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Comprehensive annotation of splice junctions supports pervasive alternative splicing at the *BRCA1* locus: a report from the ENIGMA consortium

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Loss-of-function germline mutations in *BRCA1* (MIM #113705) confer markedly increased risk of breast and ovarian cancer. The full-length transcript codifies for a protein involved in DNA repair pathways and cell-cycle checkpoints. Several *BRCA1* splicing isoforms have been described in public domain databases, but the physiological role (if any) of *BRCA1* alternative splicing remains to be established. An accurate description of 'naturally occurring' alternative splicing at this locus is a prerequisite to understand its biological significance. However, a systematic analysis of alternative splicing at the *BRCA1* locus is yet to be conducted. Here, the Evidence-Based Network for the Interpretation of Germ-Line Mutant Alleles consortium combines RT-PCR, exon scanning,

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cloning, sequencing and relative semi-quantification to describe naturally occurring *BRCA1* alternative splicing with unprecedented resolution. The study has been conducted in blood-related RNA sources, commonly used for clinical splicing assays, as well as in one healthy breast tissue. We have characterized a total of 63 *BRCA1* alternative splicing events, including 35 novel findings. A minimum of 10 splicing events (Δ1Aq, Δ5, Δ5q, Δ8p, Δ9, Δ(9,10), Δ9_11, Δ11q, Δ13p and Δ14p) represent a substantial fraction of the full-length expression level (ranging from 5 to 100%). Remarkably, our data indicate that *BRCA1* alternative splicing is similar in blood and breast, a finding supporting the clinical relevance of blood-based *in vitro* splicing assays. Overall, our data suggest an alternative splicing model in which most non-mutually exclusive alternative splicing events are randomly combined into individual mRNA molecules to produce hundreds of different *BRCA1* isoforms.

INTRODUCTION

Virtually all human multiexon *loci* are subject to alternative splicing (1–4), a biological process that can produce multiple mature RNA transcripts (RNA isoforms) from a single locus (2,5). Alternative splicing is believed to occur in all metazoan organisms, but it is more prevalent in vertebrates (in particular birds and mammals), thus suggesting a link with phenotypic complexity (6,7). However, the adaptive role of this mechanism remains elusive (8), in part because the function of many splicing isoforms is unclear (4). Indeed, many of them lack annotated coding sequences (CDSs) (9), or introduce premature termination codons (PTCs) that are predicted to induce the nonsense-mediated mRNA decay (NMD) pathway (10). In this regard, it has been suggested that alternative splicing not only increases the complexity of transcriptomes and proteomes but also plays a significant role in gene regulation (8,11). However, it has also been proposed that many alternative splicing events do not have functional significance at all, but rather represent stochastic noise in the splicing process (12).

The breast cancer predisposing gene *BRCA1* (MIM# 113705) was identified in 1994 by positional cloning in families with multiple cases of breast and ovarian cancer (13). The full-length *BRCA1* transcript includes 23 exons (22 coding exons) that encode a 1863 amino acid protein involved in multiple DNA repair pathways and cell-cycle checkpoint regulation (14). In addition to mRNA aberrations arising as a consequence of pathogenic germline mutations associated with high risk of cancer (15), several *BRCA1* alternative splicing isoforms have been described in the literature (13,16–27). Some of them, in particular those detected solely in tumor samples and/or cell lines (16,20,22), probably represent aberrant by-products of (or somatic alteration contributing to) tumorigenesis. However, others certainly represent 'naturally occurring' splicing isoforms, here defined as alternative splicing isoforms produced by wild-type alleles in non-malignant tissues. For instance, Δ(9,10), Δ11q and Δ(9,10,11q) have been described (together with the full length) as 'predominant' isoforms expressed in a wide variety of tissues (16,28). More recently, six BRCA1 transcripts (Δ5, Δ5q, Δ8p, Δ(9,10), Δ13p and Δ14p) have been reported to be 'consistently found in control samples' (29). The most comprehensive review published so far describes up to 21 *BRCA1* alternative splicing isoforms, albeit not all formally validated as 'naturally occurring' isoforms (16).

According to several reports, the relative expression levels of Δ(9,10), Δ11q and Δ(9,10,11q) are tissue specific, cell-cycle regulated and markedly altered in tumor samples, albeit conflicting results have been published (16). These observations suggest that *BRCA1* alternative splicing could play a role in certain cellular functions and might be involved in carcinogenesis (16). The Δ11q and Δ(9,10,11q) isoforms are remarkable in that they lack >50% of the full-length CDS, suggesting that the encoded proteins greatly differ from the full length in their biological activities. Apparently, engineered mice models supported specific roles for Δ11 (mice do not express Δ11q) during early embryogenesis (30–33). However, the later discovery of BRCA1-IRIS (18), a *BRCA1* locus product containing an open reading frame that extends from full-length start codon in exon 2 to the end of exon 11, continuing for 34 more triplets into intron 11 where it terminates, complicated the interpretation of previous data derived from mice models (28). Interestingly, mounting evidence indicates that BRCA1-IRIS, contrary to BRCA1 full length, has oncogenic-like activity (34). Despite these and other efforts, the physiological and pathological roles (if any) of *BRCA1* alternative splicing remain to be established.

An accurate description of 'naturally occurring' alternative splicing at the *BRCA1* locus is a prerequisite to understand its biological significance. In addition, it will become a valuable resource for the design and interpretation of *in vitro* splicing assays conducted to investigate the pathogenicity of germline genetic variants (15,26,35–37). To date, a systematic analysis of alternative splicing at the *BRCA1* locus is yet to be conducted. Here, we report a collaborative effort of the Evidence-based Network for the Interpretation of Germ-line Mutant Alleles (ENIGMA) consortium (38) conducted to comprehensively analyze *BRCA1* alternative splicing events occurring in four blood-related RNA sources, commonly used for clinical splicing assays, and one healthy breast tissue.

RESULTS

After conducting a four-stage project (Fig. 1), we have been able to annotate 63 independent *BRCA1* alternative splicing events (Tables 1–4) and Supplementary Material, Table S1), including 46 fully characterized by sequencing of the spliced junctions and 17 imputed from capillary electrophoresis (CE) alternative splicing models (Supplementary Material, Fig. S1–S3). Out of 63 events, 61 were observed in lymphoblastoid cell lines (LCLs), 53 in primary cultures of stimulated peripheral blood leukocytes (PBLs), 51 in whole blood leukocytes (LEUs), and 46 in ficoll-isolated peripheral blood mononuclear cells (PBMCs) (Supplementary Material, Table S2). The overlap was significant, with 39 events (62%) detected in all four RNA sources,

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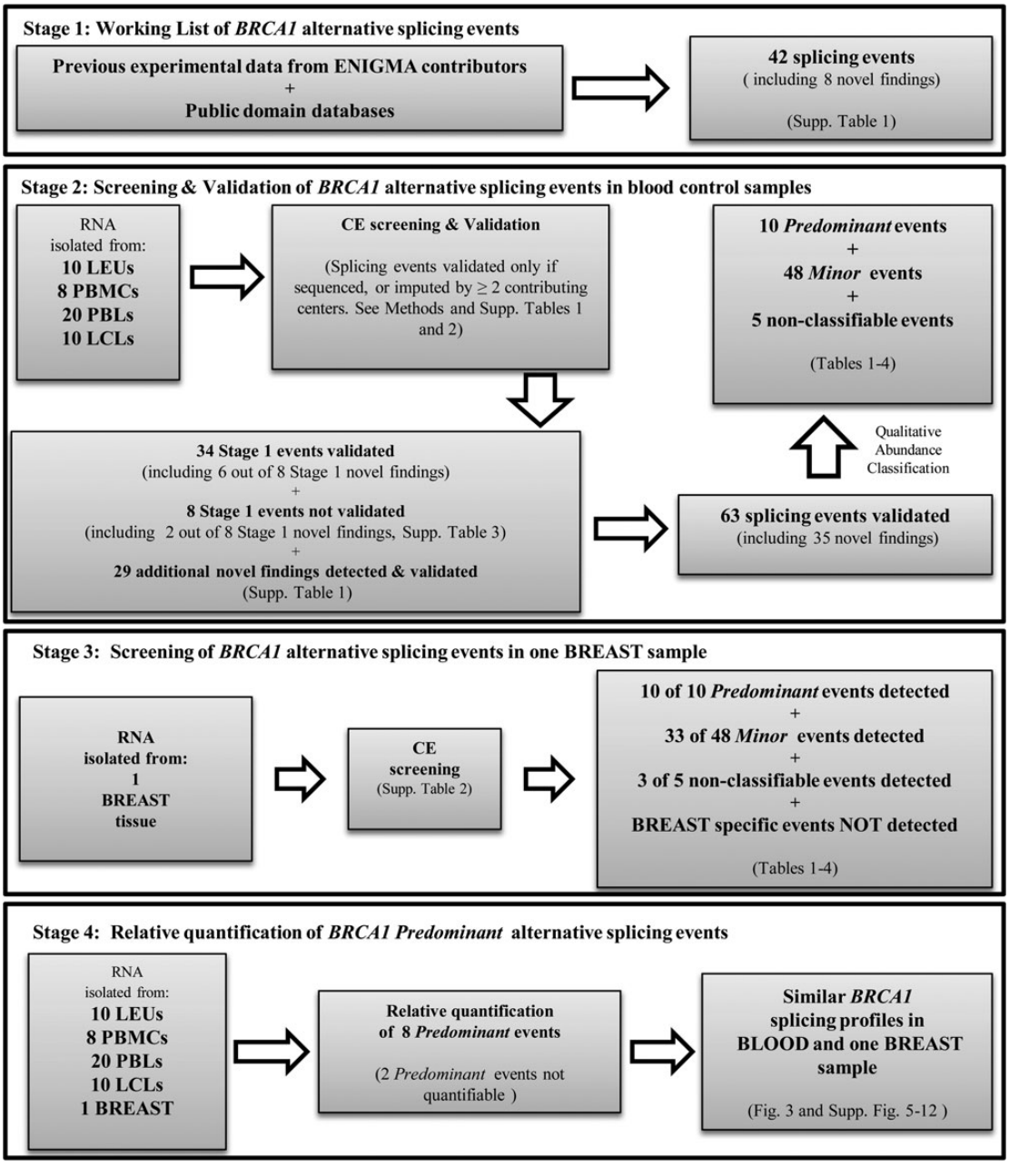

Figure 1. Workflow. We display the workflow of the four-stage study conducted by ENIGMA investigators in order to elucidate the complexity of alternative splicing at the *BRCA1* locus. Key findings have been incorporated into the figure. Note that two novel splicing events detected by ENIGMA contributors in Stage 1 were not validated in Stage 2 and have not been incorporated into the final list of 63 *BRCA1* alternative splicing events. CE, capillary electrophoresis.

and 51 events (81%) detected in at least three of them. Most discrepancies were best explained by low 'coverage' in low 'detection rate' events (see Materials and Methods and Supplementary Material, Table S2 online for further details).

We have classified these alternative splicing events into six basic structural biotypes: cassettes, multicassettes, splice donor shifts, splice acceptor shifts, terminal modifications and intronizations (Fig. 2). Splice donor shifts include the alternative use of proximal and distal sites at all *BRCA1* tandem acceptor (NAGNAG) sites (43), with the single notable exception of exon 6. The latter is probably explained by the local sequence context, as suggested by dedicated *in silico* analysis (see Supplementary Material, Fig. S4 for further details). Some splicing events are best described as mixed biotypes (Fig. 2 and Table 4). Most annotated events classify into (multi)-cassette biotypes ($N = 37$), including two inclusion events that introduce intron 3 and intron 13 genomic sequences into mature transcripts (Table 1). The former corresponds to the genomic sequence originally reported as *BRCA1* exon 4 (13), and later considered an intronic Alu element (27).

Functional annotation classifies *BRCA1* splicing events into: 23 PTC-NMDs (splicing events introducing PTCs predicted to

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Table 1. *BRCA1* alternative splicing events (cassette biotype)

Designation	RNA[a]	Status[b]	Functional annotation[c]	CDS[a]	Breast[d]	QA[e]	Previously described?[f] GENCODE[g]	Blood[h]	Others[i]
Δ2	r.-19_80del	Cloned	Non-Coding	–	Yes	Minor	–	–	BCCL (20)
Δ3	r.81_134del	Cloned	PTC-NMD[j]	–	Yes	Minor	006	LEU (17), PBMC (17,20,39)	BCCL (20)
▼4	r.134_135ins135−4047_135−3932	dir seq	PTC-NMD	–	Yes	Minor	002	–	NP (13)
Δ5	r.135 _212del	Cloned/ dir seq	No FS	p.Phe46_Arg71del	Yes	Predominant	004	LEU (13,17,25,40), LCLs(29)	NB (13)
Δ9	r.548_593del	dir seq	PTC-NMD	–	Yes	Minor	–	LEU (17), PBMC (22)	–
Δ10	r.594_670del	Cloned	PTC-NMD	–	Yes	Minor	–	LEU (17)	BCCL (20)
Δ11	r.671_4096del	Cloned	No FS	p.Ala224_Leu1365del	Yes	Minor	204	PBLs (41)	–
Δ13	r.4186_4357del	Cloned	PTC-NMD	–	–	Minor	–	–	–
▼13A	r.4357_4358ins4358−2785_4358−2719	Cloned	No FS	p.Lys1452_Ala1453ins22	Yes	Minor	005	LCLs (19,35)	NB (19)
Δ14	r.4358_c.4484del	Imputed	PTC-NMD	–	–	Minor	–	–	–
Δ15	r.4485_4675del	Cloned	PTC-NMD	–	–	Minor	–	LEU (17)	–
Δ17	r.4987_5074del	Cloned	PTC-NMD	–	–	Minor	–	LEU (17)	–
Δ18	r.5075_5152del	dir seq	No FS	p.Asp1692_Trp1718delinsGly	–	Minor	–	–	–
Δ20	r.5194_5277del	Imputed	No FS	p.His1732_Lys1759del	Yes	Minor	–	–	–
Δ21	r.5278_5332del	Cloned	PTC-NMD	–	Yes	Minor	–	LEU (17)	–
Δ22	r.5333_5406del	Cloned	FS-alternative STOP	p.Asp1778_Thr1802fs*32	Yes	Minor	007	–	–
Δ23	r.5407_5467del	Cloned/dir seq	FS-alternative STOP	p.Gly1803_Ala1823delfs*11	Yes	Minor	–	–	–

[a]According to HGVS guidelines (http://www.hgversusorg/mutnomen) last accessed December 2013. Nucleotide +1 corresponding to the A of the AUG translation initiation codon in the Ensemble reference transcript ENST00000357654. Ensemble reference protein ENSP00000350283.
[b]Events have been cloned and sequenced (cloned), directly sequenced from splicing assays (dir seq.) or imputed (see Materials and Methods).
[c]According to Mudge *et al*. (4) (see Materials and Methods for further details).
[d]Detected in normal breast tissue.
[e]Qualitative abundance (QA) based on visual inspection of splicing assays (see Materials and Methods for further details).
[f]We have excluded splicing events described as the outcome of germline mutation (i.e. for instance, Δ18 has been described previously as the outcome of various germline pathogenic mutations).
[g]GENCODE transcript IDs retrieved from Ensemble (if the corresponding splicing events is present in more than one transcript, the lowest ID number is shown).
[h]LEUs, PBMCs, PBLs, LCLs.
[i]Breast cancer cell lines (BCCLs), non-malignant placenta (NP). Non-malignant breast (NB).
[j]In-frame event generating a PTC at the splice junction.

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Table 2. *BRCA1* alternative splicing events (multicassette biotype)

Designation	RNA[a]	Status[b]	Functional annotation[c]	CDS[a]	Breast[d]	QA[e]	Previously described?[f] GENCODE[g]	Blood[h]	Others[i]
Δ2,3	r.-19_134del	Impute	Non-Coding	–	Yes	Minor	–	–	
Δ2_5	r.-19_217del	Impute	Non-Coding	–	Yes	Minor	–	–	
Δ2_10	r.-19_670del	dir seq.	Non-Coding	–	–	Minor	003	LCLs (24)	NB (24)
Δ8,9	r.442_593del	Impute	PTC-NMD	–	–	Minor	–	–	
Δ8_10	r.442_670del	Impute	PTC-NMD	–	Yes	Minor	–	–	
Δ9,10	r.548_670del	Cloned/dir seq	No FS	p.Gly183_Lys223del	Yes	Predominant	015	LEU (13,26),LCLs (29,42)	NB,NO, (13) BCCL (20)
Δ9_11	r.548_4096del	Cloned/dir seq	No FS	p.Ser184_Gly1366del	Yes	Predominant	203	LEU (17), LCL (24)	NB (13)
Δ9_12	r.548_4185del	Cloned	PTC-NMD	–	Yes	Minor	–	–	
Δ10,11	r.594_4096del	dir seq.	PTC-NMD	–	Yes	Minor	–	–	
Δ10_12	r.594_4185del	Cloned	PTC-NMD	–	Yes	Minor			
Δ11,12	r.671_4185del	Impute	PTC-NMD	–	Yes	Minor	–	–	
Δ14_15	r.4358_4675del	Cloned	No FS	p.Ala1453_Leu1558del	–	Minor	–	–	
Δ14_17	r.4358_5074del	Cloned	No FS	p.Ala1453_Thr1691del	–	Minor	202	LCLs (24)	NB (24)
Δ14_18	r.4358_5152del	Cloned	No FS	p.Ala1453_Trp1718delinsGly	–	Minor	205	LCLs (24)	NB (24)
Δ14_19	r.4358_5196del	Cloned	PTC-NMD	–	–	Minor	–	–	
Δ15_17	r.4485_5074del	Cloned	PTC-NMD	–	Yes	Minor	–	LEU (17), LCL (24)	NB (24)
Δ15_19	r.4485_5193del	Cloned	PTC-NMD	–	Yes	Minor	–	–	
Δ21,22	r.5278_5406del	Impute	No FS	p.Ile1760_Thr1802del	Yes	Minor	–	–	
Δ21_23	r.5278_5467del	Cloned	FS-alternative STOP	p.Ile1760_Ala1823delfs*11	Yes	Minor	–	–	
Δ22,23	r.5333_5467del	Impute	FS-alternative STOP	p.Asp1778_Ala1823delfs*11	Yes	Minor	–	–	

[a]According to HGVS guidelines (http://www.hgversusorg/mutnomen) last accessed December 2013. Nucleotide +1 corresponding to the A of the AUG translation initiation codon in the Ensemble reference transcript ENST00000357654. Ensemble reference protein ENSP00000350283.
[b]Events have been cloned and sequenced (cloned), directly sequenced from splicing assays (dir seq.) or imputed (see Materials and Methods).
[c]According to Mudge *et al*. (4) (see Materials and Methods for further details).
[d]Detected in normal breast tissue.
[e]Qualitative abundance (QA) based on visual inspection of splicing assays (see Materials and Methods for further details).
[f]We have excluded splicing events described as the outcome of germline mutation (i.e. for instance, Δ18 has been described previously as the outcome of various germline pathogenic mutations).
[g]GENCODE transcript IDs retrieved from Ensemble (if the corresponding splicing events is present in more than one transcript, the lowest ID number is shown).
[h]LEUs, PBMCs, PBLs, LCLs.
[i]Breast cancer cell lines (BCCLs), non-malignant ovarian (NO) and non-malignant breast (NB).

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Table 3. *BRCA1* alternative splicing events (miscellaneous biotypes)

Designation	RNA[a]	Status[b]	Functional annotation[c]	CDS[a]	Breast[d]	QA[e]	Previously described?[f] GENCODE[g]	Blood[h]	Others[i]
Splice acceptor shift									
Δ2p	r.-19_-7del	dir seq.	UTR	–	Yes	Minor	–	–	
Δ8p	r.442_444del	Cloned/ dir seq	No FS	p.Gln148del	Yes	Predominant	009	PBMC (13,20,29)	NB, NO (13)
Δ13p	r.4186_4188del	Cloned/ dir seq	No FS	p.Gln1396del	Yes	Predominant	–	LCLs (29)	
Δ14p	r.4358_4360del	Cloned/ dir seq	No FS	p.Ala1453del	Yes	Predominant	005	PBMC (13), LCLs (29)	NB (13)
Splice donor shifts									
Δ1Aq	r.-25_-20del	Cloned/ dir seq	UTR	–	Yes	Predominant	006	LCLs (21)	BCCL (20), BT (21)
▼1aA	r.-20_-19ins-20 + 1_-20 + 89	Cloned	UTR	–	Yes	Minor	010	–	
Δ5q	r.191_212del	Cloned/ dir seq	PTC-NMD	–	Yes	Predominant	010	PBMC (39), LEU (25), LCL (29)	
Δ11q	r.788_4096del	Cloned/ dir seq	No FS	p.Ser264_Gly1366del	Yes	Predominant	007	LCLs (24)	BCCL (20), NB (24)
Intronization									
11Δ3110	r.788_3897del	dir seq.	PTC-NMD	–	–	Minor	–	–	
11Δ3240	r.788_4027del	dir seq.	No FS	p.Gly263_Ser1342del	–	Minor	–	–	
Terminal modification									
(1B)		dir seq	UTR	–	Yes	–	–	–	BCCL,BT,OT,NB (23)
(IRIS)		dir seq	IntronicSTOP + polyA		Not tested	–	012	LCLs (18)	BCCL, BT (18)

[a]According to HGVS guidelines (http://www.hgversusorg/mutnomen) last accessed December 2013. Nucleotide +1 corresponding to the A of the AUG translation initiation codon in the Ensemble reference transcript ENST00000357654. Ensemble reference protein ENSP00000350283.
[b]Events have been cloned and sequenced (cloned), directly sequenced from splicing assays (dir seq.) or imputed (see Materials and Methods).
[c]According to Mudge *et al*. (4) (see Materials and Methods for further details).
[d]Detected in normal breast tissue.
[e]Qualitative abundance (QA) based on visual inspection of splicing assays (see Materials and Methods for further details).
[f]We have excluded splicing events described as the outcome of germline mutation (i.e. for instance, Δ18 has been described previously as the outcome of various germline pathogenic mutations).
[g]GENCODE transcript IDs retrieved from Ensemble (if the corresponding splicing events is present in more than one transcript, the lowest ID number is shown).
[h]LEUs, PBMCs, PBLs and LCLs.
[i]Breast cancer cell lines (BCCLs), non-malignant breast (NB), non-malignant ovarian (NO), breast tumor (BT) and ovarian tumor (OT).

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Table 4. *BRCA1* alternative splicing events (mixed biotypes)

Designation	RNA[a]	Status[b]	Functional annotation[c]	CDS[a]	Breast[d]	QA[e]	Previously described?[f] GENCODE[g]	Blood	Others
Splice donor shift + (multi)-cassette									
Δ1Aq,2	r.-25_80del	dir seq	Non-coding	–	Yes	Minor	–	–	–
Δ1Aq_3	r.-25_134del	Imputed	Non-coding	–	Yes	Minor	–	–	–
Δ1Aq_5	r.-25_217del	Imputed	Non-coding	–	Yes	Minor	–	–	–
Δ1Aq_10	r.-25_670del	dir seq	Non-coding	–	Yes	Minor	–	–	–
(Multi)-cassette + splice acceptor shift									
Δ10_13p	r.594_4188del	Imputed	PTC-NMD	–	Yes	Minor	–	–	–
Δ11_13p	r.671_4188del	Imputed	PTC-NMD	–	Yes	Minor	–	–	
Δ13_14p	r.4186_4360del	Imputed	PTC-NMD	–	–	Minor	–	–	–
▼13A,Δ14p	r.4357_4358ins4358−2785_4358−2719 + r.4358_4360del	Imputed	No FS	p.Ala1453delins22	Yes	Minor	005	–	–
Terminal modification + (multi)-cassette									
(1B),Δ2	r.-19_80del	dir seq	Non-coding	–	Yes	–	206	–	–
(1B),Δ2,3	r.-19_134del	dir seq	Non-coding	–	Yes	–	–	–	–
(1B),Δ2_5	r.-19_217del	Imputed	Non-coding	–	–	–	–	–	–
Multicassette + cassette									
Δ2,3,▼4	r.-19_134del+r.134_135ins135−4047_135−3932	Imputed	Non-coding	–	–	Minor	–	–	–
Splice donor shift + splice acceptor shift									
Δ1Aq,Δ2p	r.-25_-7del	cloned	UTR	–	Yes	Minor	–	–	–
Splice donor shift + multicassette + cassette									
Δ1Aq_3,▼4	r.-25_134del + r.134_135ins135−4047_135−3932	cloned	Non-coding	–	–	Minor	–	–	–

[a]According to HGVS guidelines (http://www.hgversusorg/mutnomen) last accessed December 2013. Nucleotide +1 corresponding to the A of the AUG translation initiation codon in the Ensemble reference transcript ENST00000357654. Ensemble reference protein ENSP00000350283.
[b]Events have been cloned and sequenced (cloned), directly sequenced from splicing assays (dir seq.) or imputed (see Materials and Methods).
[c]According to Mudge *et al*. (4) (see Materials and Methods for further details).
[d]Detected in normal breast tissue.
[e]Qualitative abundance (QA) based on visual inspection of splicing assays (see Materials and Methods for further details).
[f]We have excluded splicing events described as the outcome of germline mutation (i.e. for instance, Δ18 has been described previously as the outcome of various germline pathogenic mutations).
[g]GENCODE transcript IDs retrieved from Ensemble (if the corresponding splicing events is present in more than one transcript, the lowest ID number is shown).

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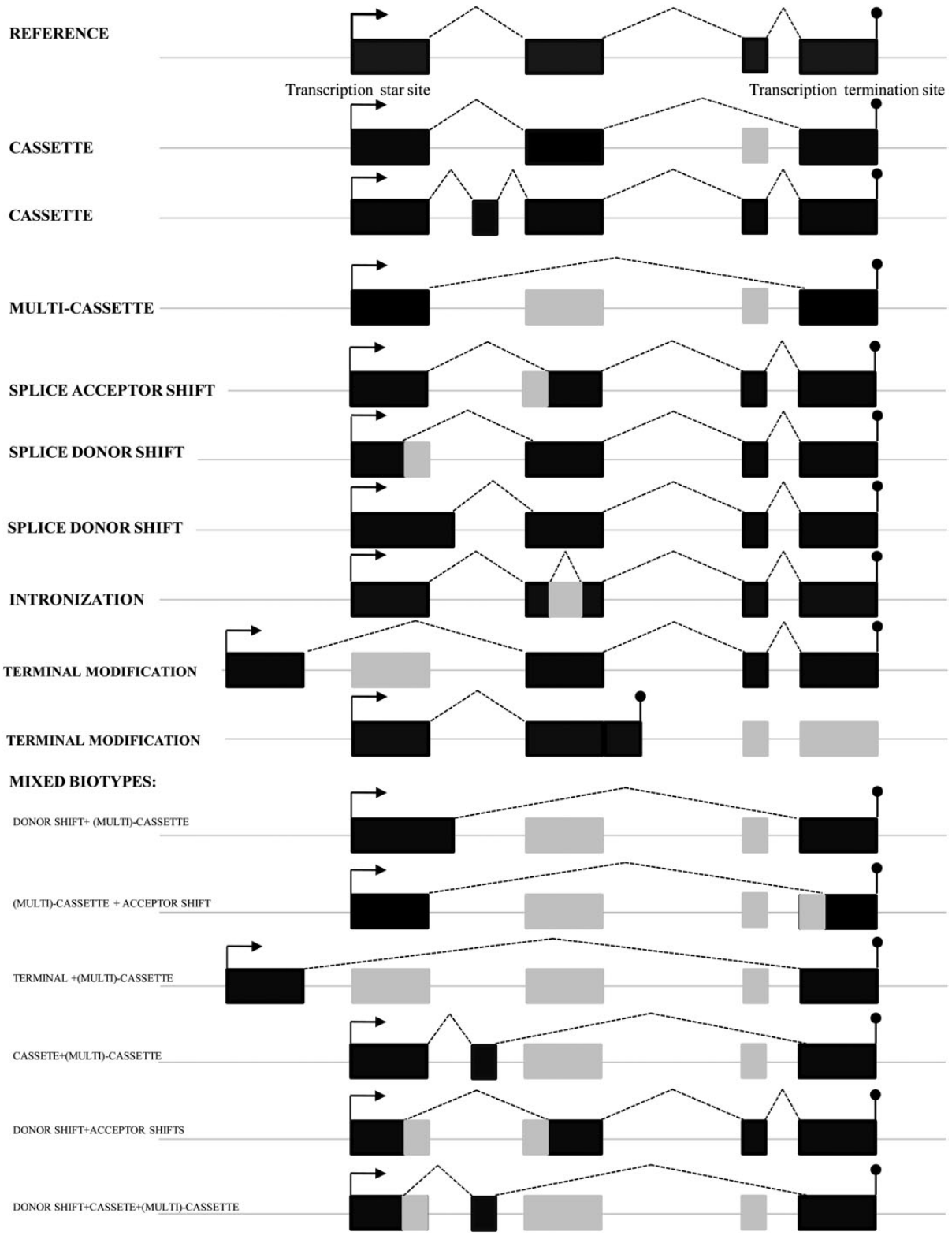

Figure 2. Splicing structural biotypes identified in the present study. For the sake of clarity, the figure represents conceptual schemes of splicing structural biotypes, not a description of the *BRCA1* locus itself. Exonic sequences are indicated by black boxes. Intronic sequences by gray lines (or boxes if exonic in the reference splicing pattern).

induce the Nonsense-Mediated RNA Decay pathway), 15 in-frames (ranging from subtle effects at NAGNAG sites to large deletions removing more than 50% of the reference CDS), 13 non-coding (eliminating the full-length start codon), 5 UTRs (splicing events modifying UnTranslated regions), 4 frame-shifts generating PTCs not predicted to induce NMD

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(PTCs located in exon 24) and 1 internal PTC with polyadenylation (IRIS) (Tables 1–4).

Splicing 'assays' were developed to detect *BRCA1* splicing events, not to address quantitative aspects. Yet, visual inspection allowed us to identify 10 'predominant BRCA1' splicing events: Δ1Aq, Δ5, Δ5q, Δ8p, Δ9, Δ(9,10), Δ9_11, Δ11q, Δ13p and Δ14p, eight of which were later analyzed (Stage 4) by semi-quantitative CE (not feasible in the case of Δ9_11 and Δ11q, see Materials and Methods for further details).

We performed a comprehensive screening of *BRCA1* alternative splicing events in one healthy breast tissue sample (BREAST). Remarkably, most splicing events previously identified in blood samples (43 out of 63) were detected, despite the lower 'coverage'. Those not detected tended to be low 'detection rate' events in blood derived samples (see Supplementary Material, Table S2 for further details). Equally relevant, our analysis did not identify any splicing events that had not been detected previously in blood. Visual inspection of saturating PCR assays detected the very same 10 'predominant' splicing events previously identified in blood-related samples.

Semi-quantitative CE indicated obvious differences among 'predominant' events both in blood and breast tissue (Fig. 3). While some events represented roughly 5% of the full-length signal (Δ5, Δ5q, Δ9, Δ13p), others represented up to 30% (Δ8p, Δ(9,10), Δ14p). Finally, we observed similar levels of Δ1Aq and full-length transcripts. Very similar splicing patterns were observed when analyzing LEU, PBMC, PBL and LCL samples separately (see Supplementary Material, Figs S5–S12). Although semi-quantitative profiling of all *BRCA1* splicing events described here is beyond the scope of the present study, there are some notable observations in relation with Stage 2 'detection rate' (Supplementary Material, Table S2). The average 'detection rate' of Stage 2 splicing events is 46%, but there is a clear distinction between the average 'detection rates' of 'predominant' versus 'minor' events (86 versus 39%). Furthermore, the 'detection' rate reaches 100% in four 'predominant' events representing $\geq$30% of the full-length signal (Δ1Aq, Δ8p, Δ(9,10), Δ14p), but decreases to 72% (range 62–87%) for those representing only 5% of the full-length signal (Δ5, Δ5q, Δ9, Δ13p). Taken together, these observations suggest that, in our experimental setting, the 'detection rate' of an individual splicing event (Supplementary Material, Table S2) is related with the actual expression level of that particular event.

Interestingly, CE analysis allowed us to identify peaks imputed to transcripts combining two or more independent splicing events. For instance, 7–11q assays (Supplementary Material, Fig. S1A) demonstrated the existence of RNA species combining Δ8p with Δ9, Δ10 and Δ(9,10) events. Similarly, 12–14 assays (Supplementary Material, Fig. S1B) demonstrated the existence of RNA species containing Δ13,▼13A, Δ13p and Δ14p events in almost all possible combinations, with the only exception being that RNA species combining Δ13 with ▼13A were not observed. Further supporting this scenario, the analysis of 7–12 assays revealed a high diversity of transcripts combining Δ8p, Δ9, Δ10, Δ(9,10), Δ11 and Δ11q splicing events (Supplementary Material, Fig. S3), including the detection of transcripts combining Δ(9,10) with Δ11q. The latter, for the sake of consistence annotated as (Δ9,10 + Δ11q) in Supplementary Material, Figure S3, but often referred to as Δ(9,10,11q) in the literature, is one of few *BRCA1* splicing isoforms described previously as predominant (16). 'Detection rate' of (Δ9,10 + Δ11q) reached 100% in Stage 2 (data not shown), further supporting a link between 'detection rate' and actual expression level.

Note that, overall, the CE signal corresponding to transcripts containing multiple splicing events is consistently lower than that of the transcripts containing the corresponding individual events, as expected from a random combination of independent elements (see several examples in Supplementary Material, Figs S1 and S3).

DISCUSSION

We have combined reverse transcription polymerase chain reaction (RT-PCR), CE, cloning and conventional sequencing to describe naturally occurring *BRCA1* alternative splicing with unprecedented resolution. To our knowledge, 34 out of the 63 splicing events reported here are novel findings, whereas only 22 events have been described previously in blood samples (see Tables 1–4 for further details). However, we have not been able to validate up to eight *BRCA1* splicing events previously reported by others (including two Stage 1 events reported by contributors of the present manuscript). While it is likely that some of these events do not qualify for 'naturally occurring' events, the data suggest that characterizing the full complexity of *BRCA1* splicing will require further studies (see Supplementary Material, Table S3 for further details). This is also suggested by the fact that we have identified several signals compatible with additional splicing events that, nonetheless, we have not been able to annotate (see Supplementary Material, Figs S1 and S3).

Overall, our data indicate that most naturally occurring *BRCA1* splicing events are rather minor if compared with the full-length signal. However, we have identified 10 'predominant' splicing events that appear to represent a substantial fraction of the full-length expression using semi-quantitative measures. Not surprisingly, all 10 'predominant' events have been described previously. Indeed, six of them (Δ5, Δ5q, Δ8p, Δ(9,10), Δ13p and Δ14p) have been described recently as *BRCA1* splicing events 'consistently found in control samples' (29).

Genome-wide analyses suggest that cassette events (30–50% of all splicing events) are the commonest alternative splicing structural biotypes observed in mammals (4,5,10). In this regard, the human *BRCA1* gene can be described as typical, since 37 out of the 63 (58%) splicing events here reported are cassette like. Remarkably, all *BRCA1* internal exons are involved in one or more cassette events so that, formally speaking, *BRCA1* lacks constitutive exons. Yet, with few exceptions (Δ5, Δ9, Δ(9,10), Δ9_11) cassette events are rather "minor events", so that most internal exons are best described as 'quasi-constitutive exons'. The number of splice site shifts ($N = 9$) is much lower, but 6 out of 10 "predominant" events correspond to this biotype. Remarkably, we have not identified structural biotypes such as mutually exclusive cassette exons or retained introns (5,10). Yet, we have identified two intronization events (Fig. 2). This would appear to be a rare structural biotype, since it is not included in a comprehensive catalog of structural biotypes (68 different biotypes) identified in human, mouse and several non-mammal vertebrates (4). Perhaps, intronization events occurring in vertebrates are associated with exceptionally

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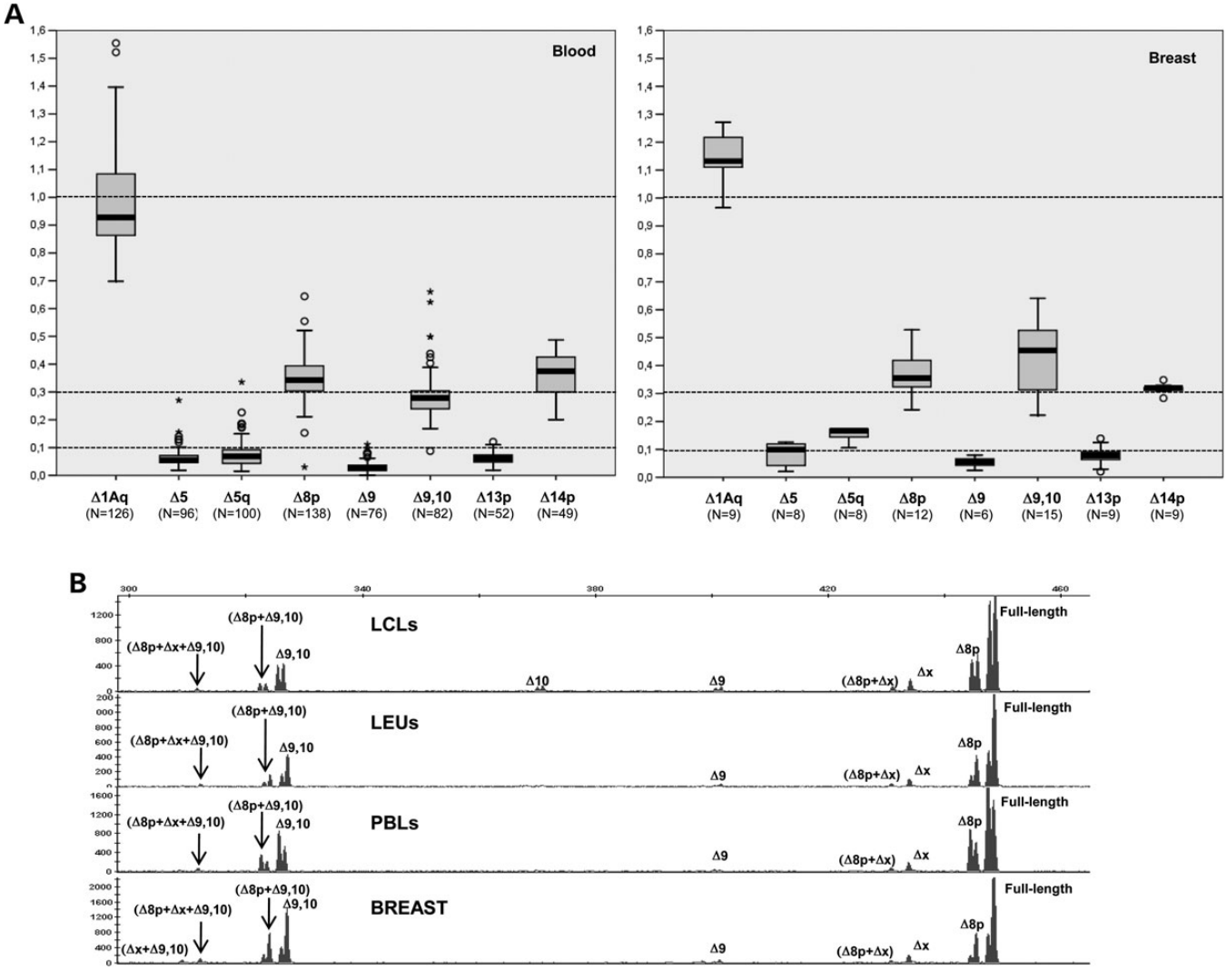

Figure 3. *BRCA1* splicing events in blood and breast tissues. (**A**) The boxplots (low, Q1, median, Q3 and high values are displayed) show the expression level of eight predominant *BRCA1* alternative splicing events relative to the full length. Relative expression level was measured by semi-quantitative CE (see Materials and Methods). BLOOD displays LEUs, PBMCs, PBMCs and LCLs data pooled together (different control samples plus technical replicates). *N* indicates the number of individual data points (different control samples plus technical replicates). BREAST displays data from one BREAST sample. In this case, *N* equals the number of technical replicates performed. Normal outliers (>1.5 inter quartile range, IQR) display a small circle. Extreme outliers (>3 IQR) display an asterisk. (**B**) Representative examples of 7–11q CE assays performed with four different RNA sources. Overall, the analysis suggests that *BRCA1* alternative splicing is similar, regardless of the RNA source analyzed. Differences are restricted to the presence/absence of 'minor' events. More important, replica experiments show that differences are not RNA source (or sample) specific, but rather the results of stochastic preferential amplification of 'minor' isoforms. Peaks representing a combination of two independent splicing events (SE) are annotated as (SE1 + SE2).

long exons, such as human *BRCA1* exon 11 (3426 bp versus an average exon length of ~180 bp in the human genome).

The spectrum of possible splicing events occurring at a single locus is so wide that any attempt to catalog it will inevitably be biased by the analytical approach employed. In our experience, CE analysis of RT-PCR products is very sensitive for detecting minor events, subtle size-effects and multicassette events (37). However, minor events involving long (≥1000 bp) intron retentions (if any) will usually escape detection, as expected product sizes are out of range of CE. Furthermore, our analytical approach does not allow discovery of novel terminal events which are not formally splicing events, but are nonetheless reported as major contributors to exon variability in mRNAs (3,5). In this regard, we have limited our study to analyze the expression of two previously reported terminal events (Exon1B and IRIS) by dedicated assays.

We have shown previously that semi-quantitative CE is able to detect splicing quantitative trait loci (sQTLs) such as rs1799965, a rare SNP [minor allele frequency (MAF) < 0.001] that is associated with an increase in expression of *BRCA1* Δ9,10 (26), and rs9534262, a common SNP (MAF > 0.40) associated with an increase in *BRCA2* Δ17,18 (44). In the present study, we have not been able to detect interindividual variability, suggesting that this is below technical replica variability. Since we have analyzed alternative splicing in a relative large number of control samples ($N = 48$), our data suggest that common sQTLs at the *BRCA1* gene (if any) are likely to induce more subtle effects than those reported for rs1799965 (c.591C > T) and rs9534262 (c.7806–14T > C).

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It is important to point out that we have produced a catalog of alternative splicing events, which may not represent a catalog of RNA isoforms (we have not cloned individual mRNAs). At present, we cannot rule out the possibility that certain splicing events tend to occur together (linked splicing model), so that the actual number of *BRCA1* mRNA isoforms might be lower than the number of splicing events reported here. Yet, overall our data favor an unlinked splicing model in which most, if not all, non-mutually exclusive alternative splicing events are randomly combined into individual mRNA molecules to potentially produce hundreds of different *BRCA1* isoforms. According to GENCODE v7, human protein-coding loci express on average 6.31 alternatively spliced transcripts (10), and those loci with >20 annotated isoforms are very rare (3). Therefore, our analysis raises the interesting possibility of *BRCA1* being a locus with particularly high levels of alternative splicing. However, global estimations of alternative splicing levels are based on genome-wide RNA-seq efforts that may underestimate the true level of alternative splicing. At least, this is supported by targeted RNA-seq experiments that identify hundreds of previously unannotated isoforms in even extensively studied protein-coding loci such as *TP53* and *HOX* (45). If proven true, the finding that *BRCA1* is a locus with a high level of alternative splicing would be consistent with recent genome-wide analyses connecting high-level alternative splicing loci with intrinsically disordered proteins/domains (IDPs/IDDs), IDPs/IDDs with Hub proteins and Hub proteins with disease (46–49). *TP53* represents a paradigm of this association (45,47,48). Similar to *TP53*, *BRCA1* is a disease associated genetic locus coding for an IDP/IDD protein with Hub properties (47). Accordingly, high-level alternative splicing would indeed be an expected feature of the *BRCA1* locus. Remarkably, CE splicing analysis at the *BRCA2* locus (a gene fairly similar to *BRCA1* in terms of overall size and exon/intron structure, but coding for a protein that lacks IDDs and/or Hub features) reveals a much lower extent of alternative splicing (ENIGMA consortium internal data).

Regardless of its biological significance, we believe that the comprehensive description of *BRCA1* alternative splicing reported here will be highly relevant for diagnosis, in particular when assessing the impact of *BRCA1* germline variants on splicing. Recently, the ENIGMA consortium conducted a multicentre investigation aimed at comparing *in vitro* splicing assay protocols and elaborating best practice guidelines (37). The study addressed analytical aspects such as primers design, reverse transcriptase protocols, NMD inhibition and detection methods, and identified primers design (positioning primers) as a major source of variability across laboratories. The study concluded that *a prior* knowledge of the expected transcripts (naturally occurring alternative splicing isoforms) was a key factor for proper primers design and clinical assessment (37). Previous studies have identified as well alternative splicing as a critical aspect to be considered in the design and analysis of *BRCA1 in vitro* splicing assays (26). In this regard, the catalog of splicing events here identified will be a valuable tool to improve the design (primers can be strategically positioned to include or exclude specific splicing events in function of the position of the variants under scrutiny) and analysis (at both qualitative and quantitative level) of future *BRCA1 in vitro* splicing assays, thus improving the clinical interpretation of the outcomes. In turn, this will facilitate the integration of *BRCA1 in vitro* splicing assays into the multifactorial likelihood models that are developed by the ENIGMA consortium to assess the clinical relevance of genetic variants (38).

Despite its comprehensiveness, the abovementioned ENIGMA study comparing *in vitro* splicing assays protocols was conducted in RNAs isolated from LCLs, so that did not evaluate the impact of using other blood-related RNA sources. Yet, LEUs or PBLs are common RNA sources for *in vitro* splicing assays in genetic testing laboratories worldwide (15). In the present study, we have shown that *BRCA1* alternative splicing is similar in four different blood RNA sources (LEUs, PBLs, PBMCs and LCLs), suggesting that the actual blood-related RNA source used for assessing the role of *BRCA1* germline variants on splicing is unlikely to represent a major contributor to variability of results. Further on, our data suggest that *BRCA1* alternative splicing is similar in blood and breast tissues, supporting that *in vitro* splicing assays performed in blood are relevant for diagnosis.

Although the biological relevance of *BRCA1* alternative splicing is largely unknown, the precise knowledge of the different splicing events will be instrumental for the definition of its functional (and clinical) relevance. Individual *BRCA1* mRNA isoforms can be monitored more closely in future splicing assays (preferably including accurate quantification), and the functional relevance of their putatively encoded proteins can be further evaluated by *in vitro* transfection of the corresponding cDNA constructs to rescue gene expression, as recently shown for *BRCA1* missense variants (50), and two *BRCA1* alternative splicing isoforms (17,51).

Finally, we believe that CE scanning, as here conducted for *BRCA1* analysis, is a feasible approach to develop accurate catalogs of locus-specific alternative splicing events that can assist the analysis and validation of data from targeted RNAseq experiments.

MATERIALS AND METHODS

Samples

We have analyzed *BRCA1* alternative splicing in RNA samples from healthy control individuals. RNA was isolated from whole blood LEUs, ficoll-isolated PBMCs, primary cultures of stimulated PBLs and LCLs. In addition, RNA was isolated from an epithelial enriched area of one healthy breast tissue obtained after cosmetic surgery (BREAST). In Stage 1 (see workflow in Fig. 1) different contributing laboratories used different isolation protocols and/or cDNA synthesis strategies, as described in a recent ENIGMA paper (37). A full description of RNA isolation and cDNA synthesis protocols used in Stages 2 and 3 (see workflow in Fig. 1) is provided in Supplementary methods. The study was approved by the Institutional Review Board of each participating center.

Identification, validation and relative quantification of 'naturally occurring' *BRCA1* alternative splicing events

For the purpose of this study, we define alternative splicing events as those incorporating splice junctions not present in the reference transcript Ensemble ENST00000357654 (hereafter referred as full-length transcript). The only exception is

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BRCA1-IRIS (see Introduction), a locus product for which no specific splice junction exists (18). Multiple combinations of forward and reverse primers located at exonic regions (as defined by the full-length transcript) were used to amplify cDNAs. A PCR performed with a particular combination of primers will be referred throughout the text as a *BRCA1* splicing 'assay'. We conducted a four-stage project as follows (see workflow in Fig. 1).

In Stage 1, contributing centers used their own control samples (blood related) and 'assays' to identify alternative splicing events at the *BRCA1* gene. All Stage 1 primers are available upon request. At this stage, splicing 'assays' were analyzed by EtBr stained agarose gel electrophoresis, CE and/or direct sequencing, depending on the contributing center. Both confirmed (sequenced) and predicted (size-matching) events were considered. In addition, we performed a comprehensive review of the literature in order to identify all *BRCA1* splicing events previously described, including 'naturally occurring' events, but also splicing events not formally validated as such (like those solely detected in tumor samples and/or cell lines). Stage 1 experimental and review data were pooled together to elaborate a working list of 42 *BRCA1* alternative splicing events (see Supplementary Material, Table S1).

In Stage 2, Stage 1 information was used to develop a panel of 18 overlapping 'assays' (all primer sequences are provided as Supplementary Material) that allowed a comprehensive scanning of *BRCA1* splicing events by CE (see Supplementary Material, Figs S1–S3 and Table S1). Thermal cycling consisted of an initial 10-min hold at 95°C, followed by 30-s hold at 95°C, 30-s hold at 58°C and 30-s hold at 72°C (increased to 2 min for exon11 containing assays) for 45 cycles to maximize sensitivity. Stage 2 screening was performed in 48 healthy control samples of European ancestry, including 10 LEUs, 8 PBMCs, 20 PBLs and 10 LCLs. Several centers contributed samples at this stage, but actual screening was centralized in one laboratory. CE analyses were performed in a 3130 Genetic Analyzer (Applied Biosystems) with GeneScan 500/1200 Size Standards (Applied Biosystems) as internal markers. Size-calling was performed with GeneMapper v4.0 Software (Applied Biosystems). Some splicing events were captured by one 'assay', while others were captured by two or more overlapping 'assays' (Supplementary Material, Table S1). Stage 2 centralized screening involved a total of 4281 CE data points (one data point defined as each technical replica of an individual splicing event assayed in one sample). 'Coverage' (defined here as data points per splicing event) ranged from 18× to 163× (67× on average). 'Detection Rate' (% of positive data points) ranged from 3 to 100%. By far, the highest 'coverage' was obtained in PBLs samples, with 2055 data points (see Supplementary Material, Table S2 for further details). None of the 'assays' listed in Supplementary Material, Table S1 allowed BRCA1-IRIS detection. For that purpose, we developed a dedicated assay that does not rely on CE analysis (see Supplementary Material). Stage 2 allowed us not only to validate 34 out of 42 Stage 1 events in a cohort of control samples but also to validate 29 additional splicing events. For the purpose of this study, we have validated findings only if sequenced, or imputed by two or more contributing centers with different primer sets.

Visual inspection of CE assays (or EtBr agarose stained gels in the case of exon 11 containing assays) revealed that most splicing events were easily classified into two categories according to their signal relative to the full-length transcript, hereafter refereed as 'predominant' and 'minor' events. BRCA1-IRIS and exon1B transcripts were not classifiable because the full-length reference transcript was not co-amplified in the corresponding assays. Later, splicing events classified as 'predominant' were further characterized by semi-quantitative CE assays (see below).

In Stage 3, screening of *BRCA1* splicing events was performed in one normal breast sample (BREAST). 'Assays' and CE protocols were as in Stage 2, although 'coverage' was much lower (see Supplementary Material, Table S2).

Finally, in Stage 4 we investigated the expression level relative to the full length in eight alternative splicing events previously annotated as 'predominant'. With this aim, LEUs, PBMCs, PBLs, LCLs and BREAST samples were reanalyzed with four splicing 'assays' (E1–E6, E7–E11q, E12–E13 and E12–E14) performed in semi-quantitative (33 PCR cycles) conditions (semi-quantitative CE). Relative quantification of individual splicing events was expressed as the average ratio between the peak area of that particular event and the peak area of the full-length signal (Fig. 3 and Supplementary Material, Figs S5–S12). Semi-quantitative CE analysis of two 'predominant' events (Δ9_11 and Δ11q) was not feasible because of the large-size difference (>3300 bp) between spliced and full-length products.

Splicing events designation

We have designated *BRCA1* exons following the Breast Core Informative database nomenclature (52), so that the 22 coding exons of the reference full-length transcript are numbered from 2 to 24 with no exon 4 defined (13). We have designated splicing events combining the following symbols: Δ (skipping), ▼ (retention), p (proximal) and q (distal). In addition, we have also used non-systematic designations previously established in the scientific literature, including IRIS, exon1A, exon1aA, exon1B, exon4 and exon13A (13,18,19,22,23).

Splice junction sequencing

Depending on the particular splicing event investigated (and/or contributing center), different approaches were followed. Direct sequencing of individual 'assays' (sometimes with internal primers at selected locations) allowed us to sequence 25 events; including 'predominant' and 'minor' events (see Tables 1–4). The latter was possible thanks to stochastic preferential amplification of 'minor' events observed in 45-cycle RT-PCR assays (an illustrative example is shown in Supplementary Material, Fig. S2). Alternatively, agarose or polyacrylamide gel excised splicing assay products were cloned into the pGEM-T vector (Promega) and sequenced. Cloning allowed us to sequence 32 events (see Tables 1–4). All sequence reactions were performed using the ABI PRISM® BigDye™ Terminator Cycle Sequencing kit (Applied Biosystems) and examined with an ABI 3130 Genetic Analyzer (Applied Biosystems), using the Sequencing Analysis software (Applied Biosystems).

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Imputation of splicing events

For this purpose, we elaborated alternative splicing models that best explained the peak pattern observed in CE analyses. Imputations were performed combining CE size-calling data, sequencing findings and GENCODE annotations retrieved through the Ensemble Genome Browser (http://www.ensembl.org, last accessed December 2013). As a rule, we imputed splicing events only if compatible with the use of canonical splice sites (GT-AG) present in the *BRCA1* reference genomic sequence GRCh37:17:41195712:41322890:-1 (http://www.ensembl.org, last accessed December 2013). The approach allowed us to annotate *BRCA1* splicing events not supported by direct sequencing evidence (referred throughout the text as imputed events). Imputation was also used to deduce the existence of transcripts combining multiple splicing events. Two representative examples of *BRCA1* splicing models are shown in Supplementary Material, Figure S1.

Structural and functional annotation of alternative splicing events

Structural and functional annotation has been performed as in Mudge *et al.* (4), although we incorporated an additional structural biotype referred throughout the text as intronization. First described in *Caenorhabditis* species (53), intronization refers to the conversion of a single exon into two exons and one intervening intron (see Fig. 2). Functional annotation of *BRCA1* splicing events includes 'non-coding' (splicing events eliminating the full-length start codon), 'PTC-NMDs' (splicing events introducing PTCs predicted to induce the nonsense-mediated RNA decay pathway), 'No-FS' (in-frame splicing events), 'FS-alternative STOP' (frame-shift events generating PTCs not predicted to induce NMD as they are located in the most downstream *BRCA1* exon), 'UTRs' (splicing events modifying UnTranslated regions) and one internal PTC with polyadenylation (IRIS).

Identification of *BRCA1* alternative splicing events in public domain databases

Studies published up to June 2013 that contained data from *BRCA1* splicing assays were identified by carrying out literature searches using the LOVD database (http://chromium.liacs.nl/LOVD2/cancer/home), PubMed (http://www.ncbi.nlm.nih.gov/pubmed) and Google Scholar (http://scholar.google.com), using the following keywords: BRCA1, BRCA2 and splicing. Each report was reviewed in detail to extract the following data: splicing events detected (excluding those directly attributed to germline pathogenic mutations) and RNA source. The data, together with information retrieved from Ensembl (BRCA1 transcripts), have been incorporated into Tables 1–4 and Supplementary Material, Tables S1 and S3.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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REFERENCES

1. Wang, E.T., Sandberg, R., Luo, S., Khrebtukova, I., Zhang, L., Mayr, C., Kingsmore, S.F., Schroth, G.P. and Burge, C.B. (2008) Alternative isoform regulation in human tissue transcriptomes. *Nature*, **456**, 470–476.
2. Chen, M. and Manley, J.L. (2009) Mechanisms of alternative splicing regulation: insights from molecular and genomics approaches. *Nat. Rev. Mol. Cell Biol.*, **10**, 741–754.
3. Djebali, S., Davis, C.A., Merkel, A., Dobin, A., Lassmann, T., Mortazavi, A., Tanzer, A., Lagarde, J., Lin, W., Schlesinger, F. *et al.* (2012) Landscape of transcription in human cells. *Nature*, **489**, 101–108.
4. Mudge, J.M., Frankish, A., Fernandez-Banet, J., Alioto, T., Derrien, T., Howald, C., Reymond, A., Guigó, R., Hubbard, T. and Harrow, J. (2011) The origins, evolution, and functional potential of alternative splicing in vertebrates. *Mol. Biol. Evol.*, **28**, 2949–2959.
5. Blencowe, B.J. (2006) Alternative splicing: new insights from global analyses. *Cell*, **126**, 37–47.
6. Kim, H., Klein, R., Majewski, J. and Ott, J. (2004) Estimating rates of alternative splicing in mammals and invertebrates. *Nat. Genet.*, **36**, 915–916.
7. Kim, E., Magen, A. and Ast, G. (2007) Different levels of alternative splicing among eukaryotes. *Nucleic Acids Res.*, **35**, 125–131.
8. Skandalis, A., Frampton, M., Seger, J. and Richards, M.H. (2010) The adaptive significance of unproductive alternative splicing in primates. *RNA*, **16**, 2014–2022.

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9. Tress, M.L., Martelli, P.L., Frankish, A., Reeves, G.A., Wesselink, J.J., Yeats, C., Olason, P.I., Albrecht, M., Hegyi, H., Giorgetti, A. *et al.* (2007) The implications of alternative splicing in the ENCODE protein complement. *Proc. Natl. Acad. Sci. USA*, **104**, 5495–5500.
10. Frankish, A., Mudge, J.M., Thomas, M. and Harrow, J. (2012) The importance of identifying alternative splicing in vertebrate genome annotation. *Database (Oxford)*, **2012**, bas014.
11. Lewis, B.P., Green, R.E. and Brenner, S.E. (2003) Evidence for the widespread coupling of alternative splicing and nonsense-mediated mRNA decay in humans. *Proc. Natl. Acad. Sci. USA*, **100**, 189–192.
12. Melamud, E. and Moult, J. (2009) Stochastic noise in splicing machinery. *Nucl. Acids Res.*, **37**, 4873–4886.
13. Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P.A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L.M. and Ding, W. (1994) A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science*, **266**, 66–71.
14. Roy, R., Chun, J. and Powell, S.N. (2012) BRCA1 and BRCA2: different roles in a common pathway of genome protection. *Nat. Rev. Cancer*, **12**, 68–78.
15. Walker, L.C., Whiley, P.J., Houdayer, C., Hansen, T.V.O., Vega, A., Santamarina, M., Blanco, A., Fachal, L., Southey, M.C., Lafferty, A. *et al.* (2013) Evaluation of a 5-tier scheme proposed for classification of sequence variants using bioinformatic and splicing assay data: inter-reviewer variability and promotion of minimum reporting guidelines. *Hum. Mutat.*, **34**, 1424–1431.
16. Orban, T.I. and Olah, E. (2003) Emerging roles of BRCA1 alternative splicing. Mol. Pathol., **56**, 191–197.
17. Sevcik, J., Falk, M., Kleiblova, P., Lhota, F., Stefancikova, L., Janatova, M., Weiterova, L., Lukasova, E., Kozubek, S., Pohlreich, P. *et al.* (2012) The BRCA1 alternative splicing variant Δ14–15 with an in-frame deletion of part of the regulatory serine-containing domain (SCD) impairs the DNA repair capacity in MCF-7 cells. *Cell Signal.*, **24**, 1023–1030.
18. ElShamy, W.M. and Livingston, D.M. (2004) Identification of BRCA1-IRIS, a BRCA1 locus product. *Nat. Cell Biol.*, **6**, 954–967.
19. Fortin, J., Moisan, A.M., Dumont, M., Leblanc, G., Labrie, Y., Durocher, F., Bessette, P., Bridge, P., Chiquette, J., Laframboise, R. *et al.* (2005) A new alternative splice variant of BRCA1 containing an additional in-frame exon. *Biochim. Biophys. Acta*, **1731**, 57–65.
20. Lixia, M., Zhijian, C., Chao, S., Chaojiang, G. and Congyi, Z. (2007) Alternative splicing of breast cancer associated gene BRCA1 from breast cancer cell line. *J. Biochem. Mol. Biol.*, **40**, 15–21.
21. Fetzer, S., Tworek, H.A., Piver, M.S. and Dicioccio, R.A. (1998) An alternative splice site junction in exon 1a of the BRCA1 gene. *Cancer Genet. Cytogenet.*, **105**, 90–92.
22. Gu, M., Li, H., Shen, C., Wu, L., Liu, W., Miao, L. and Zheng, C. (2010) Cloning and characterization of a new BRCA1 variant: a role for BRCT domains in apoptosis. *Cancer Lett.*, **295**, 205–213.
23. Xu, C.F., Brown, M.A., Chambers, J.A., Griffiths, B., Nicolai, H. and Solomon, E. (1995) Distinct transcription start sites generate two forms of BRCA1 mRNA. *Hum. Mol. Genet.*, **4**, 2259–2264.
24. Xu, C.F., Chambers, J.A., Nicolai, H., Brown, M.A., Hujeirat, Y., Mohammed, S., Hodgson, S., Kelsell, D.P., Spurr, N.K., Bishop, D.T. *et al.* (1997) Mutations and alternative splicing of the BRCA1 gene in UK breast/ovarian cancer families. *Genes Chromosomes Cancer*, **18**, 102–110.
25. Claes, K., Vandesompele, J., Poppe, B., Dahan, K., Coene, I., De Paepe, A. and Messiaen, L. (2002) Pathological splice mutations outside the invariant AG/GT splice sites of BRCA1 exon 5 increase alternative transcript levels in the 5′ end of the BRCA1 gene. *Oncogene*, **21**, 4171–4175.
26. Dosil, V., Tosar, A., Cañadas, C., Pérez-Segura, P., Díaz-Rubio, E., Caldés, T. and de la Hoya, M. (2010) Alternative splicing and molecular characterization of splice site variants: BRCA1 c.591C>T as a case study. *Clin. Chem.*, **56**, 53–61.
27. Friedman, L.S., Szabo, C.I., Ostermeyer, E.A., Dowd, P., Butler, L., Park, T., Lee, M.K., Goode, E.L., Rowell, S.E. and King, M.C. (1995) Novel inherited mutations and variable expressivity of BRCA1 alleles, including the founder mutation 185delAG in Ashkenazi Jewish families. *Am. J. Hum. Genet.*, **57**, 1284–1297.
28. Tammaro, C., Raponi, M., Wilson, D.I. and Baralle, D. (2012) BRCA1 exon 11 alternative splicing, multiple functions and the association with cancer. *Biochem. Soc. Trans.*, **40**, 768–772.
29. Houdayer, C., Caux-Moncoutier, V., Krieger, S., Barrois, M., Bonnet, F., Bourdon, V., Bronner, M., Buisson, M., Coulet, F., Gaildrat, P. *et al.* (2012) Guidelines for splicing analysis in molecular diagnosis derived from a set of 327 combined in silico/in vitro studies on BRCA1 and BRCA2 variants. *Hum. Mutat.*, **33**, 1228–1238.
30. Gowen, L.C., Johnson, B.L., Latour, A.M., Sulik, K.K. and Koller, B.H. (1996) Brca1 deficiency results in early embryonic lethality characterized by neuroepithelial abnormalities. *Nat. Genet.*, **12**, 191–194.
31. Hakem, R., de la Pompa, J.L., Sirard, C., Mo, R., Woo, M., Hakem, A., Wakeham, A., Potter, J., Reitmair, A., Billia, F. *et al.* (1996) The tumor suppressor gene Brca1 is required for embryonic cellular proliferation in the mouse. *Cell*, **85**, 1009–1023.
32. Ludwig, T., Chapman, D.L., Papaioannou, V.E. and Efstratiadis, A. (1997) Targeted mutations of breast cancer susceptibility gene homologs in mice: lethal phenotypes of Brca1, Brca2, Brca1/Brca2, Brca1/p53, and Brca2/p53 nullizygous embryos. *Genes Dev.*, **11**, 1226–1241.
33. Hakem, R., de la Pompa, J.L., Elia, A., Potter, J. and Mak, T.W. (1997) Partial rescue of Brca1 (5–6) early embryonic lethality by p53 or p21 null mutation. *Nat. Genet.*, **16**, 298–302.
34. Shimizu, Y., Luk, H., Horio, D., Miron, P., Griswold, M., Iglehart, D., Hernandez, B., Killeen, J. and ElShamy, W.M. (2012) BRCA1-IRIS overexpression promotes formation of aggressive breast cancers. *PLoS ONE*, **7**, e34102.
35. Claes, K., Poppe, B., Machackova, E., Coene, I., Foretova, L., De Paepe, A. and Messiaen, L. (2003) Differentiating pathogenic mutations from polymorphic alterations in the splice sites of BRCA1 and BRCA2. *Genes Chromosomes Cancer*, **37**, 314–320.
36. Spurdle, A.B., Couch, F.J., Hogervorst, F.B.L., Radice, P. and Sinilnikova, O.M. and IARC Unclassified Genetic Variants Working Group. (2008) Prediction and assessment of splicing alterations: implications for clinical testing. *Hum. Mutat.*, **29**, 1304–1313.
37. Whiley, P., de la Hoya, M., Thomassen, M., Becker, A., Brandão, R., Pedersen, I.S., Montagna, M., Menéndez, M., Quiles, F., Enríquez, S.G. *et al.* (2014) Comparison of mRNA splicing assay protocols across multiple laboratories: recommendations for best practice in standardized clinical testing. *Clin. Chem.*, **60**, 341–352.
38. Spurdle, A.B., Healey, S., Devereau, A., Hogervorst, F.B.L., Monteiro, A.N.A., Nathanson, K.L., Radice, P., Stoppa-Lyonnet, D., Tavtigian, S., Wappenschmidt, B. *et al.* (2012) ENIGMA – evidence-based network for the interpretation of germline mutant alleles: an international initiative to evaluate risk and clinical significance associated with sequence variation in BRCA1 and BRCA2 genes. *Hum. Mutat.*, **33**, 2–7.
39. Munnes, M., Zuther, I., Schmitz, B. and Doerfler, W. (2000) A Novel Insertional Mutation and Differentially Spliced mRNAs in the Human BRCA1 Gene. *Gene Funct Dis*, **1**, 38–47.
40. Bonnet, C., Krieger, S., Vezain, M., Rousselin, A., Tournier, I., Martins, A., Berthet, P., Chevrier, A., Dugast, C., Layet, V. *et al.* (2008) Screening BRCA1 and BRCA2 unclassified variants for splicing mutations using reverse transcription PCR on patient RNA and an ex vivo assay based on a splicing reporter minigene. *J. Med. Genet.*, **45**, 438–446.
41. Brandão, R.D., van Roozendaal, K., Tserpelis, D., Gómez García, E. and Blok, M.J. (2011) Characterisation of unclassified variants in the BRCA1/2 genes with a putative effect on splicing. *Breast Cancer Res. Treat.*, **129**, 971–982.
42. Pyne, M.T., Pruss, D., Ward, B.E. and Scholl, T. (1999) A characterization of genetic variants in BRCA1 intron 8 identifies a mutation and a polymorphism. *Mutat. Res.*, **406**, 101–107.
43. Hiller, M., Huse, K., Szafranski, K., Jahn, N., Hampe, J., Schreiber, S., Backofen, R. and Platzer, M. (2004) Widespread occurrence of alternative splicing at NAGNAG acceptors contributes to proteome plasticity. *Nat. Genet.*, **36**, 1255–1257.
44. De Garibay, G.R., Acedo, A., García-Casado, Z., Gutiérrez-Enríquez, S., Tosar, A., Romero, A., Garre, P., Llort, G., Thomassen, M., Díez, O. *et al.* (2014) Capillary electrophoresis analysis of conventional splicing assays: IARC analytical and clinical classification of 31 BRCA2 genetic variants. *Hum. Mutat.*, **35**, 53–57.
45. Mercer, T.R., Gerhardt, D.J., Dinger, M.E., Crawford, J., Trapnell, C., Jeddeloh, J.A., Mattick, J.S. and Rinn, J.L. (2012) Targeted RNA sequencing reveals the deep complexity of the human transcriptome. *Nat. Biotechnol.*, **30**, 99–104.
46. Buljan, M., Chalancon, G., Eustermann, S., Wagner, G.P., Fuxreiter, M., Bateman, A. and Babu, M.M. (2012) Tissue-specific splicing of disordered segments that embed binding motifs rewires protein interaction networks. *Mol. Cell*, **46**, 871–883.

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47. Cortese, M.S., Uversky, V.N. and Dunker, A.K. (2008) Intrinsic disorder in scaffold proteins: getting more from less. *Prog. Biophys. Mol. Biol.*, **98**, 85–106.
48. Uversky, V.N., Oldfield, C.J., Midic, U., Xie, H., Xue, B., Vucetic, S., Iakoucheva, L.M., Obradovic, Z. and Dunker, A.K. (2009) Unfoldomics of human diseases: linking protein intrinsic disorder with diseases. *BMC Genomics*, **10** (Suppl. 1), S7.
49. Kornblihtt, A.R., Schor, I.E., Alló, M., Dujardin, G., Petrillo, E. and Muñoz, M.J. (2013) Alternative splicing: a pivotal step between eukaryotic transcription and translation. *Nat. Rev. Mol. Cell Biol.*, **14**, 153–165.
50. Bouwman, P., van der Gulden, H., van der Heijden, I., Drost, R., Klijn, C.N., Prasetyanti, P., Pieterse, M., Wientjens, E., Seibler, J., Hogervorst, F.B. *et al.* (2013) A high-throughput functional complementation assay for classification of BRCA1 missense variants. *Cancer Discov.*, **3**, 42–55.
51. Sevcik, J., Falk, M., Macurek, L., Kleiblova, P., Lhota, F., Hojny, J., Stefancikova, L., Janatova, M., Bartek, J., Stribrna, J. *et al.* (2013) Expression of human BRCA1Δ17–19 alternative splicing variant with a truncated BRCT domain in MCF-7 cells results in impaired assembly of DNA repair complexes and aberrant DNA damage response. *Cell Signal.*, **25**, 1186–1193.
52. Szabo, C., Masiello, A., Ryan, J.F. and Brody, L.C. (2000) The breast cancer information core: database design, structure, and scope. *Hum. Mutat.*, **16**, 123–131.
53. Irimia, M., Rukov, J.L., Penny, D., Vinther, J., Garcia-Fernandez, J. and Roy, S.W. (2008) Origin of introns by 'intronization' of exonic sequences. *Trends Genet.*, **24**, 378–381.

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