Approved ACMG-AMP RASopathy Spectrum Disease-Related Classification Criteria

These criteria should only be used to classify germline variants potentially associated with a RASopathy phenotype. Please note that these adapted criteria are not currently designed to classify variants relative to non-RASopathy phenotypes (e.g. loss of function variants in PTPN11 related to metachondromatosis); however, information about these other genotype:phenotype correlations are noted within the supplemental material.

These criteria are also not designed to classify somatic variation in these genes. It is well-known that information about known somatic mutations can be utilized as supporting evidence for classifying variants relative to the RASopathy spectrum disorders given the disease mechanisms are directly correlated. Future initiatives in conjunction with the ClinGen somatic working group will aim to define this relationship in subsequent versions of this documentation. Currently, specific phenotype:genotype correlations regarding somatic variants should not be used as evidence to support germline pathogenicity.

VERY STRONG EVIDENCE OF PATHOGENICITY

PVS1 Null variant (nonsense, frameshift, canonical +/-1 or 2 splice sites, initiation codon, single or multi-exon deletion) in a gene where loss of function (LOF) is a known mechanism of disease

Caveats:

- Beware of genes where LOF is not a known disease mechanism (e.g. *GFAP*, *MYH7*)
- Use caution interpreting LOF variants at the extreme 3' end of a gene
- Use caution with splice variants that are predicted to lead to exon skipping but leave the remainder of the protein intact
- Use caution in the presence of multiple transcripts

RAS EP Commentary: LOF and/or haploinsufficiency has not been clearly identified as disease mechanisms for these genes *relative to the RASopathy spectrum phenotype,* therefore in general this rule is not applicable. Note that PTPN11 is currently the only gene with a confirmed association to another non-RASopathy disorder due to LOF alleles. **Variants in PTPN11 with predicted LOF should not be evaluated by these RASoathy specific criteria, but should defer to non-adjusted criteria.** Given that some historical LOF variants (e.g. canonical splice sites) could potentially result in a gain of function, users should assess using these criteria **and** non-adjusted criteria to identify the highest likelihood of pathogenicity for **all** associated diseases. We recommend that the ClinGen Dosage Sensitivity Map Status (http://www.ncbi.nlm.nih.gov/projects/dbvar/clingen/index.shtml) be reviewed for any new apparently LOF disease associations prior to classification assessment.

STRONG EVIDENCE OF PATHOGENICITY

PS1 Same amino acid change as a previously established pathogenic variant regardless of nucleotide change

Example: Val->Leu caused by either G>C or G>T in the same codon
Caveat: Beware of changes that impact splicing rather than at the amino acid/protein level

RAS EP Commentary: Previously established variant must be established as pathogenic per these criteria for germline RASopathy variants. This evidence rule can also be applied for the any observed analogous residue positions/regions throughout the gene in highly analogous groupings below:

Group 1: HRAS, NRAS, KRAS
Group 2: MAP2K1, MAP2K2
Group 3: SOS1, SOS2

PS2 *De novo* (both maternity and paternity confirmed) in a patient with the disease and no family history

Note: Confirmation of paternity only is insufficient. Egg donation, surrogate motherhood, errors in embryo transfer, *etc.* can contribute to non-maternity

PS2_Very Strong: ≥2 independent occurrences of PS2 OR ≥2 independent occurrences of PM6 and one occurrence of PS2. Evidence from literature must be fully evaluated to support independent events. Also see PM6 definition.

PS3 Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect on the gene or gene product

Note: Functional studies that have been validated and shown to be reproducible and robust in a clinical diagnostic laboratory setting are considered the most well-established

RAS EP Commentary: Approved functional studies are available for each individual gene in the supplemental material. Additional functional studies can be submitted to the expert panel for approval.

PS4 The prevalence of the variant in affected individuals is significantly increased compared to the prevalence in controls

Note 1: Relative risk (RR) or odds ratio (OR), as obtained from case-control studies, is >5.0 and the confidence interval around the estimate of RR or OR does not include 1.0. See manuscript for detailed guidance.

Note 2: In instances of very rare variants where case-control studies may not reach statistical significance, the prior observation of the variant in multiple

unrelated patients with the same phenotype, and its absence in controls, may be used as moderate level of evidence.

PS4: ≥5 independent occurrences
PS4_Moderate: 3-4 independent occurrences
PS4_Supporting: 1-2 independent occurrences

MODERATE EVIDENCE OF PATHOGENICITY

PM1 Located in a mutational hot spot and/or critical and well-established functional domain (*e.g.* active site of an enzyme) without benign variation

RAS EP Commentary: See supplemental material for approved functional domains and residues. This evidence rule can also be applied for the same analogous residue positions/regions in highly analogous groupings below:

Group 1: HRAS, NRAS, KRAS
Group 2: MAP2K1, MAP2K2
Group 3: SOS1, SOS2

PM2 Absent from controls (or at extremely low frequency if recessive) in Exome Sequencing Project, 1000 Genomes or ExAC

Caveat: Population data for indels may be poorly called by next generation sequencing

RAS EP Commentary: The variant must be completely absent from all population databases.

PM3 For recessive disorders, detected in *trans* with a pathogenic variant

Note: This requires testing of parents (or offspring) to determine phase

RAS EP Commentary: This criterion is not applicable to the RASopathies.

PM4 Protein length changes due to in-frame deletions/insertions in a non-repeat region or stop-loss variants

PM5 Missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before

Example: Arg156His is pathogenic; now you observe Arg156Cys

Caveat: Beware of changes that impact splicing rather than at the amino acid/protein level

RAS EP Commentary: Previously established variant(s) must be established as pathogenic per these criteria. Amino acid changes of variants should be concordant with pathogenicity based on how conservative or non-conservative (within the context of amino acid chain groupings) the residue change is relative to the known pathogenic residue changes. This evidence rule can also be used for pathogenic missense variants seen in the same analogous residue position in highly analogous groupings below:

Group 1: HRAS, NRAS, KRAS

Group 2: MAP2K1, MAP2K2
Group 3: SOS1, SOS2

This rule should not be used as independent criteria for calculating pathogenicity in conjunction with PM1 if the amino acid residue being interrogated is explicitly designated as a "mutational hot-spot". For example, Gly12 in HRAS is listed as a hot-spot for PM1 usage. In these situations, only PM1 should be used when combining criteria for final variant classification in order to avoid premature designation of a likely pathogenic classification in the absence of other evidence for pathogenicity.

PM5_Strong: ≥2 different pathogenic missense changes seen before at same residue of missense change.

PM6 Assumed *de novo*, but without confirmation of paternity and maternity

PM6_Strong: ≥2 independent occurrences of PM6. Evidence from literature must be fully evaluated to support independent events.

PM6_VeryStrong: ≥4 independent occurrences of PM6. Evidence from literature must be fully evaluated to support independent events.

***Also see PS2_VeryStrong*:** ≥2 independent occurrences of PS2 OR ≥2 independent occurrences of PM6 and one occurrence of PS2. Evidence from literature must be fully evaluated to support independent events.

SUPPORTING EVIDENCE OF PATHOGENICITY

PP1 Co-segregation with disease in multiple affected family members in a gene definitively known to cause the disease

Note: May be used as stronger evidence with increasing segregation data

RAS EP Commentary: Usage of PP1 requires 3-4 informative meioses. Segregation in more than one family is recommended

PP1_Moderate: 5-6 informative meioses

PP1_Strong: ≥7 informative meioses

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

RAS EP Commentary: PP2 is applicable to all RASopathy genes described and curated herein.

PP3 Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc)

Caveat: As many *in silico* algorithms use the same or very similar input for their predictions, each algorithm should not be counted as an independent criterion. PP3 can be used only once in any evaluation of a variant.

PP4 Patient's phenotype or family history is highly specific for a disease with a single genetic etiology.
RAS EP Commentary: This criterion is not applicable to the RASopathies. See PS4 criterion for proband counting options.

PP5 Reputable source recently reports variant as pathogenic but the evidence is not available to the laboratory to perform an independent evaluation
RAS EP Commentary: Currently, there are no resources that are acceptable for this criterion; however, additional groups are working on policies regarding use of somatic variation for germline disorders. Once these policies are established, the RAS EP will consider the use of other external resources (e.g. COSMIC database).

STAND ALONE EVIDENCE OF BENIGN IMPACT

BA1 Allele frequency is above 5% in Exome Sequencing Project, 1000 Genomes, or ExAC
RAS EP Commentary: An allele frequency ≥0.05% was approved. See supplemental material for additional frequency information.

STRONG EVIDENCE OF BENIGN IMPACT

BS1 Allele frequency is greater than expected for disorder
RAS EP Commentary: An allele frequency ≥0.025% was approved. See supplemental material for additional frequency information.

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.
RAS EP Commentary: Due to variable expressivity and severity, extensive clinical workup for RASopathy spectrum features is warranted, thus general population data should not be used for this criterion. Clinical laboratories are encouraged to accumulate more than 3 instances of well phenotyped family members before applying this strong criterion.

BS3 Well-established *in vitro* or *in vivo* functional studies shows no damaging effect on protein function or splicing
RAS EP Commentary: Approved functional studies are available for each individual gene in the supplemental material. Additional functional studies can be submitted to the expert panel for approval.

BS4 Lack of segregation in affected members of a family

Caveat: The presence of phenocopies for common phenotypes (*i.e.* cancer, epilepsy) can mimic lack of segregation among affected individuals. Also, families may have more than one pathogenic variant contributing to an autosomal dominant disorder, further confounding an apparent lack of segregation.

RAS EP Commentary: Requires only one informative meiosis and does *not* require an additional piece of supporting evidence to classify variant as likely benign. Due to variable expressivity and severity, individuals must be well-phenotyped.

SUPPORTING EVIDENCE FOR BENIGN IMPACT

BP1 Missense variant in a gene for which primarily truncating variants are known to cause disease

RAS EP Commentary: *This rule has contraindications for use with RASopathies*. Given the disease mechanism is gain-of-function for RASopathies, BP1 should be used for any truncating variant (nonsense, frameshift, affects canonical splice sites, initiation codon, entire gene or multi exon deletion) in genes without established LOF correlation to disease. See the supplemental material regarding dosage sensitivity information for each individual gene and potential association to disorders associated with LOF 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

BP3 In-frame deletions/insertions in a repetitive region without a known function

BP4 Multiple lines of computational evidence suggest no impact on gene or gene product (conservation, evolutionary, splicing impact, etc)

Caveat: As many *in silico* algorithms use the same or very similar input for their predictions, each algorithm cannot be counted as an independent criterion. BP4 can be used only once in any evaluation of a variant.

BP5 Variant found in a case with an alternate molecular basis for disease

BP6 Reputable source recently reports variant as benign but the evidence is not available to the laboratory to perform an independent evaluation

RAS EP Commentary: Currently, there are no resources that are acceptable for this criterion; however, additional groups are working on policies regarding use of somatic variation for germline disorders. Once these policies are established, the RAS EP will consider the use of other external resources (e.g. COSMIC database).

BP7 A synonymous (silent) 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

RAS EP Commentary: This rule is also applicable for intronic positions (except canonical splice sites) or non-coding variants and should be used in conjunction with BP4.

RULES FOR COMBINING PATHOGENIC CRITERIA

Pathogenic

1. 1 Very Strong (PVS1) AND
    a. ≥1 Strong (PS1-PS4) OR
    b. ≥2 Moderate (PM1-PM6) OR
    c. 1 Moderate (PM1-PM6) and 1 Supporting (PP1-PP5) OR
    d. ≥2 Supporting (PP1-PP5)
2. ≥2 Strong (PS1-PS4) OR
3. 1 Strong (PS1-PS4) AND
    a. ≥3 Moderate (PM1-PM6) OR
    b. 2 Moderate (PM1-PM6) AND ≥2 Supporting (PP1-PP5) OR
    c. 1 Moderate (PM1-PM6) AND ≥4 Supporting (PP1-PP5)

Likely Pathogenic

1. 1 Very Strong (PVS1) AND 1 Moderate (PM1-PM6) OR
2. 1 Strong (PS1-PS4) AND 1-2 Moderate (PM1-PM6) OR
3. 1 Strong (PS1-PS4) AND ≥2 Supporting (PP1-PP5) OR
4. ≥3 Moderate (PM1-PM6) OR
5. 2 Moderate (PM1-PM6) AND ≥2 Supporting (PP1-PP5) OR
6. 1 Moderate (PM1-PM6) AND ≥4 Supporting (PP1-PP5)

RULES FOR COMBINING BENIGN CRITERIA

Benign

1. 1 Stand-Alone (BA1) OR
2. ≥2 Strong (BS1-BS4)

Likely Benign

1. 1 Strong (BS1-BS4) and 1 Supporting (BP1-BP7) OR
2. ≥2 Supporting (BP1–BP7)

Summary of ACMG-AMP Criteria for the RASopathies

PATHOGENIC CRITERIA		
Criteria	**Criteria Description**	**Specification**
VERY STRONG CRITERIA		
PVS1	Null variant in a gene where loss of function is a known mechanism of disease.	N/A*
PS2_Very Strong	≥2 independent occurrences of PS2 OR ≥2 independent occurrences of PM6 plus 1 occurrence of PS2	Strength
PM6_Very Strong	≥4 independent occurrences of PM6	Strength
STRONG CRITERIA		
PS1	Same amino acid change as a previously established pathogenic variant regardless of nucleotide change.	Gene-Specific
PS2	De novo (paternity confirmed) in a patient with the disease and no family history.	None
PS3	Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect.	Gene-Specific
PS4	The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls. Requires ≥5 independent occurrences/ probands.	Disease-Specific
PM5_Strong	≥2 different pathogenic missense changes at residue	Strength
PM6_Strong	2-3 independent occurrences of PM6	Strength
PP1_Strong	≥7 segregations with disease	Strength
MODERATE CRITERIA		
PM1	Located in a mutational hot spot and/or critical and well-established functional domain.	Gene-specific
PM2	Absent from controls. Variant must be absent in large control population cohorts.	Disease-specific
PM3	For recessive disorders, detected in *trans* with a pathogenic variant.	N/A
PM4	Protein length changes due to in-frame deletions/insertions in a non-repeat region or stop-loss variants.	None
PM5	Missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before.	Gene-specific
PM6	Confirmed de novo without confirmation of paternity and maternity.	None
PS4_Moderate	3-4 independent occurrences/probands.	Strength
PP1_Moderate	5-6 segregations with disease	Strength

SUPPORTING CRITERIA		
PP1	Co-segregation with disease in 3-4 affected family members.	Disease-specific
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. Note: Applicable to all RASopathy genes.	Gene-specific
PP3	Multiple lines of computational evidence support a deleterious effect on the gene or gene product	None
PP4	Phenotype specific for disease with single genetic etiology.	N/A
PP5	Reputable source recently reports variant as pathogenic but the evidence is not available to the laboratory to perform an independent evaluation	N/A
PS4_ Supporting	1-2 independent occurrence/proband.	Strength

* *PTPN11* is the only gene with sufficient evidence to support haploinsufficiency associated with autosomal dominant metachondromatosis. It is recommended that predicted loss-of-function or null alleles in *PTPN11* be assessed using unmodified ACMG-AMP criteria.

BENIGN CRITERIA		
Criteria	**Criteria Description**	**Specification**
STAND ALONE CRITERIA		
BA1	Allele frequency is ≥ 0.0005 based on the filtering allele frequency (FAF) in ExAC	Disease-specific
STRONG CRITERIA		
BS1	Allele frequency is ≥ 0.00025 based on the filtering allele frequency (FAF) in ExAC	Disease-specific
BS2	Observed in ≥3 well-phenotyped unaffected individuals.	Disease-specific
BS3	Well-established *in vitro* or *in vivo* functional studies shows no damaging effect on protein function or splicing	Gene-specific
BS4	Lack of segregation in affected members of a family. Requires only one informative meiosis.	Disease-specific
SUPPORTING CRITERIA		
BP1	Loss of function or truncating variant (nonsense, frameshift, affects canonical splice sites, initiation codon, entire gene or multi exon deletion). (*Note this is a contraindication of original criteria)*	Disease-specific

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.	None
BP3	In-frame deletions/insertions in a repetitive region without a known function	None
BP4	Multiple lines of computational evidence suggest no impact on gene or gene product	None
BP5	Variant found in a case with an alternate molecular basis for disease	None
BP6	Reputable source recently reports variant as benign but the evidence is not available to the laboratory to perform an independent evaluation	N/A
BP7	A synonymous (silent) 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. Also applicable to intronic (except canonical splice sites) and non-coding variants.	Disease-specific

Key: **Gene-specific:** Specifications that are specified at the gene level; **Disease-Specific:** Disease-specific modifications based on what is known about the RASopathies; **Strength:** Increasing or decreasing strength of criteria based on the amount of evidence; **N/A:** not applicable to the RASopathies; **None:** no changes made to existing criteria definitions.