Supplemental Material for:

Long-read DNA and cDNA sequencing identifies cancer-predisposing deep intronic variation in tumor suppressor genes

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Supplemental Methods

Adaptive sampling sequencing. DNA samples sequenced by adaptive sampling were barcoded with combinations of the 24 adapters from SQK-NBD114.24 (Oxford Nanopore Technologies), as outlined in **Supplemental Table S1**. During adaptive sampling, 1MB regions around 10 breast and ovarian cancer genes were targeted with a FASTA file of 10 million bases defined by the GRCh38/hg38 regions in **Supplemental Table S2**. A zipped FASTA file is available upon request.

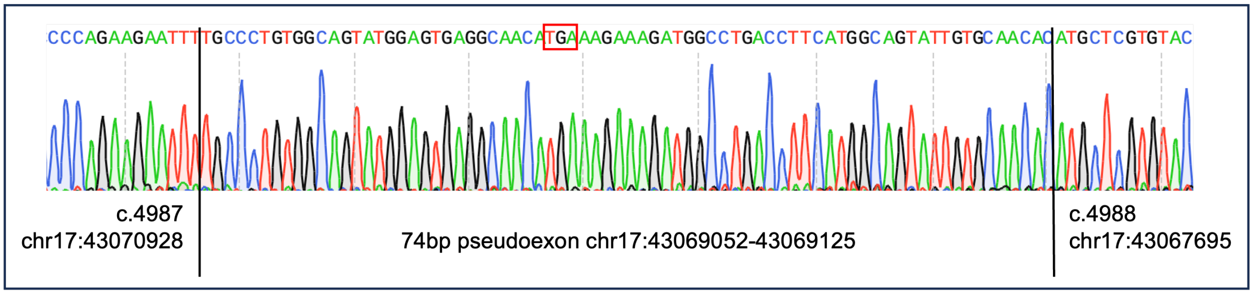
Intronic variants were defined as those >30bp from a canonical splice site. Rare variants were defined as those with ≤3 entries on gnomAD non-cancer v.3.1.2 for *BRCA1, BRCA2, PALB2, BARD1, BRIP1, RAD51C, RAD51D, TP53;* and ≤10 entries on gnomAD non-cancer v.3.1.2 for *ATM* or *CHEK2*. Rare deep intronic variants in the ten breast cancer genes in participants from severely affected families are indicated in **Supplemental Table S3**.

Gene-specific cDNA preparation. First-strand cDNA was generated with a 2uM pool of RT primers for each of the ten breast and ovarian cancer genes (**Supplementary Table S4**). Each oligo was designed to bind in the 3'UTR of its target gene, 5' of polyA signal sites and avoiding known common SNPs.

Confirmation of pseudoexon splice junctions. Long-read cDNA-derived alternate splicing events (pseudoexons) were validated by RT-PCR and Sanger sequencing. 1ug of total RNA was reverse transcribed with qScript (Quantabio) using random hexamers (Invitrogen). Following cDNA synthesis, 1ul of template was PCR-amplified with primers flanking the splice event, and PCR products were purified and Sanger sequenced as described in **Supplemental Figures S1 and S2**.

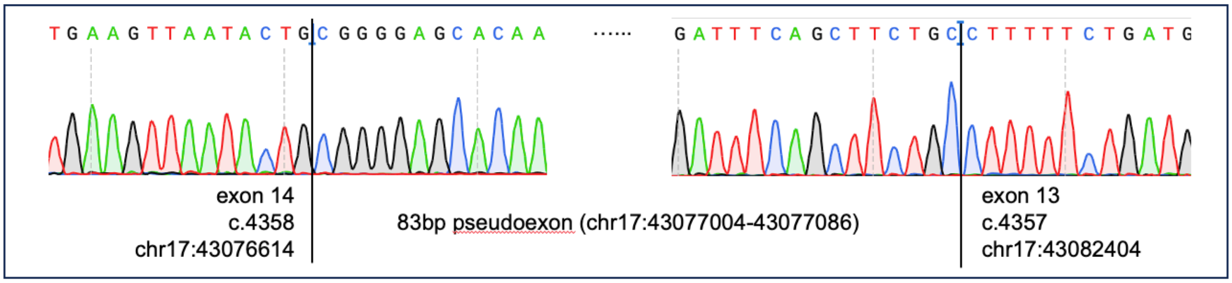
Supplementary Figure S1. Confirmation by RT-PCR and Sanger sequencing of pseudoexon splice events and junctions. All coordinates are hg38.

(**A**) *BRCA1 c.4987-1352A>G* (chr17:43,069,047 T>C) in families CF3679 and CF6196.



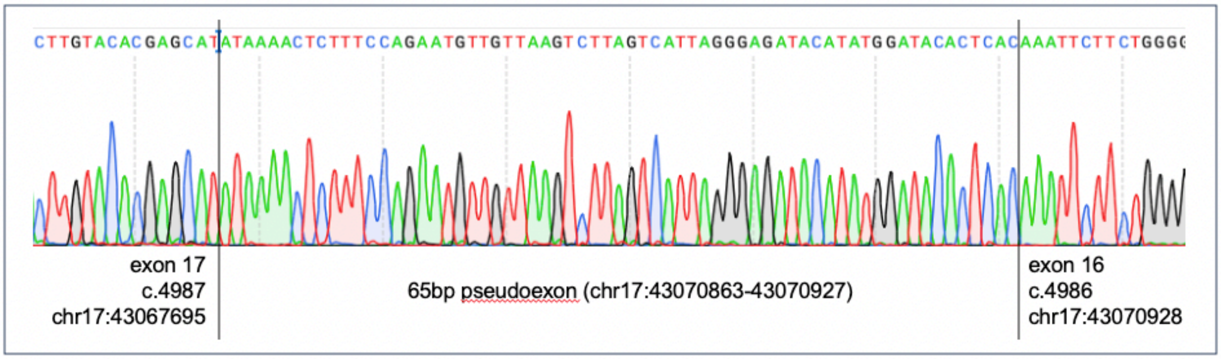
The electropherogram above illustrates the transcribed 74bp pseudoexon in *BRCA1*. Random-hexamer-primed cDNA generated from participant whole blood RNA was amplified with *BRCA1* primers: Forward, TCAGAAAAAGCAGTATTAACTTCA at c.4461–c.4484; Reverse, GGTCACCCAGAAATAGCTAAC at c.5273-c.5253 (NM_007294.4). PCR products were purified and Sanger sequenced. The pseudoexon introduces a stop at codon 1673.

(**B**) *BRCA1* c.4358-473T>G (chr17:43,077,087 A>C) in family CF4358.



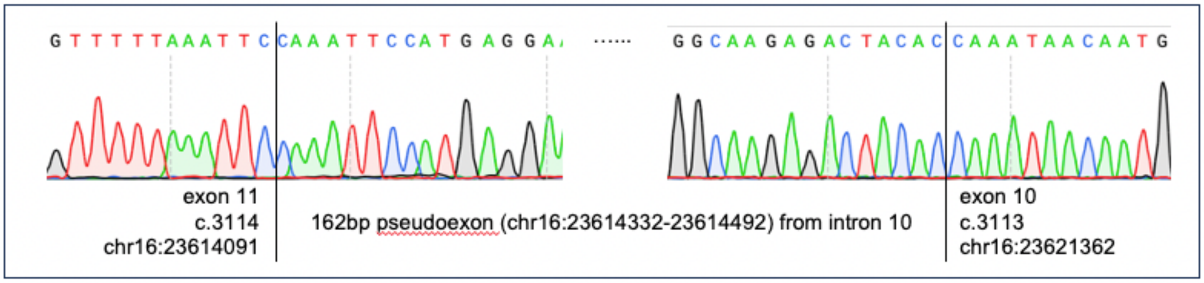
The electropherogram above illustrates the transcribed 83bp pseudoexon in *BRCA1*. Random hexamer-primed cDNA generated from participant whole blood RNA was amplified with *BRCA1* primers: Forward, GATTCAAACTTAGGTGAAGCAG at c.4197–c.4218; Reverse, TGTACACGAGCATAAATTCTTC at c.5112-c.5091 (NM_007294.4). PCR products were purified, and Sanger sequenced. The pseudoexon introduces three new codons followed by a stop after residue 1452.

(**C**) *BRCA1* c.4986+69G>A (chr17:43,070,858 C>T) in family CF4455.



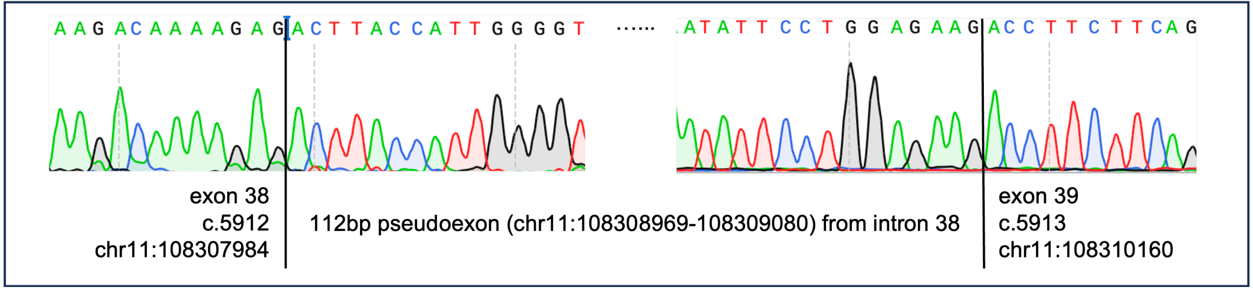
The electropherogram above illustrates the transcribed 65bp pseudoexon in *BRCA1*. Random hexamer-primed cDNA generated from participant whole blood RNA was amplified with BRCA1 primers: Forward, AAGGTCATCCCCTTCTAAAT at c.4595–c.4614; Reverse, TGTACACGAGCATAAATTCTTC at c.5198-c.5177 (NM_007294.4). PCR products were purified, and Sanger sequenced. The pseudoexon introduces 13 new codons followed by a stop after residue 1662.

(**D**) *PALB2* c.3114-239A>T (chr16:23614330 T>A) in family CF3302.



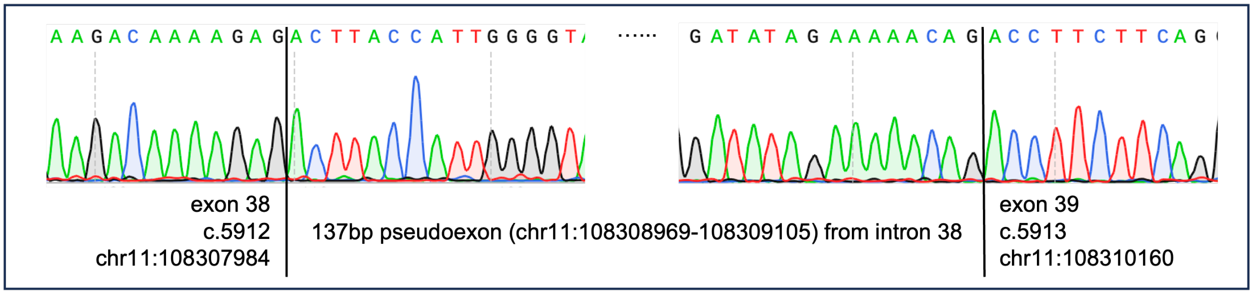
The electropherogram above illustrates the transcribed 162bp pseudoexon in *PALB2*. Random hexamer-primed cDNA generated from participant whole blood RNA was amplified with PALB2 primers: Forward, AGAGAGATCAGGGCATTGTTTT at c.2977–c.2998; Reverse, CTTCCAGGAACCTGCCAG at c.3514-c.3497 (NM_024675.4). PCR products were purified, and Sanger sequenced. The pseudoexon introduces 20 new codons followed by a stop after residue 1038

(**E**) *ATM c.5763-1080A>G* (chr11:108309080 A>G) in family CF5431.



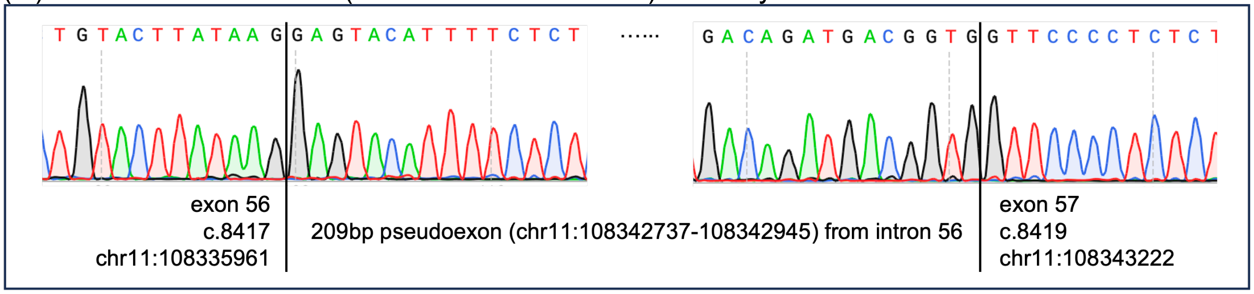
The electropherogram above illustrates the flanking splice junctions of the transcribed 112bp pseudoexon between *ATM* exons 38 and 39. Random-hexamer-primed cDNA generated from participant whole blood RNA was amplified with *ATM* primers: Forward, TAGAAGATTGTGTCAAAGTTCG at c.5318-c.5339, spanning exons 34/35; Reverse, GCAAGACTTCTTTTCTCTTGAT at c.6077-c.6056, spanning exons 39/40 (NM_000051.4). PCR products were purified and Sanger sequenced. The pseudoexon introduces a stop at codon 1929.

(**F**) *ATM c.5763-1056A>G* (chr11:108309104 G>A) in family CF6072.

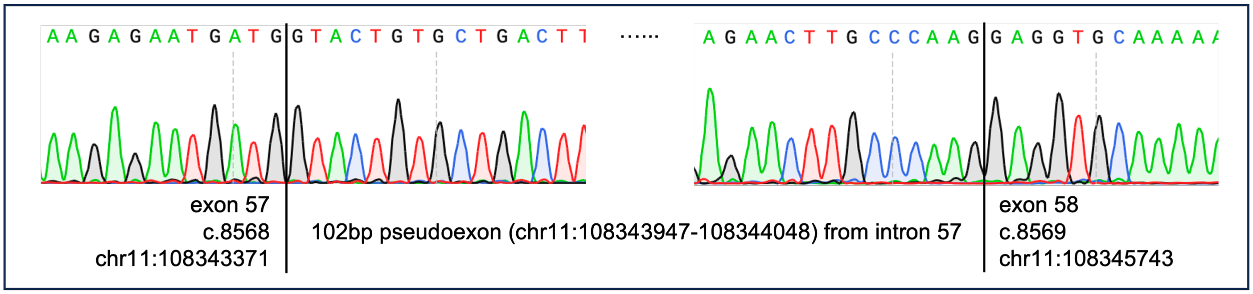


The electropherogram above illustrates the flanking splice junctions of the transcribed 137bp pseudoexon between *ATM* exons 38 and 39. Random-hexamer-primed cDNA generated from participant whole blood RNA was amplified with *ATM* primers: Forward, TAGAAGATTGTGTCAAAGTTCG at c.5318-c.5339, spanning exons 34/35; Reverse, GCAAGACTTCTTTTCTCTTGAT at c.6077-c.6056, spanning exons 39/40 (NM_000051.4). PCR products were purified, and Sanger sequenced. The pseudoexon introduces a premature stop at codon 1929 (the same as in family CF5431).

(**G**) *ATM c.8418+704G>T* (chr11:108344075 G>T) in family CF6132.



Transcript 1. The electropherogram above illustrates the flanking splice junctions of the transcribed 209bp pseudoexon between ATM exons 56 and 57. Random-hexamer-primed cDNA generated from participant whole blood RNA was amplified with *ATM* primers: Forward, AAATTAAGGTGGACCACACA at c.8153-c.8172, spanning exons 54/55; Reverse, TGTTCAAAAGCAACACCTAGA at c.8837-c.8817, spanning exons 59/60 (NM_000051.4). PCR products were purified and Sanger sequenced. The pseudoexon introduces a premature stop at codon 2762.



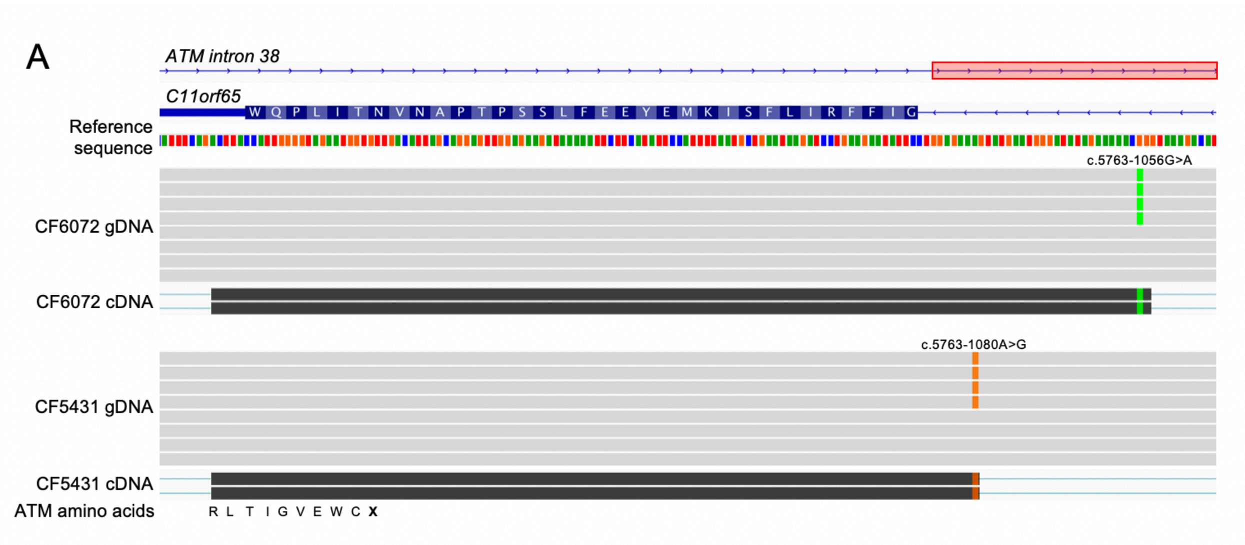
Transcript 2. The electropherogram above illustrates the flanking splice junctions of the transcribed 102bp pseudoexon between *ATM* exons 57 and 58. Random-hexamer-primed cDNA generated from participant whole blood RNA was amplified with *ATM* primers: Forward,

AAATTAAGGTGGACCACACA at c.8153-c.8172, spanning exons 54/55; Reverse, TGTTCAAAAGCAACACCTAGA at c.8837-c.8817, spanning exons 59/60 (NM_000051.4). PCR products were purified and Sanger sequenced. The pseudoexon introduces a premature stop at codon 2809.

Transcript 3. *ATM* mutant Transcript 3 includes the pseudoexon of Transcript 1 and inclusion of all of intron 57. Transcript 3 was rare but apparent from long-read cDNA sequencing, but RT-PCR and Sanger sequencing of the same cDNA samples did not reveal any copies of it. This transcript includes the premature stop at codon 2762.

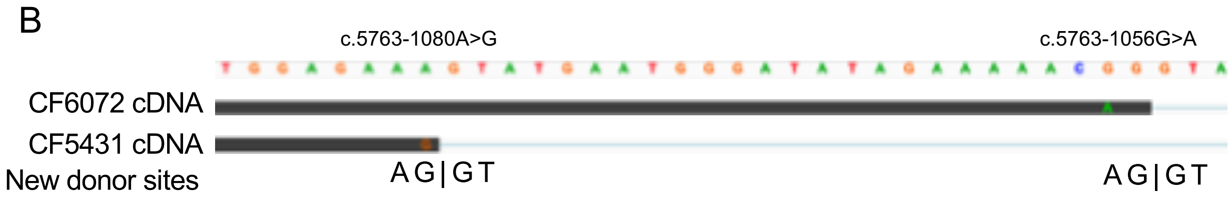
Supplemental Figure S2. *ATM* pseudoexons in families CF6072 and CF5431 in a cryptic splicing hotspot

(A) Pseudoexons in *ATM* exon 38 created by *ATM c.5763-1056G>A* in family CF6072 and *ATM c.5763-1080A>G* in family CF4531



As shown above, two variants at positions -1056 (green highlight) and -1080 (red highlight) in *ATM* intron 38 yield pseudoexons with different cryptic donors, but with the same cryptic acceptor. The red bar at the top of the figure indicates a 35bp cryptic donor hotspot predicted by SpliceAI. The hotspot is coincident with the AG-rich polypyrimidine tract in intron 6 of *C11orf65*. Pseudoexonification is possible because in addition to this feature, the 3'UTR of *C11orf65* includes a sequence that on the *ATM* strand is a near perfect acceptor motif (*TCATTCATTTCAG,* Chr11:108,308,956-108,308,968).

(**B**) Enlargement of the 3' portions of the pseudoexons shown in (**A**), indicating the basepairs of the revealed cryptic donor splice sites.


B
c.5763-1080A>G
c.5763-1056G>A
T G G A G A A A G T A T G A A T G G G A T A T A G A A A A A C G G G T A
CF6072 cDNA
CF5431 cDNA
New donor sites
AG|GT
AG|GT