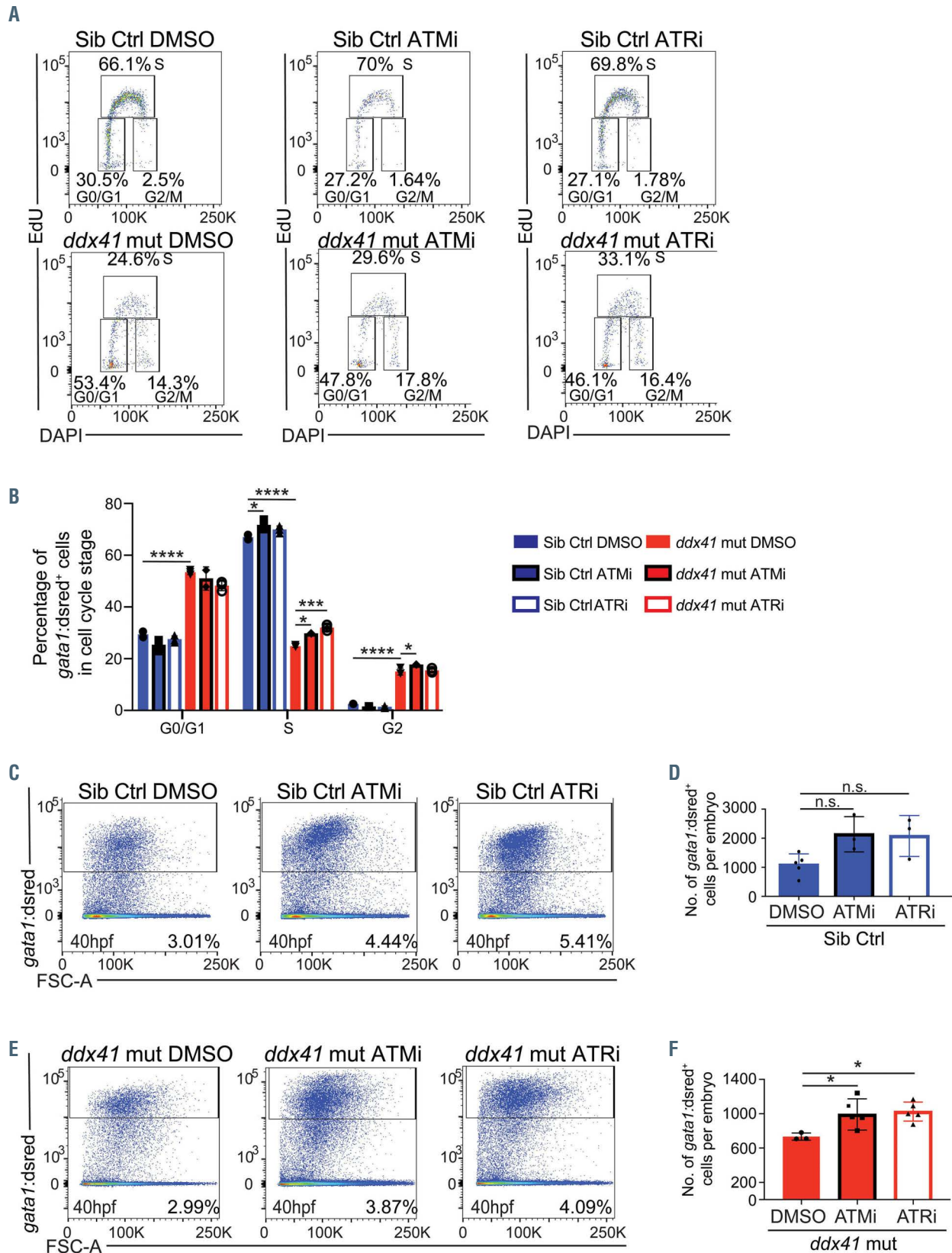


Figure 5. Ddx41 regulation of ATM/ATR signaling contributes to proper erythroid progenitor proliferation. (A) Cell cycle analysis of *gata1:dsred*⁺ erythroid cells from sibling controls (top) and *ddx41* mutants (bottom) treated with dimethyl sulfoxide (DMSO) (left), 30 nM KU60019 (Ataxia-telangiectasia-mutated [ATM] inhibitor, middle), and 30 nM AZ20 (Ataxia-telangiectasia and Rad3-related [ATR] inhibitor, right) after a 2-hour pulse of 5-ethynyl-2'-deoxyuridine (EdU) at 28 hours post fertilization (hpf). EdU incorporation (y-axis) and DAPI content (x-axis) were measured by flow cytometry at 30 hpf. (B) Quantification of the percentage of cells in each cell cycle phase from (A). (C and E) Flow cytometry plots of *gata1:dsred*⁺ erythroid cells from sibling controls (C) and *ddx41* mutants (E) treated with DMSO (left), 30 nM KU60019 (ATM inhibitor, middle), and 30 nM AZ20 (ATR inhibitor, right). (D and F). Graphs depicting the absolute number of *gata1:dsred*⁺ erythroid cells per embryo from (C) and (E). Graphs display means $\pm$ standard deviations (stds) with *P*-values calculated with a one-way ANOVA with Tukey's multiple testing correction, $P < 0.05$, *** $P \leq 0.001$, **** $P \leq 0.0001$. For flow cytometry, $n = 3-5$ pools of ~ 20 embryos per pool.

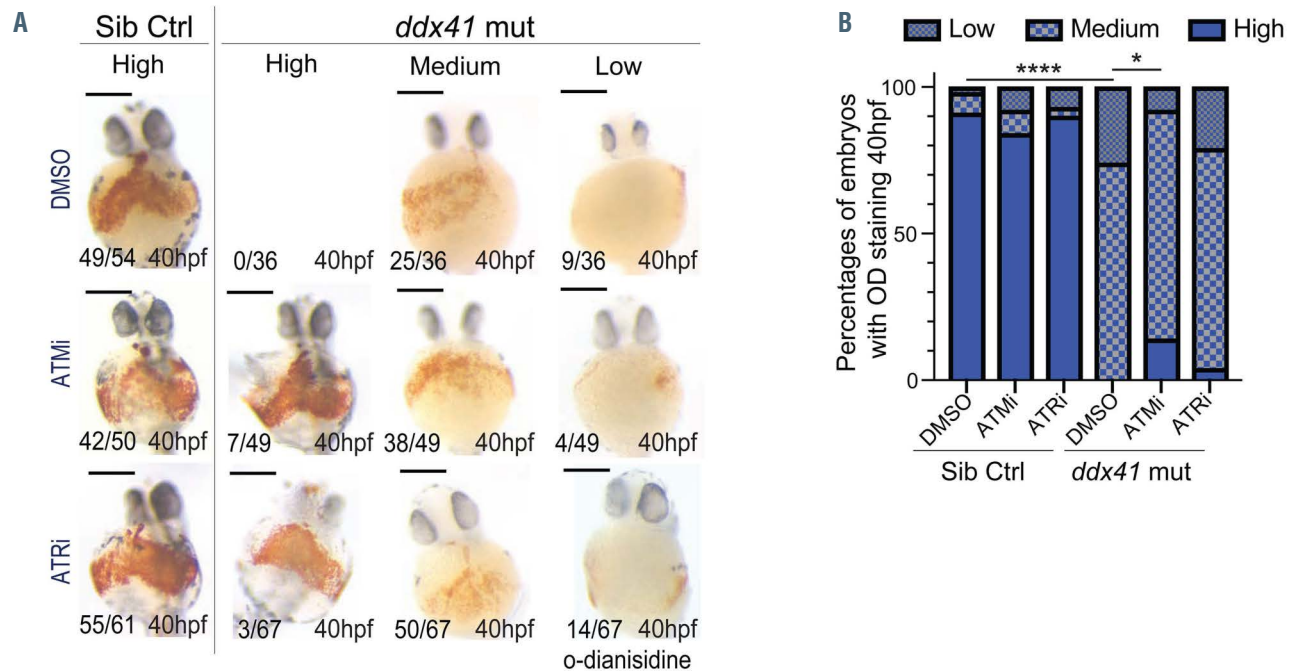


Figure 6. ATM inhibition partially suppresses *ddx41* mutant anemia. (A) Representative images of o-dianisidine staining and corresponding levels of staining from sibling controls (left) and *ddx41* mutants (right) treated with dimethyl sulfoxide (DMSO) (top), 30 nM KU60019 (Ataxia-telangiectasia-mutated inhibitor [ATMi], middle), and 30 nM AZ20 (Ataxia-telangiectasia and Rad3-related inhibitor[ATRi], bottom). Numbers on bottom left corner indicate the fraction of embryos with the same phenotype as the one depicted in the image. scale bars =100 μ m. (B) Graph depicting frequency of designated o-dianisidine staining levels in sibling controls and *ddx41* mutants at 40 hours post fertilization (hpf) treated with DMSO vehicle control, 30 nM KU60019 (ATMi), and 30 nM AZ20 (ATRi). Graphs display frequency of embryos with each phenotype with *P*-values calculated with Chi-squared test, **P*<0.05, *****P*≤0.0001. For o-dianisidine staining, n=36-67 embryos per experiment.

demonstrated that deficiency of *ddx41* triggers cell cycle arrest via activation of ATM and ATR, which ultimately mediates a decrease in proliferation and maturation of erythrocytic progenitors in *ddx41* mutants. These findings establish a critical function for Ddx41 in promoting healthy erythropoiesis by suppressing genomic stress and present a potential role for ATM and ATR signaling in DDX41-mutant pathologies.

Disclosures

No conflicts of interest to disclose.

Contributions

JTW and TVB designed the project experimental approach; JTW, ES, RW, and TVB performed the experiments; VG per-

formed bioinformatics analysis; JTW and TVB analyzed the data; JTW and TVB wrote and edited the manuscript; all authors reviewed and approved the manuscript.

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Sensing Stemness

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Abstract

Purpose of Review Hematopoietic stem cells (HSCs) are formed embryonically during a dynamic developmental process and later reside in adult hematopoietic organs in a quiescent state. In response to their changing environment, HSCs have evolved diverse mechanisms to cope with intrinsic and extrinsic challenges. This review intends to discuss how HSCs and other stem cells co-opted DNA and RNA innate immune pathways to fine-tune developmental processes.

Recent Findings Innate immune receptors for nucleic acids like the RIG-I-like family receptors and members of DNA sensing pathways are expressed in HSCs and other stem cells. Even though the “classic” role of these receptors is recognition of foreign DNA or RNA from pathogens, it was recently shown that cellular transposable element (TE) RNA or R-loops activate such receptors, serving as endogenous triggers of inflammatory signaling that can shape HSC formation during development and regeneration.

Summary Endogenous TEs and R-loops activate RNA and DNA sensors, which trigger distinct inflammatory signals to fine-tune stem cell decisions. This phenomenon could have broad implications for diverse somatic stem cells, for a variety of diseases and during aging.

Keywords Hematopoietic stem cells · DNA sensors · RNA sensors · R-loops · Transposable elements · Plasticity

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Introduction

Hematopoietic stem cells (HSCs) reside on the top of the hematopoietic hierarchy and can give rise to the majority of differentiated blood cells [1–5]. HSCs arise during development through a highly plastic process involving a dynamic cell fate and morphological transition termed the endothelial-to-hematopoietic transition or EHT [6, 7]. During this process, a specialized fraction of endothelial cells will give rise to hemogenic endothelial cells which later become HSCs. HSCs then proliferate in specified organs such as the fetal liver in mouse and the caudal hematopoietic tissue in zebrafish [8, 9]. During this plastic process, the cells undergo extensive chromatin reorganization [10] and transcriptional reprogramming that are cell-type specific. Global DNA rearrangement is also observed when quiescent adult HSCs enter a transient activated state during times of stress, including during infection, chemotherapy, and after extensive bleeding [4, 11]. Global DNA rearrangement can result in the transcription of transposable elements (TEs) and other repetitive RNAs or the formation of R-loops,

comprised of RNA:DNA hybrids and single-stranded (ss) DNA [12–15].

DNA and RNA sensing pathways are abundantly active in immune cells in order to recognize foreign DNA or RNA, but are also used as the first line of defense against pathogens in non-immune effector cells [16, 17]. Nucleic acid sensors include the endosomal Toll-like receptors (TLRs) TLR3, TLR7, TLR8, and TLR9 [18]; the nuclear and cytosolic DNA sensor cyclic GMP–AMP synthase (cGAS) [19, 20]; retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) [21]; NOD-like receptors (NLRs) [22]; AIM2-like receptors (ALRs) [23]; and C-type lectins [24]. DNA-sensing pathogen recognition receptors (PRRs) include cGAS, the endosomal TLR9, and the cytosolic ALRs AIM2 and IFI16. RNA-sensing PRRs are endosomal TLR3, TLR7, TLR8, and cytosolic RIG-I, MDA5, NLRP1 and 3, and NOD2 [17]. Interaction of these receptors with viral RNA or DNA from pathogens transduce innate immune signaling pathways that cause transcriptional activation by nuclear factor kappa B (NF- κ B), IFN-response factors (IRF), and other inflammatory transcription factors [16, 17]. Importantly, HSCs and other somatic stem cells express many of these receptors and downstream pathway components resulting in specific expression of interferon-stimulated genes (ISGs) that protects them from viral invasion.

Nucleic acid sensors recognize different types of DNA and RNA species [16, 17]. For example, RIG-I recognizes short 5' ppp-double-stranded (ds) RNA while MDA5 prefers long dsRNA. TLR3 recognizes dsRNA, TLR7/8 recognize single-stranded RNA, and TLR9 recognizes unmethylated CpG DNA. Evidence has been accumulating over the last decade demonstrating that endogenous sources of RNAs and DNAs can also stimulate nucleic acid sensors. For example, mislocalization of mitochondrial RNA or DNA in the cytosol stimulates MDA5 and cGAS, respectively, both resulting in induction of a type I IFN response [25, 26]. Mistakes in A-to-I RNA editing by adenosine deaminase acting on RNA 1 (ADAR1) or unedited *Alu* elements are also major sources of non-viral MDA5 stimulation [27–29]. Persisting double-strand breaks, micronuclei formation that occur during mitosis and accumulation of cytosolic DNA are common sources of self-activation of cGAS that lead to non-viral activation of inflammatory signaling [19].

Many pivotal studies have proven the importance of inflammatory signaling on HSCs from birth through old age and under times of stress. Only recently has the initiating, endogenous source of inflammatory signaling in HSCs started to be elucidated. In this review, we will focus mainly on the role that RLR and cGAS nucleic acid sensing pathways play in developmental decisions and stemness. We are focusing particularly on the hematopoietic system but

also presenting evidence for a non-pathogenic role of DNA/RNA sensors in other stemness contexts.

DNA Sensing-cGAS Pathway

cGAS, a recently identified DNA sensor, is thought to be one of the main responders to infectious and damaged self-DNA [19, 20]. cGAS is activated by dsDNA in both the cytoplasm and nucleus in a manner independent of specific DNA sequence [19, 20]. Upon DNA binding, cGAS produces the second messenger 2',3'-cyclic-GMP-AMP (cGAMP), which in turn binds to and activates the endoplasmic reticulum transmembrane receptor Stimulator of Interferon Genes (STING). Then, STING triggers distinct downstream mechanisms, including activation of Tank Binding Kinase 1 (TBK1); nuclear translocation of the transcription factors IRF3, IRF7, and NF- κ B; stimulation of type I IFN gene expression; and production of inflammatory cytokines (Fig. 1) [19, 20].

Although largely explored as a dsDNA sensor, recent studies revealed that cGAS can be activated by a variety of non-DNA stimuli [19]. For example, nuclear cGAS can be relocalized and activated during translational stress due to recognition of unresolved collided ribosomes as a consequence of a disruption of the ribosome-associated protein quality control [30]. Very recently, cGAS-like receptors (cGLRs) were characterized as innate immune receptors in *Drosophila melanogaster* and surprisingly were identified as RNA binders. Crystal structure studies identified differences in the ligand binding surface between cGLR1 and c-GAS that could explain the differences in nucleic acid binding preferences [31, 32]. These new findings highlight that our understanding of all the potential ligands for cGAS as well as what guides the selectivity is likely only the tip of the iceberg.

Depending on the cellular context, these effectors can result in cell proliferation, autophagy, cell senescence, or cell death [33–39]. Activation of cGAS during mitotic arrest results in accumulating, low-level IRF3 phosphorylation without inflammatory gene upregulation, but rather suppression of BCL-XL and subsequent apoptosis [39]. Additionally, STING activation can trigger autophagy via a non-canonical pathway resulting in STING translocation to the ER-to-Golgi interface and ultimately LC3 lipidation [34]. Lysosome-associated, activated STING can also trigger cell death by promoting membrane permeabilization [35]. Furthermore, exposure of mice to the STING agonist carboxymethyl-9-acridanone (CMA) results in robust pathway activation in CD4+ T cells, which promotes expression of a distinct gene set and ultimately results in apoptosis [40]. In contrast, cGAS-STING signaling in tumor-infiltrating

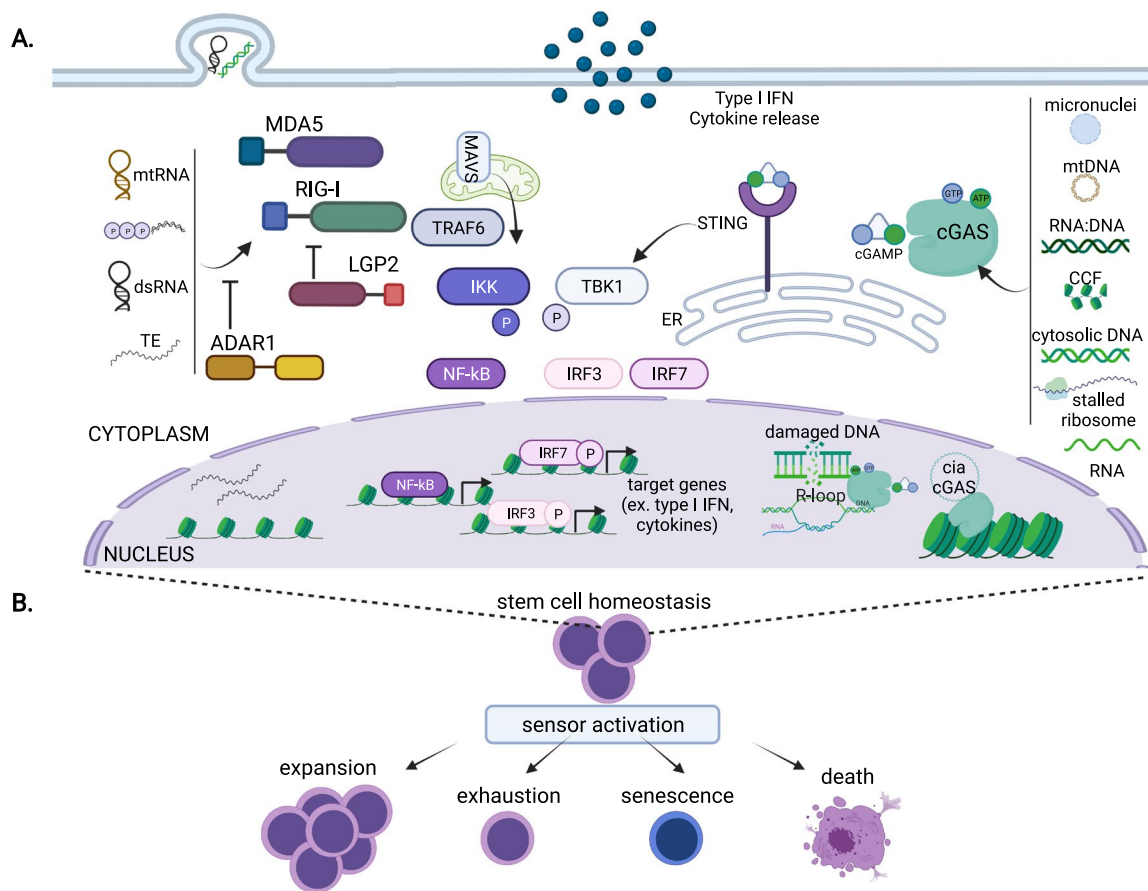


Fig. 1 **A** RIG-I-Like receptors (RLRs), such as MDA5 and RIG-I, are activated by numerous cytosolic ligands, such as mitochondrial RNA (mtRNA), 5'-triphosphate RNA, dsRNA, and transposable element transcripts (TEs). cGAS is stimulated in both the cytoplasm and nucleus by several endogenous stimuli, such as micronuclei, mitochondrial DNA (mtDNA), RNA:DNA hybrids, cytosolic chromatin fragments (CCF), naked cytosolic DNA, stalled ribosomes, and most recently RNA. Activation of RLRs promotes oligomerization with the mitochondrial anti-viral signaling protein (MAVS), which triggers activation of kinases like Tank Binding Kinase 1 (TBK1) and IκB kinase ε (IKKε). Stimuli binding to cGAS induces its synthetase activity resulting in production of the second messenger cyclic GMP-

AMP (cGAMP). STING activation by cGAMP results in induction of TBK1. Stimulation of either RNA or DNA sensor pathways can ultimately result in the translocation of Interferon Response Factor 3 (IRF3), IRF7, and nuclear factor kappa B (NF-κB) to the nucleus leading to the induction of an IFN response and the secretion of proinflammatory cytokines. Cells possess negative regulators of the sensor pathways including LGP2 and ADAR1 for RLRs and nucleosome tethering and the circular RNA cia-cGAS for nuclear cGAS. **B** Nucleic acid sensor activation alters stem cell homeostasis in cell-type and context-specific manners. Pathway activation can result in diverse cellular outcomes, such as expansion, exhaustion, senescence, or cell death. Created with BioRender.com

CD8 + T cells enhances the formation of stem-like memory T cells [41]. Signal strength and cell type differences are suggested to impart these distinct cellular outcomes [40]. There is still much to learn about the repertoire and regulation of cGAS-STING signaling and downstream outcomes.

cGAS-ing Stemness

Although mostly studied in immune effector cells, cGAS-STING signaling components are broadly expressed, comprising an ancient frontline defense of innate immunity.

Several recent findings directly demonstrate that the cGAS-STING pathway is critical for developmental and adult hematopoietic stem and progenitor cell (HSPC) homeostasis even though this field is still in its infancy.

Many years ago, it was demonstrated that polymicrobial intraperitoneal infection in mice promotes HSPC expansion in a TLR-independent manner [42]. Pathogen-associated molecular patterns or PAMPs released in this model include the TLR agonist lipopolysaccharide and the STING agonist 3',3' cyclic-di-GMP (c-di-GMP). This bacterial second messenger is structurally similar to the endogenous cGAS-generated cGAMP [43]. Similar to cGAMP, bacterial c-di-GMP binds to and activates STING. Kobayashi et al.

demonstrated that *in vivo* c-di-GMP treatment promotes multipotent progenitor expansion in a manner dependent on STING, IRF3/7, and IFNAR [44•]. In contrast, long-term HSC levels were diminished upon c-di-GMP treatment, which was dependent on STING and NF- κ B signaling but independent of IFNAR. These findings suggest that STING activation elicits distinct responses based on the cellular context. Although this work supports a role for infection-based STING activation in regulating HSPC behavior, a role for endogenous cGAS-STING signaling was unexplored.

In addition to dsDNA, cGAS can be activated by RNA:DNA hybrids [45]. RNA:DNA hybrids along with ssDNA are components of R-loops, which are nucleic acid structural variants that are essential for many cellular processes, such as transcriptional regulation, genomic stability, and immunoglobulin class switching [46]. R-loop imbalance is associated with numerous human diseases, including cancer and immune disorders. Our group recently demonstrated that R-loop imbalance can expand HSPCs [47••]. Using zebrafish, we showed that DEAD-box helicase 41 (DDX41), a factor mutated in inherited adult-onset myelodysplastic syndrome (MDS), suppresses R-loop accumulation. When DDX41 levels fall, R-loop levels rise, and trigger activation of the cGAS-STING pathway, which boosts HSPC production. R-loops induced TBK1 phosphorylation, ISG expression, and NF- κ B activation, consistent with the findings of c-di-GMP stimulation. Our findings, showing cGAS-mediated HSPC expansion in DDX41 mutant animals, indicate a link between cGAS activation and clonal hematopoiesis disorders such as MDS.

Although a role for cGAS-STING in the aging HSC compartment has not been investigated, several recent discoveries indicate that it is worth exploring. Aging is associated with low-dose chronic inflammation — inflammaging — that contributes to age-related decline in HSC function and is also linked to aberrant HSPC expansion observed in clonal hematopoiesis [48–54]. It is posited that lifelong accumulation of damaged cellular components that stimulate cGAS and STING such as micronuclei, cytosolic chromatin fragments, damaged nuclear DNA, cytosolic mtDNA, and nuclear envelope disruptions are major contributors of inflammaging [55–58]. Mitochondrial aberrations and mtDNA transfer are linked to HSC dysfunction with age, but the full underlying molecular and cellular mechanism remains unclear [59]. Future exploration into cGAS-STING as mediators of HSPC defects downstream of mitochondrial disruption is warranted.

Cellular senescence is another major cellular hallmark of aging [60]. Recent evidence demonstrated that cGAS is activated by senescence-associated damaged cellular components [33, 61]. Senescence-activated cGAS starts a feed-forward loop that stimulates STING-mediated

senescence-associated secretory phenotype (SASP), which promotes paracrine senescence. Loss of cGAS can abrogate senescence in cell culture models [33, 61], and recent *in vivo* studies indicate that age-associated phenotypes, such as decline in immune cell function and cancer development, also appear to be STING-mediated [62]. As cGAS-STING pathway signal strength elicits different responses, it will be interesting to decipher the different outcomes from acute versus low-dose chronic pathway activation on HSCs and other stem cell populations that decline with age.

New data also link the cGAS-STING pathway in skin stem cells and homeostasis. Endogenous retroviruses (ERV) produced upon microbiota stimulation in the skin and in purified keratinocytes elicit activation of the cGAS-STING pathway and establish the communication between the microbiota and the keratinocytes required for the induction of homeostatic T cell responses to a skin microbe. Mild ERV expression has a positive effect on skin tissue repair and promotes immune fitness. However, hyper-elevated levels of ERVs due to high-fat diet resulted in skin inflammation [63]. Another recent study showed that hair follicle stem cells from patients with hidradenitis suppurativa, a chronic, relapsing, inflammatory skin disease, are disturbed [64]. The patient samples had an expansion of proliferating hair follicle progenitors and decreased numbers of quiescent hair follicle stem cells due to elevated replication stress that triggered excessive STING-mediated type I IFN production. These data are highly reminiscent of the effect of cGAS-STING activation in HSPC, suggesting a potential similarity in the response of somatic stem cells to DNA-sensing pathway induction.

Mechanism of cGAS Control

cGAS is activated by dsDNA that is present in all cells, so how does it remain inactive? It was long thought that cGAS was mostly localized to the cytoplasm, such that it was only activated upon the entry of foreign DNA into the cell. In recent years that model was challenged when it was discovered that cGAS is largely confined to the nucleus [65, 66]. How does an enzyme that is activated by DNA stay silent in the sea of genomic DNA in the nucleus? Several seminal papers published in 2020 uncovered the answer to this conundrum. Within the genome, cGAS is broadly localized but enriched in heterochromatic, nucleosome-rich regions [67]. It was found that tight binding of cGAS to the histone H2A/H2B dimer in a nucleosome suppresses its activity [66, 68–71]. That said, purified chromatin can stimulate cGAS activity, potentially due to cGAS interaction with dsDNA found in nucleosome-free regions of chromatin [39, 72]. Beyond nucleosome protection of dsDNA, several non-nucleosome chromatin factors regulate cGAS accessibility to nucleosome-poor genomic regions [73]. For example, Bar-

rier-to-autoantigen 1 (BAF), another sequence-independent dsDNA binding protein, displaces cGAS from dsDNA in a competitive fashion [65]. Chromatin architecture is also suggested to modulate cGAS activity as there has been a new link between linker histones and cGAS-driven type I IFN gene expression [74]. The biophysical properties of STING can also control cGAS activation. A recent study showed that STING can condensate into puzzle-like structures in the ER via liquid–liquid phase separation of soluble proteins. These biomolecular condensates limit the immune response to pathogen invasion by sequestering STING and TBK1 [75]. Formation of biomolecular condensates was dependent on cGAMP levels suggesting a level of feedback regulation to prevent pathway overactivation.

Another level of cGAS regulation was identified in murine LT-HSCs that express high levels of *cia*-cGAS, a circular RNA that interacts with cGAS in the nucleus [76••]. Binding of *cia*-cGAS to cGAS inhibits its synthase activity and prevents cGAS recognition of self DNA. Similar to *c*-di-GMP-treated mice, *cia*-cGAS-deficient animals showed elevated expression of type I IFNs, increased HSPC proliferation, higher numbers of LSK HSPCs, and lower numbers of LT-HSCs. In these mice, HSCs eventually exhaust due to chronic cGAS activation, and subsequent type I IFN overstimulation, thus leading to a bone marrow failure phenotype. All current data support the need for tight regulation of STING-mediated pathways for cellular and tissue homeostasis.

RNA Sensing-RLR Pathway

RLRs are a family of cytoplasmic receptors that include RIG-I, melanoma differentiation-associated protein 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) [21, 77]. All RLRs have a helicase domain and carboxy-terminal domain (CTD) while RIG-I and MDA5 also include two caspase activation and recruitment domains (CARDs) that are mainly responsible for signal mediation. RNAs activate RIG-I and MDA5 which then oligomerize through the CARD domains with the mitochondrial antiviral-signaling protein (MAVS) and create a signaling platform leading to activation of kinases like TBK1 and I κ B kinase ϵ (IKK ϵ) (Fig. 1). Ultimately translocation of IRF3, IRF7, and NF- κ B to the nucleus lead to the induction of an IFN response and the secretion of proinflammatory cytokines [21].

RIG-ing Stemness

In recent years more and more evidence suggest that RLRs play critical roles in physiological or pathological processes that are not directly related to immune responses. Recent evidence from our lab suggested a role for RIG-I-like receptors

in HSC formation during development [78••]. During development, we found that RIG-I and MDA5 display low-level activity during EHT and activate NF- κ B signaling that enhances HSC formation. LGP2 on the other hand restricts NF- κ B activation and the function of RIG-I and MDA5, thus acting as a negative regulator of inflammatory signaling during HSC development. A potential source of activation of RIG-I and MDA5 comes from TE transcripts that are expressed during EHT. In adulthood, it was shown previously that RIG-I is differentially expressed between multipotent and myeloid committed progenitors [79]. Moreover, a potential antileukemic effect of RIG-I was shown in acute myeloid leukemia (AML) cells via competitive inhibition of the Src/AKT interaction. Indeed, decreased RIG-I leads to hyperactivity of Src family kinases and subsequent AKT activation [80]. This function of RIG-I is not primed by foreign RNA. We recently illustrated the function of MDA5 in hematopoietic regeneration after chemotherapy [81••]. We found that the transcription of TEs progressively increases in HSCs after chemotherapy. TE transcripts activate MDA5 to induce an inflammatory response that is necessary for HSCs to exit quiescence and proliferate in order to replenish differentiated blood cells that have been eliminated by chemotherapy. Indeed, overexpression of TE copies or knock-down of LINE1 increased or decreased cycling of HSCs *in vitro*, respectively. TE transcriptional upregulation is also observed in HSCs after irradiation while inflammatory signaling plays instrumental roles in regulating their expression [82•]. Interestingly, TE upregulation has been instrumental in cancer therapy upon treatment with demethylating agents. In these cases, demethylating agents increased transcription of TEs that were sensed by MDA5 thus leading to induction of inflammatory responses and subsequently to cell death [83–85]. Similarly, upregulation of LINE1 upon loss of MPHOSPH8 or MPP8 (M-Phase Phosphoprotein), a member of the HUSH complex, resulted in DNA damage and tumor regression or arrest [86]. The implication from these studies is that triggering RNA-sensing pathways could be instrumental for some cancer immunotherapeutic approaches.

An important role of MDA5 was also identified during the mesenchymal-to-epithelial transition (MET) during induced pluripotent stem cell (iPSC) reprogramming [87]. Loss of ADAR1-mediated RNA editing led to increased activation of MDA5 that disrupted MET. Mechanistically, they showed that ADAR1 editing of transmembrane-encoding dsRNAs is crucial to prevent MDA5 activation and to promote PERK-dependent unfolded protein response that drives MET. RLRs also play a role in mesenchymal stem cell survival [88].

LGP2, the third member of the RLR family whose function in inflammatory pathways remains controversial, has been shown to promote cell survival and fitness of CD8⁺ T cells after infection — thus, underscoring a possible role

under physiological conditions [89]. In contrast, our results showed that LGP2 acts as a negative regulator of HSPC formation during development [90]. Additionally, MAVS, the downstream adaptor of the RLR pathway, is important for mitochondrial homeostasis since it acts as a potential receptor for mitochondria-associated autophagic signaling [91].

Mechanism of RLR Stimulation Control

The high-affinity ligands for RLR receptors are viral RNAs with specific characteristics that cannot be found in host RNAs, thus providing the first layer of control of RLR and especially RIG-I activation. Additionally, the CARD domains of RIG-I interact with a domain located between the helicase domains keeping RIG-I in a closed conformation, thus preventing activation by ligands [21]. RNA binding also plays a role in controlling RIG-I signal and especially its attenuation. For example, exosome transfer of the non-coding 5'-PPP-containing *RN7SL1* RNA produced in stromal cells triggers RIG-I activation in adjacent breast cancer cells, which induces the production of a cyclic long non-coding RNA that binds to RIG-I and attenuates the signal [92]. Competition of a host-derived, IFN-inducible long noncoding RNA, *lnc-Lsm3b* with viral RNA for RIG-I binding is another example [93].

Another important regulator of RLR activity is RNA editing by ADAR1. Multiple publications have shown, especially for MDA5, that editing of RNAs by ADAR1 reduces the immunogenicity of these RNAs [27, 29]. Editing of RNA Polymerase II-transcribed *Alu* elements by human ADAR1 blocked translational shutdown by inhibiting hyperactivation of RLRs such as MDA5 or protein kinase R (PKR) in neuronal cells [27]. Additionally, Ahmad et al. showed that the amount of RNA is critical to control pathway activation as increasing the amount of RNA disrupted MDA5 signaling, presumably due to interference with filament formation [94]. RNA modifications also play a role. It was recently shown that m⁶A RNA modification of TEs reduced their half-life, and thus diminished the chances of activating the RNA-sensing pathway [95].

Other Sensors and Stimuli

As mentioned earlier, besides cGAS and RLRs many other sensors exist. Since current findings support a role for nucleic acid sensors in developmental processes, it is natural to posit that other sensors, possibly activated by diverse stimuli, could have similar roles. Indeed, it was recently shown that loss of H3K9 methylation in G9a-deficient animals results in ERV induction leading to an AIM2-mediated

inflammatory response that is devastating for mammary gland development [96]. Since these experiments were performed in the G9a knockout background, it is interesting to speculate that basal expression of ERVs may play a functional role in mammary gland development, but massive TE transcriptional upregulation impairs mammary gland development.

Metabolic regulation of NLRP3 activity and subsequent activation of interleukin-1-beta (IL-1 β) was also shown to regulate HSPC formation during development, thus pinpointing an interesting interplay between metabolism and inflammation in the regulation of cell fate decisions [97]. NLRP3 is activated by a variety of stimuli like ATP and uric acid but also mitochondrial DNA, pointing to diverse stimuli that can fine-tune the function of this receptor in physiological processes. It was also recently shown that human NLRP1 is a viral sensor of dsRNA, suggesting that NLRP1 may well sense endogenous dsRNA from TEs or other types of RNAs [98]. Of course, many other sensors could play potential roles in stem cells. For example, TLRs are known to be critical for HSC functions but their potential roles in sensing endogenous ligands remain to be fully explored [99].

Conclusions

The utility of RLRs and cGAS-STING signaling is best characterized in response to accumulation of danger signals from infectious or endogenous sources. Although RLRs and cGAS-STING are non-essential genes, animals and humans with mutations in RLRs, cGAS-STING, or downstream signaling components are prone to immune dysregulation, such as autoimmunity and infectious susceptibility [100]. Additionally, antiviral immunity plays a central role in stem cell gene engineering [101]. The recent work from our groups and others indicate that these sensing pathways could also titrate stemness properties. An innate quality of all stem cells is their ability to sense and adapt to the changing local and systemic environments while faithfully sustaining themselves and their differentiated progeny. RLRs and cGAS-STING are ancient pathways for evading foreign invaders that then evolved to cope with intrinsic damage. Similarly, we posit that the pathways adapted to modulate stem cell decisions are related to the stimulant, signal strength, and cellular context. For example, it is tantalizing to speculate that the massive nucleosomal shifting associated with cell fate transitions could stimulate low levels of cGAS activation that enforces cell fate states, similar to what was suggested for RNA sensors such as RIG-I and MDA5 [78••]. Additionally, the results discussed in this review hint to the fact that high cellular plasticity triggers nucleic acid sensors.

Hence, such mechanisms may be critical during aging or disease. It will be interesting to further investigate which endogenous ligands activate these receptors in stem cells in physiological and stress-induced contexts. TEs are gaining much attention in development, disease, and aging, but what families of TEs and which of their properties are involved still remains heavily understudied. Not to mention that a plethora of other RNAs could still stimulate nucleic acid sensors. Phenomena like hypertranscription and pervasive transcription are prevalent in cell fate changes during development and stress responses, but their importance or purpose during these times still remains to be elucidated [102, 103]. It is possible these events result in nucleic acid sensor activation. Taking into account that more and more stimuli activate DNA/RNA sensors, an emerging idea is that these receptors act as “buffers” of cellular activity during fate transitions, the response of which depends on intrinsic or extrinsic factors. Detailed studies on the direct impact of different stimuli for different sensors are still needed to fully understand this phenomenon. Is it possible that sensors sense everything? Probably not, but there is much more to discover in nucleic acid sensor biology, especially in the realm of stem cell regulation.

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Declarations

Conflict of Interest Teresa V. Bowman and Eirini Trompouki declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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●● Of major importance

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REVIEW

Clinical and mechanistic insights into the roles of DDX41 in haematological malignancies

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DEAD-box Helicase 41 (DDX41) is a member of the DExD/H-box helicase family that has a variety of cellular functions. Of note, germline and somatic mutations in the *DDX41* gene are prevalently found in myeloid malignancies. Here, we present a comprehensive and analytic review covering relevant clinical, translational and basic science findings on DDX41. We first describe the initial characterisation of *DDX41* mutations in patients affected by myelodysplastic syndromes, their associated clinical characteristics, and current treatment modalities. We then cover the known cellular functions of DDX41, spanning from its discovery in *Drosophila* as a neuroregulator through its more recently described roles in inflammatory signalling, R-loop metabolism and snoRNA processing. We end with a summary of the identified basic functions of DDX41 that when perturbed may contribute to the underlying pathology of haematologic neoplasms.

Keywords: acute myeloid leukaemia; cGAS-STING; DDX41; DEAD-box helicase; genomic instability; germline predisposition; haematopoiesis; inflammation; myelodysplastic syndrome; R-loops

Myelodysplastic syndromes: A clinical overview

Myelodysplastic syndromes (MDS) arise from clonal selection and expansion of aberrant haematopoietic stem and progenitor cells (HSPC) and are characterised by ineffective haematopoiesis and peripheral blood cytopenias [1]. MDS are among the most common haematological malignancies of the elderly, with a proclivity for malignant transformation to acute myeloid leukaemia (AML) [2–4]. Paradoxically, in MDS patients, there is a clonal selection/expansion of defective HSPCs over healthy cells [1]. Current treatment of MDS remains limited with little hope of a cure when bone marrow transplantation is not an option, which is commonly the case in elderly patients. The main reason

for this is that the factors that contribute to aberrant expansion and overall dysfunction of HSPC remain poorly understood. Recent sequencing studies on MDS patient samples have identified mutations that may contribute to disease pathogenesis and allow for more targeted and effective therapies. Some of the identified mutations occur within DEAD-Box Helicase 41 (*DDX41*) gene, which is the focus of this review.

DEAD-box helicases: Structure, functions and association with malignancies

DEAD-box helicases belong to the helicase superfamily 2, which is conserved from bacteria to mammals,

Abbreviations

AML, acute myeloid leukaemia; ATM, Ataxia-telangiectasia mutated; ATR, Ataxiatelangiectasia and Rad3-related; BTK, Bruton's tyrosine kinase; cGAS, cyclic GMP-AMP synthetase; CSNK1A1, casein kinase 1A1; DDR, DNA-damage response; DDX41, DEAD-Box Helicase 41; DEAD, Asp-Glu-Ala-Asp; HSPC, haematopoietic stem and progenitor cells; IFN- β , interferon-beta; MDM2, mouse-double minus2; MDS, myelodysplastic syndrome; NF- κ B, nuclear factor-kappa B; RB, retinoblastoma; STING, Stimulator of interferon genes.

and are characterised by a specific amino acid motif, Asp-Glu-Ala-Asp (DEAD) [5]. Structurally, these helicases contain a highly conserved DEAD-box/Helicase ATP-binding domain that allows for the binding of RNA/DNA and ATP [6], with mutations in this domain potentially disrupting helicase activity (Fig. 1) [7]. Additionally, the DEAD-box helicase family also contain a conserved Helicase C-terminal domain that plays a critical role in facilitating the ATPase activity of helicase, with mutations in this domain inhibiting nucleic-acid-dependent ATPase activity that can lead to loss-of-function of the helicase [8]. In contrast to these highly conserved domains, the N-terminal regions are disordered, highly variable in composition, and divergent in length, and are thought to contribute to specific and diverse function of these helicases [9]. These functions include transcriptional regulation, translation initiation, proper spliceosome function, and maintaining genomic stability [10]. Due to their diverse and critical cellular functions, it is not surprising that these helicases are frequently mutated and/or have altered expression levels in several different cancers [10].

Germline and somatic *DDX41* mutations associated with haematological malignancies

In 2012, *DDX41* was initially identified as a novel somatically mutated gene in AML *via* the sequencing of primary and relapsed tumour genomes from a small cohort of AML patients [11]. A few years later, germline mutations in *DDX41* were identified in a familial MDS/AML syndrome characterised by long latency, advance disease and poor prognosis [12]. This makes *DDX41* one of a handful of genes that have been implicated in causing myeloid neoplasms in adults with germline predisposition [13]. In this initial cohort of

patients, the most common germline mutation identified was a c.419 insertion of 'GATG' resulting in a p.D140 frameshift (fs) mutation within the N-terminal domain of *DDX41* resulting in loss-of-function of the mutant allele [12]. Since then, other *DDX41* germline mutations have been identified with varying frequencies in different patient populations with myeloid neoplasms including an Italian family with c.16228C > Gp.S543* nonsense mutation within the helicase C-terminal domain [14], Korean patients with p.V152G, p.Y259C, p.A500fs and p.E7* mutations [15], and Thai patients with p.F235fs and p.R339H mutations [16]. The lack of a mutational 'hotspot' coupled with the plethora of *DDX41* germline mutations identified throughout the body of the gene within haematological malignancies suggests that all these mutations result in diminished or loss-of-function of one allele of *DDX41*. Interestingly, though *DDX41* germline mutations are present in all cells, patients seem to only be at increased risk for myeloid malignancy, suggesting that the unique state and properties of the cell of origin may make it susceptible to specific perturbations.

Germline *DDX41* mutations have been shown to predispose individuals to subsequent somatic mutations on the other allele of *DDX41*, though the mechanism explaining this is currently not known. It has been noted that germline and somatic *DDX41* mutations often occur on different locations within the gene: germline mutations generally occur in the N-terminal domain (though some have been identified throughout the body of the gene) and result in a frameshift mutation resulting in loss of the allele, while somatic mutations are generally missense and occur closer to the C-terminal domain (Fig. 1) [12,17]. The current consensus in the field is that germline mutations in *DDX41* cause loss-of-function of the mutant allele, while somatic mutations result in hypomorphic

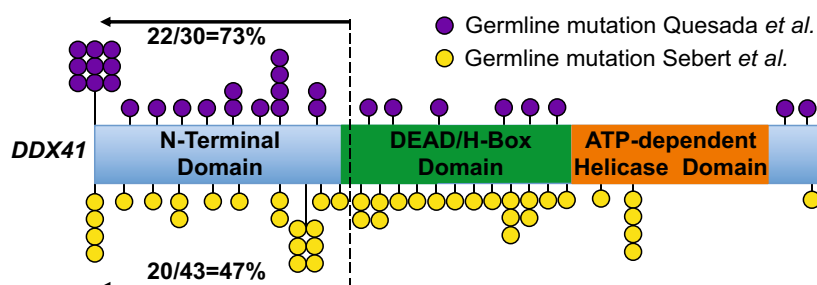


Fig. 1. Distribution of germline *DDX41* mutations in Quesada et al. versus Sebert et al. cohorts. Each dot represents the position of a germline *DDX41* mutation. Purple dots represent the mutations described in the Quesada et al. cohort and yellow dots represent the mutations described in the Sebert et al. cohort. In Quesada et al. cohort, germline *DDX41* mutations were located in the N-terminal domain upstream of the DEAD/H-Box Domain in 73% of their cohort, as compared to just 47% in the Sebert et al. cohort.

function of *DDX41* thereby acting as a 'second hit' that decreases *DDX41* functionality and predisposing individuals to myeloid neoplasm. The most common somatic *DDX41* mutation, R525H, occurs in the Helicase C-terminal domain and is thought to dampen ATPase activity and subsequent helicase activity of *DDX41* and therefore may act as a hypomorph, while maintaining other functions of *DDX41* to allow for cell viability [18]. Finally, it should be noted that biallelic loss-of-function germline mutations of *DDX41* were not found in patients, suggesting that complete loss of *DDX41* is incompatible with life [12]. However, a case report identified compound heterozygous germline missense *DDX41* mutations (P321L and G313R) in two siblings that have neurological deficits [19]. These findings suggest that these mutations do not result in full loss-of-function of *DDX41* and instead cause hypomorphic function, and that *DDX41* may play a role in neurodevelopment and/or psychomotor development, though further investigation is required. Ongoing studies are still elucidating whether germline and somatic mutations play similar or divergent roles in MDS pathogenesis.

Clinical and prognostic features of *DDX41*-mutated myeloid malignancies

More recently, the clinical and prognostic features associated with *DDX41*-mutated myeloid malignancies have been investigated [20–22]. In 2019, two separate articles examined characteristics associated with *DDX41*-mutated myeloid neoplasms. The first study identified 34 germline *DDX41* mutant patients presenting with myeloid neoplasms [17]. These patients presented with higher risk disease later in life with a significant co-occurrence of *DDX41* variants with *TP53* mutations. Additionally, they found a significantly higher incidence in males (3 : 1). All of these findings are in concordance with the data presented by Polprasert et al. in 2015. The second study identified 43 patients with *DDX41* mutations resulting in myeloid neoplasms and focused on 33 patients with causal variants [23]. Similar to the previous two studies, they reported a higher age of presentation and male predominance; however, in contrast to these studies, they also observed relatively favourable clinical outcomes with median overall survival in *DDX41* mutated haematological malignancies versus *DDX41* wild-type matched controls being 5.2 versus 2.7 years (though this was not statistically significant) [23]. This discrepancy may be due to the fact that in the previous two studies *DDX41* mutations were associated with *TP53* mutations, which are associated with inferior overall

survival [24,25], while in the report by Sebert et al. only 6% of their cohort had concurrent *TP53* mutations (note: type of *TP53* mutations not specified). Additionally, the germline *DDX41* mutations identified in Quesada et al. are more likely loss-of-function based on their positioning in the N-terminal domain, versus in Sebert et al. the germline mutations are more spread throughout the entire gene, suggesting that some of them may not result in complete loss-of-function (Fig. 1). This discrepancy suggests that the germline *DDX41* mutations identified in Quesada et al. versus Sebert et al. cohorts may not be equivalent, a finding that warrants further investigation. In a pooled qualitative analysis looking at 277 patients from 20 studies between 2015 and 2021, the incidence of any kind of *DDX41* mutation in myeloid neoplasms was 3.3%, occurring in 80% males, with *Additional Sex Combs Like 1* and *TP53* being the most frequent concomitant somatic mutations [26]. Based on these studies, understanding the effects of *DDX41* mutational status on MDS/AML prognosis and treatment is still an area of exploration. We will delve deeper into how concurrent *TP53* mutations within *DDX41* mutant MDS can mechanistically facilitate disease progression in the second last main heading of this review.

Both mutational status and *DDX41* expression levels correlate with a distinct MDS disease phenotype. Weinreb et al. compared clinical parameters in patients with low versus high *DDX41* mRNA levels as quantified by microarrays in CD34+ HSPC bone marrow cells from MDS patients [27,28]. They found that patients with lower *DDX41* levels in their HSPC has a poorer overall survival, higher bone marrow blast counts, and lower white blood cell and platelet counts compared to patients with higher *DDX41* levels, results that are largely similar to the previously reported Polprasert et al. study [12]. The most recent assessment of the molecular International Prognostic Scoring System (IPSS) for MDS was largely in agreement with these studies showing an association of high blast percentage and risk of AML progression with *DDX41* mutational status, although their analysis indicated an overall favourable prognosis for these patients [29].

In contrast to these findings in MDS, recent studies examined how *DDX41* mutational status in AML correlated with clinical features and therapeutic responses finding that patients with pathogenic loss-of-function germline variants had low blast counts, low white blood cell counts, normo- or hypocellular marrow, normal cytogenetics, few somatic mutations, and an overall favourable prognosis and therapeutic responsiveness [30,31]. *DDX41* mutations were also recently identified as a significant entity within acute

erythroleukaemia [32]. *DDX41* mutations were also seen in idiopathic cytopenia of undetermined significance [15], lymphocytic leukaemias [33] and multiple myeloma [33]. Additional large cohort studies of other haematologic neoplasms with high occurrence of *DDX41* germline mutations is needed to understand the predictive value of these mutations with disease burden, outcome and survival.

Treatment modalities that specifically target *DDX41*-mutated MDS/AML have not been reported in the literature. However, Polprasert et al. identified that *DDX41*-mutated MDS/AML had a much greater response rate to lenalidomide compared to wildtype MDS/AML, and that low *DDX41* expression correlated with positive lenalidomide response rates [12]. Subsequently, successful treatment of MDS with germline *DDX41* mutations with lenalidomide has been reported in several case reports [34]. Lenalidomide has been shown to be efficacious in the treatment of MDS with chromosome 5q deletion (del(5q) MDS) [35]. Response to lenalidomide in del(5q) MDS is mechanistically driven by recruitment of casein kinase 1A1 (CSNK1A1) to the ubiquitin ligase Cereblon resulting in its degradation. This works because CSNK1A1 is a 5q deleted gene and produced in lower quantities. Haploinsufficiency of CSNK1A1 confers a growth advantage leading to clonal expansion in in del(5q) MDS but furthering lower levels of CSNK1A1 is lethal to MDS cells [36,37]. Although *DDX41* is also present on chromosome 5 (though not in the common 5q deleted region), the underlying molecular and cellular mechanisms that contribute to the efficacy of lenalidomide in del(5q) versus *DDX41*-mutated MDS may be similar or distinct and requires substantial investigation.

DDX41 functions and their roles in haematological malignancies

Discovery of DDX41 in Drosophila

DDX41 was initially discovered in *Drosophila* and named Abstrakt [38], which is 66% identical and 80% similar to human DDX41 at the amino acid level [39], in the late 1990s. Loss of *abstrakt* led to Bolwig nerve fasciculation defects [40]. Abstrakt is essential for *Drosophila* life, with mutant embryos dying during gastrulation with numerous defects including morphological changes, elevated apoptosis and RNA mislocalisation [41]. In 2000, a report mentioned how *abstrakt* mutants develop 'melanotic' tumours and identified *abstrakt* as a homologue to a DEAD-Box RNA helicase family member [38]. Abstrakt was also shown to play

roles in post-transcriptional regulation, by modulating Inscuteable [42] and Sorting Nexin-2 [43] protein levels. Finally, while studying Abstrakt in the context of Sorting Nexin-2, it was shown that the N-terminal domain of Abstrakt allows the protein to localise to the nucleus and that Abstrakt can localise to both the nucleus and the cytoplasm [43].

DDX41 in the regulation of inflammatory signalling

Additionally, DDX41 has been implicated in the regulation of inflammatory signalling, though the directionality of how it regulates inflammatory signalling in different cellular contexts is far from resolved (Fig. 2). In mammalian cells, DDX41 was initially characterised as an intracellular DNA sensor in the context of infection [44]. Zhang et al. performed a small interfering RNA screen on all 59 members of the DEAD-Box helicase family in order to identify a member that led to the reduction in inflammatory response to DNA (poly(da:dT)) transfection [44]. DDX41 was the only member that when knocked down reduced interferon-beta (IFN- β) protein levels significantly in this context, and was also shown to reduce IFN- β level in DNA viruses-infected myeloid dendritic cells or human monocytes [44]. This function was shown to be specific to DNA and DNA viruses, as knockdown of DDX41 did not alter inflammation when RNA or RNA viruses were present. Additionally, DDX41 senses the bacterial second messengers cyclic-di-guanosine monophosphate and cyclic-di-adenosine monophosphate, leading to a type I IFN immune response [45]. These DDX41-mediated IFN responses are mediated *via* the Stimulator of interferon genes (STING also known as TMEM173, MITA, MPYS, or ERIS)- TANK-binding kinase 1 inflammatory pathway [44,45].

Several other proteins have been identified that also interact with DDX41 and regulate its role as an immune sensor. TRIM21 is an E3 ligase which interacts with DDX41 and ubiquitinates it at positions Lys9 and Lys115 [46]. TRIM21 overexpression promotes DDX41 ubiquitination and degradation and diminished type I IFN signalling in response to dsDNA, while deficiency of TRIM21 reciprocally results in enhanced type I IFN response [46]. Bruton's tyrosine kinase (BTK) also interacts with DDX41 and phosphorylates it at positions Tyr 364 and Tyr414, which are critical for DDX41 to bind dsDNA and STING [47]. Unlike TRIM21, deficiency of BTK results in reduced IFN- β production by downstream components of the DDX41-STING pathway in the context of dsDNA [47]. In combination, these proteins

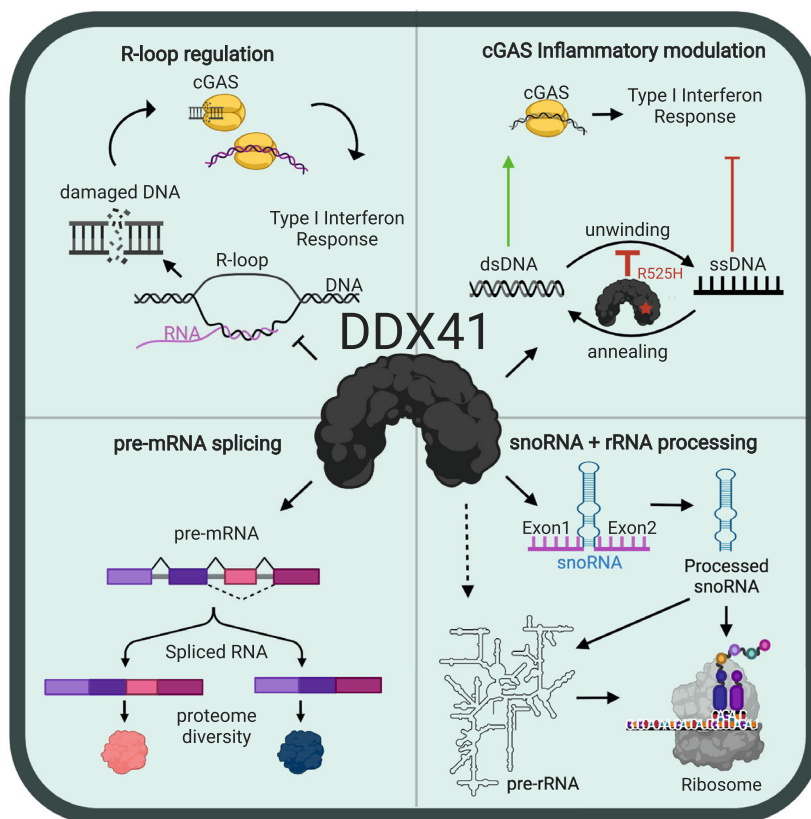


Fig. 2. Cellular activities of DDX41. DDX41 has diverse functions in the cell. Alterations in its role in R-loops regulation, cGAS inflammatory modulation, pre-mRNA splicing, and snoRNA/pre-rRNA processing have been implicated in haematologic dysfunction. *R-loop regulation*: Loss of DDX41 promotes R-loop accumulation and DNA damage resulting in cGAS pathway activation, elevated type I interferon gene expression, and increased numbers of HSPC. *cGAS inflammatory modulation*: DDX41 regulates cGAS activation through unwinding and annealing activities on dsDNA and ssDNA, respectively. The DDX41^{R525H} mutant has reduced unwinding activity but retains annealing activity, causing excessive overactivation of the innate immune response which can contribute to MDS pathogenesis. *Pre-mRNA splicing*: DDX41 is a component of the spliceosome. Loss of DDX41 can affect alternative splicing and change the proteome. *snoRNA + rRNA processing*: DDX41 loss affects snoRNA processing which can alter pre-rRNA processing and ribosomal assembly resulting in defects in protein synthesis. Aberrations of pre-mRNA splicing or protein translation can contribute to the development of haematopoietic dysfunction. Created in Biorender.

regulate DDX41 to make sure it is not promoting an overproduction of type I IFN which may result in autoimmunity.

In apparent contradiction to the studies above, recent studies have shown that loss of DDX41 may actually promote increased inflammatory signalling. Weinreb et al. investigated how insufficiency of *ddx41* in zebrafish influenced gene expression in HSPCs and reported that the expression of type I IFN genes was upregulated in *ddx41* mutant HSPCs [27]. This was attributed to diminished *ddx41* levels promoting R-loop accumulation, subsequent cGAS-STING pathway induction, and elevated nuclear factor-kappa B (NF-κB) signalling (Fig. 2). This finding has been replicated in human cells as well, with Mosler et al. showing that: (a) DDX41 binds to R-loops, unwinds R-loops *in vitro* and prevents R-loop accumulation *in vivo* and

(b) DDX41 knockdown cells show increased inflammatory/NF-κB signalling [48]. Their findings indicated an indirect effect of DDX41 and R-loops on inflammatory gene expression as neither were observed to be bound or enriched in these genes. These results are consistent with the model from Weinreb et al. that RNA:DNA hybrids or damaged DNA trigger inflammatory signalling *via* cGAS-STING pathway activation and help provide substantial corroborative evidence that R-loop accumulation caused by DDX41 deficiency leads to upregulation of inflammatory signalling is a conserved mechanism.

Out of context, the initial studies characterising DDX41 in innate immune signalling versus Weinreb et al. and Mosler et al. findings are inconsistent with one another. Why does loss of DDX41 lead to increased and decreased type I IFN signalling? We

believe that this discrepancy is due to differences in cellular context and potential pleiotropic functions of DDX41. While expression of DDX41 is ubiquitous, the studies identifying DDX41 as a cytosolic innate immune sensor were largely performed in myeloid dendritic cells or monocytes [44,45,49]. Humans with *DDX41* germline mutations carry these alterations in all cells. Similarly, the zebrafish *ddx41* mutants examined had ubiquitous loss-of-function mutations suggesting that DDX41 could have distinct immune modulatory roles depending on cell type and context. Moreover, the interaction between the pleiotropic cellular functions of DDX41 and inflammatory signalling is still mechanistically nascent and require further exploration.

A new study provides some insight on this point. Singh et al. discovered that DDX41 and cGAS can directly interact [50]. They demonstrated that DDX41-null monocytes and macrophage-like cells show diminished activation of cGAS and STING in response to dsDNA (Fig. 2). These results appear in contrast to the above studies, however they also showed that expression of the hypomorphic DDX41-R525H mutant, which has diminished helicase activity but intact annealing activity, elevated cGAS/STING pathway activation. In sum, their data suggest that the extent of DDX41 loss and the exact function that is diminished can differentially impact the activity of this highly interconnected double-stranded nucleic-acid sensing pathway.

Adding to the complexity is the report that DDX41 might have a different isoform that could have distinct functions [20,51]. A smaller 55 kDa DDX41 isoform arising from translation from a downstream methionine has also been reported. Immunofluorescence analysis demonstrated that unlike the full-length DDX41 protein that is predominantly nuclear, the new short form DDX41 had increased cytoplasmic localisation. While still speculative, it is possible that the differences in innate immune signalling with regard to DDX41 could be explained by these two isoforms as the role of DDX41 as an innate immune sensor is thought to be largely cytoplasmic.

DDX41 in RNA splicing

Beyond immune signalling, DDX41 is implicated in splicing, a process with significant relevance in MDS (Fig. 2) [1]. Polprasert et al. were the first to model the consequences of *DDX41* deficiency in a variety of haematopoietic cells using short hairpin RNAs. Reduction of *DDX41* resulted in enhanced proliferation in both *in vitro* as well as xenograft models, and

also led to reduced differentiation in *DDX41*-deficient cells, suggesting that *DDX41* acts as a tumour suppressor gene [12]. In addition to these cellular traits, they also showed that DDX41 interacts with spliceosomal components and that loss of DDX41 contributed to splicing abnormalities, specifically more avid exon skipping and exon retention [12]. They also compared the protein interactome between *DDX41* WT and R525H mutated samples and observed that the R525H mutation perturbed the interactions of DDX41 with U2 and U5 spliceosomal components [12]. Ma et al. demonstrated a requirement for DDX41 in murine HSPCs with significant changes to splicing landscape [52]. Weinreb et al. encountered similar splicing abnormalities in *ddx41* zebrafish HSPCs and red blood cells, suggesting conservation in DDX41 function in splicing regulation [27,53]. In particular, immune system-related genes, including those in the NF κ B pathway, were among the top pathways enriched in the alternatively spliced transcripts, suggesting this could be another means through which DDX41 modulates inflammatory signalling [27]. However, functional assessments that decipher if and how these splicing abnormalities could contribute to DDX41-deficient haematological abnormalities is lacking in all studies to date.

Additional studies have explored the impact of the DDX41 R525H mutant. In contrast to the results with knock down of *DDX41*, Kadono et al. showed that DDX41 R525H-overexpressing cells had decreased proliferation compared to WT DDX41-transduced cells [51]. They attributed this decreased proliferation in DDX41 R525H cells to defects in pre-rRNA processing *via* reduced helicase activity, which inhibits cell cycle progression *via* activation of the MDM2-RB-E2F axis (Fig. 2) [51]. This study provides mechanistic insights on the effect of the DDX41 R525H mutation in general but did not assess what occurs in the haematopoietic tissue. Chlon et al. established *in vivo* mammalian models of DDX41 R525H and DDX41 loss to understand how these mutations affected haematopoiesis [54]. They observed that similar to complete loss of DDX41 (*DDX41*^{-/-}), cells with one loss-of-function allele and one *DDX41* R525H mutant allele (referred to as *DDX41*^{-/R525H}) resulted in complete bone marrow failure in mice. Similar to Kadono et al. who showed that DDX41 plays a role in ribosomal biogenesis, Chlon et al. establish that DDX41 plays a role in snoRNA processing that is critical for ribosome biogenesis and proper HSPC function, and that this defect is seen in both *DDX41*^{-/-} and *DDX41*^{-/R525H} HSPCs (Fig. 2) [54]. However, some differences in *DDX41*^{-/R525H} and *DDX41*^{-/-} were

observed under specific transplantation contexts. When minor clones of *DDX41*^{-R525H} or *DDX41*^{-/-} were transplanted with a majority of *DDX41*^{+/-} bone marrow cells, the R525H mutant cells but not the null cells caused haematologic abnormalities in aged *DDX41*^{+/-} cells. These data along with the defined biochemical alterations of DDX41 R525H suggests this missense mutant imposes distinct features on the bone marrow milieu that can contribute to disease formation. The authors suggest an enticing theory that these minor biallelic mutant clones undergo premature cell death that contributes to the cytopenias observed in DDX41 mutant MDS [54]. The evidence provided by these studies suggests that the R525H somatic mutation in *DDX41* results in hypomorphic function of the protein. However, understanding how other somatic mutations effect the functions of DDX41 are still at their early beginnings.

Interplay between *TP53* and *DDX41* mutations in myeloid neoplasms

TP53 mutations are one of the top co-occurring somatic mutations in *DDX41*-mutated myeloid neoplasms [12,17,26]. Mechanistically, how do *TP53* mutations provide a competitive advantage in the context of *DDX41*-mutated myeloid neoplasms? Though no study to date has definitively answered this question, some findings by Chlon et al. and Weinreb et al. may shed some light on this matter. Chlon et al. showed that the bone marrow failure seen in *DDX41*^{-/-} and *DDX41*^{-R525H} mice was caused by cell cycle arrest and apoptosis [54]. These cellular phenotypes were also observed in the leukaemic stem and progenitor cells derived from *DDX41*^{-/-} and *DDX41*^{-R525H} mice, and they attributed these defects to the lack of mature snoRNAs in Ddx41-deficient cells. Consistent with those findings, Weinreb et al. demonstrated that anaemia developed in *ddx41* mutants caused by Ataxia-telangiectasia mutated (ATM) and Ataxia-telangiectasia and Rad3-related (ATR)-triggered cell cycle arrest in erythroid progenitors coupled with defective differentiation [53]. A common pathway activated by ribosomal stress and ATM/ATR-mediated DNA-damaged response (DDR) is *TP53*, which leads to cell cycle arrest and apoptosis. Thus, these data suggest that loss of DDX41 leads to the activation of *TP53* resulting in cell cycle arrest and apoptosis, contributing to cytopenias and loss of HSPC clone fitness. However, if these DDX41-deficient cells also have concurrent *TP53* mutations, they may be able to avoid activation of DDR pathways, cell cycle arrest, and apoptosis. This would allow for survival and

further clonal selection that could allow for malignant transformation to occur.

Another mechanistic connection that may explain the selection of *TP53* mutations in *DDX41*-mutated neoplasms is genomic instability caused by R-loop accumulation. Weinreb et al. showed that R-loops accrued in Ddx41 deficient cells, which promoted HSPC expansion in *ddx41* loss-of-function zebrafish [27]. Many aspects of this model such as elevated R-loop levels and pro-inflammatory gene expression, were conserved in human cells with *DDX41* knock-down [27]. Mosler et al. further established DDX41 as a *bona fide* regulator of R-loops in human cells, by identifying it as a component of the R-loop proximal proteome and showing that DDX41 can suppress R-loop accumulation and consequent genomic-instability in human cells [48]. Their findings support a model through which DDX41 insufficiency-driven R-loop accumulation and genomic instability indirectly promote inflammatory signalling [48]. This mechanism could allow *DDX41*;*TP53* double mutant cells to reduce the negative consequences of the DDR pathway (such as cell cycle arrest and apoptosis), while allowing for increased inflammatory signalling that may drive clonal selection and ultimately leukemic progression. Deciphering how these two mutant factors synergise could guide more effective therapeutic strategies to eradicate these aggressive clones in myeloid neoplasms.

Conclusions and future perspectives

Since the discovery of *DDX41* mutations in MDS, there have been great strides in the field characterising DDX41's biological functions and role in the pathogenesis of haematologic malignancies. However, many questions remain unanswered. Why do patients with germline DDX41 mutations develop haematopoietic disease in the sixth and seventh decade of life as opposed to in their childhood or late teens and why is it more prevalent in males? In the realm of clinical characteristics, why does DDX41 mutational status generally confer a good prognosis in MDS, except when co-occurring with *TP53* mutations? Since DDX41 germline mutations are present in every cell in the body, why are patients only prone to haematologic malignancies, and what dictates if lymphoid or myeloid leukaemia will arise? Furthermore, do mutations in niche cells also cause dysregulation of the bone marrow microenvironment and could this contribute to subsequent leukaemogenesis? Another major conundrum is what drives the selection of second-hit mutations in DDX41 that paradoxically seem to decrease cellular fitness. Hopefully these and similarly

unexplored questions will be answered in the years to come.

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Data Availability

Not applicable.

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DDX41-associated susceptibility to myeloid neoplasms

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Abstract:

Deleterious germline *DDX41* variants confer risk for myeloid neoplasms (MNs), and less frequently to lymphoid malignancies, with autosomal dominant inheritance and an estimated prevalence of 3% among MNs. Germline *DDX41* variants include truncating alleles that comprise about two thirds of all alleles; missense variants located preferentially within the DEAD box domain; and deletion variants. The identification of a truncating allele on tumor-based molecular profiling should prompt germline genetic testing, since virtually all such alleles are germline. Somatic mutation of the wild-type *DDX41* allele occurs in about half of MNs with germline *DDX41* alleles, typically in exons encoding the helicase domain and most frequently as R525H. Several aspects of deleterious germline *DDX41* alleles are noteworthy: (i) Certain variants are common in particular populations; (ii) MNs develop at older ages typical of *de novo* disease, challenging the paradigm that inherited cancer risk always causes disease in young people; (iii) Despite equal frequencies of these variants in men and women, men progress to MNs more frequently, suggesting a gender-specific effect on myeloid leukemogenesis; and (iv) Individuals with deleterious germline *DDX41* variants develop acute severe graft versus host disease after allogeneic hematopoietic cell transplantation with wild-type donors more than others unless they receive post-transplant cyclophosphamide, suggesting a pro-inflammatory milieu that stimulates donor-derived T-cells. Biochemical studies and animal models have identified *DDX41*'s ability to interact with double stranded DNA and RNA:DNA hybrids with roles in mRNA splicing, rRNAs/snoRNAs processing, and modulation of innate immunity, disruption of which could promote inflammation and drive tumorigenesis.

Conflict of interest: COI declared - see note

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DDX41-associated susceptibility to myeloid neoplasms

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Abstract

Deleterious germline *DDX41* variants confer risk for myeloid neoplasms (MNs), and less frequently to lymphoid malignancies, with autosomal dominant inheritance and an estimated prevalence of 3% among MNs. Germline *DDX41* variants include truncating alleles that comprise about two thirds of all alleles; missense variants located preferentially within the DEAD box domain; and deletion variants. The identification of a truncating allele on tumor-based molecular profiling should prompt germline genetic testing, since virtually all such alleles are germline. Somatic mutation of the wild-type *DDX41* allele occurs in about half of MNs with germline *DDX41* alleles, typically in exons encoding the helicase domain and most frequently as R525H. Several aspects of deleterious germline *DDX41* alleles are noteworthy: (i) Certain variants are common in particular populations; (ii) MNs develop at older ages typical of *de novo* disease, challenging the paradigm that inherited cancer risk always causes disease in young people; (iii) Despite equal frequencies of these variants in men and women, men progress to MNs more frequently, suggesting a gender-specific effect on myeloid leukemogenesis; and (iv) Individuals with deleterious germline *DDX41* variants develop acute severe graft versus host disease after allogeneic hematopoietic cell transplantation with wild-type donors more than others unless they receive post-transplant cyclophosphamide, suggesting a pro-inflammatory milieu that stimulates donor-derived T-cells. Biochemical studies and animal models have identified *DDX41*'s ability to interact with double stranded DNA and RNA:DNA hybrids with roles in mRNA splicing, rRNAs/snoRNAs processing, and modulation of innate immunity, disruption of which could promote inflammation and drive tumorigenesis.

Clinical Observations (Table 1)

Myeloid neoplasms (MNs) with deleterious germline *DDX41* variants constitute an autosomal dominant familial myelodysplastic syndrome (MDS)/acute myeloid leukemia (AML) disorder as described by the World Health Organization ¹, occurring at an estimated prevalence of 3% among all MNs regardless of the family history ². As such, *DDX41* is the most common germline predisposition gene leading to MNs in adults. Importantly, the majority of cases with this disorder report no family history of hematopoietic malignancy (HM) at diagnosis ²⁻⁷. After the initial descriptions ^{8,9}, germline and somatic *DDX41* mutations have garnered the attention of hematologists throughout the world, identifying both deleterious and benign variants and unique clinical features of the MNs that develop in these individuals ^{2-7,10-12}. Although a complete understanding of the disease risk and prognostication is lacking currently, accumulated genetic data afford informed clinical decisions for these patients and their families.

DDX41 variant types

DDX41-mutated myeloid neoplasms define a unique clinical subtype that is distinct from wild-type ones ². Among *DDX41* mutations identified in thousands of MNs, more than 80% are germline in nature and about 15% are somatic ². Deleterious germline *DDX41* variants include: truncating alleles, which comprise about two thirds of such variants; missense variants encoding changes within the DEAD box domain; and deletions (Figure 1) ^{2,5,13-15}. Interestingly, very rare congenital cases caused by bi-allelic deleterious germline variants have been reported ¹⁶. When molecular profiling panels for MNs include *DDX41*, the identification of a *DDX41* variant should prompt further assessment. Because virtually all truncating alleles are germline, such alleles should prioritize that individual for germline genetic testing. Increasingly, germline missense *DDX41* variants are recognized as deleterious ², so these should also prompt further testing. Importantly, the c.3G>A, p.M1? start loss allele, one that to date has only been seen as a germline allele, often gets detected at a variant allele frequency (VAF) of about 30% due to a

fairly common polymorphism that results in decreased binding of PCR primers made according to the standard DNA sequence. Therefore, the identification of this allele at VAFs in this range should also prompt proper germline testing.

As with other cancer predisposition genes, *DDX41* variants within MNs are often bi-allelic, with somatic mutations being detected in about half of the cases with germline *DDX41* predisposition variants ². When they occur, missense somatic mutations are mainly found within the helicase domain ^{2-8,10-12}. Among somatic *DDX41* mutations, R525H is identified in 80% of MNs; G530D/S in 9%, P321L in 7%, and T227A/M in 3%; with less than 5% being truncating alleles ². For individuals in whom only somatic *DDX41* mutations are identified within MNs, testing for germline risk alleles should include the capacity to detect large deletions of multiple exons to avoid missing a deleterious germline *DDX41* allele ².

Population distribution of *DDX41* variants

Deleterious germline *DDX41* variants have been reported throughout the world, including from Europe, the United States, Australia, the United Kingdom, France, Japan, Korea, and Thailand (Figure 2) ^{3-11,17-21}. Certain variants are more frequent within particular populations. For example, the c.3G>A, p.M1? and c.415_418dup, p.D140Gfs*2 alleles are more common in people with Northern European descent, whereas the c.1496dup, p.Ala500Cysfs*9 allele is more frequent in Japanese and Korean individuals, suggesting that these are founder variants. With the availability of large-scale public sequencing databases of healthy people, several novel rare truncating *DDX41* variants can be seen, such as in South America, Africa, and India, which likely represent additional germline variants more common in those populations (Figure 2, olive font). We look forward to germline testing within those populations for confirmation of those alleles as germline variants. Most of the germline variants are not found in both Asian and European populations, but both Asian and European variants are identified in people living in the United Kingdom and the United States, probably because of immigration from Asian areas.

For this reason, enrichment of germline variants should be calculated from patients with MNs versus healthy controls while controlling for the genetic background. Recently, the enrichment of Asian pathogenic variants was assessed by comparing more than 4,000 MNs to more than 20,000 healthy controls within Japanese individuals, yielding an enrichment of deleterious germline *DDX41* variants with an odds ratio (OR) of 10.6 (95%CI: 4.5-22.8)².

Age distribution MNs derived from deleterious germline *DDX41* variants

Deleterious germline *DDX41* variants challenge the notion that inheritance of a cancer predisposition allele incites tumorigenesis at a younger age. Typically, germline *DDX41*-mutated MNs develop with long latency at an average age of about 68 years old (yo), which is similar to *de novo* AML^{3-12,14,17,19,20}. For this reason, age of onset of MNs should not be a criterion for prioritizing germline genetic testing.

MN penetrance

Importantly however, the penetrance of MNs derived from deleterious germline *DDX41* variants has not been established²². Because such information is necessary for appropriate genetic counseling, researchers have used the Kin (first-degree relatives) cohort method recently to estimate the penetrance of *DDX41*-mutated myeloid neoplasms, as was performed for *BRCA^{mut}*-associated breast cancer. This calculation of disease incidence considers competing risks associated with other causes of death using the Fine-Gray model, which theoretically attributes more precise risks typically in older populations. For deleterious *DDX41* germline variants, the risk of any myeloid neoplasm is almost negligible before 40yo, but rapidly increases to 49% by the age of 90².

Gender distribution of MNs derived from deleterious germline *DDX41* variants

Several groups have demonstrated that men with deleterious *DDX41* variants are more likely to develop MNs than women with such alleles^{3,12} despite equal frequencies of pathogenic germline *DDX41* variants in men versus women, suggesting a gender-specific effect on myeloid leukemogenesis. Male gender is more frequent in *DDX41*-mutated cases compared to wild-type ones, with an OR 1.7-3.1. Accordingly, enrichment of *DDX41* pathogenic variants is more remarkable in men (OR 20.7) than in women (OR 5.0), although both OR are significant compared to healthy individuals. The penetrance is also (approximately 4 times) higher in men compared to women².

Disease phenotypes and co-occurring secondary mutations

Within specific types of MNs, deleterious germline *DDX41* variants are found in: 3-5% in MDS, 5-9% in secondary AML, 1-2% in MDS/myeloproliferative neoplasm (MPN), and <0.5% in MPN. Compared to individuals without deleterious germline *DDX41* variants, MDS and AML patients are 10 times more enriched for such alleles². The most common preceding diagnosis in those who developed secondary AML was MDS². Patients with such *DDX41* alleles who develop MNs usually present with leukopenia and hypocellular bone marrow with prominent erythroid dysplasia. An initial sequencing study of erythroleukemia (AEL) revealed that the category of *DDX41* mutant cases is a subset with distinct genetic profile²³, but such variants were less frequent in another study of AEL²⁴. Among MDS subtypes, refractory anemia with excess blasts is the most frequent. In accordance with the phenotypic features, deleterious germline *DDX41* variants are significant risk factors of leukemic evolution²⁵, which is confined to truncating alleles, but very rarely observed in the cases with missense variants². Despite higher risk phenotypes, the most frequent cytogenetic pattern in the MNs that develop is a normal karyotype, confirmed in phenotype-matched subgroup analysis^{2,8,11,12}. Most frequent co-occurring somatic mutations within the MNs occur in *ASXL1*, *CUX1*, *TP53*, *DNMT3A*, and *TET2*

^{3,8,10,26}, rather than mutations in genes typically associated with secondary AML, such as *NRAS*, *FLT3*, and *NPM1*. Among these, only *CUX1* mutations are preferentially observed in *DDX41*-mutated cases compared to wild-type ones ². Of note, the clone sizes of co-occurring somatic mutations are smaller than somatic *DDX41* mutations, suggesting that their acquisition occurs after the somatic *DDX41* mutation ². Although MNs are the overwhelming majority of HMs diagnosed in these individuals, lymphoid malignancies have been observed, also in the adult age range ^{3,4,9,19,27}. Solid tumors have also been observed in individuals with deleterious germline *DDX41* variants, although the contributions of these variants to tumorigenesis is unclear.

Cellular Functions of DDX41

DDX41 is a member of the DEAD-box RNA helicase family, although unlike the name implies, it interacts with double stranded DNA (dsDNA) and RNA:DNA hybrids ²⁸. Expressed widely across many tissues ²⁹, DDX41 is found in both the cytoplasm and nucleus with distinct and pleiotropic functions in each cellular compartment (Figure 3) ³⁰. Our understanding of how DDX41 insufficiency contributes to the development of HMs is still in its infancy.

DDX41 as a splicing regulator

DDX41 is a metazoan-specific spliceosomal component that is part of the catalytically active Complex C spliceosome (Figure 3) ⁸. In line with this connection, spliceosomal components are among the top DDX41-interacting proteins identified in both *Caenorhabditis (C.) elegans* and human cells (Table 2) ^{8,31}. Several groups have identified significant alternative splicing changes in *DDX41* mutant or knockdown hematopoietic (HSPC, erythroid progenitors, and leukemic cells) and non-hematopoietic cell types across multiple organisms (*C. elegans*, zebrafish, mice, and human) indicating high conservation for DDX41 in splicing regulation ^{8,31-34}. The functional significance of the specific splicing alterations identified in these studies remains

untested. However, one study showed patients with deleterious *DDX41* variants rarely have somatic mutations in spliceosomal components⁸. This negative selection for mutational co-occurrence supports the model that the function of DDX41 in splicing is critical for hematopoiesis. Consistent with the mutual exclusivity observed in patients, genetic interaction studies in *C. elegans* *sacy-1* (*DDX41* homolog) mutants revealed synthetic lethality with splicing factor mutants³¹. Combined, these findings indicate that although the precise function of DDX41 in pre-mRNA processing is unknown, it is essential for cellular survival.

DDX41 in rRNA and snoRNA processing

In addition to a role in pre-mRNA splicing, DDX41 affects processing of RNAs critical for ribosome assembly and function (Figure 3)^{30,35}. Human cells overexpressing a common somatic hypomorphic mutant form of DDX41 (R525H) displayed defects in pre-ribosomal RNA (pre-rRNA) processing, which correlated with diminished cellular proliferation³⁰. Pre-rRNAs are transcribed and processed in the nucleolus where they are then assembled into ribosomes. Part of their processing and maturation is mediated by small nucleolar RNAs (snoRNA). Defects in snoRNAs will alter pre-rRNA cleavage and pseudouridylation required for proper ribosome functioning³⁶. As an evolutionarily conserved coupling mechanism, most snoRNAs are located within introns of ribosome protein genes³⁷. Thus, the first step in snoRNA processing requires proper splicing. A recent study demonstrated that DDX41 could be a previously unrecognized player involved in snoRNA processing³⁵. *Ddx41*-deficient murine hematopoietic stem and progenitor cells (HSPCs) displayed a marked increase in snoRNA levels. Moreover, identification of DDX41-interacting RNAs revealed a strong enrichment for snoRNAs suggesting the effect could be direct, potentially via removal of snoRNA-containing introns via splicing regulation. Consistent with this function being important for hematopoiesis, knockdown of several snoRNAs in murine HSPCs diminished cell viability and differentiation³⁵. Currently, it remains incompletely understood if the effect of DDX41 on ribosome biogenesis is via direct

regulation to both snoRNAs and pre-rRNAs or due to snoRNA defects that then alter pre-rRNA processing. Also, the relative contribution of splicing defects in protein coding versus non-coding genes on *DDX41*-mutant hematopoietic pathology is an unanswered question in the field.

DDX41 in innate immunity

DEAD-box helicases also play critical roles in immunity³⁸. This is best characterized in myeloid effector cells such as macrophages and dendritic cells that activate type I interferon (IFN) signaling in response to either infection or exposure to DNA-type pathogen-associated molecular patterns (PAMPs)³⁸. DDX41 is one of the pattern recognition receptors (PRR) that is stimulated by these PAMPs, specifically bacterial cyclic dinucleotides as well as viral dsDNA and RNA:DNA hybrids^{28,39,40}. It is thought that cytoplasmically localized DDX41 initiates the type I IFN signaling cascade starting with the Stimulator of interferon genes (STING, also known as TMEM173, MITA, MPYS, or ERIS), which then activates TANK-binding kinase 1, Interferon response factor 3, and nuclear factor kB (Figure 3)^{39,40}.

HMs, especially MNs, have a strong connection to inflammatory dysfunction⁴¹. Because of the connection of DDX41 to type I IFN, this pathway has been examined in DDX41 mutant hematopoiesis^{32,34,42}. Functionally hypomorphic zebrafish *ddx41* mutants display aberrant HSPC expansion³². Gene expression analysis of *ddx41* mutant compared to normal HSPCs revealed deregulation of type I IFN pathway components and targets. Similar results were observed in human hematopoietic cells upon *DDX41* knockdown⁴². Surprisingly, type I IFN signaling appeared upregulated, not down in these studies. This result is seemingly contradictory with a function of DDX41 as an activator of this pathway, thus another role of DDX41 might be driving this inflammation. Within the nucleus, DDX41 interacts with R-loops, which are structural DNA variants comprised of single stranded DNAs (ssDNAs) and RNA:DNA hybrids (Figure 3)^{42,43}. DDX41 is a helicase that is stimulated by RNA:DNA hybrid binding suggesting it could unwind and destroy these molecules²⁸. In line with this model, both

zebrafish and human cells with insufficient DDX41 levels accumulate RNA:DNA hybrids which results in elevated double-stranded (ds) DNA breaks^{32,42}. Since the discovery of DDX41 as a DNA PRR, new data indicate the main DNA sensor in most cells is cyclic GMP-AMP synthase (cGAS)⁴⁴. cGAS is activated by many infectious and damage-associated stimuli, including dsDNA, RNA:DNA hybrids, micronuclei, and damaged mitochondria among others⁴⁴. Ligand binding to cGAS stimulates its enzymatic activity leading to the production of the second messenger cyclic GMP-AMP (cGAMP), which can bind to several proteins, including STING, leading to an inflammatory signaling cascade that results ultimately in activation of the type I IFN pathway⁴⁴. In the zebrafish *ddx41* mutant model, R-loops and/or the ensuing damaged DNA orchestrated a cGAS-STING-mediated inflammatory response that promoted HSPC expansion³². These data imply that DDX41 regulation of R-loop levels is important in hematopoietic homeostasis.

Other connections between DDX41 and cGAS are emerging. DDX41 binds the bacterial second messengers cyclic-di-guanosine monophosphate (c-di-GMP) and cyclic-di-adenosine monophosphate (c-di-AMP), which are structurally similar to cGAS-produced cGAMP, suggesting cGAS could modulate DDX41 activity³⁹. Additionally, a recent study uncovered a mechanism for DDX41 modulation of cGAS activity⁴⁵. Loss of DDX41 in monocytic cells lowered dsDNA-triggered activation of the cGAS-STING pathway. In contrast, expression of the R525H hypomorphic mutant DDX41, which displays dampened helicase activity with intact dsDNA binding, increased pathway activation (Figure 3). The findings indicate a complex interplay among the nucleic-acid sensing pathways that could have significant impact on hematopoiesis and immunity.

Interplay of DDX41 functions in hematopoiesis

The examination of how the pleiotropic cellular functions of DDX41 impact hematopoiesis and how they crosstalk is in its infancy. The differences among the studies in

various hematopoietic and non-hematopoietic cells could be explained by cell-type selective functions and dependencies. Additionally, DDX41 isoforms may localize to distinct cellular compartments^{9,30}. Full-length DDX41 appears largely nuclear, whereas a shorter isoform with a truncated N-terminus is found predominantly in the cytoplasm³⁰. The relative levels of these two isoforms could alter how a particular cell deploys DDX41. Expression of the corresponding transcripts across hematopoietic cell types and during HSPC development is unknown.

Management Implications

MN Pathogenesis

Deleterious germline *DDX41* variants challenge the accepted paradigm that germline predisposition variants cause cancer at ages younger than expected, since MNs rarely develop in people younger than 40yo and typically in the late 60's^{3-8,10-12}. This inverted age distribution of MNs suggests a unique pathogenesis. Cascade testing in families has identified many relatives who share the deleterious familial *DDX41* allele. These individuals are being followed closely decades before MNs develop, which will allow the natural history and clonal progression of these malignancies to be described. Because we postulate that MNs arise as the sequential acquisition of somatic mutations, beginning with the phenomenon of clonal hematopoiesis (CH), an open question is whether CH precedes the development of MNs in those with deleterious germline *DDX41* alleles. If so, it is possible that the presence of CH represents a molecular clock that marks the initiation of MN development. Future studies will demonstrate whether CH-associated somatic mutations occur within expected genes (e.g., *DNMT3A*, *TET2*, *ASXL1*, and others) or within a gene set unique to germline *DDX41*-associated MNs. Since MNs occur within the expected age range, the role of the pathogenic germline *DDX41* variant throughout most of an individual's lifetime and its impact on any CH that might develop remain a mystery. If these questions could be answered by future studies, we might be able to design specific

interventions that could be instituted decades before MN development to delay or prevent these cancers.

Multiple recent studies have shown that deleterious germline *DDX41* variants are associated with better prognosis than *de novo* MNs^{3,5,12,20,21}. Initially, however, MNs arising in those with a deleterious germline *DDX41* variants were described as having a poor prognosis, likely because cases with somatic deletion of the *DDX41* locus, typically caused by deletion of the telomeric region of chromosome 5q with complex karyotype and biallelic *TP53* mutations, were included in the mutated cohort. Table 1 contrasts initial descriptions of MNs arising in those with deleterious germline *DDX41* variants⁴⁶ to more updated understanding of the features of these malignancies^{3-7,10-12}. A recent detailed study showed that the bone marrow blast percent is higher in *DDX41*-mutated MDS compared to *DDX41*-wild-type MDS. In contrast, the bone marrow blast percent is lower in *DDX41*-mutated secondary AML compared to such cases with wild-type *DDX41*, suggesting that with deleterious germline *DDX41* variants, bone marrow blasts increase until leukemic evolution (at ~20%), but tend not to progress rapidly after that point².

Therefore, the discrepancy between frequent leukemic evolution and better prognosis should be addressed. Another possible explanation of this discrepancy between frequent leukemic evolution and better overall survival is that *DDX41*-mutated cases respond to specific therapy. Early data suggest that patients may respond to lenalidomide⁸, and the most recent study showed that hypomethylating agents might be effective and improve overall survival in *DDX41*-mutant myeloid neoplasms cases (hazard ratios: 0.25-0.60) compared to wild-type *DDX41* ones². This retrospective study also showed that treatment with hypomethylating agents before hematopoietic stem cell transplantation (HSCT) dramatically improved overall survival in *DDX41*-mutated cases compared to wild-type cases, which should be validated by prospective clinical trials.

Implications for allogeneic HSCT

For most individuals with germline predisposition variants, the risk of developing a HM is relieved by allogeneic HSCT using a donor who lacks the deleterious familial allele. However, given the common nature of some germline *DDX41* variants within particular populations, several instances have been described in which allogeneic donors, either related or unrelated, had a deleterious germline *DDX41* variant that was transferred to the recipient during allogeneic HSCT⁴⁷⁻⁵⁰. For this reason, we advocate: (i) the addition of *DDX41* to all molecular profiling panels being used to prognosticate for HMs, since the identification of a truncating *DDX41* allele at VAF >30% is likely to be germline. However, the use of such panels should not replace proper germline testing as these panels generally are unable to detect all types of germline alleles^{51,52}. (ii) testing of all allogeneic stem cell donors for germline predisposition variants including *DDX41*.

We advocate transplantation of individuals diagnosed with MNs in clinical remission when they have deleterious germline *DDX41* variants (*i.e.*, likely pathogenic or pathogenic variants). Like in other germline cancer predisposition disorders, only deleterious germline variants are clinically actionable. Germline variants deemed to be of uncertain significance, likely benign, or benign do not prompt further studies or cascade testing in families unless the variant gets upgraded into likely pathogenic or pathogenic classification with additional functional or segregation data.

Observational studies have shown that after allogeneic HSCT using wild-type donors, individuals with deleterious germline *DDX41* variants suffer more graft-versus-host disease (GVHD) than others^{53,54}. One study showed that patients with deleterious germline *DDX41* variants had more stage 3-4 acute GVHD (38%) compared to those with deleterious *CHEK2* variants (0%), other hereditary HM variants (12%), or patients without such germline variants (9%) unless they had received post-transplant cyclophosphamide as GVHD prophylaxis⁵³. We postulate that individuals with deleterious germline *DDX41* variants have a pro-inflammatory

milieu generated from the germline allele that remains in all of their non-transplanted organs, which stimulates donor-derived T-cells and causes severe GVHD. Based on these observations, we advocate consideration of lower intensity transplantation regimens, optimizing donor selection, and use of post-transplant cyclophosphamide as GVHD prophylaxis when individuals with deleterious germline *DDX41* variants undergo allogeneic HSCT, even when using wild-type donors.

Population screening and monitoring

Because truncating and deleterious germline *DDX41* variants can be identified in screening sequencing databases from populations across the world (Figure 2), we recognize that it is possible to do population-based screening for deleterious germline *DDX41* variants. Currently, we advocate that once an individual is identified as having a deleterious germline *DDX41* variant, baseline blood work, including a complete blood cell count and differential (CBC/diff), and a bone marrow biopsy be performed to test for the presence of cytopenias and/or bone marrow dysplasia, respectively. It is important to establish the degree of bone marrow dysplasia prior to MN progression so that an accurate diagnosis of MDS can be made over time for patients. We advise including molecular profiling that includes *DDX41* and cytogenetic analysis at the time of bone marrow biopsy since the presence of the *DDX41* R525H somatic mutation and/or del(5q) could signal impending or overt MN. The age at which baseline studies are performed and when surveillance monitoring is begun should be decided ideally in collaboration with genetic counselors experienced in hereditary hematopoietic malignancies and discussions that consider the family history and the personal preferences of the affected individuals. Once the baseline studies have been performed, we advise repeating a CBC/diff 3-4 times/year with consideration of an annual bone marrow examination. Evidence for progression toward MN includes identification of somatic mutations and/or karyotypic abnormalities, and some advocate for pre-emptive allogeneic HSCT if such changes are

demonstrated above baseline studies even in the absence of increasing blast burden in the bone marrow. The hope is that future studies will define the molecular progression to MNs for individuals with germline *DDX41* predisposition alleles, allowing us to intercede and slow or prevent MN development without the need for allogeneic HSCT.

Conclusion

Identified in about 3% of MNs, deleterious germline *DDX41* variants constitute the most common germline predisposition disorder causing myeloid, and less often lymphoid, malignancies in adults. Several unique features of these alleles are important for optimal patient management: (i) The vast majority of truncating *DDX41* alleles are germline, and others, like the c.3G>A, p.M1? start loss allele, have only been seen as germline alleles. Therefore, when such alleles are detected on tumor-based molecular profiling platforms, they should prioritize that individual for proper germline testing. (ii) MNs that develop people with deleterious germline *DDX41* alleles occur at ages typical of *de novo* disease, challenging the paradigm that inherited cancer risk always causes disease at young ages. (iii) Men with these alleles progress to MNs more frequently, suggesting a gender-specific effect on myeloid leukemogenesis. (iv) Because certain variants are quite common in particular populations, unrelated allogeneic HSCT donors with these alleles have inadvertently been used, resulting in transfer of a deleterious germline *DDX41* variant into recipients. The presence of these common *DDX41* variants may allow donor-based and/or population-based screening in the future. (v) When wild-type donors are used in allogeneic HSCT of those with deleterious germline *DDX41* variants, post-transplant cyclophosphamide appears to be an effective means of post-transplant GVHD prophylaxis that prevents the otherwise severe GVHD that occurs in these individuals. The exact mechanism(s) by which these variants confer risk to MNs is unclear, but biochemical studies and animal models demonstrate that *DDX41* interacts with double stranded DNA and RNA:DNA hybrids

with roles in mRNA splicing, rRNAs/snoRNAs processing, and modulation of innate immunity, which could lead to an inflammatory milieu that drives tumorigenesis.

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Authorship Contributions

H.M., T.V.B., and L.A.G. wrote and edited the manuscript and its tables and figures.

Conflicts of Interest

L.A.G. receives royalties from UptoDate, Inc. for a co-authored article on germline predisposition to hematopoietic malignancies. H.M. and T.V.B. and report no conflicts of interest.

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Tables and Figure Legends

Table 1. Description of HMs arising from deleterious germline *DDX41* variants in WHO classification versus recent updates.

Topics	WHO classification	Updates by recent studies
Prevalence	1.5% of myeloid neoplasms	High in higher risk MDS /sAML (5-8%), low in MPN (<1%) (Ref #2-10) / 10 times more enrichment in myeloid neoplasms (ref 2)
Germline mutations	Half of all the mutations	70-80%, 2 thirds are truncating (ref 5, 13-15)
Age at onset	Long latency (mean 62 years old)	Long latency (mean 66 years old) (ref 2-12, 14, 17, 19-20)
Male predominance	No description	Yes (1.7-3.1 times), compared with WT cases in any disease phenotype (ref 2-3, 12)
Hematologic findings	Leukopenia/erythroid dysplasia	Hypocellular bone marrow/Higher blast count in MDS (ref 2)
Penetrance	Not fully established	0% of penetrance at 40 years old / 50% life long penetrance (ref 2)
Karyotype	Frequent normal karyotype	Normal karyotype is 2-4 times more frequent, compared with WT cases (ref 2, 8, 11-12)
Concomitant mutations	No description	Mutations in <i>CUX1</i> and <i>GNAS</i> are more frequent compared to WT cases. (ref 2) Mutations in <i>ASXL1</i> , <i>TET2</i> , <i>DNMT3A</i> , and <i>TP53</i> are frequent but the frequencies are not significantly different compared to those in WT cases. (ref 2-3, 8, 26)
Leukemic evolution	No description	Faster leukemic evolution (ref 2, 25)
Prognosis	Poor in cases with somatic deletion	Better in cases with pathogenic variants (ref 2, 3, 5, 12, 20-21)
Therapeutics	Lenalidomide may be effective	Hypomethylating agents may be effective (ref 2)

Table 2. Contrast of phenotypes in animal versus human studies.

	Animal Studies	Human Observations
Effect on HSPCs	Hypomorphic zebrafish <i>ddx41</i> mutants display aberrant HSPC expansion, with deregulation of type I IFN pathway components and targets (ref 32)	Patients present with leukopenia, hypocellular bone marrow, and erythroid dysplasia (ref 2)
	Mouse <i>Ddx41</i> KO HSPC show diminished survival and transplantation capacity (ref 34-35)	
	Mouse HSPCs with <i>Ddx41</i> heterozygous KO show competitive advantage (ref 35).	
	Mice with <i>Ddx41</i> heterozygous KO show age-dependent hematopoietic defects including anemia, thrombocytopenia, and BM hypocellularity (ref 35)	
	Animals with heterozygous KO of <i>Ddx41</i> function do not develop hematopoietic malignancies (ref 32, 34-35)	Patients with deleterious germline <i>DDX41</i> variants develop hematopoietic malignancies, mostly of myeloid lineage, with late onset (ref 2-22)
DDX41-interacting proteins	spliceosomal components [<i>C elegans</i>] (ref 31)	spliceosomal components (ref 8)
		R-loop-interacting proteins (ref 42-43)
Alternative splicing changes in <i>DDX41</i>-mutant or knock-down cells	Increased alternative exon usage, intron retention, alternative 5' and 3' splice site usage [<i>C. elegans</i> , zebrafish, mice] (ref 31-33)	Increased exon inclusion and intron retention (ref 8)
Mutual exclusivity with splicing mutations?	Yes. <i>C. elegans</i> <i>sacy-1</i> mutants show synthetic lethality with germline splicing factor mutants (ref 31)	Yes. Co-occurrence of somatic mutations in genes encoding splicing factors is rare, suggesting that <i>DDX41</i> 's splicing function is critical (ref 8)
Role in ribosome biogenesis (snoRNA and pre-rRNA processing)	<i>Ddx41</i> -deficient HSPCs display increased snoRNA levels (ref 35)	Cells overexpressing <i>DDX41</i> R525H display defects in pre-rRNA processing and had diminished cellular proliferation (ref 30)
		<i>DDX41</i> -interacting RNAs are

		enriched for snoRNAs, suggesting that DDX41 could remove snoRNA-containing introns via splicing regulation (ref 35)
R-loop interactions	Increased RNA:DNA hybrids result from insufficient Ddx41 levels, which induces a cGAS-STING-mediated inflammatory response that directs HSPC expansion [zebrafish] (ref 32)	Increased RNA:DNA hybrids and consequently elevated dsDNA breaks result from insufficient DDX41 levels (ref 42)
cGAS-STING interactions	Murine fetal liver Ddx41-null HSPCs display diminished inflammatory gene expression (ref 34)	Loss of DDX41 in monocytic cells lowered dsDNA-triggered activation of the cGAS-STING pathway. However, expression of DDX41 R525H, with intact dsDNA binding but dampened helicase activity, activates the STING pathway (ref 45)
		Human hematopoietic cells show paradoxical increased type I IFN signaling upon DDX41 knockdown (ref 42)

Abbreviations used: DDX41, DEAD-box helicase 41; KO, knockout; HSPCs, hematopoietic stem and progenitor cells; IFN, interferon; snoRNA, small nucleolar RNA; rRNA, ribosomal RNA; dsDNA, double-stranded DNA; cGAS, cyclic GMP-AMP synthase; STING, stimulator of interferon genes.

Figure Legends

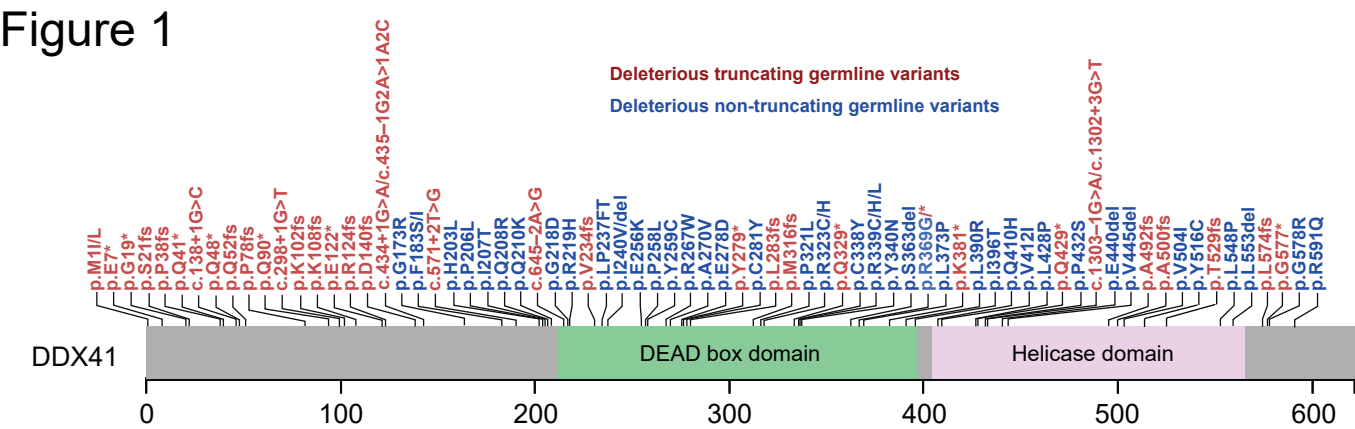
Figure 1. Deleterious DDX41 germline variants that confer risk to MNs. The DDX41 protein schematic is shown, including the DEAD box (green) and helicase (lilac) domains. Truncating variants are shown in red, and missense variants in blue.

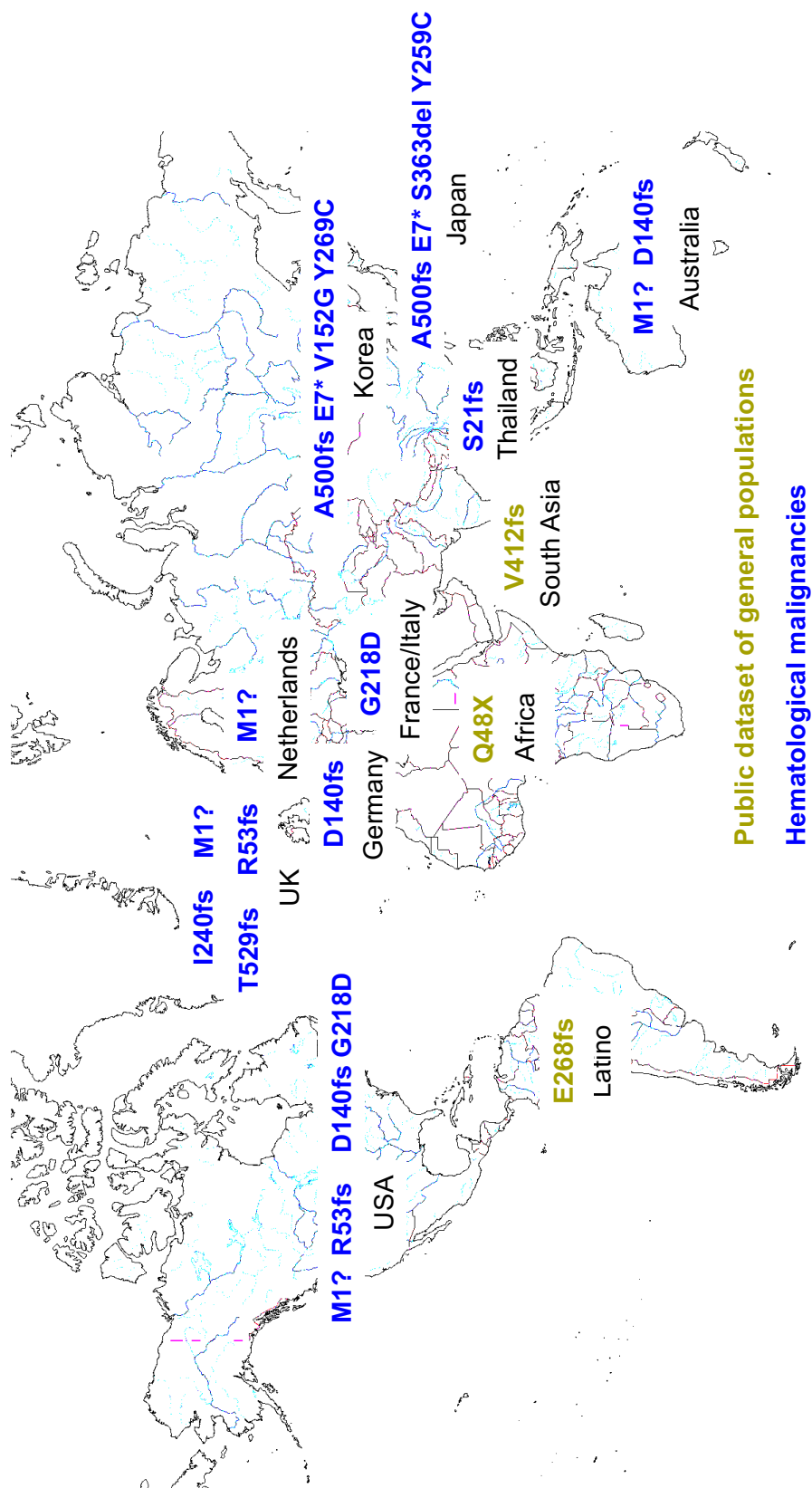
Figure 2. Ethnic diversity of deleterious germline DDX41 alleles. A world map is displayed with common deleterious germline *DDX41* alleles present within particular geographic areas. Standard variant nomenclature is used. Deleterious germline variants identified within HMs are shown in blue font, and those identified from public databases are given in olive font.

Figure 3. Master of many: cellular functions of DDX41. DDX41 localizes to both cytoplasmic and nuclear compartments and plays distinct roles in each location. In the nucleus, DDX41 functions in at least three processes. Via its association with the catalytically-active spliceosome, DDX41 regulates pre-mRNA splicing. Here, it also modulates snoRNA processing, potentially via promoting excision of snoRNA-containing introns found in ribosome-protein genes. DDX41 can also modify pre-rRNA processing, either indirectly via its control of snoRNA processing or perhaps directly via an unknown mechanism. In the nucleus, DDX41 also interacts with R-loops, which are comprised of RNA:DNA hybrids and ssDNAs. DDX41 insufficiency leads to R-loop accumulation and subsequent increased dsDNA breaks that trigger a cGAS-STING-mediated type I interferon (IFN) response. Cytoplasmically localized DDX41 can bind to infection-derived or damaged endogenous dsDNA and then promote STING activation and type I IFN response. Additionally, DDX41 can modulate cGAS activity via its dsDNA unwinding capabilities by altering the relative amounts of dsDNA-to-ssDNA. For example, the R525H DDX41 mutant, which has intact dsDNA binding activity but diminished unwinding activity, can overstimulate cGAS leading to a heightened type I IFN response. Created with

BioRender.com Abbreviations used: DDX41, DEAD-box helicase 41; mRNA, messenger ribonucleic acid; snoRNA, small nucleolar RNA; rRNA, ribosomal RNA; DNA, deoxyribonucleic acid; dsDNA, double stranded DNA; ssDNA, single stranded DNA; cGAS, cyclic GMP-AMP synthase; STING, Stimulator of interferon genes; IFN- interferon.

Figure 1





Master of many: Cellular functions of DDX41

