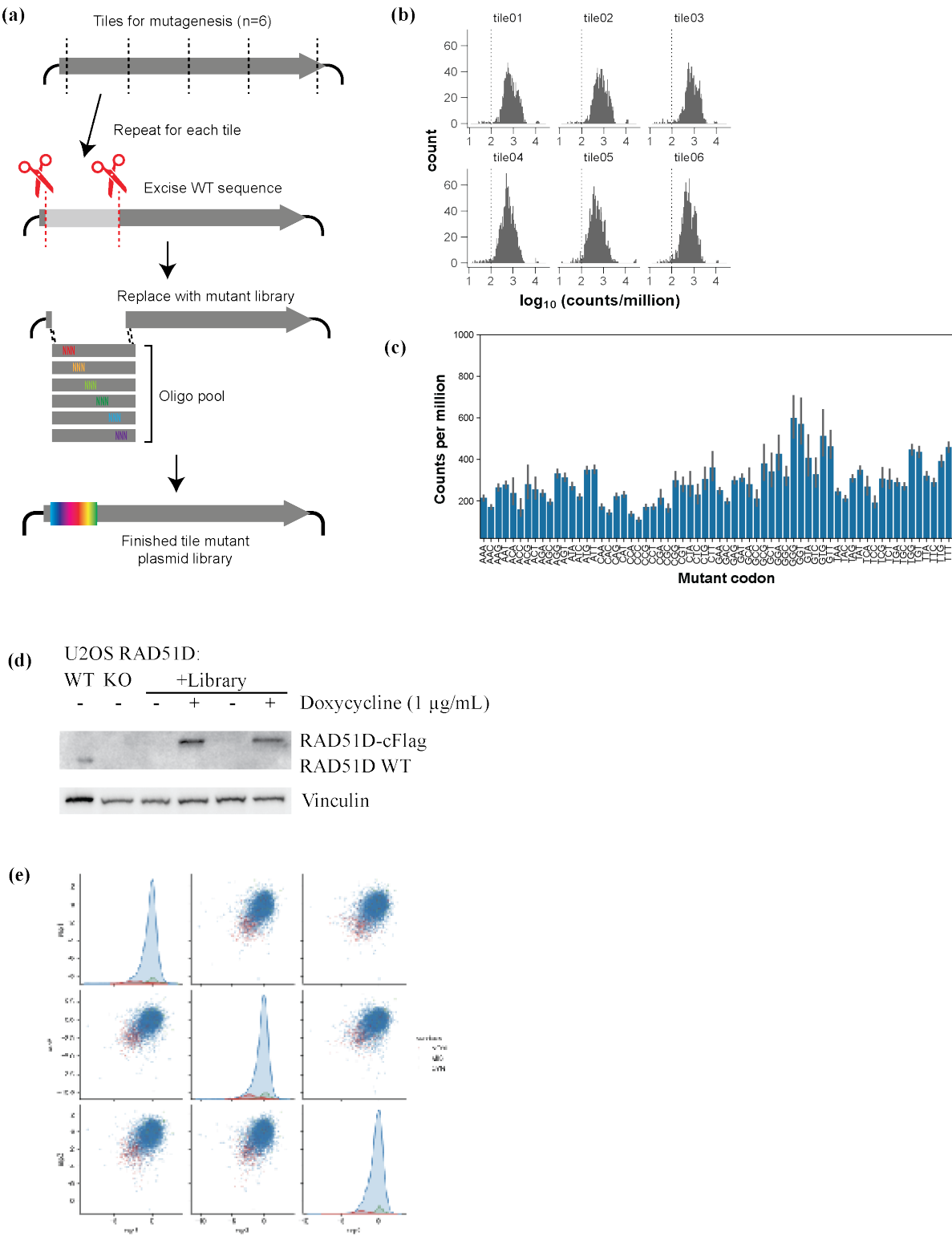


**Supplemental Information:**

**Supplementary Table 1: RAD51D Tiles**

| <b>Tile ID</b> | <b>Codon Start</b> | <b>Codon End</b> |
|----------------|--------------------|------------------|
| 1              | 1                  | 46               |
| 2              | 47                 | 93               |
| 3              | 94                 | 139              |
| 4              | 140                | 202              |
| 5              | 203                | 265              |
| 6              | 266                | 328              |

Supplementary Figure 1

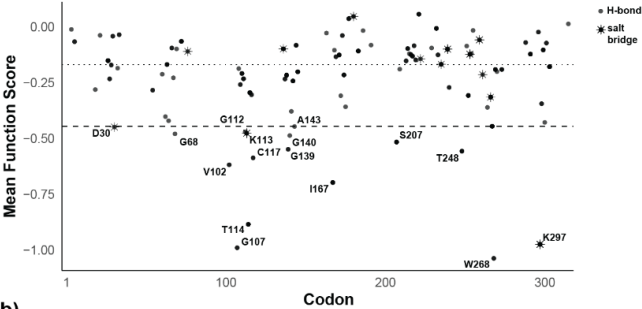


Supplementary Figure 1: Mutant library uniformity.

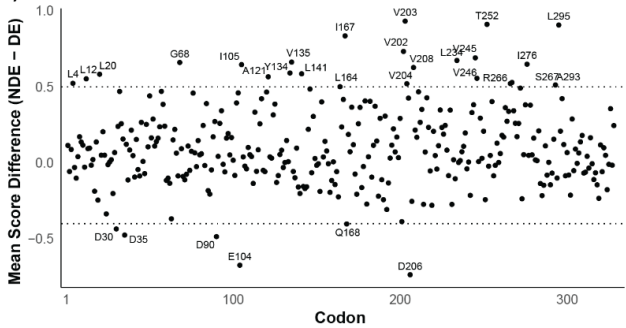
a) Diagram of the tile-based mutagenesis strategy. b) Histograms of *RAD51D* variant abundance,  $\log_{10}(\text{counts}/\text{million counts})$ , within pre-selection libraries for each mutagenesis tile. Cutoff line is at 100 cpm (1/10,000). c) Variant abundance, shown by mutant codon sequence. Overall,  $\geq 97\%$  of designed mutations are recovered, with a skew towards A+T-rich codons (mean of 310, 292, 284, and 280 cpm for codons with 0, 1, 2, or 3 G+C bases, respectively) d) Western showing doxycycline induced expression of two RAD51D tiles. e) Scatterplots showing correlation between function scores of experimental triplicates (mean pairwise Pearson's  $r=0.47$ )

Supplementary Figure 2

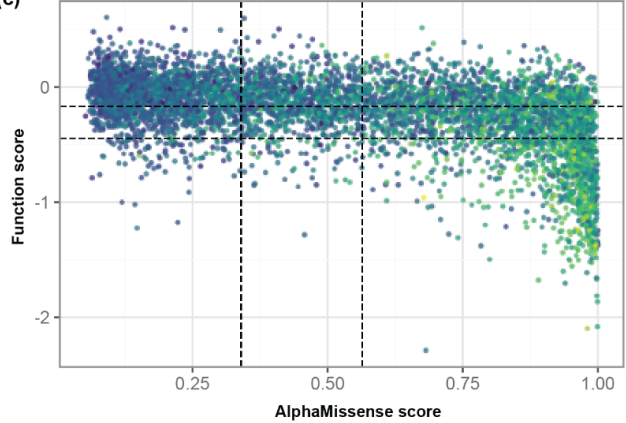
(a)



(b)



(c)



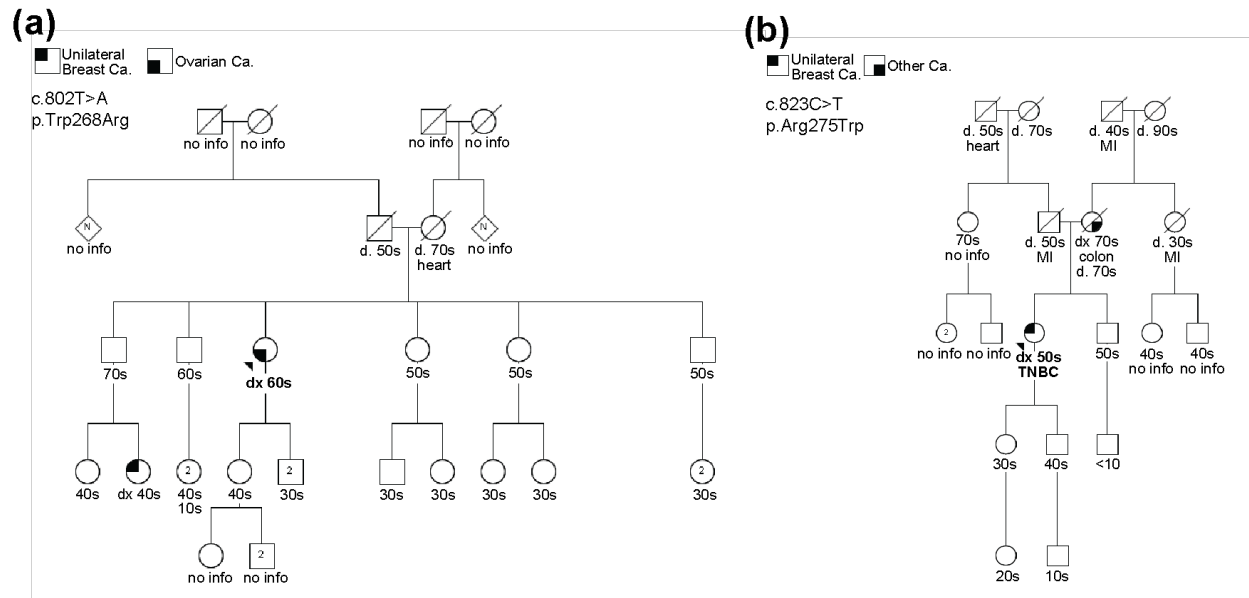
| AlphaMis Call | MAVE Call | n    | % of MIS |
|---------------|-----------|------|----------|
| benign        | NEU       | 1926 | 33.3     |
|               | INT       | 106  | 1.8      |
|               | LOF       | 92   | 1.6      |
| ambiguous     | NEU       | 814  | 14.1     |
|               | INT       | 96   | 1.7      |
|               | LOF       | 66   | 1.1      |
| pathogenic    | NEU       | 1503 | 26.0     |
|               | INT       | 331  | 5.7      |
|               | LOF       | 854  | 14.8     |

Supplementary Figure 2: MAVE analysis of unique LOF residues.

- (a) Mean function scores of residues making contact with other proteins, ATP, or DNA via H-bonds or salt bridges (noted with circles or starbursts, respectively) in the BCDX2 complex (as predicted in PDB: 8GBJ).
- (b) Intolerance of negatively charged (i.e. Asp (D) and Glu (E)) residues throughout RAD51D, as measured by the difference between the mean function scores of D/E and non-D/E mutations. Residues with a difference  $\pm 0.5$  are labeled.

(c) AlphaMissense pathogenicity scores compared to MAVE function scores. Each missense variant is one point, shaded by stability prediction ( $\Delta\Delta G$ ) from ThermoMPNN<sup>55</sup>. AlphaMissense scores were binned as benign, pathogenic, and ambiguous using the published cutoff values. Counts of each plotted category are reported in the table.

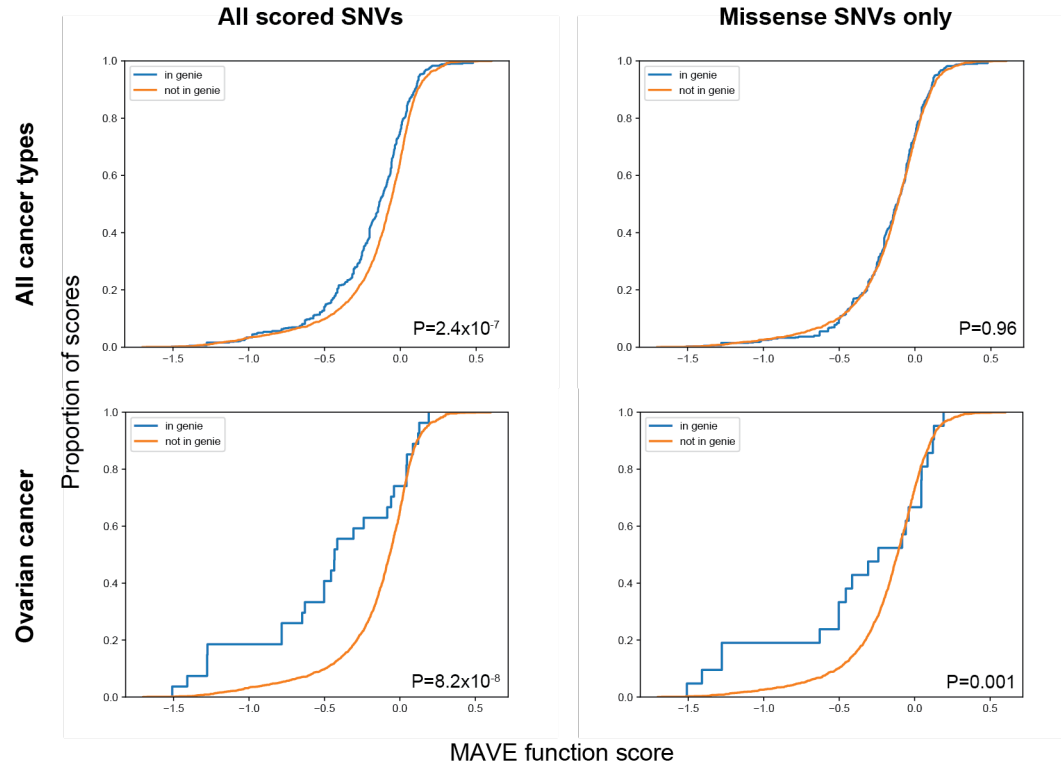
### Supplementary Figure 3



**Supplementary Figure 3. Pedigrees with HBOC and germline functionally abnormal *RAD51D* variants.**

Pedigrees for probands with HBOC and *RAD51D* VUS classified as functionally abnormal in our assay: **(a)** c.802T>A (p.Trp268Arg) and **(b)** c.823C>T (p.Arg275Trp). TNBC = Triple Negative Breast Cancer; MI = Myocardial Infarction

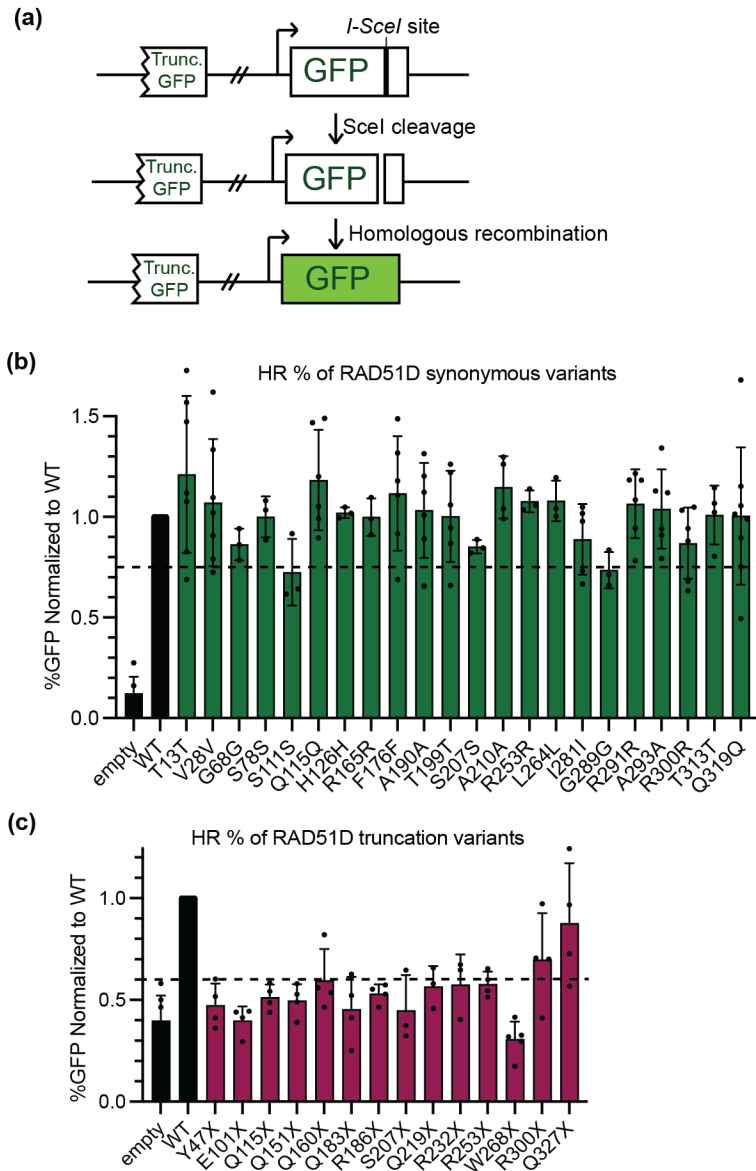
## Supplementary Figure 4



**Supplementary Figure 4. Somatic *RAD51D* variants in tumor genomes are enriched for LOF.**

Empirical cumulative distribution functions (ecdfs) of MAVE function scores, for SNVs present in AACR Project GENIE (blue) or absent (orange). Left column: all SNVs; right column: missense variants only. Upper row: all cancer types; lower row: ovarian cancer only. P values from *t*-test of AACR Project GENIE individuals' somatic variants vs scored SNVs (left: all; right: missense only) not present in GENIE.

## Supplementary Figure 5



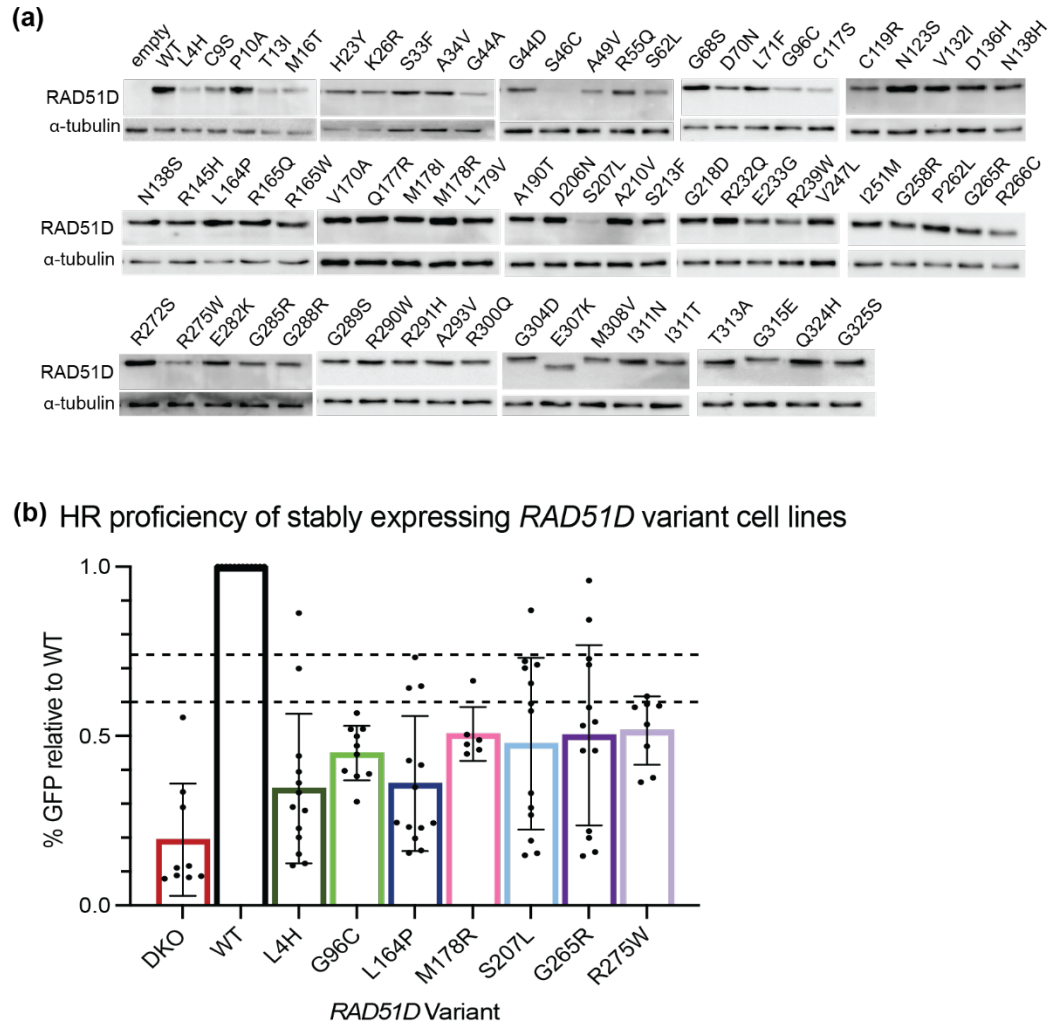
**Supplementary Figure 5: Series of synonymous and truncation variants used to determine HR thresholding for benign and pathogenic variants**

(a) Schematic of sister chromatid exchange assay to measure homologous recombination. In this assay, a cassette with a non-functional copy of GFP is integrated into *RAD51D* CRISPR/Cas9 U2OS cells. This GFP has a unique *I-SceI* restriction cut site. A DSB can be induced by expression

of a plasmid expressing the I-SceI restriction enzyme. GFP expression is restored by use of a homologous template provided on the cassette following homologous recombination.

**(b-c)** A plasmid with indicated synonymous or truncation variant was transiently infected into *RAD51D* CRISPR/Cas9 U2OS cells with a plasmid coding for the I-SceI restriction enzyme. The percentage of GFP<sup>+</sup> cells was measured after three days, indicating a recombination event using a GFP fragment on the cassette. The HR proficiency threshold was determined based on comparison with the range of synonymous variants (green bars) normalized to pcDNA3-RAD51D WT (HR >0.75). The threshold for loss of HR function was calculated using the range of truncation variants compared to a wild-type *RAD51D* expressing plasmid, as <0.6 (indicated in red for the variants). Note that a subset of those variants analyzed here are replotted in **Figure 5** as representative variants. Means of 3-7 trials are plotted  $\pm$  s.d. An empty vector was used as a negative control. Source data are provided as a Source Data file.

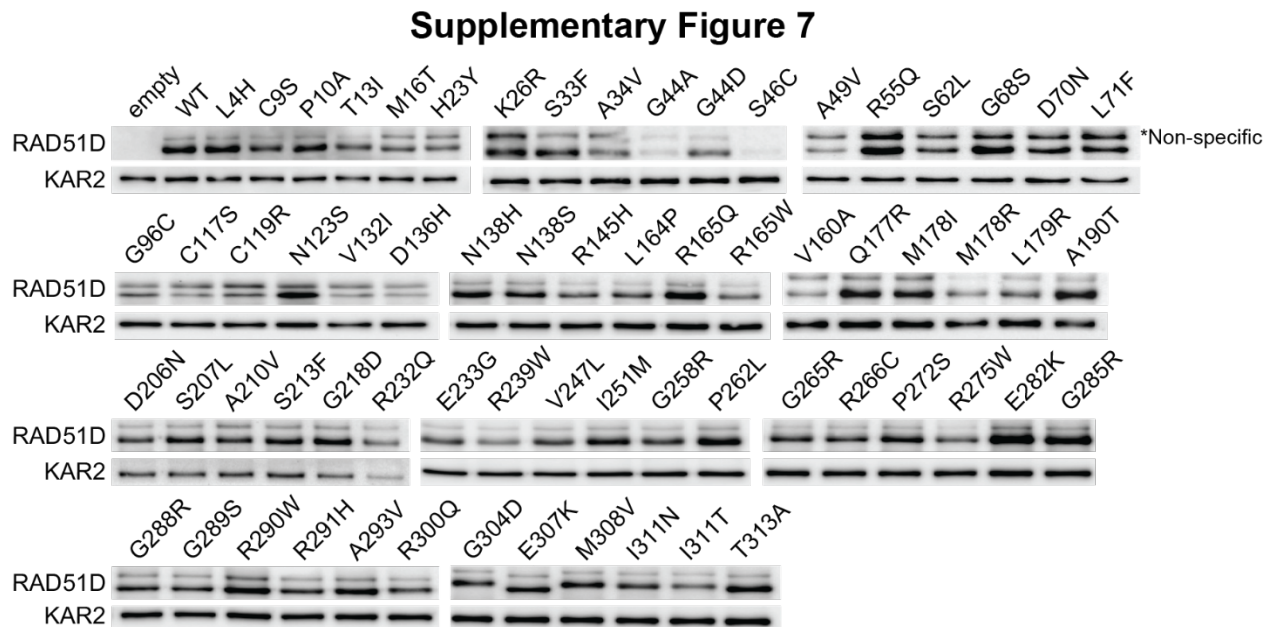
## Supplementary Figure 6



**Supplementary Figure 6. Expression and HR function of *RAD51D* variants in *RAD51D* CRISPR/Cas9 U2OS cells.**

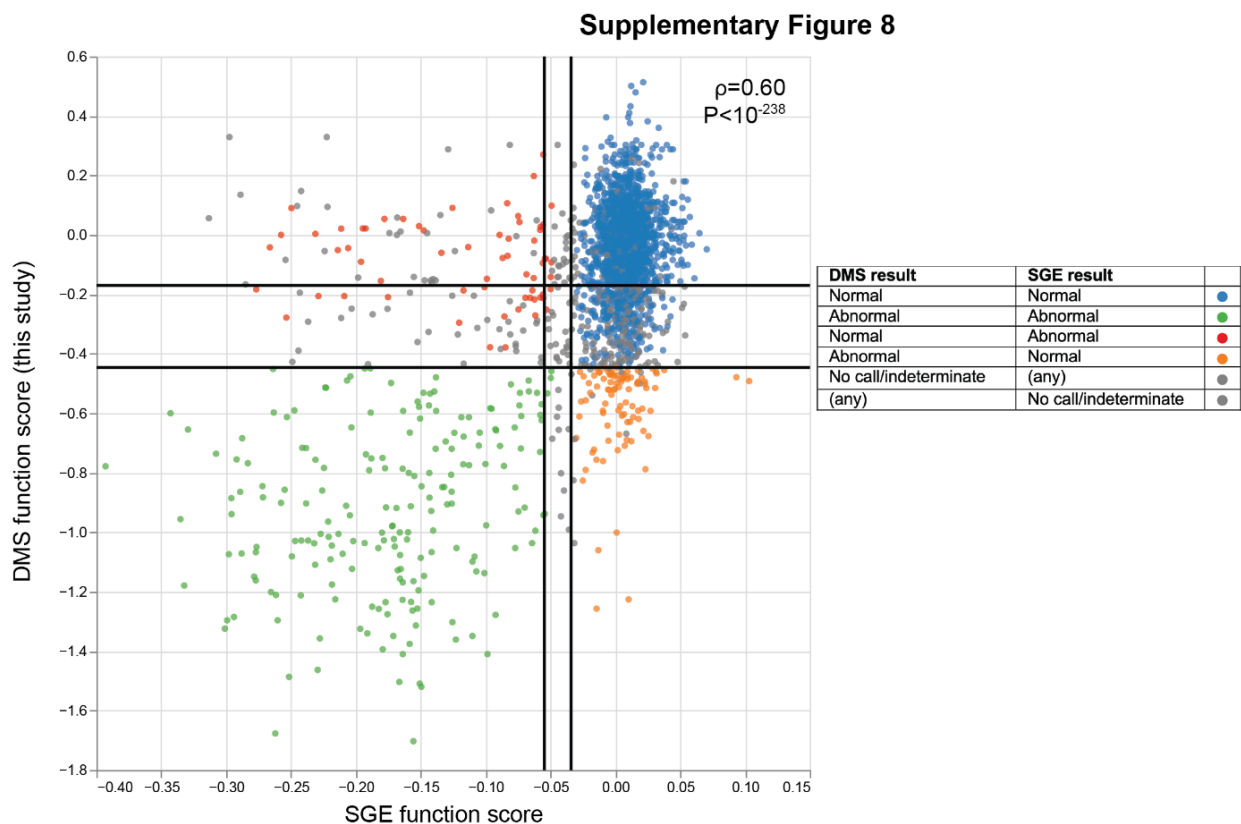
**(a)** Western blot analysis of protein extract from U2OS *RAD51D* KO cells transiently expressing pcDNA3-*RAD51D* WT or the indicated *RAD51D* variants was assessed using an anti-*RAD51D* antibody, and equal protein loading was assessed using an anti-tubulin antibody. Note that a subset of variants shows reduced protein expression compared with pcDNA3-*RAD51D* WT. Experiment performed in triplicate.

(b) HR analysis of RAD51D variants that are stably expressed in the *RAD51D* KO cell line used for the clonogenic survival assays shown in **Figure 4**. Source data are provided as a Source Data file.



**Supplementary Figure 7. Western blot showing expression of RAD51D variants from the Y3H experiments.**

Western blot analysis of protein extract from yeast cells expressing pGAD-C1-RAD51D WT or the indicated *RAD51D* variants was assessed using an anti-RAD51D antibody, and equal protein loading was assessed using an anti-KAR2 antibody. Note that a subset of variants has reduced protein expression relative to WT RAD51D. Experiment performed in triplicate. Source data are provided as a Source Data file.



**Supplementary Figure 8. MAVE analysis from this study and Saturation Genome Editing (SGE) analysis on *RAD51D* are highly concordant.**

The MAVE (DMS) function score from this study was plotted relative to the SGE function score<sup>46</sup>. Variants that both studies find to be normal are indicated in blue circles, variants that both studies find to be abnormal are indicated in green circles, conflicting results are shown in red or orange circles, and grey circles denote variants that received function scores from both studies but were deemed no-call and did not receive a classification from either one or both studies.