Germline IKAROS dimerization haploinsufficiency causes hematologic cytopenias and malignancies

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Abstract:
IKAROS is a transcription factor forming homo/hetero-dimers and regulating lymphocyte development and function. Germline mutations affecting *IKAROS* N-terminal DNA binding domain, acting in a haploinsufficient or dominant negative manner, cause immunodeficiency. Herein we describe four germline heterozygous *IKAROS* variants affecting its C-terminal dimerization domain by haploinsufficiency, in four unrelated families. Index patients presented with hematologic disease consisting of cytopenias (thrombocytopenia, anemia, neutropenia)/Evans syndrome, and malignancies (T-ALL, Burkitt lymphoma). These mutations are partially or completely deficient to form homo- and hetero-dimers, but do not affect the wild type allele function. Moreover, they alter key mechanisms of IKAROS gene regulation including sumoylation, protein stability, and the recruitment of the nucleosome remodeling and deacetylase complex; none of them affected the N-terminal DNA binding. These C-terminal dimerization mutations are largely associated with hematologic disorders, display dimerization haploinsufficency, incomplete clinical penetrance, and differ from previously reported allelic variants in their mechanism of action. Dimerization mutants contribute to the growing spectrum of *IKAROS*-associated diseases displaying a genotype-phenotype correlation.

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Key Points

- We report *IKAROS* allelic variants disrupting dimerization of the IKAROS family of transcription factors.

- IKAROS dimerization mutants act through a distinctive mechanism and manifest predominantly as hematologic diseases, with limited infections.

Abstract

IKAROS is a transcription factor forming homo/hetero-dimers and regulating lymphocyte development and function. Germline mutations affecting *IKAROS* N-terminal DNA binding domain, acting in a haploinsufficient or dominant negative manner, cause immunodeficiency. Herein we describe four germline heterozygous *IKAROS* variants affecting its C-terminal dimerization domain by haploinsufficiency, in four unrelated families. Index patients presented with hematologic disease consisting of cytopenias (thrombocytopenia, anemia, neutropenia)/Evans syndrome, and malignancies (T-ALL, Burkitt lymphoma). These mutations are partially or completely deficient to form homo- and hetero-dimers, but do not affect the wild type allele function. Moreover, they alter key mechanisms of IKAROS gene regulation including sumoylation, protein stability, and the recruitment of the nucleosome remodeling and deacetylase complex; none of them affected the N-terminal DNA binding. These C-terminal dimerization mutations are largely associated with hematologic disorders, display dimerization haploinsufficiency, incomplete clinical penetrance, and differ from previously reported allelic variants in their mechanism of action. Dimerization mutants contribute to the growing spectrum of *IKAROS*-associated diseases displaying a genotype-phenotype correlation.

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Keywords

Primary immunodeficiency; inborn errors of immunity; transcription factors; cancer susceptibility; penetrance; expressivity; infections

Introduction

IKAROS is a hematopoietic, cell-specific zinc finger transcription factor that plays a crucial role in lymphocyte differentiation and development[1,2]. These effects are mediated through its interaction with the nucleosome remodeling deacetylase (Mi-2/NuRD) and Sin3 histone deacetylase complexes, and the Polycomb repressive complex 2 proteins[1,3-6]. The IKAROS family consists of IKAROS/IKZF1, HELIOS/IKZF2, AIOLOS/IKZF3, EOS/IKZF4, and PEGASUS/IKZF5, each of which has multiple isoforms[7-9]. They all contain a conserved N-terminal DNA binding domain (zinc fingers [ZF]1-4) and a C-terminal dimerization domain (ZF5-6). The N-terminal ZFs are required for binding to specific DNA sequences containing the core GGGAA motif, while the C-terminal ZFs mediate homo- and heterodimerization between IKAROS isoforms and other IKAROS family members[7].

Mouse models have demonstrated that Ikaros is essential for commitment of early hemopoietic progenitors of the B and T lymphoid lineages, and mature lymphoid and myeloid cells[10,11]. In humans, somatic *IKZF1* mutations leading to functional defects or reduced protein expression were originally observed in patients with B-cell precursor acute lymphoblastic leukemia (ALL) and rarely T-cell ALL, and were associated with poor prognosis[12-15]. Moreover, germline heterozygous *IKZF1* mutations were detected in 0.9 % of all B-ALL, the most common malignancy in children[12].

Recently, we and others reported germline heterozygous *IKZF1* allelic variants associated with specific immunological phenotypes. The phenotypes consisted of a common variable immunodeficiency (CVID)-like presentation with progressive B-cell loss,

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hypogammaglobulinemia and antibody dysfunction, along with recurrent and severe bacterial infections; B-ALL susceptibility and autoimmune manifestations were also observed[12,16-22]. These patients carried heterozygous loss-of-function mutations that primarily affected the DNA binding domain of *IKZF1*, impairing its function and displaying haploinsufficiency (HI), *i.e.*, the mutant allele did not affect the function of the wildtype (WT) allele. Alternatively, some DNA binding domain loss-of-function mutations acted in a dominant negative (DN) manner, *i.e.*, the mutant allele negatively affected the function of the WT allele. The latter patients presented with more serious clinical and immunological phenotypes, including severe T cell differentiation and functional defects, as well as absent B cells, mild to severe neutropenia, and eosinopenia. Their clinical findings included susceptibility to *Pneumocystis jirovecii* pneumonia; recurrent bacterial, viral, and/or fungal infection; and predisposition to T-ALL[23].

Herein we describe novel missense and nonsense *IKZF1* allelic variants in four unrelated families primarily affecting dimerization and acting by a previously unreported mechanism. The affected individuals presented with some distinctive clinical features, including hematopoietic cytopenias presenting as Evans syndrome and hematologic malignancies including T-cell ALL and Burkitt lymphoma; other manifestations were B-cell lymphopenia and hypogammaglobinemia, but recurrent or severe infections were not highly prevalent. Our data demonstrate that dimerization defective (DD) mutants acting by haploinsufficiency can also disrupt sumoylation, protein stability, and binding to histone deacetylase 1 (HDAC1) that alters the recruitment of the nucleosome remodeling and deacetylase (NuRD) complex affecting gene regulation by this transcription factor. These results demonstrate that mutations affecting the dimerization domain, as well as those involving the DNA binding domain, all lead to IKAROS

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dysfunction. Furthermore, each genotype has a unique mechanism of action likely defining the

clinical phenotypes in the growing spectrum of defects producing IKAROS-associated diseases.

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Materials and methods

Patients and samples

All patients or their guardians provided written informed consent in accordance with the Declaration of Helsinki under institutional review board−approved protocols of the National Institute of Allergy and Infectious Diseases, NIH. Blood from healthy donors and patients was obtained under approved protocols, which also allowed for the collection and use of patients' family history and pedigrees for publication. All procedures were based on standard of care and established clinical guidelines were followed.

Sequencing

Selected next-generation sequencing results were confirmed by Sanger sequencing in index cases, as well as in all relatives available. Sanger sequencing, whole exome sequencing (WES), and RNASeq methods are provided in the Supplementary methods.

Protein expression, localization and interaction

IKAROS protein expression, co-immunoprecipitations, immunofluorescence and EMSA were performed as previously described[23] and are detailed in the Supplementary methods.

Luciferase reporter assay

The IKBS1, high affinity IKAROS binding site (TCAGCTTTTGGGAATACCCTGTCA) was used to assess IKAROS binding activity. Luciferase activity was measured using the Dual-

Luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Additional information is provided in the Supplementary methods.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism 6.0 (GraphPad). Differences were considered significant when $P < 0.05$.

Clinical histories

See complete clinical summaries and laboratory results in Supplemental file and Table 1.

Family A (p.R213*)

Patient A.III.1 is a 17-years-old male, healthy until 9 years of age when he was diagnosed with immune thrombocytopenia (ITP). Laboratory evaluation showed mild leukopenia, thrombocytopenia and hypogammaglobulinemia. He did not have a history of recurrent or severe infections. Family history was remarkable for his father (A.II.1) reporting one episode of pneumonia in adulthood, and pernicious anemia.

Family B (p.S427*)

Patient B.II.1 is a 31-year-old male, healthy until 2.5 years of age when he was diagnosed and successfully treated for T-ALL. He remained asymptomatic until 27 years of age when he developed recurrent episodes of watery diarrhea. No severe or recurrent infections were reported. Immune evaluation at age 28 showed hypogammaglobulinemia and non-protective titers to childhood vaccines. Family history was remarkable for his mother (B.I.1) reporting hypothyroidism and type II diabetes.

Family C (p.C467R)

Patient C.III.1 is a 18-year-old female, healthy until 9 years of age when she was diagnosed with ITP. Pre-rituximab treatment laboratory studies demonstrated slightly diminished Ig levels and a poor response to pneumococcal antigens. At age 13, she was diagnosed with Coombs positive, autoimmune hemolytic anemia and relapsing ITP. Family history was remarkable for her sister

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(C.III.2) reporting Hashimoto's disease; her maternal aunt (maternal aunt C.II.3) with childhood onset CVID, and her maternal grandmother (C.I.1) with adult onset specific antibody deficiency (SAD).

Family D (p.R502L)

Patient D.II.1 is a 15-year-old male, healthy until 9 years of age when he was diagnosed and successfully treated for a disseminated Burkitt lymphoma. At age 12, a non-malignant 5-6 cm diameter intra-abdominal lymph node was diagnosed. Soon after, he developed ITP and neutropenia. Family history was unremarkable.

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Results

IKZF1 mutations, familial segregation, transcription, and protein expression

Four individual germline heterozygous mutations in *IKZF1* (NM_006060) were identified by WES in each family's index patient, *i.e.*, c.637C>T (p.R213*) in A.III.1, c.1280C>A (p.S427*) in B.II.1, c.1399T>C (p.C467R) in C.III.1, and c.1505G>T (p.R502L) in D.II.1 (Fig. 1A, B); results were confirmed by Sanger sequencing. The index patient's mutation was detected in other relatives of all four families (A.I.1, A.II.1, B.I.1, C.I.1, C.II.1, C.II.2, C.II.3, C.III.2 and D.I.1) (Fig. 1A). Amino acids C467 and R502 are conserved through evolution (Fig. 1C). None of these variants are reported in the Genome Aggregation Database (Supplemental table 1).

Mutant allele transcripts were detected in the index patients and mutation-positive family members tested (Fig 2A). IKAROS expression was evaluated in T and B cells by flow cytometry using a C-terminus directed antibody aimed to detect both the WT and the missense, but not the C-terminus truncated forms of the protein. In this setting, index patients with missense mutations (C.III.1 and D.II.1) and healthy donors showed comparable IKAROS levels (Fig. 2B). Conversely, patients with nonsense mutations (A.III.1, and B.II.1) demonstrated reduced IKAROS expression as only one copy of the WT protein was expressed and detected (Fig. 2B). When an N-terminus directed antibody aimed to detect the WT and both mutant forms of the protein was tested by western blot, truncated mutant proteins R213* and S427* were discriminated at their predicted molecular weights of 25 kDa and 45 kDa, respectively (*i.e.*, lower than the 60 kDa molecular weight of WT (isoform 1) or missense mutated IKAROS) in the nuclear extracts of T-cell blasts of index patients A.III.1 and B.II.1 (Fig. 2C). Since the protein

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expression level of truncated mutants R213* and S427* was lower than WT in T-cell blasts (overexposed blots shown), DD mutant protein stability was evaluated by a cycloheximide chase assay. When compared to each respective cycloheximide-untreated sample, WT IKAROS protein expression was reduced by 32%; whereas DD mutants were 51-89 % degraded (R213* 72%, S427* 70%, C467R 89%, R502L 51%, Y462* [laboratory-generated vector lacking ZF5 & ZF6] 80%) (Fig. 2D and E). Previously described HI mutant R162Q and DN mutant N159S showed similar degradation levels as WT IKAROS. These results show that, despite their *in vitro* reduced protein stability that may have contributed to the decreased amount of mutant protein in patients A.III.1 and B.II.1 (Fig. 2C), DD mutants are indeed transcribed and expressed in both primary and transfected cells.

IKAROS homo/heterodimerization

All four DD mutations are predicted to affect either ZF5 and/or ZF6, which are essential for homo/heterodimerization with IKAROS and its family members. We therefore examined the ability of the mutant proteins to homodimerize with WT IKAROS and heterodimerize with IKAROS family members, AIOLOS and HELIOS. Mutants R213*, S427*, C467R completely failed to bind WT IKAROS; mutant R502L showed partial binding (~25% compared to WT/WT binding). Truncated IKAROS mutant proteins R213* and S427* completely failed to bind WT AIOLOS or WT HELIOS; minimal binding (<5%) was detected with the C467R mutant, and ~60% binding was observed with the R502L mutant protein (Fig. 3). These results demonstrate that all four IKAROS DD mutations disrupt homo- and heterodimerization, either completely (R213*, S427* and C467R) or partially (R502L).

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DNA binding and pericentromeric heterochromatin localization

WT or mutant IKAROS proteins were expressed in NIH3T3 cells, known to lack endogenous expression of IKAROS or its family members, for the evaluation of pericentric-heterochromatin (PC-HC) localization[9,24,25]. WT IKAROS exhibited the characteristic punctate staining pattern of PC-HC localization. Complete DD mutants R213*, S427*, Y462* and C467R exhibited a diffuse distribution in the nucleus without PC-HC localization; patial DD mutant R502L showed a punctate staining pattern similar to that of WT (Fig 4A). These data suggests that partial but not completely defective dimerization can support normal PC-HC DNA binding. Co-expressing mutant proteins with WT IKAROS to mimick heterozygosity did not affect WT IKAROS PC-HC targeting, ruling out a DN effect. Likewise, known IKAROS HI mutant R162Q did not have a DN effect; however, previously reported DN mutant N159S did affect WT IKAROS PC-HC targeting (Fig. 4B).

EMSA assays were performed to evaluate IKAROS' ability to specifically bind its DNA target sequences. WT IKAROS recognized DNA target baits and bound as monomers, dimers, and multimers, that were discriminated based on their molecular weights. Mutants R213*, S427* and Y462* showed strong monomer but no dimer or multimer binding, suggesting that failed IKAROS homodimerization still allows monomer protein-target binding (Fig. 5A). Mutant C467R showed overall reduced binding to all DNA/IKAROS containing complexes, and mutant R502L showed low to normal binding as a monomer and weak binding as dimers and multimers (Fig. 5A). Previously reported HI and DN mutants showed complete absence of DNA binding as monomers, dimers or multimers. Co-expression of DD mutants with WT IKAROS demonstrated no effect on its DNA binding to target probes, ruling out a DN effect (Fig 5B).

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Gene transcription regulation by IKAROS DD mutations was assed by a luciferase assay (vector carrying the IKAROS consensus sequence, IKBS1). Ectopic expression of WT IKAROS repressed ~60% of basal transcriptional activity (Fig 5C). Complete dimerization defective mutants R213*, S427*, Y462* and C467R minimally repressed the basal transcriptional activity; partial DD mutant R502L repressed levels similar to WT IKAROS. When WT and DD IKAROS mutants were co-expressed, luciferase activity was repressed to the levels of WT alone, suggesting no DN effect by the DD mutants. Noteworthy, DNA-binding defective HI mutant R162Q completely failed to repress the basal transcriptional activity but DN mutant N159S repressed the transctiptional activity by 46%, despite its inability to bind to its specific DNA binding sites, highlighting the intrinsic limitations of this type of study (Fig 5A, C). Altogether, as complete DD mutants R213*, S427*, and C467R have lost PC-HC localization (Fig 4A), binding their target sequence as monomers seems insufficient to localize to PC-HC or repress transcription, suggesting that binding as dimers and/or multimers is required for the pericentromeric localization and transcription regulation.

Post-translational modification and transcriptional regulation

Sumoylation, a post-translational process that involves the conjugation of small ubiquitin-like modifiers (SUMOs, 12 kDa), is a major regulator of protein functions, including cell-cycling, cell survival, DNA repair, protein stability, nuclear transport, and transcription[26]. IKAROS harbors four SUMO acceptor sites (K58, K240, K425, K459), and its sumoylation disrupts the interaction between IKAROS and co-repressors of the NuRD complex[27]. We detected multiple shifted bands of single and multi-symoylated IKAROS WT protein (Fig. 6A). Complete DD

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mutants (R213*, S427*, C467R, and Y462*) showed markedly reduced sumoylated proteins (Fig. 6A) either by missed sumoylation sites (*e.g.*, R213*, S427*) and/or likely structural/functional defects associated with each particular DD mutation (*e.g.*, C467R, Y462*). Partial DD mutant R502L, and DNA binding defective mutants HI R162Q and DN N159S, did not affect sumoylation. This data suggest that IKAROS dimerization (fully or partially preserved) is necessary for IKAROS sumoylation.

Both the N- and C-terminal IKAROS regions are associated with the NuRD complex, regulating the repression of transcriptional activity via histone deacetylase (HDAC)-dependent or independent mechanisms. IKAROS sumoylation also affects its interaction with the NuRD complex[27]. Mutants lacking the dimerization domain (R213*, S427*, Y462*) showed markedly decreased interaction with HDAC1 (a core subunit of the NuRD complex); missense mutants (C467R, R502L) showed markedly increased HDAC1 binding (Fig. 6B). No HDAC1 binding defects were detected with HI mutant R162Q or DN mutant N159S. When we tested the interaction between IKAROS and the C-terminal binding protein (CtBP), a HDAC-independent corepressor known to associate with IKAROS through a PEDLS motif located at IKAROS N-terminus domain [28], both truncated and missense IKAROS DD mutants showed similar levels of binding (Supplemental figure 1). This result suggests that IKAROS-HDAC1 interaction is likely independent of transcriptional regulators binding to the N-terminus of IKAROS such as CtBP, and mostly dependent on the structural/functional integrity of IKAROS C-terminal region. These results also suggest that the dysregulated HDAC1 recruitment by DD mutants is unlikely dependent upon PC-HC localization or sumoylation: mutants C467R and R502L, presenting opposite effects on these functions, showed similarly increased HDAC1 binding. Different

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structural/functional defects associated with each particular DD mutation likely determine their dysregulated HDAC1 binding through C-terminus region of the protein.

The effect of the DD variants on gene transcription was evaluated by RNASeq analysis on T-cell blasts, and compared to healthy donors (HD) and patients with DNA binding defective HI (H167R) and DN (N159S) allelic variants (1 of each). Correlation analysis with unsupervised clustering showed that gene expression signatures in all allelic variants clustered together and separated from HD (Fig. 7A). An allelic variant-focused evaluation confirmed that when compared to HD, each mutation type was associated with a particular set of up- and down-dysregulated genes: 42 in the DD patients (12 up-, 30 down-regulated), 63 in the DNA binding HI patients (19 up, 44 down) and 534 in the DNA binding DN patients (315 up-, 219 down). A more discrete number of genes was commonly dysregulated among all allelic variants (Fig. 7B and C). Data analysis through the Ingenuity pathway analysis (IPA) showed that the functions related to development of malignant tumor and differentiation of leukemic cell lines were activated in DD patient cells, whereas functions associated with infection, inflammatory response, T cell homeostasis, development and differentiation were not (Fig. 7D and Supplemental figure 2). Interestingly, while malignant tumor-related pathway is largely up-regulated in DD patients, less canonical pathways were affected compared to the HI and DN patients, suggesting that the different regulation of genes, at least in part, is associated with different clinical and immunological phenotypes in the three groups of IKAROS allelic variants. To evaluate the IKAROS-specific NuRD and Sin3-related gene transcription, a PCR gene array methodological approach was tested in patients for all known IKAROS allelic variants. Patients carrying DD (complete and partial), HI and DN DNA binding defective variants could be

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discriminated upon analysis. Among the NuRD and Sin3-related gene transcripts, genes in which

mutations have been described to be associated with T-ALL and Burkitt lymphoma in the

COSMIC database (*i.e.*, *CREBBP, NCOR1, NCOR2*) were dysregulated in the DD patients.

Moreover, known cancer-associated genes (i.e., CHD, MTA, NCOR, HDAC etc.) [29-32] and genes

associated with immune dysregulation or autoimmune diseases (*i.e.*, ARID4A, Histone H4

[*HIST4H4, HIST1H4I*]*, MTA2*) [33-35] were also dysregulated in DD patients (Supplemental figure

3). Taken together, these results suggest that DD mutations dysregulate IKAROS function and

transcription through a mechanism different from that of DNA binding defective mutations, and

a subset of the dysregulated genes are closely associated with the hematologic manifestations

presented by the DD patients.

Baseline lymphocyte phenotype and immune functions were evaluated in the DD patients (Table

1) and results were compared to previously reported allelic variants. The overall B-cell and T-

cell immunophenotype was closer to DNA binding defective HI patients (*i.e.*, progressive B cell

lymphopenia, elevated CD8^{+} T cells) than that of DN patients (*i.e.*, early absence of B cells,

severe T cell maturation and functional defects)[16,23,36]. However, distinctive immunophenotypic

features could also be identified between DD and HI allelic variants. Dimerization defects

displayed a more limited impact on B cells than the other allelic variants as B cells were ≤ 2% in

1/13 (7.6 %) DD patients, compared to 16/26 (61.5 %) in HI patients, and 7/7 (100%) in DN

patients[16,23]. Moreover, CD8^{+} T cells were increased in 14/26 (54%) HI patients, but this feature

was less prevalent in the DD (3/13, 23%) or DN (2/7, 29%) cases studied. Furthermore, whereas

patients carrying DN IKAROS mutations had defects in memory T-cell differentiation and

impaired T-cell activation and proliferation, all four DD index patients had normal percentages

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of memory T cells and normal T-cell proliferation (Table 1 and Supplemental figure 4). When B cell function was tested in the four index patients, BCR or CD40L-induced B-cell proliferation was comparable to the normal controls in three (A.III.1, C.III.1, D.II.1) and slightly/moderately decreased in one patient (B.II.1). To further evaluate B cell functions, we tested immunoglobulin secretion from naïve B cells and total PBMCs. While markedly decreased IgG generation was observed in index patients B.II.1 and C.III.1 both in naïve B cells and total PBMCs (A.III.1 was not tested due to limited sample availability), slightly low and normal levels of IgG secretion were detected in patient D.II.1's naïve B cells and PBMCs, respectively (Supplemental figure 5). This result correlates with the serum immunoglobulin levels in index patients (B.II.1, C.III.1, and D.II.1), and explains the milder impact of B cell function in family D. Interestingly, variable results were observed among family members carrying the same mutations, reinforcing the variable penetrant and expressivity impact of IKZF1 DD mutants.

Discussion

We report thirteen individuals in four unrelated families carrying four novel germline heterozygous *IKZF1* mutations primarily affecting dimerization. We demonstrated that DD mutants were completely or partially incapable of binding to WT IKAROS, AIOLOS and HELIOS. Besides the dimerization defect, several other downstream functions were also affected. These mutant proteins could not bind to their consensus sequences in target DNA as dimers or multimers, but two of them (*i.e.*, R213* and S427*) were still able to bind as monomers, and all but mutant R502L failed to localize to PC-HC where IKAROS recruits its target genes for transcription regulation. Noteworthy, the four mutants showed a range of impact degrees depending on the function evaluated, being the nonsense mutations the most deleterious and R502L the least, based on the experiments performed. Under a structural/protein modeling analysis it is predicted that the polar basic to nonpolar missense change associated with mutation R502L within the alpha helix of the IKAROS C2H2-6 domain, may abolish cation-pi interactions thereby destabilizing the IKAROS C2H2 alpha helix and disrupting the homodimerization interface as the other DD mutants also do (data not shown). On a genotype/phenotype perspective, the R502L dychotomy between reduced biologic impact with still an aggressive clinical presentation is likely a matter of functional threshold (or gene dosage) associated with the intrinsic biology of DD haploinsufficiency. The performance of R502L, as seen with the other DD mutants, falls under the haploinsufficiency range of activity for multiple IKAROS functions. Once this threshold is trespassed (either by a little or a lot), the function seems compromised and results in a biologic, and in its turn, clinical phenotype. More difficult to define is the exact haploinsufficiency range or threshold for each IKAROS function, particularly

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considering this transcription factor includes at least 17 isoforms acting by homo-/heterodimerization with other family members. Nonetheless, mutant protein R502L was still able to generate a robust biologic and clinical impact, despite a reduced functional effect of PC-HC binding and sumoylation. The R502L mutant, associated with hematologic cytopenias and malignancy, showed reduced IKAROS homo/heterodimerization (~25% and ~60% of WT, respectively), decreased protein stability, affected IKAROS dimer/multimer binding to DNA consensus sequences, dysregulated general and NuRD/Sin3-related specific gene transcription and HDAC1 binding.

 In fact, all of the DD mutants altered all these functions, highlighting their common underlying pathophysiologic mechanisms. Moreover, these features also distinguish them from the HI and DN allelic variants that share defective DNA binding, but do not display any of the DD-related defects mentioned above (Table 2).

Post-translational modification of transcription factors has been proprosed to modulate activation or repression of numerous genes during lymphocyte development[27,37]. IKAROS is required to facilitate chromosome accessibility on target genes, where it recruits the NuRD complex for gene expression regulation. Sumoylation, a post-translational change affecting IKAROS, has been shown to interfere with the recruitment of gene transcription co-repressors[27]. Impaired DNA binding of IKAROS is associated with release of the NuRD complex from IKAROS target genes. Released NuRD complexes cause lymphocyte maturation arrest which promotes leukemogenesis[38]. Our study shows that germline mutants that lack IKAROS dimerization domain (R213*, S427*) result in a marked reduction of sumoylation and HDAC1 binding. These findings indicate that IKAROS' N-terminal domain is involved in recruitment of HDAC1

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but depends on the C-terminal domain to be functionally intact (Fig 6A and B). In contrast, missense mutants that maintain the IKAROS dimerization domain but still demonstrate defective dimerization (C467R and R502L) show markedly increased binding to HDAC1, suggesting that the C-terminal domain can recruit more HDAC1 when not occupied by IKAROS family members. To address how dimerization defects and alteration of HDAC1 recruitment affect transcription activity, we tested luciferase activity using a pGL4.11 vector containing IKBS1 repeats. While complete DD mutants R213*, S427*, C467R, and Y462*, and HI mutant R162Q failed to repress the transcription activity, partial DD mutant R502L did not show any defect in repression of transcription activity. This shows that binding as polymers is more important for the transcription repression than the alteration of NuRD/HDAC1 complex recruitment, which was increased by C467R and R502L but decreased by R213* and S427*. Based on our experience with this technique, we acknowledge that luciferase results using an extrachromosomal reporter plasmid containing a small part of the IKAROS binding region may not accurately reproduce long distance interactions that may be essential for IKAROS function, chromatin assembly or recruitment of NuRD complex. Therefore, the detailed mechanisms by which altered NuRD complex recruitment of DD mutants affect transcription repression need to be further studied for full elucidation.

When we assessed gene level transcription by RNASeq, we found that all IKAROS allelic variants tested (complete and partial DD mutants and DNA binding defective HI and DN mutants) shared an abnormal gene expression pattern. However, comparing HD vs. each IKAROS mutation-type determined that each allelic variant dysregulated a unique set of genes. IPA pathway analysis revealed that while functions associated with malignant tumor

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development were largely dysregulated in DD and DN patients, functions related with T cell development and differentiation were preferentially dysregulated in HI and DN patients. These results suggest a corelation between genotypes and clinical/immunological phenotypes, as hematologic malignancies were more prevlent in DD (2/13) and DN (1/8) patients when compared to HI patients (3/59), wheras abnormal T cell lineage differentiation and/or function were more frequently reported in DN (8/8), and HI (13/26) patients but were not that prevalent in DD mutation carriers (3/13) patients analyzed[16,36]. Moreover, we performed an IKAROS-associated NuRD and Sin3-related gene transcription evaluation using a different technical approach to achieve a more in depth assessment. This PCR gene array-based analysis further discriminated the mutation types at the transcriptomic level and linked the DD mutants to the patients' clinical presentations (hematologic cytopenias due to immunedysregulation and malignancies) (Fig 7 and Supplemental figure 3). Our data show that DD mutations affect the chromatin remodeling complex of particular genes and modify the chromatin landscape, likely leading to oncogenesis and a break in immunological tolerance highly compatible with the clinical phenotype described in our patients.

In terms of clinical presentations, four of thirteen mutation-positive individuals reported herein were asymptomatic (A.I.1, C.II.1, C.II.2 and D.I.1), and one (B.I.1) presented with hypothyroidism and type 2 diabetes, symptoms questionably related to the IKAROS defect. On the other hand, two presented with tissue-specific autoimmunity, *i.e.*, pernicious anemia in A.II.1 and Hashimoto's disease in C.III.2, and two presented with recurrent infections associated with either late onset specific antibody deficiency (C.I.1) or early onset CVID-like disease (C.II.3). Interestingly, the four index cases (A.III.1, B.II.1, C.III.1, and D.II.1) all presented with hematologic cytopenias or malignancies but no, or very limited, history of infections.

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Manifestations in the index patients started during their first decade of life either as T-ALL, Burkitt lymphoma or cytopenias resembling Evans syndrome, a rare autoimmune disorder of AIHA, ITP, and/or autoimmune neutropenia. A recent study demonstrated that 49 of 80 pediatric patients with Evans syndrome had germline variants in primary immunodeficiency (PID)-causing genes[39]. Although that study did not provide biologic validation for the variants found, 29 were linked to muations that had been previously proven deleterious, while 20 were not. Among the latter, two patients with *IKZF1* and one with *IKZF2* mutations presented with Evans syndrome during childhood[39]. Our work not only reinforces the genetic and clinical relationship between IKAROS and Evans syndrome as recently reported, but also provides biologic validation of this genotype-phenotype correlation describing a novel dimerization defective mechanism for IKAROS-associated diseases. Other germline heterozygous mutations predicted to negatively affect IKAROS' dimerization domain (K286*, D186fs, M306*, and C394*) have been recently published[12,21]. Although the intrinsic dimerization defect and mechanism of action were not part of the scope of those manuscripts, these mutants appeared to exhibit incomplete clinical penetrance and were mainly associated with hematologic manifestations (*i.e.*, B-ALL and different degrees of B-cell deficiency); recurrent infections likely occurred in only one of those cases[12,21].

All germline heterozygous *IKZF1* allelic variants reported so far (*i.e.*, HI, DN) and herein (*i.e.*, DD), are associated with certain clinical phenotypes. While patients with DN mutations present with a T- and B-cell CID phenotype mainly characterized by opportunistic infections (7/7)[23], patients carrying HI mutations present primarily with a CVID picture of increased susceptibility to B-cell immunity-controlled infections (~60%)[16-22,40]; patients with DD mutations, most

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typically present with hematologic dyscracias (38%, 5/13; cytopenias in 4, malignancies in 2). Although the clinical presentations of the three allelic variants cannot be completely differentiated from each other, they largely segregate with a particular pathophysiologic mechanism (HI, DN or DD) affecting IKAROS function.

Similar to patients carrying HI defects but in contrast to those with DN allelic variants, we detected virtually asymptomatic DD mutation-positive relatives of the index cases in at least three of the four extended families evaluated. Some of the asymptomatic relatives showed signs that could be interpreted as immunologic penetrance despite the lack of symptoms (*e.g.*, B.I.1, had slightly diminished IgG levels; C.II.1 had reduced switched and non-switched meomory B cells). This type of presentation with incomplete immunologic and/or clinical penetrance is common in autosomal dominant PIDs[16,41]. Moreover, although all index cases clinically manifested symptoms within the first decade of life, late onset disease rather than non-clinical penetrance cannot be ruled out

In summary, germline heterozygous IKAROS dimerization defects acting through haploinsufficiency, work via a unique pathophysiologic mechanism and may manifest during childhood with hematologic diseases consisting of cytopenias (ITP, AIHA and/or neutropenia)/Evans syndrome and malignancies (T-ALL and Burkitt lymphoma) with limited infections and what appears to be incomplete clinical penetrance. These findings, together with previous reports of DNA binding haploinsufficiency (HI) mutations presenting primarily as CVID-like disease and dominant negative (DN) defects presenting as CID, expand the genotype-

phenotype spectrum of IKAROS-associated diseases with underlying novel pathophysiology

mechanisms and distinctive immunological and clinical features.

Acknowledgments

Author contributions

HSK and SDR designed the project and wrote the manuscript. CCR, SL, JR, AT, WAG, SS, ZJ, EG, KB, TAF, JV and SC were involved in collecting biologic and clinical data from the patients and analyzing data. HSK, SCM, SG performed experiments and analyzed data. JEN and JS performed RNASeq and analyzed data. JEN, JS, RJH, TS performed next generation sequencing and/or analyzed data. MG, MH, LL assists informed consent process and arrange the sample delivery. EG, JR, CCR, and SC provided samples, clinical information and analyzed data.

Disclosure of Conflicts of Interest

The authors declare no competing financial interests.

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Figure and table legends

Figure 1. Genetics and pedigrees of families with *IKZF1* mutations. (A) Segregation of the *IKZF1* mutations and clinical and immunological phenotypes. Squares and circles indicate male and female family members, respectively. Question mark indicates an unscreened individual. (B) Schematic presentation of the structure of IKAROS isoform 1 (NM_006060). Dark gray indicates the zinc fingers (ZF) and arrows indicate the site of the mutations. Numbers indicate amino acid location. Previously reported germline IKAROS mutations in CVID or CID patients are indicated below the domain. Blue color indicates haploinsufficient mutations (DNA binding defective mutations and loss of one *IKZF1* allele); dominant negative mutations are shown in red; dimerization defective mutations are shown in green; gray color indicates the variants whose function are unclear. (C) Sequence conservation of ZF5 and ZF6 in IKAROS. The mutated amino acid sites found in Families C and D (C467 and R502, respectively) are indicated in the green box.

Figure 2. IKAROS transcripts and protein expression in patients (A) The chromatograms show the sequence of the indicated heterozygous mutation in cDNA prepared from PBMCs. WT indicates wild type of IKAROS's reference sequence. Arrows indicate the site of mutations. (B) IKAROS protein expression levels were tested in T and B cells from PBMCs from index patients and a paired healthy donor control (HD). (C) IKAROS expression in nuclear extracts of T cell blasts. Triangles indicate R213* and S427* mutant protein. Bottom blots were overexposed to visualize the low levels of truncated IKAROS protein (R213* and S427*). Data shown are representative of two independent experiments. (D, E) HEK293T cells were transfected with a vector expressing IKAROS WT or mutant. After 24 hours, cells were treated with cycloheximide

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(CHX, 20 μg/ml) for further 24h. Whole cell lysates were prepared and analyzed by immunoblot analysis using HA antibodies to test IKAROS expression. Vinculin was used as a loading control. The relative IKAROS protein stability was calculated by dividing CHX treated sample by untreated sample (X100) after normalization with Vinculin to show the remaining protein amount after CHX treatment. Data are mean value $\pm$ SEM of 3-4 independent experiments. Data (WT vs. each mutant) were analyzed using unpaired *t*-test utilizing the GraphPad Prism software. *p<0.05.

Figure 3. Impaired interaction of mutant protein with the IKAROS family. (A-C) 293T cells were transfected with Flag-tagged wild type (WT) IKAROS, AIOLOS, HELIOS and HA-tagged IKAROS WT or mutants (R213*, S427*, C467R, R502L, Y462* [a laboratory-generated mutant with deletion of ZF5 and 6]). EV indicates empty vector control. Immunoprecipitations were performed using anti-Flag antibodies. Western blot analysis of the IP samples with anti-HA and anti-Flag or AIOLOS or HELIOS antibodies is shown. 5% of the total volumes of the whole cellular lysates used for each IP reaction were loaded as input controls. Data shown are representative of 3 independent experiments. Representative densitometry result of immunoprecipiated HA-IKAROS was performed using ImageStudio.

Figure 4. Pericentromeric targeting of the mutant IKAROS. (A-B) NIH3T3 cells were transfected with a HA-tagged WT or mutant expression vector alone (A) or together with Flag-tagged WT IKAROS (B). Cells were labeled with anti-HA or together with anti-Flag antibodies, followed by Alexa 488-conjugated (green) and Alexa 568-conjugated (red) secondary antibodies. Cells were visualized by using a ZOE fluorescence microscope (original magnification is X175).

Data shown are representative of 3 independent experiments. The scale bar indicates 25 μm. HI

and DN indicate DNA binding defective haploinsufficient mutation and dominant negative

mutation, respectively.

Figure 5. DNA binding and transcription activity of the mutant IKAROS protein. (A-B) EMSA

assays were performed using nuclear extracts from HEK293T cells transfected with indicated

IKAROS mutation alone (A) or together with WT IKAROS (B). Numbers indicates the ratio of

amount of plasmid DNA used for the co-transfection. The nuclear extracts were allowed to bind

to two different IKAROS probes: IK-bs1, an IKAROS consensus binding sequence and γ-Sat 8,

a sequence from the pericentromeric region of human Chromosome 8. IKAROS containing

complexes are indicated with arrows. Triangles indicate mutant IKAROS protein binding to the

DNA as a monomer. Data shown are representative of 3 independent experiments. (C). Four

repeats of IKBS1 were inserted into the pGL4.11 vector (pGL4.11-IKBS1) and co-transfected

with pcDNA3-HA IKAROS wt or mutants and pRL-TK (Renilla luciferase) as indicated.

Twenty hours later, cells were lysed and luciferase activity was measured using the Dual-

Luciferase reporter assay system. The firefly luciferase activity was normalized to the *Renilla*

luciferase activity and then the results were normalized to the empty vector control. Each

experiment was carried out in duplicate. Data are means ± SEM from three independent

experiments.

Figure 6. Sumoylation of IKAROS WT and mutant. (A) HEK293T cells were co-transfected

with GFP-sumo1 and HA-tagged IKAROS WT or mutant. Protein extracts were

immunoprecipitated with anti-HA antibodies, and probed with anti-Sumo1 antibodies to see the

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sumoylation, and anti-HA antibodies to see the immunoprecipitated IKAROS. Arrows near 100-200 kDa indicate single or multiple sumoylated IKAROS protein. (B) HEK293T cells were co-transfected with Flag-tagged IKAROS WT or mutant and HA-tagged HDAC1. Protein lysates were immunoprecipitated with antibodies to the Flag epitope. Western blot analysis of the IP samples with anti-HA (for HDAC1) and anti-Flag (for IKAROS) antibodies is shown. 5% of the total volumes of the whole cellular lysates used for IP reactions were loaded as input controls. Data shown are representative of 3 independent experiments.

Figure 7. Correlation analysis of targeted RNA-seq data on T-cell blasts from healthy donor (HD) controls (n=5) and patients with DD, HI, and DN mutations. RNASeq was performed using RNA extracted from enriched blasted T cells. Scale bar indicates Pearson correlation coefficient for variance stabilized data for differentially expressed mRNA. (B) Venn diagram analysis comparing lists of differentially expressed genes for each IKAROS study group [*i.e.*, DD (n=4), HI (n=1), DN (n=1)] vs. HD controls (n=5). (C) Table of differentially expressed genes for the DD study group only and differentially expressed genes common among groups. Genes overexpressed compared to HD controls are in red text; genes underexpressed compared to HD controls are in blue text. (D) IPA Diseases and Functions Analysis heatmap showing a comparison of the predicted effected cellular processes and biological functions based on gene expression. The activation z-score represents the bias in gene regulation that predicts whether the biological function exists in an activated (red) or inactivated (blue) state.

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ID Mutation	A.I.1 (Grandma) p.R213*	A.II.1 (Father) p.R213*	A.III.1 (Index Pt) p.R213*	B.I.1 (Mother) p.S427*	B.II.1 (Index Pt) p.S427*	C.I.1 (Grandma) p.C467R	C.II.1 (Mother) p.C467R	C.II.2 (Aunt) p.C467R	C.II.3 (Aunt) p.C467R	C.III.1 (Index Pt) p.C467R	C.III.2 (Sister) p.C467R	D.I.1 (Father) p.R502L	D.II.1 (Index Pt) p.R502L	Reference (% of total lymphocytes)
Age and gender	74y, F	48y, M	17y, M	74y, F	31y, M	66y, F	44y, F	36y, F	38y, F	18y, F	21y, F	50y, M	15y, M	
Age at onset			9y/ITP		2.5y/T-ALL					9y/ITP			9y/Burkitt lymphoma	
Lymphocyte phenotyping														
CD3 T (ALC)	812	1384	970	797	1348	2510 (H)	1196	1821	1004	1394	1556	2095	4181 (H)	714-2266
CD3 T (%)	81.2	87.6 (H)	85.8 (H)	57 (L)	89.3 (H)	91.6 (H)	87.3 (H)	80.2	85.8 (H)	91.1 (H)	77.4	77.6	88.2 (H)	60-83.7
CD4 T (ALC)	346 (L)	272 (L)	421	558	477	1121	523	742	580	915	824	1450	2176 (H)	359-1565
CD4 T (%)	34.7	17.2 (L)	37.3	39.9	31.6 (L)	40.9	38.2	32.7	49.6	59.8	41	53.7	45.9	31.9-62.2
CD4+CD62L+CD45RA+(%)	n.d.	3.8 (L)	11.6	n.d.	3.4 (L)	3.2 (L)	7.7	4.9 (L)	14.2	20.4	8.5	18.2	25.6	7.6-37.7
CD4+CD62L+CD45RA- (%)	n.d.	10.2 (L)	21.5	n.d.	24.7	34 (H)	23	25	31.3 (H)	33.9 (H)	25.4	22.8	15.1	10.4-30.7
CD4+CD62L-CD45RA- (%)	n.d.	3.1	4	n.d.	3.5	3.7	6.8	2.7	4	5.2	6.6	11.7	4.6	2.3-15.6
CD4+CD62L-CD45RA+ (%)	n.d.	0.2	0.3	n.d.	0	0	0.6	0	0.1	0.3	0.5	1.1	0.6	0-1.5
CD8 T (ALC)	441	1063 (H)	483	230	808	1145 (H)	564	826	335	412	607	508	1673 (H)	178-853
CD8 (%)	44.1 (H)	67.3 (H)	42.7 (H)	16.5	53.5 (H)	41.8 (H)	41.2 (H)	36.4 (H)	28.6	26.9	30.2	18.8	35.3 (H)	11.2-34.8
CD8+CD62L+CD45RA+(%)	n.d.	26.8 (H)	20.7 (H)	n.d.	22 (H)	18.8	11.3	16.2	16.4	20.2 (H)	16	7.2	26.2 (H)	5.7-19.7
CD8+CD62L+CD45RA- (%)	n.d.	6.9	6.1	n.d.	9.1	11.5 (H)	5.2	14 (H)	7.7	1.9	6.1	3.1	2.3	1.5-10.3
CD8+CD62L-CD45RA- (%)	n.d.	4.7	7.2	n.d.	3.2	3.1	5.4	5.2	2.6	2.3	3	6	3.8	1.1-9.2
CD8+CD62L-CD45RA+ (%)	n.d.	28.8 (H)	8.7 (H)	n.d.	19.2 (H)	8.4 (H)	19.3 (H)	1	1.9	2.5	5.1	2.4	3	0.7-7.8
CD4/CD8 ratio	0.79 (L)	0.26 (L)	0.87 (L)	2.42	0.59 (L)	0.98 (L)	0.93 (L)	0.9 (L)	1.73	2.22	1.36	2.86	1.3	1.11-5.17
CD4+CD45RA+CD31+(%)	n.d.	n.d.	8.6	n.d.	2.1	n.d.	4.8	n.d.	n.d.	13.9	4.6	9.5	18.6	1.6-20.2
CD4+CD45RA-CXCR5+(%)	n.d.	n.d.	3.4	n.d.	5	n.d.	7.3	n.d.	n.d.	7.6	8.5	3.2	2.9	1.8-8.9
CD4+CD25+FOXP3+ (%)	n.d.	n.d.	2	n.d.	2	n.d.	0.6 (L)	n.d.	n.d.	1.6	0.9 (L)	2.3	1.7	1.6-4.3

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CD20 B (ALC)	76	38 (L)	35 (L)	247	27 (L)	121	92	118	41 (L)	44 (L)	217	154	351 (H)	59-329
CD20 B (%)	7.6	2.4 (L)	3.1	17.7	1.8 (L)	4.4	6.7	5.2	3.5	2.9 (L)	10.8	5.7	7.4	3-19
CD20+CD27-IgM+ (%)	n.d.	78.8	84.5	n.d.	39.2 (L)	84.2	91.2	84.8	98.7 (H)	98.8 (H)	89.3	53.3 (L)	86.6	56.2-91.5 (% of B cells)
CD20+CD27+IgM+ (%)	n.d.	12.0	12.5	n.d.	45.19 (H)	4.3 (L)	3.9 (L)	5.7	0.5 (L)	0.9 (L)	6.9	29.9	7.9	4.8-38.5 (% of B cells)
CD20+CD27+IgM- (%)	n.d.	6.6	2 (L)	n.d.	6.5	6.1	3.2 (L)	6.2	0 (L)	0.2 (L)	2.7 (L)	15.4	2.6 (L)	4.3-19.9 (% of B cells)
CD19+CD24++CD38++ (%)	n.d.	6.1	18.7	n.d.	0 (L)	16.8	7.7	4.9	11.1	3.2 (L)	10.6	4.2 (L)	4.1 (L)	4.7-18.7 (% of B cells)
CD19+CD24-CD38++ (%)	n.d.	0.6	0.3	n.d.	0 (L)	0.1 (L)	0.1 (L)	0.4	0 (L)	0 (L)	0.1 (L)	1.5	0.7	0.3-3.6 (% of B cells)
NK (ALC)	106 (L)	158	130	353	134	104 (L)	85 (L)	336	125 (L)	95 (L)	233	454	209	126-729
NK (%)	10.61	10	11.5	25.2	8.9	3.8 (L)	6.2	14.8	10.7	6.2	11.6	16.8	4.4 (L)	6.2-34.6
Immunoglobulins (mg/dL)														
IgG	n.d.	516 (L)	1001*	672.7 (L)	349 (L)	1147	n.d.	n.d.	59.8 (L)	984*	n.d.	n.d.	740	700-1600
IgA	n.d.	36 (L)	49 (L)	90.5	28 (L)	98	n.d.	n.d.	<10 (L)	12 (L)	n.d.	n.d.	112	70-400
IgM	n.d.	15 (L)	14 (L)	54.3	<7 (L)	<10 (L)	n.d.	n.d.	<10 (L)	6 (L)	n.d.	n.d.	98	40-230
Auto antibodies														
	n.d.	n.d.	Anti-platelet Ab positive, Coombs negative.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	Coombs positive ANA positive	n.d.	n.d.	ANA positive, Anti-dsDNA negative, Coombs negative.	
Vaccine response														
	n.d.	n.d.	Progressive loss of protective titers towards pneumococcal and HiB antigens	n.d.	1/14 pneumococcal, non protective titers to tetanus toxoid, diphtheria toxoid, measles, mumps, rubella or varicella	5/23 to pneumococcal vaccine response (Pre), 8/23 to pneumococcal vaccine response (Post), Tetanus protective	n.d.	n.d.	n.d.	2/14 pneumococcal, protected to diphtheria and tetanus	n.d.	n.d.	n.d.	
Clinical history														
	Asymptomatic	1 pneumonia (age 25y), pernicious anemia	ITP, progressive hypogamma-globulinemia	Type 2 diabetes, hypothyroidism	T-ALL, Recurrent diarrhea, Hypogamma-globulinemia	Pneumonia (x 2, starting in her 50's)	Asymptomatic	Asymptomatic	Recurrent URIs, Hypogamma-globulinemia, fibromyalgia	ITP, AIHA, Hypogamma-globulinemia s/p Rituximab	Hashimoto's	Asymptomatic	Burkitt lymphoma, Lymphadeno-pathy, ITP, Neutropenia	

Table 1. Clinical and laboratory feature in patients with heterozygous *IKZF1* mutations

H and L indicate values above/below the normal range (respectively) compared to adult controls in NIH Clinical Center.
The B cells subsets are expressed as the percentage of CD19$^+$ cells.
*The patient was receiving IgG replacement therapy.
n.d indicates not determined.
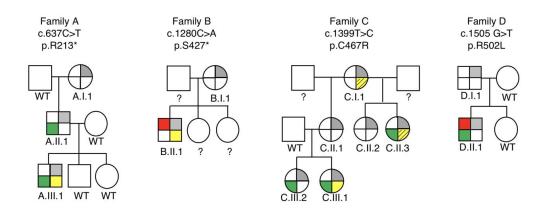ITP: Idiopathic thrombocytopenic purpura
AIHA: Autoimmune hemolytic anemia

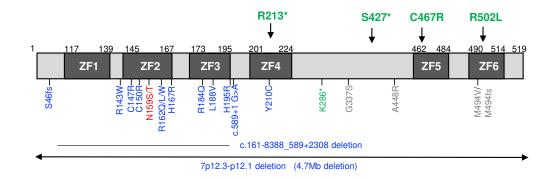| Functional test		WT	R213* (DD)	S427* (DD)	C467R (DD)	R502L (DD)	Y462* (Del ZF5+6)	R162Q (HI)	N159S (DN)
Dimerization		+++	-	-	-	+	-	+++	+++
PC-HC (WT or Mutant alone)		+++	-	-	-	+++	-	-	-
PC-HC (co-expression with WT)	Mut-Mut	NA	-	-	-	+++	-	-	-
	WT-Mut	NA	-	-	-	+++	-	++	-
	WT-WT	+++	++	++	++	+++	++	++	-
DNA binding	Monomer	+/++	+++	+++	+	++	+++	-	-
	Multimer	+++	-	-	+	+	-	-	-
Sumoylation		+++	+	+	+	+++	+	+++	+++
HDAC1 binding		+	+/-	+/-	+++	++	+/-	+	+
Protein stability (CHX treated)		+++	+	+	+	++	+	+++	+++
T/B/Myeloid abnormalities		NA	T-/B++/M-				NA	T-/B+++/M-	T+++/B+++/M+++
Infections		NA	-/+				NA	+++	+++
Autoimmune cytopenias		NA	++/+++				NA	+	-
Hematologic malignancies		NA	++/+++				NA	+	+/++

Table 2. Summary of various effects of germline heterozygous *IKZF1* mutations

All mutations were found in the patients, except Y462* (a laboratory-generated mutant to test the effect of deletion of ZF5 and ZF6, gray color). R162Q and N159S are previously reported DNA binding defective *IKZF1* mutations acting by haploinsufficiency (HI) or dominant negative (DN) effect, respectively. "+/++/+++" indicates the strength of each IKAROS function test, being "+++" the strongest and "-" not detected (all compared to WT); NA, indicates not applicable.

Figure 1

A

Legend:
- ■ (gray) Mutation positive (Heterozygous)
- ■ (yellow) Antibody deficiency
- ▨ Recurrent infection
- ■ (green) Autoimmune/immune dysregulation
- ■ (red) Malignancy

Family A
c.637C>T
p.R213*

Family B
c.1280C>A
p.S427*

Family C
c.1399T>C
p.C467R

Family D
c.1505 G>T
p.R502L

B

R213*   S427*   C467R   R502L

1   117   139   145   167   173   195   201   224   462   484   490   514   519

ZF1   ZF2   ZF3   ZF4   ZF5   ZF6

S46fs

R143W
C147R
C150R
N159S/T
R162Q/L/W
H167R

R184Q
L188V
H195R
c.589+1 G>A

Y210C

K286*

G337S

A448R

M494V/
M494fs

c.161-8388_589+2308 deletion

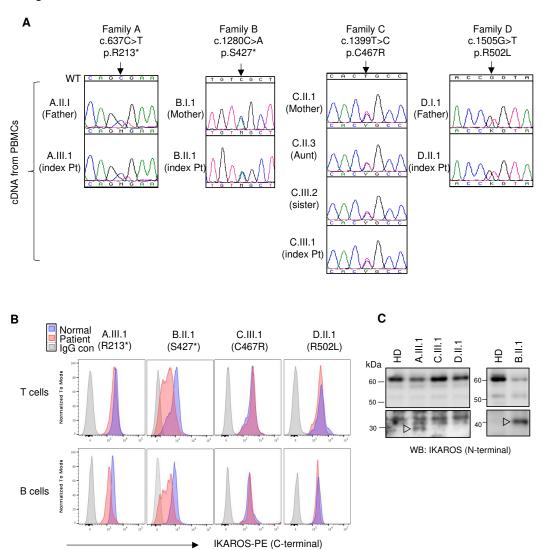7p12.3-p12.1 deletion   (4.7Mb deletion)

C

	ZF5	ZF6

Homo.sapiens   RVVSTSGEQMKVYKCEHCRVLFLDHVMYTIHMGCHGFRDPFECNMCGYHSQDRYEFSSHITRGEHRFHMS
P.Troglodytes   RVVSTSGEQMKVYKCEHCRVLFLDHVMYTIHMGCHGFRDPFECNMCGYHSQDRYEFSSHITRGEHRFHMS
M.Mulatta   RVVSTSGEQMKVYKCEHCRVLFLDHVMYTIHMGCHGFRDPFECNMCGYHSQDRYEFSSHITRGEHRFHMS
C.Lupus   RVIGTSGEPMKVYKCEHCRVLFLDHVMYTIHMGCHGFRDPFECNMCGYHSQDRYEFSSHITRGEHRFHMS
B.Taurus   RVIGTSGEALKVYRCEHCRVLFLDHVMYTIHMGCHGFRDPFECNMCGYHSQDRYEFSSHITRGEHRFHMS
M.Musculus   RVVSTSGEQLKVYKCEHCRVLFLDHVMYTIHMGCHGFRDPFECNMCGYHSQDRYEFSSHITRGEHRYHLS
G.Gallus   KVISSNGEQVRVYKCEHCRVLFLDHVMYTIHMGCHGFRDPFECNMCGYHSQDRYEFSSHITRGEHRFHMS
D.Rerio   KVLSGDGEELRAYRCIHCRVLFLDHVMYTIHMGCHGFRDPFECNLCGYRSQDRYEFSSHITRGEHRI---
X.Tropicalis   KVVSGSGESLKVHKCEHCRVIFLDHVMYTIHMGCHGFRDPFECNMCGYQSQDRYEFSSHITRGEHRFNMG
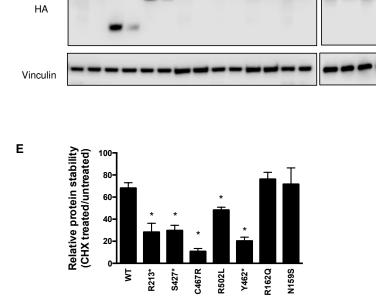
C467R   R502L

Figure 2

A

Family A
c.637C>T
p.R213*

Family B
c.1280C>A
p.S427*

Family C
c.1399T>C
p.C467R

Family D
c.1505G>T
p.R502L

cDNA from PBMCs

WT

A.II.I (Father)

A.III.1 (index Pt)

B.I.1 (Mother)

B.II.1 (index Pt)

C.II.1 (Mother)

C.II.3 (Aunt)

C.III.2 (sister)

C.III.1 (index Pt)

D.I.1 (Father)

D.II.1 (index Pt)

B

Normal
Patient
IgG con

A.III.1 (R213*)

B.II.1 (S427*)

C.III.1 (C467R)

D.II.1 (R502L)

T cells

B cells

IKAROS-PE (C-terminal)

C

kDa

HD   A.III.1   C.III.1   D.II.1

HD   B.II.1

60

50

30

60

50

40

WB: IKAROS (N-terminal)

Figure 2

D

E

Figure 3

A

+Flag-**IKAROS**-WT

HA: EV, WT, R213*, S427*, C467R, R502L, Y462*

IP: Flag
- HA
- Flag

Input
- HA
- Flag

B

+Flag-**AIOLOS**-WT

HA: EV, WT, R213*, S427*, C467R, R502L, Y462*

IP: Flag
- HA
- AIOLOS

Input
- HA
- AIOLOS

C

+Flag-**HELIOS**-WT

HA: EV, WT, R213*, S427*, C467R, R502L, Y462*

IP: Flag
- HA
- HELIOS

Input
- HA
- HELIOS

Figure 4

A

	WT	R213*	S427*	C467R	R502L	Del ZF5+6 Y462*	HI R162Q	DN N159S
HA								

B

+ Flag-IKAROS WT

	HA- WT	HA- R213*	HA-S427*	HA-C467R	HA-R502L	HA-Y462*	HA-R162Q	HA-N159S
HA								
Flag (WT)								
Merged								

Figure 5

A

IKBS1

γSat8

B

IKBS1

γSat8

C.

Figure 6

A

B

Figure 7

A

B

C

	DD only		DD & DN	HI & DD	DD, HI, & DN
MED12L	TSHR	PELI2	ZNF346	ADD2	IL6R
BEND3	LGALS17A	ABCA1	ATP6V0A4		AQP3
OFD1	EPHA4	KIR2DL4	TMEM213		ZNF215
PML	ZNF208	LILRB1	CXCL13		CNTLN
LRRC2	SLC22A23	TAGLN3	IGFBP3		ZNF629
MCU	ADAM23	HK3	KLRC3		TUSC3
CTSL	SGSM1	NCAM1-AS1	IKZF2		CCL2
ENOX1	LYZ	NCAM1	GAS7		
PIGH	CAMSAP3	KIAA1804	C10orf10		
UBE2Q2P1	BAIAP2L1	SNPH	IFI27		
CDH1	FAM110C	PCOLCE	TIE1		
KLF4	HSD11B1	PASK	LTBR		
MT1H	RAB31	PLXNB2	CTSG		
FAM3A	FAAH2	PTGDR	KHK		

D