Overall cancer risk in people with deleterious germline *DDX41* variants

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Abstract

Germline loss-of-function (LoF) *DDX41* variants predispose to late-onset hematopoietic malignancies (HM), predominantly of myeloid lineage. Among 43 families with germline *DDX41*^{LoF} variants, bone marrow (BM) biopsies in those without (N=8) or with malignancies (N=21) revealed mild dysplasia in peripheral blood (57%) and BM (88%), long before the average age of *DDX41*-related HM onset. Therefore, we recommend baseline BM biopsies in people with germline *DDX41*^{LoF} alleles to avoid over-diagnosis of myelodysplastic syndromes. A variety of solid tumors were also observed in our cohort, with 24% penetrance by age 75. Although acquired *DDX41* mutations are common in HM, we failed to identify such alleles in solid tumors arising in those with germline *DDX41*^{LoF} variants (N=15), suggesting an alternative mechanism driving solid tumor development. Furthermore, 33% of pedigrees in which ≥15% of first-degree relatives including the proband were diagnosed with a solid tumor had second germline deleterious variants in other cancer-predisposition genes, likely serving as primary cancer drivers. Finally, both lymphoblastoid cell lines and primary peripheral blood from individuals with germline *DDX41*^{LoF} variants exhibited differential levels of inflammation-associated proteins. These data provide evidence of inflammatory dysfunction mediated by germline *DDX41*^{LoF} alleles that may contribute to solid tumor growth in the context of additional germline cancer-associated variants. For those with HM and personal/family histories of solid tumors, we recommend broad germline testing. *DDX41* may be an indirect modifier of solid tumor pathogenesis compared to its tumor suppressor function within hematopoietic tissues, a hypothesis that can be addressed in future work.

Introduction

Germline deleterious variants in many genes are known to predispose to hematopoietic malignancies (HM), and classification schemes for leukemias now include these entities.[1-3] *DDX41*, encoding DEAD-box RNA helicase 41, is the most common germline-mutated gene in adult myeloid neoplasms (MN), driving approximately 3% of MN.[4] Germline *DDX41* loss-of-function (LoF) alleles predispose to late-onset MN.[4-16] Fifty-four percent of *DDX41*-mutated neoplasms acquire a somatic mutation on the wild-type (WT) allele, usually the "hotspot" variant *DDX41* c.1574G>A (p.Arg525His), suggesting that DDX41 acts as a tumor suppressor for MN.[4-9,11,12,14,15,17] Multiple *in vivo* studies show an association between germline *DDX41*^{LOF} variants and hematopoietic stem cell expansion,[18-21] with R-loop accumulation[18] and a genomic instability-associated inflammatory response.[20,21] However, the exact mechanism by which germline *DDX41*^{LOF} alleles contribute to malignancies is unclear.

Those with germline *DDX41*^{LoF} variants develop MN at a me-

dian age of 68, generally with favorable overall survival.[4,8,13-15] However, these individuals are at risk for severe acute graft-*versus*-host disease (GVHD) when they undergo allogeneic hematopoietic cell transplantation even with WT donors, unless they receive post-transplant cyclophosphamide,[22] suggesting an activated inflammatory milieu.[4] Furthermore, solid tumors have been reported in families with germline *DDX41*^{LoF} alleles, but it is unknown if that allele contributes directly to their development.[10,13] DDX41 is an RNA helicase required for activation of the cGAS-stimulator of IFN genes (STING)-type I interferon pathway in response to DNA virus invasion, which suppresses R-loop accumulation.[18,20,23] Furthermore, DDX41 can activate cGAS-STING in response to R-loop accumulation.[18] Therefore, DDX41 plays an important role in immune regulation even in the absence of viral DNA invasion. Additionally, the *DDX41* "hotspot" variant Arg525His increases STING activation, while knockout of *DDX41* decreases STING activation, suggesting that different *DDX41* alleles differentially affect DDX41-mediated immune processes and cancer predisposition.[18,23] However, the impact of different germline *DDX41* variants on immunity and inflammation has yet to be investigated in patients or human-derived cell lines.

Methods

Additional details are provided in the *Online Supplementary Appendix*.

Patients

All individuals signed written informed consent to participate in research approved by Institutional Review Boards at the University of Chicago and Northwestern University, conducted in accordance with the Declaration of Helsinki, and protected by National Institutes of Health Certificates of Confidentiality.

Germline sequencing

Individuals with personal and/or family histories consistent with a deleterious germline *DDX41* variant or those with such alleles identified via tumor profiling[24] underwent clinical germline genetic testing (*Online Supplementary Table S1*). DNA was sequenced using an augmented whole exome sequencing platform[25] in the University of Chicago Genetic Services Laboratory (https://genes.uchicago.edu/clinical-genetics). DNA variants in 139 cancer-predisposing genes (*Online Supplementary Table S2*) were analyzed. A custom bioinformatic pipeline capable of detecting single nucleotide variants and copy number variants was used (https://github.com/LucyGodley/Pipeline/blob/main/Variant_Calling/WES/hg/Automated/WES_Pipeline.sh).[16] Variants were curated according to the American College of Medical Genetics and Genomics/Association of Molecular Pathology.[26] Deleterious variants in cancer-causing genes were confirmed by Sanger sequencing.

Somatic solid tumor sequencing

DNA derived from formalin-fixed, paraffin-embedded solid tumor tissue derived from eight patients with germline *DDX41*^{LOF} variants was sequenced via the OncoPlus next-generation panel, which includes *DDX41*.[27] Additional tumor-derived sequencing data from the Cancer Genome Atlas (TCGA; https://portal.gdc.cancer.gov/) were acquired for 11 additional patients with truncating *DDX41* alleles that are likely to be germline based on the frequency with which such alleles are inherited.[17]

Lymphoblastoid cell line preparation

Lymphoblastoid cell lines (LCL) were derived from peripheral blood B cells from individuals with deleterious germline *DDX41*^{LOF} variants (*DDX41*$^{var/+}$), which were transformed using Epstein-Barr virus cultured in standard LCL growth media. *DDX41*^{WT} LCL were purchased from the Coriell Institute for Medical Research (https://www.coriell.org/), which were derived using a virtually identical transformation protocol from three individuals: a 44-year-old (yo) man; a 25yo man; and a 42yo woman.

Protein isolation and western blotting

Whole-cell protein lysates were prepared from *DDX41*^{WT} and *DDX41*$^{var/+}$ LCL 2 days after passaging. Nuclear and cytoplasmic fractions were prepared from DDX41^{WT} and *DDX41*$^{var/+}$ LCL 2 days after passaging using the Pierce "NE-PER Nuclear and Cytoplasmic Extraction Reagents" kit (Thermo Fisher Scientific). A standard SDS-PAGE western blotting protocol was performed to quantify total DDX41 in whole cell lysates and NF-κB in nuclear and cytoplasmic fractions.

RNA sequencing

RNA sequencing was performed at the University of Chicago Functional Genomics Laboratory, and data were analyzed using the Cufflinks pipeline (https://cole-trapnell-lab.github.io/cufflinks/manual/; *Online Supplementary Figure S1*). Genes of interest were validated using real-time qualitative reverse transcriptase polymerase chain reaction (qRT-PCR).

Measurement of cytokine levels

Quantification of 105 unique cytokines from conditioned LCL growth medium was performed using the "Proteome Profiler Human XL Cytokine Array Kit" (R&D Systems). Quantification of 65 unique cytokines (43 of which were also assessed in the cytokine arrays; *Online Supplementary Figure S2*) from conditioned LCL growth medium was performed using the "Human Magnetic Luminex Multiplex Cytokine/Chemokine Array Kit-65 Plex" (Creative Biolabs). Quantification of transforming growth factor-β (TGF-β) was performed using the "Human/Mouse/Rat/Porcine/Canine TGF-β 1 Quantikine ELISA" (R&D Systems). Levels of ANG, CXCL13, CXCL8, and IL-9 were confirmed using a custom

"ProcartaPlex" Luminex panel (Thermo Fisher Scientific) and normalized to a GDF-15 internal control. Conditioned LCL growth media from *DDX41*^{WT} and *DDX41*$^{var/+}$ LCL was eight-times concentrated for all assays.

UK Biobank proteomics analysis

Proteomics data from blood plasma in a cohort of 49 individuals with deleterious likely germline *DDX41* variants (cases) were compared to that of 98 age- and sex-matched controls available in the UK Biobank[28] (https://biobank.ndph.ox.ac.uk/ukb/field.cgi?id=30900, project ID 83200). At the time the peripheral blood was collected, none of these individuals had been diagnosed with cancer. Protein interaction analysis was performed using STRING (https://string-db.org/) with the minimum required interaction score set to "high confidence" (0.700). Pathway enrichment analysis was performed using the STRING database, the Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.genome.jp/kegg/pathway.html), and the DISEASES database (https://diseases.jensenlab.org/Search).

Results

Mild dysplasia in patients with germline *DDX41*^{LoF} variants at baseline

Germline *DDX41* variants were identified in 102 individuals from 52 families (*Online Supplementary Table S1; Online Supplementary Figures S3, S4*). Germline pathogenic (P) and likely pathogenic (LP) *DDX41* variants were identified in 93 individuals (91%) from 43 families (83%; Table 1). Germline *DDX41* variants of uncertain significance (VUS) were identified in 11 individuals (11%) from 11 families (21%). Two families (9 and 26; *Online Supplementary Table S1*) had both a germline P/LP (deleterious) *DDX41* variant and a VUS. Among the 28 distinct deleterious variants identified, two (7%) were novel. Of the nine distinct *DDX41* VUS identified, five (56%) were novel (Figure 1A).

Bone marrow (BM) biopsies were reviewed from 29 individuals (8 without malignancies and 21 with malignancies) with germline deleterious *DDX41* variants. Both peripheral blood and BM demonstrated dysplasia commonly at baseline regardless of age compared to *DDX41*^{WT} individuals (Table 2; Figure 1B-Q). The most frequent morphologies observed at baseline were small, hypolobated megakaryocytes (75%) and macrocytic erythrocytes (50%; *Online Supplementary Table S3*), even in individuals as young as 17yo (Figure 1B-E). A 46yo woman with a germline *DDX41* deletion of exons 12-17 displayed similar, but more severe, dysplastic characteristics at baseline (Figure 1F-I). In contrast, BM from this patient's 73yo father with myelodysplastic syndrome (MDS) showed dysplasia in all three lineages and increased blasts (Figure 1J-M). Similar observations of multilineage dysplasia and 18% blasts were made in an unrelated 66yo with MDS and a familial *DDX41* allele encoding p.P258L (Figure 1N-Q).

DDX41 is not a tumor suppressor in solid tumor development

Our cohort of 43 pedigrees with deleterious germline *DDX41* variants allowed us to characterize the tumor spectrum and age of diagnosis in those with *DDX41*^{LoF} alleles. Penetrance of HM in individuals carrying germline P/LP *DDX41* variants was 54% (N=50/93) by 90yo, similar to what has been observed in other cohorts.[6,8,17] HM included MDS (N=22), acute myeloid leukemia (AML, N=28), chronic myeloid leukemia (CML, N=1), Hodgkin lymphoma (HL, N=1), and non-Hodgkin lymphoma (NHL, N=1; *Online Supplementary Table S1*). The average age of onset for HM in this cohort was 64yo, consistent with the well-known late onset of *DDX41*-related neoplasms.[4,16] Additionally, there was higher HM penetrance in individuals ≥50yo compared to those <50yo (P=0.0005), consistent with the well-known late-onset of HM associated with *DDX41*.

As in other patient cohorts,[10,13] we observed solid tumors in people with germline *DDX41*^{LOF} variants: breast (N=3), melanoma (N=3), prostate (N=3), colon (N=3), basal cell carcinoma (N=3), ovarian (N=2), gastric (N=1), endometrial (N=1), tonsillar (N=1), mesothelioma (N=1), renal (N=1), head and neck (N=1), lung (N=1), and vulvar (N=1; Figure 2A). Penetrance of solid tumors in individuals with germline P/LP *DDX41* variants was 24% (N=22/93) by 75yo, with an average age of onset of 62yo. Fifty-five percent (N=12/22) of these individuals also developed HM, and in those cases, the solid tumor preceded the HM (mean latency =8 years; range, 2-13 years) in all but one individual (N=11/12, 92%; Figure 2B). Methods used to treat these solid tumors included radiation and chemotherapy, suggesting that these HM may have been treatment-related (Figure 2B). BM biopsies were performed on seven of the 12 patients with HM and solid tumors and pathologic findings (such as *TP53* mutations and complex karyotypes including t(11;16)(q23;p13)) from five (71%), supported considering these HM as treatment-related (*Online Supplementary Table S3*).

Acquisition of a somatic *DDX41* mutation, typically p.R525H, occurs in 54% of MN associated with deleterious germline *DDX41* alleles,[17] suggesting that *DDX41* often acts as a tumor suppressor gene in MN. To determine if *DDX41* has a similar role in solid tumor development, DNA derived from solid tumors was sequenced from individuals with germline P/LP *DDX41* variants (N=5) or VUS (N=3). No somatic *DDX41*^{LoF} variants were identified (Table 3), suggesting that somatic mutations like p.R525H are less common in solid tumors or that *DDX41* has an alternative role in the development of these tumors. Because some *DDX41* variants have only been observed as germline alleles and others, like truncating variants are virtually always germline,[17] we searched the TCGA database for solid tumors with those *DDX41* alleles (N=10) and failed to identify any additional somatic *DDX41* mutations (Table 3), again suggesting that these solid tu-

Table 1. Deleterious germline variants detected in pedigrees with hematopoietic malignancies with and without solid tumors.

			Family #	Germline *DDX41* variant (P/LP) NM_016222.4	DDX41-encoded protein variant NP_057306.2	# Primary relatives	# Primary relatives with ST	% Primary relatives with ST	ST in primary relatives	Additional germline-mutated gene*	Additional germline variant (P/LP)	Additional-encoded protein variant
Families with germline P/LP *DDX41* variants* (N=43)	HM+ST (N=21/43, 49%)	Additional germline variant (N=7/21, 33%)	9	c.142C>T	p.Gln48*	7	2	29	Breast x2	*PALB2*	c.2938del (P) NM_024675.3	p.Ser980Alafs*10 NP_078951.2
			11	c.268C>T	p.Gln90*	9	3	33	Endometrial, lung x2	*ATRX*	c.7219C>T (P) NM_000489.6	p.Arg2407* NP_000480.3
			26	c. 490C>T	p.Arg164Trp	7	2	29	Basal cell carcinoma, colon x2	*APC*	c.3920T>A (LP) NM_000038.6]	p.Ile1307Lys NP_000029.2
			28	c. 490C>T	p.Arg164Trp	7	2	29	Colon, ovarian, prostate, spinal	*BRCA1*	c.68_69delAG (P) NM_007294.4	p.Glu23Valfs*17 NP_009225.1
			30	c.653G>A	p.Gly218Asp	5	2	40	Lung, neuroendocrine carcinoma	*ATM*	c.2921+1G>A (P) NM_000051.4	p.?
			31	c.766G>A	p.Glu256Lys	8	4	50	Basal cell carcinoma, melanoma, prostate x2, renal	*CDKN2A*	c.9_32dup (LP) NM_000077.5	p.Ala4_Pro11dup NP_000068.1
			38	c.1187T>C	p.Ile396Thr	5	1	20	Breast	*BRCA2*	c.6174delT (P) NM_000059.4	p.Phe2058LeufsTer12 NP_000050.3
		DDX41 only (N=14/21, 67%)	6	c.3G>A	p.Met1?	6	2	33	Breast, pancreatic	-	-	-
			8	c.121C>T	p.Gln41*	4	2	50	Lung	-	-	-
			12	c.323del	p.Lys108Serfs*3	13	2	15	Breast, colon, liver, meningioma	-		-
			13	c.415_418dupGATG	p.Asp140Glyfs*2	4	1	25	Gastric	-	-	-
			14	c.415_418dupGATG	p.Asp140Glyfs*2	13	5	38	Colon, melanoma x2, uterine	-	-	-
			15	c.415_418dupGATG	p.Asp140Glyfs*2	3	2	67	Bladder, breast, melanoma, prostate	-	-	-
			19	c.1141A>T	p.Lys381*	6	2	33	Clear cell renal, prostate	-		-
			23	c.386dup	p.Lys130Glufs*5	13	2	15	Colon, ovarian	-	-	-
			27	c. 490C>T	p.Arg164Trp	5	2	40	Bladder, breast, tonsillar	-	-	-

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			Family #	Germline *DDX41* variant (P/LP) NM_016222.4	DDX41-encoded protein variant NP_057306.2	# Primary relatives	# Primary relatives with ST	% Primary relatives with ST	ST in primary relatives	Additional germline-mutated gene*	Additional germline variant (P/LP)	Additional-encoded protein variant
Families with germline P/LP *DDX41* variants* (N=43)	HM+ST (N=21/43, 49%)	*DDX41* only (N=14/21, 67%)	29	c.653G>A	p.Gly218Asp	6	1	17	Tonsillar	-	-	-
			32	c.773C>T	p.Pro258Leu	6	2	33	Melanoma, prostate	-	-	-
			34	c.1013G>A	p.Cys338Tyr	6	1	17	Breast	-	-	-
			39	c.1187T>C	p.Ile396Thr	5	1	20	Breast, endometrial/ uterine	-	-	-
			41	c.1474dup	p.Ala492Glyfs*17	6	1	17	Breast	-	-	-
	HM only (N=22/43, 51%)	Additional germline variant (N=1/22, 5%)	24	c.435-2_435-1delinsCA	p.?	6	0	0	-	*CHEK2*	c.470T>C (P) NM_007194.4	p.Ile200Thr NP_009125.1
		DDX41 only (N=21/22, 95%)	1	c.3G>A	p.Met1?	10	0	0	-	-	-	-
			2	c.3G>A	p.Met1?	9	0	0	-	-	-	-
			3	c.3G>A	p.Met1?	12	1	8	-	-	-	-
			4	c.3G>A	p.Met1?	9	1	11	-	-	-	-
			5	c.3G>A	p.Met1?	8	1	13	-	-	-	-
			7	c.121C>T	p.Gln41*	8	1	13	-	-	-	-
			10	c.232_233insAA	p.Pro78Glnfs*3	6	0	0	-	-	-	-
			16	c.415_418dupGATG	p.Asp140Glyfs*2	8	1	13	-	-	-	-
			17	c.415_418dupGATG	p.Asp140Glyfs*2	7	0	0	-	-	-	-
			18	c.946_947del	p.Met316Asp*31	3	0	0	-	-	-	-
			20	c.1285C>T	p.Gln429*	7	0	0	-	-	-	-
			21	c.1496dup	p.Ala500Cysfs*9	8	0	0	-	-	-	-
			22	c.108T>A	p.Tyr36*	3	0	0	-	-	-	-
			25	c. 490C>T	p.Arg164Trp	7	1	14	-	-	-	-
			33	c.847delC	p.Leu283Cysfs*21	6	0	0	-	-	-	-
			35	c.1016G>T	p.Arg339Leu	7	1	14	-	-	-	-
			36	c.1105C>G	p.Arg369Gly	11	0	0	-	-	-	-
			37	c.1118T>C	p.Leu373Pro	7	0	0	-	-	-	-
			40	c.1283T>C	p.Leu428Pro	4	0	0	-	-	-	-
			42	c.1721del	p.Leu574Arg*fs143	6	0	0	-	-	-	-
			43	c.1721del	p.Leu574Arg*fs143	5	0	0	-	-	-	-

*Pedigrees in which ≥15% of first-degree relatives including the proband were diagnosed with a solid tumor were more likely to have second germline deleterious variants in other cancer-predisposition genes (P=0.0212). HM: hematopoietic malignancy; LP: likely pathogenic; P: pathogenic; ST: solid tumor.

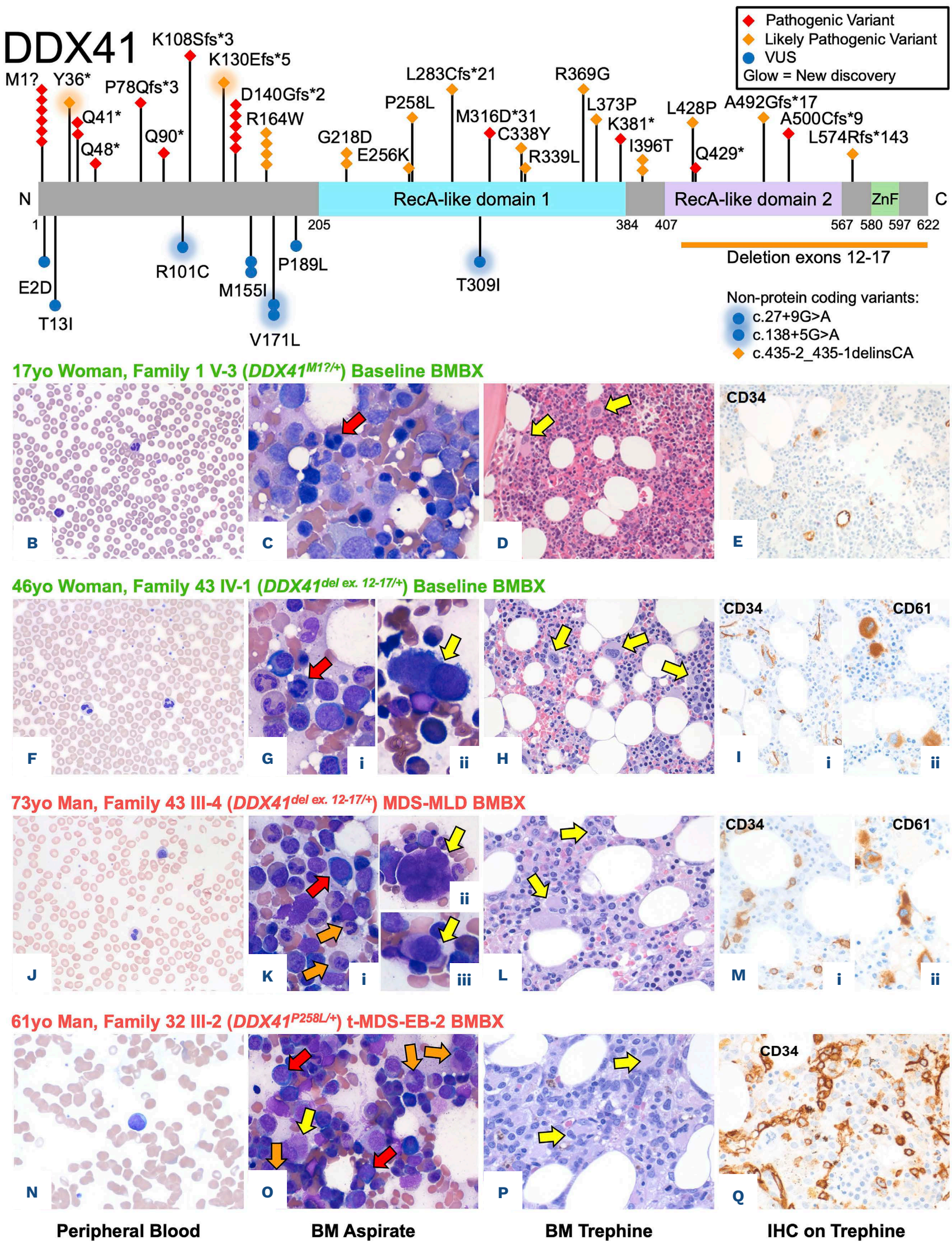

Figure 1. Family-associated germline *DDX41* variants and morphologic features of baseline and malignant peripheral blood and bone marrow in individuals with deleterious germline *DDX41* variants at different ages. (A) Deleterious germline *DDX41* variants identified in patients and families with hematopoietic malignancies (HM) are shown above the protein schematic, and variants

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of uncertain significance (VUS; blue circles) are shown below. Pathogenic variants are indicated by red diamonds, and likely pathogenic variants by orange diamonds. Non-protein coding variants are listed in the bottom right. The likely pathogenic copy number variant (CNV) is indicated by an orange line. Novel variants are shown with glow and previously identified variants are shown without glow. DDX41 protein domains are indicated by color: RecA-like domain 1 (light blue), RecA-like domain 2 (lilac), and zinc finger (ZnF, light green). (B-Q) Images shown include (B, F, J, N) peripheral blood, (C, G, K, O) bone marrow (BM) aspirate, (D, H, L, P) BM trephine, and (E, I, M, Q) immunohistochemistry on trephine. (B-E) A 17-year-old (yo) female with a pathogenic *DDX41* variant (p.M1?), mild dysplastic changes in erythroid lineage (red arrow) and megakaryocytic lineage (yellow arrow), but insufficient for diagnosis of myelodysplastic syndromes (MDS). (F-I) A 46yo female with a likely pathogenic *DDX41* deletion of exons 12-17, significant (>10%) dysplastic changes in both erythroid and megakaryocytic lineages, but no granulocytic dysplasia. (J-M) A 73yo male (father of (F-I)) with a likely pathogenic *DDX41* deletion of exons 12-17, 4.6% blasts and significant dyserythropoiesis and dysmegakaryopoiesis as well as dysgranulopoiesis manifested mainly by abnormal nuclear morphology including hyposegmentation, dense chromatin and nuclear membrane projections (orange arrow), but not cytoplasmic hypogranulation, diagnosed with MDS with multilineage dysplasia. (N-Q) A 61yo male with a likely pathogenic *DDX41* variant (p.P258L), 18% blasts, and multilineage dysplasia (particularly prominent in granulocytes), diagnosed MDS with excess blasts-2 progressing toward acute myeloid leukemia.

Table 2. Dysplasia identified in peripheral blood and bone marrow examinations in those with deleterious germline *DDX41* variants from our cohort.

	Peripheral blood			Core biopsy/aspirate smear		
	Dysplasia	No dysplasia	% with dysplasia	Dysplasia	No dysplasia	% with dysplasia
Baseline	4	3	57	7	1	88
Malignant	20	0	100	21	0	100
			P=0.0120*			*P*=0.2759* (NS)

**P* values determined by two-tailed Fisher's exact tests to determine association between malignancy and presence of dysplasia. BM: bone marrow; NS: not significant.

mors may have been driven by an alternative mechanism.

Second cancer-risk alleles in those with solid tumors

To test if second germline P/LP variants could drive the formation of solid tumors within the 43 families comprising our cohort, we analyzed DNA variants from 139 cancer-predisposition genes using augmented whole exome sequencing from germline tissue. We divided these pedigrees into those that had solid tumors and HM, defined as those with a ≥15% prevalence of solid tumors in first-degree relatives of the proband, including the proband (N=21/43), *versus* those with only HM (N=22/43; Table 1). Among the 21 pedigrees with solid tumors and HM, seven (33%) had second deleterious germline variants in other cancer risk genes: *APC, ATM, ATRX, BRCA1, BRCA2, CDKN2A,* and *PALB2* (Table 1; *Online Supplementary Tables S4, S5*). The solid tumors in these pedigrees were consistent with the expected tumor spectra of each disorder (Table 1; *Online Supplementary Table S1*). In five of these pedigrees (N=5/7, 71%), the additional cancer-predisposing allele was identified in an individual with both the familial *DDX41*^{LoF} allele and a solid tumor(s). In contrast, we identified only one family with a second deleterious germline variant among the 22 pedigrees with only HM (5%; Table 1; *Online Supplementary Tables S4, S5*). These findings demonstrate that germline *DDX41*-mutated families with solid tumors are more likely to have germline pathogenic variants in other cancer-predisposition genes than families with only HM (*P*=0.02; *Online Supplementary Figure S3*), providing support for the recommendation that families with germline *DDX41*^{LOF} alleles with a ≥15% prevalence of solid tumors among primary relatives including the proband should have comprehensive testing for cancer risk alleles.

DDX41$^{var/+}$ patient-derived lymphoblastoid cell lines exhibit inflammatory dysregulation

The prevalence of solid tumors in our family cohort as well as prior *in vivo* studies and clinical observations of severe GVHD disease in those with germline *DDX41*^{LOF} alleles suggest an important role for DDX41 in regulating inflammation.[4,18-20,23] First, we quantified total DDX41 protein levels by western blotting using *DDX41*^{WT} LCL derived from three sex-matched individuals as negative controls and five germline *DDX41*$^{var/+}$ LCL derived from: a 65yo man with a *DDX41* allele encoding a start-loss variant, p.M1? (family #6 individual III-6); a 66yo man with a *DDX41* allele encoding p.P258L (family #32 individual III-2); a 73yo man with a truncating *DDX41* allele, p.A492Gfs*17 (family #41 individual III-3); a 65yo woman with a similar truncating *DDX41* allele, p.A500Cfs*9 (family #21 individual III-1); and a 74yo man with a *DDX41* allele encoding a deletion of exons 12-17 (del ex. 12-17, family #43 individual III-4; *Online Supplementary Table S1*). We found lower DDX41 levels in the context of germline variants associated with nonsense-mediated mRNA decay (p.A492Gfs*17, p.A500Cfs*9, and del ex. 12-17), but relatively unchanged DDX41 levels in the absence of such variants (*P*=0.04; *Online Supplementary Figure S5*). To determine how different patient-associated germline *DDX41* variants affect

gene expression, we performed RNA sequencing revealing differential gene expression between *DDX41*^{WT} and *DDX41*$^{var/+}$ LCL, and among individual patient-derived *DDX41*$^{var/+}$ cell lines, suggesting that each germline *DDX41* variant may differentially disrupt *DDX41*-mediated functions. Furthermore, principal component analysis revealed clustering of *DDX41*$^{P258L/+}$ with *DDX41*$^{A500Cfs*9/+}$, and *DDX41*$^{A492Gfs*17/+}$ with *DDX41*$^{del\ ex.12\text{-}17/+}$, suggesting similar effects of these variants on DDX41 protein function (Figure 3A). Eight genes known to be associated with inflammation (*e.g., CDC14B, CD244, CD9, IL1R1, IL23R, IL32, LTBR,* and *PTPN14*) were upregulated across all five *DDX41*$^{var/+}$ LCL (Figure 3B). Additionally, upreg-

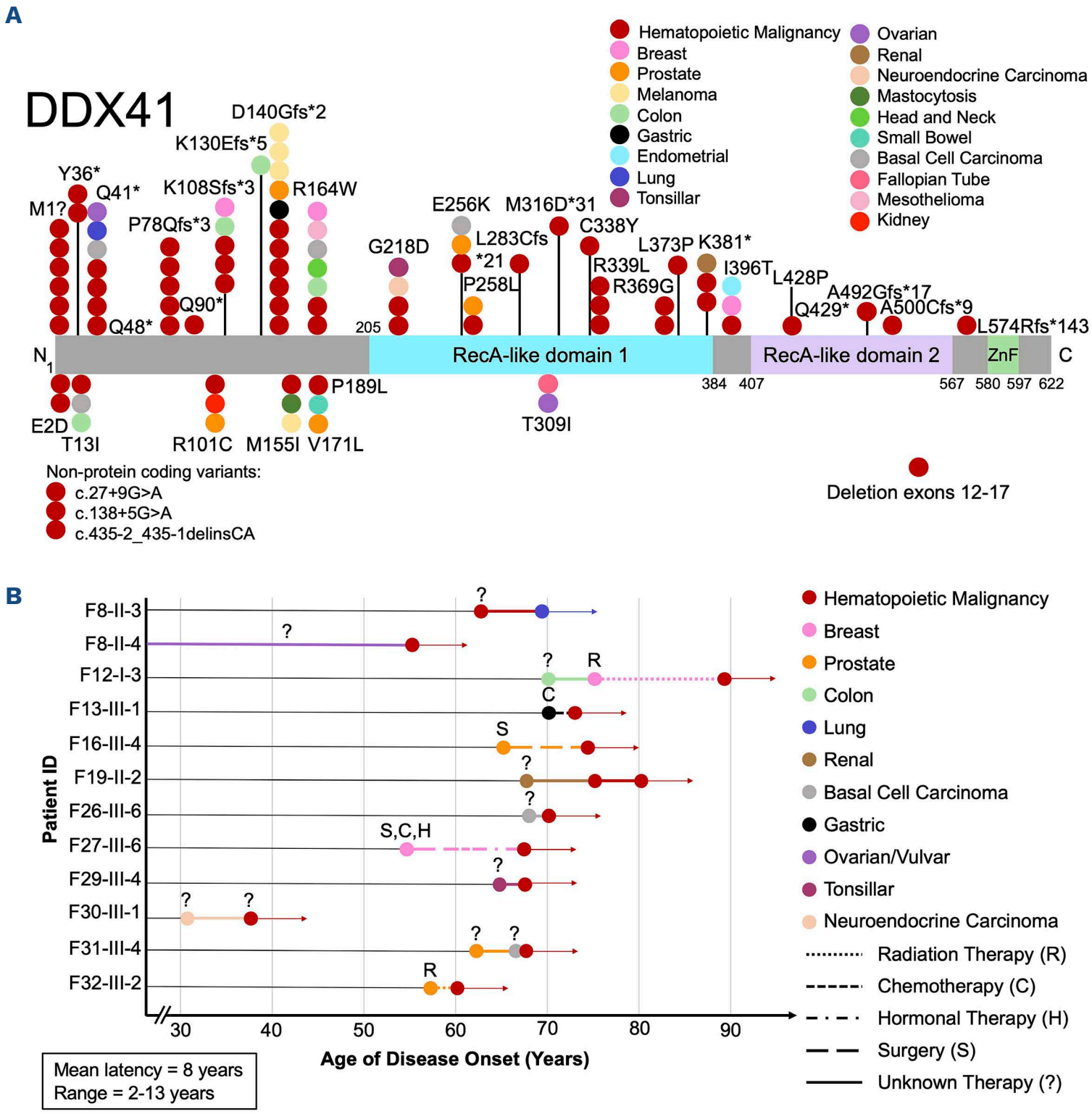


Figure 2. Disease breakdown by *DDX41* variant and timelines of solid tumors and hematopoietic malignancies in patients with multiple malignancies. (A) DDX41 protein schematic showing all malignancies identified in individuals with loss-of-function (LoF) *DDX41* alleles plotted by corresponding variant. Diseases represented include hematopoietic malignancies (HM) (red), and solid tumors such as breast (pink), prostate (orange), melanoma (yellow), colon (light green), gastric (black), endometrial (light blue), lung (dark blue), tonsillar (magenta), ovarian/vulvar (purple), renal (brown), neuroendocrine carcinoma (peach), mastocytosis (dark green), head and neck (green), small bowel (teal), basal cell carcinoma (gray), fallopian tube (fuchsia), mesothelioma (light pink), and kidney (salmon) cancers. DDX41 protein domains are indicated by color: RecA-like domain 1 (light blue), RecA-like domain 2 (lilac), and zinc finger (ZnF) (light green). (B) Age at cancer diagnoses and treatments in individuals with *DDX41*^{LoF} alleles who were diagnosed with more than 1 cancer. HM are shown in red. Solid tumors represented are breast (pink), prostate (orange), colon (light green), lung (dark blue), renal (brown), basal cell carcinoma (grey), gastric (black), ovarian/vulvar (purple), tonsillar (magenta), and neuroendocrine carcinoma (peach). Solid tumor cancer therapies are indicated by: radiation therapy (R); chemotherapy (C); hormonal therapy (H); surgery (S); and unknown (?). Mean latency refers to the average years between the onsets of solid tumors and HM, whereas the range refers to the minimum and maximum latencies present.

ulation of 22 hallmark pathways was observed in *DDX41*$^{var/+}$ LCL, including TNF-α signaling via NF-κB (P=1.3x10^{-9}), hypoxia response (P=1.9x10^{-8}), epithelial-mesenchymal transition (P=7.6x10^{-7}), early estrogen response (P=5.2x10^{-6}), IL-2/STAT5 signaling (P=5.1x10^{-6}), angiogenesis (P=3.4x10^{-3}), ultraviolet (UV)-response down (P=1.2x10^{-4}), and inflammatory response signaling (P=5.0x10^{-5}; Figure 3C). Two pathways were down-regulated: E2F (P=2.1x10^{-4}) and MYC signaling (P=1.5x10^{-3}; Figure 3C). These findings were validated by qRT-PCR (*Online Supplementary Table S6; Online Supplementary Figure S6*). Taken together, RNA sequencing and qRT-PCR data suggest that patient-derived *DDX41*$^{var/+}$ LCL exhibit differential expression of immune-related genes and processes.

Next, we used cytokine arrays and Luminex assays to assess the levels of 127 unique cytokines in *DDX41*$^{var/+}$ and *DDX41*^{WT} LCL-conditioned growth media to investigate inflammatory signaling at the protein level (*Online Supplementary Figure S2*). ANG, CXCL13, CXCL8, and IL9 levels were higher in *DDX41*$^{var/+}$ LCL-conditioned media than in *DDX41*^{WT} LCL-conditioned media (Figure 3D, E), validated by a Luminex panel (Figure 3F-I). Interestingly, RNA expression of these inflammatory cytokines showed no significant overall increases or decreases in *DDX41*$^{var/+}$ LCL from WT, suggesting that translation and/or protein level regulation may be important in DDX41-mediated inflammatory changes (*Online Supplementary Table S6*). Although RNA sequencing revealed upregulation of several

Table 3. Somatic sequencing from solid tumor tissue from patients with germline deleterious *DDX41* variants.

Pedigree ID	Sex	Age in years	Germline variant classification	*DDX41* germline variant NM_016222.4	DDX41 encoded protein variant NP_057306.2	Solid tumor, age in years at dx.	Presence of "hotspot" (p.Arg525His) or other DDX41 variant
From TCGA	F	57	LP/P	c.C1105T	p.Arg36*	Breast invasive carcinoma, 57	No
From TCGA	M		P	c.C142T	p.Q48*	Prostate adenocarcinoma	No
F23-III-1	M	69	LP	c.386dup	p.Lys130Glufs*5	Colon, 69	No
F13-III-1	F	72	P	c.415_418dup	p.Asp140Glyfs*2	Gastric, 70	No
From TCGA	M	77	LP/P	c.418_419insGATG	p.Asp140_Pro141delinsGly*	Bladder urothelial carcinoma, 77	No
From TCGA	M	56	LP/P	c.418_419insGATG	p.Asp140_Pro141delinsGly*	Esophageal carcinoma, 56	No
From TCGA	M	32	LP/P	c.418_419insGATG	p.Asp140_Pro141delinsGly*	Pheochromocytoma and paraganglioma, 32	No
From TCGA	F	73	LP/P	c.C475T	p.Arg159*	Lung adenocarcinoma 73	No
From TCGA	M	59	LP/P	c.C475T	p.Arg159*	Head and Neck squamous cell carcinoma, 59	No
From TCGA	F	57	LP/P	c.C475T	p.Arg159*	Cervical squamous cell carcinoma and endocervical adenocarcinoma, 57	No
F27-III-6	F	69	P	c.490C>T	p.Arg164Trp	Breast, 54	No
F29-III-4	M	66	LP	c.653G>A	p.Gly218Asp	Tonsillar, 64	No
F30-III-1	F	37	LP	c.653G>A	p.Gly218Asp	Neuroendocrine carcinoma, 31	No
From TCGA	F	64	LP/P	c.946_947del	p.Met316fs	Liver hepatocellular carcinoma, 64	No
From TCGA	M	46	LP/P	c.A1789T	p.Lys597*	Bladder urothelial carcinoma, 46	No
F48-III-1	M	73	VUS	c.465G>A	p.Met155Ile	Melanoma, 72	No
F51-III-2	M	77	VUS	c.511G>C	p.Val171Leu	Prostate, 72	No
F52-III-2	F	67	VUS	c.926C>T	p.Thr309Ile	Ovarian, 67	No

F: family (pedigree ID)/female(sex); ID: identification; LP: likely pathogenic; M: male; P: pathogenic; VUS: variant of uncertain significance; dx: diagnosis.

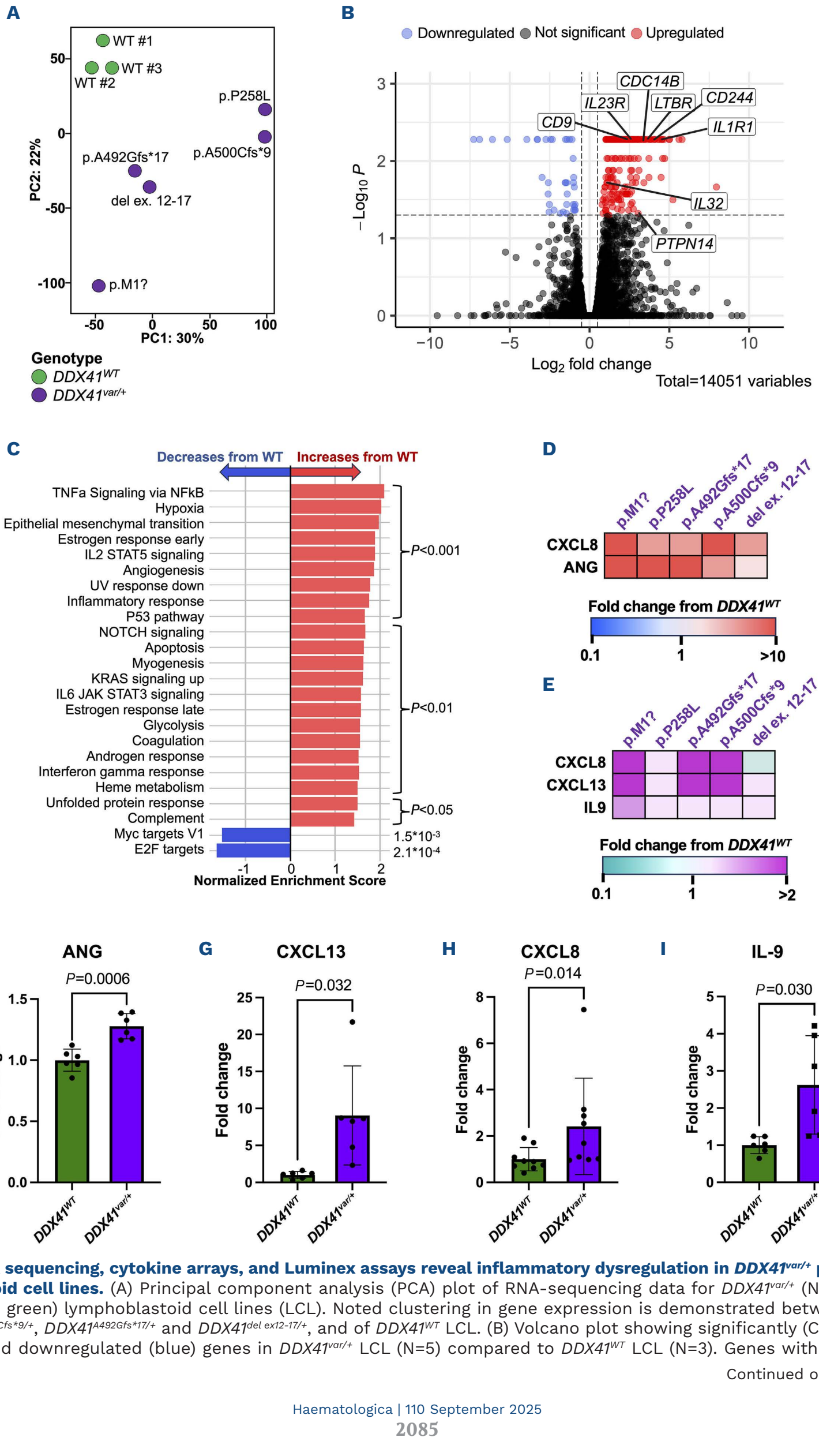
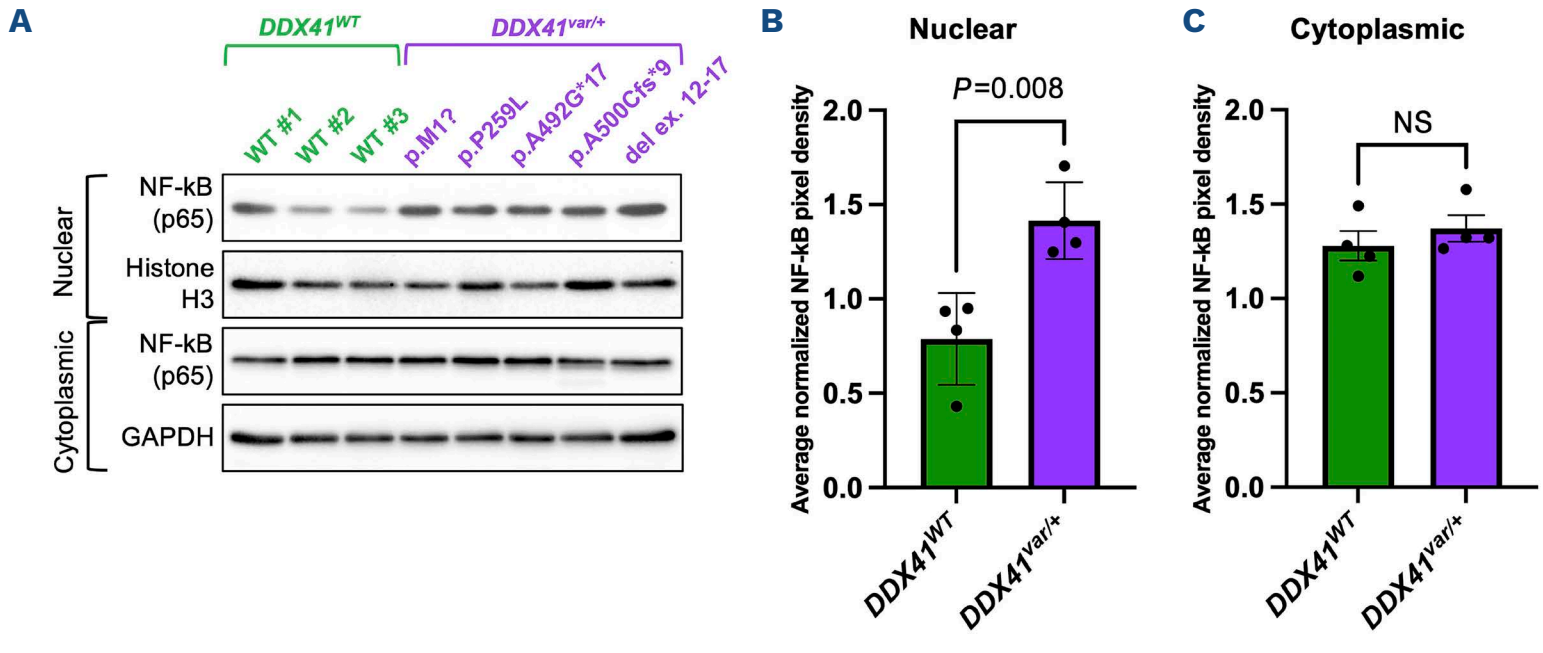


Figure 3. RNA sequencing, cytokine arrays, and Luminex assays reveal inflammatory dysregulation in *DDX41*$^{var/+}$ patient-derived lymphoblastoid cell lines. (A) Principal component analysis (PCA) plot of RNA-sequencing data for *DDX41*$^{var/+}$ (N=5, purple) and *DDX41*^{WT} (N=3, green) lymphoblastoid cell lines (LCL). Noted clustering in gene expression is demonstrated between *DDX41*$^{P258L/+}$ and *DDX41*$^{A500Cfs*9/+}$, *DDX41*$^{A492Gfs*17/+}$ and *DDX41*$^{del\ ex12\text{-}17/+}$, and of *DDX41*^{WT} LCL. (B) Volcano plot showing significantly (CI=95%) upregulated (red) and downregulated (blue) genes in *DDX41*$^{var/+}$ LCL (N=5) compared to *DDX41*^{WT} LCL (N=3). Genes with no statistically

Continued on following page.

significant change are in grey. (C) Normalized enrichment plot of genes from 24 hallmark signaling pathways. Increases in overall gene expression in *DDX41*$^{var/+}$ LCL from wild-type (WT) are in red, while decreases from WT are in blue. *P* values were determined by Pearson's correlation. (D) Heat map of cytokine array data showing fold changes in pixel densities of increased cytokines in patient-derived *DDX41*$^{var/+}$ (purple) LCL-conditioned media compared to *DDX41*^{WT}. Fold changes range from 0.1 (blue) to >10 (red). (E) Heat map of commercial Luminex data showing fold changes in pixel densities of increased cytokines in patient-derived *DDX41*$^{var/+}$ (purple) LCL-conditioned media compared to *DDX41*^{WT}. Fold changes range from 0.1 (turquoise) to >2 (magenta). (F-I) Bar graphs showing data from a custom Luminex panel. *P* values were determined using two-tailed *t* tests with Welch's correction and confirm higher levels of (F) ANG, (G) CXCL13, (H) CXCL8, and (I) IL-9 in *DDX41*$^{var/+}$ (purple) LCL-conditioned media compared to *DDX41*^{WT} (green).



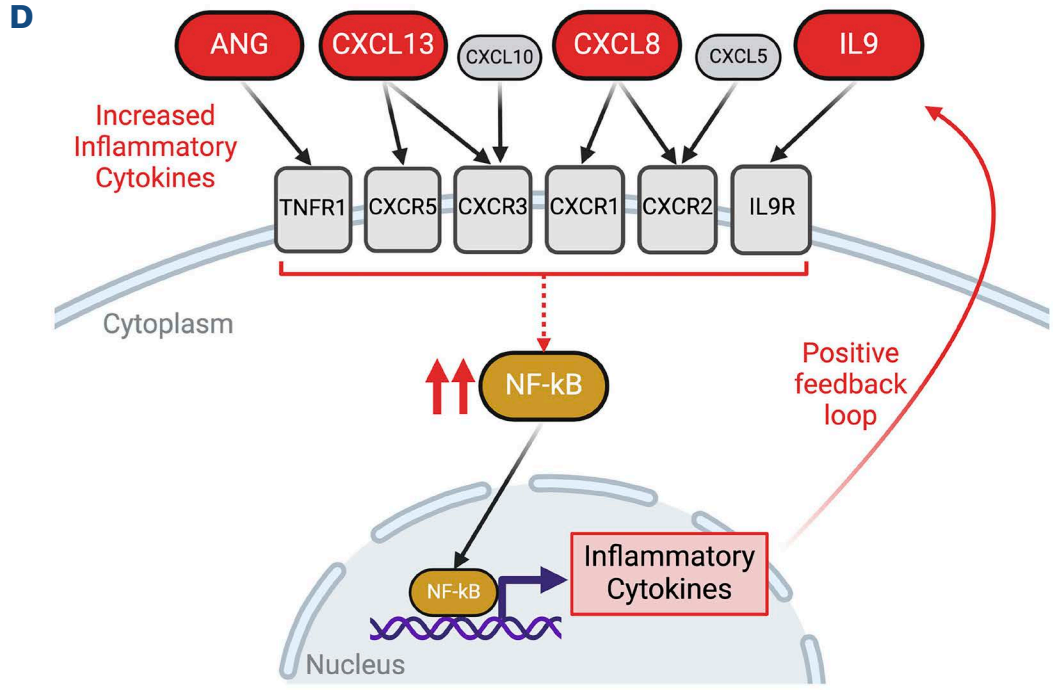


Figure 4. Testing the proposed mechanism of inflammatory dysregulation in germline *DDX41*$^{var/+}$ lymphoblastoid cell lines. (A) Western blots to quantify NF-κB (p65 subunit) in nuclear and cytoplasmic protein fractions from patient-derived *DDX41*$^{var/+}$ (purple) and *DDX41*^{WT} (green) lymphoblastoid cell lines (LCL). Histone H3 was used as a nuclear marker and loading control while GAPDH was used as a cytoplasmic marker and loading control. (B) Average NF-κB pixel densities in nuclear protein fractions from patient-derived *DDX41*$^{var/+}$ (purple) and *DDX41*^{WT} (green) LCL normalized to histone H3. Higher levels of NF-κB were detected in *DDX41*$^{var/+}$ LCL compared to *DDX41*^{WT} (*P*=0.008) according to a two-tailed *t* test with Welch's correction. (C) Average NF-κB pixel densities in cytoplasmic protein fractions from patient-derived *DDX41*$^{var/+}$ (purple) and *DDX41*^{WT} (green) LCL normalized to GAPDH. No significant change in NF-κB was detected according to a two-tailed *t* test with Welch's correction. (D) Visual summary of cytokine array, Luminex, and western blot data. Cytokines whose levels were higher in *DDX41*$^{var/+}$ than in *DDX41*^{WT} LCL-conditioned media according to cytokine array and Luminex are shown in red. Increased activation and translocation of NF-κB (gold) is indicated by red upward arrows. Direct protein interactions are indicated by solid black arrows. Indirect activation of NF-κB by inflammatory cytokine signaling is indicated by a dotted red arrow. Proteins/receptors that were identified in literature but were not quantified are shown in grey. Created in https://BioRender.com.

TGF-β-associated pathways, direct measurement of TGF-β levels by enzyme-linked immunosorbant assay did not show elevation in *DDX41*$^{var/+}$ LCL-conditioned media (*Online Supplementary Figure 7*). Overall, assessment of inflammatory cytokines in patient-derived LCL-conditioned media suggests there is inflammatory dysregulation in the context of deleterious germline *DDX41* variants.

A mechanism for inflammatory dysregulation in germline *DDX41*$^{var/+}$ lymphoblastoid cell lines

Well-studied signaling pathways involving DDX41 and the key inflammatory cytokines, ANG, CXCL13, CXCL8, and IL9, intersect at the transcription factor complex NF-κB (Figure 4). The p65 and p60 subunits of NF-κB translocate to the nucleus upon activation of the NF-κB complex.[29] Therefore, to determine if NF-κB activity increases in the context of germline *DDX41*^{LOF} alleles, we measured NF-κB p65 subunit levels by western blotting in nuclear and cytoplasmic cellular fractions of patient-derived *DDX41*$^{var/+}$ (N=5) *versus DDX41*^{WT} (N=3) LCL. Overall, increased NF-κB levels were observed in the nuclear fractions of *DDX41*$^{var/+}$ LCL compared to WT (P=0.008) while cytoplasmic levels were unchanged (Figure 4), confirming activation of NF-κB in *DDX41*$^{var/+}$ LCL and suggesting that NF-κB activity may be involved in DDX41-mediated inflammatory dysregulation (Figure 4D).

Inflammatory dysregulation in individuals with likely germline *DDX41*^{LoF} variants

To determine if there is inflammatory dysregulation in individuals with germline *DDX41*^{LoF} alleles, we analyzed UK Biobank proteomic data available for 2,922 proteins measured from participants' primary peripheral blood.[28] We compared protein levels in individuals with likely germline *DDX41*^{LoF} variants without cancer (N=49) to twice the number of age and sex-matched controls (N=98; *Online Supplementary Tables S7, S8*). Levels of 30 proteins increased in the context of germline *DDX41*^{LoF} alleles, including stress antigens MICA and MICB (P=0.04; Figure 5A). Levels of 114 proteins decreased in the context of germline *DDX41*^{LoF} alleles, including immune-signaling proteins CD79B (P=0.0004), HLA-E (P=0.0004), CD4 (P=0.003), CD28 (P=0.005), and CD80 (P=0.02; Figure 5A). Protein interaction analysis of proteins found to decrease in germline *DDX41*^{LoF} cases compared to WT controls revealed 30 proteins with high confidence (95% confidence interval [CI]=0.700) interactions in which CD4, CD28, and CD80 appear to be central (Figure 5B). STRING pathway enrichment analysis of proteins found to decrease in the context of germline *DDX41*^{LoF} alleles showed that nine of the top ten diminished pathways (90%) involve inflammation (Figure 5C). KEGG pathway enrichment analysis revealed dysregulated NF-κB signaling (false discovery rate [FDR]=1.0x10^{-2}) and disease-gene associations showed "immune system disease" (FDR=4.0x10^{-8}), "autoimmune disease" (FDR=5.0x10^{-7}), and "primary immunodeficiency disease" (FDR=0.7x10^{-6}) as most likely to be present among our *DDX41*^{LoF} cases (*Online Supplementary Figure S8*). Overall, these data suggest that individuals with deleterious likely germline *DDX41*^{LoF} variants have dysregulation of inflammatory proteins years before cancer develops, which could contribute to tumor pathogenesis.

Discussion

Our cohort of 52 families with germline variants in *DDX41* is the largest published to date. Importantly, these families reflect what is known of germline *DDX41*^{LoF} allele carriers in other cohorts such as a 54% HM penetrance by 90yo and an average age of HM onset of 64yo.[4,6,8,17] The penetrance of malignancies in an unselected population is lower and has been investigated previously.[30] However, extensive study of the 43 families with deleterious germline *DDX41* variants and molecular studies on patient-derived tissues allowed us to reveal that the phenotypes and cancer risks within such families may be more complex than previously appreciated. Baseline biopsies in individuals with germline deleterious *DDX41* variants revealed distinct dysplasia in the peripheral blood and BM, particularly in megakaryocytic and erythroid lineages. Most notably, these changes were observed in a 17yo individual indicating that mild dysplasia may be characteristic of individuals with *DDX41*^{LoF} variants many decades before the expected age of onset of *DDX41*-related HM. Importantly, the two baseline cases discussed are representative of many clinical cases we have observed over the past decade. We caution against overinterpretation of bone marrow dysplasia and misdiagnosis of MDS in individuals with germline *DDX41*^{LoF} alleles, since baseline dysplasia in people with *DDX41*^{LoF} alleles must be distinguished carefully from malignancy-associated changes. We suggest performing a baseline BM biopsy when an individual is diagnosed with a germline *DDX41*^{LoF} variant to provide a comparator for subsequent BM examinations to allow assessment of dysplastic changes over time.

Although *DDX41* has long been associated with HM, the presence of solid tumors in our cohort of 43 families with deleterious germline *DDX41* variants and as has been reported previously[10,13] warranted deeper investigation. The spectrum of solid tumors observed in our cohort shares similarities with previous studies: Bannon *et al.* reported an 18% prevalence of solid tumors in individuals with germline *DDX41*^{LoF} alleles,[10] similar to our cohort with a 24% penetrance by 75yo. The same study reported prostate cancer and melanoma,[10] which were observed frequently in our cohort as well (prostate, N=3; melanoma, N=3). Additionally, our observation that over half of germline *DDX41*^{LoF} carriers who developed solid tumors developed HM an average of 8 years later suggests a potential compounded effect of the germline cancer-risk allele(s) with the therapies used to treat the solid tumors. We recommend increased surveillance of individuals with germline *DDX41*^{LoF} alleles treated for solid tumors. We recognize the challenge this presents, because currently genetic cancer risk testing for solid tumors often lacks coverage of *DDX41*.

We recommend inclusion of *DDX41* in cancer risk testing for families with both HM and solid tumors.

We failed to identify any somatic *DDX41* mutations in patient-derived solid tumor tissue (N=8) or in TCGA data (N=10). Moreover, we observed that among families with HM and solid tumors (N=21/43), ~30% had second germline deleterious variants in other cancer-associated genes. Low numbers of tumors identified in those with likely germline *DDX41*^{LoF} alleles with or without additional cancer-risk alleles in public tumor databases precluded our ability to assess differenc-

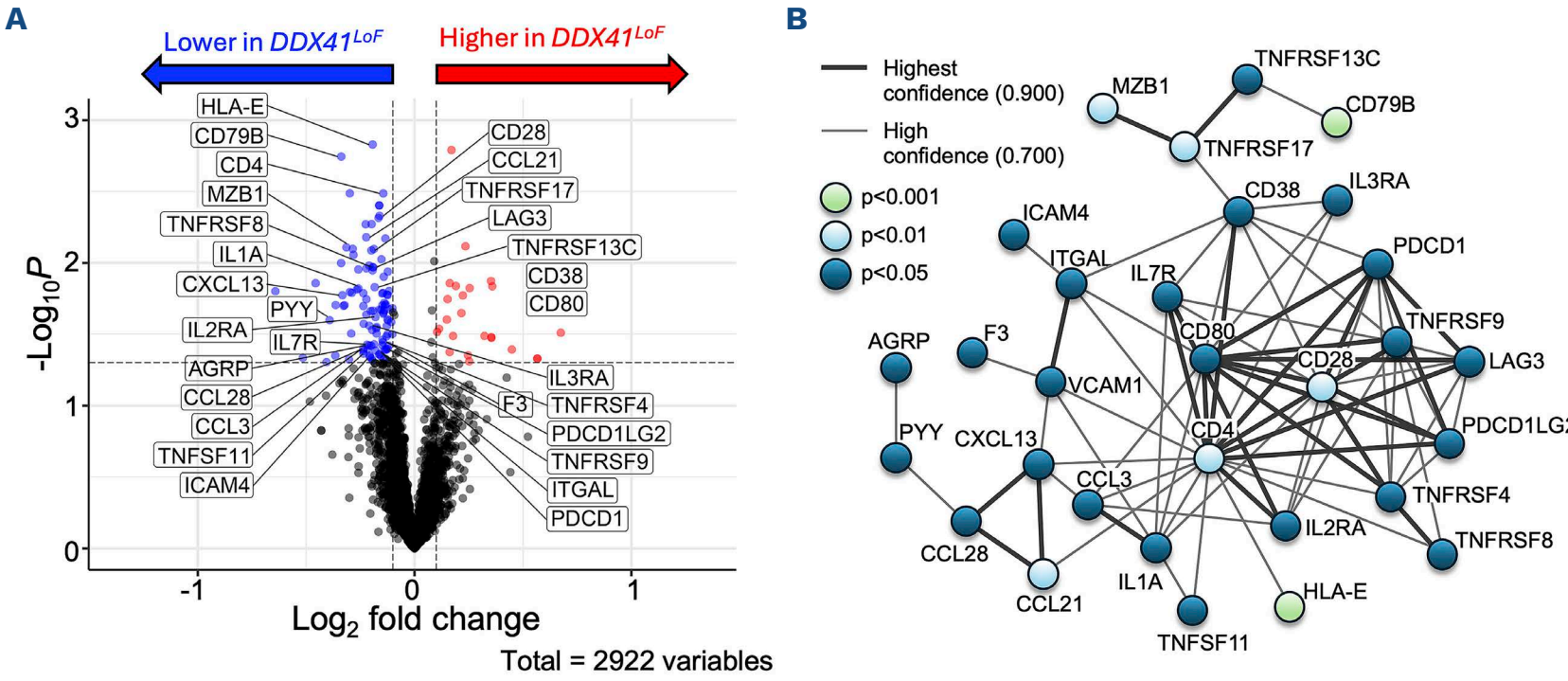


Figure 5. Inflammatory dysregulation in UK Biobank participants with likely germline *DDX41*^{LoF} variants. (A) Volcano plot showing proteins that are increased (red; 95% confidence interval [CI]) or decreased (blue) in individuals with likely germline loss-of-function (LoF) *DDX41* variants compared to wild-type (WT) controls. (B) Protein-protein interaction network showing proteins found to decrease in individuals with likely germline *DDX41*^{LoF} variants compared to WT controls. Only proteins with "high confidence" (0.700) or "highest confidence" (0.900) interactions (N=30) according to the STING database are shown. The level of significance with which proteins were found to decrease are indicated by color: *P*<0.001 (green), *P*<0.01 (light blue), or *P*<0.05 (dark blue). (C) Enrichment plot showing pathways enriched among the 114 proteins found to decrease in individuals with likely germline *DDX41*^{LoF} variants compared to WT controls according to the STRING database.

es in tumor prevalence. We hope that broader testing and expansion of public databases will allow this analysis in the future. Together, these data suggest that *DDX41* may contribute indirectly to solid tumor development, arguing for broad comprehensive germline cancer risk testing in families with solid tumors in ≥15% of primary relatives including the proband and deleterious germline *DDX41* variants.

Since DDX41 regulates innate immunity, we hypothesized that it might contribute to solid tumor development via inflammation. We generated LCL from five individuals in our cohort with distinct germline deleterious *DDX41* variants to investigate this hypothesis. Recognizing that each *DDX41*$^{var/+}$ LCL has a different genetic background and that each *DDX41* mutation was shown to affect protein expression differently, we searched for inflammatory phenotypes that were shared and distinguished *DDX41*$^{var/+}$ from *DDX41*^{WT} controls. We identified upregulation of inflammation-associated genes and several pathways, including TNF-κ signaling via NF-κB (P=1.3x10^{-9}) and inflammatory response signaling (P=5.0x10^{-5}) across all five *DDX41*$^{var/+}$ LCL, indicating an inflammatory phenotype at the transcription level. Analysis of cytokines present in LCL-conditioned growth media demonstrated elevated levels of four cytokines, ANG, CXCL13, CXCL8, and IL9, suggesting dysregulated inflammatory signaling in the presence of a deleterious germline *DDX41* variant. Increased levels of NF-κB in *DDX41*$^{var/+}$ compared to *DDX41*^{WT} LCL nuclear extracts and by our RNA-sequencing data provide further evidence for an inflammatory imbalance driven by these cytokines, which are known to signal through NF-κB. NF-κB signaling is known to promote tumor proliferation, induce epithelial-mesenchymal transition, and stimulate the immune system in favor of tumor growth, consistent with our hypothesis that the *DDX41*^{LoF}-mediated inflammatory signature modifies solid tumor pathogenesis.[29] Moreover, NF-κB is activated in response to the cGAS-STING-TBK1 axis in *Ddx41*-deficient zebrafish.[18,31,32] Our proteomics analysis of individuals with likely germline *DDX41*^{LoF} variants compared to age and sex-matched controls revealed inflammatory dysregulation as well. Interaction analysis of proteins found to decrease in germline *DDX41*^{LoF} cases compared to WT controls revealed many high confidence interactions, particularly involving immune cell receptors CD4, CD28, and CD80. STRING pathway enrichment analyses showed that 90% of the most dysregulated pathways involved inflammation. Enriched KEGG pathways included NF-κB signaling, supporting our hypothesis that germline *DDX41*^{LoF} variants are associated with dysregulated NF-κB signaling. Interestingly, JAK/STAT signaling was also enriched according to the STRING database. Since NF-κB is known to contribute to JAK/STAT signaling in response to inflammatory cytokines,[33] it is possible these pathways are central to DDX41-mediated inflammatory dysregulation. The proteomics data currently available within the UK Biobank[28] are obtained from a single time point. We advocate for similar studies to be performed prospectively in a cohort of individuals with germline *DDX41*^{LoF} alleles compared to familial controls to assess changes in the inflammatory milieu over their lifetimes.

The lack of somatic *DDX41* mutations in solid tumors of those with germline P/LP *DDX41* variants, the presence of other cancer-associated germline pathogenic variants, and inflammatory dysregulation in patient-derived cells and proteomic data suggest that *DDX41* may be an indirect modifier of solid tumor pathogenesis compared to its tumor suppressor function as seen in HM.[5-9,11,12,14,15,17] Based on our data, we advocate for broad cancer risk testing for families with HM and solid tumors that includes *DDX41*. We also advocate for screening of other cancer-risk alleles in families known to have a *DDX41*^{LOF} allele with a history that includes solid tumors in ≥15% of primary relatives including the proband. We hope our observations are hypothesis-generating and encourage further research on the mechanism by which germline deleterious *DDX41* variants contribute to malignancies.

Disclosures

LAG receives royalties from UptoDate, Inc. for a co-authored article on hereditary HM. All other authors have no conflicts of interest to disclose.

Contributions

LAG conceived and supervised the project. SCK, MP, MP, AK, and LM assembled the pedigrees. SCK, CR, HB, SK, ST, SG, PK, and HPS compiled the clinical data. JC and PJ provided expert hematopathologic assessment. SD, ST, SG, PK, and HS performed clinical augmented whole exome sequencing. AK, CR, and HB curated DNA variants. YH and SCK validated variants by Sanger sequencing. RS, SO, and HM analyzed TCGA data. SCK, YH, and JS performed molecular studies on patient-derived cells. AA and MM contributed Luminex data. PJ, KR, and HB performed and SD analyzed single-cell RNA sequencing. SCK designed the figures and compiled the tables. SCK and LAG wrote the manuscript. SCK, JC, YH, JS, SD, AK, MP, MP, LM, AK, HB, CR, SK, RS, HM, ST, SG, PK, HPS, KR, HB, MM, SO, PJ, AA, SD, and LAG edited the manuscript.

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Data-sharing statement

Online Supplementary Data are provided in the Online Supplementary Appendix. For original data, please contact the corresponding author.

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