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

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Abstract

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 characterization 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 signaling, 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 hematologic neoplasms.

Graphical Abstract

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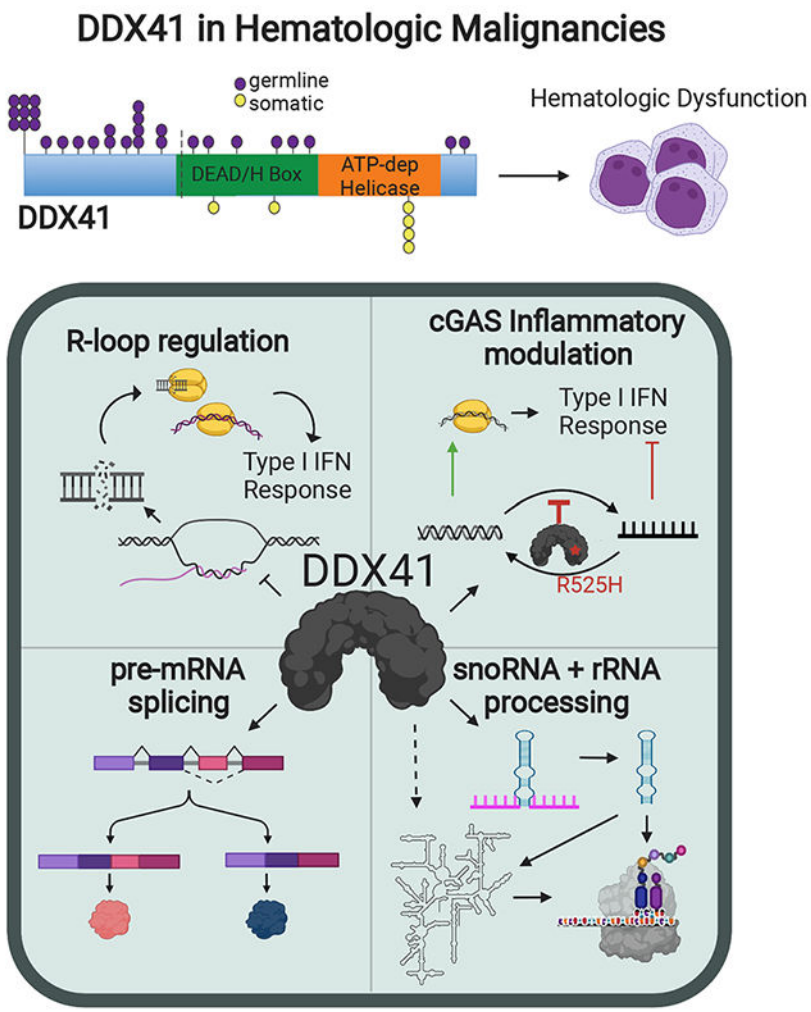
Declaration of interests

The authors declare no competing interests.



The *DEAD-box Helicase 41* (*DDX41*) gene is commonly mutated in inherited and sporadic hematologic malignancies. Germline and somatic mutations diminish DDX41 levels or activity, suggesting that DDX41 inhibition promotes hematologic malignancy. Deficits in R-loop regulation, cGAS inflammatory modulation, pre-mRNA splicing, and snoRNA/rRNA processing by DDX41 have also been implicated in the initiation of hematopoietic dysfunction.

Keywords

DDX41; hematopoiesis; myelodysplastic syndrome; acute myeloid leukemia; inflammation; DEAD-box helicase; R-loops; genomic instability; germline predisposition; cGAS-STING

Introduction

Myelodysplastic syndromes: A clinical overview

Myelodysplastic syndromes (MDS) arise from clonal selection and expansion of aberrant hematopoietic stem and progenitor cells (HSPC) and are characterized by ineffective hematopoiesis and peripheral blood cytopenias [1]. MDS are amongst the most common

hematological malignancies of the elderly, with a proclivity for malignant transformation to Acute Myeloid Leukemia (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 (SF2), which is conserved from bacteria to mammals, and are characterized 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 (Figure 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 hematological malignancies

In 2012, *DDX41* was initially identified as a novel somatically mutated gene in AML via the sequencing of primary and relapsed tumor 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 characterized 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 hematological 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 (Figure 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 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 favorable clinical outcomes with median overall survival in *DDX41* mutated hematological malignancies versus *DDX41* wild-type matched controls being 5.2 vs 2.7 years (though this was not statistically significant) [24]. 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 [25, 26], 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 (Figure 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 2015-2021, the incidence of any kind of *DDX41* mutation in myeloid neoplasms was 3.3%, occurring in 80% males, with *ASXL1* and *TP53* being the most frequent concomitant somatic mutations [27]. 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 [28, 29]. 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 favorable prognosis for these patients [30].

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 favorable prognosis and therapeutic responsiveness [31, 32]. *DDX41* mutations were also recently identified as a significant entity within acute erythroleukemia (AEL) [33]. *DDX41* mutations were also seen in idiopathic cytopenia of undetermined significance (ICUS) [15], lymphocytic leukemias [34], and multiple myeloma [34]. Additional large cohort studies of other hematologic 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 [35]. Lenalidomide has been shown to be efficacious in the treatment of MDS with chromosome 5q deletion (del(5q) MDS) [36]. 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 [37, 38]. 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 hematological malignancies

Discovery of DDX41 in Drosophila

DDX41 was initially discovered in Drosophila and named Abstrakt [39], which is 66% identical and 80% similar to human DDX41 at the amino acid level [40], in the late 1990's. Loss of *abstrakt* led to Bolwig nerve fasciculation defects [41]. Abstrakt is essential for Drosophila life, with mutant embryos dying during gastrulation with numerous defects including morphological changes, elevated apoptosis, and RNA mislocalization [42]. In 2000, a report mentioned how *abstrakt* mutants develop "melanotic" tumors and identified *abstrakt* as a homolog to a DEAD-Box RNA helicase family member [39]. Abstrakt was also shown to play roles in post-transcriptional regulation, by modulating Inscuteable [43] and Sorting Nexin-2 [44] 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 localize to the nucleus and that Abstrakt can localize to both the nucleus and the cytoplasm [44].

DDX41 in the regulation of inflammatory signaling

Additionally, DDX41 has been implicated in the regulation of inflammatory signaling, though the directionality of how it regulates inflammatory signaling in different cellular contexts is far from resolved (Figure 2). In mammalian cells, DDX41 was initially characterized as an intracellular DNA sensor in the context of infection [45]. Zhang *et al.* performed a small interfering RNA (siRNA) 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 [45]. 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 [45]. 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 (c-di-GMP) and cyclic-di-adenosine monophosphate (c-di-AMP), leading to a type I IFN immune response [46]. 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 (TBK1) inflammatory pathway [45, 46].

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 [47]. TRIM21 overexpression promotes DDX41 ubiquitination and degradation and diminished type I IFN signaling in response to double-stranded DNA (dsDNA), while deficiency of TRIM21 reciprocally results in enhanced type I IFN response [47]. 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 [48]. Unlike TRIM21, deficiency of BTK results in reduced IFN-β production by downstream components of the DDX41-STING pathway in the context of dsDNA [48]. In combination, these proteins 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 signaling. 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 [28]. This was attributed to diminished *ddx41* levels promoting R-loop accumulation, subsequent cGAS-STING pathway induction, and elevated NF-κB signaling (Figure 2). This finding has been replicated in human cells as well, with Mosler *et al.* showing that: 1) DDX41 binds to R-loops, unwinds R-loops *in vitro*, and prevents R-loop accumulation *in vivo*, and 2) DDX41 knockdown cells show increased inflammatory/NF-κB signaling [49]. 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 signaling via cGAS-STING pathway activation and help provide substantial corroborative evidence that R-loop accumulation caused by DDX41 deficiency leads to upregulation of inflammatory signaling is a conserved mechanism.

Out of context, the initial studies characterizing DDX41 in innate immune signaling 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 signaling? 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 [45, 46, 50]. 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 signaling 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 [51]. They demonstrated that DDX41-null monocytes and macrophage-like cells show diminished activation of cGAS and STING in response to dsDNA (Figure 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, 52]. 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 localization. While still speculative, it is possible that the differences in innate immune signaling 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 signaling, DDX41 is implicated in splicing, a process with significant relevance in MDS (Figure 2) [1]. Polprasert *et al.* were the first to model the consequences of *DDX41* deficiency in a variety of hematopoietic cells using shRNAs. 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 tumor 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 [53]. Weinreb *et al.* encountered similar splicing abnormalities in *ddx41* zebrafish HSPCs and red blood cells, suggesting conservation in DDX41 function in splicing regulation [28, 54]. In particular, immune system-related genes, including those in the NFκB pathway, were among the top pathways enriched in the alternatively spliced transcripts, suggesting this could be another means through which DDX41 modulates inflammatory signaling [28]. However, functional assessments that decipher if and how these splicing abnormalities could contribute to DDX41-deficient hematological 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 [52]. They attributed this decreased proliferation in DDX41 R525H cells to defects in pre-rRNA (ribosomal RNA) processing via reduced helicase activity, which inhibits cell cycle progression via activation of the MDM2-RB-E2F axis (Figure 2) [52]. This study provides mechanistic insights on the effect of the DDX41 R525H mutation in general but did not assess what occurs in the hematopoietic tissue. Chlon *et al.* established *in vivo* mammalian models of DDX41 R525H and DDX41 loss to understand how these mutations affected hematopoiesis [55]. 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 (Figure 2) [55]. 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 hematologic 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 [55]. 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, 27]. 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 questioned, 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 [55]. These cellular phenotypes were also observed in the leukemic 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 anemia developed in *ddx41* mutants caused by Ataxia-telangiectasia mutated (ATM) and Ataxiatelangiectasia and Rad3-related (ATR)-triggered cell cycle arrest in erythroid progenitors coupled with defective differentiation [54]. 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 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 [28]. Many aspects of this model such as elevated R-loop levels and pro-inflammatory gene expression, were conserved in human cells with *DDX41* knockdown [28]. 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 [49]. Their findings support a model through which DDX41 insufficiency-driven R-loop accumulation and genomic instability indirectly promote inflammatory signaling [49]. 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 signaling that may drive clonal selection and ultimately leukemic progression. Deciphering how these two mutant factors synergize 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 characterizing DDX41's biological functions and role in the pathogenesis of hematologic malignancies. However, many questions remain unanswered. Why do patients with germline DDX41 mutations develop hematopoietic disease in the 6^{th} and 7^{th} 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 hematologic malignancies, and what dictates if lymphoid or myeloid leukemia will arise? Furthermore, do mutations in niche cells also cause dysregulation of the bone marrow microenvironment and could this contribute to subsequent leukemogenesis? 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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Abbreviations:

MDS	myelodysplastic syndrome
AML	acute myeloid leukemia
HSPC	hematopoietic stem and progenitor cells
DEAD	Asp-Glu-Ala-Asp
SF2	superfamily 2
DDX41	*DEAD-Box Helicase* 41
ASXL1	Additional Sex Combs Like 1
AEL	acute erythroleukemia

ICUS	idiopathic cytopenia of undetermined significance
CSNK1A1	casein kinase 1A1
IFN-β	interferon-beta
c-di-GMP	cyclic-di-guanosine monophosphate
c-di-AMP	cyclic-di-adenosine monophosphate
STING	Stimulator of interferon genes
TBK1	TANK-binding kinase 1
BTK	Bruton's tyrosine kinase
NF-κB	nuclear factor-kappa B
cGAS	cyclic GMP-AMP synthetase
shRNA	short hairpin RNA
pre-rRNA	pre-ribosomal RNA
MDM2	mouse-double minus2
RB	retinoblastoma
ATM	Ataxia-telangiectasia mutated
ATR	Ataxiatelangiectasia and Rad3-related
DDR	DNA-damage response

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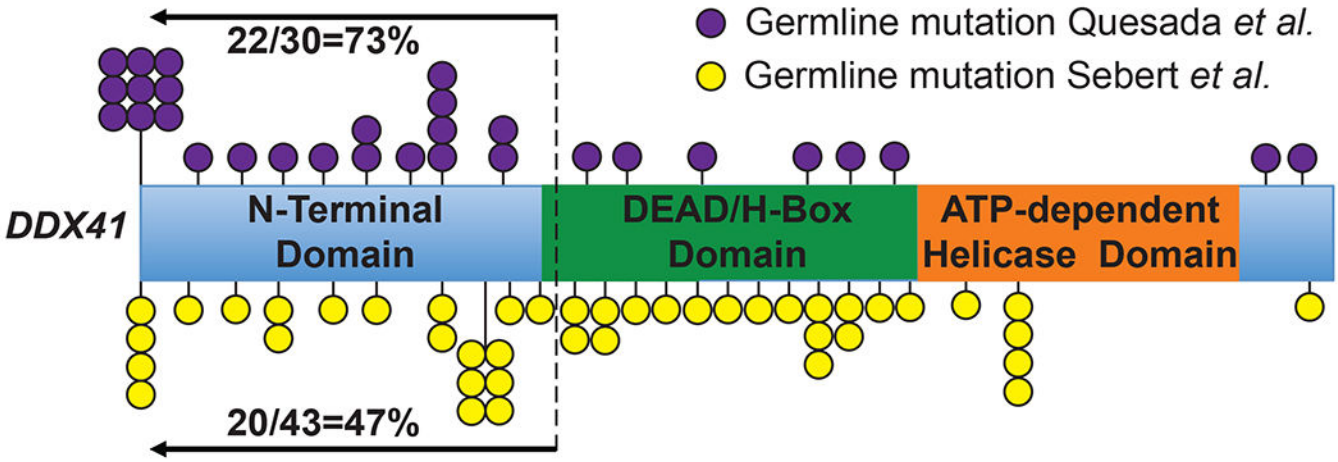

Figure 1. Distribution of germline *DDX41* mutations in Quesada *et al*. versus Sebert *et al*. cohorts.

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.

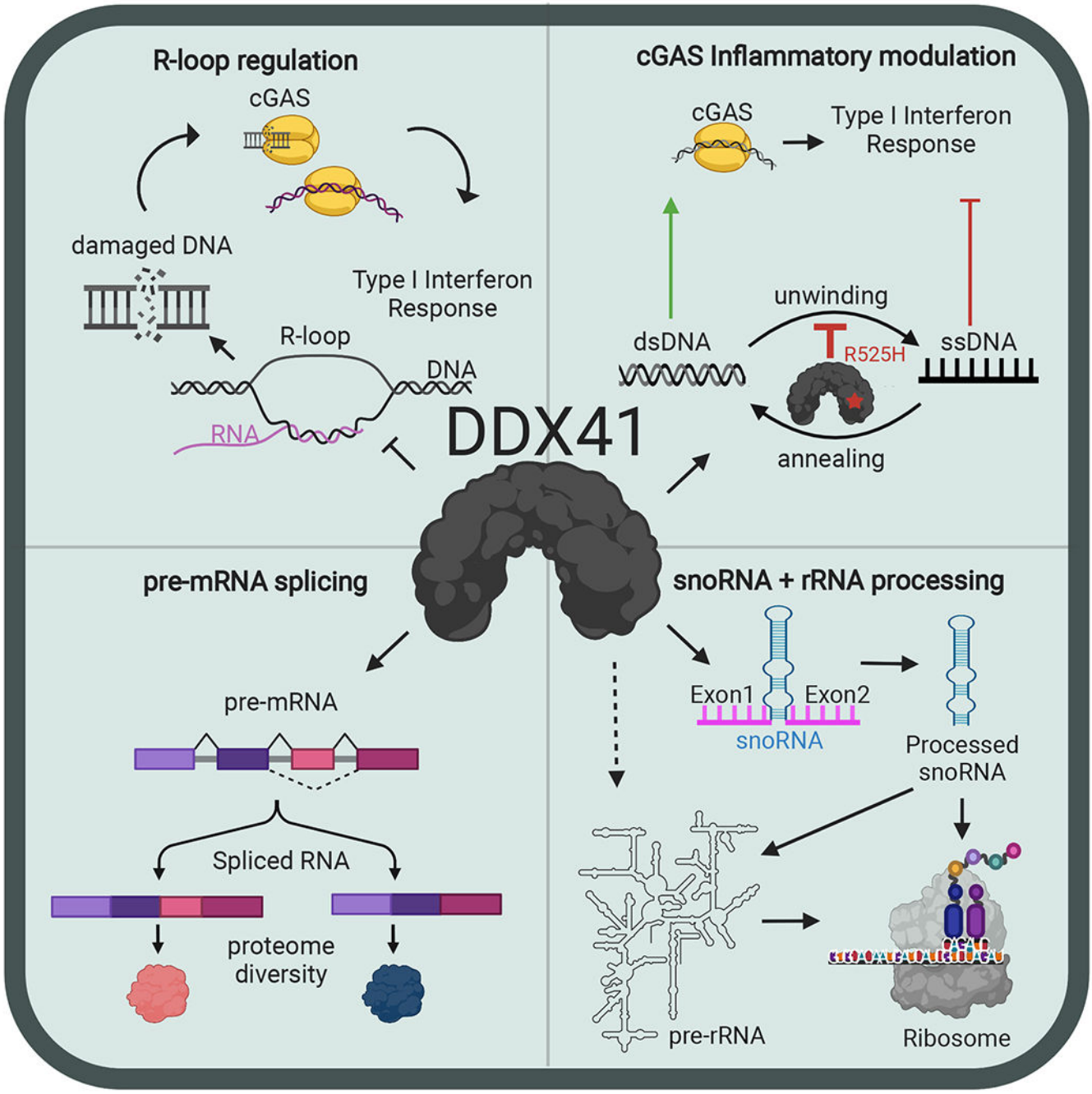

Figure 2. Cellular activities of DDX41.

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 hematologic 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 hematopoietic dysfunction. Created in Biorender.