Saturation editing of genomic regions by multiplex homology-directed repair

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Saturation mutagenesis1,2—coupled to an appropriate biological assay—represents a fundamental means of achieving a high-resolution understanding of regulatory3 and protein-coding protein nucleic acid sequences of interest. However, mutagenized sequences introduced in trans on episomes or via random or “safe-harbour” integration fail to capture the native context of the endogenous chromosomal locus. This shortcoming markedly limits the interpretability of the resulting measurements of mutational impact. Here, we couple CRISPR/Cas9 RNA-guided cleavage6 with multiplex homology-directed repair using a complex library of donor templates to demonstrate saturation editing of genomic regions. In exon 18 of BRCA1, we replace a six-base-pair (bp) genomic region with all possible hexamers, or the full exon with all possible single nucleotide variants (SNVs), and measure strong effects on transcript abundance attributable to nonsense-mediated decay and exonic splicing elements. We similarly perform saturation genome editing of a well-conserved coding region of an essential gene, DBR1, and measure relative effects on growth that correlate with functional impact. Measurement of the functional consequences of large numbers of mutations with saturation genome editing will potentially facilitate high-resolution functional dissection of both cis-regulatory elements and trans-acting factors, as well as the interpretation of variants of uncertain significance observed in clinical sequencing.

Functional consequences of genetic variants are best studied by manipulating the endogenous locus, which provides the native chromosomal context with respect to DNA sequence and epigenetic milieu, and for proteins, endogenous levels and patterns of expression. Programable endonucleases, for example, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) or clustered regularly interspaced short palindromic repeat (CRISPR)/Cas-based RNA-guided DNA endonucleases, enable direct genome editing with increasing practicality. However, genome editing has primarily been applied to introduce single changes to one or a few genomic loci, rather than many programmed changes to a single genomic locus.

We sought to leverage CRISPR/Cas9 to introduce saturating sets of programmed edits to a specific locus via multiplex homology-directed repair (HDR). We first targeted six bases of a BRCA1 exon (Supplementary Table 1). Five days post-transfection with pCas9-sgBRCA1x18 and the HDR library into ~800,000 HEK293T cells, achieving 3.33% HDR efficiency. We performed two independent transfections with the same HDR library and edited gDNA were consistent with limited ‘bottlenecking’ of hexamers within replicates and the correlation between the HDR library and edited gDNA was consistent with limited ‘bottlenecking’ during transfection and minimal influence of hexamer identity on HDR efficiency (Extended Data Figs 1 and 2).

We estimated the effect of introducing each hexamer to these genomic coordinates on transcript abundance by calculating enrichment scores (cDNA divided by gDNA counts, calibrated to wild type). These enrichment scores were well correlated between biological replicates (Fig. 1b, a vs 2a: R = 0.659) and between D3 replicates (Extended Data Fig. 2c; a vs 1b: R = 0.662). When we pooled read counts from D3 replicates, correlation between biological replicates improved (Extended Data Fig. 2d; 1 vs 2: R = 0.706).

To maximize precision (see Supplementary Note 1 for discussion of reproducibility), we merged data across all four replicates for 4,048 exons (Fig. 1c; Supplementary Table 2). Several results support the biological validity of the resulting enrichment scores. First, as anticipated by nonsense-mediated decay (NMD), hexamers introducing stop codons were associated with markedly reduced mRNA levels (Fig. 1c; Wilcoxon rank sum test (WRST) P = 9.7 × 10⁻⁸⁴; median for nonsense hexamers 12-fold below overall median). Second, previous studies measured hexamer influence on splicing at analogous coordinates of different exons via a plasmid minigene assay. Despite these contextual differences, the strongest exonic splicing silencers (ESSs) (bottom 2% in ref. 14) scored strongly below median (Fig. 1c; WRST P = 84; median for nonsense hexamers 12-fold below overall median). In clinical sequencing.

We next sought to assay the effects of SNVs across the full 78 base pairs of BRCA1 exon 18 (Extended Data Fig. 4). We cloned three HDR libraries with selective PCR sites in either the 5' or 3' region and 3% wild type (WT):1:1:1) in the other half of the exon (L: 5' degeneracy, 3' nonsynonymous selective PCR site; R: 3' degeneracy, 5' nonsynonymous selective PCR site; R2: 3' degeneracy, 5' synonymous selective PCR site) (Supplementary Table 1). Five days post-transfection with pCas9-sgBRCA1x18 (1.02–1.29% HDR efficiency), we selectively amplified and deeply sequenced gDNA and cDNA.

Using data from all edited exons with ≥ 1 mutation and ≥ 10 gDNA counts, we estimated effect sizes of all possible SNVs using a weighted linear model. Estimated effect sizes were reproducible (R = 0.846 (R), 0.853 (R2), and 0.686 (L); Fig. 2a, Extended Data Figs 5 and 6, Supplementary Table 3). Effect sizes for the same SNVs interrogated with different selective PCR strategies (R vs R2) were also well correlated (R = 0.847; Fig. 2b).

The estimated effect sizes reflect empirically measured changes in transcript abundance resulting from programmed edits (Fig. 2c). As expected with NMD, nonsense mutations reduced transcript abundance.
**Figure 1** | Saturation genome editing and multiplex functional analysis of a hexamer region influencing BRCA1 splicing. a, Experimental schematic. Cultured cells were co-transfected with a single Cas9-sgRNA construct (CRISPR) and a complex homology-directed repair (HDR) library containing an edited exon that harbours a random hexamer (blue, green, orange) and a fixed selective PCR site (red). CRISPR-induced cutting stimulated homologous recombination with the HDR library, inserting mutant exons into the genomes of many cells. At five days post-transfection, cells were harvested for gDNA and RNA. After reverse transcription, selective PCR was performed followed by sequencing of gDNA- and cDNA-derived amplicons. Hexamer enrichment scores were calculated by dividing cDNA counts by gDNA counts. b, Correlation of enrichment scores between biological replicates for hexamers observed in each experiment with positions of previously identified exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs) and stop codons indicated. c, Rank-ordered plot of enrichment scores with positions of ESEs, ESSs and stop codons indicated.

**Figure 2** | Multiplex homology-directed repair reveals effects of single nucleotide variants on transcript abundance. Three separate HDR libraries (R, R2, and L) containing a 3% mutation rate (97% WT, 1% each non-WT base) in either half of BRCA1 exon 18 were introduced to the genome via co-transfection with pCas9-sgBRCA1x18. Enrichment scores were calculated for each haplotype observed at least 10 times in gDNA sequencing, and effect sizes of SNVs were determined by weighted linear regression modelling. ‘Sense’ includes both missense and synonymous SNVs. a, Effect sizes calculated from replicate transfections of HDR library R, consisting of a 3% per-nucleotide mutation rate in the 3’-most 39 bases and the same selective PCR site used in Fig. 1, were highly correlated ($R = 0.846$). b, Library R2 harboured a selective PCR site composed of 5 synonymous changes, none of which are present in library R. When effect sizes derived from experiments with library R2 were plotted against those from library R, there was a strong correlation ($R = 0.847$), indicating reproducibility and demonstrating that differences between selective PCR sites did not strongly influence scores. c, Effect sizes for SNVs across the exon are displayed. Data sets from libraries R and L were combined to span the entire exon. Dashed lines represent SNVs that introduce nonsense codons.
Figure 3 | Saturation genome editing and multiplex functional analysis at an essential gene, DBR1, in Hap1 cells. An HDR library targeting a highly conserved region of DBR1 exon 2 was used with pCas9-EGFP-sgDBr1x2 to introduce point mutations across 75 bp and all possible codon substitutions at three residues believed to participate at the enzyme’s active site. a, Sequencing of gDNA from the HDR library and populations of edited cells at D5, D8 and D11 reveals selection for synonymous mutations, and depletion of frameshift, nonsense and missense variants. b, Mean D11 enrichment scores are plotted as line segments for SNVs in the 3′-most 73 bases of exon 2 and the 2 bases of intron 2. Above the enrichment scores in ascending order are the WT nucleotide at each position, each 1-bp genome edit, the wild-type amino acid (AA), and the AA derived from each genome edit (asterisk indicates a stop codon). Segment colour indicates mutation type, faded segments indicate discordant effects between replicates, and AAs are coloured according to the Lesk colour scheme (orange, small nonpolar; green, hydrophobic; magenta, polar; red, negatively-charged; blue, positively charged). The first nine bases shown correspond to the active site residues. c, D8 (top) and D11 (bottom) amino acid level enrichment scores were calculated for active site residues N84, H85, E86 after excluding discordant observations between replicates (Extended Data Fig. 10c). On both D8 and D11 we observe strong selective effects and tolerance of only synonymous (green boxes) and a few missense variants.

An optimized single-guide RNA (sgRNA) sequence was cloned into a bicistronic sgRNA/Cas9-2A-EGFP vector (pCas9-EGFP-sgDBr1x2). Five million haploid human cells (Hap1) were co-transfected with the DBR1 HDR library and pCas9-EGFP-sgDBr1x2. On D2, ~250,000 enhanced green fluorescent protein-positive (EGFP⁺) cells were sorted by fluorescence-activated cell sorting (FACS) and further cultured, taking samples on D5, D8 and D11 (1.14% HDR efficiency, estimated on D8). Following gDNA isolation and selective PCR, deep sequencing was performed to quantify the relative abundance of edited haplotypes in each sample.

We first examined the relative proportions of mutation classes at each time point (Fig. 3a). The strong enrichment of synonymous mutations and depletion of nonsense and frameshifting mutations over time indicated that selection was acting on edited cells in culture, consistent with DBR1 essentiality. We calculated enrichment scores (D8 or D11 counts divided by D5 counts) for 365 of the 388 (94%) programmed edits and 12 single base deletions (the subset with relative abundance >5 x 10⁻⁵ on D5) (Fig. 3b; Extended Data Fig. 9; Supplementary Table 4). Enrichment scores strongly correlated with functional consequence. The median enrichment score for synonymous edits was nearly identical to wild-type (1.006-fold lower), but 73-fold lower for missense edits (P = 1.7 x 10⁻⁸, WRST against synonymous edits), 207-fold lower for nonsense edits (P = 1.9 x 10⁻⁸), and 211-fold lower for frameshifting single base deletion edits (P = 1.5 x 10⁻⁸). Furthermore, enrichment scores for SNVs were inversely correlated with metrics of predicted deleteriousness like CADD (ρ = -0.295; P = 1.2 x 10⁻⁵), and 211-fold lower for frameshifting single base deletion edits (P = 1.5 x 10⁻⁸). Residues N84, H85 and E86 of DBR1 were edited to all 63 possible non-wild-type codons. Consistent with their predicted role in the active site of an essential enzyme, only synonymous mutations and a few missense substitutions were tolerated (Fig. 3c).

Amino acid level enrichment scores were well correlated between D11 biological replicates (R = 0.752; P = 2.6 x 10⁻⁵; Extended Data Fig. 10c), and were bimodally distributed in each replicate, allowing broad classification of changes as tolerated or deleterious. The small proportion of discordantly classified variants might be explained by Hap1 reversion to diploidy or off-target effects, highlighting the importance of G-rich biological replicates for this experimental design (Supplementary Note 1). Notably, there were no reproducibly tolerated nonsense or frameshifting edits. Overall, these data support the conclusion that our empirically derived enrichment scores reflect true biological effects of specific genomic point mutations within DBR1.

We demonstrate that it is feasible to generate and functionally analyse hundreds to thousands of programmed genome edits at a single

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locus in a single experiment. We emphasize three major limitations of the method as it stands. First, we only introduced programmed edits to the immediate vicinity of coordinates targeted by the endonuclease (Extended Data Figs 5a and 9a), and the narrow window associated with HDR mechanisms in mammalian cells may fundamentally limit the size of the region that can be subjected to multiplex editing in one experiment. Saturation genome editing of a full gene—for example, to measure functional consequences of all possible variants of uncertain significance—will require multiple experiments tiling along its exons.

Second, only a small proportion of cells were successfully edited in each experiment, bottlenecking complexity, limiting reproducibility (Supplementary Note 1), and necessitating the selective PCR site. Looking forward, a variety of techniques, for example, transient hypothermia or oligonucleotide-based HDR, may improve editing efficiency. Consistent with this, we note that ZFNs and TALENs have demonstrated efficiencies up to 50% in some studies. Also, although the low editing efficiency necessitated using haploid cells for DBR1 mutagenesis, this could potentially have been performed in diploid cells by knocking out one allele via NHEJ and then knocking in the HDR library to the other allele.

Finally, the development of functional assays that are biologically relevant and technically viable remains a challenge. Here, we exploited strategies that directly linked genotype to phenotype—for example, targeted RNA sequencing to measure transcript abundance or targeted DNA sequencing to measure reduced cellular fitness. Analogous approaches can be taken in other contexts—for example, targeted chromatin immunoprecipitation-sequencing (ChIP-seq) of co-activators to assay enhancers, increased cellular growth rate to assay cancer drivers or drug resistance, or FACS-based phenotypic sorting for cellular assays more generally (Supplementary Note 2).

There is a strong demand for techniques that accurately and scalably measure mutational consequences, and a dearth of experimental data measuring distributions of effect sizes or corresponding to direct manipulation of the genome. By multiplexing both the introduction and assaying of mutations in their native context, we anticipate that saturation genome editing will accelerate our ability to measure and interpret the functional consequences of genetic variation.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 6 May; accepted 18 July 2014.

Published online 20 August 2014.

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Supplementary Information is available in the online version of the paper.

Acknowledgements We thank F. Zhang and his laboratory for the CRISPR/Cas9 backbone constructs used in this study and G. Church and his laboratory for providing reagents used to establish CRISPR/Cas9 editing techniques in our lab. We also thank members of the Shendure laboratory for helpful discussions and D. Prunkard for assistance with FACS. This work was supported by the National Institutes of Health (DP1HG007811 to J.S.) and the UW Medical Scientist Training Program (G.M.F. and J.K.).

Author Contributions The project was conceived and designed by G.M.F. and J.S. G.M.F. and E.A.B. performed experiments. E.A.B. and R.J.H. performed data analysis and generated data figures. G.M.F. generated schematic figures. G.M.F., E.A.B., R.J.H. and J.S. wrote the manuscript. J.C.K. assisted G.M.F to establish genome editing techniques in our lab. We also thank members of the Shendure laboratory for helpful discussions and D. Prunkard for assistance with FACS. This work was supported by the National Institutes of Health (DP1HG007811 to J.S.) and the UW Medical Scientist Training Program (G.M.F. and J.K.).

Author Information Sequence data used for this analysis are available in SRA under accession number SRP044126. Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to J.S. (shendure@uw.edu) or G.M.F. (g2f@uw.edu).
The DBR1 HDR library was cloned as above except with the following differences. HDR library variants were derived from 388 oligonucleotides synthesized on a microarray (CustomArray) to include all possible single base pair changes in a 75-bp region comprising part of DBR1 exon 2 (chr3:13789234–137892416), all coding variants at the first three residues of the 75-bp region (chr3:137892408–137892416), and the reference 75-bp sequence. All DBR1 HDR library sequences also included two synonymous mutations designed to prevent re-cutting of edited genomes by disrupting PAM and protospacer sequences (chr3:137892424 and chr3:137892421), and a 6-bp selective PCR site in intron 2 of DBR1 (chr3:137892331–137892336). The library was cloned into a pUC19-DBR1ex2 backbone, a vector containing the surrounding DBR1 sequence cloned from hap1 gDNA (chr3:137891573–137892939).

A bicistronic Cas9-sgRNA vector designed to cleave within DBR1 exon 18 (‘pCas9-sgBRCA1x18’) was cloned according to a published protocol20 by ligating annealed oligonucleotides into a human codon-optimized Streptococcus pyogenes Cas9-sgRNA vector from the lab of Feng Zhang (pX330-U6-Chimeric_BB-CBh-hSpCas9; Addgene plasmid #42230). The same protocol was followed to create pCas9-EGFP-sgDBR1x2 from a similar Zhang lab vector that allows for fluorescent identification of Cas9-expressing cells (pSpCas9(BB)-2A-GFP (pX458); Addgene plasmid #48138).

Cell culture and transfection. For BRCA1 experiments, HEK293T cells were cultured in Dulbecco’s modified Eagle medium (Life Technologies) supplemented with 10% FBS and 100 U ml

\[^{-1}\] penicillin + 100 µg ml

\[^{-1}\] streptomycin. ~3 × 10

\[^{6}\] Hap1 cells were passaged to a 60-mm dish in antibiotic-free media one day before co-transfection with 3 µg each of pCas9-EGFP-sgDbr1x2 and the DBR1 HDR library via Transfection 8.0 (OriGene) according to protocol. On D2, FACS was performed (BD FACSaria III) to isolate ~250,000 EGFP

\[^{+}\] cells which were then expanded in culture with samples taken of ~1 × 10

\[^{6}\] cells on D5, and 4–8 × 10

\[^{6}\] cells on D8 and D11. gDNA was isolated according to protocol with the QiaAmp Kit (Qiagen). A biological replicate was performed, as well as negative controls in which the HDR library was transfections with the empty pSpCas9(BB)-2A-GFP construct (to enable FACS of transected cells without editing).

Reverse transcription, selective PCR and sequencing. For BRCA1 experiments, reverse transcription (RT) was performed using SuperScriptIII (Invitrogen) with a general primer (primer located in their BRCA1 exon 18 [10,000 bp from the start] or exon 21 [whole exon experiments]). Initial rounds of PCR were performed on large quantities of sample gDNA (8–12 µg gDNA, 100–150 ng per reaction) and cDNA (25 µg total RNA reverse transcribed and split into 45–47 reactions) using the KAPA HiFi HotStart ReadyMix PCR kit. In the first gDNA PCR, a primer external to the HDR library was used to prevent amplification of plasmid DNA. cDNA reactions were either primed from exons 16 and 18 (hexamer experiment; Library L) or exons 18 and 20 (Libraries R, R2). After the initial gDNA and cDNA reactions, all PCR products from a single sample were pooled and purified using the QIAquick PCR Purification Kit (Qiagen).

For both cDNA and gDNA reactions, a primer designed to selectively amplify edited products bearing the selective PCR site was used either in the first or second reaction. Optimal temperature and annealing time for each primer was determined via gradient PCR, and negative control reactions were performed using input from HDR library-only transfections to ensure products were derived from edited genomes as opposed to the HDR library. Negative controls failed to amplify for all experiments. Two subsequent PCRs were performed to add sequencing adaptors (‘PU1’ and ‘PU2’), sample indices, and flow cell adapters.

For the DBR1 experiment, 30 cycles of selective PCR were performed on gDNA (300 ng per reaction) from D5 (3 µg), D8 and D11 (27 µg) each. Wells from each sample were pooled, PCR purified, and then re-amplified for 15 additional cycles. The 1,055 bp product was gel-purified (QIAquick Gel Extraction Kit, Qiagen), and two subsequent PCRs were performed to incorporate sequencing and flow cell adapters before sequencing as above.

After final reactions were purified (AMPure XP beads, Agencourt), paired-end sequencing was performed on all samples with the Illumina MiSeq to quantify gDNA and/or cDNA abundances for each edited haplotype. All primer sequences for reverse transcription, selective PCR, and sequencing library preparation are provided in Supplementary Table 1.
HDR efficiencies were estimated for all experiments via deep sequencing of target loci by performing PCR on 150–300 ng of gDNA using primers external to the region of editing and the selective PCR site. Reported HDR efficiencies were conservatively calculated as the fraction of sequencing reads containing the selective PCR site and bearing at least one variant represented in the HDR library. Analysis of sequencing data. For quality control, fully overlapping paired-end reads were merged with PEAR30 (Paired-End reAd mergE) and discordant pairs were eliminated. By design, the mutagenized region is covered by both the forward and reverse reads on the Illumina platform, resulting in high-confidence calls per site.

For BRCA1 hexamer reads to be included, the six bases on either side of the hexamer were required to match the reference sequence, and every base call in the hexamer required a quality score of at least Q30. For BRCA1 whole-exon mutagenesis, the full read was required to be the correct length and match the library consensus sequence outside of the mutagenized region, every base quality score inside the mutagenized region was required to be at least Q30, and no indels were tolerated in alignment with BWA-MEM33. cDNA reads not matching any gDNA inside the mutagenized region was required to be at least Q30, and no indels were tolerated. Based on the bimodal distribution observed in each replicate, mutations with fold change relative to the wild type sequence was taken to calculate an enrichment score. In each replicate, the abundance of each haplotype, the null expectation of equal variance (\( \sigma^2 \)) for each cDNA/gDNA ratio was violated. Because each effect size (\( \sigma^2 \)) was the average of \( n_{ij} \) observations (reads), then \( \text{var}(y_{ij}) = \frac{\text{var}(y)}{n_{ij}} = \sigma^2/n_{ij} \), suggesting that the weight for each variable should be \( n_{ij} \). To predict single nucleotide effect sizes across exon 18 of BRCA1, we then fit the weighted linear model:

\[
y_{ij} = \beta_0 + w_{ij} \beta_1 X_{ij}
\]

in which \( y_{ij} \) is the loge enrichment score for a given haplotype, \( w_{ij} \) is the number of gDNA reads for a given haplotype, \( \beta_1 \) is the effect of nucleotide i at position j relative to the wild-type allele, and \( X_{ij} \) is a dummy variable indicating the presence or absence of a particular nucleotide change i at position j relative to the wild-type allele. Regression analyses were performed in R 3.0.0 using the lm() function. The resulting coefficients of the model adjusted for the intercepts (\( \beta_0 + \beta_1 X_{ij} \)) were interpreted as effect sizes of the individual SNVs on exon splicing/stability. To merge data across replicates, effect sizes were averaged (including across overlapping bases between libraries L and R in the BRCA1 exon).

Comparisons to other metrics of functional impact. For comparison to plasmid studies, ESR-seq scores were taken from ref. 14. Hexamers with positive ESR-seq scores are deemed exonic splicing enhancers, whereas negative ESR-seq scores denote exonic splicing silencers. For comparison of BRCA1 exon 18’s SNV effect sizes to an in silico method, all SNVs were queried on MutPredSplice’s web server (http://mutdb.org/mutpredsplice/submit.htm). MutPredSplice reports a single score estimating the likelihood that a variant will disrupt splicing at any genomic locus. Absolute values of BRCA1 exon 18 splicing effect sizes were then correlated with MutPredSplice scores to determine concordance between our data and predicted effects on splicing.

For DBR1, calculated enrichment scores were compared to BLOSUM62 substitution34 (obtained from NCBI), PolyPhen-2 (ref. 35), and CADD22 (PolyPhen-2 and CADD scores obtained from querying genomic coordinates from CADD’s pre-computed genomic annotations (http://cadd.gs.washington.edu/download).
Extended Data Figure 1 | Distributions and pair-wise correlations of hexamer abundances. a, The relative abundance of hexamers within the HDR library (red), gDNA (blue), cDNA data (green) are shown for a single experiment. The vertical black line represents our threshold of 10 gDNA reads. b–d, Scatterplots from a single replicate show pair-wise correlations between sequencing counts for the HDR library, gDNA, and cDNA for hexamers with at least 10 observations in the gDNA library, excluding wild type and control hexamers ($n = 3,633$). The HDR library and the gDNA data are most highly correlated ($R$ 95% confidence interval (CI): 0.596–0.636), followed by the gDNA and cDNA ($R$ 95% CI: 0.419–0.471) and the HDR library and cDNA ($R$ 95% CI: 0.341–0.394).
Extended Data Figure 2 | Correlations for hexamer genome editing efficiency and enrichment scores between replicates.  

**a**, gDNA counts for all hexamers with at least ten reads in each of two gDNA preps from separate transfections with the same HDR library (n = 2,980) exhibited moderate correlation (R 95% CI: 0.355–0.416). **b**, However, hexamer editing rates, defined as gDNA counts normalized to HDR library counts, were substantially less correlated (R 95% CI: 0.084–0.155), consistent with a hexamer’s HDR library abundance contributing more to its gDNA abundance than systematic differences in HDR efficiency secondary to the hexamer sequence itself.  

**c**, Hexamer enrichment scores for two pools of cells from a single transfection split on D3 were well-correlated (R 95% CI: 0.643–0.681).  

**d**, Pooling data from cells split on D3 replicates from a single transfection yielded an improved correlation between biological replicates (that is, independent transfections; R 95% CI: 0.690–0.722).
Extended Data Figure 3 | Comparison of genome-based hexamer enrichment scores to plasmid-based hexamer scores. a, There was a modest correlation between ESS and ESE hexamers defined by a previous study $^{14}$ (x-axis) and the enrichment scores calculated here (y-axis; Spearman $\rho = 0.524$). The previous study also interrogated hexamers positioned +5 to +10 nucleotides relative to a splice junction, but was plasmid-based rather than genome-based and in the context of different exons. b, To reveal effects of GC content on hexamer abundance, histograms display the distribution of enrichment scores for each possible G+C level (0–6). Hexamers containing two or fewer G+C base pairs exhibited broadly lower enrichment scores than hexamers containing three or more G+C base pairs.
Extended Data Figure 4 | Experimental schematic for genome editing and functional analysis of BRCA1 exon 18. Cultured cells were co-transfected with a single Cas9-sgRNA construct (CRISPR) and an HDR library. Each HDR library was generated from cloning of an oligonucleotide synthesized with 3% nucleotide degeneracy (97WT:1:1:1) for approximately half of the exon and a selective PCR site introduced to the other (fixed) half of the exon (red).

CRISPR-induced HDR integrates mutant exons into the genome. Cells were cultured for five days post-transfection, and then harvested for gDNA and total RNA. After reverse transcription, selective PCR was performed before sequencing the edited pools of gDNA and cDNA. Each exon haplotype’s enrichment score was measured by dividing cDNA reads by gDNA reads, and effect sizes for each SNV were calculated via weighted linear regression.
Extended Data Figure 5 | Positional SNV editing rates and replication of effect sizes. 

a, Editing rates for each SNV in BRCA1 exon 18 were calculated by dividing each SNV’s gDNA sequencing abundance by its HDR library abundance. Editing rates were then plotted across the exon for each library (red = L, blue = R, green = R2) with locations of their selective PCR sites and the CRISPR-targeted PAM illustrated below. For HDR libraries R and R2, there was a subtle decrease in editing rate with increasing distance from the Cas9 cleavage site ($\rho^R = -0.264$, $p^R = 4.1 \times 10^{-3}$; $\rho^{R2} = -0.361$, $p^{R2} = 4.8 \times 10^{-5}$). For library L, which allowed re-cutting by not destroying the PAM, there was a sharp peak of editing centred on the Cas9 cleavage site, and a rapid decline in efficiencies in the 5’ direction (further from the 3’ selective PCR handle). 

b–c, SNV effect sizes were concordant across biological replicates for libraries R2 (b) and L (c) (library R shown in Fig. 2). Notably, variants of high effect size scored similarly across independent transfections.
Extended Data Figure 6 | Biological replicate effect size reproducibility for all libraries. Three separate HDR libraries (R, R2, and L) containing 3% nucleotide degeneracy in either half of BRCA1 exon 18 were introduced into the genome via co-transfection with pCas9-sgBRCA1x18. Enrichment scores were calculated for each haplotype observed at least ten times in the gDNA, and effect sizes of SNVs were determined by weighted linear regression. Effect sizes of individual variants for libraries R2 (left), R (middle), and L (right) were well correlated between biological replicates. Dashed lines represent SNVs that introduce nonsense codons.
Extended Data Figure 7 | Correlation between effect sizes and predicted disruption of splicing motifs and indel effects. a, MutPred Splice was used to predict the functional impact of all 234 single nucleotide substitutions on splicing in BRCA1 exon 18 (x-axis), and these scores were compared to absolute values of our empirically measured effect sizes (y-axis; \( r = 0.322 \)). Although nonsense variants contributed to this trend, the sense variants with the largest effect sizes generally had high MutPred Splice scores. b, For indels observed in gDNA from library 2 (virtually all of which occur at the Cas9 cleavage site), size frequencies are plotted. Indel size \( = 0 \) includes all haplotypes with wild type length. c, For each indel size, enrichment scores were calculated and normalized to that of the average full length exon. As predicted by nonsense-mediated decay, indels that shift the coding frame were associated with low transcript abundance.
Extended Data Figure 8 | Experimental schematic for saturation genome editing and multiplex functional analysis of DBR1 exon 2. Hap1 cells were co-transfected with a single Cas9-2A-EGFP-sgRNA construct (CRISPR) and an HDR library cloned from array-synthesized oligonucleotides containing programmed SNVs (orange, blue) and active site codon substitutions (green). The HDR library exon haplotypes also included two synonymous mutations (red) to disrupt PAM and protospacer sequences to prevent Cas9 re-cutting, and a 6 bp selective PCR site (light blue) substituted in the downstream intron. Successfully transfected cells (EGFP+) were selected on D2 by FACS, and cultured. On D5, D8, and D11, samples of cells were taken and selective PCR was performed before targeted sequencing of gDNA. Each haplotype’s enrichment score, a measure of the haplotype’s fitness in cell culture, was calculated by dividing D8 or D11 abundance by D5 abundance.
Extended Data Figure 9  | DBR1 editing rates by position and comparison of haplotype abundances between D5 and the HDR library, D8, and D11.

a, Editing rates for programmed SNVs represented in the DBR1 gDNA library above threshold (n = 216) were calculated by normalizing each SNV’s gDNA abundance by its HDR library abundance. Rates are plotted by position, with the locations of the targeted PAM (orange) and selective PCR site (purple) indicated below. The editing rate did not significantly change with position (P > 0.05), consistent with positional effects being negated by eliminating re-cutting and performing selective PCR from a distal site.

b, Scatterplots display the frequencies at which each haplotype was observed in the D5 sample vs the HDR library, D8, and D11 samples. To account for bottlenecks from editing of a limited number of cells in this representative experiment, analysis of individual haplotypes was restricted to those present at frequencies above 5 × 10^-5 in the D5 sample (n = 377; represented by the vertical line). Selection was evident by the depletion of many haplotypes in D8 and D11 samples.
Extended Data Figure 10 | Performance of computational predictions of deleterious DBR1 mutations and reproducibility between biological replicates. 

**a**, D11 enrichment scores from a single experiment were used to empirically define deleterious mutations as those with scores fourfold below wild type (vertical line).

**b**, Three in silico metrics of functional impairment were tested for their ability to anticipate the deleteriousness of these mutations as indicated by the area under the receiver operating characteristic curve (AUC): BLOSUM62 (AUC = 0.672, 214 SNVs), PolyPhen-2 (AUC = 0.671, 155 non-synonymous SNVs), and CADD (AUC = 0.701, 214 SNVs). Despite the different approaches of these algorithms, all three exhibited comparably moderate predictive power.

**c**, A biological replicate of the DBR1 experiment was performed and D11 enrichment scores for amino acid substitutions were well correlated (grey lines on scatterplot indicate the 'deleteriousness' threshold of fourfold depletion). The distribution of amino acid level enrichment scores for each experiment is displayed along each axis, reflecting bimodality. Notably, unexpected effects (that is, nonsense mutations scoring as tolerated) were among the relatively small percentage of effects not consistent between replicates.