

Accurate classification of *BRCA1* variants with saturation genome editing

Gregory M. Findlay¹, Riza M. Daza¹, Beth Martin¹, Melissa D. Zhang¹, Anh P. Leith¹, Molly Gasperini¹, Joseph D. Janizek¹, Xingfan Huang¹, Lea M. Starita^{1,2*} & Jay Shendure^{1,2,3*}

Variants of uncertain significance fundamentally limit the clinical utility of genetic information. The challenge they pose is epitomized by *BRCA1*, a tumour suppressor gene in which germline loss-of-function variants predispose women to breast and ovarian cancer. Although *BRCA1* has been sequenced in millions of women, the risk associated with most newly observed variants cannot be definitively assigned. Here we use saturation genome editing to assay 96.5% of all possible single-nucleotide variants (SNVs) in 13 exons that encode functionally critical domains of *BRCA1*. Functional effects for nearly 4,000 SNVs are bimodally distributed and almost perfectly concordant with established assessments of pathogenicity. Over 400 non-functional missense SNVs are identified, as well as around 300 SNVs that disrupt expression. We predict that these results will be immediately useful for the clinical interpretation of *BRCA1* variants, and that this approach can be extended to overcome the challenge of variants of uncertain significance in additional clinically actionable genes.

Our ability to predict the phenotypic consequences of an arbitrary genetic variant in a human genome remains poor. This problem is evidenced by the large numbers of variants of uncertain significance (VUS) identified in 'actionable' genes, that is, genes in which the definitive identification of a pathogenic variant would alter clinical management¹. For example, heterozygous germline variants that disrupt *BRCA1* markedly increase the risk of early-onset breast and ovarian cancer^{2,3} and are actionable, as more frequent screening or prophylactic surgery can lead to improved outcomes^{4,5}. Clinical sequencing can identify specific variants as risk-conferring⁶. However, as of January 2018, most *BRCA1* SNVs are classified as VUS⁷. VUS are typified by rare missense SNVs, but also include variants potentially affecting messenger RNA (mRNA) levels. Further illustrating the challenge associated with VUS, there are hundreds of *BRCA1* SNVs that have received conflicting interpretations⁷.

There are two main approaches for resolving VUS. The first approach, data sharing, relies on the expectation that as *BRCA1* is sequenced in more individuals, the recurrent observation of a variant in individuals who either have or have not developed cancer will enable its interpretation. However, given that the majority of potential variants in *BRCA1* are extremely rare and that the phenotype is incompletely penetrant, it is unclear whether sufficient numbers of humans will ever be sequenced to accurately quantify cancer risk for each possible variant.

The second approach, functional assessment, has spurred the development of diverse in vitro assays for *BRCA1*⁸. As the homology-directed DNA repair (HDR) function of *BRCA1* is key for tumour suppression, one commonly used assay measures whether expression of a *BRCA1* variant can rescue HDR integrity^{9,10}. Other *BRCA1* assays evaluate embryonic stem cell viability¹¹, transcriptional activation¹², drug sensitivity¹¹, protein-protein interaction^{9,13} or splicing^{14,15}. Computational predictions based on features such as conservation can be informative but are insufficiently accurate to be used in the absence of genetic or experimental evidence¹⁶.

Experimental assessments of *BRCA1* variants have been limited in several ways. First, they are typically performed post hoc and have not kept pace with the discovery of VUS. Second, assays expressing variants

as cDNA-based transgenes removed from their genomic context^{9,13} fail to assess the effects on splicing or transcript stability, and risk artefacts of overexpression¹⁷. Genome editing provides a potential means to overcome these challenges, but has yet to be applied to characterize any appreciable number of VUS in *BRCA1* or other genes similarly linked to cancer predisposition.

Here we set out to apply genome editing to measure the functional consequences of all possible SNVs in key regions of *BRCA1*, regardless of whether they have been previously observed in a human. Given the large size of *BRCA1*, we prioritized 13 exons that encode the RING and BRCT domains, which critically underlie its role as a tumour suppressor^{18–20}. In addition to around 400 VUS or variants with conflicting interpretations, all 21 *BRCA1* missense SNVs classified by a ClinVar-approved expert panel as pathogenic reside in these exons⁷, as do missense and splice variants shown to disrupt *BRCA1* in functional assays^{11,21} (ClinVar is a widely used database of clinical variant interpretations submitted by clinical testing laboratories). In each experiment, a single exon is subjected to saturation genome editing (SGE)²², wherein all possible SNVs are simultaneously introduced and concurrently assayed. We used SGE to measure functional effects for 3,893 SNVs, comprising 96.5% of all possible SNVs in the targeted exons. These scores are bimodally distributed and nearly perfectly concordant with expert-based assessments of pathogenicity. We predict that our functional classifications will be of immediate clinical utility, and that scaling this approach to additional genes will substantially enhance the utility of genetic testing.

Saturation genome editing of *BRCA1* exons

Many genes in the HDR pathway, including the hereditary cancer predisposition genes *BRCA1*, *BRCA2*, *PALB2* and *BARD1*⁶, have been deemed essential in the human haploid cell line HAP1²³ (Fig. 1a). To confirm this, we transfected HAP1 cells with a plasmid co-expressing Cas9 and guide RNAs (gRNAs) targeting each of these genes²⁴. High cell death was evident by light microscopy, and a luminescence-based survival assay established that targeting any of these genes substantially reduces HAP1 viability (Extended Data Fig. 1a–c). Deep sequencing of

¹Department of Genome Sciences, University of Washington, Seattle, WA, USA. ²Brotman Baty Institute for Precision Medicine, Seattle, WA, USA. ³Howard Hughes Medical Institute, University of Washington, Seattle, WA, USA. *e-mail: lstarita@uw.edu; shendure@uw.edu

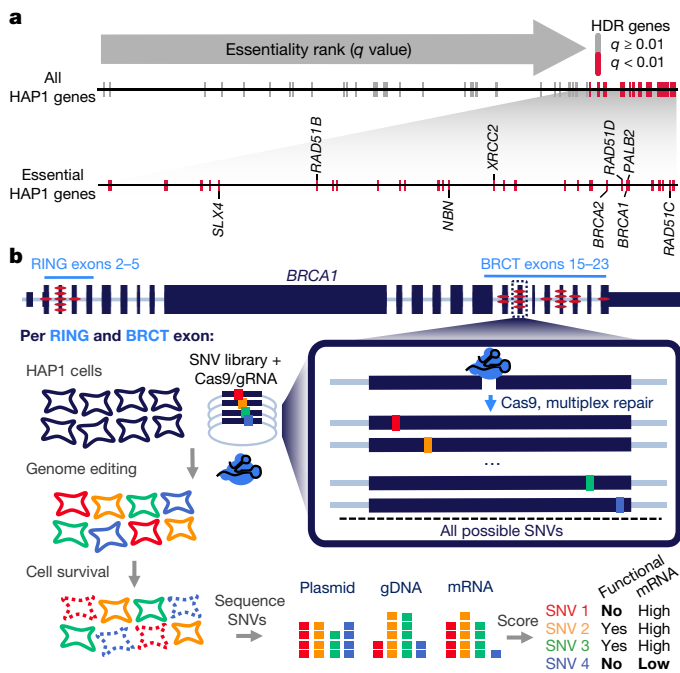


Fig. 1 | *BRCA1* and other HDR pathway genes are essential in HAP1 cells. **a**, The q -value rankings²³ of HDR pathway genes ($n = 66$) among 14,306 genes scored in a HAP1 gene trap screen for essentiality are indicated with tick marks. Essential HDR genes are coloured red and those implicated in cancer predisposition are labelled in the enlargement below. Of the 66 HDR pathway genes scored, 34 including *BRCA1* were 'essential', a 3.4-fold enrichment compared to non-HDR genes (Fisher's exact test, $P = 6.1 \times 10^{-12}$). **b**, SGE experiments were designed to introduce all possible SNVs across 13 *BRCA1* exons encoding the RING (exons 2–5, NCBI, NM_007294.3) and BRCT domains (exons 15–23). The exonic locations of all 21 *BRCA1* missense variants in ClinVar deemed pathogenic by an expert panel are indicated by red ovals. For each exon, a Cas9/gRNA construct was transfected with a library of plasmids containing all SNVs within approximately 100 bp of genomic sequence (the 'SNV library'). SNV library plasmids contained homology arms, as well as fixed synonymous variants within the CRISPR target site to prevent re-cutting. Upon transfection, successfully edited cells carried a single *BRCA1* SNV from the library. Cells were sampled 5 and 11 days after transfection and targeted gDNA and RNA sequencing was performed to quantify SNV abundances. SNVs compromising *BRCA1* function were selected against, manifesting in reduced gDNA representation, and SNVs that affect mRNA production were depleted in RNA relative to gDNA.

the edited loci of *BRCA1*-targeted cells confirmed that cell death was consequent to mutations, as there was widespread selection against frameshifting indels (Extended Data Fig. 1d). Overall, these results confirm the importance of HDR pathway components in HAP1 cells.

We next designed and optimized experiments for SGE²² (Fig. 1b), focusing on the 13 exons of *BRCA1* that encode the RING and BRCT domains (exons 2–5 and 15–23, respectively; NCBI, NM_007294.3). To create libraries of repair templates, we used array-synthesized oligonucleotide pools containing all possible SNVs spanning each exon and around 10 base pairs (bp) of adjacent intronic sequence. Oligonucleotide pools for each exon were cloned into plasmids with homology arms ('SNV libraries'). Each design also included a fixed synonymous substitution at the Cas9 target site to reduce re-cutting after successful HDR²². Each SGE experiment targeted one exon. A population of 20 million HAP1 cells was co-transfected on day 0 with a corresponding SNV library and Cas9/gRNA plasmid. Variant frequencies were quantified by targeted sequencing of the edited exon from genomic DNA (gDNA) collected on day 5 and day 11.

We initially performed SGE in replicate for each exon in wild-type HAP1 cells. In each exon, we observed the expected depletion of frameshifting indels (Extended Data Fig. 2). However, to achieve

more robust data, we optimized SGE in HAP1 cells in two ways. First, to increase HDR rates²⁵, we generated a monoclonal *LIG4*-knockout HAP1 line (HAP1-LIG4^{KO}) (Extended Data Fig. 3a, g). Second, as HAP1 cells can spontaneously revert to diploidy²⁶, sorting HAP1 cells for 1*n* ploidy before editing improved reproducibility (Extended Data Fig. 3b, h).

We performed optimized SGE on each of the 13 exons in 1*n*-sorted HAP1-LIG4^{KO} cells. We observed a median 3.6-fold increase in HDR rates on day 5 in HAP1-LIG4^{KO} relative to wild-type HAP1 cells (Fig. 2a), allowing us to test nearly every SNV in replicate (Extended Data Fig. 3c). Because these optimizations increased reproducibility without substantially altering SNV effects on survival (Fig. 2b, Extended Data Figs. 3, 4), we proceeded with data from the 1*n*-sorted HAP1-LIG4^{KO} cells. Additionally, targeted RNA sequencing of day 5 HAP1-LIG4^{KO} samples was used to determine the abundance of exonic SNVs in *BRCA1* mRNA (Fig. 2c).

Function scores for 3,893 *BRCA1* SNVs

To calculate function scores for each SNV, we first calculated the log₂ ratio of the frequency of a SNV on day 11 to its frequency in the plasmid library. Second, positional biases in editing rates were modelled using day 5 SNV frequencies and subtracted (Extended Data Fig. 5). Third, to enable comparisons between exons, we normalized function scores such that the median synonymous and nonsense SNV in each experiment matched global medians. Lastly, a small number of SNVs that could not confidently be scored were filtered out (Extended Data Fig. 6). Altogether, we obtained function scores for 3,893 SNVs, which comprise 96.5% of all possible SNVs within or immediately intronic to these exons (Supplementary Table 1; <https://sge.gs.washington.edu/BRCA1/>).

Function scores were bimodally distributed (Fig. 2d). All nonsense SNVs scored below -1.25 ($n = 138$, median = -2.12), whereas 98.7% of synonymous SNVs more than 3 bp from splice junctions scored above -1.25 ($n = 544$, median = 0.00). We classified all SNVs as 'functional', 'non-functional', or 'intermediate' by fitting a two-component Gaussian mixture model (Extended Data Fig. 7). We categorized 72.5% of SNVs as functional, 21.1% as non-functional and 6.4% as intermediate.

It is particularly challenging to interpret the clinical importance of rare missense variants in *BRCA1*. Of the missense SNVs assayed, 21.1% (441 out of 2,086) were non-functional (Fig. 2e). Although most remaining missense SNVs were functional (70.6%), there was an enrichment for missense SNVs with intermediate effects (8.1% compared with 4.4% of all other SNVs; Fisher's exact test, $P = 2.7 \times 10^{-6}$).

An advantage of genome editing is that the effect of variants on native regulatory mechanisms such as splicing can be ascertained²². Whereas SNVs disrupting canonical splice sites (the two intronic positions immediately flanking each exon) were mostly non-functional (89.5%) or intermediate (5.5%) (Fig. 2e), SNVs positioned 1–3 bp into the exon or 3–8 bp into the intron had variable effects. We defined SNVs in these regions that did not alter the amino acid sequence as 'splice region' variants, of which 22.9% were non-functional (Fig. 2e). SNVs positioned more deeply in introns or in the 5' untranslated region (UTR) were similar to non-splice-region synonymous SNVs, in that they were much less likely to score as non-functional (intronic, 1.8%; 5' UTR, 0.0%; and synonymous, 1.3%, as non-functional).

Function scores accurately predict pathogenicity

We next investigated how well our function scores agreed with clinical variant interpretations present in ClinVar. Of 169 SNVs deemed 'pathogenic' in ClinVar that overlapped with our classifications, 162 were designated 'non-functional', two 'functional', and the remaining five 'intermediate'. By contrast, of 22 SNVs deemed 'benign' in ClinVar, 20 were designated 'functional', one 'non-functional', and one 'intermediate' (Fig. 3a). Three SNVs that scored unambiguously discordant with ClinVar suggest potential errors in the available clinical variant interpretations (Supplementary Note 1). A receiver operating

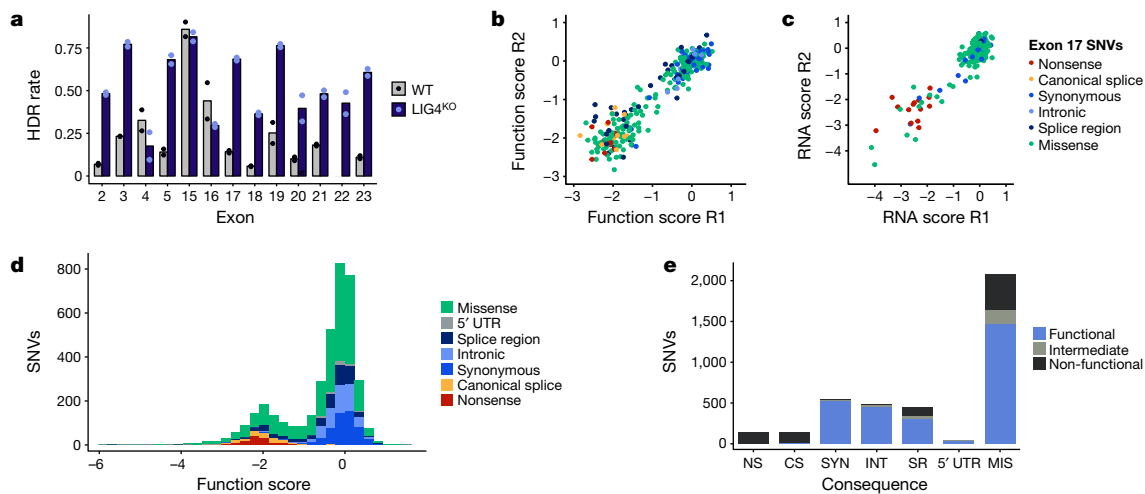


Fig. 2 | Saturation genome editing enables functional classification of 3,893 *BRCA1* SNVs. **a**, HDR editing rates were calculated for each exon as the fraction of day 5 reads containing the SNV library's fixed synonymous variant (an 'HDR marker' edit). The average of two wild-type HAP1 replicates and two HAP1-LIG4^{KO} replicates is plotted, with points indicating rates for each replicate. (Asterisk denotes missing exon 22 data.) **b**, **c**, Measurements for exon 17 SNVs assayed in HAP1-LIG4^{KO} cells are plotted to show correlations of function scores (**b**, $n = 291$, Spearman's

$\rho = 0.88$) and RNA expression scores (**c**, $n = 231$, Spearman's $\rho = 0.61$). Reproducibility is detailed further in Extended Data Fig. 4. **d**, A histogram of 3,893 SNV function scores (averaged from $n = 2$ replicates and normalized across exons) shows how each category of mutation compares to the overall distribution. **e**, The number of SNVs within each category is plotted and coloured by functional classification. (NS, nonsense; CS, canonical splice; SYN, synonymous; INT, intronic; SR, splice region; MIS, missense.)

characteristic (ROC) curve showed a sensitivity of 96.7% at 98.2% specificity when we treat 'likely pathogenic' and 'likely benign' ClinVar annotations as pathogenic and benign, respectively (Fig. 3b). Importantly, sensitivity and specificity are high for missense and splice region SNVs (Extended Data Fig. 7f).

We scored 25.0% (64 out of 256) of VUS and 49.2% (60 out of 122) of SNVs with conflicting interpretations as non-functional (Fig. 3c). Missense VUS from ClinVar were more likely to score as non-functional than missense SNVs that were absent from ClinVar (25.9% compared with 17.2%, Fisher's exact test, $P = 0.002$). Of 3,140 assayed SNVs that were absent from ClinVar, 498 (15.9%) scored as non-functional. The distribution of function scores for the 29 firmly 'pathogenic' missense SNVs confirmed here to be non-functional does not significantly differ from that of the 296 non-functional missense SNVs absent from ClinVar (median -2.05 versus -1.97 ; Wilcoxon rank-sum test, $P = 0.35$).

We investigated the relationship between our function scores and allele frequencies in large-scale variant databases, such as gnomAD (The Genome Aggregation Database; whole-exome and whole-genome sequencing data from over 120,000 individuals)²⁷. Among 302 assayed SNVs that overlap with gnomAD, higher allele frequencies were associated with higher function scores (Extended Data Fig. 8a). For instance, 33 out of 166 (19.9%) of singleton variants were non-functional, whereas only 8 out of 136 (5.9%) non-singleton variants were non-functional (Fisher's exact test, $P = 3 \times 10^{-4}$). A similar trend was observed with the Bravo database (Extended Data Fig. 8b). The FLOSSIES database contains variants observed in around 10,000 women over seventy years old who have not developed breast or ovarian cancer (<https://whi.color.com/gene/ENSG00000012048>). Of 39 intersecting *BRCA1* SNVs in FLOSSIES, only one scored as non-functional (Extended Data Fig. 8c). Collectively, these observations confirm that *BRCA1* SNVs with higher allele frequencies are more likely to be functional.

Several computational metrics are currently used to assess deleteriousness of variants and are often included in genetic testing reports. Although our function scores correlate with metrics such as CADD²⁸, phyloP²⁹ and Align-GVGD³⁰, the modesty of these correlations underscores the value of functional assays (Fig. 3d, Extended Data Fig. 9a–g). ROC curve analysis restricted to the 46 missense SNVs deemed 'pathogenic' or 'benign' in ClinVar reveals that SGE function scores outperform these metrics (Extended Data Fig. 9h–l).

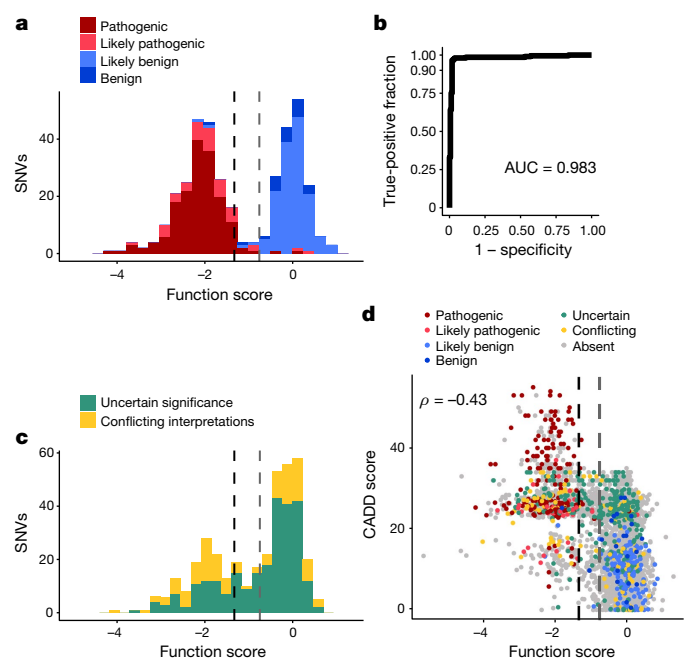


Fig. 3 | SGE function scores are highly accurate at predicting clinical interpretations of *BRCA1* SNVs. **a**, The distribution of SNV function scores coloured by ClinVar interpretation. Scores are shown for $n = 375$ SNVs with at least a 'one-star' review status in ClinVar and either a 'pathogenic' or 'benign' interpretation (including 'likely'). The dashed lines indicate the functional classification thresholds determined by mixture modelling. Grey divides 'functional' and 'intermediate' (function score $= -0.748$), and black divides 'intermediate' and 'non-functional' (function score $= -1.328$). **b**, An ROC curve reveals optimal sensitivity and specificity for classifying the same 375 SNVs in **a** at SGE function score cutoffs from -1.03 to -1.22 . **c**, The distribution of scores plotted as in **a** for the 378 SNVs annotated as variants of uncertain significance or with conflicting interpretations. 91.3% of such variants are classified as 'functional' or 'non-functional' by SGE. **d**, CADD scores, which predict deleteriousness, inversely correlate with function scores (Spearman's $\rho = -0.43$, $n = 3,893$ SNVs). SNVs are coloured by ClinVar annotation.

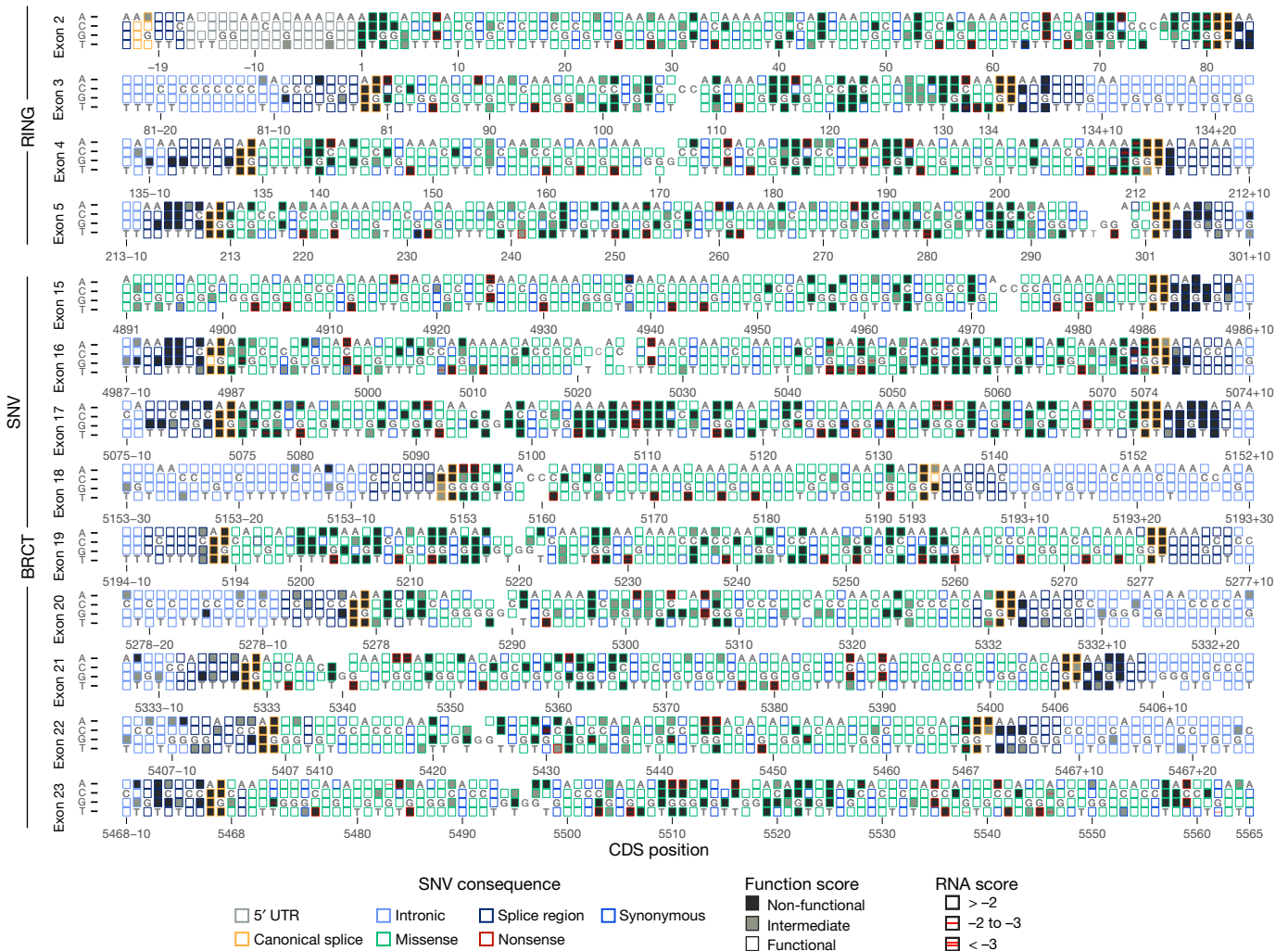


Fig. 4 | Sequence-function maps for 13 *BRCA1* exons. The 3,893 SNVs scored with SGE are each represented by a box corresponding to coding sequence position (NCBI, NM_007294.3) and nucleotide identity. Boxes are filled corresponding to functional classification, and outlined corresponding to the mutational consequence of the SNV. Red lines within

SGE function scores also strongly correlate with the results of assays designed to test particular aspects of *BRCA1* activity. For example, they are highly concordant with assays specific for the role of *BRCA1* in HDR^{9,13,31} and transcriptional activation¹² (Extended Data Fig. 9m, n), as well as with the results of a multiplexed assay that assesses the function of *BRCA1* variants in HDR³².

Mechanisms of *BRCA1* loss-of-function

To gain insights into the various mechanisms by which SNVs in *BRCA1* compromise function, we performed targeted RNA sequencing of *BRCA1* transcripts from edited day 5 cells. We normalized SNV frequencies in cDNA to their frequencies in gDNA to produce mRNA expression scores ('RNA scores') for 96% of the functionally characterized exonic SNVs. Together with function scores, RNA scores enable fine mapping of molecular consequences of SNVs (Fig. 4).

Overall, 89% of non-functional missense SNVs did not reduce RNA levels substantially, suggesting that their effects are mediated at the protein level (Fig. 5a, Supplementary Note 2). Many residues that are sensitive to missense SNVs that do not affect RNA levels map to buried hydrophobic residues or to the zinc-coordinating loops required for RING domain folding³³ (Fig. 5b, c). For example, 20 out of 21 missense SNVs in c.5104–c.5112 were scored as non-functional, including four VUS (Fig. 4). This intolerance to variation is probably due to the hydrophobicity and internal position of Y1703 and F1704, and the

boxes mark SNVs depleted in RNA; one line indicates an RNA score between -2 and -3 (log₂ scale) and two lines indicate a score below -3 . RNA measurements were determined only for exonic SNVs, excluding exon 18. Reference nucleotides are indicated; blank boxes indicate missing data.

polar contacts made between K1702 and a phosphorylated binding partner³⁴. This contrasts with a 51-bp stretch spanning exons 21 and 22 (c.5368–c.5418, p.1790–1806) in which none of the 104 missense SNVs assayed were non-functional.

SGE also implicates numerous SNVs that affect expression. For example, all SNVs that disrupt the translation initiation codon score as non-functional, and certain SNVs at the -3 , $+4$ and $+5$ positions predicted to decrease translational efficiency³⁵ score as intermediate or non-functional. In addition, 11% of non-functional missense SNVs are depleted from RNA by at least 75%, many of which map to unstructured regions (Fig. 5b, c), suggesting loss-of-function is consequent to reduced mRNA levels rather than disrupted protein function. Consistent with this, the 12 synonymous SNVs classified as non-functional also tended to markedly reduce mRNA levels (median 5.4-fold reduction).

Variants depleted in mRNA probably affect RNA splicing. This is evidenced by an overrepresentation of non-functional exonic SNVs near splice junctions, including low scores for many SNVs at terminal G nucleotides of exons (Fig. 4), non-functional exonic SNVs with low mRNA levels that create new acceptor or donor sequences (Fig. 5d), and the presence of 6–8 bp regions wherein many SNVs have strong effects on mRNA levels, suggestive of exonic splice enhancers³⁶ (Extended Data Fig. 10a). Certain exons were particularly prone to harbour non-functional SNVs with low RNA scores. In exon 16, for

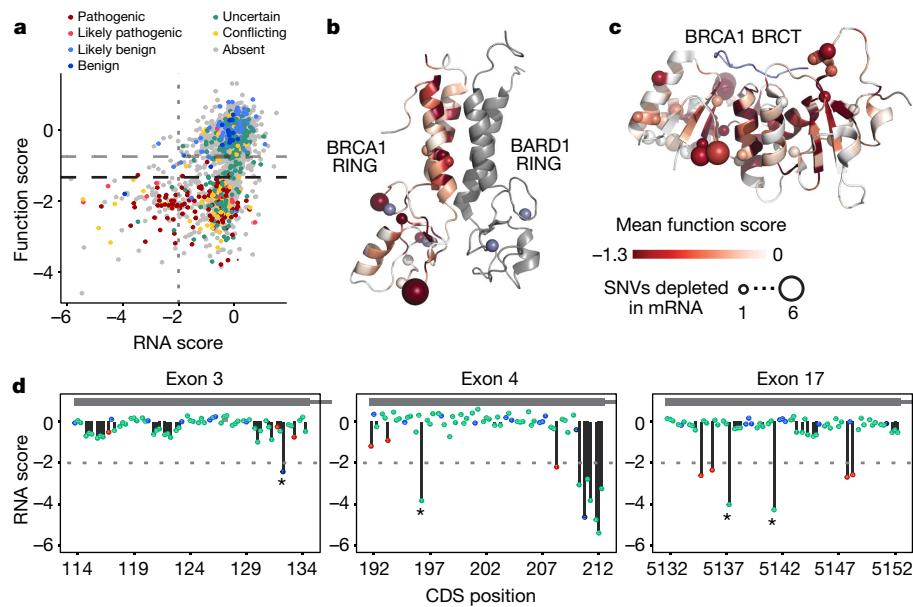


Fig. 5 | Measuring SNV mRNA abundance and function in parallel delineates mechanisms of variant effect. **a**, Function scores are plotted against RNA scores for all exonic synonymous and missense SNVs scored ($n = 2,646$). Horizontal dashed lines indicate functional thresholds, and the vertical dotted line marks an RNA score of -2 . **b**, **c**, Function scores for all SNVs were mapped onto the structures of the RING (**b**, PDB 1JM7) and BRCT (**c**, PDB 1T29) domains in shades of red by averaging missense SNV scores at each amino acid position. The number of SNVs that cause more than 75% reduction in mRNA levels at each amino acid position is represented by the size of the sphere at the alpha-carbon of each residue.

Grey denotes residues not assayed and the BACH1 peptide bound to the BRCT structure is coloured blue. **d**, SNV RNA scores are plotted by transcript position, with lines to the x axis denoting SNV functional classifications (no line, functional; grey line, intermediate; black line, non-functional; SNVs coloured by consequence as in Fig. 2c). The horizontal dashed line in each plot marks an RNA score of -2 , corresponding to 75% reduction in mRNA. Examples of non-functional SNVs with low RNA scores that create new 5'-GU splice donor motifs are indicated with asterisks.

instance, 46 of 244 SNVs (excluding nonsense) were non-functional (Extended Data Fig. 10a). Most of these (26 out of 46) reduced RNA levels by ≥ 2 -fold, and 15 by ≥ 4 -fold. By contrast, in exon 19, 55 of 234 SNVs (excluding nonsense) were non-functional, but none lowered expression by ≥ 2 -fold (Extended Data Fig. 10b). Exon 19 also completely lacks non-functional SNVs in its flanking intronic regions (apart from the acceptor and donor sites), suggesting it is robustly spliced.

Discussion

Here we applied SGE to critical domains of *BRCA1*, characterizing the consequences of nearly 4,000 SNVs in their native genomic context and obtaining a bimodal distribution of functional effects. A benefit of functional data is that measurements are systematically derived, independent of prior expectation³⁷. Because we measured cell survival, the effects of SNVs on multiple layers of gene function (for example, splicing, translation, and protein activity) are effectively integrated. Our study has several caveats (Supplementary Note 3), most notably that we used a survival assay in HAP1 cells as opposed to a more physiologically appropriate model. However, our data are validated by high concordance with the available evidence for clinical pathogenicity.

High sensitivity and specificity were obtained for both missense and splice region SNVs, the classes of variants that are most problematic for clinical interpretation. Our review of firmly discordant SNVs suggests that our true accuracy may be higher than calculated using ClinVar assertions as a gold standard (Supplementary Note 1). These discordances highlight the importance of integrating new evidence as it becomes available and updating databases accordingly. For instance, the submissions in the Breast Cancer Information Core, which mostly date to the early 2000s, underlie 51 conflicting interpretations. SGE scores support the more recent classification in the vast majority of such conflicts (Supplementary Table 2).

The interpretation of genetic variation is presently the rate-limiting step for genomic medicine. The fact that more than 70% of ClinVar variants and more than 95% of non-ClinVar variants assayed here

have never been observed in more than 120,000 humans represented in gnomAD illustrates the challenges facing observational approaches to variant interpretation. Given this, a pressing question is how best to integrate functional data into existing clinical variant classification schemes³⁸. The predictive power demonstrated here suggests that SGE function scores classify variants with more than 95% accuracy. As current standards for defining 'likely' pathogenic and benign variants accept comparable uncertainty³⁹, we argue that a failure to incorporate function scores would be a missed opportunity.

Optimal weighting of different approaches might further improve classification of variants lacking genetic evidence. For unexpected functional classifications, such as synonymous SNVs with low scores, and for cases in which the clinical evidence is contradictory, functional data can provide specific hypotheses to test. For example, c.5044G>A, for which our data contradicts ClinVar, could be disambiguated by testing *BRCA1* mRNA levels in individuals carrying this SNV. The approximately 6% of SNVs exhibiting intermediate function scores remain beyond definitive interpretation. The fact that we observe an excess of missense SNVs with intermediate scores suggests that some of these may be hypomorphic *BRCA1* alleles⁴⁰. Further studies will be necessary to assess the risk conferred by these variants.

We prioritized the RING and BRCT domains, but SGE of all exons of *BRCA1* is justified, and the essentiality of *BRCA2*, *PALB2*, *BARD1* and *RAD51C* in HAP1 cells suggests that these genes are assayable by the same method. For other genes, assays compatible with saturation genome editing (for example, drug selection, FACS on phenotypic markers) may need to be developed and validated. Scaling SGE to many loci also promises to improve our understanding of how diverse biological functions are encoded by the genome.

Here we show that SGE is a viable strategy for functionally classifying thousands of variants in a clinically actionable gene, most of which have yet to be observed in a human. We anticipate function scores will prove valuable, both for adjudicating hundreds of observed *BRCA1* variants for which the interpretation is currently ambiguous, as well as

for providing immediate functional assessments for newly observed variants. This work may also serve as a blueprint for the comprehensive functional analysis of all potential SNVs in clinically actionable genes.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at <https://doi.org/10.1038/s41586-018-0461-z>.

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Correspondence and requests for materials should be addressed to L.M.S. or J.S.

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METHODS

Data reporting. No statistical methods were used to predetermine sample size. The experiments were not randomized; the investigators were not blinded to allocation during experiments and outcome assessment.

HDR pathway essentiality analysis in HAP1 cells. HAP1 cells were derived from KBM7 cells (a near-haploid immortalized chronic myelogenous leukaemia line) by introduction of induced pluripotent stem cell factors⁴¹. HAP1 gene essentiality scores were obtained²³ and filtered on genes with more than 20 mapped gene-trap insertions ($n = 14,306$). Of 78 HDR genes defined by the Gene Ontology term 'double-strand break repair via homologous recombination' (GO:0000724), 66 were among the 14,306 genes included in analysis. To rank genes by essentiality, they were first ordered by q value (low to high) and second by the proportion of gene-trap insertions in the sense orientation (low to high). HDR pathway genes implicated in cancer (labelled in Fig. 1a) were defined as those included on the University of Washington BROCA sequencing panel⁴².

gRNA design and cloning. All CRISPR gRNAs used in SGE and essentiality experiments were cloned into pX459²⁴. This plasmid expresses the gRNA from a U6 promoter, as well as a Cas9-2A-puromycin resistance (-puroR) cassette. *S. pyogenes* Cas9 target sites were chosen for SGE experiments on multiple criteria, assessed in the following order: (i) to induce cleavage within *BRCA1* coding sequence, (ii) to target a genomic site permissive to synonymous substitution within the guanine dinucleotide of the PAM or the protospacer, (iii) to have minimal predicted off-target activity⁴³, (iv) to have maximal predicted on-target activity⁴⁴.

Complementary oligonucleotides ordered from Integrated DNA Technologies (IDT) were annealed, phosphorylated, diluted and ligated into BbsI-digested and gel-purified pX459, as described previously²⁴. Ligation reactions were transformed into *Escherichia coli* (Stellar competent cells, Takara), which were plated on ampicillin. Colonies were cultured and Sanger-sequenced to confirm correct gRNA sequences. Purification of sequence-verified plasmids for transfection was performed with the ZymoPure Maxiprep kit (ZymoResearch). For targeting *LIG4* in HAP1 cells, pX458²⁴ was used instead of pX459, which expresses EGFP in lieu of puroR.

HDR library design and cloning. Array-synthesized oligonucleotides were designed as follows for each saturation genome editing region (that is, a *BRCA1* exon). The sequence to be mutated (~100 bp) was obtained from the human genome (hg19) and a synonymous substitution was introduced at the chosen Cas9 target site (for example, a substitution at the PAM site). This 'fixed' substitution in the library was included in design to serve multiple purposes: (i) plasmid library molecules harbouring the substitution are predicted to be cleaved less frequently by Cas9-gRNA complexes, (ii) SNVs introduced to cells are predicted to be depleted via Cas9 re-cutting less frequently as a consequence of the fixed substitution, and (iii) sequencing reads can be filtered on the fixed substitution to distinguish true SNVs introduced via HDR from sequencing errors. A second synonymous substitution at an alternative CRISPR target site was introduced to the sequence as well, such that the SNV library for each exon would be compatible with multiple gRNAs. Next, a sequence was created for every single nucleotide substitution on this template. For all sequences, adapters were added to both ends to enable PCR amplification from the oligonucleotide pool. For each SGE region, the total number of oligonucleotides designed was three times the length of the region, plus the oligonucleotide template without any SNV (for example, for a 100-bp SGE region, 301 total oligonucleotides were designed).

Pooled oligonucleotides were synthesized (Agilent Technologies). Primers designed to amplify the subset of oligonucleotides corresponding to a single region of an exon were used to perform PCR with Kapa HiFi Hot-start Ready Mix (Kapa HiFi, Kapa Biosystems). PCR products were purified with Ampure beads (Agencourt) to be used in subsequent library cloning reactions.

Homology arms were cloned into pUC19 by PCR-amplifying (Kapa HiFi) regions surrounding each targeted exon from HAP1 gDNA. Primers for these reactions were designed such that homology arms would be between 600 bp and 1,000 bp on both sides of the targeted region. Adapters homologous to pUC19 were added to primers to facilitate NEBuilder HiFi Assembly cloning (NEB) into a linearized pUC19 vector. Cloning reactions were transformed into Stellar competent cells and selected with ampicillin. Plasmid DNA was isolated from colonies (Qiagen MiniPrep kit) and sequence-verified.

To construct the HDR library, homology arm plasmids were linearized via PCR using primers that conferred 15–20 bp of terminal overlap with the adapter sequences flanking each PCR-amplified oligonucleotide pool. This sequence overlap enabled cloning via the NEBuilder HiFi Assembly Cloning Kit (NEB). Cloning reactions were transformed into Stellar competent cells, and a small proportion (1%) of the transformation was plated on ampicillin-containing plates to assess efficiency. All remaining transformed cells were grown directly in 100 ml of medium with ampicillin for 16–18 h, and plasmid DNA from the culture was isolated (ZymoPure Maxiprep kit) to produce each final HDR library.

HAP1 cell culture. Quality-controlled wild-type HAP1 cells were purchased (Haplogen/Horizon Discovery) and cultured in medium comprising Iscove's Modified Dulbecco's Medium (IMDM) with L-glutamine and 25 mM HEPES (GIBCO) supplemented with 10% fetal bovine serum (Rocky Mountain Biologicals) and 1% penicillin–streptomycin (GIBCO). Cells were grown on plates at 37°C with 5% CO₂, and passed before becoming confluent. For routine passaging, cells were washed once with 1× phosphate-buffered saline (PBS, Gibco), trypsinized with 0.25% trypsin with EDTA (Gibco), resuspended in medium, centrifuged for 5 min at 300g, and then resuspended and plated.

A monoclonal *LIG4*-knockout HAP1 line (HAP1-LIG4^{KO}) was generated by transfecting a plasmid expressing a Cas9-2A-GFP cassette and a gRNA targeting the human *LIG4* coding sequence (gRNA sequence: 5'-GCATAATGT CACTACAGATC-3') into wild-type HAP1 cells. Single GFP-expressing HAP1 cells were sorted into wells of a 96-well plate and cultured. After two weeks, gDNA was collected and Sanger sequencing was performed to assess *LIG4* editing. A clone with a 4-bp deletion was identified and expanded further for use in saturation genome editing experiments.

HAP1 cells can spontaneously revert to a diploid state in cell culture. Therefore, to sort a 1n-enriched population of cells before transfection, cells were stained for DNA content with Hoechst 34580 (BD Biosciences) at 5 µg ml⁻¹ medium for 1 h at 37°C. FACS was performed to isolate 1–2 × 10⁶ cells from the lowest intensity Hoechst peak, corresponding to 1n ploidy. These cells were expanded for seven days before transfection.

Transfection of HAP1 cells. For all experiments, HAP1 cells were transfected using TurboFectin 8.0 (Origen) according to manufacturer's protocol. A 2.5 × volume of Turbofectin was added to the transfection mix for each µg of plasmid DNA in Opti-Mem (Life Technologies). For each SGE transfection, 10 million cells were passaged to a 10-cm dish. The next day (day 0), cells were co-transfected with 12 µg of the Cas9/gRNA plasmid (pX459) and 3 µg of the SNV library corresponding to a single exon. Negative control transfections were performed for each library using a pX459 vector targeting *HPRT1* instead of *BRCA1*, thus preventing genomic integration of the library. On day 1, cells were passaged into medium supplemented with puromycin (1 µg ml⁻¹) to select for successfully transfected cells. On day 4, cells were washed twice and passaged to 6-cm plates in regular media.

Cell populations were sampled on day 5 and day 11 for all SGE experiments. On day 5, half of the cells were pelleted and frozen and the other half passaged. The cells were passaged on day 8 into 15-cm dishes and then harvested on day 11. Negative control transfections were harvested on day 5 and used to confirm that PCR amplicons were not derived from the plasmid DNA of the SNV library.

For the luminescence-based viability assay, HAP1 cells were plated at 35–40% confluency in a 6-well dish (approximately 1.2 million cells per well per target) then transfected with 1.5 µg Cas9/gRNA plasmid targeting coding exons of HDR genes or controls the following day. After 24 h of transfection, the cells were plated in time-point triplicates at 20,000 cells per well in 96-well clear bottom plates in medium with and without puromycin. Cells without puromycin were assessed 4 h after plating to establish baseline absorbance for each target. Cell survival was assessed at day 2, day 5, and day 7 after transfection using the CellTiterGlow reagent (Promega, 1:10 dilution of suggested reagent). Luminescence at 135-nm absorbance was measured using a Synergy plate reader (Biotek Instruments).

Nucleic acid sampling and sequencing library production. For obtaining wild-type HAP1 genomic DNA for cloning homology arms and for genotyping the HAP1-LIG4^{KO} cell line, DNA was isolated using the DNeasy kit (Qiagen). For each SGE experiment, DNA and total RNA were purified using the AllPrep kit (Qiagen). DNA samples were quantified with the Qubit dsDNA Broad Range kit (Thermo Fisher) and RNA samples by UV spectrometry (Nanodrop). PCR primers for genomic DNA were designed such that one primer would anneal outside of the homology arm sequence, thereby selecting for amplicons derived from gDNA and not plasmid DNA. PCR conditions were optimized using gradient qPCR on wild-type HAP1 gDNA.

All gDNA collected from the population of day-5 cells was sampled by performing many PCR reactions in parallel on a 96-well plate, using 250 ng of gDNA per 50 µl reaction such that all day-5 gDNA was used in PCR (Kapa HiFi). At least as many PCR reactions were performed for day-11 samples (which yielded more gDNA) to ensure adequate sampling. PCRs were performed for the minimal number of cycles needed to complete amplification, with cycling conditions as specified in the Kapa HiFi protocol. An additional PCR was performed using day-5 gDNA from negative control transfections for each exon.

After PCR, multiple wells of amplicons from the same sample were pooled and purified using Ampure beads. Next, a nested qPCR was performed using the first reaction as template to produce a smaller amplicon with custom sequencing adapters ('PU1L' and 'PU1R'), which was likewise purified with Ampure beads. The SGE libraries were also PCR-amplified at this step, starting from 50 ng of plasmid DNA. Lastly, a final qPCR was performed using purified products from the second reaction as template to add dual sample indexes and flow cell adapters.

RNA was sampled from day-5 HAP1-LIG4^{KO} cells (AllPrep, Qiagen). Reverse transcription followed by RNase H treatment was performed on all collected RNA or a maximum of 5 µg per sample (Superscript IV Kit, Life Technologies). This reaction was primed with a gene-specific primer complementary to the 3' UTR in exon 23 of *BRCA1*. Primers were designed for each exon to amplify across exon junctions, and reaction conditions were optimized using gradient PCR. cDNA was distributed into five equal PCR reactions, which were run on a qPCR machine and then pooled in equal ratios. Flow cell adapters and sample indexes were added in an additional reaction (as for gDNA samples).

All sequencing libraries were purified with Ampure beads, quantified with the Qubit dsDNA High Sensitivity kit (Life Technologies), diluted and denatured for sequencing in accordance with protocols for the Illumina NextSeq or MiSeq machines.

Sequencing and data analysis. Sequencing was performed on an Illumina NextSeq or MiSeq instrument, allocating about 3 million reads to each gDNA and cDNA sample, 1 million reads for each HDR library, and 500,000 reads for each negative control sample. gDNA samples for individual exons were sequenced on the same run. In total, 300 cycle kits were used, with 150 cycles for read 1 and read 2 each, and 19 cycles for dual index reads. Custom sequencing primers and indexing primers are provided in Supplementary Table 3. Illumina PhiX control DNA was added to each sequencing run (around 10% MiSeq, around 30–40% NextSeq) to improve base calling.

We used bcl2fastq 2.16 (Illumina) to call bases and perform sample demultiplexing and fastqc 0.11.3 was run on all samples to assess sequencing quality. SeqPrep was used with the following parameters to perform adapter trimming and to merge perfectly matched overlapping read pairs: '-A GGTGGAGCGAGATTGATAAAGT -B CTGAGCTCTCTACAGCCATTAG -M 0.1 -m 0.001 -q 20 -o 20'. Merged reads containing 'N' bases were removed. Reads from cDNA samples were removed if they contained indels or did not perfectly match transcript sequence flanking each targeted exon. Remaining cDNA reads were processed to match genomic DNA amplicons by removing flanking exonic sequence and replacing it with the exon's corresponding intronic sequence. All reads were then aligned to reference gDNA amplicons for each exon using the needle command in the EMBOSS 6.4.0 package with the following parameters: '-gapopen 10 -gapextend 0.5 -format sam'. Reads not aligning to the reference amplicon (alignment score, <300) were removed from analysis. To analyse indels, unique cigar counts were quantified from day-5 and day-11 samples using a custom Python script. Reads were classified as HDR events for rate calculations if the programmed edit or edits to the PAM or protospacer (HDR marker edits) were observed in the alignment. Variants without identifiable markers of HDR were not used. Abundances of SNVs were quantified only from aligned reads that had no other mismatches or indels, with the exception of the HDR markers. SNV reads with only the cut-site proximal HDR marker were summed with reads that had both HDR markers to get total abundances for each SNV in each sample, to which a pseudocount of 1 was added to all variants present in either the library, day-5 or day-11 sample. Frequencies for each SNV were calculated as SNV reads over total reads. SNV measurements from wild-type HAP1 cells and HAP1-LIG4^{KO} cells were processed separately at all steps.

Specific exon 2 splice junctions were queried by counting the number of reads from cDNA samples that perfectly matched specific isoform junctions. Two 14-bp sequences spanning the end of exon 1 and the beginning of exon 2 were counted to measure use of the canonical junction (5'-TCTGGTTCATTGGA-3' and 5'-TCTGGTTCAGTGA-3'; the latter of which contains an HDR marker introduced during editing). The 14-bp sequence spanning the end of exon 1 and the portion of exon 2 corresponding to the reported alternative AG acceptor site^{45,46} was (5'-TAAAGAAAGAAATG-3'). The proportion of the total reads counted containing the latter sequence was used to approximate the relative contribution of the alternative acceptor site.

Modelling positional biases of library integration. Positional biases in editing rates were modelled for each SNV by using a LOESS regression to fit the log₂ day 5 over library ratios as a function of chromosomal position. To avoid modelling biological effects instead of positional effects, the model was fit only on the subset of SNVs that were not substantially depleted between any two time points in the experiment (that is, SNVs with day 5 over library ratios greater than 0.5 and day 11 over day 5 ratios greater than 0.8). The regression was performed for each exon replicate, using the 'loess' function in R with span = 0.15. Each model was extended flatly outward to include any positions not fit (a total of 22 nucleotides of sequence on the edges of the edited regions). We subtracted positional fit (the model's output) for each SNV from its log₂ day 11 over library ratio to get position-adjusted ratios for each SNV.

Normalizing scores within and across exons. Position-adjusted log₂ day 11 over library ratios were normalized first across exon replicates, and then across all assayed exons. To do this, scores from within each replicate were linearly scaled such that the median synonymous and median nonsense SNVs within the replicate would match the median synonymous and median nonsense SNV values averaged

across replicate experiments. The ensuing SNV scores for each replicate were then normalized across all exons in the same manner, such that each exon's median synonymous and median nonsense SNV scores would match the global median synonymous and the global median nonsense SNV scores, respectively.

SNV functional class assignment. Function scores were averaged across replicates and a mixture model was used to estimate the probability that each SNV's score was drawn from the non-functional distribution of scores. The non-functional distribution was defined as nonsense SNVs across all exons. The functional distribution was defined as exonic synonymous SNVs not within 3 bp of splice junctions and with RNA scores within 1 standard deviation of the median synonymous SNV. This definition does not fully guarantee that these SNVs have no functional consequence. The means and variances of the 'non-functional' and 'functional' groups were fixed and a model was fit using the normalmixEM function of the mixtools package in R, with starting component proportions set to 0.5. The posterior probabilities generated from the model were used as point estimates of the probability of drawing each SNV's score from the non-functional distribution (P_{nf}). Functional classifications were made by setting thresholds for P_{nf} as follows: $P_{nf} > 0.99 =$ 'non-functional', $0.01 < P_{nf} < 0.99 =$ 'intermediate', $P_{nf} < 0.01 =$ 'functional'.

Independent of mixture modelling, ROC curves were used to assess performance of SGE data and other metrics' ability to predict assigned ClinVar classifications. These analyses were performed with the 'plotROC' package in R, and Youden's *J*-statistic (sensitivity plus specificity minus 1) was calculated to determine optimal values reported in text.

Variant filtering. A small minority of SNVs that could not be accurately scored were removed from analysis. If a SNV was not present in the HDR library at a frequency over 1 in 10⁴, it was presumed to have been lost in oligonucleotide synthesis or cloning and was removed. Further, if a SNV was not observed with complete HDR markers at a frequency over 1 in 10⁵ in day-5 genomic DNA samples from both replicate experiments, it was removed. SNVs introduced near the CRISPR recognition site have the potential to facilitate Cas9 re-cutting of the locus (for example, by replacing the PAM edit or introducing an alternative PAM site). Because these SNVs are likely to score lower consequent to Cas9 editing biases and not their effects on gene function, SNVs were filtered that created increased potential for re-cutting as follows: When an HDR marker mutation used to disrupt editing occurred at position 2 of the PAM (for example, 'NGG' to 'NCG'), SNVs that replaced this marker with an alternate base were removed to prevent biases introduced by re-cutting non-canonical *S. pyogenes* Cas9 PAMs (for example, 'NAG', 'NTG'). Additionally, variants that created a new PAM 1 bp 3' of the mutated PAM were excluded owing to the potential for re-cutting (for example, unedited PAM: 5'-NCGA, edited PAM with HDR marker: 5'-NCGA, filtered out SNV that creates new PAM +1 bp 3': 5'-NCGG). (Extended Data Figure 6 describes re-cutting observed at alternative PAMs.) To prevent misinterpretation, we also removed SNVs that created amino acid changes specific to the context of the library's fixed edits (for example, if in the unedited background, the SNV causes an X to Y change, but with a fixed edit in the same codon, the SNV causes an X to Z change). We also applied this logic to remove SNVs that introduced splice donor sites only in the context of the edited PAM, and SNVs that create splice donor sites in the unedited context but not in the context of the edited PAM.

The RNA scores for exon 18 samples were neither well-correlated across replicates nor with SNV abundances in genomic DNA, indicating probable bottlenecking in library preparation. Therefore, RNA data from exon 18 was excluded. Wild-type HAP1 function scores from exon 22 were excluded because there was an unusually high correlation between SNV frequencies sampled from the plasmid library and from day-5 gDNA, suggesting plasmid contamination in gDNA sequencing. This problem was fixed by designing a new primer to prepare gDNA sequencing samples from HAP1-LIG4^{KO} cells.

External data sources. Variant annotations were downloaded from CADD²⁸ version 1.3 (<http://cadd.gs.washington.edu/download>). This included the following scores: mammalian phyloP, Grantham deviation, SIFT, Polyphen-2 and CADD. Align-GVGD scores were obtained by running the Align-GVGD program on *BRCA1* sequences conserved to sea urchin. ClinVar data were downloaded on 2 January 2018 for all germline SNVs with at least a 1-star annotation. SNVs annotated as 'Benign/Likely benign' were grouped with 'Likely benign' SNVs and SNVs classified 'Pathogenic/Likely pathogenic' were grouped with 'Likely pathogenic' SNVs. SNV allele frequencies were obtained from <http://gnomad.broadinstitute.org/> on 26 December 2017 for gnomAD²⁷, from <https://bravo.sph.umich.edu/freeze5/hg38/> on 19 November 2017 for Bravo, and from <https://whi.color.com/> on 9 October 2017 for FLOSSIES data. The hg19 UCSC Genome Browser was accessed from <https://genome.ucsc.edu/> on 1 May 2018 for chr17:41,276,108–41,276,139. Throughout this study, *BRCA1* exons, coding nucleotide positions, and amino acid positions are referenced by the ClinVar transcript annotation for *BRCA1*, NCBI transcript NM_007294.3.

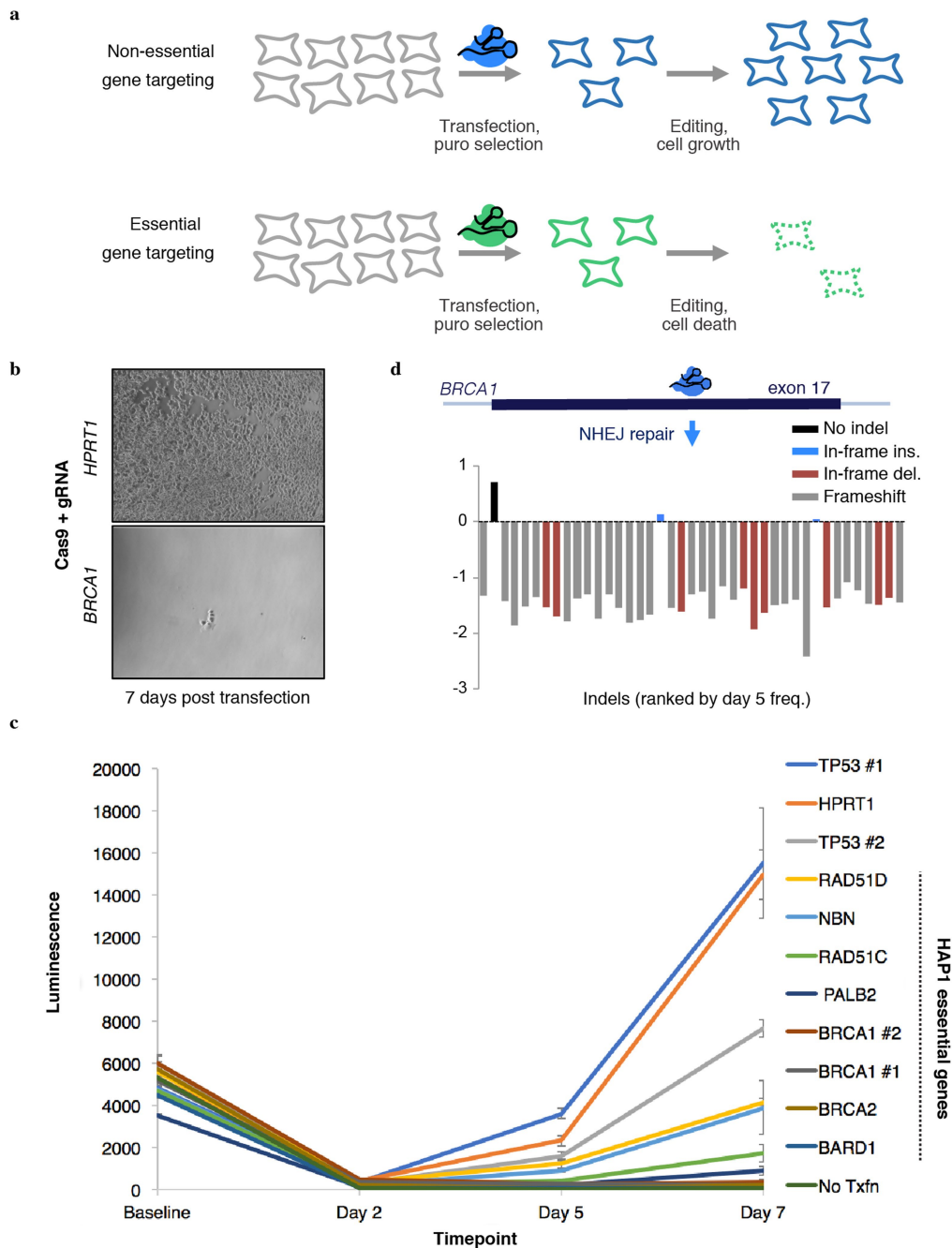
Statistical reporting. All statistical tests described were performed as two-tailed tests using the R software package.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Code availability. Custom scripts for analysing sequencing data were written in Python and R. All code is available at: https://github.com/shendurelab/saturation-GenomeEditing_pipeline.

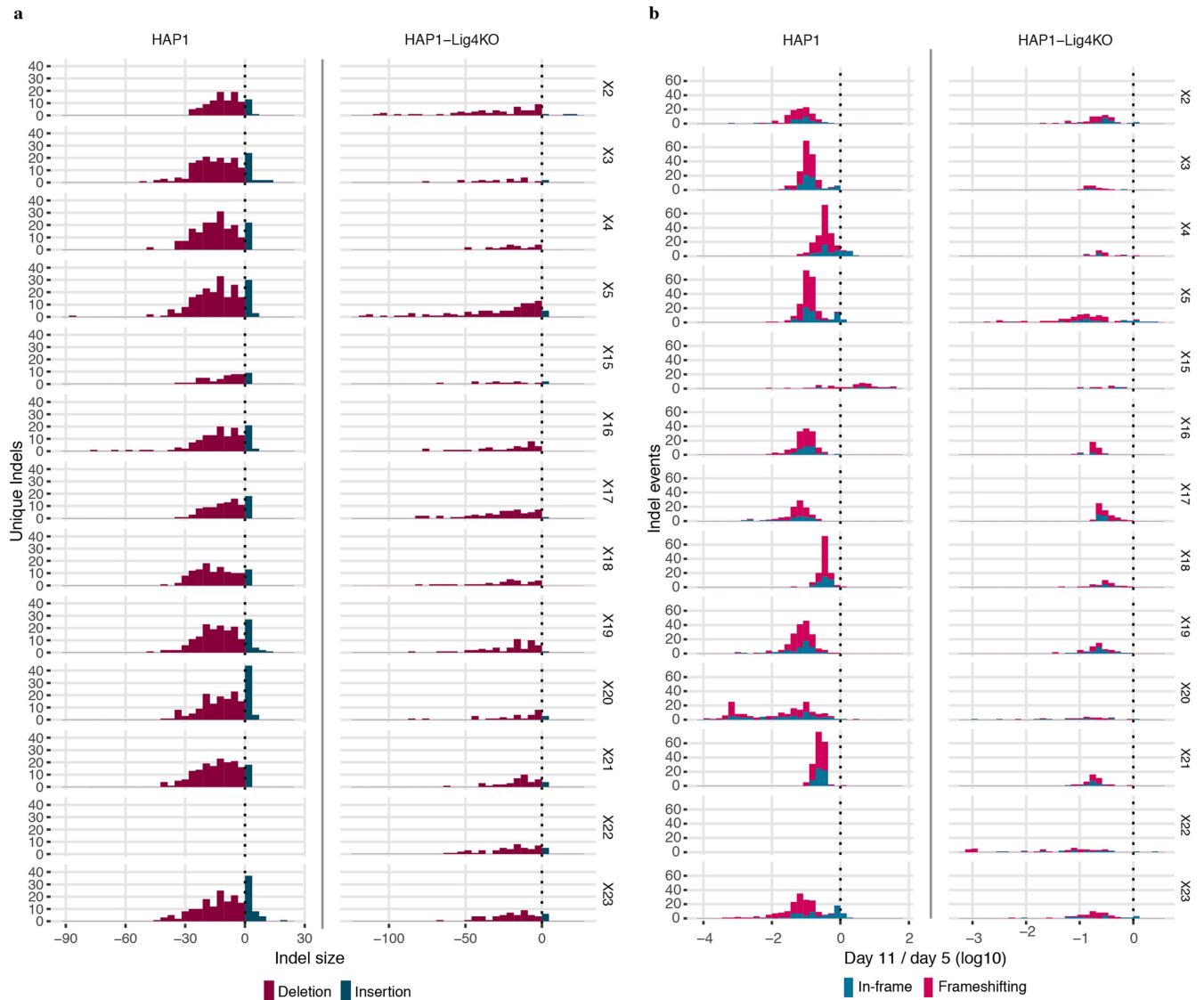
Data availability. Function scores are freely available for all non-profit uses (see <https://sge.gs.washington.edu/BRCA1/>), as well as by non-exclusive license under reasonable terms to commercial entities that have committed to open sharing of *BRCA1* sequence variants. Sequencing data are available at Gene Expression Omnibus under accession GSE117159.

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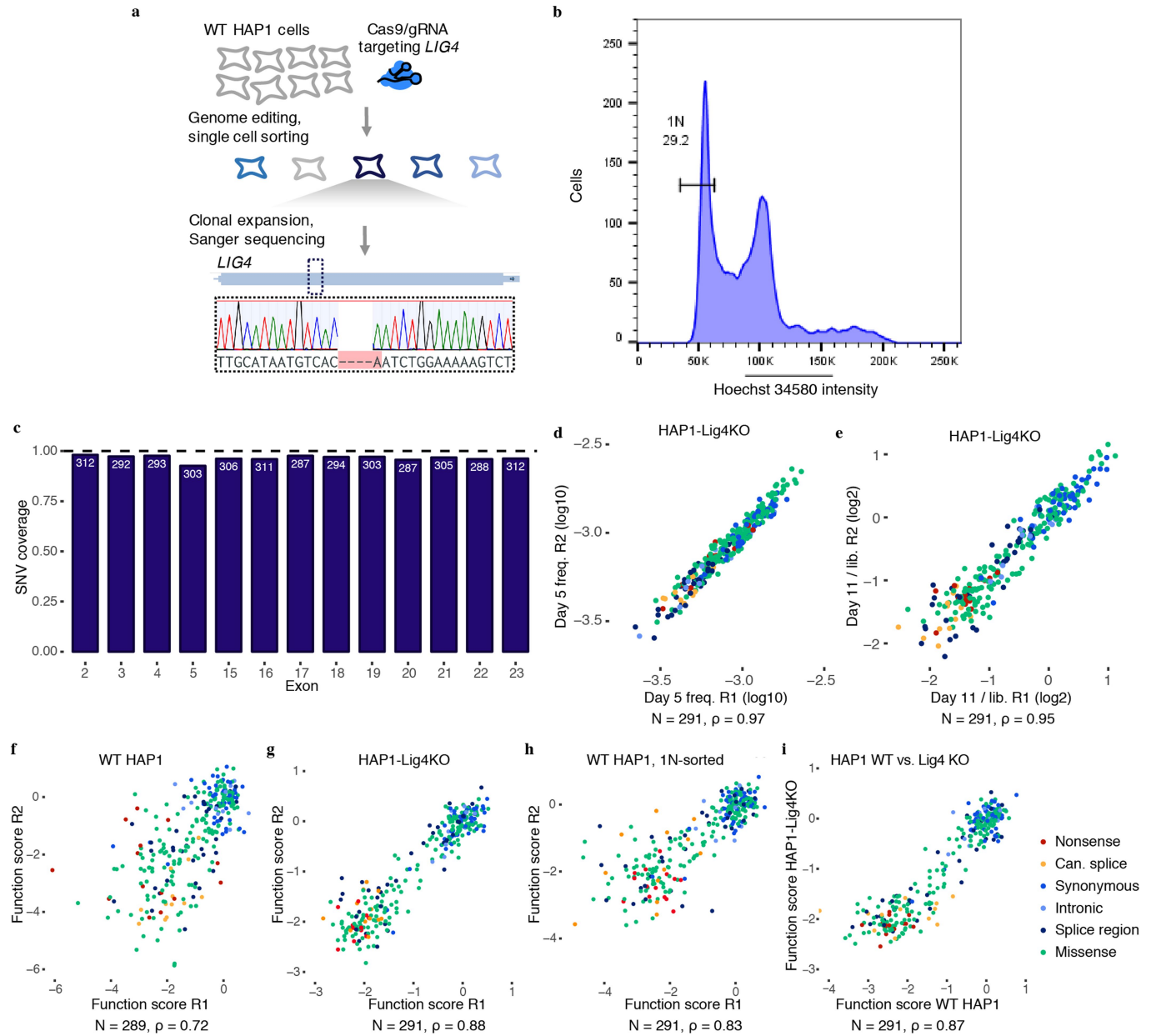
Extended Data Fig. 1 | CRISPR targeting of HDR pathway genes to confirm essentiality in HAP1 cells. **a**, Schematic, HAP1 cells are transfected with a plasmid expressing a gRNA and a Cas9-2A-puromycin cassette²⁴. Owing to low transfection rates for HAP1 cells, puromycin selection reduces viable cells in all transfections. Over time, however, CRISPR targeting of non-essential genes leads to increased cell growth compared to CRISPR targeting of essential genes. **b**, HAP1 cell populations were transfected with a Cas9/gRNA plasmid either targeting the non-essential gene *HPRT1* (control) or exon 17 of *BRCA1* on day 0. Successfully transfected cells were selected with puromycin (days 1–4) and cultured until imaging on day 7, at which point cells were imaged. Images are

representative of two transfection replicates. **c**, Cell viability of HAP1 cells transfected with Cas9/gRNA constructs targeting different HDR genes and controls (*HPRT1*, *TP53*) was measured using the CellTiterGlow assay. Luminescence is proportional to the number of living cells in each well when the assay is performed. Triplicate wells for each gRNA at each time point were processed, quantified on a plate reader and averaged. Error bars show the standard error of the mean. gRNA sequences are included in Supplementary Table 3. **d**, The targeted *BRCA1* exon 17 locus was deeply sequenced from a population of transfected cells sampled on day 5 and day 11. The fold-change from day 5 to day 11 for each editing outcome observed at a frequency over 0.001 in day 5 sequencing reads is plotted.



Extended Data Fig. 2 | Analysis of Cas9-induced indels observed in *BRCA1* SGE experiments. Variants observed in gDNA sequencing were included in this analysis if (i) they aligned to the reference with either a single insertion or deletion within 15 bp of the predicted Cas9 cleavage site and (ii) were observed at a frequency greater than 1 in 10,000 reads in both replicates. **a**, Histograms show the number of unique indels observed of each size, with negative sizes corresponding to deletions. More unique indels were observed in wild-type HAP1 cells compared to

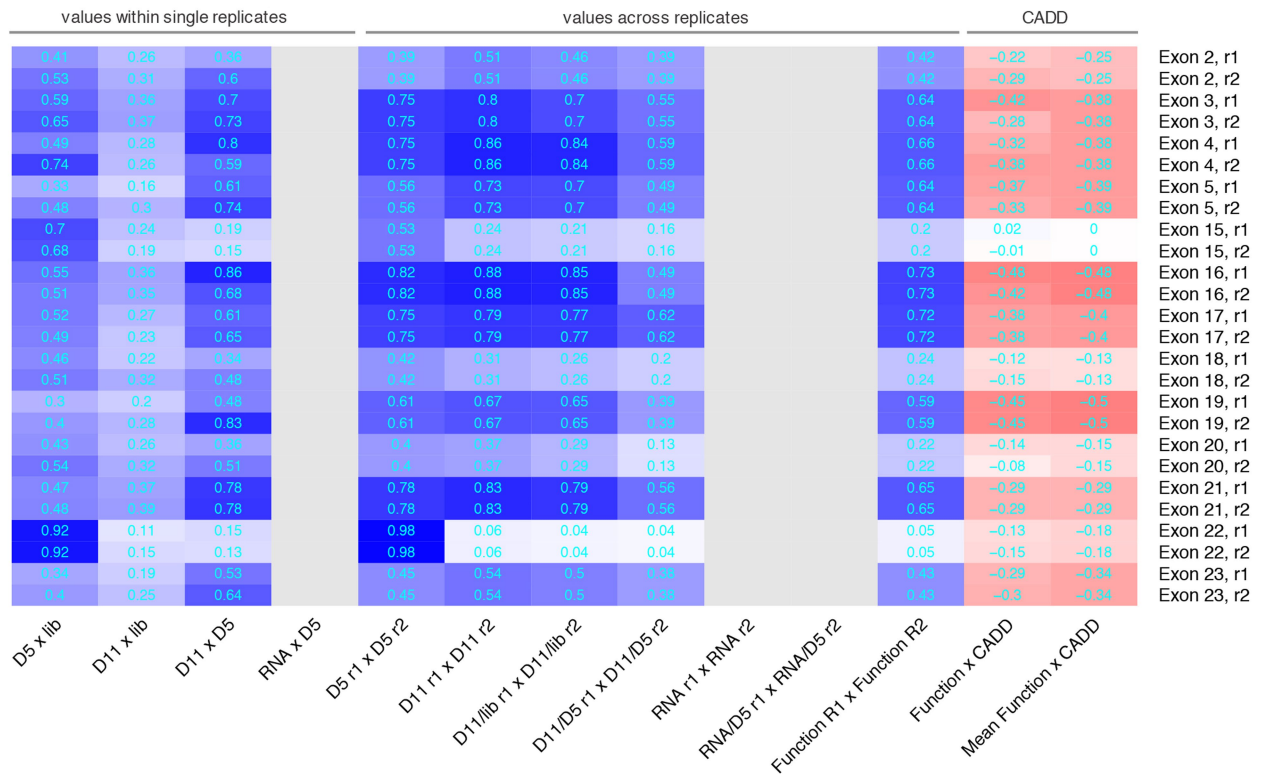
HAP1-LIG4^{KO} cells for exons compared (wild-type data for exon 22 was excluded). **b**, Day 11 over day 5 indel frequencies were normalized to the median synonymous SNV in each replicate and then averaged across replicates to measure selection on each indel. The distribution of selective effects is shown for each experiment as a histogram, in which indels are coloured by whether their size was divisible by 3 (that is, ‘in-frame’ versus ‘frameshifting’). Whereas frameshifting variants were consistently depleted, some exons were tolerant to in-frame indels.



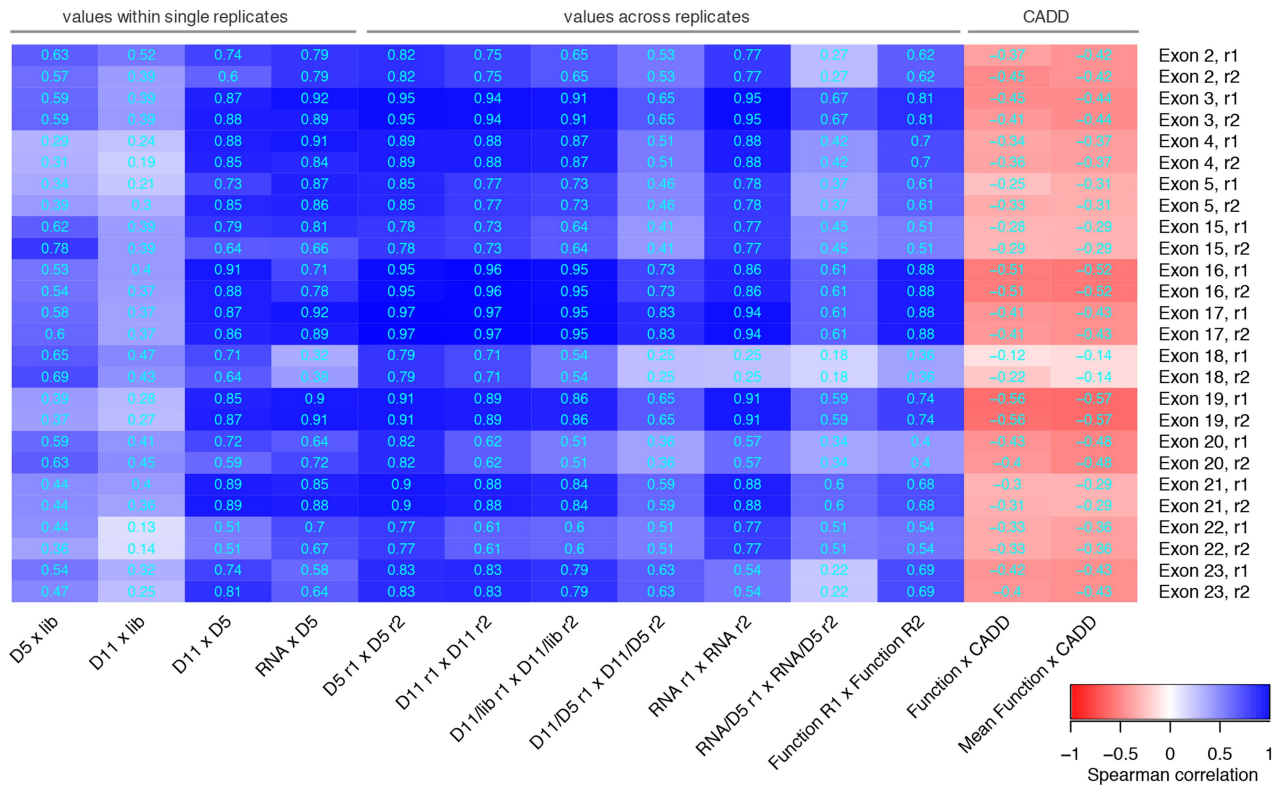
Extended Data Fig. 3 | HAP1 cell line optimizations for saturation genome editing to assay essential genes. **a**, A gRNA targeting Cas9 to the coding sequence of *LIG4*, a gene integral to the non-homologous end-joining pathway, was cloned into a vector co-expressing Cas9-2A-GFP²⁴. Wild-type HAP1 cells were transfected, and single GFP-expressing cells were sorted into wells of a 96-well plate. Eight monoclonal lines were grown out over a period of three weeks and screened using Sanger sequencing for frameshifting indels in *LIG4*. The Sanger trace shows the frameshifting deletion present in the clonal line chosen for subsequent experiments, referred to as HAP1-LIG4^{KO}. **b**, To purify HAP1 cells for haploid cells, live cells were stained for DNA content with Hoechst 34580 and sorted using a gate to select cells with the lowest DNA content, corresponding to 1n cells in G1. **c**, The fraction of all possible

SNVs scored is shown for each exon. SNVs were excluded mainly due to proximity to the HDR marker and/or poor sampling (Methods). **d**, **e**, Measurements across replicates are plotted for exon 17 SNVs assayed in HAP1-LIG4^{KO} cells to show correlations of day 5 frequencies (**d**) and day 11 over library ratios (**e**). **f–h**, Plots comparing SNV function scores across replicate experiments for exon 17 saturation genome editing experiments performed in unsorted wild-type HAP1 cells (**f**), HAP1-LIG4^{KO} cells (**g**), and wild-type HAP1 cells sorted on 1n ploidy (**h**). **i**, Function scores (averaged across replicates) are plotted to compare results for exon 17 experiments performed in wild-type 1n-sorted HAP1 cells and HAP1-LIG4^{KO} cells. The number of SNVs plotted and the Spearman correlation is displayed for each plot (**d–i**).

a WT HAP1



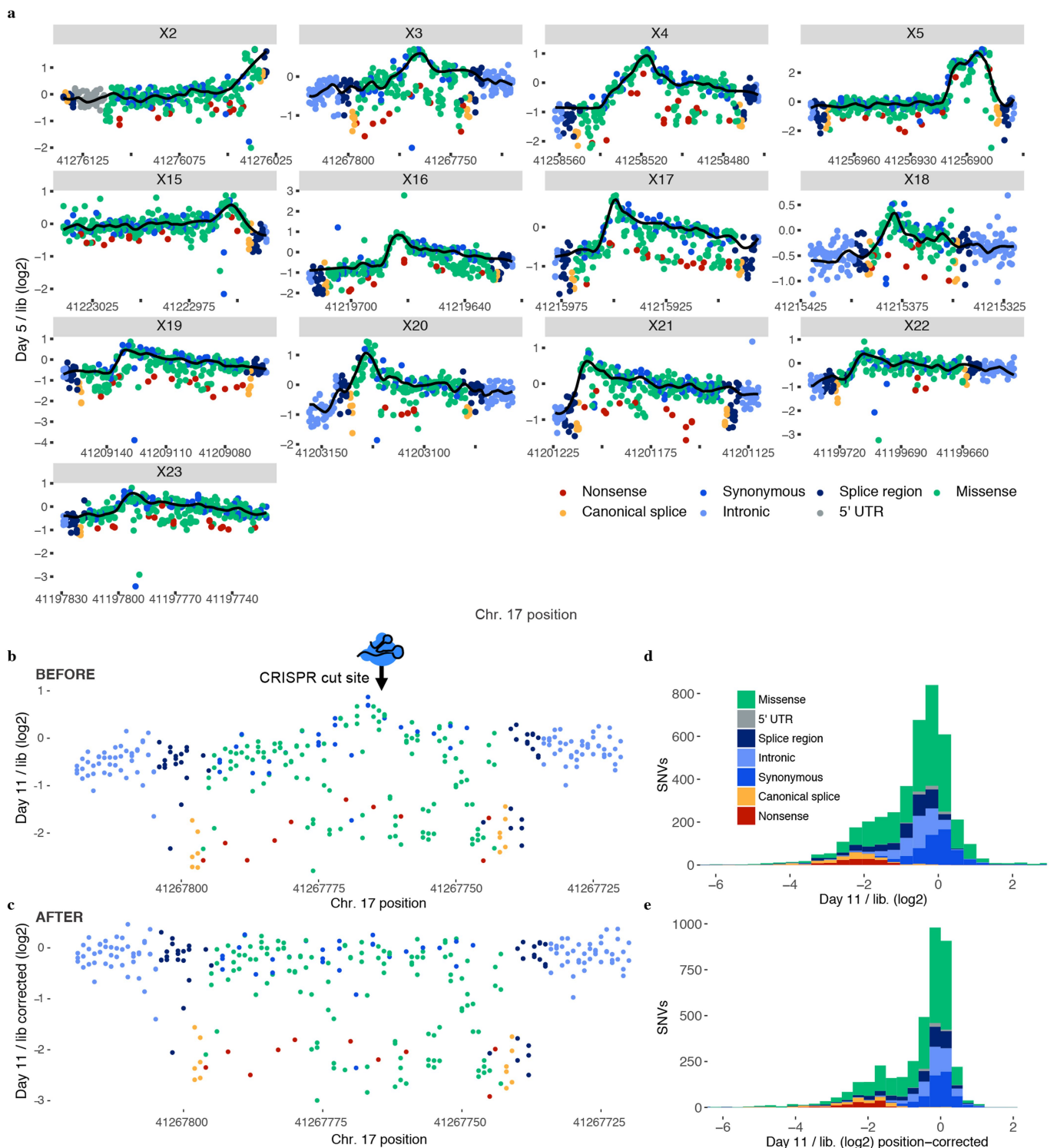
b HAP1-Lig4KO



Extended Data Fig. 4 | See next page for caption.

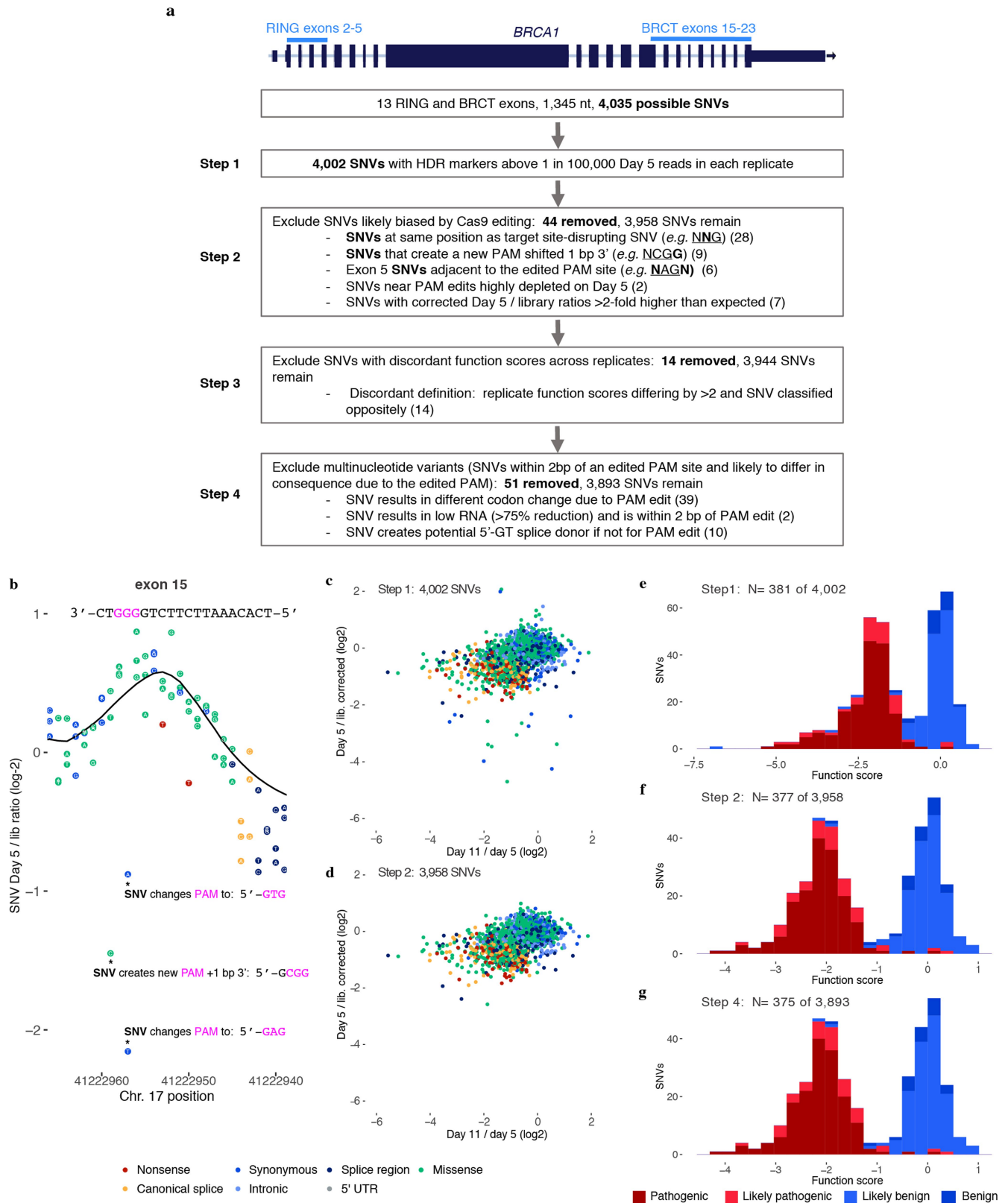
Extended Data Fig. 4 | Correlations for SNV measurements within single experiments, across transfection replicates, and to CADD scores for all SGE experiments. Heat maps indicate Spearman correlation coefficients for SNV measurements from experiments in wild-type HAP1 cells (a) and in HAP1-LIG4^{KO} cells (b). Grey boxes indicate absent RNA data from wild-type HAP1 cells. The four leftmost columns show how SNV frequencies correlate between samples from within a single replicate experiment. The unusually high correlations between exon 22 SNV frequencies in the plasmid library and in day 5 gDNA samples from wild-type HAP1 cells suggests plasmid contamination in gDNA. Indeed, primer homology to a repetitive element in the exon 22 library was

identified. Consequently, the wild-type HAP1 exon 22 data was removed from analysis and a different primer specific to gDNA was used to prepare exon 22 sequencing amplicons from HAP1-LIG4^{KO} cells. The low HAP1-LIG4^{KO} correlations between exon 18 SNV frequencies in day 5 gDNA and RNA and between RNA replicates suggests RNA sample bottlenecking consequential to low RNA yields. Therefore, exon 18 RNA was also excluded from analysis. Consistent with the higher rates of HDR-mediated genome editing (Fig. 2a), replicate correlations (middle columns) were generally higher in HAP1-LIG4^{KO} cells than wild-type HAP1 cells. CADD scores predict the deleteriousness of each SNV, and are therefore negatively correlated with function scores (rightmost columns).



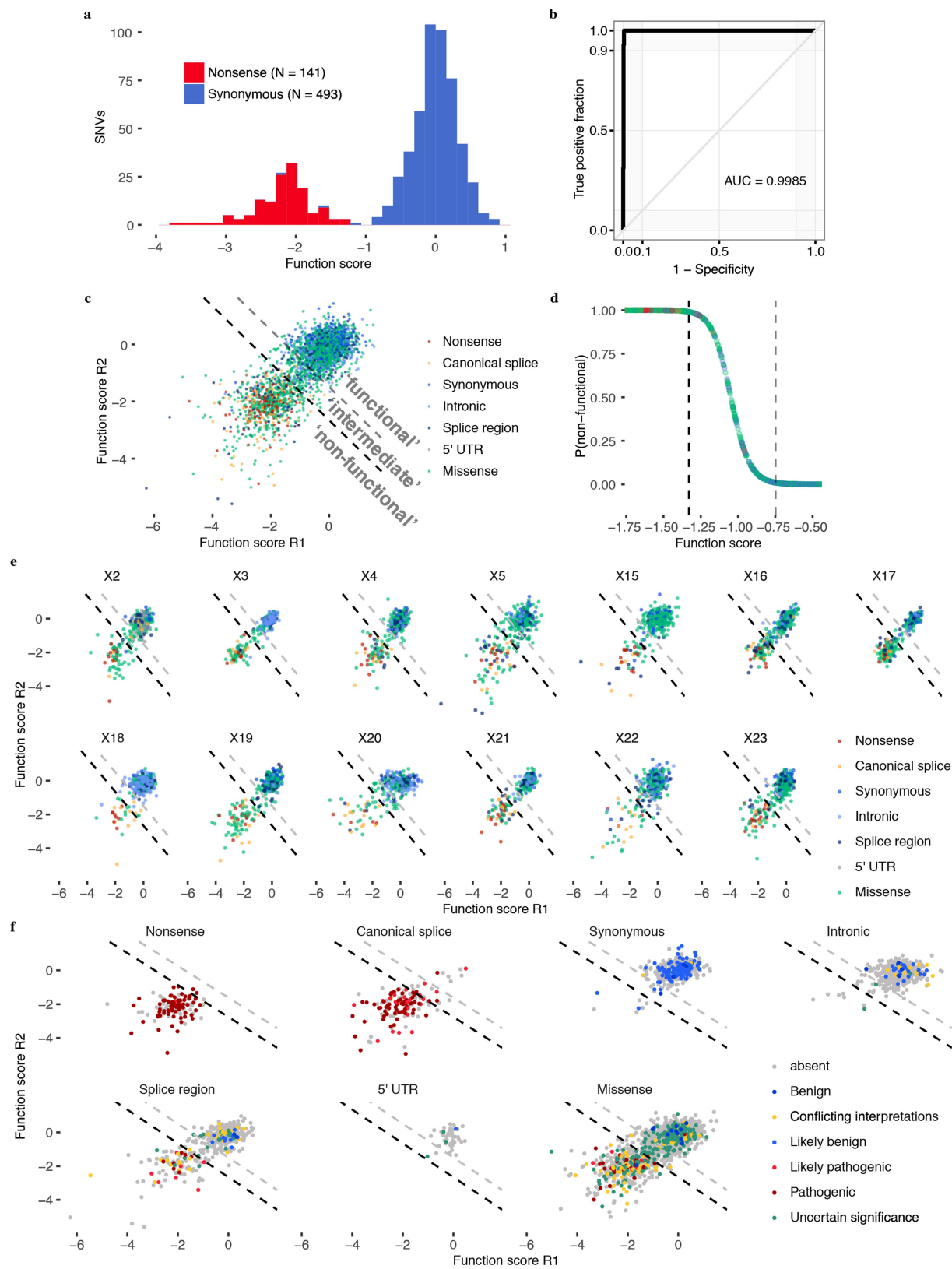
Extended Data Fig. 5 | Models of SNV editing rates across *BRCA1* exons to account for positional biases. Gene conversion tracts arising during HDR in human cells are short such that library SNVs are introduced to the genome more frequently near the CRISPR target site. We modelled this positional effect in our data for $n = 4,002$ SNVs (pre-filtering) using a LOESS regression fit on day 5 over library SNV ratios. **a**, Plots shown here are of the average of $n = 2$ replicates per exon, with the black line indicating the LOESS regression. By day 5, selective effects on gene function are evidenced by nonsense SNVs (red) appearing at lower frequencies compared to neighbouring SNVs. Therefore, to best approximate the SNV editing rate as a function of position alone (that is, the 'baseline'), the regression excluded SNVs that were selected against

between day 11 and day 5 (see Methods). **b, c**, Day 11 over library SNV ratios were adjusted by the positional fit for each experiment in calculating function scores. This adjustment is illustrated here for an exon 3 replicate by plotting the day 11 over library ratio as a function of position before (**b**) and after (**c**) adjustment for ($n = 298$ SNVs). The elevated day 11 over library ratios for SNVs near the CRISPR cleavage site (indicated with an arrow) are corrected to achieve a more uniform baseline across the mutagenized region. **d, e**, The distributions of SNV day 11 over library ratios before and after accounting for positional effects are shown, coloured by mutational consequence ($n = 4,002$ SNVs, averaged across $n = 2$ replicates).



Extended Data Fig. 6 | SNV filtering to prevent erroneous functional classification. **a**, The flow chart describes filters used to produce the final SNV dataset and shows how many SNVs were removed at each step. **b**, Raw day 5 over library SNV ratios are shown for a portion of exon 15 to illustrate how re-editing biases necessitate filtering. The three depleted SNVs marked with asterisks create alternative PAM sequences that probably allow the Cas9-gRNA complex to re-cut the locus and cause their removal. For other SNVs, the fixed PAM edit (a GGG to GCG synonymous change) minimizes re-editing. Alternative PAM sequences created by

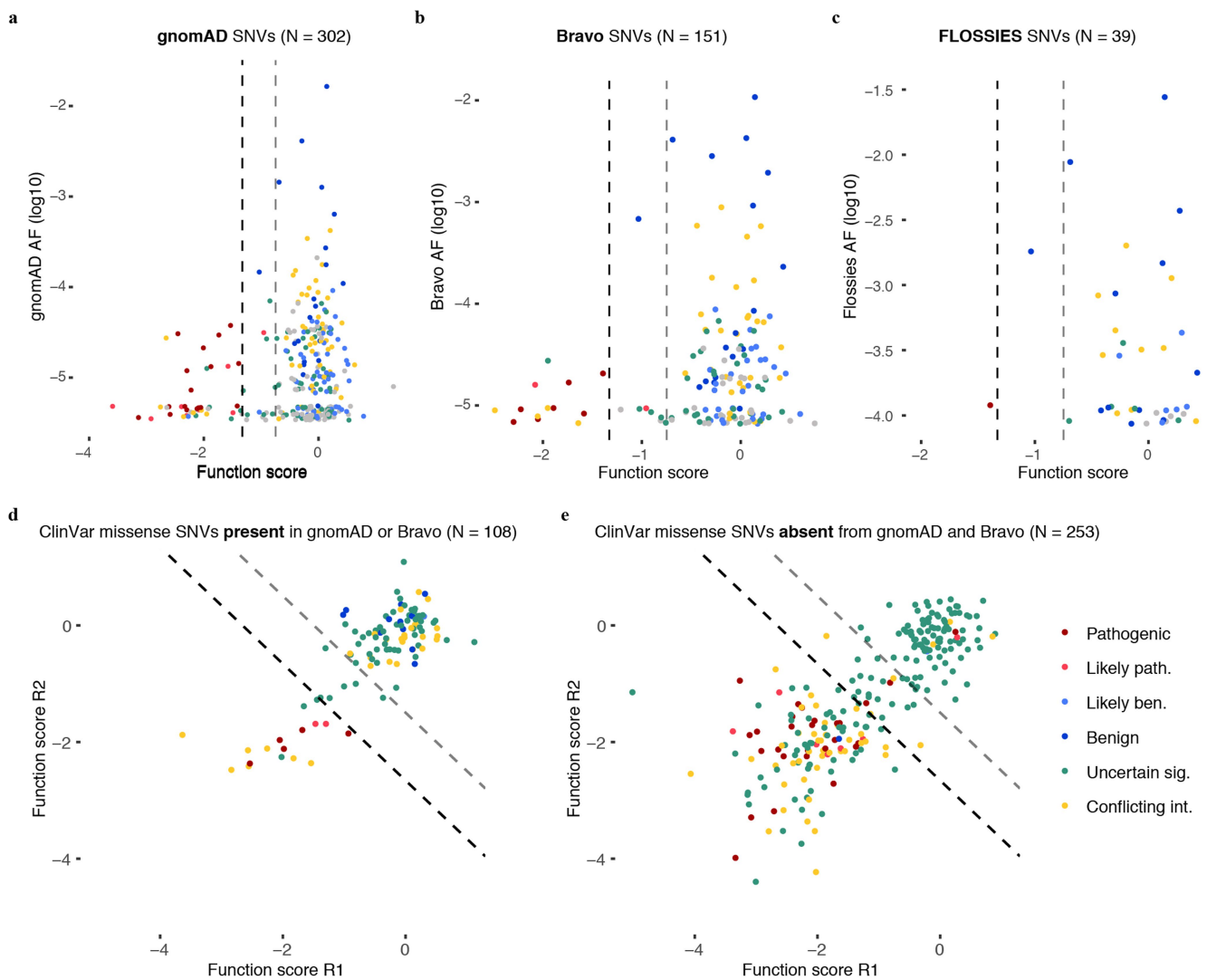
each indicated SNV are shown in magenta. The LOESS regression curve in shown in black. **c**, **d**, Plots show the relationship between day 5 over library and day 11 over day 5 ratios before (**c**) and after (**d**) filtering steps 1 and 2. Filtering removes outliers because editing biases primarily affect the day 5 over library ratio. **e-g**, Histograms show the distributions of function scores for SNVs deemed 'pathogenic' or 'benign' in ClinVar at different stages of filtering. Scores in **e** are derived before normalization across exons.



Extended Data Fig. 7 | See next page for caption.

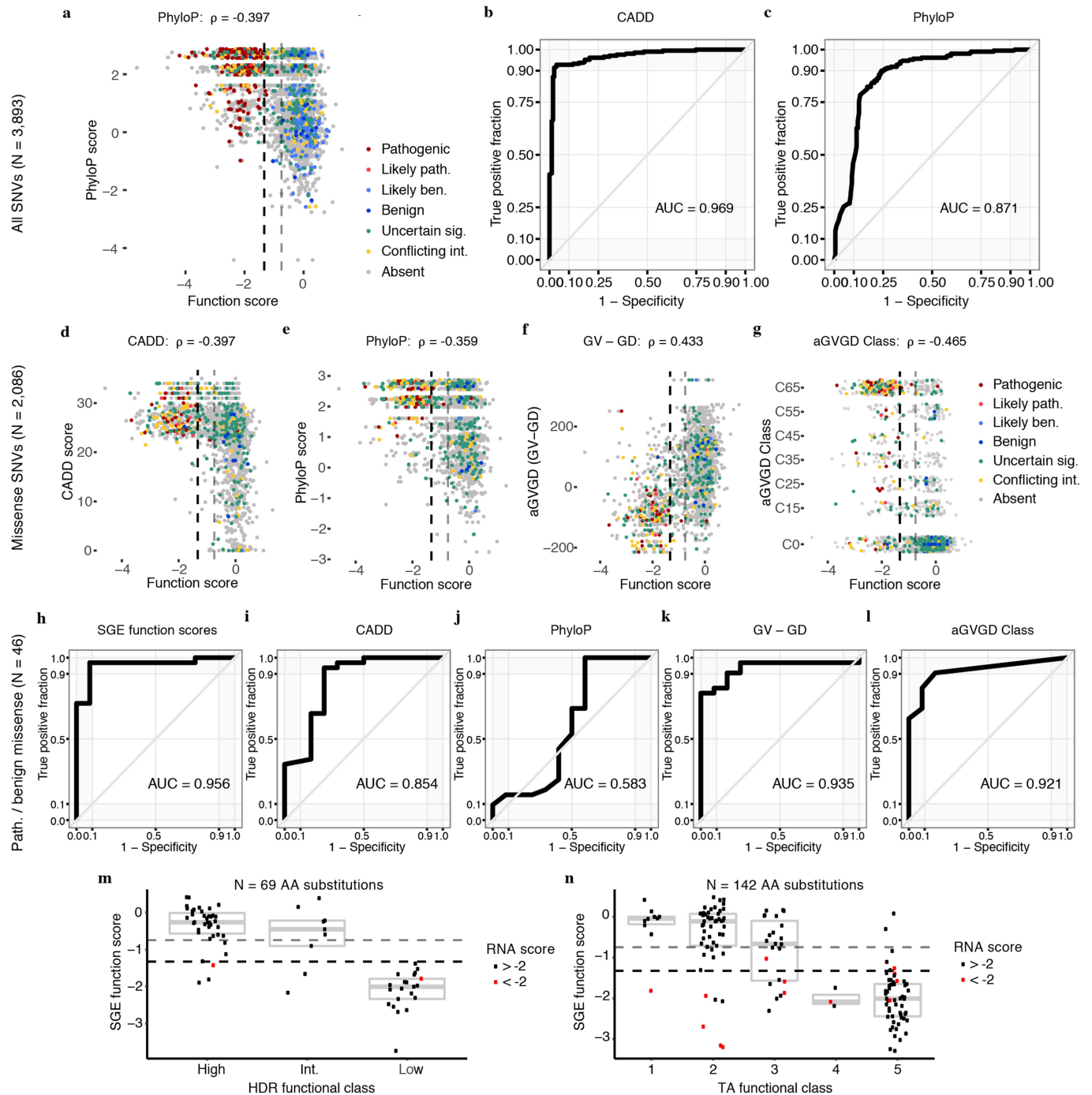
Extended Data Fig. 7 | Mixture modelling of scores to classify SNVs by functional effect. **a**, Distributions of ‘non-functional’ and ‘functional’ SNVs plotted here were defined respectively as all nonsense SNVs and all synonymous SNVs with RNA scores within 1 standard deviation of the median synonymous SNV. **b**, An ROC curve was generated using SGE function scores to distinguish the 634 ‘functional’ and ‘non-functional’ SNVs defined in **a**. **c**, A two-component Gaussian mixture model was used to produce point estimates of the probability that each SNV was ‘non-functional’, P_{nf} , given its average function score across replicates. These P values are plotted in **d** against function scores for a subset of the data. Thresholds were set such that $P_{nf} < 0.01$ corresponds to ‘functional’, and $P_{nf} > 0.99$ corresponds to ‘non-functional’, and $0.01 < P_{nf} < 0.99$

corresponds to ‘intermediate’ classification. Functional classification thresholds are drawn as dashed lines; black denotes the non-functional threshold and grey the intermediate threshold. **e**, **f**, SNV function scores across replicates are plotted for each exon with SNVs coloured by mutational consequence (**e**), and for each type of mutational consequence with SNVs coloured by ClinVar status (**f**). Using the optimal function score cutoff for all SNVs tested (Fig. 3b), sensitivities and specificities for distinguishing ‘Pathogenic’/‘Likely pathogenic’ from ‘Benign’/‘Likely benign’ ClinVar annotations for each type of mutation are as follows: 92.7% and 92.9% for missense SNVs ($n = 55$), 100% and 100% for splice region SNVs ($n = 23$), and 95.2% sensitivity for canonical splice site SNVs ($n = 83$; specificity not calculable).



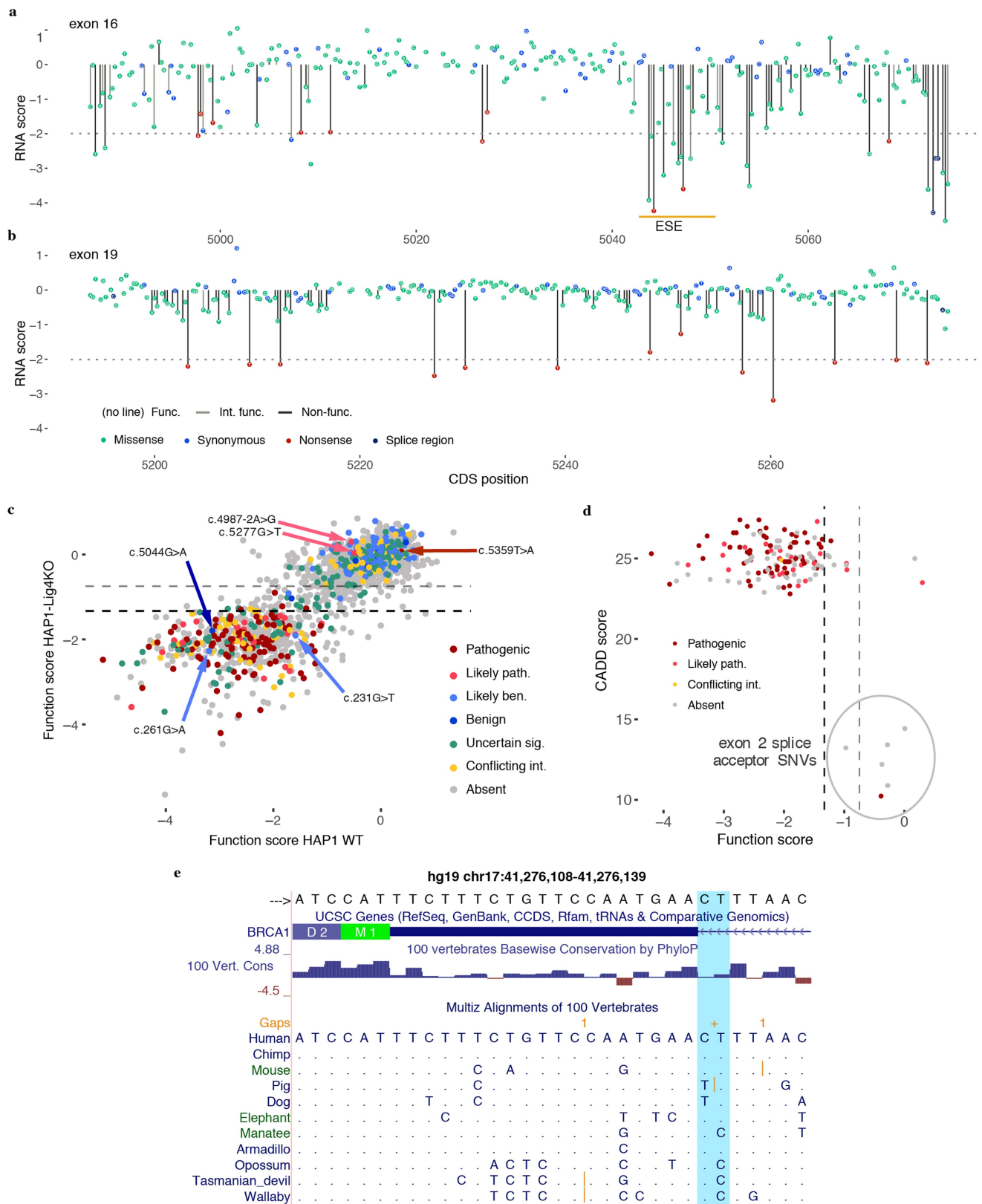
Extended Data Fig. 8 | *BRCA1* SNVs observed more frequently in large-scale population sequencing are more likely to score as functional. **a–c**, SNV function scores are plotted against gnomAD (**a**), Bravo (**b**), and FLOSSIES (**c**) allele frequencies. **a**, Among the 302 SNVs assayed also present in gnomAD, higher allele frequencies associate with higher function scores (Wilcoxon signed-rank test, $P = 3.7 \times 10^{-12}$). **b**, Bravo is a collection of whole-genome sequences ascertained from 62,784 individuals through the NHLBI TOPMed program. Similarly to SNVs present in gnomAD, higher allele frequencies in Bravo correlate with higher function

scores. **c**, FLOSSIES is a database of variants seen in targeted sequencing of breast cancer genes sampled from approximately 10,000 cancer-free women who are at least 70 years old. Only 1 of 39 assayed SNVs present in FLOSSIES scored as non-functional. **c, d**, Missense SNVs in ClinVar are separated by whether they have (**c**) or have not (**d**) been seen in either gnomAD or Bravo and function scores across replicates are plotted, with dashed lines demarcating functional classes. A higher proportion of ClinVar missense SNVs absent from gnomAD and Bravo score as non-functional (50.6% versus 15.7%; Fisher's exact test, $P = 1.80 \times 10^{-17}$).



Extended Data Fig. 9 | SGE function scores correlate with computational metrics and perform favourably at predicting ClinVar annotations. **a**, SNV function scores are plotted against mammalian phyloP scores, with colours indicative of ClinVar status (Spearman's correlation shown). **b**, **c**, ROC curves show the performance of CADD scores and phyloP scores for discriminating ClinVar 'pathogenic' and 'benign' SNVs (including 'likely'), as described in Fig. 3b for SGE data. **d**–**g** Plots as in **a**, but for missense SNVs only, showing correlations between SGE function scores and CADD²⁸ scores, phyloP scores³⁹, Grantham differences (Grantham amino acid variation minus Grantham amino acid deviation; GV - GD), and align-GVGD classifications⁴⁷.

Missense SNV function scores also correlate with SIFT scores⁴⁸ ($\rho = 0.363$) and PolyPhen-2 scores⁴⁹ ($\rho = -0.277$). (Spearman's correlation, $P < 1 \times 10^{-37}$ for all correlations). **h**–**l**, ROC curves assess the performance of SGE function scores and each indicated metric at distinguishing firmly 'pathogenic' and 'benign' missense SNVs (not including 'likely'). **m**, **n**, SGE scores for missense variants are plotted against results from homology-directed repair assays^{9,31} (**m**) and results from transcriptional activation assays¹² (**n**). In cases where multiple SNVs assayed lead to same amino acid substitution, function scores were averaged and coloured red if either SNV had an RNA score less than -2 . Box plots depict the sample median (line) and the interquartile range (box).



Extended Data Fig. 10 | See next page for caption.

Extended Data Fig. 10 | Evidence supporting SNV scores in discordance with ClinVar classifications. **a, b**, Complete maps of RNA scores for exons 16 (**a**) and exon 19 (**b**) reveal highly variable sensitivity to RNA depletion. The location of the strongest predicted exonic splice enhancer in exon 16 is indicated by the orange line³⁶. **c**, Function scores (means from two replicates) are plotted to compare results from preliminary experiments in wild-type HAP1 to those in HAP1-LIG4^{KO}. Data are shown only for experiments with Spearman's correlations between replicates greater than 0.50 in wild-type HAP1 cells ($n = 2,096$ SNVs; exons 3, 4, 5, 16, 17, 19, 21). Discordantly classified SNVs are indicated with arrows. **c.19-2A>G** was the only firmly discordant SNV for which the function score could not be corroborated in wild-type HAP1, consequent to low reproducibility of exon 2 wild-type function scores. Indeed, **c.19-2A>G** scored highly variably between wild-type replicates. **d**, The sequence-

function map of exon 21 is shown with the function scores for the two 'pathogenic' SNVs observed in linkage indicated. Dashed lines demarcate functional classifications. **e**, Function scores are plotted against CADD scores for all canonical splice SNVs assayed, coloured by ClinVar status. The six possible exon 2 splice acceptor SNVs (circled) have the lowest CADD scores among all canonical splice SNVs assayed, and none score as 'non-functional'. **e**, A USCS Genome Browser shot shows the PhyloP conservation track and selected mammalian sequence alignments for the exon 2 acceptor region, with the canonical acceptor site nucleotides highlighted in light blue (hg19 chr17:41,276,108-41,276,139). Multiple mammalian species are identified that have a G at position c.19-2 of the human transcript (corresponding to a C in the plus-strand orientation shown).

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When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

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- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
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Only common tests should be described solely by name; describe more complex techniques in the Methods section.
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- A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
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- Clearly defined error bars
State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on [statistics for biologists](#) may be useful.

Software and code

Policy information about [availability of computer code](#)

Data collection

No software was used except for Illumina RTA basecalling.

Data analysis

Common, freely available DNA sequencing data analysis software was used to analyze data, as described in Methods: bcl2fastq v2.16, Python 2.7.3, SeqPrep (available at <https://github.com/jstjohn/SeqPrep>), fastqc v0.11.3, EMBOSS v6.4.0, R v3.1.3, RStudio v1.0.153.

Custom scripts were written in Python and R to analyze data, and are available at: https://github.com/shendurelab/saturationGenomeEditing_pipeline.

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All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
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Function scores are freely available for all nonprofit uses (see <https://sge.gs.washington.edu/BRCA1/>), as well as by nonexclusive license under reasonable terms to commercial entities that have committed to open sharing of BRCA1 sequence variants. Sequencing data is available at GEO under accession GSE117159. Custom scripts for analyzing sequencing data were written in Python and R. All code is available at: https://github.com/shendurelab/saturationGenomeEditing_pipeline.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	<p>No explicit calculations were performed to determine sample size. Rather, we aimed to test every possible nucleotide in each of the 13 BRCA1 exons that encode for the RING and BRCT domains. Therefore, in each transfection, the number of SNVs tested was roughly 3-fold the number of nucleotides in the exon tested (including some adjacent intron sequence), corresponding to all possible SNVs. Our achieved sampling is shown in Extended Data Fig. 3c.</p> <p>To effectively sample each SNV in each experiment, we transfected cell populations large enough to maximize the chance every library SNV was introduced to the genome hundreds of times each (approximately 20,000,000 cells per experiment).</p>
Data exclusions	<p>Exclusion criteria were not pre-established. Exclusions were performed prior to analyzing the accuracy of the data at predicting clinical variant interpretations. Conclusions remain the same with or without exclusions, as shown in Extended Data Fig. 6e-g.</p> <p>Approximately 3.5% of SNV measurements were excluded. In short, data were excluded according to uniformly applied rules to ensure scores were reflective of experimental selection and not poor sampling and/or artifacts resulting from the genome editing process (Extended Data Fig. 6a).</p> <p>More specifically, to rule out artificial sampling from sequencing error, SNVs not adequately sampled on Day 5 (over 1 in 100,000 reads) were excluded. Certain SNVs located near Cas9 target sites made the enzyme more likely to re-cut the genome after editing, thereby artificially lowering the SNVs abundance (Extended Data Fig. 6b,c). Additionally, SNVs that occurred near HDR marker mutations and due to this, caused different amino acid sequence changes or had greater potential to disrupt splicing were also excluded.</p> <p>RNA data for exon 18 was excluded due to a lack of reproducibility across replicates (Extended Data Fig. 4), and exon 22 WT data was excluded due to primer cross-reactivity that led to an uncharacteristically high correlation between plasmid library measurements and Day 5 measurements (Extended Data Fig. 4a).</p>
Replication	<p>All SGE experiments were performed at least two times to assess reproducibility, which is described in Extended Data Fig. 4. A small fraction of SNVs scored discordantly between replicates were removed from the data set (n=14 SNVs, Extended Data Fig. 6a).</p> <p>Two replicates were obtained in both WT HAP1 cells, and HAP1-Lig4KO cells. Results between cell lines also showed a high correlation (Extended Data Figs. 3i and 10c).</p>
Randomization	Variants were tested in multiplex, with hundreds of variants related by physical proximity in the genome per sample.
Blinding	Function scores for each variant were determined without knowledge of known pathogenicity status, which was only assessed after.

Reporting for specific materials, systems and methods

Materials & experimental systems

- | | |
|-------------------------------------|---|
| n/a | Involvement in the study |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Unique biological materials |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Antibodies |
| <input type="checkbox"/> | <input checked="" type="checkbox"/> Eukaryotic cell lines |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Palaeontology |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Animals and other organisms |
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Methods

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|-------------------------------------|--|
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| <input checked="" type="checkbox"/> | <input type="checkbox"/> ChIP-seq |
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| <input checked="" type="checkbox"/> | <input type="checkbox"/> MRI-based neuroimaging |

Eukaryotic cell lines

Policy information about [cell lines](#)

- | | |
|--|---|
| Cell line source(s) | HAP1 cells were obtained from Haplogen (now Horizon Discovery). |
| Authentication | Cells lines were authenticated by karyotype. |
| Mycoplasma contamination | Cell lines were not tested for mycoplasma. |
| Commonly misidentified lines
(See ICLAC register) | No commonly misidentified cell lines were used. |

Flow Cytometry

Plots

Confirm that:

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

- | | |
|---------------------------|---|
| Sample preparation | Live cell, DNA content staining with Hoescht dye |
| Instrument | BD FACS Aria II |
| Software | FlowJo10 |
| Cell population abundance | The haploid, 1N population was 29% |
| Gating strategy | The 1N population was identified by staining for DNA content. |

- Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.