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ClinGen Myeloid Malignancy Variant Curation Expert Panel recommendations for germline RUNX1 variants

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Abstract:
Standardized variant curation is essential for clinical care recommendations for patients with inherited disorders. Clinical Genome Resource (ClinGen) variant curation expert panels are developing disease-associated gene specifications using the 2015 American College of Medical Genetics and Genomics (ACMG) and Association for Molecular Pathology (AMP) guidelines to reduce curation discrepancies. The ClinGen Myeloid Malignancy Variant Curation Expert Panel (MM-VCEP) was created collaboratively between the American Society of Hematology (ASH) and ClinGen to perform gene and disease-specific modifications for inherited myeloid malignancies. The MM-VCEP began optimizing ACMG/AMP rules for RUNX1, since many germline variants have been described in patients with familial platelet disorder with predisposition to acute myeloid leukemia (FPD/AML), characterized by thrombocytopenia, platelet functional/ultrastructural defects, and a predisposition to hematologic malignancies. The 28 ACMG/AMP codes were tailored for RUNX1 variants by modifying gene/disease specifications, incorporating strength adjustments of existing rules, or both. Key specifications included calculation of minor allele frequency thresholds, formulating a semiquantitative approach to counting multiple independent variant occurrences, identification of functional domains and mutational hot spots, establishing functional assay thresholds, and characterizing phenotype-specific guidelines. Preliminary rules were tested using a pilot set of 52 variants, among which 50 were previously classified as benign/likely benign (BEN/LBEN), pathogenic/likely pathogenic (PATH/LPATH), variant of unknown significance (VUS), or conflicting interpretations (CONF) in ClinVar. The application of RUNX1-specific criteria resulted in a reduction of CONF variants and VUS by 33%, emphasizing the benefit of gene-specific criteria and sharing internal laboratory data.

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Running Title: ClinGen curation rules for germline *RUNX1* variants

Key points

- The ClinGen MM-VCEP has specified *RUNX1*-specific curation rules to address gene function, gene-specific domains, and phenotypic criteria.
- *RUNX1*-specific criteria resulted in a reduction of CONF variants and VUS by 33%, emphasizing the need for expert variant curation.

Abstract

Standardized variant curation is essential for clinical care recommendations for patients with inherited disorders. Clinical Genome Resource (ClinGen) variant curation expert panels are developing disease-associated gene specifications using the 2015 American College of Medical Genetics and Genomics (ACMG) and Association for Molecular Pathology (AMP) guidelines to reduce curation discrepancies. The ClinGen Myeloid Malignancy Variant Curation Expert Panel (MM-VCEP) was created collaboratively between the American Society of Hematology (ASH) and ClinGen to perform gene and disease-specific modifications for inherited myeloid malignancies. The MM-VCEP began optimizing ACMG/AMP rules for *RUNX1*, since many germline variants have been described in patients with familial platelet disorder with predisposition to acute myeloid leukemia (FPD/AML), characterized by thrombocytopenia, platelet functional/ultrastructural defects, and a predisposition to hematologic malignancies. The 28 ACMG/AMP codes were tailored for *RUNX1* variants by modifying gene/disease specifications, incorporating strength adjustments of existing rules, or both. Key specifications included calculation of minor allele frequency thresholds, formulating a semiquantitative approach to counting multiple independent variant occurrences, identification of functional domains and mutational hot spots, establishing functional assay thresholds, and characterizing phenotype-specific guidelines. Preliminary rules were tested using a pilot set of 52 variants, among which 50 were previously classified as benign/likely benign (BEN/LBEN), pathogenic/likely pathogenic (PATH/LPATH), variant of unknown significance (VUS), or conflicting interpretations (CONF) in ClinVar. The application of *RUNX1*-specific criteria resulted in a reduction of CONF variants and VUS by 33%, emphasizing the benefit of gene-specific criteria and sharing internal laboratory data.

Introduction

In 2015, the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) released a landmark document providing guidance on variant classification, which is now adopted by many international diagnostic laboratories. It was designed to have universal applicability to all Mendelian disorders, using several types of weighted and categorized evidence, and therefore requires significant expertise as well as gene- and disease-specific knowledge to be correctly applied.[1] Variable application of functional and domain-related evidence and inconsistent interpretation and use of the ACMG/AMP criteria are key contributors to incorrect classifications of variants and significant discrepancies among laboratories highlight the utility of expert guidance.[2–4] A few studies have proposed approaches to one or more aspects of variant interpretation, such as quantitative criteria for co-segregation, use of population databases, adaptation of minor allele frequency (MAF), classes of evidence, and gene-level implications.[2–8] However, due to the unique characteristics of every gene and its disease correlates, along with the variability in the application of classification criteria and evidence interpretation, there is still a lack of comprehensive guidance for variant interpretation.

This need for expert involvement and gene-specific guidance has been addressed by the National Institutes of Health funded Clinical Genome Resource (ClinGen, https://clinicalgenome.org), which serves as a body for managing and centralizing clinically relevant genomic knowledge, providing guidance and tools for defining the clinical validity of gene and variant contribution to disease. Several working groups and expert panels were created within ClinGen, including gene and disease-specific Variant Curation Expert Panels (VCEPs).[9,10] Moreover, the ClinGen Sequence Variant Interpretation Working Group (SVI, https://clinicalgenome.org/working-groups/sequence-variant-interpretation/) aims to provide general recommendations for the refinement and evolution of the ACMG/AMP guidelines then to be specialized further by the gene-specific VCEP.

The publicly available ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/), launched in 2013, serves as a valuable centralized resource for documenting the clinical significance of genetic variants submitted by clinical and research laboratories and databases such as OMIM and GeneReviews.[11] ClinVar utilizes the ACMG-recommended five-level scoring system to indicate the level of evidence supporting the assertion of clinical significance of a variant. Human variant data curated by ClinGen expert panels are submitted to ClinVar with a three-star status (reviewed by expert panel) including a designation that the ClinGen VCEP process has been recognized by the Food and Drug Administration.

The general work-flow of a VCEP is to define its leadership/membership and scope of focus as well as conflicts of interest.[9] Once approved, this group develops disease-specific variant classification rules, based on curation of gene-specific features, published literature, and evidence thresholds that are consistent with gene-disease associations. ACMG and AMP have defined 28 codes that address specific evidence, including population data, segregation data, functional data, computational predictions, and allelic data.[3] Each code is weighted according to the strength of the evidence: stand-alone, very strong, strong, moderate, or supporting. Codes are also designated as defining the direction of clinical significance: benign or pathogenic. These evidence codes applied to variants are then combined to arrive at a single designation of clinical significance: pathogenic (PATH), likely pathogenic (LPATH), variant of uncertain significance (VUS), likely benign (LBEN), or benign (BEN). Once preliminary rules are specified, they are pilot tested on a collection of variants with existing assertions of clinical significance, and based on the results of this preliminary testing, the VCEP may adjust some of its rules to optimize variant classification. Once final rules have been approved, they are published and implemented, with VCEP-curated assertions disseminated via the ClinVar database.

A Myeloid Malignancy VCEP, hereafter referred to as MM-VCEP, was formed in 2018, as a collaboration between the American Society of Hematology (ASH) and ClinGen. The MM-

VCEP began adapting the ACMG/AMP framework for *RUNX1* variant classification, because as the first germline predisposition syndrome identified for myeloid malignancies, there were many variants already deposited in the ClinVar repository. Germline pathogenic variants in *RUNX1*, first described in 1999, cause dominantly inherited familial platelet disorder with predisposition to acute myeloid leukemia (FPD/AML), characterized by mild to moderate thrombocytopenia, functional and ultrastructural platelet defects, and a predisposition to myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) and less frequently to T-cell acute lymphoblastic leukemia (T-ALL).[12–14] In 2016, the revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia included myeloid malignancies arising from germline pathogenic variants in *ANKRD26*, *ETV6,* and *RUNX1* in a new category defined as 'Myeloid neoplasms with germline predisposition and pre-existing platelet disorder'.[15] Reported inherited and *de novo RUNX1* variants include missense, nonsense, and splice site single-nucleotide variants (SNVs), small in- or out-of-frame insertions and deletions (indels), as well as copy number variants (CNVs), such as intragenic or whole gene-deletions.[16–18] The prevalence of pathogenic *RUNX1* germline variants is unknown, but presumed to be rare. The disease shows high penetrance with variable expressivity and genotype/phenotype correlation, and the life-time risk of hematologic malignancies is approximately 44%, with an average age of onset at 33 years.[19–21] More than half of germline *RUNX1* variants are reported in single probands/families,[13] leading to a high allelic heterogeneity that restricts the collection of data from segregation analyses and functional analyses across several affected families. Individuals with a hematologic malignancy are often candidates for hematopoietic stem cell transplantation. The identification of patients with a pathogenic germline variant in *RUNX1* and its correct classification of the variant are imperative to the selection of potential related donors, among other clinical implications.[22–26]

Here, we present the *RUNX1*-specific guidelines generated by the MM-VCEP. The MM-VCEP adapted the ACMG/AMP framework for *RUNX1* variant classification with the aim of

improving consistency in variant classification and curating *RUNX1* variants for three-star submission to ClinVar. We have used multiple lines of evidence, showing the rationale and data supporting each criterion's modification, and the results from pilot testing the criteria on variants with BEN/LBEN, PATH/LPATH, VUS, and conflicting (CONF) ClinVar assertions. The application of rules for *RUNX1* variant curation will serve as a model for the curation of variants in other genes that also cause inherited myeloid hematologic malignancies, such as *ANKRD26*, *ETV6, DDX41,* and *GATA2*. The ClinGen's website contains the MM-VCEP variant classification recommendations and any subsequent modifications to these codes over time (https://www.clinicalgenome.org/affiliation/50034).

Methods

ClinGen Myeloid Malignancy Variant Curation Expert Panel

The MM-VCEP is sponsored by ASH through its partnership with ClinGen and is described at https://clinicalgenome.org/affiliation/50034/. The MM-VCEP team is comprised of 34 professionals with expertise in key domains and includes clinical geneticists, genetic counselors, hematologists with professional training in genetics, laboratory and research scientists, and variant curation experts. Additional emphasis was placed on global representation, with 23 participating international institutions in six countries: Australia, France, Italy, Sweden, the United Kingdom, and the United States. The MM-VCEP meets regularly via bi-weekly teleconferences and corresponds via email on a regular basis. Approval of MM-VCEP is overseen by ClinGen and consists of four steps: (1) defining the group/members and scope of the VCEP, (2) developing gene/disease-specific classification rules, (3) optimization of rules using pilot variants, and (4) MM-VCEP approval by ClinGen, implementation of rules in the ClinGen Variant Curation Interface (VCI) and submission of curated variants to the ClinVar database. For step two, members were divided into three subgroups that focused on the modification of functional/computational/splicing criteria (Team F), population/phenotypic criteria

(Team P), and segregation/allelic/*de novo* criteria (Team S). All members disclosed potential conflicts of interest as required by ClinGen.

ACMG/AMP specifications for RUNX1

MM-VCEP members proposed and discussed changes to the existing ACMG/AMP classifications for *RUNX1* germline variants and arrived at consensus decisions via teleconference calls and email. Criteria modifications included gene or disease-specific modifications, strength-level adjustments, general recommendations, and certain criteria being deemed 'not applicable'. Publicly available databases, predictive software and published data obtained from relevant manuscripts were used for criteria specifications. For BA1/BS1 *RUNX1* specific population MAF, calculations were made assuming Hardy-Weinberg equilibrium using the recently published Whiffin/Ware online calculator.[6] Additional efforts included identification of key functional domains and mutational hot spots within *RUNX1*, defining informative functional assays, and characterizing phenotypic criteria. Recommendations for using ACMG/AMP criteria from the ClinGen's SVI working group were also incorporated.[27–29] Preliminary and final ACMG/AMP specifications required complete consensus of the MM-VCEP.

Pilot variants

All pilot variants are annotated using RefSeq IDs NM_001754.4 and NC_000021.9 (GRCh38/hg38). Variants submitted to ClinVar by a variety of clinical laboratories were prioritized for classification. Preliminary rules were refined by interpreting a set of 52 *RUNX1* variants, which were selected to represent the spectrum of variants in *RUNX1*, covering various types of SNVs such as missense, nonsense, splice site, synonymous and intronic variants, indels such as in-frame duplications and out-of-frame deletions, and CNVs such as intragenic deletions. Similarly, the pilot variants covered a diverse range of classifications in ClinVar including discrepant assertions (twelve BEN/LBEN, fourteen VUS, twenty PATH/LPATH, four CONF, two with no ClinVar assertions). The variant classification and rules applied were reviewed on conference calls to resolve discrepancies and reach consensus. Basic information

regarding individual phenotypes and segregation with disease was obtained from the literature and ClinVar submitters. Statistical approaches for calculations of PS4 are available in the Supplementary Methods. Further optimization of rules was performed, and a discussion with the entire MM-VCEP was triggered whenever members disagreed or raised concerns regarding the applicability of a given rule. Curators utilized ClinGen's VCI (VCI, https://curation.clinicalgenome.org) to assess and document the applicable rules for each variant. Once the MM-VCEP was approved, the classified *RUNX1* variants with the adapted evidence code framework applied to the variants were submitted to ClinVar and were designated with a 3-star evidence code and FDA recognition flag. The first 52 *RUNX1* variant curations are now available in ClinVar and can be accessed at: https://www.ncbi.nlm.nih.gov/clinvar/submitters/507107/

Results

Summary of rule specifications

The final MM-VCEP ACMG/AMP specifications for *RUNX1* were approved by ClinGen and are outlined in Table 1. Six out of the original 28 ACMG/AMP criteria had general recommendations on the application of the rule (PM2, PP3, BS4, BP2, BP4, BP7), two required gene- or disease-based specifications (BA1, BS1), and two rules were adjusted in their level of strength (PS1, PM5). Both gene- or disease-based and strength-level specifications were made to nine rules (PVS1, PS2, PS3, PS4, PM1, PM4, PM6, PP1, BS3). Five rules required exceptions for combinations with other rules (PS2, PS3, PM5, PM6, PP3), and nine rules were deemed not applicable (PM3, PP2, PP4, PP5, BS2, BP1, BP3, BP5, BP6). One change to the ACMG/AMP combination of criteria for classification of clinical significance was made in the case of BS1 which can be used as a stand-alone criterion for LBEN classification in the absence of any supporting pathogenic evidence. The following section highlights the approaches and rationale behind key specifications such as phenotypic criteria, MAF thresholds and validity of

functional assays. Of note, germline material for FPD/AML patients or patients with suspected inherited hematologic malignancies cannot include blood or bone marrow from these patients since this is the affected tissue harboring somatic mutations. We recommend using cultured skin fibroblasts as the gold standard, or alternatively DNA from hair roots or cultured mesenchymal stromal cells.[19,25]

Phenotypic criteria for FPD/AML

FPD/AML is characterized by mild to moderate thrombocytopenia, platelet functional and/or ultrastructural defects, and a predisposition to hematologic malignancies, most often AML and MDS, and less frequently T-ALL (Table 2). The penetrance is high; however, not all individuals carrying the pathogenic variant display the FPD/AML phenotype. Thrombocytopenia is the most common clinical presentation, followed by hematologic malignancies in approximately 44% of these patients.[19–21] The MM-VCEP defined that in order to fit the FPD/AML phenotype, the patient must show at least one of the following phenotypic criteria: (1) mild to moderate thrombocytopenia with normal platelet size and volume in the absence of other causative factors such as autoimmune (e.g. antibodies against platelet surface antigens) or drug-related thrombocytopenia;[30] (2) platelet ultrastructural and/or functional defects, including platelet alpha[31] or dense granule secretion defects[30,32,33] or impaired platelet aggregation – particularly in response to collagen and epinephrine;[34,35] (3) diagnosis of a hematologic malignancy, most commonly affecting the myeloid lineage causing AML or MDS, less frequently involving the lymphoid lineage and manifesting as T-ALL.[26,30,36,37] There are rare case reports of patients with germline *RUNX1* variants and mixed myeloproliferative syndromes/MDS such as chronic myelomonocytic leukemia,[26,38] as well as case reports of patients with B-ALL[39] and hairy-cell leukemia.[40]

Population data (BA1, BS1, PM2, PS4, PS4_moderate, PS4_supporting, BP2)

FPD/AML is a rare disorder. The prevalence of the disease-associated *RUNX1* variants is unknown, with an estimated 5,515 families worldwide based on a population incidence generated from a survey of centers with FPD/AML patients (personal communication) which is likely an underestimate of the true prevalence. Among the three phenotypic features seen in individuals with germline *RUNX1* variants (Table 2), thrombocytopenia is the most common. We conservatively estimated the prevalence of thrombocytopenia for use in the BA1/BS1 calculations. Most clinical laboratories establish their reference values for platelet counts by measuring samples from at least 120 healthy individuals and identifying the most outlying 5% of observed values. Most often, these outlying observations are split evenly between the ends of the test result distribution in the reference population, 2.5% at each end of the distribution, resulting in a two-sided reference interval.[41] Using this approach, the prevalence of thrombocytopenia can be defined as 1 in 40. The penetrance in families with *RUNX1* germline variant is high to near-complete with 85% being the lowest penetrance reported to date ([13,19–21],unpublished internal data). So far, no founder variants in *RUNX1* have been reported. *De novo* variants are rare, but have been described.[16–18] The MM-VCEP modified BA1 using these conservative assumptions and corresponding values to account for the unknown prevalence and disease contribution of *RUNX1*. In order to obtain a *RUNX1*-specific population MAF threshold for BA1, we utilized the Whiffin/Ware calculator[6] (http://cardiodb.org/allelefrequencyapp/) with a prevalence of 1 in 40, a conservative unascertained penetrance estimate of 85%, an allelic heterogeneity of 100% and a maximum genetic heterogeneity of 10%. The MM-VCEP also adopted the SVI recommendation that the variant be present in any general continental population dataset with a minimum number of 2,000 alleles and the variant is present in ≥ 5 alleles.[42] A 95% confidence interval was used to develop the thresholds. The threshold developed for application of BA1 as a stand-alone criterion is a MAF equal to or higher than 0.0015 (0.15%). For BS1, a maximum genetic heterogeneity contribution of 1% (one magnitude lower) was used, which led to a range of

0.00015 (0.015%) up to 0.0015 (0.15%) for application of BS1. Since we have used conservative values for the calculation, we allow a variant to reach a LBEN classification based on BS1 alone if there is no contradictory evidence supporting pathogenicity (as outlined in a recent SVI revision).[29] For this work, the gnomAD population database was mostly used, although other databases with a minimum of 2,000 alleles are also sufficient. However, we encourage the use of a large dataset such as gnomAD, ExAC, or ESP.

Since most *RUNX1* variants are unique to probands or families,[13] it was determined that the variant must be completely absent from all population databases in order to apply PM2. The MM-VCEP tested pilot PATH/LPATH variants with this rule, and validated this determination. The MM-VCEP further recommends that the mean coverage of exome and genome sequencing data for *RUNX1* in the population databases used should be at least 20x.

Criterion PS4 is based on the significantly higher prevalence of a variant in cases versus controls, which is considered strong evidence for pathogenicity. Ideally, published case-control studies are used as evidence. Given the rarity of FPD/AML, an existing case-control study for *RUNX1* variants could not be identified. The original ACMG/AMP guideline states that odds ratio (OR), measuring an association between a genotype and phenotype, can be used for Mendelian diseases. Accordingly, in the absence of a published case-control study, the MM-VCEP created a 'quasi-case-control study' with the estimated number of probands worldwide and the overall gnomAD population as the control cohort. In order to apply this code, the proband has to meet at least one of the *RUNX1*-phenotypic criteria (Table 2), and the variant has to be either absent from gnomAD or only present once. This code has a sliding weight scale to account for the number of unrelated probands who meet the *RUNX1*-phenotypic criteria. PS4 is applied with ≥ 4 probands (OR 100.6), PS4_moderate with 2-3 probands (OR 50.3-75.5) and PS4_supporting with 1 proband (OR 25.1) (Table S1).

BP2, supporting evidence for a benign code, can be applied in the context of autosomal dominant FPD/AML when the variant is found *in trans* with a known pathogenic variant. Since

there is no evidence in the literature of probands with a homozygous pathogenic *RUNX1* variant, and lack of Runx1 is embryonically lethal in mice, the MM-VCEP recommends that BP2 also be applied when a variant is found in a confirmed homozygous state in population databases or internal laboratories.[43,44]

Segregation data (PP1_strong, PP1_moderate, PP1, BS4)

Segregation with disease (PP1) is used as evidence for pathogenicity and with increasing number of meioses, a stronger level of evidence can be applied. The MM-VCEP adopted the approach taken by other ClinGen EPs,[45–48] and supported by the SVI and others,[49] that additional meioses support higher levels of evidence. Thus, based on calculated logarithm of the odds (LOD) score thresholds of 0.9, 1.5, and 2.1, respectively, three or four meioses fulfill criteria for PP1, five or six meioses for PP1_moderate and seven or more meioses for PP1_strong. Of note, only individuals well documented as having a *RUNX1* phenotype (Table 2) and a positive genotype or obligate carriers are included when counting segregations. The phenotype of those individuals should be well described. We waived the ACMG/AMP recommendation for demonstrating co-segregation in more than one family, given that many *RUNX1* variants are unique to a single family[13] and have not been reported in other unrelated families, which would severely affect the utility of segregation data. We acknowledge that by waiving this recommendation, there is a possibility of the identified variant being in a linkage disequilibrium with a truly causative variant.

Lack of segregation in affected family members (BS4) can be used as a benign criterion when a *RUNX1* variant is present and non-segregation with disease occurred in at least two or more informative meioses. BS4 should only be applied for genotype-positive, phenotype-negative family members and there must be confidence that the family members do not meet any of our *RUNX1*-phenotypic criteria, taking into account ages of individuals.

De novo occurrence (PS2_moderate, PS2_supporting, PM6, PM6_supporting)

De novo RUNX1 variants are rare, but have been reported in the literature.[16–18]

The two *de novo* criteria are applied when both maternity and paternity are confirmed (PS2) or assumed (PM6), and the variant has been assessed as *de novo* in a patient with the disease and no family history. The following specifications were added by our MM-VCEP: (1) No family history is defined by the absence of the FPD/AML specific phenotype in first- and/or second-degree relatives, and (2) the proband must exhibit at least one phenotypic FPD/AML criterion (Table 2). PS2/PM6 were further specified using the SVI recommendation of a point-based scoring system to determine the level of strength. The FPD/AML phenotype is not highly specific, and there is substantial genetic heterogeneity - the same phenotype can be caused by other underlying germline conditions such as pathogenic variants in *ANKRD26*[50] or *ETV6.*[51] We thus concluded that due to the lack of a highly specific phenotype and the presence of genetic heterogeneity, the maximum allowable value is 1 point contributing to the overall score. Due to this restriction, these two criteria do not have a strong or very strong level of evidence. PS2_moderate is reached with a score of 1 point (two or more proven *de novo* occurrences) and PS2_supporting is utilized when reaching a score of 0.5 points (one proven *de novo* occurrence). Likewise, PM6_moderate is met when four assumed *de novo* occurrences are present (score of 1), and PM6_supporting is applicable with two to three assumed *de novo* cases (score of 0.5). Combining these two criteria (e.g. in the case of the same variant having both confirmed and assumed *de novo* evidence) is possible with the recognition that the maximum allowable value is still 1 point, which effectively leads to the application of one moderate or two supporting rules (Table S2).

Computational and predictive data (PVS1, PVS1_strong, PVS1_moderate, PS1, PS1_moderate, PM1, PM1_supporting, PM4, PM4_supporting, PM5_strong, PM5, PM5_supporting, PP3, BP4, BP7)

RUNX1 germline variants have been well described as being dominant-negative, loss-of-function (LOF), or hypermorphic.[13,31,52,53] Three major isoforms (A, B, C) are expressed by the use of two promoters and alternative splicing (Figure 1B). Expression of the short human

RUNX1A isoform has been shown to favor expansion of the hematopoietic stem cell pool, whereas expression of the full length RUNX1B and RUNX1C isoforms, which only differ by 33 amino acids (AA) at the N-terminus of isoform C (exons 2-3 in NM_001754.4), function to promote hematopoietic differentiation.[54–61] The differential function and expression of these isoforms in hematopoietic tissue is not fully understood. The MM-VCEP recommends using RUNX1 isoform C as the default transcript (NM_001754.4), since this is the isoform used for annotation by most clinical laboratories. The MM-VCEP decision tree for SNVs/indels (Figure 2, Table S3) and CNVs (Figure S1) refined the PVS1 criterion across all LOF variant types previously reported for *RUNX1* (nonsense, frameshift, canonical splice site variants and single- or multi-exon deletions) by using the SVI recommendations[27] and gene-specific adjustments. We recommend down-grading the strength level from *very strong* to *strong* for C-terminal variants that are not predicted to undergo nonsense mediated decay (NMD) but affect the transactivation domain (TAD), inhibitory domain and/or the VWRPY motif (Figure 1).[62,63] NMD is not predicted if the premature termination codon occurs in the 3' most exon or within the 3' most 50 nucleotides of the penultimate exon.[64,65] Deletions of exon 2-3, presumably only affecting RUNX1 isoform C, have been reported in four families (unpublished data,[66]) displaying a typical FPD/AML phenotype and segregation with disease. Although the functional effects of the exon 2-3 deletions on isoform C and potential effects on isoforms A and B require further investigations, we recommend applying PVS1_moderate according to the PVS1 CNV decision tree (Figure S1). The ClinGen CNV interpretation working group is currently developing a systematic framework for the clinical interpretation of CNVs, which will then benefit the curation of *RUNX1* CNVs in the future.

A variant affecting the same AA residue as a previously established pathogenic variant can either lead to the same AA change (PS1) or a different AA change (PM5). The MM-VCEP added the following recommendations for both rules: RNA data, or agreement in splicing predictors show no splicing effects, which was defined as Splice Site Finder (SSF) and

MaxEntScan (MES) predicting either an increase in the canonical splice site score or a decrease of the canonical splice site score by no more than 10% and no putative cryptic splice sites are created. In addition, the previously established PATH/LPATH variant must be asserted PATH/LPATH based on MM-VCEP rules for *RUNX1* before this rule can be applied. A strength modification was established for PS1 (same AA as previously established pathogenic variant) and PS1_moderate (same AA as previously established likely pathogenic variant). Likewise, PM5_strong is applied when two or more different pathogenic missense changes have been detected previously at the same AA residue, PM5 is used when a different pathogenic missense change has been seen previously at the same residue, and PM5_supporting is utilized when one missense change at the same residue has previously been determined to be likely pathogenic.

For *in-silico* evaluation of missense variants, the MM-VCEP recommends using REVEL, a meta-predictor that combines 13 individual tools with high sensitivity and specificity and has recently demonstrated the highest performance compared to any individual tool or other ensemble methods.[67,68] For splicing predictions, we recommend using the SSF and MES, both of which have been shown to predict splicing effects with high accuracy.[69–71] PP3, defined as multiple lines of computational evidence supporting a deleterious effect, can be applied for missense variants with a REVEL score of >0.75. It can also be applied for missense or synonymous variants if the variant alters the last three bases of an exon preceding a splice donor site or the first three bases of an exon following a splice acceptor site[69] and the predicted decrease in the score of the canonical splice site (measured by both MES and SSF) is at least 75% regardless of the predicted creation/presence of a putative cryptic splice site. PP3 should be applied for intronic variants (in introns 4-8) located in reference to exons at positions +3 to +5 for splice donor sites or -3 to -5 for splice acceptor sites[69,72] for which the predicted decrease in the score of the canonical splice site is at least 75% (measured by both MES and SSF)

regardless of the predicted creation/presence of a putative cryptic splice site. PP3 cannot be used for canonical splice site variants.

The benign criterion BP4 should be applied for missense variants if all of the following criteria apply: the variant's REVEL score is < 0.15, SSF and MES predict either an increase in the canonical splice site score or a decrease of the canonical splice site score by no more than 10%, and no putative cryptic splice sites are created. BP4 should also be applied for synonymous, intronic, and non-coding variants for which SSF and MES predict either an increase in the canonical splice site score or a decrease of the canonical splice site score by no more than 10%, and no putative cryptic splice sites are created.

The original PM1 code can be applied for variants affecting mutational hotspots and/or functional domains without benign variation. The Runt homology domain (RHD), spanning from AA 77-204, has been established as a highly conserved DNA binding domain without any benign variation in ClinVar. Thirteen somatic and/or germline mutational hotspots within the RHD have been identified: R107, K110, A134, R162, R166, S167, R169, G170, K194, T196, D198, R210, R204.[12,20,73,74] The MM-VCEP recommends using PM1 for variants affecting these thirteen AA residues. For variants in other parts of the RHD for which germline variants have been previously reported (AA 105-204), a reduced strength-level (PM1_supporting) is recommended. For other residues within the RHD (AA 77-104), no germline *RUNX1* pathogenic variants have been reported to date. In the future, the AA range under PM1_supporting may be expanded to other parts of the protein if more evidence emerges. Analogous to PM1, PM4 (protein length changes due to in-frame deletions/insertions in a non-repeat region or stop-loss variants) is applied to in-frame deletions/insertions impacting the same thirteen AA residues (listed above) and, likewise, PM4_supporting can be utilized for in-frame deletions/insertions impacting at least one of the other parts of the RHD where germline variants have been previously reported (AA 105-204).

The MM-VCEP agreed to extend BP7 (synonymous variant with no splicing effect and position is not highly conserved) to apply to intronic/non-coding variants at or beyond positions +7/-21 for which SSF and MES predict either an increase in the canonical splice site score or a decrease of the canonical splice site score by no more than 10%, no putative cryptic splice sites are created and the position is not conserved, e.g. PhyloP score < 0.1[75] or the variant is the reference nucleotide in one primate and/or three mammal species.[76]

Functional data (PS3, PS3_moderate, PS3_supporting, BS3, BS3_supporting)

The evolutionarily conserved 128 AA RHD, present in most of the RUNX1 isoforms (Figure 1B), is involved in DNA binding and heterodimerization with CBFβ. Heterodimerization of RUNX1 with CBFβ promotes DNA binding by stabilizing the interaction of the complex with the DNA. RUNX1 regulates the activity of several important hematopoietic genes, such as the granulocyte-macrophage colony-stimulating factor,[77,78] T-cell receptor,[79,80] myeloperoxidase,[81,82] and neutrophil elastase,[82] by binding to a core sequence (TGTGGT) found in their promoters or enhancers.

Transactivation assays demonstrating altered transactivation compared to wild type are often performed as functional studies to evaluate the pathogenicity of a *RUNX1* variant. Promoter sequences of *M-CSFR*, *PF4*, *C-FMS* and *GZMB*, containing consensus RUNX1 binding sites TGTGGT have been used for this purpose.[31,83–87] Data from secondary assays are frequently used to evaluate an altered function of mutant RUNX1. Electrophoretic mobility shift assays[31,87–90] and yeast hybrid assays[88,89] are performed to demonstrate decreased DNA binding affinity, and co-immunoprecipitation assays,[85,90,91] fluorescence resonance energy transfer assays[88] and affinity assays[31] can demonstrate diminished heterodimerization ability of mutant RUNX1 with CBFβ. Abnormal cellular localization of mutant RUNX1 can be shown by immunofluorescence[31,53,83] and cell-fractionation with Western blot.[85,90] Sorted primary hematopoietic stem and progenitor cells can be used to demonstrate reduced colony-forming

potential,[53,92] and xenotransplantation experiments may reveal abnormal function of mutant RUNX1 *in vivo.*[53]

The MM-VCEP defined the strong pathogenic code PS3 as the combination of reduced transactivation (<20% of wild type and/or reduced to levels similar to well-established pathogenic variants such as R201Q or R166Q) and data from a secondary assay that demonstrate altered function of mutant RUNX1. The transactivation assay should include wild type and known pathogenic controls as well as co-expression with CBFβ. PS3 can also be applied for evidence of very low or abnormal mRNA/protein expression of the variant allele as a functional consequence of a null variant or incorrect mRNA/protein products. The MM-VCEP further stipulates that PS3 cannot be applied if the variant meets PVS1. If the variant meets PVS1_strong and PS3, we recommend applying either PVS1_strong and PS3_moderate or upgrading PVS1_strong to PVS1 without applying PS3. PS3_moderate is applied when data from transactivation assays show reduced transactivation (<20% of wild type and/or reduced to levels similar to well-established pathogenic variants such as R201Q or R166Q) or two or more secondary assays demonstrating altered function. PS3_supporting can be applied for transactivation assays demonstrating enhanced transactivation (>115% of wild type) as has been shown previously for the hypermorphic RUNX1 mutant, S388X.[52]

Likewise, the BS3 requirements (functional studies show no damaging effect on protein function) are a normal transactivation (80-115% of wild type) and data from a secondary assay that demonstrate normal function. BS3_supporting can be applied when there is evidence of normal transactivation (80-115% of wild type) while data from secondary assays are not required.

Rules deemed not applicable

Four rules of the pathogenic framework (PM3, PP2, PP4, PP5) and five rules of the benign framework (BS2, BP1, BP3, BP5, BP6) were deemed not applicable. The reasoning behind the decision for each code is explained briefly below:

Because the FPD/AML phenotype is associated with autosomal dominant transmission, PM3 (detected *in trans* with a pathogenic variant in a recessive gene) cannot be applied for FPD/AML.

The recommended cutoff for PP2 (missense variant in a gene with low rate of missense variants) is a constraint z score of ≥ 3.09,[28] which was not met by *RUNX1*.

The phenotype observed in FPD/AML is rather non-specific and can be caused by a number of other inherited predisposition syndromes, somatic variants, or environmental factors, which make the original ACMG/AMP rule PP4 for a highly specific phenotype not applicable to *RUNX1*.

Incomplete penetrance, an average age of onset of 33 years for hematologic malignancies,[19–21] and the lack of sufficient clinical data to exclude a *RUNX1*-related phenotype render BS2 (observed in a healthy individual with full penetrance at an early age) not applicable.

Both missense and truncating variants have been described as causative in FPD/AML, making BP1 (missense variant in a gene with primarily truncating variants) not applicable.

Similarly, *RUNX1* does not contain a repetitive region without known function used to apply BP3.

BP5 can be applied when the variant is found in a case with an alternate molecular basis for disease. The MM-VCEP concluded that this rule is not applicable because in rare circumstances, a patient can carry variants in two genes predisposing to hematologic malignancies, as has been described in case-reports. In addition, variants in other genes presenting as low-penetrance risk factors, modifier genes, and/or somatic mutations in hematopoietic stem and progenitor cells may contribute to the clinical presentation and complicate the search for the causative variant.[93,94]

Following recommendations from the SVI, the MM-VCEP agreed not to use the two variant classifications from reputable sources evidence codes (PP5 and BP6) based on the published rationale.[95]

Performance of the MM-VCEP specifications in pilot variant classification

For pilot testing, 52 variants with a broad spectrum of ClinVar assertions (twelve BEN/LBEN, fourteen VUS, twenty PATH/LPATH, four CONF, and two variants with no ClinVar assertions) were selected. The MM-VCEP applied the *RUNX1*-modified ACMG/AMP criteria to all pilot variants. During testing, experts were able to provide feedback on the usability of the evidence codes, comment on the weight of certain lines of evidence and suggest further modifications of the rule. A list of all pilot variants, the ClinVar submitters' variant classification, and the classifications made by our MM-VCEP are presented in Table S4. Figure 3 compares the original ClinVar classifications with our MM-VCEP classifications grouped by PATH/LPATH, BEN/LBEN, VUS and CONF variants. Of the fourteen VUS, two were upgraded into the LPATH category. Of the four CONF variants, one was upgraded to PATH and three were downgraded to LBEN. Two of eighteen variants previously listed as PATH/LPATH in ClinVar were downgraded to VUS after applying the *RUNX1*-specific codes. MM-VCEP members with knowledge of the criteria applied by the ClinVar submitters were able to corroborate the VUS classifications. A detailed schematic of the *RUNX1* gene and the newly classified pilot variants is shown in Figure 1A. Overall, applying the *RUNX1* specifications to the VUS/CONF variants resulted in a reduction in VUS/CONF classifications of 33%. All of the twelve variants that were submitted in ClinVar as BEN/LBEN remained in this category, with most LBEN variants being downgraded to BEN and only two remaining LBEN. An overview of the frequency of pathogenic and benign evidence codes applied is given in Figure S2. The test set received a final concordance of 92% with consensus ClinVar classifications (90% for the PATH/LPATH test set, 86% with the VUS test set, and 100% for the BEN/LBEN test set).

Discussion

RUNX1 is commonly mutated in hematologic malignancies with high rates of somatic variants in MDS/AML.[73,96] Tumor-based next-generation sequencing panels covering *RUNX1*

among other genes are implicated in the molecular diagnostic process of MDS/AML in most treatment centers. Some of these somatic *RUNX1* variants are subsequently determined to be germline.[97] In addition, recent achievements such as the inclusion of inherited hematologic malignancies into the revised WHO classification of myeloid neoplasms and acute leukemia[15] and a more standardized evaluation of the family history have raised awareness of these syndromes among physicians. This will increase the identification of patients with FPD/AML. Accurate *RUNX1* variant curation is fundamental for the appropriate clinical care of these patients, especially when considering a related donor for hematopoietic stem cell transplantation. In addition, FPD/AML with thrombocytopenia may be misdiagnosed as immune thrombocytopenic purpura and the correlating dysmegakaryopoiesis in the bone marrow can be mistaken as an early-stage-MDS, underscoring the importance of adequate *RUNX1* variant curation.[98,99]

Our curation of pilot variants demonstrated the impact of our proposed rules to improve variant classification, resulting in a reduction of VUS/CONF variants by 33%. Further use of these rules should continue to reduce the number of VUS and lead to fewer number of variants with VUS/CONF assertion within ClinVar. Being able to reclassify a variant from VUS/CONF assertions has a significant impact on patient care as it provides patients and physicians with the definitive data to guide treatment decisions, including donor selection amongst matched relatives. As we implement these *RUNX1*-specific rules, the variant annotation in ClinVar will contain a link to the specific version of the MM-VCEP *RUNX1* evidence rules, a summary of the specific evidence codes used for that variant, and a link to the ClinGen evidence repository where all the evidence evaluated for that variant is found. Given these detailed expert reviewed curations, MM-VCEP curated variants will be submitted under a “three-star expert panel reviewed” FDA recognized designation. We expect that our *RUNX1*-specific rules will require further updating as additional data become available, or at a minimum every two years, and will address improved computational modeling, functional assays, and larger and more ethnically

diverse population databases. Per ClinGen policy, *RUNX1* VUS and LPATH variants will be reassessed by the expert panel every two years and other variants may be re-curated if discrepancies in the variant classification or new evidence emerge over time. At any time, a link to the most up-to-date recommendations of *RUNX1* evidence codes can be found on the MM-VCEP homepage [https://www.clinicalgenome.org/affiliation/50034]. Furthermore, ongoing general refinements to the ACMG/AMP guidelines made by the ClinGen SVI will need to be addressed, particularly for the curation of intragenic *RUNX1* deletions and consensus rules for evaluation of splicing predictions. The next step of the MM-VCEP will be the curation of all current ClinVar deposited *RUNX1* variants. Further work will extend this study to other genes causing inherited hematologic malignancies.

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Authorship Contributions

All of the authors participated in the construction and pilot testing of the *RUNX1* curation rules and edited the manuscript. X.L., S.F., S.M., D.W., and L.A.G. participated in the majority of the manuscript writing.

Disclosure of Conflicts of Interest

SEP is a member of the scientific advisory panel of Baylor Genetics Laboratories. LAG is a member of the scientific advisory board for Invitae, Inc.

Tables

Table 1- MM-VCEP ACMG/AMP specifications for *RUNX1* variants.

Table 2- FPD/AML phenotypic criteria.

Figure Legends

Figure 1- Schematic of *RUNX1* exonic distribution, protein isoforms and functional domain structure with all 52 pilot variants and their final MM-VCEP classification. A Isoform C with Runt homology domain (RHD), transactivation domain (TAD) and the VWRPY motif and location of all 49 single-nucleotide pilot variants with their final MM-VCEP classification. Pathogenic (PATH) and likely pathogenic (LPATH) variants are shown at the top, variants with unknown significance (VUS), likely benign (LBEN), and benign (BEN) variants are shown at the bottom. The exonic distribution of isoform C is displayed below. **B** Schematic of RUNX1 isoforms A, B, and C and their functional domains. Regions in gray are unique to one isoform. The three pilot copy number variants are shown at the bottom with the deletion of exons 2 and 3 exclusively affecting the N-terminal 33 amino acids of isoform C.

Figure 2- PVS1 decision tree for SNVs/indels**.** Application of different levels of strength for PVS1 depending on the prediction of non-sense mediated decay (NMD), the location within a known critical protein domain and the expression of alternative isoforms.

Figure 3- Comparison of ClinVar and MM-VCEP classifications. Fifty previously asserted and ClinVar deposited *RUNX1* variants are shown on the x axis. Final MM-VCEP classifications are color-coded (see legend on the right). ClinVar variants with previous LPATH, CONF and VUS assertions were most often re-classified using MM-VCEP specified rules for *RUNX1*.

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Table 1

Table 1- MM-VCEP ACMG/AMP specifications for *RUNX1 variants*.

		***RUNX1* rule specifications**						
ACMG/AMP criteria code	**Original ACMG/AMP rule summary**	**Specification**	**Stand alone**	**Very strong**	**Strong**	**Moderate**	**Supporting**	**Comments**
PVS1	Null variant in a gene where LOF is a known mechanism of disease.	gene-specific, strength	na	Per modified *RUNX1* PVS1 decision tree for SNVs, indels and CNVs and table of splicing effects.			na	*RUNX1* LOF variants are a common mechanism of disease in FPD/AML. Three major isoforms (A, B, C) are expressed by use of two promotors and alternative splicing. C-terminal variants not predicted to undergo NMD are classified as **PVS1_strong**, deletions of exons 2 and 3, presumably only affecting RUNX1 isoform 1C, meet **PVS1_moderate**.
PS1	Same AA change as a previously established pathogenic variant regardless of nucleotide change.	strength	na	na	Same AA change as a previously established pathogenic variant regardless of nucleotide change.	Same AA change as a previously established likely pathogenic variant regardless of nucleotide change.	na	(1) RNA data or agreement in splicing predictors show no splicing effects (SSF and MES predict either increase in canonical splice site score or decrease of canonical splice score by no more than 10% and no putative splice site are created). (2) The previously established PATH/LPATH variant must be asserted pathogenic/likely pathogenic based on MM-VCEP rules for *RUNX1* before this rule can be applied
PS2	*De novo* (maternity and paternity confirmed) in a patient with the disease and no family history.	disease-specific, strength	na	na	na	≥ 2 proven *de novo* occurrences (maternity and paternity confirmed) in patients with the *RUNX1*-phenotype.	1 proven *de novo* occurrence (maternity and paternity confirmed) in a patient with the *RUNX1*-phenotype.	(1) No family history is defined as: absence of the variant and any of the *RUNX1*-phenotypic criteria in first- and/or second-degree relatives. (2) The proband must exhibit at least one phenotypic FPD/AML criterion. (3) The maximum allowable strength by combining **PS2** and **PM6** criteria is to apply one moderate or two supporting rules.
PS3	Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect.	gene-specific, strength	na	na	Transactivation assays demonstrating altered transactivation (<20% of wt, and/or reduced to levels similar to well-established pathogenic variants such as R201Q or R166Q) AND data from a secondary assay demonstrating altered function. **PS3** cannot be applied if the variant meets **PVS1**. If the variant meets criteria for **PVS1_strong** and **PS3**, we recommend either applying **PVS1_strong and PS3_moderate** or **upgrading PVS1_strong** to **PVS1** without applying **PS3.**	Transactivation assays demonstrating altered transactivation (<20% of wt, and/or reduced to levels similar to well-established pathogenic variants such as R201Q or R166Q) OR ≥ 2 secondary assays demonstrating altered function.	Transactivation assays demonstrating enhanced transactivation (>115% of wt).	(1) Transactivation assays should include wt and known pathogenic controls, as well as co-expression with CBFβ. Promoter sequences of CSF1R (M-CSF-R), PF4, C-FMS and GZMB, containing consensus RUNX1 binding sites have been used for transactivation assays. (2) The following secondary assays have been performed: EMSA and yeast hybrid assays (decreased DNA-binding affinity), co-IP, FRET and affinity assays (diminished heterodimerization ability with CBFβ), IF and WB with cell fractionation (abnormal cellular localization), colony forming assays (reduced colony-forming potential), xenotransplantation experiments (abnormal function of mutant RUNX1 *in vivo*). (3) **PS3** can also be applied for evidence of very low or abnormal mRNA/protein expression of the variant allele as a functional consequence of a null variant or incorrect mRNA/protein products
PS4	The prevalence of the variant in affected individuals is significantly increased compared to the prevalence in controls.	disease-specific, strength	na	na	≥ 4 probands meeting *RUNX1*-phenotypic criteria.	2-3 probands meeting *RUNX1*-phenotypic criteria.	1 proband meeting *RUNX1*-phenotypic criteria.	The affected individual has to fit at least one of the *RUNX1*-phenotypic criteria AND variant has to be either absent from gnomAD (overall population) or only present once.
PM1	Located in a mutational hot spot and/or critical and well-established functional domain without benign variation.	gene-specific, strength	na	na	na	Variant affecting one of the following 13 hotspot residues: R107, K110, A134, R162, R166, S167, R169, G170, K194, T196, D198, R201, R204.	Variant affecting one of the other AA residues 105-204 within the RHD.	The RHD (AA 77-204) has been established as highly conserved DNA-binding domain without any benign variation in ClinVar. No germline pathogenic variants have been reported in residues in the region (AA 77-104) to date. The AA range under **PM1_supporting** may be expanded in the future to other parts of the protein if more evidence emerges
PM2	Absent from controls.	general recommendation	na	na	na	Per original ACMG/AMP guidelines.	na	Variant must be completely absent from all population databases. The mean coverage of *RUNX1* in the population database used should be at least 20x.
PM3	For recessive disorders, detected *in trans* with a pathogenic variant.	na						FPD/AML is inherited in an autosomal dominant manner.
PM4	Protein length changes due to in-frame deletions/insertions in a non-repeat region or stop-loss variants.	gene-specific, strength	na	na	na	In-frame deletion/insertion impacting at least one of the 13 hotspot residues R107, K110, A134, R162, R166, S167, R169, G170, K194, T196, D198, R201, R204	Other in-frame deletion/insertion impacting residues 105-204 within the RHD.	see PM1
PM5	Missense change at AA residue where a different missense change determined to be pathogenic has been seen before.	strength	na	na	Missense change at the same residue where ≥ 2 different missense changes have previously been determined to be pathogenic. **PM5_strong** cannot be applied together with **PM1**.	Missense change at the same residue where a different missense change has previously been determined to be pathogenic.	Missense change at the same residue where a different missense change has previously been determined to be likely pathogenic.	see PS1
PM6	Assumed *de novo* (but without confirmation of maternity and paternity) in a patient with the disease and no family history.	disease-specific, strength	na	na	na	≥ 4 assumed *de novo* occurrences (without confirmation of maternity and paternity) in patients with the *RUNX1*-phenotype.	2 or 3 assumed *de novo* occurrences (without confirmation of maternity and paternity) in patients with the *RUNX1*-phenotype.	see PS2
PP1	Co-segregation with disease in multiple affected family members.	disease-specific, strength	na	na	≥ 7 meioses observed within one or across multiple families.	5 or 6 meioses observed within one or across multiple families.	3 or 4 meioses observed within one or across multiple families.	(1) Affected individuals show at least one of the *RUNX1*-specific phenotypic criteria. (2) Only genotype and phenotype positive individuals and obligate carriers are counted. (3) Demonstration of co-segregation in multiple families is not required since many *RUNX1* variants are unique and only occur in one family.
PP2	Missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease.	na						Missense constraint z-score for *RUNX1* is < 3.09.
PP3	Multiple lines of computational evidence support a deleterious effect on the gene or gene product.	general recommendation	na	na	na	na	Per original ACMG/AMP guidelines.	(1) **PP3** should be applied for missense variants with a REVEL score of >0.75 (2) **PP3** should be applied for missense or synonymous variants if the variant alters the last three bases of an exon preceding a donor splice site or the first three bases of an exon following a splice acceptor site and the predicted decrease in the score of the canonical splice site (measured by both MES and SSF) is at least 75% regardless of the predicted creation/presence of a putative cryptic splice site. (3) **PP3** should also be applied for intronic variants (in introns 4-8) located in reference to exons at positions +3 to +5 for splice donor sites or -3 to -5 for splice acceptor sites for which the predicted decrease in the score is at least 75% (measured by both MES and SSF) regardless of the predicted creation/presence of a putative cryptic splice site. (4) **PP3** cannot be applied for canonical splice site variants.
PP4	Patient's phenotype or family history is highly specific for a disease with a single genetic etiology.	na						FPD/AML does not exhibit a highly-specific phenotype and there is substantial genetic heterogeneity.
PP5	Reputable source recently reports variant as pathogenic, but the evidence is not available to the laboratory to perform an independent analysis.	na						According to SVI recommendations.
BA1	Allele frequency is >5% in ESP, 1000G, or ExAC.	disease-specific	MAF ≥ 0.0015 (0.15%)	na	na	na	na	(1) The variant is present in any general continental population dataset with a minimum number of 2,000 alleles and variant present in ≥ 5 alleles.
BS1	Allele frequency is greater than expected for disorder.	disease-specific	na	na	MAF between 0.00015 (0.015%) and 0.0015 (0.15%)	na	na	(1) The variant is present in any general continental population dataset with a minimum number of 2,000 alleles and variant present in ≥ 5 alleles. (2) Variant can be classified as likely benign based on **BS1** alone if there is no contradictory evidence supporting pathogenicity.
BS2	Observed in a healthy adult individual for a recessive (homozygous), dominant (heterozygous), or X-linked (hemizygous) disorder, with full penetrance expected at an early age.	na						FPD/AML patients display incomplete penetrance and the average age of onset of hematologic malignancies is 33 years.
BS3	Well-established *in vitro* or *in vivo* functional studies show no damaging effect on protein function or splicing.	gene-specific, strength	na	na	(1) Transactivation assays demonstrating normal transactivation (80-115% of wt) AND (2) data from a secondary assay demonstrating normal function.	na	Transactivation assays demonstrating normal transactivation (80-115% of wt).	see PS3 (1) and (2)
BS4	Lack of segregation in affected members of a family.	general recommendation	na	na	Applied when seen in ≥ 2 informative meioses.	na	na	This code should only be applied for genotype-positive, phenotype-negative (with sufficient laboratory evidence) family members.
BP1	Missense variant in a gene for which primarily truncating variants are known to cause disease.	na						FDP/AML is caused by both pathogenic missense and truncating variants.
BP2	Observed *in trans* with a pathogenic variant for a fully penetrant dominant gene/disorder or observed *in cis* with a pathogenic variant in any inheritance pattern.	general recommendation	na	na	na	na	Per original ACMG/AMP guidelines.	**BP2** can also be applied if the variant is detected in a homozygous state.
BP3	In frame-deletions/insertions in a repetitive region without a known function	na						RUNX1 does not contain a repetitive region without known function.
BP4	Multiple lines of computational evidence suggest no impact on gene or gene product.	general recommendation	na	na	na	na	Per original ACMG/AMP guidelines.	**BP4** should be applied for missense variants if all of the following apply: (1) REVEL score < 0.15, (2) SSF and MES predict either an increase in the canonical splice site score or a decrease of the canonical splice site score by no more than 10%, and (3) no putative cryptic splice sites are created. **BP4** should also be applied for synonymous, intronic and non-coding variants for which SSF and MES predict either an increase in the canonical splice site score or a decrease of the canonical splice site score by no more than 10% and no putative cryptic splice sites are created.
BP5	Variant found in a case with an alternate molecular basis for disease.	na						In rare circumstances, a patient can carry two variants in genes predisposing to hematologic malignancies.
BP6	Reputable source recently reports variants as benign, but the evidence is not available to the laboratory to perform an independent evaluation.	na						According to SVI recommendations.
BP7	A synonymous variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not highly conserved.	general recommendation	na	na	na	na	Per original ACMG/AMP guidelines. **BP7** can not be applied in combination with **PP3**.	Also applicable to intronic/non-coding variants at or beyond positions +7/-21 for which (1) SSF and MES predict either an increase in the canonical splice site score or a decrease of the canonical splice site score by no more than 10% and no putative cryptic splice sites are created AND (2) evolutionary conservation prediction algorithms predict the site as not conserved (e.g. PhyloP score < 0.1) or the variant is the reference nucleotide in one primate and/or three mammal species.).

Abbreviations: AA: Amino Acid, ACMG: American College of Medical Genetics, AMP: Association for Molecular Pathology, CNV: Copy Number Variant, EMSA: Electrophoretic Mobility Shift Assay, FPD/AML: Familial Platelet Disorder with predisposition to Acute Myeloid Leukemia, FRET: Fluorescence Resonance Energy Transfer, IF: Immunofluorescence, IP: Immunoprecipitation, LOF: Loss-Of-Function, MAF: Minor Allele Frequency, MES: MaxEntScan, MMCEP: Myeloid Malignancy Variant Curation Expert Panel, na: not applicable, NMD: Nonsense-Mediated Decay, RHD: Runt Homology Domain, SNV: Single-Nucleotide Variant, SSF: Splice Site Finder, SVI: ClinGen Sequence Variant Interpretation Working Group, WB: Western Blot, wt: wild type.

Table 2- FPD/AML phenotypic criteria.

Feature	Details	Life-time risk
Thrombocytopenia	Mild to moderate, normal platelet size and volume, absence of other causes for thrombocytopenia.	in most patients
Platelet ultrastructural and/or functional defects	Includes platelet alpha or dense granule secretion defects and impaired platelet aggregation (particularly in response to collagen and epinephrine).	unknown
Hematologic malignancy	Most commonly AML or MDS, less frequently T-ALL. There are rare case-reports of patients with germline *RUNX1* mutations and mixed MPN/MDS such as CMML, as well as case-reports of patients with B-ALL, and hairy-cell leukemia.	~44%

Abbreviations: ALL: Acute Lymphoblastic Leukemia, AML: Acute Myeloid Leukemia, CMML: Chronic Myelomonocytic Leukemia, FPD/AML: Familial Platelet Disorder with predisposition to Acute Myeloid Leukemia, MDS: Myelodysplastic Syndrome, MPN: Myeloproliferative Syndrome.

Figure 1

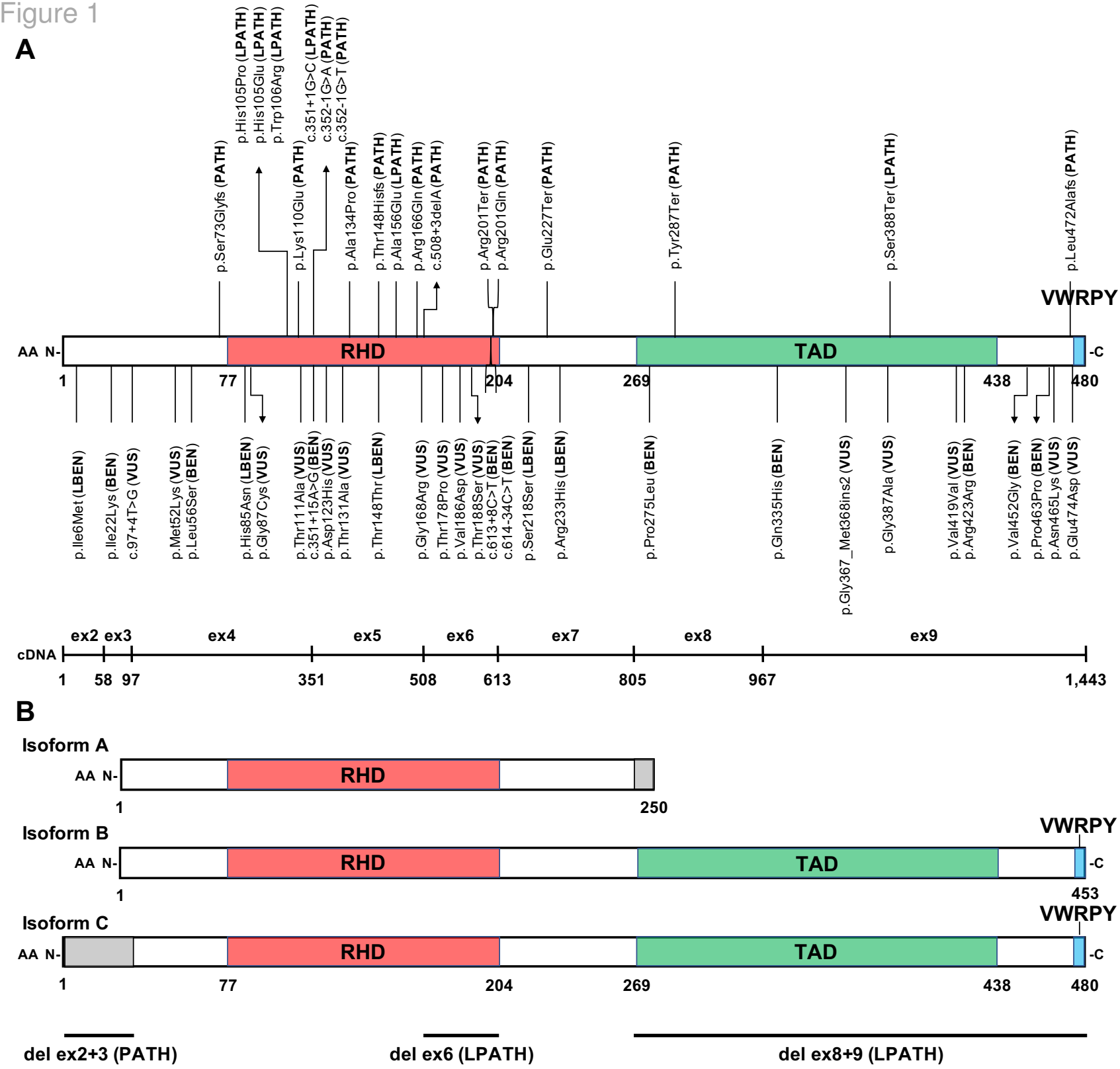

A
p.Ser73Glyfs (PATH)
p.His105Pro (LPATH)
p.His105Glu (LPATH)
p.Trp106Arg (LPATH)
p.Lys110Glu (PATH)
c.351+1G>C (LPATH)
c.352-1G>A (PATH)
c.352-1G>T (PATH)
p.Ala134Pro (PATH)
p.Thr148Hisfs (PATH)
p.Ala156Glu (LPATH)
p.Arg166Gln (PATH)
c.508+3delA (PATH)
p.Arg201Ter (PATH)
p.Arg201Gln (PATH)
p.Glu227Ter (PATH)
p.Tyr287Ter (PATH)
p.Ser388Ter (LPATH)
p.Leu472Alafs (PATH)
VWRPY
AA N-
RHD
TAD
-C
1
77
204
269
438
480
p.Ile6Met (LBEN)
p.Ile22Lys (BEN)
c.97+4T>G (VUS)
p.Met52Lys (VUS)
p.Leu56Ser (BEN)
p.His85Asn (LBEN)
p.Gly87Cys (VUS)
p.Thr111Ala (VUS)
c.351+15A>G (BEN)
p.Asp123His (VUS)
p.Thr131Ala (VUS)
p.Thr148Thr (LBEN)
p.Gly168Arg (VUS)
p.Thr178Pro (VUS)
p.Val186Asp (VUS)
p.Thr188Ser (VUS)
c.613+8C>T (BEN)
c.614-34C>T (BEN)
p.Ser218Ser (LBEN)
p.Arg233His (LBEN)
p.Pro275Leu (BEN)
p.Gln335His (BEN)
p.Gly367_Met368ins2 (VUS)
p.Gly387Ala (VUS)
p.Val419Val (VUS)
p.Arg423Arg (BEN)
p.Val452Gly (BEN)
p.Pro463Pro (BEN)
p.Asn465Lys (VUS)
p.Glu474Asp (VUS)
cDNA
ex2 ex3
ex4
ex5
ex6
ex7
ex8
ex9
1
58
97
351
508
613
805
967
1,443
B
Isoform A
AA N-
RHD
1
250
Isoform B
VWRPY
AA N-
RHD
TAD
-C
1
453
Isoform C
VWRPY
AA N-
RHD
TAD
-C
1
77
204
269
438
480
del ex2+3 (PATH)
del ex6 (LPATH)
del ex8+9 (LPATH)

Figure 2

RUNX1 PVS1 decision tree for SNVs/Indels

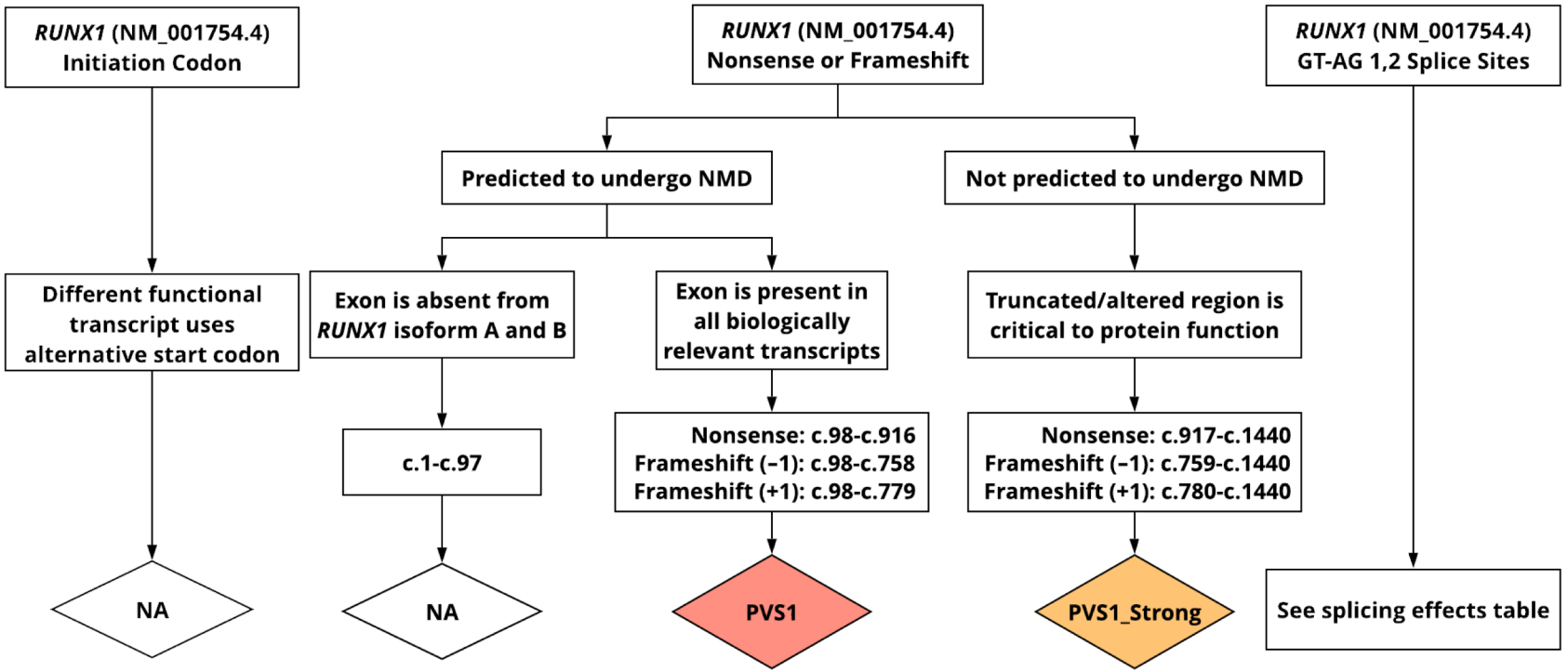
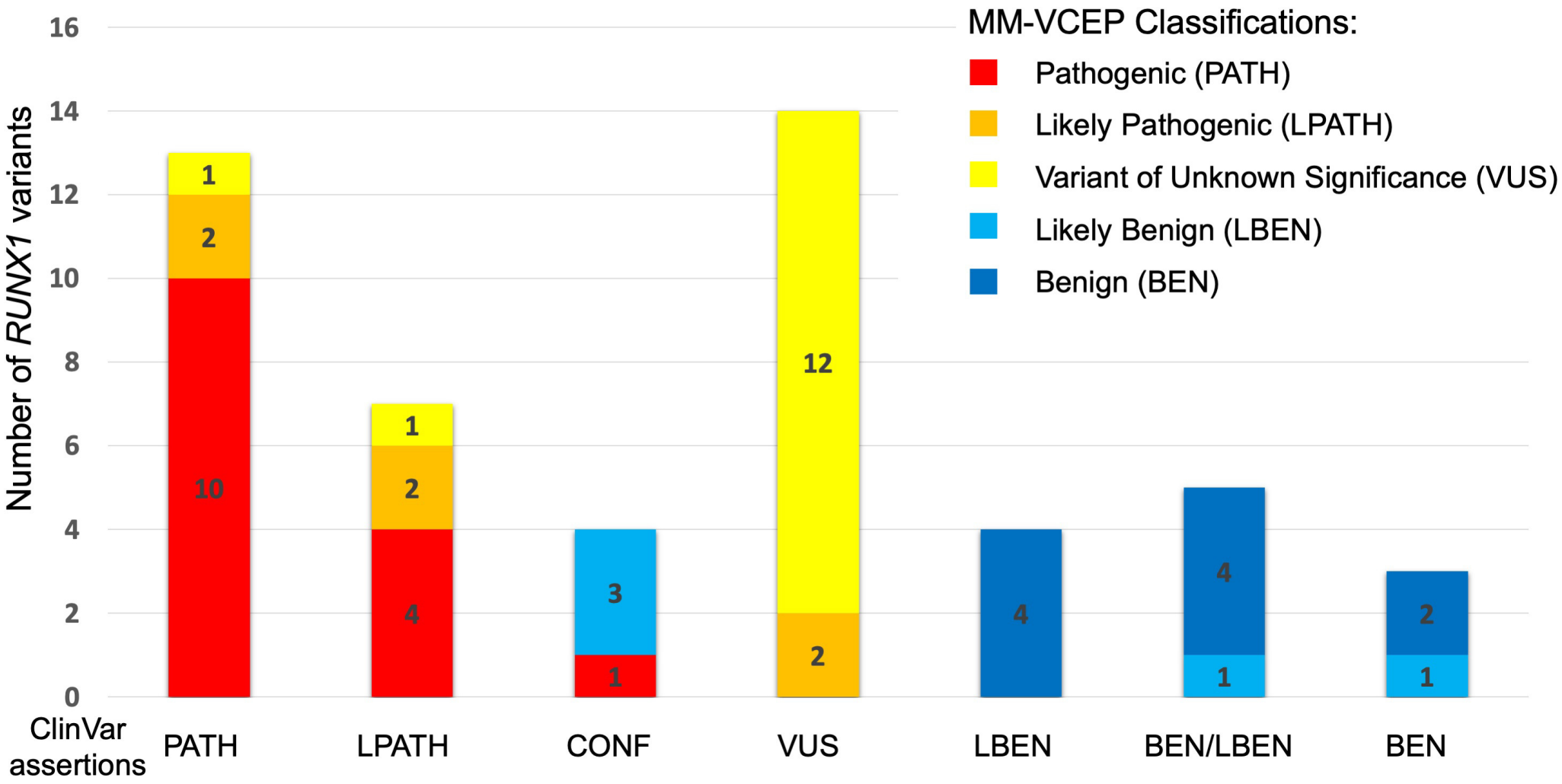


Figure 3


MM-VCEP Classifications:
Pathogenic (PATH)
Likely Pathogenic (LPATH)
Variant of Unknown Significance (VUS)
Likely Benign (LBEN)
Benign (BEN)
Number of RUNX1 variants
16
14
12
10
8
6
4
2
0
1
2
10
1
2
4
3
1
12
2
4
4
1
2
1
ClinVar assertions
PATH
LPATH
CONF
VUS
LBEN
BEN/LBEN
BEN