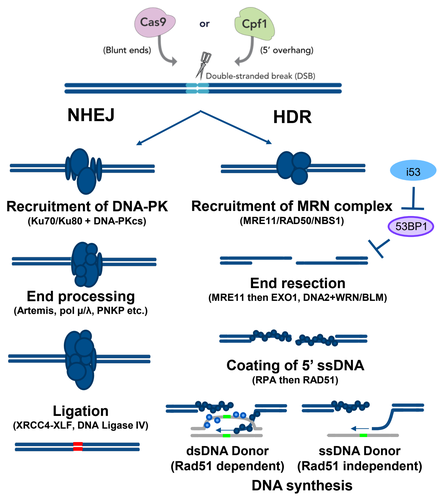
Engineering an improved inhibitor of 53BP1 to enhance HDR efficiency Introducing Alt-R™ HDR enhancer Protein

Steve E. Glenn*, Adam Brainard, Karthik Murugan, Vaughn Thada, and Christopher A. Vakulskas, Integrated DNA Technologies, Coralville, IA, USA

* Corresponding author: sglenn@idtdna.com

Introduction

One method of attaining precise genome editing is by creating a double-strand break using a sequence-specific nuclease while providing a single or double-stranded DNA sequence containing a desired modified sequence as a template for the endogenous cellular DNA damage repair machinery. Cells repair double-strand breaks primarily via non-homologous end joining (NHEJ) in which broken DNA ends are directly joined together, and homology directed repair (HDR), which encompasses several pathways that use a DNA template to facilitate repair. The efficiency of HDR varies by cell type and target site within the genome. p53-binding protein 1 (53BP1) plays a critical role in determining if a break is repaired by HDR or NHEJ. 53BP1 is recruited to sites of double-strand breaks and inhibits end resection, a key initial step in HDR. A modified variant of ubiquitin (i53) was shown recently to block 53BP1 recruitment to the broken DNA ends and to enhance the rate of HDR when delivered as mRNA or plasmid. We tested i53 and found that it did not function as well when delivered as purified protein. To improve the efficiency of 53BP1 inhibition, we performed a two-hybrid screen to identify ubiquitin variants with improved affinity for 53BP1. Based on this screen, we developed ubiquitin variants with improved efficacy for enhancing HDR when delivered as protein during nucleofection. We found that our improved ubiquitin variant (Alt-R™ HDR enhancer Protein: HEP) was able to improve rates of HDR in multiple cell types and using multiple types of donor template. We also did not observe the cytotoxicity or off target effects we observe with our small molecule Alt-R™ HDR enhancer V2 NHEJ inhibitor. We envision that our ubiquitin variants targeting 53BP1 can be a valuable tool for *ex vivo* genome editing, where both high efficiency and cell viability are needed and the effects of blocking NHEJ are a potential concern.



Alt-R™ HDR enhancer Protein causes ~2-fold increase in HDR across most sites

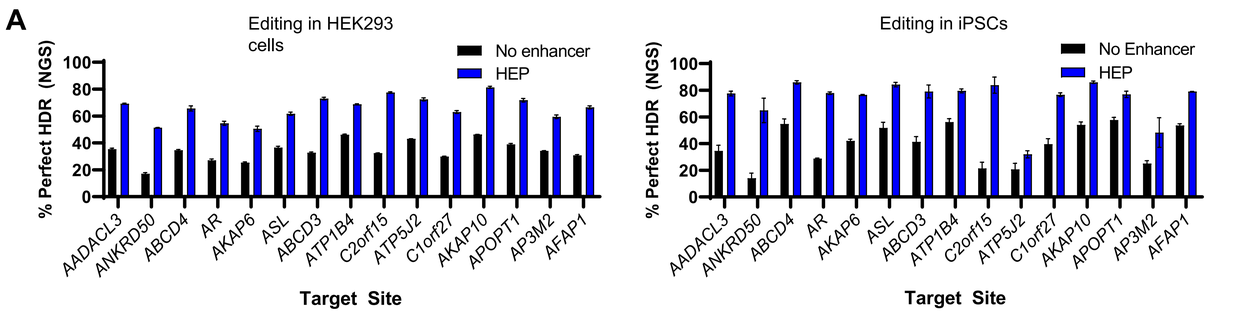


Figure 3 Alt-R™ HDR enhancer Protein is effective across various target sites in multiple cell types. Percent perfect HDR insertion of a 6 bp EcoRI cut site measured by NGS at fifteen sites in HEK293 (Left) or iPSCs (Coriell Institute) (Right) cells treated with Cas9 RNP, ssDNA donor, and either 0 or 25 μM HEP. Error bars indicate SD, n=2-3. **B.** Percent KI of GFP CDS (~740 bp) at *SERPINC1* in HEK293 cells treated with Cas9 RNP, dsDNA Alt-R donor block, and either 0 or 25 μM HEP. KI measured by MinION® sequencing. Error bars indicate SD, n=3. **C.** Percent KI for a ~2 kb T2A-CAR insert at the TRAC locus in iPSCs using Cas9 mRNA, sgRNA, and varying amounts of Nanoplasmid donor (Aldevron®) with or without 25 μM HEP delivered by Lonza nucleofection. KI was calculated using PCR followed by analysis using an Agilent Fragment Analyzer. Error bars indicate SD, n=3.

A two-hybrid screen for mutations that improve affinity

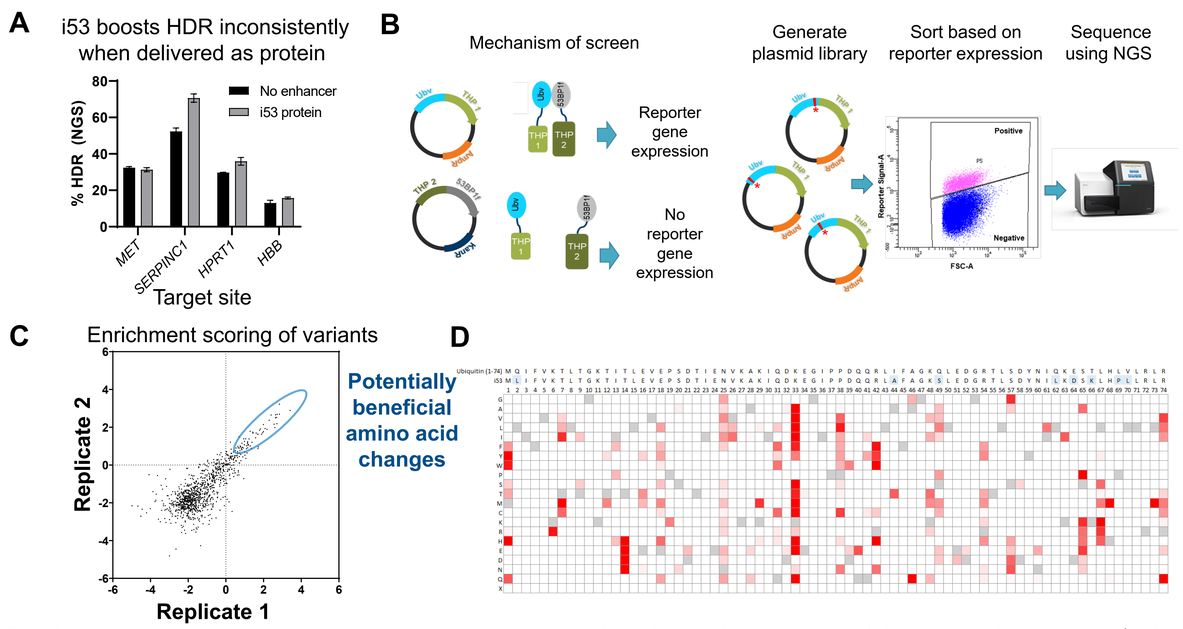


Figure 1. Overview of screen to identify novel 53BP1 binding ubiquitin variants. A. Percent perfect HDR insertion of a 6-bp EcoRI cut site measured by NGS in HEK293 cells (ATCC) treated with Cas9 RNP targeting the indicated gene alongside a ssDNA donor template plus 100 μM i53 protein delivered by Lonza® nucleofection. n=2. **B.** Mechanism of the two-hybrid screen to measure the affinity of 53BP1-Ubiquitin interaction. Plasmids for expression of i53 and a fragment of 53BP1 containing the tudor domain (53BP1f) fused to components of the two-hybrid system (THP1 and THP2) were generated. If a ubiquitin variant (Ubv) interacts with 53BP1f then reporter gene expression is induced. A plasmid library was generated consisting of plasmids with all possible single amino acid substitutions across i53. The plasmid library was delivered into cells which were then sorted based on reporter gene expression. Plasmid DNA from the original and positive populations was sequenced on a MiSeq® platform (Illumina®) using 2 × 150 bp PE sequencing. **C.** The graph shows the log2 enrichment of individual amino acid changes. Enrichment score of each ubiquitin variant was calculated by comparing the read counts between the two-hybrid reporter positive and input fractions, and then normalized to the average score of all synonymous changes. The data shown is for the 1059 single amino acid changes with read counts of more than 50 in the input for both replicates. Enrich2 was used to compile reads for each variant. **D.** Heatmap showing the average log2 enrichment from two replicates for beneficial (average enrichment>0) amino acid changes in the high selection pressure experiment. Scale 0 to 2, white to red. The sequences of human ubiquitin (a.a. 1-74) and i53 are shown above, with i53 mutations indicated in light blue. Gray boxes indicate no amino acid change.

Higher affinity variants are better able to enhance HDR

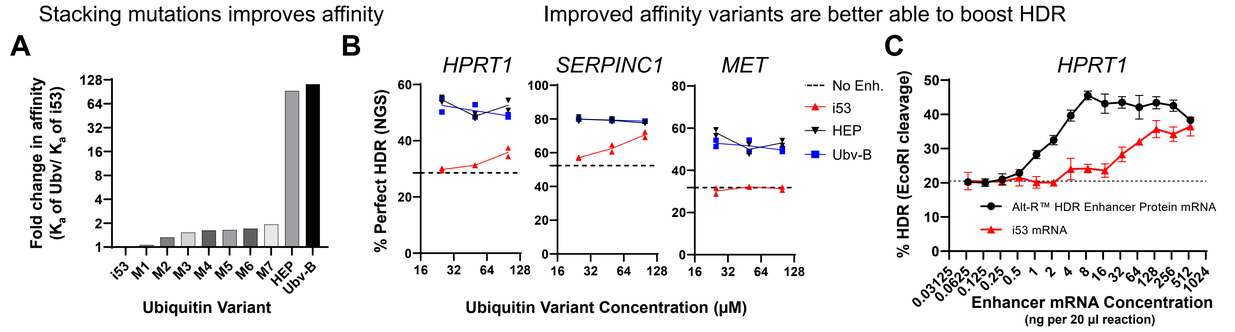


Figure 2. Higher affinity ubiquitin variants have an improved ability to enhance HDR. A. Fold change relative to i53 of the *in vitro* binding affinity of i53, i53 plus single mutations (M1-M7) with enrichment scores of >0.5 (Figure 1D), or ubiquitin variants (HEP and Ubv-B) with a combination of mutations (HEP: multiple IDT identified mutations stacked on i53, Ubv-B: IDT identified mutations stacked on human ubiquitin) to a Tudor domain containing fragment of 53BP1 measured by bio-layer interferometry (n = 1). **B.** Percent perfect HDR in HEK293 cells using 2 μM Cas9 RNP and 2 μM Alt-R HDR donor oligo for the introduction of an EcoRI cut site in the *SERPINC1, HPRT1,* and *MET* genes. Ubiquitin variants were delivered alongside donor and RNP at 25, 50, or 100 μM final concentrations using the Lonza® Nucleofector® system. Precent perfect HDR after 48 hours was determined by NGS on a MiSeq® platform (Illumina®) using 2 × 150 bp PE sequencing. The black, blue, and red symbols indicate the HDR rates using His-tagged HEP, Ubv-B, or i53, respectively (n=2). The dashed lines indicates the average percent perfect HDR in controls with no HDR enhancer (n = 3, standard deviation <1.5%). **C.** Percent HDR measured by EcoRI cleavage in HEK293 cells treated with varying concentrations of HEP (black) or i53 (red) mRNA alongside 4.8 μM sgRNA, 1 μg Cas9 mRNA (Aldevron), and 2 μM donor oligo for the insertion of an EcoRI cut site at *HPRT1* delivered using the Lonza® Nucleofector® system (n=3). The dashed line indicates average percent HDR with no ubiquitin variant mRNA (n=3, standard deviation <2%).

Alt-R™ HDR enhancer Protein boosts HDR added to NHEJ and MMEJ inhibition

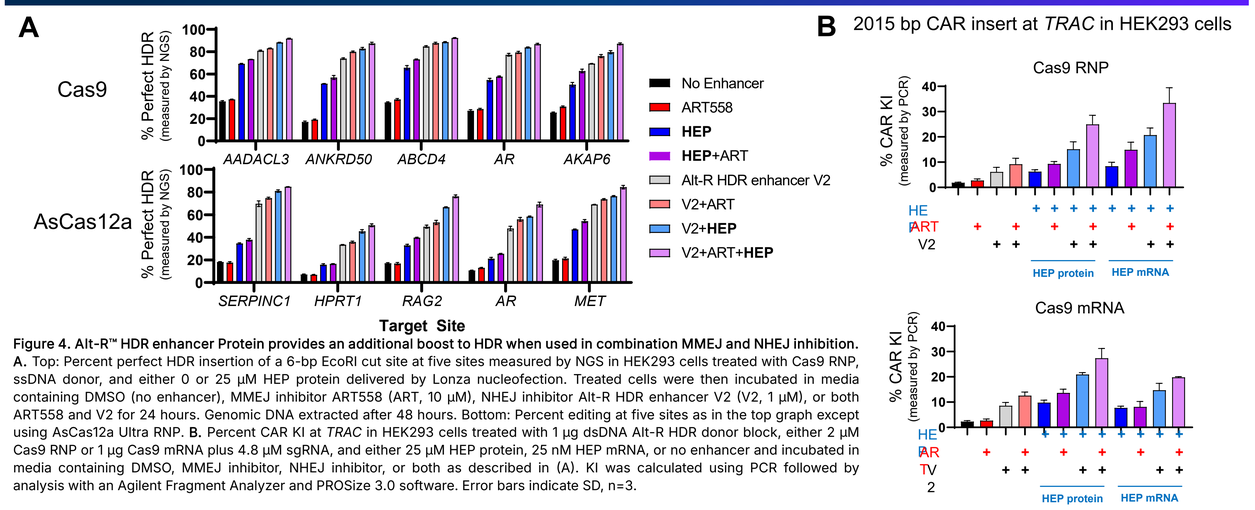


Figure 4. Alt-R™ HDR enhancer Protein provides an additional boost to HDR when used in combination MMEJ and NHEJ inhibition. A. Top: Percent perfect HDR insertion of a 6-bp EcoRI cut site at five sites measured by NGS in HEK293 cells treated with Cas9 RNP, ssDNA donor, and either 0 or 25 μM HEP protein delivered by Lonza nucleofection. Treated cells were then incubated in media containing DMSO (no enhancer), MMEJ inhibitor ART558 (ART, 10 μM), NHEJ inhibitor Alt-R HDR enhancer V2 (V2, 1 μM), or both ART558 and V2 for 24 hours. Genomic DNA extracted after 48 hours. Bottom: Percent editing at five sites as in the top graph except using AsCas12a Ultra RNP. **B.** Percent CAR KI at *TRAC* in HEK293 cells treated with 1 μg dsDNA Alt-R HDR donor block, either 2 μM Cas9 RNP or 1 μg Cas9 mRNA plus 4.8 μM sgRNA, and either 25 μM HEP protein, 25 nM HEP mRNA, or no enhancer and incubated in media containing DMSO, MMEJ inhibitor, NHEJ inhibitor, or both as described in (A). KI was calculated using PCR followed by analysis with an Agilent Fragment Analyzer and PROSize 3.0 software. Error bars indicate SD, n=3.

Alt-R™ HDR enhancer Protein effects are distinct from NHEJ inhibition

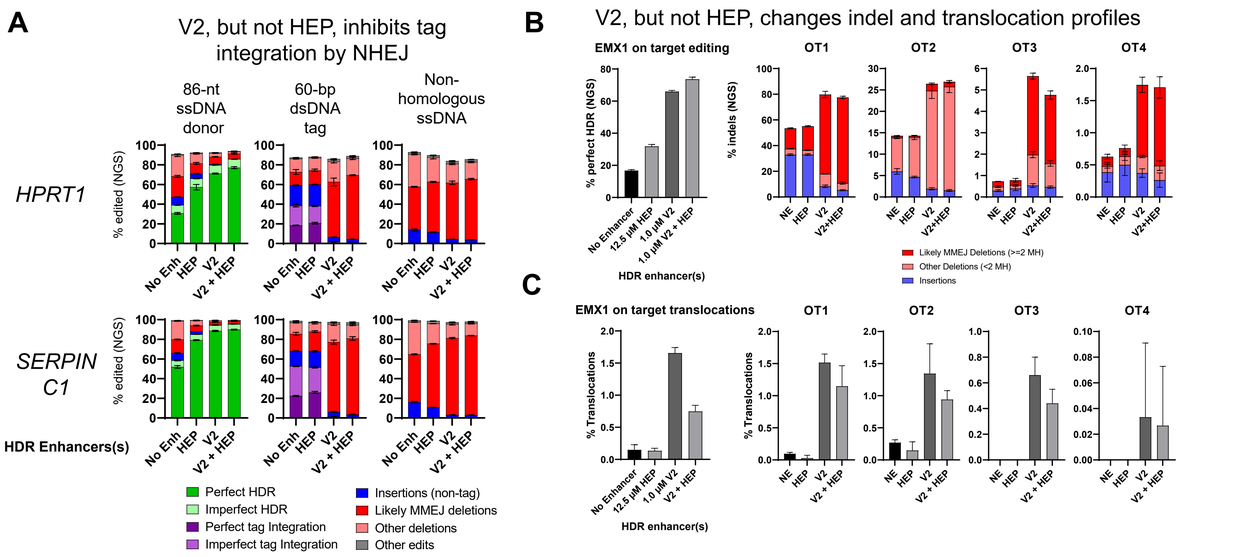


Figure 5. The effects of transient 53BP1 inhibition by Alt-R™ HDR enhancer Protein on DNA repair are distinct from the effects of small molecule-based inhibition of NHEJ. A. Breakdown of repair events at *HPRT1* and *SERPINC1* in HEK293 cells treated with Cas9 RNP, 0 or 25 μM HEP, and either 2 μM ssDNA donor (86-nt – 40-nt homology arms), 2 μM dsDNA (60 bp) tag without homology to the target site, or 2 μM ssDNA Alt-R Cas9 Electroporation Enhancer (IDT). Cells were then incubated either with or without 1 μM Alt-R enhancer V2 NHEJ inhibitor. Error bars indicate SD, n=3. **B.** Breakdown of repair events at *EMX1* on- and off-target sites in HEK293 cells treated with Cas9 RNP targeting EMX1, ssDNA donor, and 0 or 12.5 μM HEP and incubated with 0 or 1 μM V2 for 24 hours. Editing measured using rhAmpSeq™ using a pool of NGS primers targeting the EMX1 on-target site and a set of known off-target sites. The graphs show the rate of perfect HDR at the on-target site and the distribution of repair events at the most frequently edited off-target sites with likely MMEJ repair events being deletions with microhomology lengths of at least 2 bp. **C.** Percent translocations to the on and off target sites, as calculated by summing all reads using the forward amplicon primer for each site that paired with a reverse primer for a different site in the primer pool for translocations events that reached a 0.05% FDR, divided by all reads that included the forward primer.

Conclusions: Alt-R™ HDR enhancer Protein (HEP)

- HEP boosts HDR in multiple cell types
- HEP used with MMEJ and NHEJ inhibitors can further boost HDR.
- HEP does not cause off target effects and translocations typical of NHEJ inhibition
- HEP offers a safer method for enhancing HDR over NHEJ inhibitors

Joseph OBrien
Integrated DNA Technologies
jobrien@idtdna.com| +1 (319) 626-8438