GUCY2D-specific PVS1 criteria – based on ClinGen SVI Working Group publication of Tayoun 2018 (PMID:30192042) and the PVS1 decision tree of ClinGen SVI Splicing Subgroup (Walker 2023, PMID: 37352859)

Nonsense or Frameshift

- Nonsense or frameshift variants that terminate at codons 2 through 1058 are expected to produce transcripts that will undergo NMD → PVS1
- Nonsense or frameshift variants from codon 1059 through 1104 are expected to produce transcripts that will not undergo NMD
  - Nonsense or frameshift variants from p.Arg1059 through p.Lys1068 may disrupt critical elements of the RD3 binding site → PVS1
  - Nonsense or frameshift variants beyond p.Lys1068 likely to disrupt critical function → PVS1_Strong

Deletion
(Single exon to full gene)

- Full gene deletion → PVS1
- Single to multi exon deletion – Disrupts reading frame and is predicted to undergo NMD
  - Exon is present in biologically-relevant transcript NM_000180.4 → PVS1
  - Exon is absent from biologically-relevant transcript NM_000180.4 → N/A
- Single to multi exon deletion – Disrupts reading frame and is **NOT** predicted to undergo NMD → Exons 2-19 harbor pathogenic variants and all are considered critical to protein function → PVS1
- Single to multi exon deletion – Preserves reading frame → Exons 2-19 harbor pathogenic variants and all are considered critical to protein function → PVS1

Duplication
(≥1 exon in size and must be completely contained within gene)

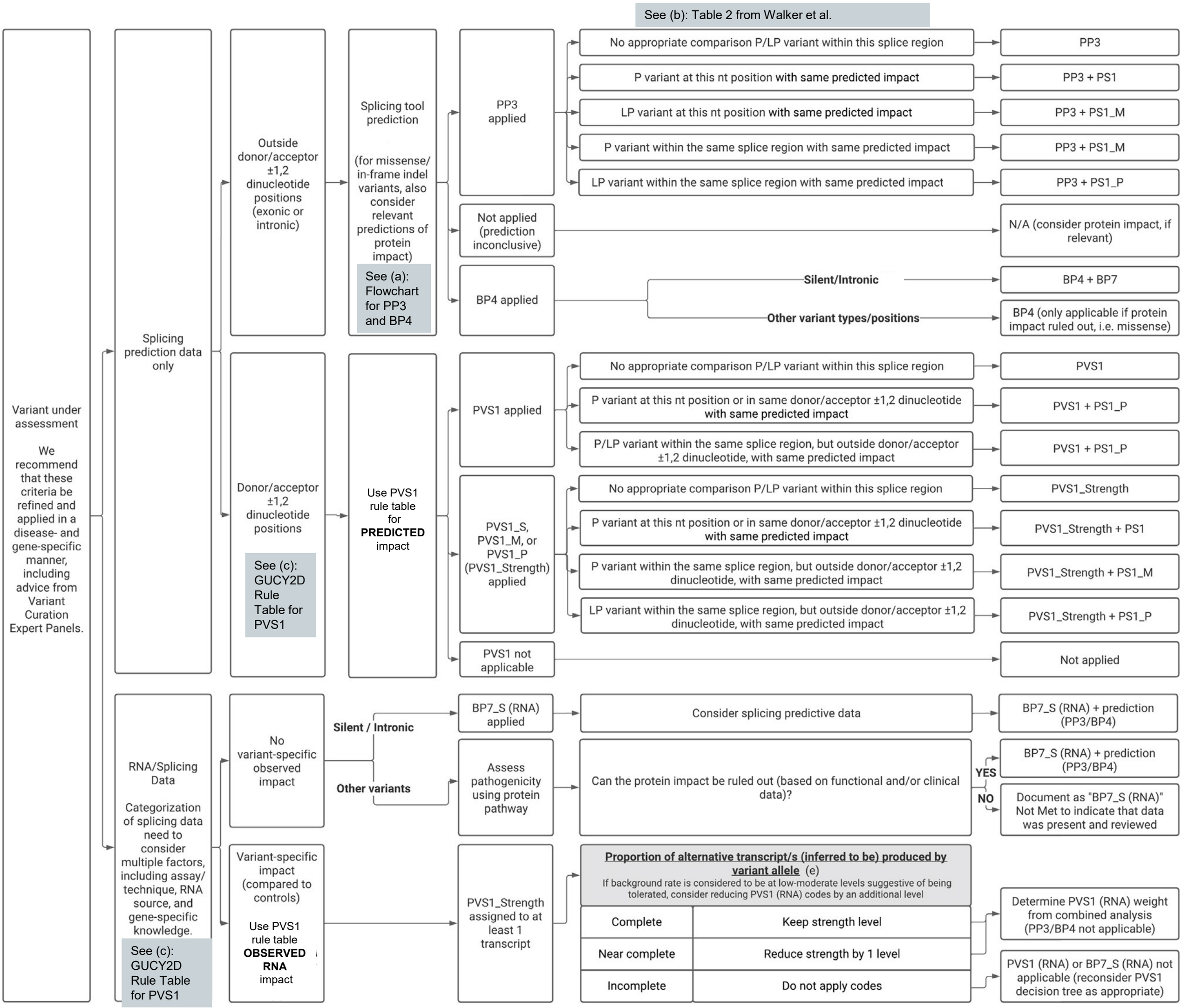
- Proven in tandem
  - Reading frame disrupted and NMD predicted to occur → PVS1
  - No or unknown impact on reading frame and NMD → N/A
- Presumed in tandem
  - Reading frame disrupted and NMD predicted to occur → PVS1
  - Reading frame presumed disrupted and NMD predicted to occur → PVS1_Strong
- Proven not in tandem → N/A

Initiation Codon

- No known alternative start codon in other transcripts → ≥1 pathogenic variant(s) upstream of closest potential in-frame start codon. (closest in-frame Met is p.Met218) → PVS1
- Different functional transcript uses alternative start codon → N/A

Variants with evidence of splicogenicity → See expanded GUCY2D-specific PVS1 criteria on the following page

GUCY2D-specific PVS1 decision tree for splicing

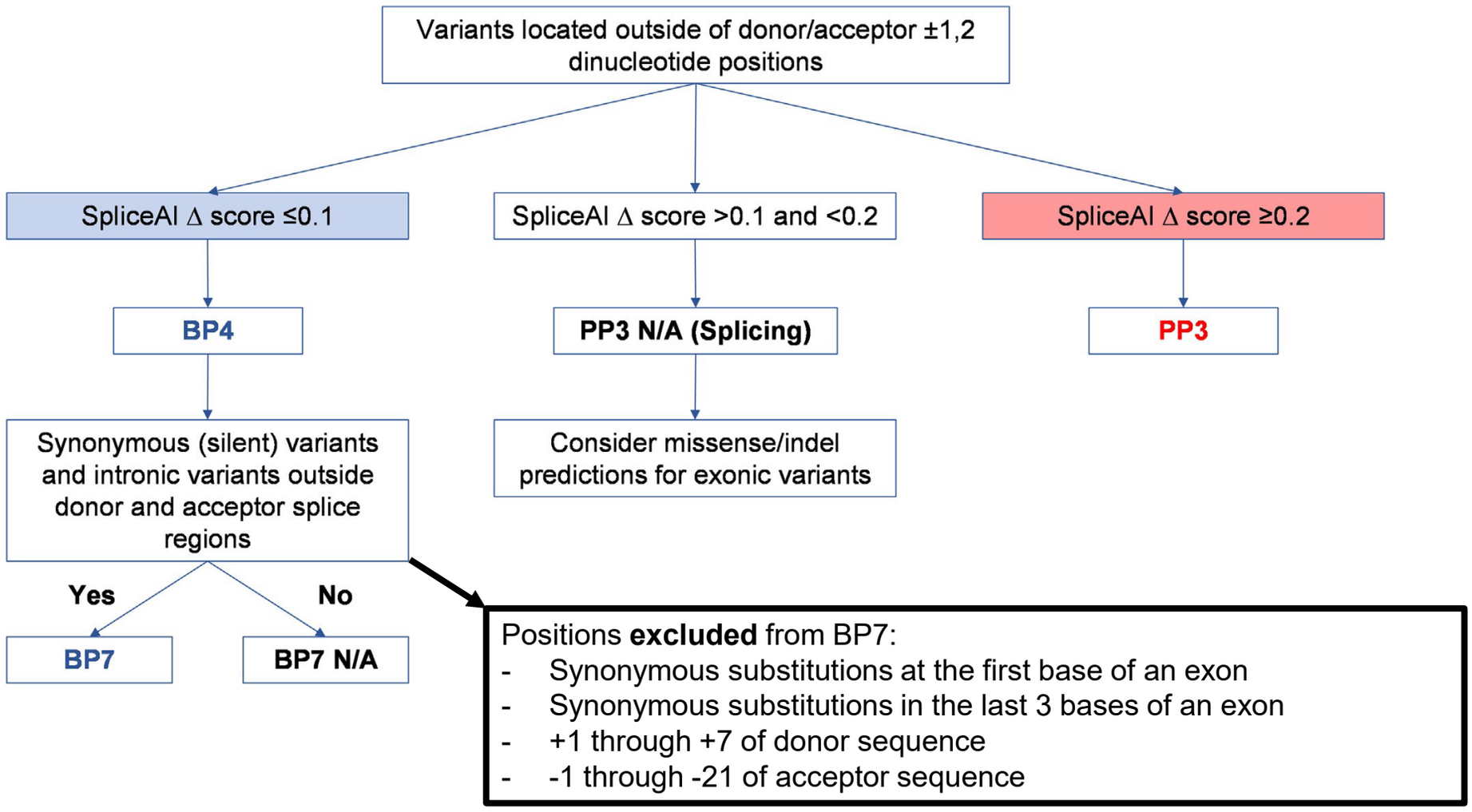


Gene-specific modifications for GUCY2D (additional details on following pages):

(a) SpliceAI Flowchart (based on Walker et al., 2023) for PP3 and BP4 – calibrated cutoff scores for SpliceAI splicing prediction. If BP4 applies, consider BP7 based on location of variant.
(b) Table 2 from Walker et al., 2023: If PP3 applies, consider PS1 code weights for variants with the same predicted splicing event as a known (likely) pathogenic variant.
(c) GUCY2D rule table for PVS1 – based on Walker et al. but GUCY2D-specific.

- Use when variant under assessment affects the donor/acceptor +/- 1,2 dinucleotide positions. As noted in Walker et al., PS1 code can be added where applicable.
- Use to evaluate RNA splicing data - **observed** results of the splicing assay are classified in the same manner as predicted results. As detailed in flowchart above, use PVS1(RNA) if there is evidence of impact on splicing or BP7_Strong(RNA) if evidence suggests no impact.

(a) SpliceAI Flowchart (based on Walker 2023, Figure 4)



(b) Table 2 from Walker 2023

Table 2. PS1 code weights for variants with same predicted splicing event as a known (likely) pathogenic variant

Variant under assessment (VUA)	Baseline computational/ predictive code applicable to VUA	Position of comparison variant relative to VUA	PS1 code applicable to VUA	
			with P comparison variant	with LP comparison variant
Located outside splice donor/ acceptor ±1,2 dinucleotide positions	PP3	same nucleotide	PS1	PS1_Moderate
	PP3	within same splice donor/acceptor motif (including at ±1,2 positions)	PS1_Moderate	PS1_Supporting
Located at splice donor/acceptor ±1,2 dinucleotide positions	PVS1	within same splice donor/ acceptor ±1,2 dinucleotide	PS1_Supporting	N/A
	PVS1	within same splice donor/acceptor region, but outside ±1,2 dinucleotide[a]	PS1_Supporting	PS1_Supporting
	PVS1_Strong, PVS1_Moderate, or PVS1_Supporting	within same splice donor/ acceptor ±1,2 dinucleotide	PS1	N/A
	PVS1_Strong, PVS1_Moderate, or PVS1_Supporting	within same splice donor/acceptor motif, but outside ±1,2 dinucleotide[a]	PS1_Moderate	PS1_Supporting

Prerequisite for all: the predicted event of the VUA must precisely match the predicted event of the comparison (likely) pathogenic variant (e.g., both predicted to lead to exon skipping, or both to lead to enhanced use of a cryptic splice motif, AND the strength of the prediction for the VUA must be of similar or higher strength than the strength of the prediction for the comparison [likely] pathogenic variant). For an exonic variant, predicted or proven functional effect of missense substitution(s) encoded by the VUA and (likely) pathogenic variant should also be considered before application of this code. Dinucleotide positions refer to donor and acceptor dinucleotides in reference transcript(s) used for curation. Designated donor and acceptor motif ranges should be based on position weight matrices for intron category (see methods). For GT-AG introns these are defined as follows: the donor motif, last 3 bases of the exon and 6 nucleotides of intronic sequence adjacent to the exon; acceptor motif, first base of the exon and 20 nucleotides upstream from the exon boundary. Consider other motif ranges for non-GT-AG introns.

[a]If relevant, splicing assay data for a pathogenic variant outside a ±1,2 dinucleotide position may be used to update a PVS1 decision tree and hence the applicable PVS1 code for a ±1,2 dinucleotide variant.

(c) GUCY2D PVS1 rule table (for +- 1,2 changes and RNA splicing assays):

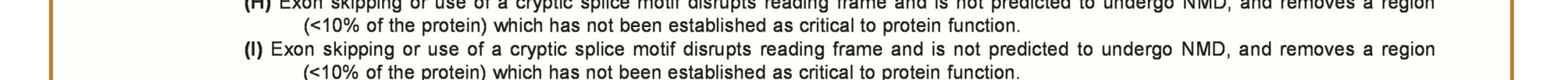
Based on generic gene schematic (shown below) proposed by Walker et al with the following modifications:

1. Pathogenic variants have been identified in every exon so all exons are considered to be “critical to protein function”, requirement for being more than 10% of total protein length does not apply.
2. ATG initiation site is located in exon 2 so 5’ UTR recommendation (A) applies
3. No potential “rescue isoforms” are known
4. **Use this table to assign appropriate PVS1 code and rationale**:

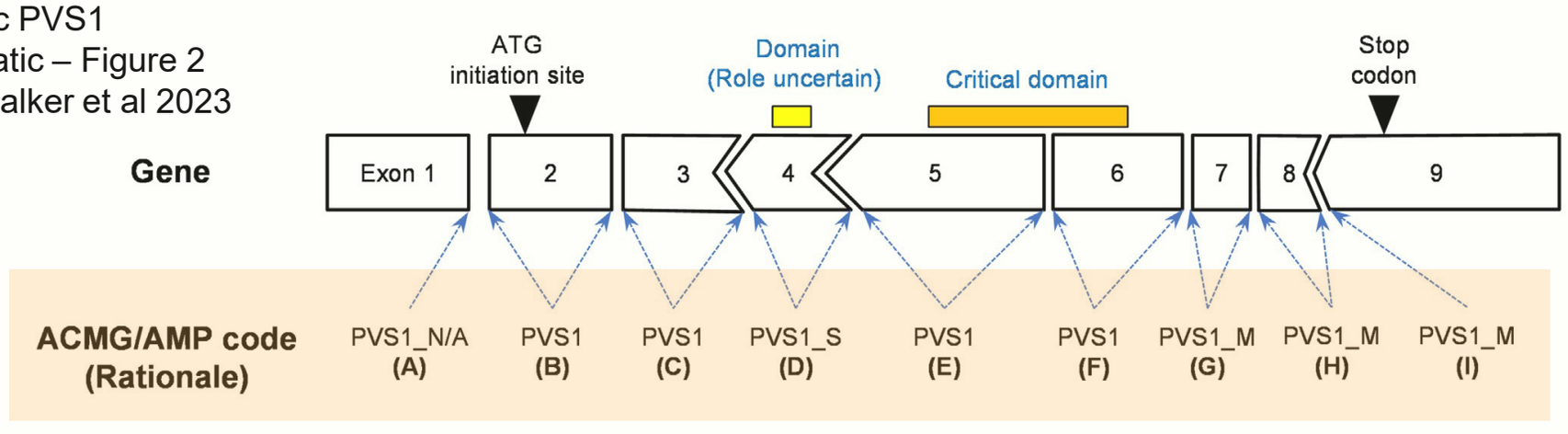
	3' acceptor position	5' donor position	exon skipping leads to preserved reading frame or frameshift (fs) with NMD?	PVS1 code for +- 1,2 dinucleotide change and rationale from Walker et al	exon known to be critical to protein function
exon 1	NA	-10	NA	PVS1_N/A (A)	NA
exon 2	-9	721	next in-frame Met codon is at 244 in exon 3, no NMD but would make a truncated protein	PVS1 (B)	LS, ECD
exon 3	722	1026	fs/NMD	PVS1 (C)	ECD
exon 4	1027	1378	fs/NMD	PVS1 (C)	ECD
exon 5	1379	1463	in-frame	PVS1 (F)	ECD, TM
exon 6	1464	1566	fs/NMD	PVS1 (C)	JMD
exon 7	1567	1668	in-frame	PVS1 (F)	JMD
exon 8	1669	1749	in-frame	PVS1 (F)	JMD
exon 9	1750	1956	in-frame	PVS1 (F)	JMD, KHD
exon 10	1957	2113	fs/NMD	PVS1 (C)	KHD
exon 11	2114	2263	in-frame	PVS1 (F)	KHD
exon 12	2264	2412	fs/NMD	PVS1 (C)	KHD
exon 13	2413	2576	fs/NMD	PVS1 (C)	KHD, DD
exon 14	2577	2769	fs/NMD	PVS1 (C)	CCD
exon 15	2770	2944	fs/NMD	PVS1 (C)	CCD
exon 16	2945	3043	in-frame	PVS1 (F)	CCD
exon 17	3044	3138	fs/NMD	PVS1 (C)	CCD
exon 18	3139	3224	fs/NMD	PVS1 (H) - exon is known to be critical so upgraded to standard PVS1	CCD
exon 19	3225	3312+24	fs/NMD	PVS1 (I) - exon is known to be critical so upgraded to standard PVS1	CCD
exon 20	*25	*237	NA		NA

LS: leader sequence; ECD: extracellular domain; TM: transmembrane domain; JMD: juxtamembrane domain; KHD: kinase homology domain; DD: dimerization domain; CCD:cyclase catalytic domain

GUCY2D exon map: overhang on top is a two-nt overhang, overhang on bottom is a one-nt overhang. Parallel lines represent in-frame junctions (eg. del of exons 8+9 is in frame, del of 6+7 is out of frame)



Generic PVS1 schematic – Figure 2 from Walker et al 2023



(A) 5’ UTR region - No splicing alteration predicted or use of a cryptic splice motif does not affect the coding sequence.
(B) Exon skipping or use of a cryptic splice motif eliminates the initiation codon and there are no alternative start codons.
(C) Exon skipping or use of a cryptic splice motif disrupts reading frame and is predicted to undergo NMD
(D) Exon skipping or use of a cryptic splice motif preserves reading frame, and removes a region (>10% of the protein) which has not been established as critical to protein function.
(E) Exon skipping or use of a cryptic splice motif disrupts reading frame and is predicted to undergo NMD
(F) Exon skipping or use of a cryptic splice motif preserves reading frame, and removes a region which has been established as critical to protein function
(G) Exon skipping or use of a cryptic splice motif preserves reading frame, and removes a region (<10% of the protein) which has not been established as critical to protein function.
(H) Exon skipping or use of a cryptic splice motif disrupts reading frame and is not predicted to undergo NMD, and removes a region (<10% of the protein) which has not been established as critical to protein function.
(I) Exon skipping or use of a cryptic splice motif disrupts reading frame and is not predicted to undergo NMD, and removes a region (<10% of the protein) which has not been established as critical to protein function.