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Systematic functional validation of IKAROS variants from patients and laboratory-generated mutations

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The full-text version of this article contains a data supplement.

Key Points

- Deleterious germ line mutations in *IKAROS* can cause immunodeficiency and leukemia; functional validation is critical to assign causality.
- Systematic, multifunctional evaluation of *IKAROS* variants helped to confirm, exclude, or anticipate IKAROS-associated diseases.

The transcription factor IKAROS plays an important role in lymphocyte development, differentiation, and as a tumor suppressor. To date, >70 *IKAROS* germ line heterozygous variants have been reported in patients with primary immunodeficiency (PID)/inborn errors of immunity (IEI) and leukemia, and this number continues to grow. Germ line *IKAROS* loss- and gain-of-function mutations have been linked to immunodeficiency, immune dysregulation, and hematologic malignancies, with a broad spectrum of clinical manifestations. Routine next-generation sequencing approaches in patients with PID/IEI have facilitated the identification of *IKAROS* variants, including several cases with variants of uncertain significance (VUS). To determine the VUS' functional behavior, we systematically generated constructs recapitulating those changes and tested IKAROS functions in vitro. We also conducted an in-depth examination of the C-terminal dimerization domain using alanine-scanning mutagenesis to identify amino acids critical for dimerization and other functions. This work provides a comprehensive description of the biologic impact of 81 previously unreported and/or untested *IKAROS* variants, including 33 patient-detected germ line VUS and 48 laboratory-generated mutations in the dimerization domain. Among them, 15 of the patient-detected variants, primarily mapping to IKAROS DNA-binding or dimerization domains, and at least 21 of the laboratory-generated mutations, impaired IKAROS function and could explain or result in human disease. VUS located in between *IKAROS* DNA binding and dimerization domains were less likely to be functionally deleterious. Of note, both positive and negative functional data herein generated can be relevant for patients carrying these *IKAROS* variants, helping to establish a diagnosis and guide treatment decisions.

Introduction

IKAROS, a member of the IKAROS family of DNA-binding zinc-finger (ZF) transcription factors, was first described in 1992 as a regulatory factor in T-cell development.[1] IKAROS has 2 main functional domains, an N-terminal DNA binding and a C-terminal dimerization domain, which are crucial not only for T-cell but also for B-cell and natural killer lymphocytes, dendritic cells, myeloid, and erythroid lineages development and function.[2-5] IKAROS' tumor suppressor activity and the contribution of somatic and germ line *IKAROS/IKZF1* genetic defects have been linked to B-cell acute lymphoblastic leukemia (B-ALL) and T-cell acute lymphoblastic leukemia (T-ALL), although these alterations are less frequent in T-ALL.[6-8] Germ line IKAROS loss-of-function (LOF) mutations have been reported in patients with primary immunodeficiency (PID)/inborn errors of immunity (IEI), primarily presenting with common variable immunodeficiency (CVID)–like phenotypes characterized by recurrent bacterial infections, autoimmunity, and malignancy. Functional studies revealed that LOF defects can be caused by 3 different mechanisms:[9-13] (1) haploinsufficiency (HI) because of large deletions involving the *IKZF1* gene, missense variants mainly affecting the DNA binding and resulting in functional defects without affecting the wild type (WT) allele function, or reduced IKAROS protein expression because of mutant protein instability, (2) dominant negative (DN) which are missense variants that interfere with the WT protein function, and (3) dimerization defective (DD) variants that primarily affect IKAROS dimerization. Depending on the mechanism of action, the immunological and clinical phenotypes vary. Patients with HI present with progressive loss of B cells, hypogammaglobulinemia, recurrent bacterial infections, immune dysregulation, and B-ALL (~5%) with incomplete penetrance.[9,10,14] Patients with DN mutations (N159S/T) exhibit severe T-cell, B-cell, myeloid, and erythroid cell defects, hypogammaglobulinemia with life-threatening/opportunistic bacterial, viral, and fungal infections (ie, *Pneumocystis jirovecii*); 1 case of T-ALL has been reported.[11,15,16] Patients with DD changes show low to normal B cell counts, mild hypogammaglobulinemia, and an increased risk of autoimmune diseases and malignancies, but a more limited infectious disease susceptibility.[12,17] Although *IKAROS* HI and DD mutations are associated with autoimmunity and/or immune dysregulation, none of the patients with DN mutations have been reported to present autoimmune diseases so far. More recently, IKAROS gain-of-function (GOF; R183C/H and T398M) defects have been reported in patients with PID/IEI primarily presenting with immune dysregulation symptoms (inflammation, autoimmunity, allergy, and atopy), abnormal plasma cell proliferation, and malignancies.[18-22]

Next-generation sequencing panels, whole exome sequencing (WES), and whole genome sequencing, are nowadays routinely performed for diagnostic and research purposes on patients with PID/IEI. This provides an unbiased genetic approach to efficiently identify variants in PID/IEI-associated disease-causing genes. However, the pathogenic significance of many of these variants, including those affecting *IKZF1*, remains uncertain or unexplored, as challenging in vitro functional tests are required to validate their

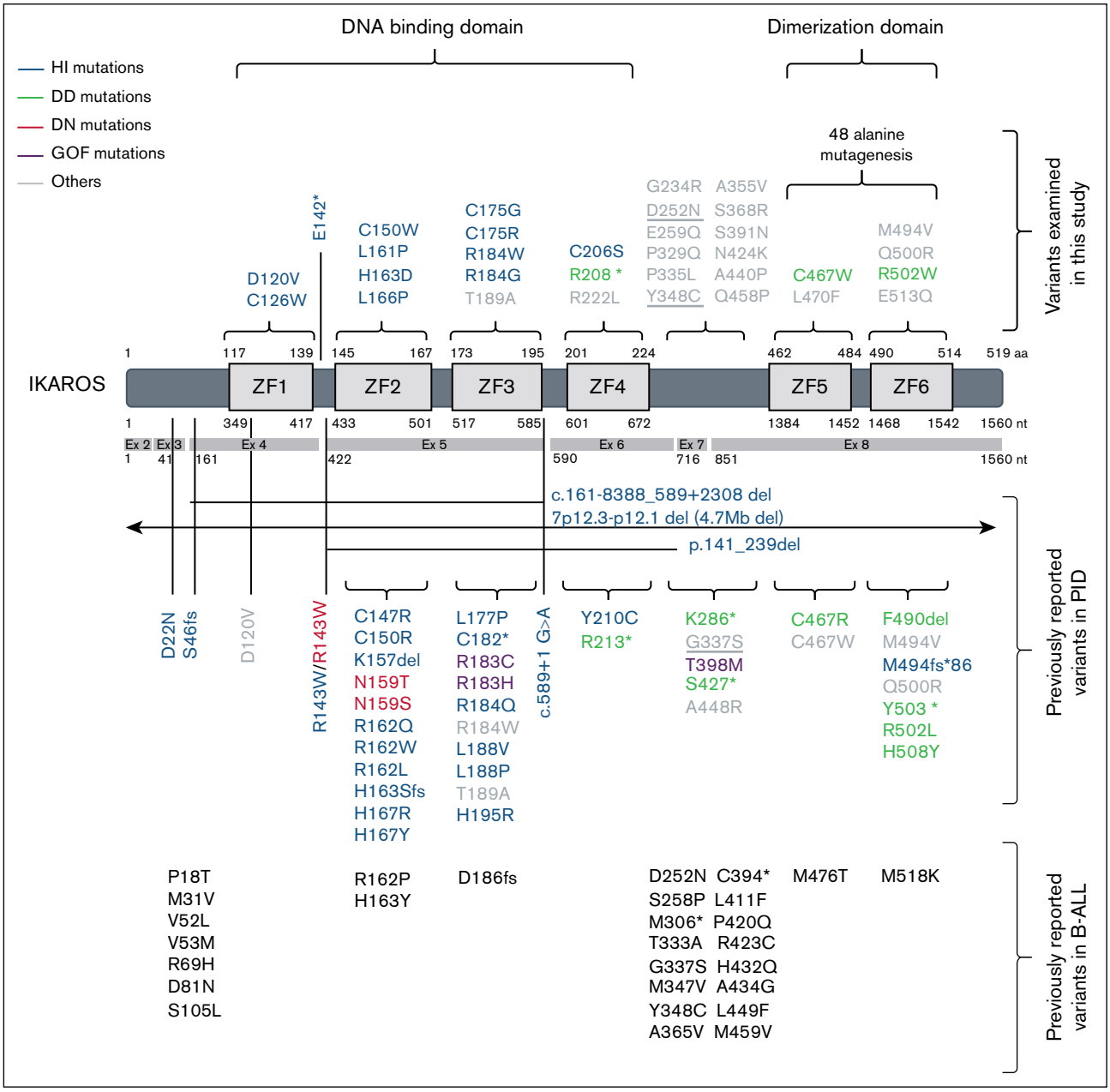

Figure 1. Germ line IKAROS variants identified in patients with primary immune deficiency and B-ALL. The schematic displays IKAROS isoform1 (NM_006060) with coding exons and ZFs. Germ line variants identified in patients with PID/IEI[9,13,20,27,28] and B-ALL,[8] with previously reported variants shown at the bottom and the variants examined in this study (top). LOF mutations, reduced protein expression due to protein instability, or large deletions that do not affect WT function, leading to haploinsufficiency, are indicated in blue. Mutations causing dimerization defects are marked in green, mutations presenting a dominant-negative effect over WT IKAROS protein are depicted in red, and mutations associated with IKAROS GOF are indicated in purple. Variants found in patients with PID that are either untested, show no difference, or do not belong to the listed mechanisms (LOF, DN, GOF) are indicated in gray (others). Underlined gray indicates variants that were tested elsewhere and showed impaired function.[8]

functional defects. Our group uses a systematic approach for validating *IKAROS* variants of uncertain significance (VUS) in vitro that includes immunohistochemistry, electrophoretic mobility shift assays (EMSA), and reporter assays to analyze their impact on pericentromeric targeting, DNA binding, and transcriptional activity.

In this study, we characterized 33 previously unreported and/or functionally unexplored *IKAROS* VUS in patients with immunodeficiency, immune dysregulation, and/or malignancy (Figure 1; supplemental Table 1).[17,23-26] We also performed alanine scanning mutagenesis of all 48 amino acids in IKAROS zinc finger 5 (ZF5) and ZF6, the dimerization domain, to evaluate their individual functional role. This combination of patient-based retrospective genetics (patient-detected germ line heterozygous VUS) and prospective genetics (unbiased dimerization domain alanine scanning) is aimed at helping researchers and clinicians

better understand the role of *IKAROS* variants, their impact on IKAROS function and contribution to clinical manifestations.

Materials and methods

Detailed descriptions of the methods are provided in the supplemental Materials and Methods.

Patients and samples

Blood from healthy donors was obtained under approved protocols.

EMSA

Nuclear extracts from HEK293T cells overexpressing either IKAROS WT and/or mutant constructs were subjected to EMSA (LightShift Chemiluminescent EMSA Kit, Thermo Fisher Scientific; catalog no. 20148), as described previously.[11]

Immunoprecipitation and immunoblotting

Protein lysates from HEK293T cells overexpressing the indicated plasmids were subjected to immunoprecipitation using anti-Flag or anti-hemagglutinin (HA) antibodies and Protein A/G agarose beads (Pierce, catalog no. 20421) for 4 to 5 hours. The beads were washed 3 times with lysis buffer, and immunoprecipitates and total lysates were analyzed by immunoblotting. Protein lysates from enriched CD3 T-cells, T blasts, transfected HEK293T cells were subjected to immunoblotting.

Immunofluorescence staining

NIH3T3 cells were transfected with indicated plasmids using Effectene or Nucleofector Kit R (Amaxa, program A-24), and immunofluorescence staining was performed as previously described.[12,29] Images were taken from 5 to 10 different fields in each of the 3 independent experiments, and representative examples are shown in the Figures 2-4. Pericentromeric-heterochromatin (PC-HC) foci in each transfected cell were counted using the image processing package ImageJ find maxima command.

Luciferase reporter assay

HEK293T cells were cotransfected with pGL4.11-IKBS1 or pGL3-HES1, along with pRL-TK (Renilla luciferase) and IKAROS (WT or mutants) using Effectene. Luciferase activity was measured using the Dual-Luciferase reporter assay system (Promega). For ITGA5 luciferase activity, HEK293T cells were transfected with pEZX-PG04-ITGA5 promoter (GeneCopoeia-HPRM69441-PG04) and IKAROS WT and/or mutant constructs, and luciferase activity was measured using the Secrete-Pair Dual Luminescence Assay Kit (GeneCopoeia) on a Glomax GM3000 plate reader (Promega).

Statistical analysis

When indicated, data were analyzed using 2-tailed Student *t* test utilizing the GraphPad Prism software (GraphPad, version 10.3.1). The differences were considered significant when $P < .05$.

All patients and/or their guardians provided written informed consent in accordance with the Declaration of Helsinki under institutional review board–approved protocols at the collaborating institutions and/or the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Results

Functional testing of patient-detected VUS within IKAROS ZF domains

After dimerizing and multimerizing, IKAROS targets to foci of PC-HC through direct DNA binding to regulate gene transcription.[30] To investigate how variants affect these IKAROS functions (Figure 1; Table 1; supplemental Table 1), we generated mutant constructs recapitulating patient defects located both within or outside the ZF (the latter described in the next paragraph) and analyzed such functions. WT IKAROS localized to the nucleus and showed a punctate staining pattern, which is characteristic of pericentromeric targeting. In contrast, IKAROS variants D120V and C126W mapping to ZF1; C150W, L161P, H163D, and L166P located in ZF2; and C175G, C175R, R184G, and R184W mapping within ZF3, completely disrupted both pericentromeric targeting and DNA binding. An exception to the ZF-mapping variants, ZF3-located T189A behaved as WT (Figure 2A-B; supplemental Figure 1). Of note, T189A was identified in WES of a sporadic PID cohort,[23] and its function has not been previously validated.

Although most PID-associated variants are in ZF2 and ZF3, only 1 missense variant (Y210C) has been previously reported and functionally tested in ZF4.[10,14,31] In our study, 2 more missense variants, C206S and R222L, were identified in the patients and tested for their functional effects. The results showed that R222L had no defect on IKAROS function, but C206S displayed a partial DNA-binding defect with normal punctate staining pattern, similar to what was previously reported for Y210C.[10,14,31]

Among the missense variants in the dimerization domain, including C467W and L470F in ZF5, M494V, Q500R, R502W, and E513Q in ZF6, only C467W showed a complete dimerization defect (Figure 2C). Variant C467W also showed a diffuse nuclear localization pattern, suggesting impaired pericentromeric targeting as well as impaired DNA binding ability (Figure 2A-C). R502W showed a partial dimerization defect with abnormal DNA binding and minimal changes in pericentromeric targeting, similar to the previously described variant at the same site (R502L).[12]

Early truncating IKAROS variants E142* and R208*, that partially remove the DNA binding domain and fully remove the dimerization domain, behaved similar to C467W, showing impaired dimerization as well as diffuse nuclear staining pattern (Figure 2A,C). Different from C467W, mutants E142* and R208* showed aberrant cytoplasmic accumulation, likely due to the partial to complete loss of the DNA binding domain, which is necessary for proper nuclear localization (Figure 2A). EMSA data showed that C467W exhibited reduced DNA binding particularly at dimer and multimer sites, whereas R208* exhibited a faint mutant band compared to the previously reported DD mutant R213* (Figure 2B). In a previous study we showed that WT IKAROS requires dimerization/multimerization for proper DNA target recognition; on the other hand, mutant R213* strongly binds to DNA as a monomer, likely in a nonphysiological manner because of partial ZF4 deletion and complete loss of the dimerization domain.[12] Despite only 5 amino acids distinguishing the 2 ZF4-located variants R213* and R208*, the latter showed markedly diminished DNA binding and partial cytoplasmic localization compared to R213*, suggesting that this short 5-amino acid sequence in ZF4 can influence nuclear localization and DNA binding. Cobb et al showed that deletion of ZF2 or

Table 1. Clinical and immunological features and treatment of patients with IKAROS variants analyzed in this study

Protein change	Singleton (S)/familial case (F1/2/3)	Age of onset/sex (age at last evaluation)	Infections severe/ recurrent/opportunistic Y/N	AI/ID Y/N	Allergy Y/N	Malignancy Y/N	Low B cells Y/N	Hypogam Y/N	Poor vaccine response Y/N	IgRT Y/N	Antimicrobial prophylaxis Y/N	Immune modulator Y/N	Clinical immune phenotype	HSCT Y/N	Status alive (A)/ deceased (D)
C126W	S	7 years/F (8 years)	Y	Y	Y	N	Y	Y	n.d.	Y	N	Y	CVID	N	A
E142*	S	14 years/F (60 years)	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	CVID/cancer susceptibility	N	A
C150W	F1.1	3 years/M (11 years)	Y	N	N	N	Y	Y	Y	Y	N	N	CVID	N	A
C150W	F1.2	4 years/F (8 years)	Y	N	N	N	N	Y	Y	N	Y	N	CVID	N	A
C150W	F1.3	n.a./M (father)	N	N	N	N	n.d.	n.d.	n.d.	N	N	N	Asympt	N	A
L161P	F2.1	2 years/M (5 years)	Y	N	N	N	Y	Y	n.d.	Y	Y	N	CVID	N	A
L161P	F2.2	n.a./M (38 years)	N	Y	N	N	N	N	n.d.	N	N	Y	PIRD	N	A
L161P	F2.3	11 months/M (3 years)	N	Y	N	N	n.d.	n.d.	n.d.	N	N	Y	PIRD	N	A
H163D	S	5 years/F (19 years)	Y	N	N	N	Y	Y	Y	Y	N	N	CVID	N	A
L166P	S	3 years/M (13 years)	Y	N	Y	N	Y	Y	Y	Y	Y	N	CVID	N	A
C175G	F3.1	8 years/F (43 years)	Y	N	N	N	Y	Y	Y	Y	N	N	CVID	N	A
C175G	F3.2	6 months/F (18 years)	Y	N	N	N	N	Y	Y	Y	N	N	CVID	N	A
C175G	F3.3	6 months/M (17 years)	Y	N	N	N	Y	Y	Y	Y	N	N	CVID	N	A
C175R	F4.1	n.a./F (8 years)	Y	Y	N	N	Y	Y	Y	Y	N	N	CVID	N	A
C175R	F4.2	n.a./F (11 years)	Y	N	N	Y	Y	Y	Y	Y	N	N	CVID/cancer susceptibility	Y	A
C175R	F4.3	n.a./M (6 years)	Y	Y	N	N	Y	Y	Y	Y	N	N	CVID	N	A
C175R	F4.4	n.a./F (32 years)	Y	N	N	N	N	Y	Y	Y	N	N	CVID	N	A
R184W	F5.1	12 years/F (13 years)	N	Y	N	N	N	Y	Y	N	N	N	PIRD	N	A
R184W	F5.2	7 years/M (46 years)	Y	Y	N	N	n.d.	Y	n.d.	Y	N	N	CVID	N	A
R184G	S	3/F (14 years)	N	Y	N	N	N	N	n.d.	N	N	Y	PIRD	N	A
C206S	F6.1	1 month/F (9 months)	Y	N	N	N	N	Y	Y	Y	Y	N	CVID	N	A
C206S	F6.2	3 years/F (27 years)	Y	N	N	Y	N	N	Y	N	N	N	SAD/cancer susceptibility	N	A
R208*	S	4 years/F (24 years)	N	Y	N	N	Y	Y	Y	Y	N	Y	PIRD	Y	A
R222L	S	3 years/M (16 years)	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	CVID/PIRD	N	A
G234R	S	n.a./F (44 years)	Y	Y	N	Y	N	Y	n.d.	Y	Y	Y	CVID/cancer susceptibility	N	A
D252N	S	3 years/M (4 years)	Y	Y	Y	N	Y	N	n.d.	Y	Y	Y	PIRD	Y	D
E259Q	S	14 years/F (25 years)	Y	Y	Y	Y	N	Y	N	Y	N	Y	CVID/cancer susceptibility	N	A
P329Q	F7.1	n.a./F (19 years)	Y	Y	N	N	N	N	N	N	N	N	PIRD	N	A
P329Q	F7.2	n.a./F (mother)	N	N	N	N	n.d.	n.d.	n.d.	N	N	N	Asympt	N	A
P329Q	S	n.a./M (45 years)	Y	N	N	N	N	N	n.d.	N	N	N	Infection susceptibility	N	A
P335L	S	7 years/F (13 years)	N	Y	N	N	N	N	N	N	N	Y	PIRD	N	A
Y348C	F8.1	10 years/F (15 years)	N	Y	N	N	N	N	N	N	N	N	PIRD	N	A
Y348C	F8.2	n.a./F (mother)	N	N	N	N	n.d.	n.d.	n.d.	N	N	N	Asympt	N	A
A355V	S	2 years/M (12 years)	Y	Y	n.a.	N	N	Y	Y	n.a.	n.a.	n.a.	CVID	N	A
A355V	S	3 years/M (11 years)	N	Y	N	N	N	N	N	N	N	Y	PIRD	N	A

AI/ID, autoimmunity/immune dysregulation; Asympt, asymptomatic; CVID, common variable immunodeficiency; F, female; IgRT, immunoglobulin replacement therapy; M, male; N, no; n.a., not available; n.d., not determined; PIRD, primary immune regulatory disorders; SAD, specific antibody deficiency; Y, yes.

Table 1 (continued)

Protein change	Singleton (S)/familial case (F1/2/3)	Age of onset/sex (age at last evaluation)	Infections severe/ recurrent/opportunistic Y/N	AI/ID Y/N	Allergy Y/N	Malignancy Y/N	Low B cells Y/N	Hypogam Y/N	Poor vaccine response Y/N	IgRT Y/N	Antimicrobial prophylaxis Y/N	Immune modulator Y/N	Clinical immune phenotype	HSCT Y/N	Status alive (A)/ deceased (D)
S368R	F9.1	1 years/M (22 years)	Y	Y	N	N	Y	Y	Y	Y	Y	N	CVID	N	A
S368R	F9.2	n.a./M (54 years)	N	N	N	N	n.d.	n.d.	n.d.	N	N	N	Asympt	N	A
S391N	S	14 years/M (17 years)	Y	Y	N	N	N	N	Y	N	N	Y	SAD, PIRD	N	A
N424K	S	35 years/M (38 years)	Y	Y	Y	N	Y	Y	n.d.	N	Y	Y	CVID, PIRD	N	D
A440P	S	2 years/F (32 years)	Y	Y	N	N	N	Y	n.d.	Y	Y	Y	CVID	N	A
Q458P	S	n.a./F (56 years)	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	PIRD	N	A
Q458P	S	4 years/M (17 years)	Y	Y	N	N	n.d.	Y	n.d.	n.a.	n.a.	n.a.	CVID	N	A
L470F	S	41 years/F (58 years)	Y	Y	N	Y	N	Y	Y	Y	Y	N	CVID/cancer susceptibility	N	A
R502W	S	6 years/F (29 years)	Y	Y	N	N	N	N		N	Y	Y	PIRD	N	A
E513Q	F10.1	6 months/F (4 years)	Y	Y	N	N	N	N	Y	N	N	N	SAD	N	A
E513Q	F10.2	n.a./F (31 years)	N	N	N	N	n.d.	n.d.	n.d.	N	N	N	Asympt	N	A

AI/ID, autoimmunity/immune dysregulation; Asympt, asymptomatic; CVID, common variable immunodeficiency; F, female; IgRT, immunoglobulin replacement therapy; M, male; N, no; n.a., not available; n.d., not determined; PIRD, primary immune regulatory disorders; SAD, specific antibody deficiency; Y, yes.

ZF3 resulted in complete abrogation of DNA binding and pericentromeric targeting, accompanied by partial localization to the cytoplasm.[30] On the other hand, a mutant vector with a deleted ZF4 exhibited the characteristic WT punctate staining pattern, along with partial localization to the cytoplasm, and showed slightly reduced to normal DNA binding capacity.[30] These and our findings suggest that ZF4 plays a limited role in DNA binding, is not necessary for pericentromeric targeting, but does contribute to proper nuclear localization (as R208* showed partial cytoplasmic localization).

Mutant E142* completely abolished DNA binding, likely due to the lack of ZF2 and ZF3, the primary drivers of this function. When IKAROS protein expression was tested in primary cells from available samples, protein levels were comparable between IKAROS missense variant carriers (C175R and C206S) and healthy controls (supplemental Figure 2A). However, in the patient carrying the E142*, IKAROS protein expression was reduced and the mutant E142* protein was undetectable, even though the mutant allele was transcribed, as confirmed by Sanger sequencing of complementary DNA prepared from the patient's peripheral blood mononuclear cells (supplemental Figure 2B-C). Our group previously demonstrated that the IKAROS dimerization domain is critical for protein stability.[12,13] E142* mutant showed low protein expression and reduced protein stability in a cycloheximide chase assay performed in HEK293T (supplemental Figure 2D). Therefore, the lack of mutant protein expression in primary cells is likely dependent on its decreased protein stability. Both reduced IKAROS protein expression in the patient's primary cells and impaired IKAROS function suggest that E142* likely causes haploinsufficiency.

Next, we tested whether the IKAROS variants with impaired homodimerization also affected heterodimerization with AIOLOS WT and HELIOS WT by coimmunoprecipitation (co-IP) evaluation. The truncation mutants E142* and R208*, both lacking the dimerization domain, impaired heterodimerization with AIOLOS and HELIOS (Figure 2C; supplemental Figure 3A). On the other hand, the missense mutants with complete -C467W- or partial -R502W- homodimerization defects, showed a similar heterodimerization impairment with HELIOS, but normal heterodimerization with AIOLOS (Figure 2C; supplemental Figure 3A-B). These findings suggest that a homodimerization defect does not always affect heterodimerization in the same way, warranting further tests to determine whether new variants in dimerization domain affect homodimerization and also heterodimerization.

To further investigate the mutants' mechanism of action, those exhibiting a diffuse nuclear staining pattern were evaluated in WT and mutant cotransfection experiments to mimic the heterozygous native condition and determine whether the mutations have a dominant-negative effect. A previous study demonstrated that IKAROS DN mutations (N159S/T) dominantly inhibit WT pericentromeric targeting when coexpressed with WT.[11] When we performed cotransfection studies, none of the mutants tested including D120V, C126W, E142*, C150W, L161P, H163D, L166P, C175G/R, R184G/W, R208*, and C467W, interfered with WT's pericentromeric localization (supplemental Figure 4A). For this evaluation, we quantified the number of foci-per-cell in single-transfected or cotransfected cells. In cotransfected cells, foci counts were partially reduced to levels comparable to the WT

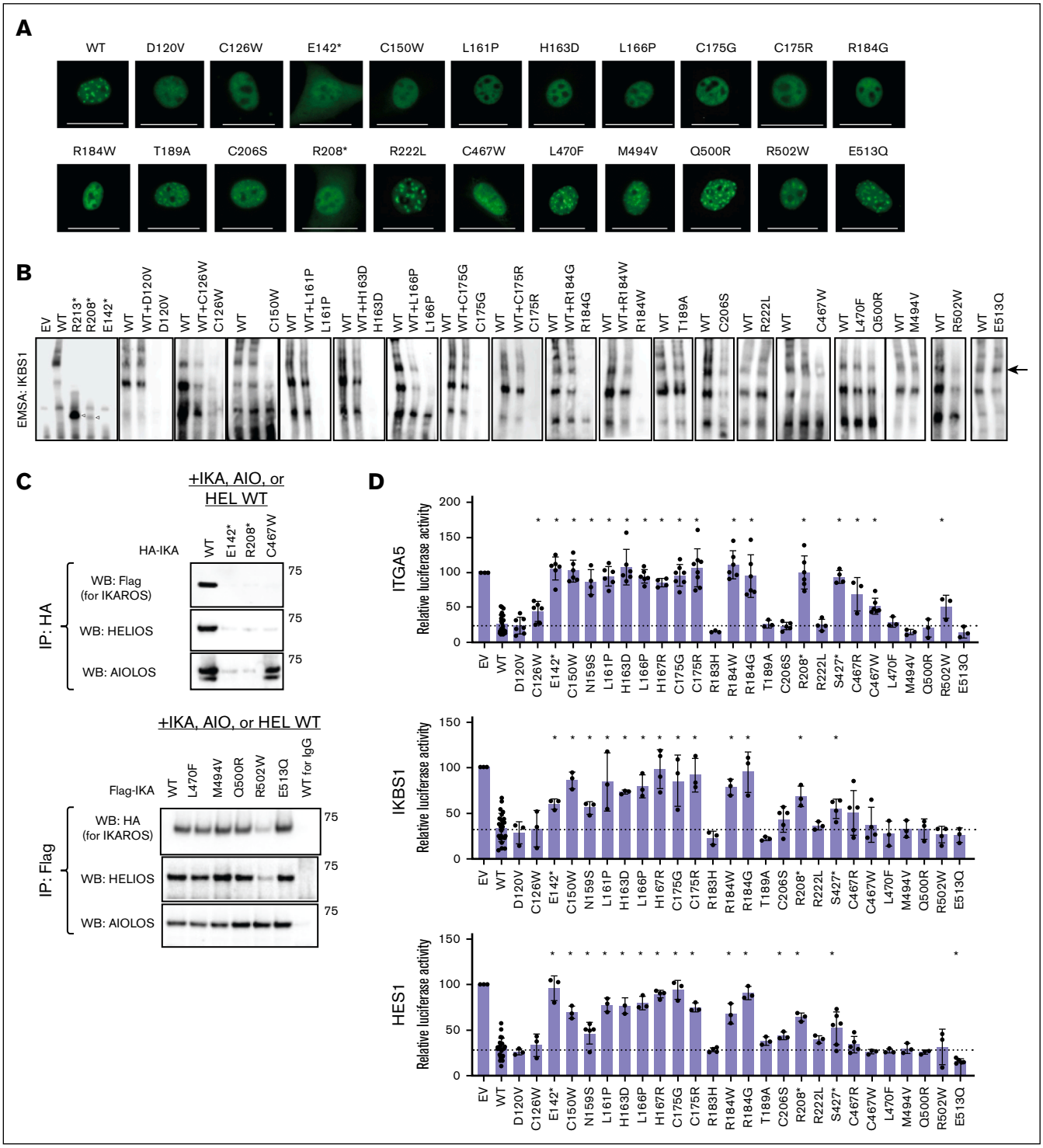

Figure 2. IKAROS function tests. (A) NIH3T3 cells transfected with HA- or Flag-tagged IKAROS WT or mutant were fixed, permeabilized, and stained with anti-HA or Flag antibodies, followed by Alexa Fluor 488–conjugated secondary antibodies. Images were obtained with an EVOS M5000 fluorescence microscope (40× objective). Scale bars, 25 μm. (B) EMSAs were performed using nuclear extracts from HEK293T cells transfected with the indicated IKAROS mutation alone or together with WT IKAROS. Equal amounts of IKAROS protein were used in the assay (see supplemental Figure 1 for immunoblotting data). The arrow indicates IKAROS-containing complexes, likely representing dimers. Open triangles indicate mutant IKAROS protein (R213*, R208*) binding to the DNA. (C) HEK293T cells were transfected with the indicated IKAROS WT or mutant along with IKAROS WT, AIOLOS WT or HELIOS WT. Immunoprecipitations were performed with the indicated antibody. Normal rabbit IgG was used as a negative control for nonspecific binding (see supplemental Figure 3 for IP input and pulldown data). (D) Three different luciferase reporter assays (ITGA5, IKBS1, and HES1) in HEK293T cells are shown. Firefly luciferase reporter activity (IKBS1 and HES1) or Gaussia luciferase (ITGA5) activity was normalized to Renilla luciferase activity or secreted alkaline phosphatase,

control, suggesting that these mutants do not exert a DN effect (supplemental Figure 4B). We also performed luciferase assays cotransfecting WT and mutant constructs at varying ratios and found no evidence of a DN effect (supplemental Figure 5). These findings strongly suggest that the LOF variants tested in this study likely act by haploinsufficiency because of either an impaired DNA binding and/or a dimerization defect.

IKAROS regulates various genes related to hematopoiesis, immune cell differentiation, leukemogenesis, as well as cell signaling and adhesion, including the integrin subunit alpha 5 (*ITGA5*) gene.[8,32,33] When ITGA5 luciferase activity was analyzed, mutants that impaired DNA binding, dimerization, and/or pericentromeric targeting failed to repress its activity, ranging from partial loss (C126W, C467W, R502W) to complete loss (E142*, C150W, L161P, H163D, L166P, C175G/R, R184G/W, R208*) (Figure 2D). We also included previously defined LOF (DN-N159S,[11] HI-H167R,[10] DD-S427* and C467R[12]) as well as GOF (R183H[18]) variants as assay controls. Although known LOF mutations failed to suppress luciferase activity, the GOF mutant exhibited a trend of greater suppression when compared with the WT control. The C206S mutant, with a partial but not absent reduction in DNA binding, was still able to suppress ITGA5 luciferase activity similar to levels of the WT control, suggesting that when some residual DNA binding is maintained, the luciferase activity is not sensitive enough to distinguish between different levels of transcriptional regulation. When the luciferase activity of the variants was tested on Hes family bHLH transcription factor 1 (HES1) and IKBS1 (IKAROS consensus binding sequence), 2 well-established IKAROS targets,[34,35] the results were overall similar to those observed when evaluating ITGA5 luciferase activity. Interestingly, variants D120V, C126W, C467W, and R502W that impaired DNA binding and/or pericentromeric targeting, were still able to suppress HES1 and IKBS1 luciferase activity (Figure 2D). Luciferase data suggest that the loss of the DNA binding and/or pericentromeric targeting because of variants in ZF2 and ZF3 mostly correlates with a failure to regulate transcriptional activity with the reduction approaching levels observed with the empty vector control. In contrast, LOF missense variants involving ZF1 (D120V, C126W), ZF4 (C206S), ZF5 (C467W) and ZF6 (R502W) had minimal or no impact on luciferase suppression (Figure 2D; supplemental Table 2). Altogether, this suggests that IKAROS transcriptional suppression may depend on ZF2-ZF3, in addition to an intact dimerization domain or interactions with other transcription factors. Although the luciferase assay offers insight into how the mutant affects transcriptional activity, particularly if the mutations are within core DNA-binding domain (ZF2, ZF3) or severely disrupt dimerization, it cannot readily determine whether changes in transcriptional activity arise directly from impaired DNA binding rather than other mechanisms, especially when DNA binding is only partially decreased or increased.

Functional testing of patient-detected VUS outside IKAROS ZF domains

Unbiased genetic testing, either as panels, WES or whole genome sequencing, is commonly used for diagnostic purposes in patients with PID/IEI. Through these methods, known pathogenic mutations as well as VUS that require functional validation are frequently identified. When we tested 12 missense VUS located outside IKAROS functional domains, more specifically between ZF4 and ZF5, all displayed normal punctate staining pattern and DNA binding comparable to the WT control (Figure 3). While germ line heterozygous missense variants reported in patients with PID/IEI are primarily located in the IKAROS DNA-binding or dimerization domains, several other variants reported to be associated with B-ALL are located outside those functional domains.[8,36] Churchman et al evaluated 22 germ line variants outside of the ZF domains using EMSA, luciferase assay, cellular localization, adhesion, and drug responsiveness assays in either HEK293T or primary mouse BCR-ABL1+B-ALL cells.[8] Of the 22 variants, 16 tested were functionally damaging (M31V, V52L, V53M, D81N, S105L, D252N, S258P, T333A, G337S, M347V, Y348C, A365V, R423C, A434G, L449F, M459V), including D252N and Y348C, which were also found in patients with PID and tested in this study. These variants showed IKAROS cellular mislocalization, pronounced aggregations, or reduced drug sensitivity in primary mouse BCR-ABL1+B-ALL cells.[8] Despite being shown to be deleterious in studies using these murine cells, most missense variants located between ZF4 and ZF5 in our study showed no defects in the canonical IKAROS functions tested: that is, DNA binding, pericentromeric targeting, protein localization and transcription activity were unaffected (Figure 3).

More recently, and by focusing on the IKAROS ZF4-ZF5 region, Zhang et al identified helical motifs spanning amino acids 252 to 321, including a critical KRK motif that mediates interaction with the nucleosome remodeling and deacetylase (NuRD) complex, essential for transcriptional repression and antiproliferative function.[37] Moreover, several SUMOylation sites (K240, K425 and K459) are located in between ZF4 and ZF5, which have been shown to disrupt IKAROS-NuRD complex interaction.[38]

Altogether, IKAROS missense variants in the ZF4-ZF5 region may not only disrupt interactions with transcriptional regulators and affect posttranslational modifications but also affect cell adhesion and drug responses. While some of these tests are technically challenging to perform, some are also difficult to relate to disease causality (ie, cell adhesion and drug responsiveness defects). As all these functional tests were not included in our systematic evaluation, we cannot formally exclude any of these functions were impaired on the patient-detected mutations evaluated in our work. Further and more complex studies to functionally and reproducibly evaluate this region are therefore warranted.

Systematic alanine scanning and functional testing of IKAROS dimerization domain

IKAROS protein consists of 6 C2H2 ZFs, 4 N-terminus ZF1-4, that are involved in DNA binding, and 2 C-terminus ZF5-6 that facilitate homodimerization and heterodimerization with the corresponding ZFs of IKAROS or other IKAROS family members.[39-41] The C2H2 ZFs are the most common DNA-binding motifs in eukaryotes. C2H2 motifs are characterized by conserved cysteines, histidines,

Figure 2 (continued) respectively. The results were further normalized to the empty vector, set as 100%. The dotted line indicates the average value of the WT control. Data are mean + standard deviation from at least 3 independent experiments. Significance was determined by two-tailed Student *t* test comparing WT to the indicated mutant, $*P < .05$. IgG, immunoglobulin G.

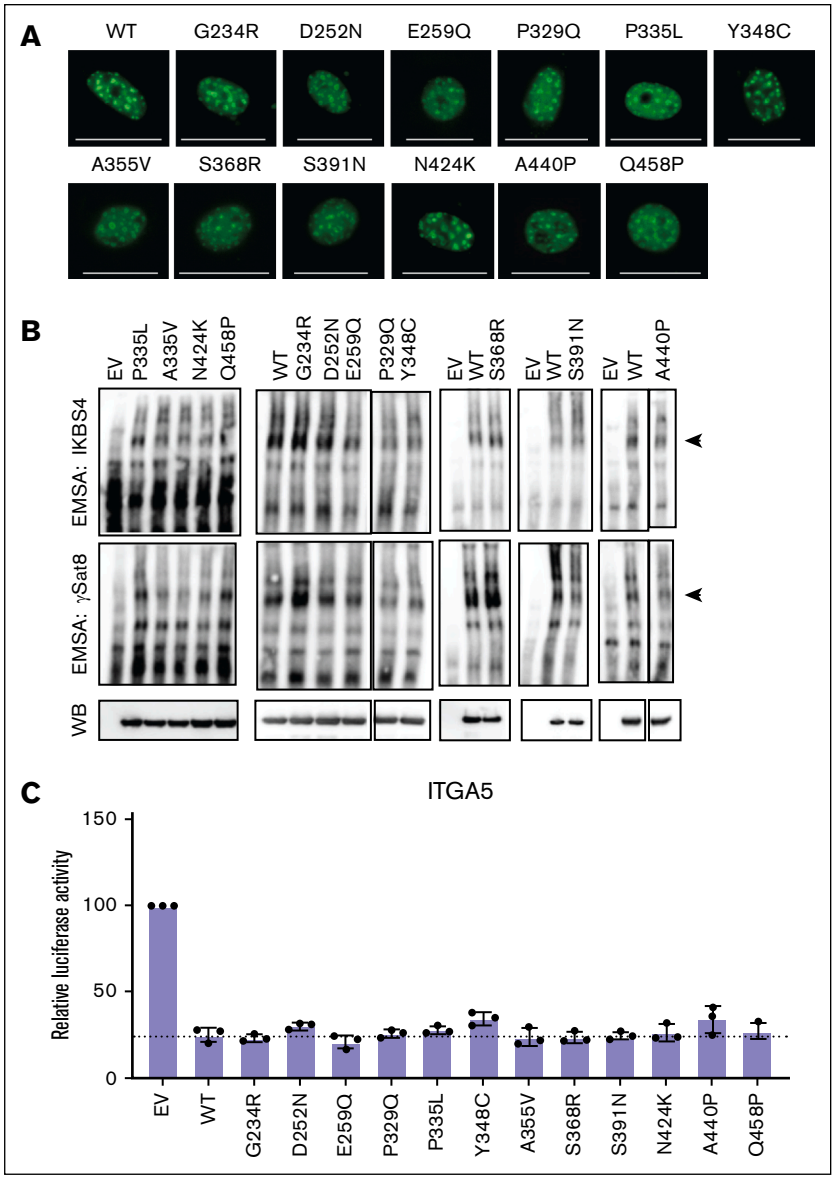

Figure 3. Analysis of IKAROS variants located between ZF4 and ZF5. (A) NIH3T3 cells overexpressing IKAROS WT or the indicated mutants were stained with either HA or Flag antibodies to assess pericentromeric heterochromatin localization. Images were obtained with an EVOS M5000 fluorescence microscope (40× objective). Scale bars, 25 μm. (B) EMSAs were performed using nuclear extracts from HEK293T cells overexpressing IKAROS WT or mutants. Protein expression was tested from the nuclear extracts using a Flag-antibody, and equal amounts of IKAROS protein were used for the assay. The arrow indicates IKAROS-containing complexes, likely representing dimers. (C) ITGA5 luciferase activity was assessed in HEK293T cells overexpressing IKAROS WT or the indicated mutants. The dotted line indicates the average value of the WT control. Data represent the mean ± standard deviation from 3 independent experiments.

and hydrophobic residues, with their consensus sequence defined as (F/Y)-X-C-X_{2-5}-C-X_3-(F/Y)-X_5-ψ-X_2-H-X_{3-5}-H (X, any amino acid; ψ, hydrophobic residue) (Figure 4A).[42] Detailed substitution mutants have shown that IKAROS ZF2 and ZF3 are essential for both pericentromeric targeting and DNA binding, aiding in the prediction of functional defects when IKAROS variants in this region are identified in patients.[30] In addition to the DNA-binding domain, IKAROS dimerization domain is also critical for pericentromeric targeting as this targeting is abolished by disruption of the C-terminal mediated dimerization, and several dimerization-defective variants have been reported in PID/IEI patients (eg, R213*, S427*, C467R, F490del, R520L, and H508Y).[12,30] To gain further insight into the functions of the dimerization domain and to predict the impact of missense variants affecting individual amino acids in ZF5 and ZF6 in patients, we generated alanine substitutions and conducted immunoprecipitation assays to determine the mutants' effects on homodimerization and other IKAROS functions. Flag-tagged mutants were cotransfected with HA-tagged IKAROS WT into HEK293T cells, and co-IPs were performed using an anti-Flag antibody. The alanine substitutions at core cysteine and histidine residues in both ZF5 and ZF6 (C464A, C467A, H480A, H484A, C492A, C495A, H508A, and H514A) impaired homodimerization (Figure 4B). In addition to these residues, several other sites also showed mild to severe homodimerization defects (F471A, D473A, M476A, I479A,N493A, M494A, G496A, R502A, Y503A, F505A I509A, R511A, and G512A). Impaired homodimerization affects IKAROS' punctate staining pattern and DNA binding. Mutations with severely impaired dimerization showed a diffuse staining pattern and reduced DNA binding (mutations marked in red; Figure 4A). Mutations causing a partial defect in homodimerization (marked in blue) also exhibited abnormal DNA binding, either overall diminished or impaired binding at multimer sites (Figures 4 and 5A). However, most of these partial DD mutations retained normal punctate staining

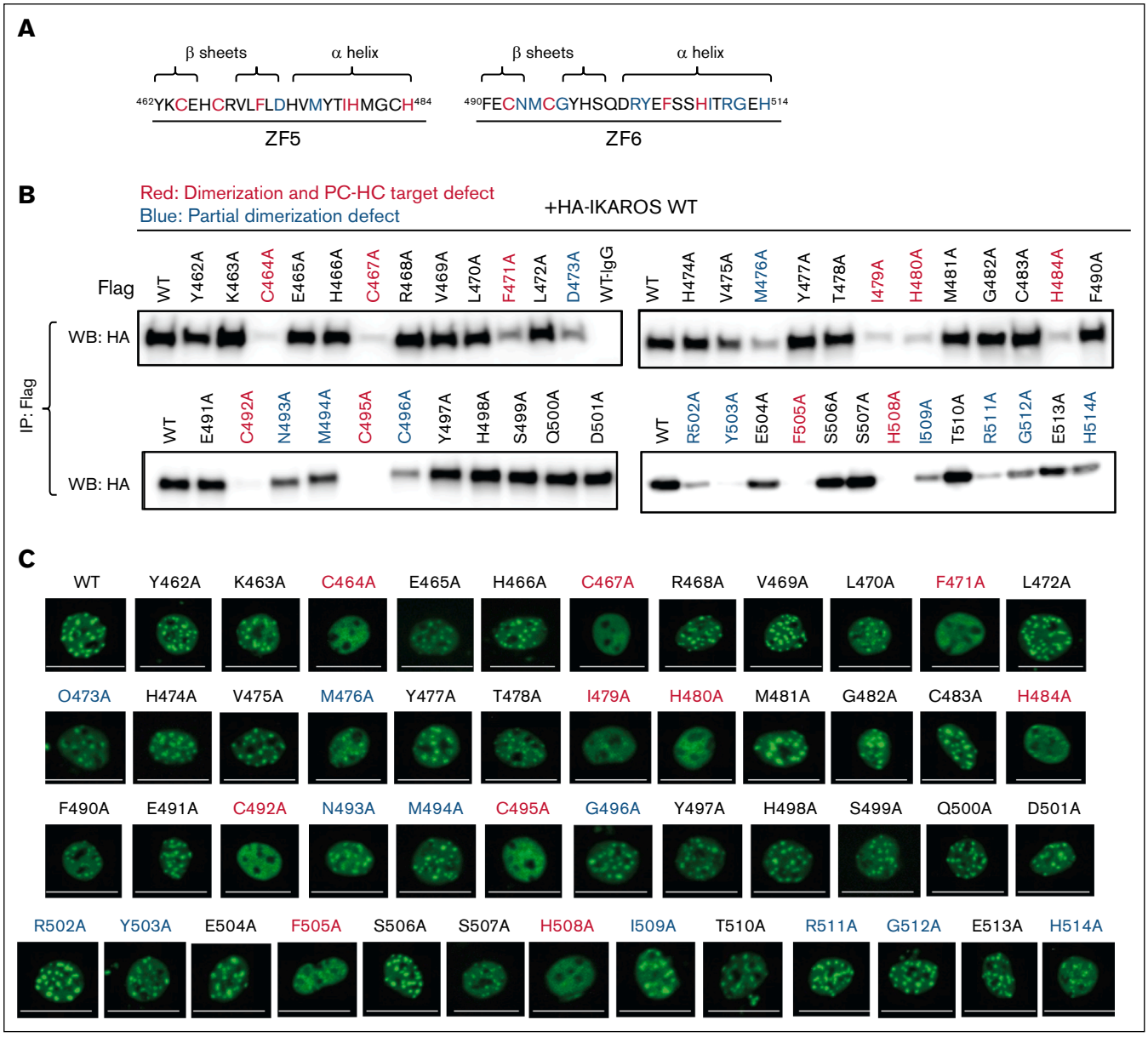

Figure 4. Analysis of scanning mutagenesis within ZF5 and ZF6. (A) ZF5 and ZF6 protein sequences are shown.[41,43] Residues essential for dimerization and pericentromeric targeting are highlighted in red, while residues with intermediate dimerization defects but normal pericentromeric targeting are shown in blue. (B) Interactions between Flag-tagged IKAROS WT or alanine substitution mutants with HA-tagged IKAROS WT were analyzed using coimmunoprecipitation assays (see supplemental Figure 4 for IP input and pulldown data). (C) NIH3T3 cells overexpressing Flag-tagged IKAROS WT or mutants were stained with Flag antibody, followed by Alexa Fluor 488–conjugated secondary antibody to assess pericentromeric heterochromatin localization. Cells were visualized using a ZOE fluorescence microscope (original magnification, ×175). Scale bars, 25 μm. Data shown are representative of 2 to 3 independent experiments.

pattern, similar to what was previously reported with mutant R502L, suggesting that partial dimerization is sufficient to maintain proper pericentromeric localization.[12] In addition to the 21 severe/partial DD mutations described earlier, 10 other mutants that retained normal homodimerization and punctate staining pattern showed abnormal DNA binding to both probes tested in EMSA assays (Y462A, V469A, L470A, L472A, V475A, Y477A, G482A, F490A, Y497A, and D501A), particularly dimer and/or multimer binding sites. The ITGA5 luciferase test revealed that both complete and partial dimerization-defective mutations showed varying effects on transcriptional repression, with some exhibiting impaired and others no transcriptional defects (Figure 5B). The latter primarily involved partial dimerization defects in ZF6 (M494A, R502A, Y503A, I509A, R511A, G512A, and H514A).

Then, we further assessed the heterodimerization capacity of IKAROS mutants exhibiting either partial or severe homodimerization defects. Heterodimerization with HELIOS WT showed patterns similar to homodimerization, with mutations with partial IKAROS homodimerization defects generally exhibiting partial

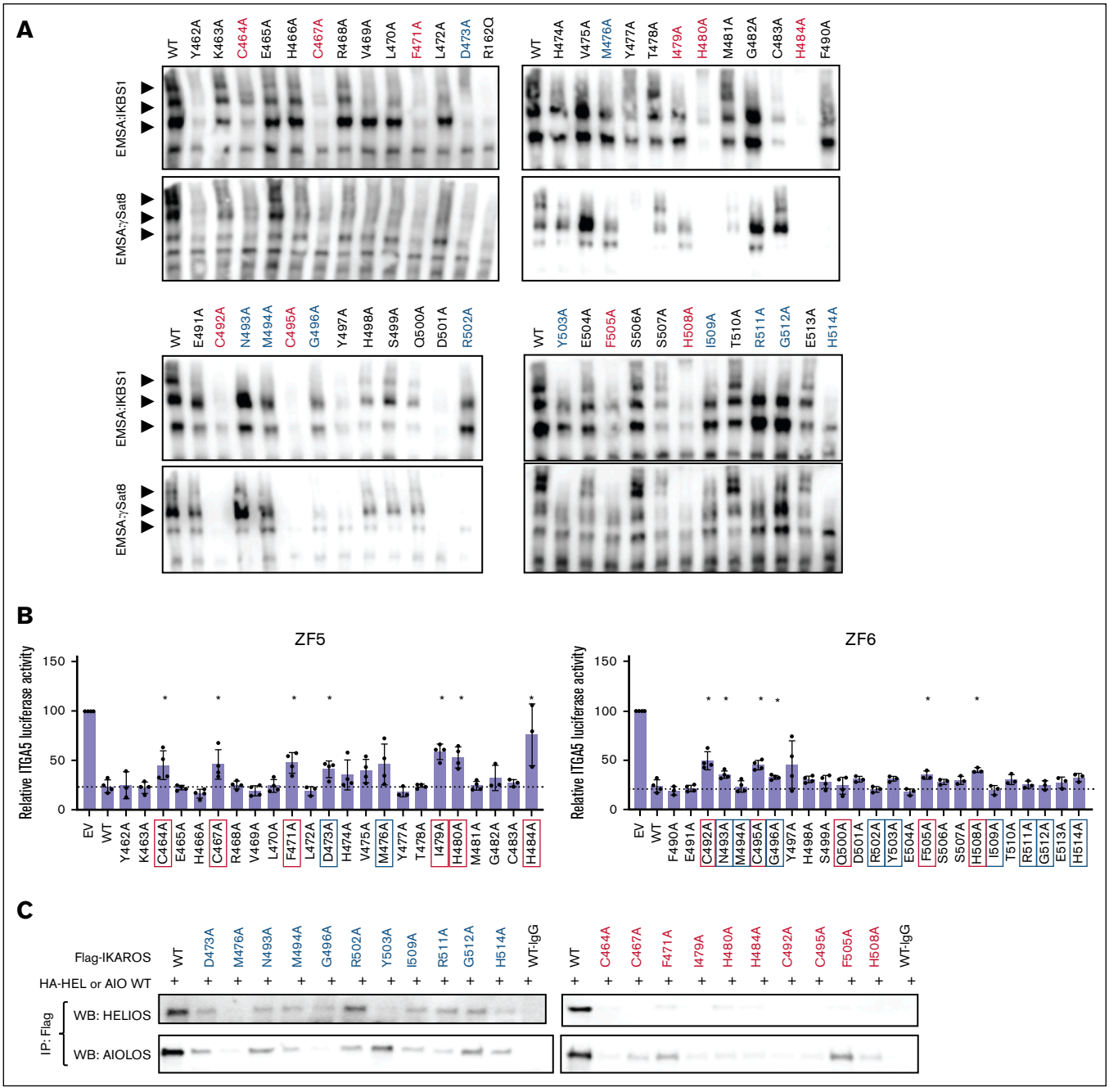

Figure 5. EMSA and luciferase activity with alanine substitution mutants within ZF5 and ZF6. (A) EMSAs were performed using nuclear extracts from HEK293T cells overexpressing IKAROS WT or mutants. Equal amounts of IKAROS protein were used for the assay. R162Q was used as a IKAROS LOF control. The 3 arrows indicate IKAROS-containing complexes: the upper, middle, and lower bands likely correspond to multimer, dimer, and monomer, respectively. (B) HEK293T cells were cotransfected with ITGA5 and IKAROS plasmids. The next day, Gaussia luciferase activity was normalized to secreted alkaline phosphatase. Relative luciferase activity is shown after normalization to the empty vector. The dotted line indicates the average value of the WT control. Residues essential for dimerization and pericentromeric targeting are highlighted in red, and residues with intermediate dimerization defects but normal pericentromeric targeting are shown in blue. Data represent the mean ± standard deviation from at least 3 independent experiments. Significance was determined by 2-tailed Student *t* test comparing WT to the indicated mutant, $^*P < .05$. (C) HEK293T cells were transfected with the indicated IKAROS WT or mutant along with HELIOS WT or AIOLOS WT. Immunoprecipitations were performed with an anti-Flag antibody, with anti-IgG serving as a negative assay control for nonspecific binding (see supplemental Figure 7 for IP input and pulldown data).

HELIOS heterodimerization impairment; except for M476A and Y503A, which showed a more severe deficiency. Moreover, most IKAROS mutants with severe homodimerization defects resulted in uniformly impaired HELIOS heterodimerization. Interestingly, heterodimerization with AIOLOS varied, ranging from partial to severe levels (Figure 5C). Altogether, our data demonstrate that mutants

with severe homodimerization defects are accompanied by a range of heterodimerization impairments, faulty pericentromeric targeting and transcriptional repression. However, mutants with partial dimerization defects exhibit varying levels of functional impairments, which need to be evaluated using diverse assays, as no single test can definitively characterize their functional defects.

Of note, while the M494A showed reduced dimerization and abnormal DNA-binding patterns, the M494V variant, reported in patients with PID,[17] demonstrated results largely comparable to those of the WT control (Figure 2). This difference may be attributed to the distinct properties of valine compared with alanine, which could influence structural stability, or the integrity of the dimerization interface required for effective DNA-binding.

Discussion

Dysregulation of IKAROS expression and function plays a well-established role in the disrupted hematopoiesis observed in both mice and humans with IKAROS-associated diseases.[8,9,44] Mutations in this gene have been linked to a wide range of clinical phenotypes, including CVID-like disease, combined immunodeficiency, primary immune regulatory disorders, and lymphoproliferative disorders including leukemia and lymphoma. As the number of IKAROS-associated diseases continues to grow, studies are increasingly uncovering correlations between genotypes and phenotypes, as well as clinical variability between individual patients. In the present work, we investigated 33 VUS identified in patients with PID/IEI that were previously unreported or had not yet been functionally validated for pathogenicity. Like other already published IKAROS-associated diseases, patients carrying LOF variants acting by haploinsufficiency primarily presented as PID/IEI with a CVID-like phenotype consisting of recurrent respiratory infections and/or autoimmunity, along with low B cell counts, hypogammaglobulinemia and poor antibody responses (Table 1). Of note, patients presenting with clear primary immune regulatory disorders-like or cancer susceptibility disease patterns but without increased infectious susceptibility were also detected; asymptomatic individuals were reported as well in this cohort. Altogether, these findings reinforce the intrinsic expression variability and incomplete penetrance linked to IKAROS-associate diseases driven by DNA-binding and dimerization HI, as well as reduced protein expression caused by large deletions or protein instability.[9,10,12]

IKAROS mutations, either somatic or germ line, have also been extensively associated with different forms of hematologic malignancies.[6-8,12,45,46] In this cohort of 46 patients carrying 33 functionally evaluated novel *IKAROS* variants, 5 hematopoietic cancers were reported in 4 patients. B-ALL was reported in patients carrying mutations C175R (ZF3) and C206S (ZF4); Hodgkin lymphoma was diagnosed in the patient with the G234R variant (located between ZF4 and ZF5), and both non-Hodgkin lymphoma, as well as a cutaneous follicular lymphoma in the patient carrying the L470F variant (ZF5). As previously reported, *IKAROS* variants associated with hematologic malignancy are scattered throughout the gene and related to different mechanisms of disease.[8,9] Of note, an apparently increased prevalence of non-hematopoietic cancers is also present in this cohort of patients, including colon cancer and squamous cell carcinoma associated with mutation E142* (early truncating), dysgerminoma linked to variant E259Q, osteochondroma to variant A440P, and vulvar cancer to variant Q458P, the last 3 changes mapping to the area between ZF4 and ZF5. As we did not detect any functional defects for several of these variants (ie, G234R, A440P, Q458P, and L470F), we can neither confirm nor rule out a causal association between them and the malignancies the patients developed, whether through direct carcinogenic effects or immune-related mechanisms (eg, defective immune surveillance[47]).

The presence of IKAROS somatic deletions or missense mutations in patients with B-ALL is associated with poor prognosis.[48] More recent studies have shown that patients with IKZF1plus (defined as IKAROS deletions accompanied by codeletions in CDKN2A/B, PAX5, or the PAR1 region, in the absence of ERG deletion) experience poorer outcomes and a higher risk of relapse, with postinduction minimal residual disease levels serving as a major modifier of its prognostic impact.[49,50] In cases of IKAROS-associated diseases in PID/IEI, treatment with broad spectrum antimicrobial or immunoglobulin replacement therapy is commonly employed due to predominant humoral immune defects and high susceptibility to recurrent bacterial infections. Allogeneic hematopoietic stem cell transplantation (HSCT) is a potential curative treatment option, particularly for patients with the most severe phenotypes.[11,15,19,21] Among the patients we studied, 3 of them underwent HSCT. The patient with the C175R (ZF3) variant received the graft from his mutation-positive asymptomatic mother, before the etiology of his disease was determined. The patient and his mother have been under regular monitoring since then. The patient with the R208* (ZF4) mutation underwent liver transplantation because of acute liver failure at age 17 years and 6 years later received an HSCT to correct the IKAROS defect. The patient with the likely de novo D252N variant (area between ZF4 and ZF5) received an HSCT because of a myelodysplastic syndrome; unfortunately, the patient passed away 2 months after the procedure because of sepsis, grade 4 gastrointestinal graft-versus-host disease/grade 2 skin graft-versus-host disease. These findings highlight the importance of accurate genetic diagnosis and timely intervention to minimize late-onset complications and improve clinical outcomes.

Despite the important role of IKAROS in PID/IEI and hematologic malignancies, the pathogenic significance of many VUS remains unsolved or untested, largely due to the need for detailed molecular testing to establish causality. In this study, we functionally characterized 33 IKAROS missense variants detected in patients within or outside the functional ZF domains. Using a transient transfection system, we introduced either WT or mutant plasmids and conducted a series of assays to assess the impact of each variant on pericentromeric targeting, DNA binding, and transcriptional activity of IKAROS target promoters. Our results demonstrated that 15 of these missense variants impaired IKAROS protein function, suggesting potential pathogenicity, whereas the remaining 18 variants showed no detectable differences from WT assay controls and thus remain of uncertain clinical relevance. Furthermore, we performed an alanine-scanning mutagenesis of 48 residues within the dimerization domain and identified at least 21 critical amino acids required to support IKAROS proper function. This approach allowed us to map regions essential for dimerization and downstream regulatory activities, as well as to anticipate the significance of these variants that may eventually be identified in patients. While the variants resulting in nondeleterious biologic effects have, in general, a less robust diagnostic/clinical

management impact, these data also suggest that non–IKAROS-related causes should be considered in disease causality.

Overall, our study provides important functional insights into the pathogenic potential of IKAROS variants, both clinical VUS and anticipatory variants. Although there are limitations in validating functional defect, particularly for variants between ZF4 and ZF5, our findings contribute to the understanding of the genetic, immunological, and clinical features related to IKAROS-associated diseases and may also help guide a more targeted patient management and care.

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Authorship

Contribution: H.S.K. and S.D.R. designed the study, analyzed data, and wrote the manuscript; H.S.K., A.A.G.S., N.K., A.E.-S., S.G., J.E.N., and J.L.S. performed experiments; H.C., K.W., W.I., M.F.S., S.K.L., A.A., A.G.-S., S.S., C.B., P.M., R.Z.M., H.A., D.P.H.v.K., A.V.G., D.C., E.Z.-H., A.R., S.T., C.M., A.G., P.O., J.C.A.B., J.J.L., A.A.W., M.C., M.-A.D., B.D.M., J.D.M., X.S., J.F.N., C.D.P., L.H., J.M.R., J.V., M.J.B., H.L., W.J.H.G., D.K., F.H., L.M.A., C.R.-G., R.E., T.R., and K.B. collected clinical and immunological data, and family history; and T.A.F. contributed to clinical insight and scientific discussion.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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