Supplementary Material and Methods

Construction of the BRCA1 BRCT variant set

All the BRCA1 BRCT missense variants recorded (as of November, 2008) in the Breast Cancer Information Core (BIC) Database (http://research.nhgri.nih.gov/bic/) and eleven additional patient-derived variants were generated. The eleven additional variants are: T1700A (1), A1708V (2), D1739V, R1753T (3), M1775K (4), D1778Y, M1783I, Q1785H (3), E1794D (3), H1805P and R1835P.

Coding sequences for the tandem BRCT repeats of BRCA1 were generated with PCR primers FT7 (5'-gga cga gaa ttc tta acc agg gag ctg att atg gtg aac aaa aga atg tcc atg-3') and CD6 (5'-gat ctg gga tcc tca ggg gat ctg ggg tat cag-3'). The 5' primer FT7 includes a ribosome binding site and an *Eco*RI restriction site for cloning. The 3' primer CD6 includes stop codons and a *Bam*HI restriction site for cloning. The mutant PCR products were then cloned into the T7 promoter based expression vector, pLM1-BRCA1-BRCT (1646-1858), as previously reported (5). For P1859R, P1856S, L1854P, Y1853C, D1851E, L1844R, A1843P, S1841R, S1841N, V1838E, W1837C, W1837G, W1837R, E1836K, R1835P and V1833M, coding sequences were amplified using FT7 and modified CD6 primers that incorporated relevant mutations. For N1647K, S1651F, M1652T, M1652I, V1653M, S1655F, G1656D, F1662S, M1663L, M1663K, L1664P, V1665M and A1669S, coding sequences were amplified using CD6 and modified FT7 that included relevant mutations. All other missense substitutions were engineered using PCR splicing methods (6). All the vectors were sequenced to confirm presence of mutagenesis.

To generate plasmid constructs for use in transcriptional assays, pLM1 vectors containing the BRCA1 variants were digested with *Eco*RI and *Xba*I and the

0.7 Kb fragment containing the variants in the context of BRCA1 aa 1646-1859 was isolated. The fragments containing the variants were ligated to a pCDNA3 vector containing a GAL4 DNA Binding Domain digested with the same enzymes (7-9).

1. Abkevich V, Zharkikh A, Deffenbaugh AM, Frank D, Chen Y, Shattuck D, et al. Analysis of missense variation in human BRCA1 in the context of interspecific sequence variation. J Med Genet. 2004;41:492-507.
2. Lovelock PK, Spurdle AB, Mok MT, Farrugia DJ, Lakhani SR, Healey S, et al. Identification of BRCA1 missense substitutions that confer partial functional activity: potential moderate risk variants? Breast Cancer Res. 2007;9:R82.
3. Carvalho MA, Marsillac SM, Karchin R, Manoukian S, Grist S, Swaby RF, et al. Determination of cancer risk associated with germ line BRCA1 missense variants by functional analysis. Cancer Res. 2007;67:1494-501.
4. Tischkowitz M, Hamel N, Carvalho MA, Birrane G, Soni A, van Beers EH, et al. Pathogenicity of the BRCA1 missense variant M1775K is determined by the disruption of the BRCT phosphopeptide-binding pocket: a multi-modal approach. Eur J Hum Genet. 2008;16:820-32.
5. Williams RS, Chasman DI, Hau DD, Hui B, Lau AY, Glover JN. Detection of protein folding defects caused by BRCA1-BRCT truncation and missense mutations. J Biol Chem. 2003;278:53007-16.
6. Horton RM, Ho SN, Pullen JK, Hunt HD, Cai Z, Pease LR. Gene splicing by overlap extension. Methods Enzymol. 1993;217:270-9.
7. Carvalho MA, Couch FJ, Monteiro AN. Functional assays for BRCA1 and BRCA2. Int J Biochem Cell Biol. 2007;39:298-310.
8. Vallon-Christersson J, Cayanan C, Haraldsson K, Loman N, Bergthorsson JT, Brondum-Nielsen K, et al. Functional analysis of BRCA1 C-terminal missense mutations identified in breast and ovarian cancer families. Hum Mol Genet. 2001;10:353-60.
9. Phelan CM, Dapic V, Tice B, Favis R, Kwan E, Barany F, et al. Classification of BRCA1 missense variants of unknown clinical significance. J Med Genet. 2005;42:138-46.

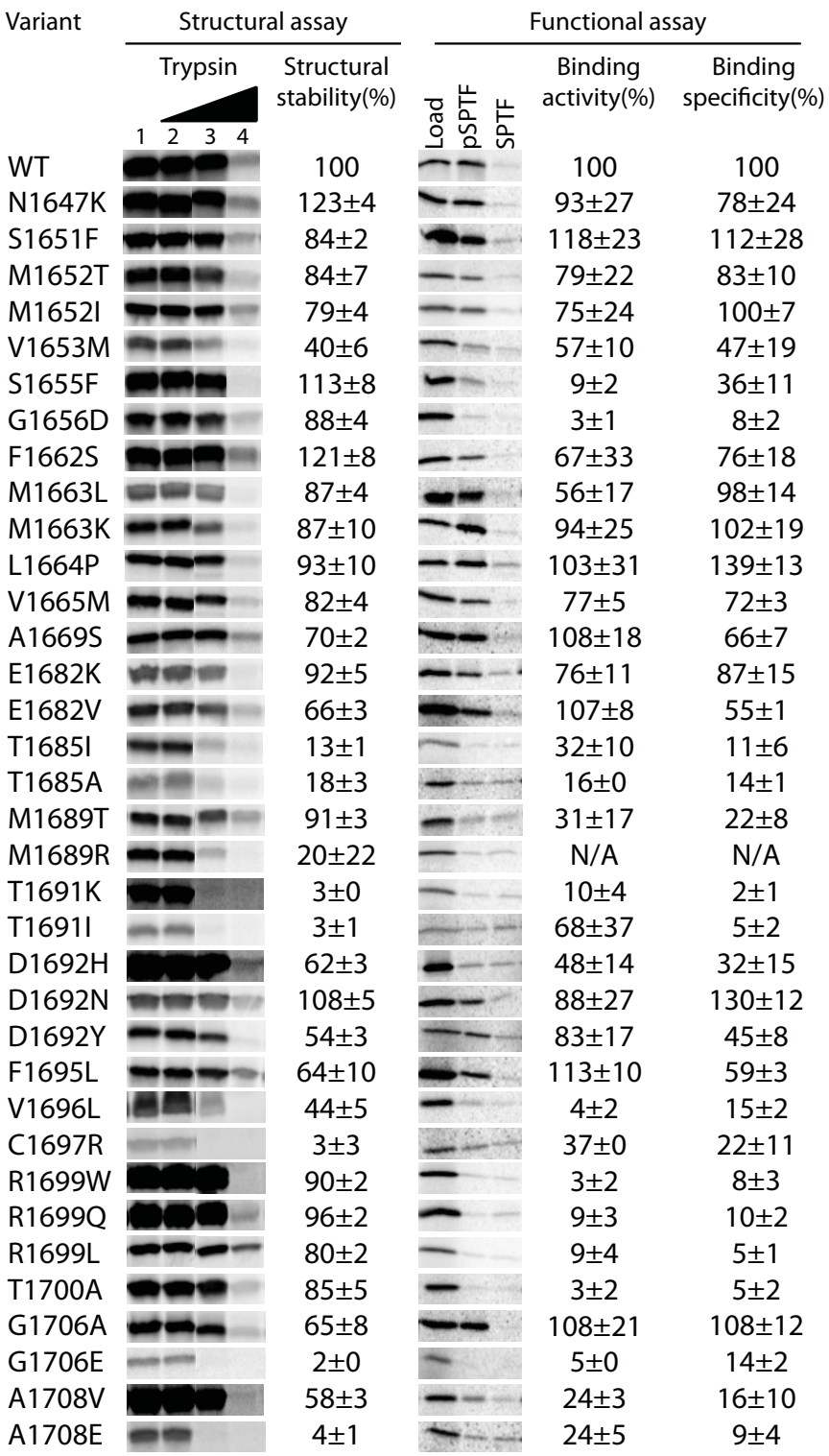
Prediction of pre-mRNA splicing defects in BRCA1 BRCT variants

In addition to the introduction of missense or nonsense mutations, single nucleotide polymorphisms can also affect pre-mRNA splicing. In theory, splicing can be disrupted through mutation of either the 5' donor, 3' acceptor or branch sites, or through creation of cryptic splice sites (1, 2). To probe the possibility that any of the BRCA1 BRCT missense variants could affect normal splicing patterns, we used the NNSplice 0.9 algorithm (3) to assess the effects of the BRCA1 SNPs on splice junction scores. The default threshold of 0.4 was used to find splice sites. Variant scores at least 20% lower or higher that the wild type score were marked as deleterious (4).

Fourteen of the tested mutants were predicted to potentially lead to splicing defects (Table 1). Nine variants (M1663L, M1663K, D1692H, D1692N, D1692Y M1775R, M1775K, D1778Y and D1778N) gave significantly reduced splice junction scores, and may be associated with an increased risk of exon skipping. Five mutations (V1714G, S1715R, W1718C, A1752V and V1809F) were predicted to potentially lead to novel cryptic splice sites.

Lee, M. *et al.* Supplemtary Figure 1a

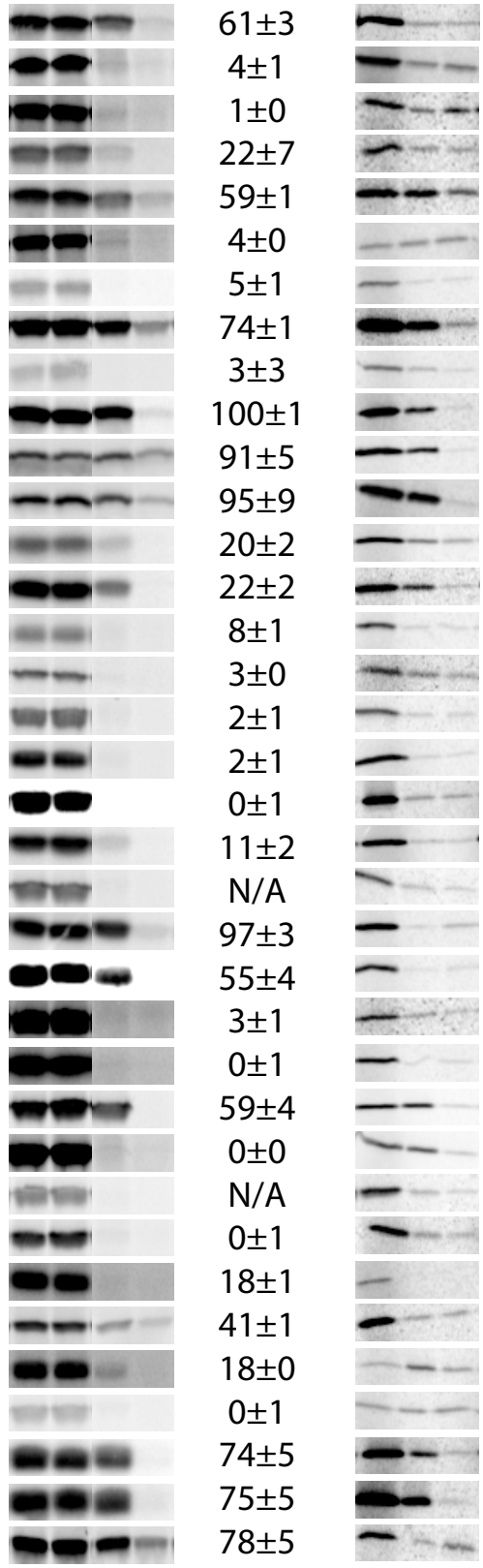
Evaluation of BRCA1 missense variants



Structural stability for each variant was assessed by digestion of trypsin at 1 μg/ml (lane 2), 10 μg/ml (lane 3) and 100 μg/ml (lane 4). The amount of full-length variant remaining after digestion with 10 μg/ml (lane 3) was expressed as a percentage of the input protein (lane 1). Standard deviations represent results from at least 3 independent experiments.

Lee, M. *et al.* Supplementary Figure 1b

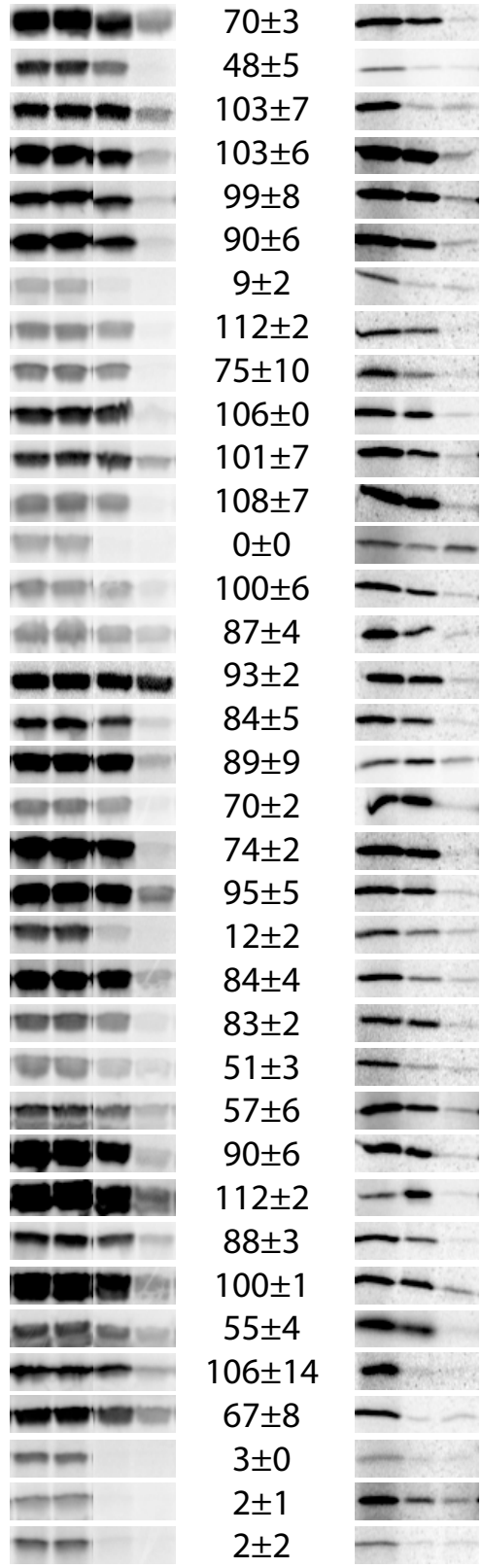
Evaluation of BRCA1 missense variants



Variant	Structural assay: Structural stability(%)	Functional assay: Binding activity(%)	Functional assay: Binding specificity(%)
V1713A	61±3	25±7	3±3
V1714G	4±1	13±3	9±1
S1715R	1±0	10±4	9±1
S1715N	22±7	42±25	7±3
S1715C	59±1	91±28	39±27
W1718C	4±0	97±3	11±1
W1718S	5±1	18±5	8±5
T1720A	74±1	87±4	56±4
S1722F	3±3	19±2	12±6
R1726G	100±1	84±11	126±27
N1730S	91±5	114±43	94±37
D1733G	95±9	113±15	99±5
F1734S	20±2	55±16	20±5
V1736A	22±2	72±5	49±3
V1736G	8±1	14±1	5±1
G1738E	3±0	71±3	3±3
G1738R	2±1	15±10	3±0
D1739Y	2±1	10±8	5±1
D1739V	0±1	5±4	11±2
D1739E	11±2	6±2	20±5
D1739G	N/A	16±7	1±0
V1741G	97±3	5±3	8±9
H1746N	55±4	9±7	10±1
P1749R	3±1	29±18	18±9
R1751P	0±1	6±3	3±1
R1751Q	59±4	76±12	58±10
A1752V	0±0	89±7	31±8
A1752P	N/A	16±12	5±1
R1753T	0±1	42±9	16±9
F1761S	18±1	N/A	N/A
G1763V	41±1	19±1	15±7
L1764P	18±0	23±9	19±6
I1766S	0±1	94±11	5±1
P1771L	74±5	75±15	87±38
P1771R	75±5	97±31	85±16
T1773I	78±5	63±5	15±7

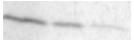
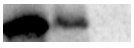
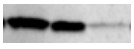
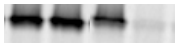
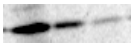
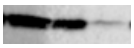
Lee, M. *et al.* Supplementary Figure 1c

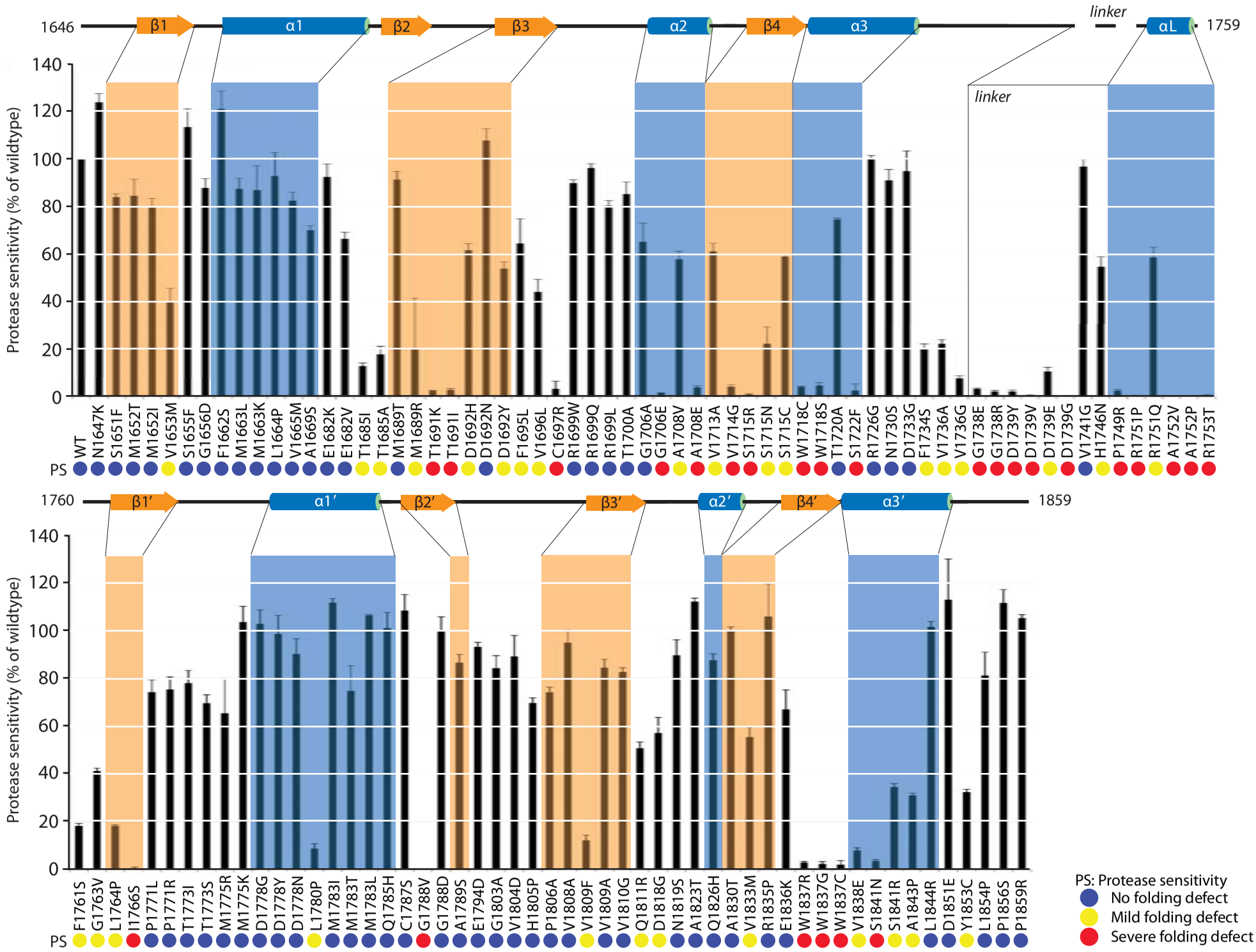
Evaluation of BRCA1 missense variants



Lee, M. *et al*. Supplementary Figure 1d

Evaluation of BRCA1 missense variants

Variant	Structural assay		Functional assay		
	Trypsin (1 2 3 4)	Structural stability(%)	Load / pSPTF / SPTF	Binding activity(%)	Binding specificity(%)
V1838E		8±1		22±10	11±4
S1841N		3±1		26±1	5±1
S1841R		35±1		17±3	7±1
A1843P		31±1		27±10	16±2
L1844R		101±2		42±11	67±2
D1851E		113±17		71±8	89±6
Y1853C		32±1		14±3	4±3
L1854P		81±10		78±31	100±20
P1856S		112±6		82±12	84±25
P1859R		105±2		67±12	127±10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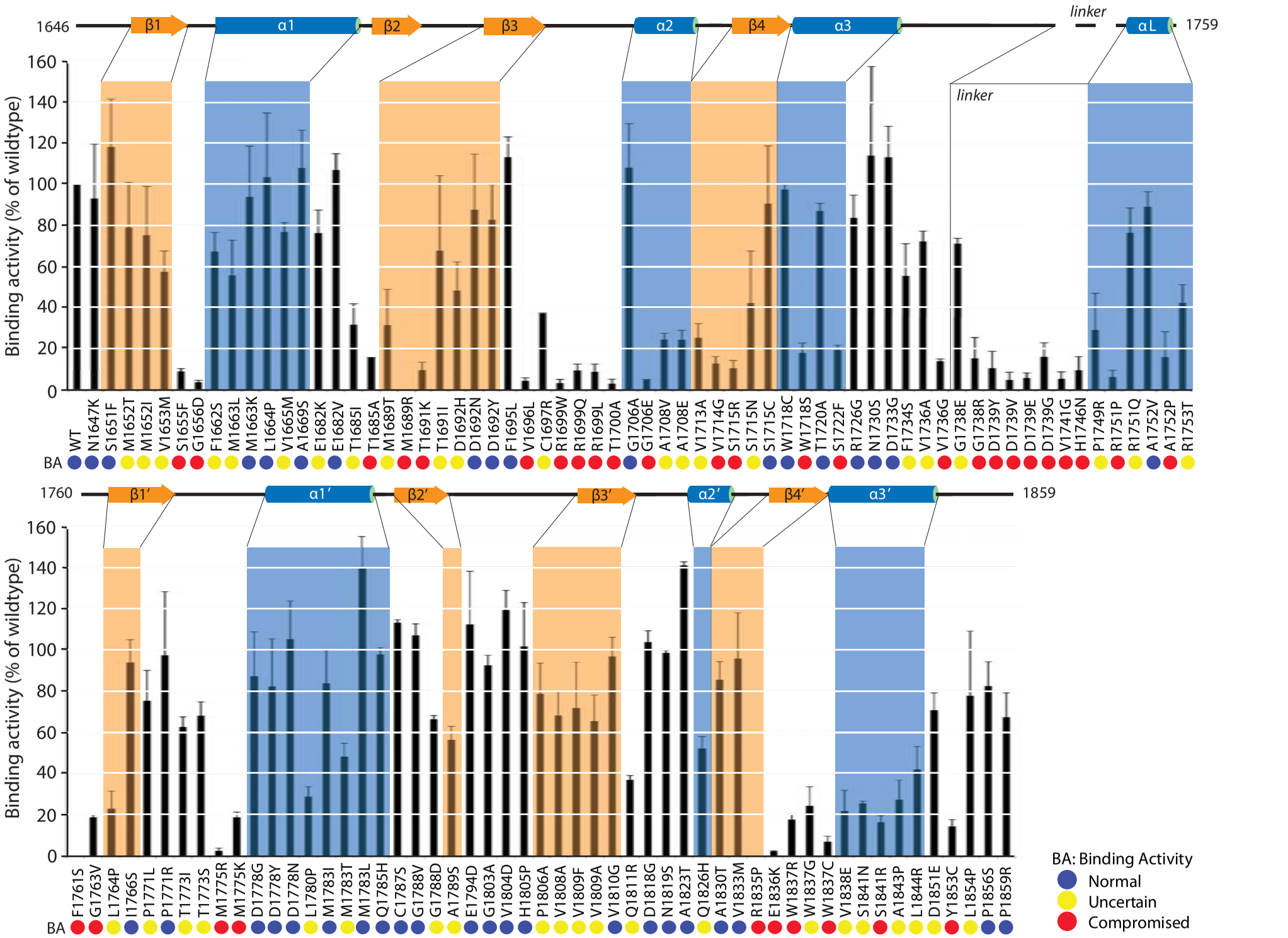
β1
α1
β2
β3
α2
β4
α3
linker
αL
1759
linker
Protease sensitivity (% of wildtype)
0
20
40
60
80
100
120
140
WT N1647K S1651F M1652T M1652I V1653M S1655F G1656D F1662S M1663L M1663K L1664P V1665M A1669S E1682K E1682V T1685I T1685A M1689T M1689R T1691K T1691I D1692H D1692N D1692Y F1695L V1696L C1697R R1699W R1699Q R1699L T1700A G1706A G1706E A1708V A1708E V1713A V1714G S1715R S1715N S1715C W1718C W1718S T1720A S1722F R1726G N1730S D1733G F1734S V1736A V1736G G1738E G1738R D1739Y D1739V D1739E D1739G V1741G H1746N P1749R R1751P R1751Q A1752V A1752P R1753T
PS
1760
β1′
α1′
β2′
β3′
α2′
β4′
α3′
1859
Protease sensitivity (% of wildtype)
0
20
40
60
80
100
120
140
F1761S G1763V L1764P I1766S P1771L P1771R T1773I T1773S M1775R M1775K D1778G D1778Y D1778N L1780P M1783I M1783T M1783L Q1785H C1787S G1788V G1788D A1789S E1794D G1803A V1804D H1805P P1806A V1808A V1809F V1809A V1810G Q1811R D1818G N1819S A1823T Q1826H A1830T V1833M R1835P E1836K W1837R W1837G W1837C V1838E S1841N S1841R A1843P L1844R D1851E Y1853C L1854P P1856S P1859R
PS
PS: Protease sensitivity
No folding defect
Mild folding defect
Severe folding defect


1646
β1
α1
β2
β3
α2
β4
α3
linker
αL
1759
linker
Binding activity (% of wildtype)
0
20
40
60
80
100
120
140
160
WT
N1647K
S1651F
M1652T
M1652I
V1653M
S1655F
G1656D
F1662S
M1663L
M1663K
L1664P
V1665M
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D1692N
D1692Y
F1695L
V1696L
C1697R
R1699W
R1699Q
R1699L
T1700A
G1706A
G1706E
A1708V
A1708E
V1713A
V1714G
S1715R
S1715N
S1715C
W1718C
W1718S
T1720A
S1722F
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A1752P
R1753T
BA
1760
β1′
α1′
β2′
β3′
α2′
β4′
α3′
1859
Binding activity (% of wildtype)
0
20
40
60
80
100
120
140
160
F1761S
G1763V
L1764P
I1766S
P1771L
P1771R
T1773I
T1773S
M1775R
M1775K
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D1778Y
D1778N
L1780P
M1783I
M1783T
M1783L
Q1785H
C1787S
G1788V
G1788D
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E1794D
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V1833M
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W1837R
W1837G
W1837C
V1838E
S1841N
S1841R
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Y1853C
L1854P
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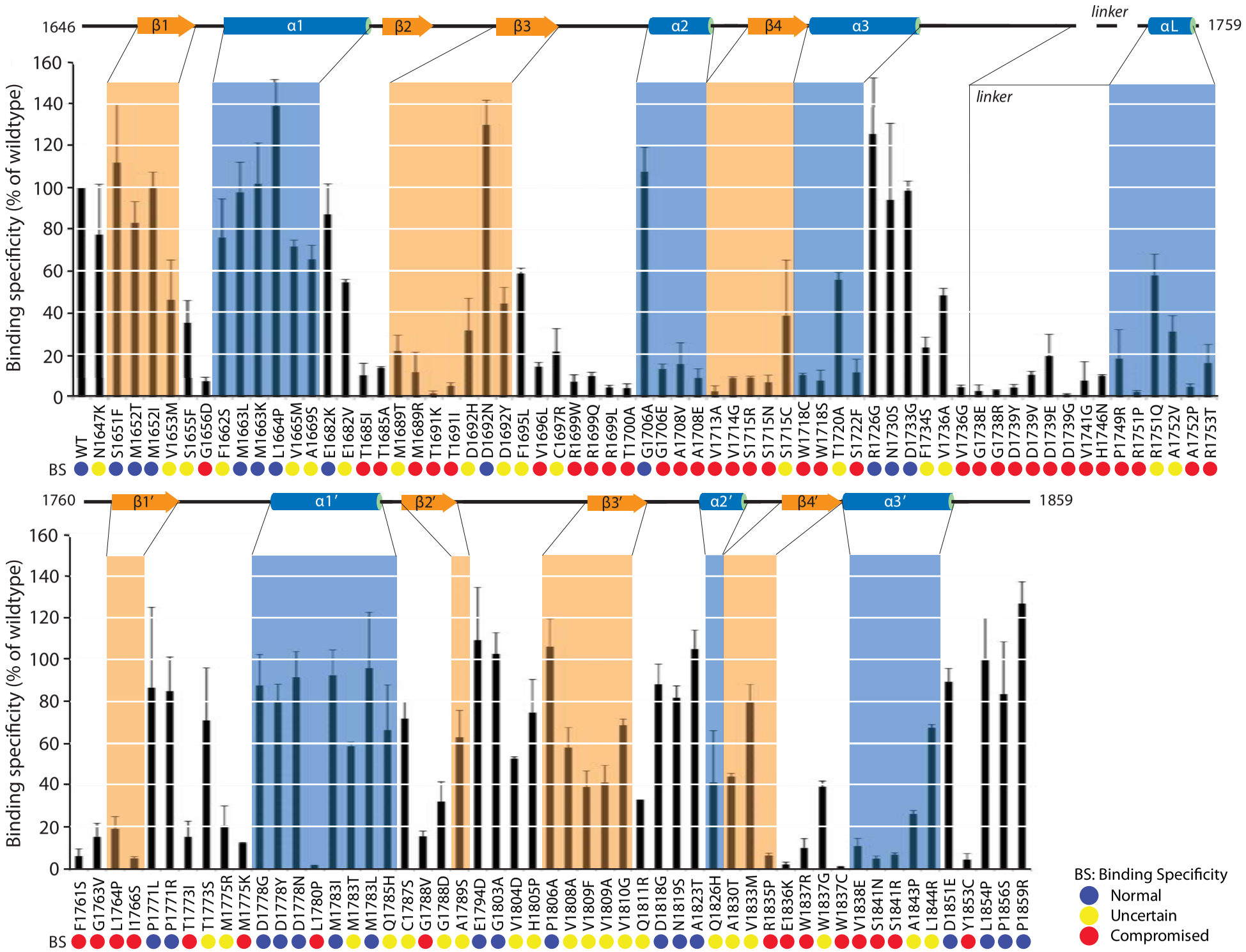
P1859R
BA
BA: Binding Activity
Normal
Uncertain
Compromised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β1
α1
β2
β3
α2
β4
α3
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αL
1759
linker
Binding specificity (% of wildtype)
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20
40
60
80
100
120
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160
WT N1647K S1651F M1652T M1652I V1653M S1655F G1656D F1662S M1663L M1663K L1664P V1665M A1669S E1682K E1682V T1685I T1685A M1689T M1689R T1691K T1691I D1692H D1692N D1692Y F1695L V1696L C1697R R1699W R1699Q R1699L T1700A G1706A G1706E A1708V A1708E V1713A V1714G S1715R S1715N S1715C W1718C W1718S T1720A S1722F R1726G N1730S D1733G F1734S V1736A V1736G G1738E G1738R D1739Y D1739V D1739E D1739G V1741G H1746N P1749R R1751P R1751Q A1752V A1752P R1753T
BS
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β1′
α1′
β2′
β3′
α2′
β4′
α3′
1859
Binding specificity (% of wildtype)
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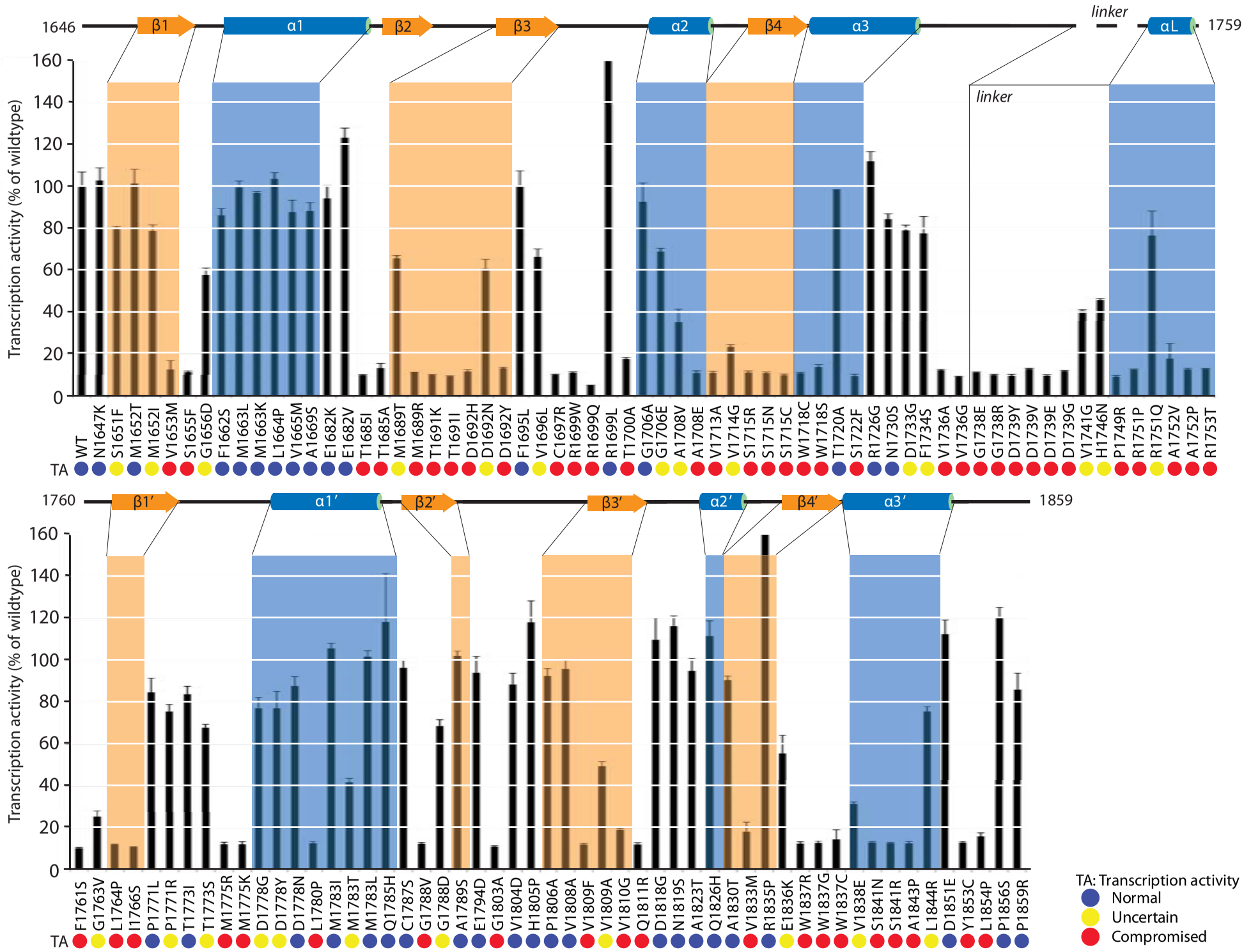
60
80
100
120
140
160
F1761S G1763V L1764P I1766S P1771L P1771R T1773I T1773S M1775R M1775K D1778G D1778Y D1778N L1780P M1783I M1783T M1783L Q1785H C1787S G1788V G1788D A1789S E1794D G1803A V1804D H1805P P1806A V1808A V1809F V1809A V1810G Q1811R D1818G N1819S A1823T Q1826H A1830T V1833M R1835P E1836K W1837R W1837G W1837C V1838E S1841N S1841R A1843P L1844R D1851E Y1853C L1854P P1856S P1859R
BS
BS: Binding Specificity
Normal
Uncertain
Compromised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β2
β3
α2
β4
α3
linker
αL
1759
linker
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M1652T
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V1653M
S1655F
G1656D
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R1753T
TA
1760
β1′
α1′
β2′
β3′
α2′
β4′
α3′
1859
Transcription activity (% of wildtype)
0
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40
60
80
100
120
140
160
F1761S
G1763V
L1764P
I1766S
P1771L
P1771R
T1773I
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W1837G
W1837C
V1838E
S1841N
S1841R
A1843P
L1844R
D1851E
Y1853C
L1854P
P1856S
P1859R
TA
TA: Transcription activity
Normal
Uncertain
Compromised

Lee, M. *et al.* Supplementary Figure 3