1   **CRISPR-Cas9 genome editing in human cells works via the Fanconi Anemia pathway**

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3   **Author List**

4   Chris D Richardson[1,2]; Katelynn R Kazane[1,2]; Sharon J Feng[1,2]; Nicholas L Bray[1,2]; Axel J Schäfer[2,3];

5   Stephen Floor[2,3,4]; Jacob E Corn[1,2]*

6   [1]Innovative Genomics Institute, University of California, Berkeley, 94720

7   [2]Department of Molecular and Cell Biology, University of California, Berkeley, CA, 94720

8   [3]Howard Hughes Medical Institute, University of California, Berkeley, Berkeley, United States

9   [4]Current address: Department of Cell and Tissue Biology, University of California, San Francisco, California

10   94143

11   *Correspondence: jcorn@berkeley.edu

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14   **Abstract**

15   CRISPR-Cas9 genome editing creates targeted double strand breaks (DSBs) in eukaryotic cells that are

16   processed by cellular DNA repair pathways. Co-administration of single stranded oligonucleotide donor

17   DNA (ssODN) during editing can result in high-efficiency (>20%) incorporation of ssODN sequences into

18   the break site. This process is commonly referred to as homology directed repair (HDR) and here referred

19   to as single stranded template repair (SSTR) to distinguish it from repair using a double stranded DNA

20   donor (dsDonor). The high efficacy of SSTR makes it a promising avenue for the treatment of genetic

21   diseases[1,2], but the genetic basis of SSTR editing is still unclear, leaving its use a mostly empiric process.

22   To determine the pathways underlying SSTR in human cells, we developed a coupled knockdown-editing

23   screening system capable of interrogating multiple editing outcomes in the context of thousands of

24   individual gene knockdowns. Unexpectedly, we found that SSTR requires multiple components of the

25   Fanconi Anemia (FA) repair pathway, but does not require Rad51-mediated homologous recombination,

26   distinguishing SSTR from repair using dsDonors. Knockdown of FA genes impacts SSTR without altering

27   break repair by non-homologous end joining (NHEJ) in multiple human cell lines and in neonatal dermal

28   fibroblasts. Our results establish an unanticipated and central role for the FA pathway in templated repair

29   from single stranded DNA by human cells. Therapeutic genome editing has been proposed to treat genetic

30   disorders caused by deficiencies in DNA repair, including Fanconi Anemia. Our data imply that patient

31   genotype and/or transcriptome profoundly impact the effectiveness of gene editing treatments and that

32   adjuvant treatments to bias cells towards FA repair pathways could have considerable therapeutic value.

33

34   **Main Text**

35   The type II CRISPR endonuclease Cas9 and engineered guide RNA (gRNA) form a

36   ribonucleoprotein (RNP) complex that introduces double stranded breaks (DSBs) at DNA sequences

37   complementary to the 23 bp protospacer-PAM sequence. This activity stimulates two major types of DNA

38   repair within a host cell that are relevant to genome editing: genetic disruption, which creates insertions or

39   deletions (indels) at the cut site and can disrupt functional sequences; and genetic replacement, which

40   incorporates exogenous donor DNA sequences at the cut site, allowing the correction of dysfunctional

41   elements or insertion of new information[3]. Efficient and targeted genetic replacement is particularly exciting,

42   as it holds great promise for the cure of myriad genetic diseases.

43   Despite the rapid adoption of CRISPR-Cas9 genome editing, relatively little is known about which

44   cellular DSB repair pathways underlie Cas9-mediated genetic replacement. This lack of clarity has

45   complicated efforts to better understand and rationally improve the process of genome editing. The

46   pathways responsible for genetic replacement are frequently referred to in aggregate as HDR, which

47   includes DSB repair programmed from dsDonors (both linear and plasmid) requiring several kilobases of

48   homology to the targeted site, as well as synthetic ssODNs with only 100-200 bases of homology to the

49   target[4]. Repair from dsDonors is relatively inefficient in most cell types[5] and is assumed to utilize a repair

50   mechanism paralleling meiotic homologous recombination (HR)[6]. By contrast, SSTR is highly effective in

51   human cells (>20% of alleles)[1,5,7] and broadly conserved among metazoans[8], but very little is known about

52   the mechanism responsible. While screening human cancer cell lines, we found that SSTR-based genome

53   editing at a given locus can vary from completely ineffective (0% SSTR) to extremely efficient (30% SSTR)

54   depending on the cell background [**Extended Data Figure 1**]. This implies genetic or transcriptional

55   differences that up- or down-regulate gene editing in different contexts.

56   To map the pathways involved in SSTR, we developed a coupled inhibition-editing screening

57   platform that combines individual CRISPR inhibition (CRISPRi) of thousands of genes with Cas9 editing at

58   a single-copy genomically integrated BFP reporter **[Figure 1A].** Each cell in the screening pool stably

59   expresses a dCas9-KRAB CRISPRi construct as well as a gRNA targeting the TSS of a single gene. This

60   pool is then nucleofected with preformed Cas9-gRNA ribonucleoprotein complex (RNP) targeting the BFP

61   reporter, as well as an ssODN that programs a 3 basepair codon-swap that converts BFP to GFP[7]. The

62   femtomolar affinity between *S. pyogenes* Cas9 and the gRNA[9], along with the transient nature of the Cas9

63   RNP[10] strongly disfavors guide swapping between Cas9 molecules, preserving separation between

64   CRISPRi and targeted gene editing. Editing outcomes in each cell are separated by fluorescence activated

65   cell sorting (FACS) and next generation sequencing is used to determine genes whose knockdown leads to

66   enrichment or depletion from each sorted population. [**Figure 1A**].

67   To enable discovery of relatively low frequency events, we created a focused CRISPRi lentiviral

68   library containing 2,000 genes (10,000 gRNAs, 5 gRNAs targeting each primary gene transcript) with gene

69   ontology terms related to DNA processing **[Document S2 GUIDES]**[11]. This library was stably transduced at

70   low multiplicity of infection into cells expressing dCas9-KRAB and selected for the gRNA construct for ten

71   days to allow gRNA populations to reach equilibrium and for gRNAs targeting essential genes to drop out

72   of the population. We harvested a sample of cells at this point as a control for comparison with previously

73   published essentiality screens. We then electroporated cells with Cas9 RNP and the BFP-to-GFP ssODN[7].

74   Under unperturbed conditions, this combination of reporter, RNP, and ssODN yields ~70% gene disruption

75   (no longer BFP+) and ~20% SSTR (BFP edited to GFP) [**Extended Data Figure 2**]. We harvested another

76   sample of cells seven days after electroporation to identify genes whose knockdown is synthetic lethal with

77   a Cas9-induced DSB, as measured by depletion only after introduction of Cas9. To identify genes involved

78   in editing events, we used FACS to separate cells into unedited (BFP+/GFP-), Indel (BFP-/GFP-) and

79   SSTR edited (BFP-/GFP+) populations **[Figure 1A, Extended Data Figure 2].** We used Illumina

80   sequencing to measure gRNA abundances in each population, and compared these distributions to the

81   edited unsorted cell population to reveal which target genes promote (gRNA depleted from edited

82   population) or restrict (gRNA enriched from edited population) specific genome editing activities.

83   To benchmark our screening system, we identified essential genes by comparing the library-

84   infected dCas9-KRAB CRISPRi cells with cells infected with only the gRNA library and no dCas9-KRAB

85   **[Extended Data Figure 3A, Document S3 Essential Genes]**. Genes that were depleted after 14 days

86   from the functional CRISPRi cells as compared to the gRNA-only control cells were significantly enriched

87   for critical biological processes (DAVID[12] analysis: proteasome core complex p=8.6e-14 and DNA

88   replication p=1.7e-11). Furthermore, genes we identified as essential reproduced previously published

89   essentiality screens **[Extended Data Figure 4A]**[11], demonstrating that we had achieved stable gene

90   knockdown and robust hit calling from the cell pools.

91   We next investigated genes whose knockdown was synthetic lethal with a Cas9-induced DSB.

92   gRNAs targeting these genes should be depleted after Cas9 editing as compared to unedited cells

93   **[Extended Data Figure 3B]**. While essential genes are progressively lost from the cell population over

94   time **[Extended Data Figure 4B]**, genes whose knockdown is synthetic lethal with DNA damage are lost

95   only after a Cas9 DSB **[Extended Data Figure 4C]**. Genes required to survive a single Cas9-induced DSB

96   were enriched for the GO terms such as cell cycle arrest (p=3.3E-05) and response to DNA damage (p=

97   3.4E-22) and include several factors previously reported to be synthetic lethal with other DNA damaging

98   agents **[Document S3 SurviveDSB]**. For example, knockdown of MYBBP1A has recently been reported to

99   cause senescence in combination with nonspecific DNA damage[13]. Our screening cell line contains a

100   single copy of the targeted BFP allele, which suggests that a single DSB is sufficient to trigger genotoxic-

101   induced senescence when these synthetic lethal genes are depleted. Together, our results indicate that our

102   coupled inhibition-editing strategy performs well in identifying not only essential genes, but also genes

103   involved in DNA repair pathways required to survive a Cas9 DSB. Future investigation of novel genes

104   identified as synthetic lethal with a DSB could provide new insight into mechanisms of genome

105   surveillance.

106   To identify factors required for SSTR editing, we used FACS to isolate GFP+ cells (that had

107   undergone BFP-to-GFP conversion via SSTR) and measured depletion of gRNAs relative to the unsorted

108   edited pool **[Figure 1B]**. Strikingly, 70% (28/40) of genes annotated in the Fanconi Anemia (FA) pathway

109   were robustly and consistently depleted from the GFP+ population relative to unsorted edited cells. Gene

110   set enrichment analysis[14] verified that DNA repair in general and the FA pathway in particular was a

111 defining feature of SSTR **[Figure 1C]**. Several distinct functional groups within the FA repair pathway were

112 identified as required for SSTR: multiple components of the FA core complex that senses lesions, FA core

113 regulatory components that activate the FANCD2-FANCI heterodimer, downstream effector proteins that

114 repair lesions, and associated proteins that interact with canonical FA repair factors **[Figure 1D]**. Our

115 identification of the FA pathway as central to SSTR was striking, as to the best of our knowledge the FA

116 pathway has not previously been investigated for its role in Cas9 gene editing.

117 We used individual knockdown of FANCA, RAD51, and other DNA repair genes to further

118 investigate the genetic basis of SSTR and dsDonor HDR. Previous reports have indicated the editing

119 outcomes of SSTR are RAD51-independent and ineffective during G2/M[15,16], while dsDonor HDR is

120 RAD51-dependent[17], FANCA-dependent[18], and active during G2/M[19]. Using Cas9 RNPs to edit the same

121 locus with dsDonors and ssODNs in K562 human erythroleukemia cells, we found approximately four-fold

122 higher gene replacement efficiency with ssODNs. Knockdown of FANCA caused statistically significant

123 four-fold reduction in SSTR (p<0.05, Welch's two-sided t-test) and a non-significant two-fold reduction in

124 dsDonor HDR (p=0.22, Welch's two-sided t-test) **[Figure 1E]**. These results highlight the unexpected role

125 of FANCA in SSTR and suggest it might also play some role in HDR. As expected, neither SSTR nor HDR

126 required NHEJ (mediated by LIG4), or the related Alternative End Joining (Alt-EJ) pathway (mediated by

127 PARP1). Notably, we found that knockdown of RAD51 abolished dsDonor HDR but had no effect upon

128 SSTR **[Extended Data Figure 5]**. Taken together, individual knockdown bolsters the hypothesis derived

129 from the primary screen that SSTR is a genetically distinct pathway from dsDonor-mediated HDR.

130 The FA repair pathway is best understood in its capacity to identify and repair interstrand crosslinks

131 (ICLs) throughout the genome, but has recently gained attention for its role in protecting stalled replication

132 forks[20-22]. In the presence of ICLs or a stalled fork, the FA core complex (comprised of FANCA, B, C, E, F,

133 G, L, M, FAAP100, FAAP20, and FAAP24) is required for monoubiquitination and activation of the

134 FANCD2-FANCI heterodimer by UBE2T and FANCL. Monoubiquitination then leads to recruitment of

135 downstream factors that repair the lesion via nucleotide excision repair (NER) or specialized homologous

136 recombination sub-pathways. Subsequent to repair, FANCD2/FANCI is recycled through deubiquitination

137 by USP1 and WDR48. Deactivation of FANCD2/FANCI appears to be a key step in restoring homeostasis,

138 as mutants in USP1 and WDR48 phenocopy classical FA mutants with an increased sensitivity to ICL-

139   causing agents. Notably, our screen identified that SSTR depends upon genes that act in every functional

140   category of the FA repair pathway **[Figure 1D].** SSTR is therefore likely to be a central activity of the FA

141   repair pathway as opposed to a moonlighting activity of one or more FA genes.

142   To further explore the genetic basis of SSTR, we used CRISPRi to stably knock down seven

143   separate FA repair genes that operate at different places in the FA pathway and quantified the frequency of

144   Cas9-mediated SSTR at multiple loci. Knockdown of FANCA, FANCD2, FANCE, FANCF, FANCL, HELQ,

145   UBE2T, USP1, and WDR48 all substantially decreased SSTR at a stably integrated BFP reporter, as

146   measured by flow cytometry **[Figure 2A]**. Stable cDNA re-expression of each factor restored wildtype

147   levels of SSTR, demonstrating that CRISPRi was specific to the targeted gene and that ablation of each

148   gene was solely responsible for the loss of SSTR. Re-expression of an FA factor in the context of its

149   knockdown increased editing efficiency up to 8-fold. These results demonstrate that multiple genes in

150   different parts of the FA repair pathway are required for SSTR editing, that their presence is necessary for

151   efficient SSTR, and that re-expression is sufficient to restore SSTR.

152   In addition to the FA pathway's well-characterized roles in ICL repair, there is an emerging view that

153   it plays additional roles in preserving genome stability. FA genes protect against aberrant chromosomal

154   structures and replication stress via specialized subcomplexes that in part depend upon particular

155   helicases, including Bloom's helicase (BLM) and the 3'-5' ssDNA helicase HELQ. We found that siRNA

156   knockdown of BLM had no effect on SSTR **[Extended Data Figure 7],** but knockdown of HELQ markedly

157   reduced SSTR **[Fig 2A**, **Extended Data Figure 7]**. BLM and its interaction partner RMI2 exhibited strong

158   phenotypes in the primary screen **[Document S3, SSTR]**. However, both of these factors were required

159   (p<0.01) for survival of a Cas9-induced DSB **[Document S3, SurviveDSB]**, which suggests a role for the

160   BLM complex in surviving a DSB instead of SSTR itself. While BLM has been linked to FA-mediated

161   resolution of replication stress[23], HELQ directly interacts with FANCD2/FANCI with unknown functional

162   significance[24]. HELQ also interacts with multiple recombination subcomplexes, including BCDX2 (RAD51B,

163   RAD51C, RAD5D, and XRCC2) and CX3 (RAD51C-XRCC3). These complexes could promote

164   recombination between the ssODN and genomic DNA, and we asked if these complexes also impact

165   SSTR.

166   We found that RAD51C is required for SSTR, but RAD51B and XRCC2 are not. This suggests that

167   BCDX2 does not play a role in SSTR [Extended Data Figure 7]. Conversely, both RAD51C and XRCC3

168   are required for SSTR, implicating the CX3 complex in SSTR [Extended Data Figure 7]. Intriguingly, CX3

169   has been reported to act downstream of RAD51 filament formation[25], but we found that RAD51 itself is

170   dispensable for SSTR [Figure 1B, Extended Data Figure 5]. We anticipate that future work to

171   characterize how the FA pathway interacts with downstream effectors, especially polymerases and genes

172   that mediate recombination, will provide valuable insights into the mechanism of SSTR and its interaction

173   with other pathways that maintain genome stability.

174   Inhibition of SSTR by interfering with the FA pathway could work by globally reconfiguring DNA

175   repair pathway preference or by specifically inhibiting SSTR. We investigated how the FA pathway

176   influences repair pathway choice by inhibiting several FA genes and measuring editing outcomes using

177   Illumina sequencing [Extended Data Figure 8]. When editing the endogenous hemoglobin β (HBB) locus

178   at the causative amino acid (Glu6) for sickle cell disease, we found that all seven FA factors are required

179   for SSTR editing [Figure 2B]. Notably, knockdown of FA genes decreased levels of SSTR while

180   simultaneously increasing levels of NHEJ, such that total editing (SSTR + gene disruption) remained

181   relatively constant [Figure 2B, Extended Data Figure 9]. However, when we edited the HBB locus in the

182   absence of an ssODN, we found that knockdown of FA repair genes did not significantly increase NHEJ

183   frequency on its own [Extended Data Figure 9]. We found similar results at the BFP locus when

184   measuring editing outcomes by Illumina sequencing. These results imply that the FA repair pathway acts to

185   divert repair events that would otherwise be repaired by NHEJ into SSTR outcomes. This model parallels

186   proposed roles for the FA pathway in balancing NHEJ and HR repair frequencies during ICL repair[26,27] and

187   balancing Alt-EJ, NHEJ, and HDR repair outcomes near DSBs[28].

188   To determine if FA repair genes are responsible for SSTR in primary human cells, we edited human

189   neonatal dermal fibroblasts at HBB Glu6. These fibroblasts have previously been shown to be capable of

190   SSTR repair, albeit at lower levels than many cell lines[15]. Untreated or mock siRNA treated fibroblasts

191   exhibited approximately 5% SSTR at the HBB locus, as measured by Illumina sequencing. siRNA

192   knockdown of either FANCA or FANCE led to an approximately five-fold reduction in SSTR [Figure 2C].

193   Therefore, the FA repair pathway is tightly linked to SSTR in at least one primary human cell type.

194   The sequence outcomes of genomic disruption (indels) following Cas9-induced DSBs are often

195   nonrandom and surprisingly consistent at individual loci, leading to an emerging model that repair

196   outcomes are determined by the intrinsic repair pathway preferences of the edited cell and the sequence

197   immediately adjacent to the cut site[29]. To determine how FA pathway disruption affects the characteristic

198   spectrum of indels as a Cas9-induced break, we characterized individual allele frequencies in unperturbed

199   and FA knockdown CRISPRi cell lines using Illumina sequencing at both BFP and HBB Glu6. We also

200   examined SSTR conversion tracts, a function of SNP integration relative to distance from the Cas9-induced

201   break, by following the incorporation of several single nucleotide polymorphisms (SNPs) encoded by the

202   ssODN into the genomic sequence.

203   In the absence of an ssODN to program SSTR, neither the overall frequency nor the pattern of

204   indels at the Cas9 cut site was affected by disruption of the FA repair pathway **[Figure 3A]**. We

205   furthermore observed no change in indel spectra upon FA knockdown when editing cells in the presence of

206   an SSTR-templating ssODN. However, when editing with an ssODN, SSTR decreased dramatically upon

207   disruption of the FA pathway. This decrease was remarkably uniform across SNPs within the ssODN and

208   did not measurably alter the SNP conversion tracts. These results reinforce our earlier observation that FA

209   repair pathway inactivation specifically inhibits SSTR without altering the frequency of indels. Additionally,

210   the molecular sequence outcomes of NHEJ are unaffected by the FA pathway. Instead, the FA pathway is

211   restricted to SSTR repair and the balance between NHEJ and SSTR, but does not play a direct role in

212   error-prone end-joining pathways.

213   In sum, we have found that multiple functional complexes within the FA repair pathway are

214   necessary for Cas9-mediated SSTR. Genome editing is commonly grouped into two categories, genetic

215   disruption and genetic replacement, based on sequence outcomes[2]. Our results demonstrate that final

216   genetic replacement outcomes using different templates (ssODN vs dsDonors) are identical at the

217   sequence level but stem from completely different pathways **[Figure 4]**. Specifically, information from

218   double stranded DNA templates and genomic DNA are incorporated using Rad51-dependent processes,

219   but single stranded DNA templates are incorporated through the FA pathway. A great deal of work has

220   focused on improving HDR during gene editing by activating Rad51-mediated processes, including Rad51

221   agonist small molecules[30] and strategies to stimulate recruitment of Rad51 throughout the cell cyle[31]. Our

222   results indicate that future efforts to the activate FA pathway could be invaluable during gene editing for

223   research or therapeutic uses.

224   Cas9-mediated genome editing holds great promise for the treatment of genetic diseases such as

225   sickle cell disease and Fanconi Anemia. High rates of gene editing are typically required for therapeutic

226   editing applications, but editing efficiencies can differ greatly between cells. Without knowledge of the

227   pathways responsible for genetic replacement outcomes and the activity of those pathways in the targeted

228   cell type, it was previously difficult to rationalize why editing might fail in one application while succeeding

229   in another. Our results predict that human cell types with intrinsic repair preferences that impact the FA

230   pathway will be more or less capable of SSTR **[Extended Data Figure 1].** The expression level of FA-

231   related factors could in future be useful as a biomarker for patient cell "editability", and treatments that

232   enhance the activity of the FA pathway could be especially valuable in difficult to edit cells. For example,

233   we found that complementing FA pathway knockdown yields up to 8-fold increase in editing efficiency in

234   cell lines **[Figure 2A]**. This suggests that reactivating the FA pathway could be valuable in cases where it

235   has been disrupted, such as in Fanconi Anemia itself. Small molecule activators of the FA pathway remain

236   to be identified, but our results suggest that transiently increasing the levels of FA proteins could

237   complement patient-specific defects to enable lasting gene editing cures. More broadly, our results suggest

238   that patient genotype or transcriptome could increase or decrease the effectiveness of therapeutic

239   treatments in previously unanticipated ways. Deeper understanding of the molecular basis of SSTR and

240   dsDonor HDR is likely to suggest new biomarkers to 'match' patient genotype with therapeutic editing

241   strategy.

242   Finally, our data imply that the default repair pathway for DSBs, especially DSBs introduced by

243   Cas9, is end joining, and that the activity of the FA repair pathway determines whether many events will

244   instead be repaired by SSTR **[Figure 2A, Figure 4]**. Cas9 is very stable on genomic targets[7,32], and so it is

245   possible that Cas9 itself is recognized as an interstrand crosslink or roadblock within the genome.

246   However, we disfavor this hypothesis because FA knockdown only impacts SSTR without directly affecting

247   indels. Instead, we hypothesize that Cas9-stimulated repair using an ssODN template mimics some

248   substrate of the FA pathway, such as a stalled replication fork. We note that SSTR is much more efficient

249   than HDR from a double stranded DNA template **[Figure 1E]**, to the extent that in many cell lines, the most

250 common single allele at an edited locus is the product of SSTR **[Figure 3B]**. This ability raises intriguing

251 questions about genome integrity in the presence of single stranded DNA exposed by R-loops, replication

252 crises, or viral infection. The FA pathway has already been implicated in replication crises, and future work

253 to address remaining questions could provide insight into mechanisms by which human cells maintain their

254 genomes.

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256

257 **Figure Legends**

258 **Figure 1: A coupled knockdown-editing screen identifies multiple Fanconia Anemia factors**

259 **necessary for SSTR. (A)** Schematic of the coupled knockdown-editing screening strategy. Lentiviruses

260 comprising a BFP reporter and a gRNA are pooled and transduced into dCas9-KRAB K562 cells to

261 produce a knockdown cell library. The gRNA library used here targets 2,000 distinct genes with gene

262 ontology terms related to DNA (10,000 total gRNAs, 5 gRNAs per gene). This knockdown cell library is

263 nucleofected with a pre-formed Cas9 RNP targeting BFP and an ssODN programming a 3bp mutation that

264 converts BFP to GFP. Edited unsorted cells can be separated into three populations by FACS: Unedited

265 (BFP+/GFP-); Indel (BFP-/GFP) and SSTR (BFP-/GFP+). Genes involved in regulating SSTR are identified

266 by measuring guides that are significantly enriched or depleted in the SSTR population relative to the

267 unsorted population by Illumina sequencing. **(B)** Multiple genes in the Fanconi Anemia pathway are

268 depleted from the SSTR population. Representative SSTR genes are highlighted in blue, negative control

269 untargeted gRNAs are shown in black, and targeted gRNAs are shown in orange. **(C)** GSEA analysis

270 reveals that genes depleted from the SSTR population are significantly enriched in the FA repair pathway.

271 **(D)** Multiple components of the FA repair pathway play a role in SSTR. The FA pathway can be separated

272 into four functional categories: the FA Core complex, Core regulator factors involved in FANCD2-FANCI

273 ubiquitination, Downstream repair effectors, and Associated factors involved in regulating FA outcomes.

274 Genes are colored by log_2 depletion of the indicated genes from each complex. Asterisk denotes gene

275 scores that have been adjusted based on secondary analyses. Raw data is available in **[Document S3]**.

276 **(E)** Individual CRISPRi knockdown of FANCA prevents SSTR. Representative BFP-GFP flow cytometry

277 profiles are shown for untargeted (WT) or FANCA CRISPRi edited by co-administration of RNP targeting

278 the BFP locus and ssODN or dsDonor. qPCR verifying knockdown of genes can be found in [**Extended**

279 **Data Figure 6**].

280

281 **Figure 2: Knockdown of FA genes specifically inhibits SSTR. (A)** Knockdown of multiple FA repair

282 genes prevents SSTR at a single copy genomically integrated BFP reporter. Stable cDNA re-expression

283 rescues this phenotype. Stable CRISPRi cell lines with untargeted (NT, grey) gRNA or gRNA targeting

284 specific genes were edited by co-administration of RNP targeting the BFP reporter and an ssODN

285 containing a BFP→GFP mutation (see **[Extended Data Figure 6]** for transcript levels)**.** The frequency of

286 GFP+ cells was quantified by flow cytometry, normalized to untargeted controls, and %SSTR was plotted

287 for knockdown of FA factors (light blue) or knockdown of FA factors in the context of cDNA re-expression

288 (dark blue). Data are presented as mean±sd of at least two biological replicates. **(B)** Knockdown of FA

289 repair genes prevents SSTR and upregulates NHEJ at the HBB Glu6 codon. Cell lines with untargeted

290 control gRNA (NT, grey), or gRNAs targeting each FA gene (red) were edited using Cas9 targeting the

291 HBB locus and a ssDNA donor that introduces the Glu6Val sickle mutation. NHEJ (light columns) and

292 SSTR (dark columns) were measured by amplicon Illumina sequencing of edited alleles **[Extended Data**

293 **Figure 8]** and normalized to untargeted controls. Data presented are the mean±sd of at least two biological

294 replicates. **(C)** Inhibition of FA repair genes prevents SSTR in primary fibroblasts. Human neonatal dermal

295 fibroblasts were treated without (WT) or with siRNA targeting the indicated genes and edited as described

296 in **[Figure 2B]**. Verification of knockdown can be found in **[Extended Data Figure 5C]**.

297

298 **Figure 3: FA gene knockdown rebalances edited alleles without affecting the spectra of molecular**

299 **outcomes. (A)** FA repair genes are required for SSTR but not end-joining. Cell lines with stable

300 knockdown of control (untargeted gRNA, NT), FANCA, or FANCE were edited at the BFP (blue) or HBB

301 Glu6 (red) loci. Editing was performed without (RNP only, left panels) or with (RNP+ssODN, right panels)

302 ssODN containing three trackable SNPs and editing outcomes were measured using amplicon sequencing.

303 The frequency of sequence alteration by insertion/deletion (indel) or SSTR is plotted as a function of

304 distance from the Cas9 cut site (diamond). Frequency of sequence alteration is displayed from 30bp PAM

305 proximal (-) to 30bp PAM distal (+) of the Cas9 cut site. Data displayed are representative of biological

306 replicates. **(B)** SSTR is the most common allele in Cas9-edited cell populations and is lost when FA genes

307 are knocked down. Allele frequencies were calculated for the editing experiments presented in **[Figure**

308 **3A]**. Wildtype sequences with PAM underlined are presented for BFP and HBB Glu6 along with the

309 sequence alignments of the five most common alleles produced when editing the BFP or HBB loci in the

310 context of control (NT) or FANCA knockdown. Alleles are categorized as SSTR, wildtype (WT), or Indel.

311 Percentages of total reads are presented for each allele.

312

313 **Figure 4:** Cas9-mediated genomic replacement can be separated into two distinct pathways based on the

314 type of template used for repair. Cas9-induced DSBs can be repaired using donor-dependent or end

315 joining repair events. Donor-dependent events can be separated into Rad51-dependent FANCA-

316 independent (HR) or Rad51-independent FA-dependent (SSTR) repair. Sequence replacement outcomes

317 are in competition with end-joining, whose outcomes include perfect repair of the original sequence,

318 genetic replacement, and genetic disruption.

319

320 **Extended Data Figure 1:** SSTR efficiency varies in different human cell lines. Nine cell lines were edited

321 using RNP targeting the EMX1 locus either without or with ssODN containing PciI sequence. The edited

322 locus was amplified, re-annealed, and digested using T7 Endonuclease I (t), which quantifies gene

323 disruption, or the restriction enzyme PciI (R), which quantifies SSTR.

324

325 **Extended Data Figure 2:** BFP, GFP, and Non-fluorescent populations are effectively resolved during a

326 pooled CRISPRi screen. **(A)** Replicate one of dCas9-KRAB cells infected with gRNA library and edited at

327 the BFP locus. Live single cells were plotted to compare BFP and GFP intensities. Three populations were

328 sorted: BFP+/GFP- (BFP), BFP-/GFP+ (GFP), and BFP-/GFP- (UN). Percentages in each gate are

329 presented for 100,000 cells. **(B)** Total cells sorted for each edited population.

330

331 **Extended Data Figure 3:** Pooled CRISPRi screens identify DNA repair genes that contribute to multiple

332 phenotypes. (A) Essential genes in the K562 cell background. A volcano plot identifies genes from the DNA

333 repair library enriched or depleted in CRISPRi cells relative to gRNA-only controls. Representative

334 essential genes are highlighted in blue, negative control untargeted gRNAs are shown in black, and

335 targeted gRNAs are shown in orange. (B) Genes synthetic lethal with a DSB. A volcano plot identifies

336 genes from the DNA repair library enriched or depleted in CRISPRi cells treated with Cas9 relative to

337 untreated cell populations. Representative synthetic lethal genes are highlighted in blue, negative control

338 untargeted gRNAs are shown in black, and targeted gRNAs are shown in orange.

339

340 **Extended Data Figure 4:** (A) Essential genes identified in this study substantially overlap with previous

341 studies. The distribution of gRNAs in two unedited dCas9-KRAB cell libraries was separately compared to

342 the gRNA distribution in an unedited K562 cell library and gene scores for each target genes were

343 calculated. Essential genes were defined as those showing significant (p<0.05) depletion (log$_2$(fold

344 change)<-0.5) in the dCas9-KRAB population. These essential genes were compared to the K562 dataset

345 generated in (Horlbeck et al, 2016), filtered for significance (p<0.05) and magnitude of effect (phenotype<-

346 0.2). Overlap between gene lists is presented as a Venn diagram. (B) Essential genes are progressively

347 lost from library. (C) Genes that are synthetically lethal with a single DSB are abruptly lost from library

348 following Cas9 treatment.

349

350 **Extended Data Figure 5:** Effective knockdown of DNA repair factors using siRNA. **(A)** Knockdown and

351 editing of DSB repair factors reveals genetic differences between HDR and SSTR. K562 cells expressing a

352 BFP reporter were treated with the indicated siRNAs and edited by co-administration of RNP targeting the

353 BFP reporter with ssODN or dsDonor containing a BFP->GFP mutation. **(B)** siRNA of RAD51, PARP1, and

354 LIG4 in K562 cells **[Extended Data Figure 5A]**. Cells were siRNA treated for 48 hours prior to editing. Fold

355 depletion of the siRNA-target transcript over controls (ACTB, GAPDH) was measured by qPCR. Data

356 presented represent the transcriptional state of the cells at the time of editing. All data were calculated from

357 technical triplicate and biological replicate. **(C)** siRNA knockdown of FANCA and FANCF in human dermal

358 fibroblasts. Cells were siRNA treated prior to editing **[Figure 2C]** as described in **[Extended Data Figure**

359 **5B]**.

360

361   **Extended Data Figure 6:** Effective knockdown of DNA repair factors using CRISPRi. **(A)** Stable CRISPRi

362   cells targeting the indicated gene with or without re-expression of a cDNA of the indicated gene were

363   harvested **[Figures 2A-B]** and fold depletion of the indicated transcripts over control transcripts (ACTB,

364   GAPDH) was measured by qPCR. Data presented were calculated from technical triplicate and biological

365   replicate.

366

367   **Extended Data Figure 7:** HELQ interaction partners play a role in SSTR. **(A)** Interaction map of HELQ

368   reproduced from an earlier study (Adelman 2013) illustrates direct physical interactions between HELQ and

369   other complexes and reported interactions from BIOGRID, STRING and MINT databases. **(B)** HELQ

370   interaction partners play a role in SSTR. K562 cells were siRNA or CRISPRi treated against the indicated

371   target genes prior to editing at the BFP locus. Data presented are the mean±sd of biological duplicate. **(C)**

372   HELQ interaction partners can be effectively depleted by siRNA or CRISPRi. Fold depletion of the target

373   transcript over controls (ACTB, GAPDH) was measured by qPCR. Data presented were calculated from

374   technical triplicate and biological replicate.

375

376   **Extended Data Figure 8:** Schematic of amplicon sequencing used at BFP or HBB loci. Protospacer (grey)

377   and PAM (black) are presented within amplified sequence. ssODN size and orientation is also displayed.

378   Vertical hash marks indicate locations where ssODN and genomic sequences differ.

379

380   **Extended Data Figure 9:** Total editing remains consistent when SSTR is disrupted. Indel (NHEJ, light

381   blue), SSTR (medium blue), and total (dark blue) editing events were quantified for the indicated CRISPRi

382   cell lines and normalized to untargeted controls. **(A)** RNP targeting the HBB locus with donor ssODN. **(B)**

383   RNP targeting the HBB locus without ssODN. **(C)** RNP targeting the BFP locus with donor ssODN. **(D)**

384   RNP targeting the BFP locus without ssODN. Data presented are the mean±sd of biological duplicates.

385

386   **Extended Data Figure 10:** Flow cytometry and amplicon sequencing produce similar editing rates. Editing

387   at the BFP locus was quantified by flow cytometry (light blue), amplicon sequencing followed by in-house

388   bioinformatic analysis (medium blue), or amplicon sequencing followed by CRISPRessoPOOL[33] analysis

389   (dark blue). Data presented are biological duplicate.

390

391   **Document S1:**   Materials and Methods (Word doc)

392   **Document S2:**   Molecular biology reagents (Excel doc)

393   **Document S3:**   Processed screen data – collapsed gene scores (Excel doc)

394

395   **Acknowledgements**

401

402   **Author Contributions**

403   CDR and JEC designed experiments; CDR, AJS, and SF performed pooled screens; CDR, KRK, and SJF

404   performed follow-up experiments; CDR, NLB, and JEC analyzed data; CDR and JEC wrote the manuscript.

405

406   **References**

407   1.   DeWitt, M. A. *et al.* Selection-free genome editing of the sickle mutation in human adult
408   hematopoietic stem/progenitor cells. *Sci Transl Med* **8,** 360ra134–360ra134 (2016).
409   2.   Cox, D. B. T., Platt, R. J. & Zhang, F. Therapeutic genome editing: prospects and challenges. *Nat*
410   *Med* **21,** 121–131 (2015).
411   3.   Sternberg, S. H. & Doudna, J. A. Expanding the Biologist's Toolkit with CRISPR-Cas9. *Mol Cell* **58,**
412   568–574 (2015).
413   4.   Maggio, I. & Gonçalves, M. A. F. V. Genome editing at the crossroads of delivery, specificity, and
414   fidelity. *Trends in biotechnology* **33,** 280–291 (2015).
415   5.   Chen, F. *et al.* High-frequency genome editing using ssDNA oligonucleotides with zinc-finger
416   nucleases. *Nat Meth* **8,** 753–755 (2011).
417   6.   Jasin, M. Genetic manipulation of genomes with rare-cutting endonucleases. *Trends Genet* **12,** 224–
418   228 (1996).
419   7.   Richardson, C. D., Ray, G. J., DeWitt, M. A., Curie, G. L. & Corn, J. E. Enhancing homology-directed
420   genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA. *Nat*
421   *Biotechnol* (2016). doi:10.1038/nbt.3481
422   8.   Sander, J. D. & Joung, J. K. CRISPR-Cas systems for editing, regulating and targeting genomes.
423   *Nat Biotechnol* **32,** 347–355 (2014).
424   9.   Wright, A. V. *et al.* Rational design of a split-Cas9 enzyme complex. *Proceedings of the National*

Academy of Sciences **112,** 2984–2989 (2015).

10. Kim, S., Kim, D., Cho, S. W., Kim, J. & Kim, J.-S. Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. *Genome Research* **24,** 1012–1019 (2014).

11. Horlbeck, M. A., Gilbert, L. A., Villalta, J. E. & Adamson, B. Compact and highly active next-generation libraries for CRISPR-mediated gene repression and activation. *eLife* (2016). doi:10.7554/eLife.19760.001

12. Huang, D. W., Sherman, B. T. & Lempicki, R. A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *UNKNOWN* **4,** 44–57 (2008).

13. George, B. *et al.* Regulation and function of Myb-binding protein 1A (MYBBP1A) in cellular senescence and pathogenesis of head and neck cancer. *Cancer Lett* **358,** 191–199 (2015).

14. Subramanian, A. *et al.* Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* **102,** 15545–15550 (2005).

15. Lin, S., Staahl, B. T., Alla, R. K. & Doudna, J. A. Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. *eLife* **4,** (2014).

16. Bothmer, A. *et al.* Characterization of the interplay between DNA repair and CRISPR/Cas9-induced DNA lesions at an endogenous locus. *Nat Commun* **8,** 1–12 (2016).

17. Davis, L. & Maizels, N. Homology-directed repair of DNA nicks via pathways distinct from canonical double-strand break repair. *Proc Natl Acad Sci USA* **111,** E924–32 (2014).

18. Yang, Y.-G. *et al.* The Fanconi anemia group A protein modulates homologous repair of DNA double-strand breaks in mammalian cells. *Carcinogenesis* **26,** 1731–1740 (2005).

19. Hustedt, N. & Durocher, D. The control of DNA repair by the cell cycle. *Nat Cell Biol* (2017). doi:10.1038/ncb3452

20. Walden, H. & Deans, A. J. The Fanconi anemia DNA repair pathway: structural and functional insights into a complex disorder. *Annu Rev Biophys* **43,** 257–278 (2014).

21. Moldovan, G.-L. & D'Andrea, A. D. How the fanconi anemia pathway guards the genome. *Annu Rev Genet* **43,** 223–249 (2009).

22. Palovcak, A., Liu, W., Yuan, F. & Zhang, Y. Maintenance of genome stability by Fanconi anemia proteins. *Cell Biosci* **7,** 8 (2017).

23. Ellis, N. A., Groden, J., Ye, T. Z., Straughen, J. & Lennon, D. J. The Bloom's syndrome gene product is homologous to RecQ helicases. *Cell* **83,** 655–666 (1995).

24. Adelman, C. A. *et al.* HELQ promotes RAD51 paralogue-dependent repair to avert germ cell loss and tumorigenesis. *Nature* **502,** 381–384 (2013).

25. Chun, J., Buechelmaier, E. S. & Powell, S. N. Rad51 paralog complexes BCDX2 and CX3 act at different stages in the BRCA1-BRCA2-dependent homologous recombination pathway. *Mol Cell Biol* **33,** 387–395 (2013).

26. Adamo, A. *et al.* Preventing nonhomologous end joining suppresses DNA repair defects of Fanconi anemia. *Mol Cell* **39,** 25–35 (2010).

27. Renaud, E., Barascu, A. & Rosselli, F. Impaired TIP60-mediated H4K16 acetylation accounts for the aberrant chromatin accumulation of 53BP1 and RAP80 in Fanconi anemia pathway-deficient cells. *Nucleic Acids Research* **44,** 648–656 (2016).

28. Howard, S. M., Yanez, D. A. & Stark, J. M. DNA damage response factors from diverse pathways, including DNA crosslink repair, mediate alternative end joining. *PLoS Genet.* **11,** e1004943 (2015).

29. van Overbeek, M. *et al.* DNA Repair Profiling Reveals Nonrandom Outcomes at Cas9-Mediated Breaks. *Mol Cell* 1–30 (2016). doi:10.1016/j.molcel.2016.06.037

30. Song, J. *et al.* RS-1 enhances CRISPR/Cas9- and TALEN-mediated knock-in efficiency. *Nat Commun* **7,** 10548 (2016).

31. Orthwein, A. *et al.* A mechanism for the suppression of homologous recombination in G1 cells. *Nature* (2015). doi:10.1038/nature16142

32. Knight, S. C. *et al.* Dynamics of CRISPR-Cas9 genome interrogation in living cells. **350,** 823–826 (2015).

33. Pinello, L. *et al.* Analyzing CRISPR genome-editing experiments with CRISPResso. *Nat Biotechnol* **34,** 695–697 (2016).
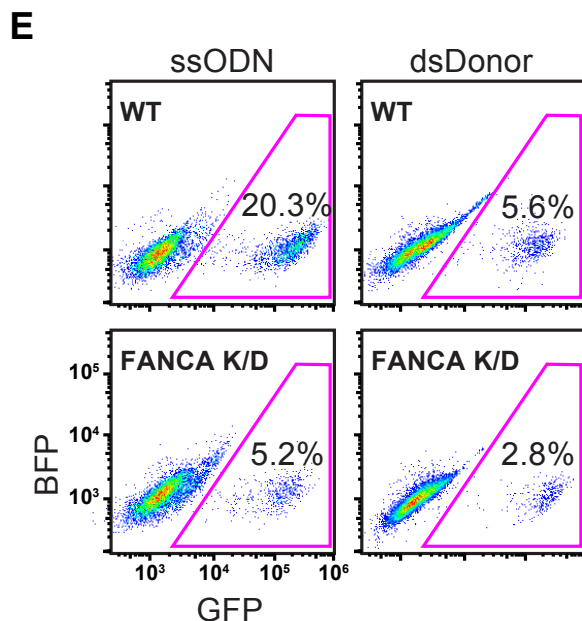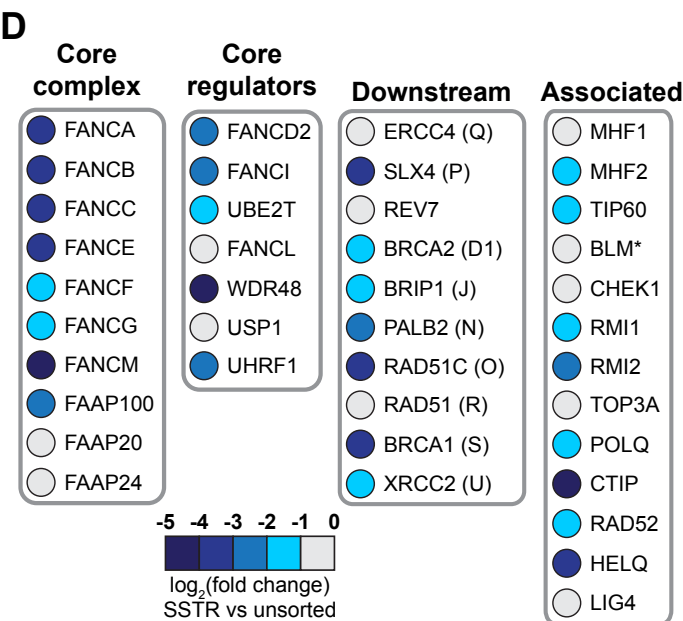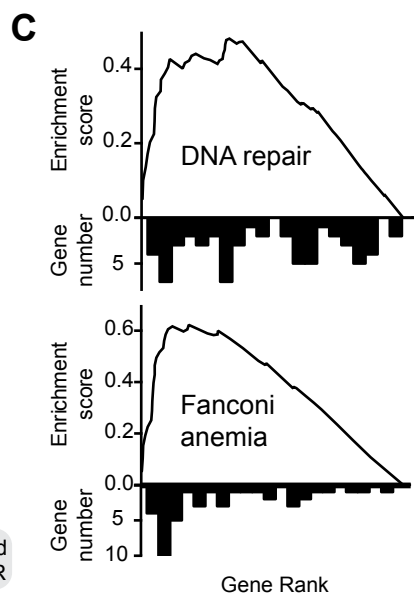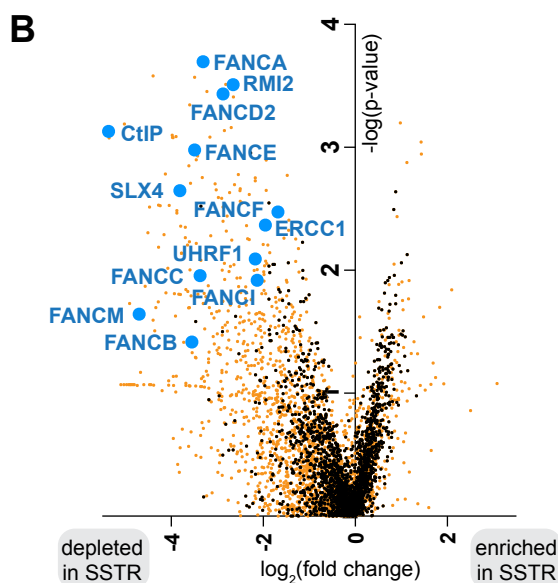
Figure 1

Figure 2

A

BFP His67Tyr

Fold Change SSTR

K/D: NT, FANCA, FANCD2, FANCE, FANCF, FANCL, HELQ, UBE2T, USP1, WDR48

cDNA: ● ● ● ● ● ● ● ● ●

B

HBB Glu6Val

Fold Change Editing

Legend: NHEJ, SSTR

K/D: NT, FANCA, FANCD2, FANCE, FANCF, FANCL, HELQ, UBE2T, USP1, WDR48

C

HBB Glu6Val

Fold Change SSTR

K/D: NT, FANCA, FANCF
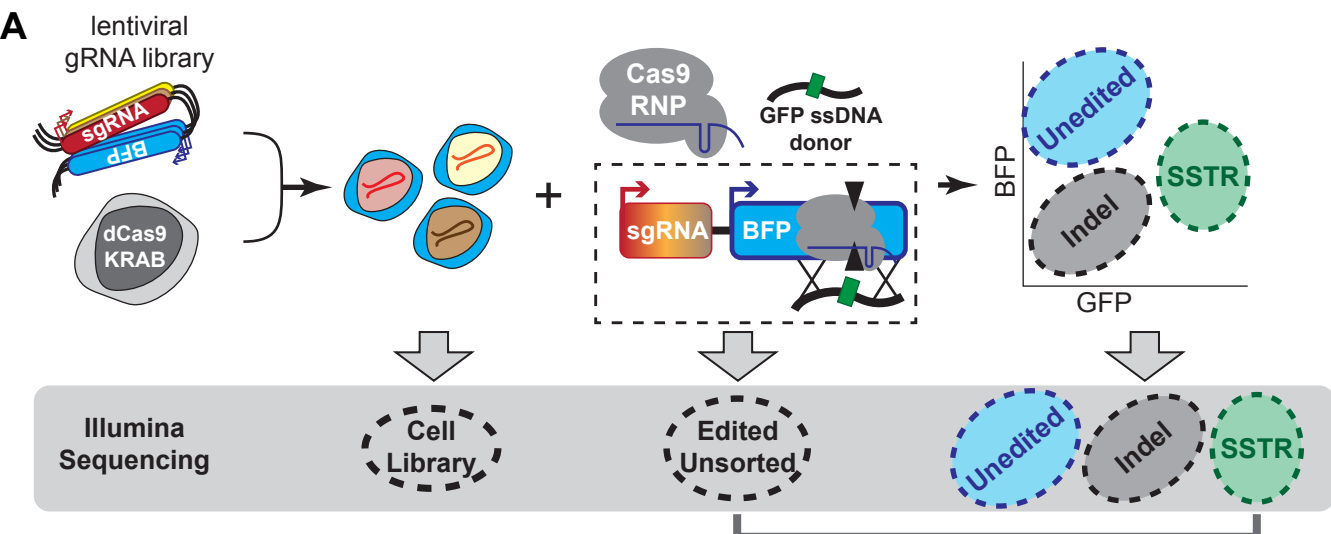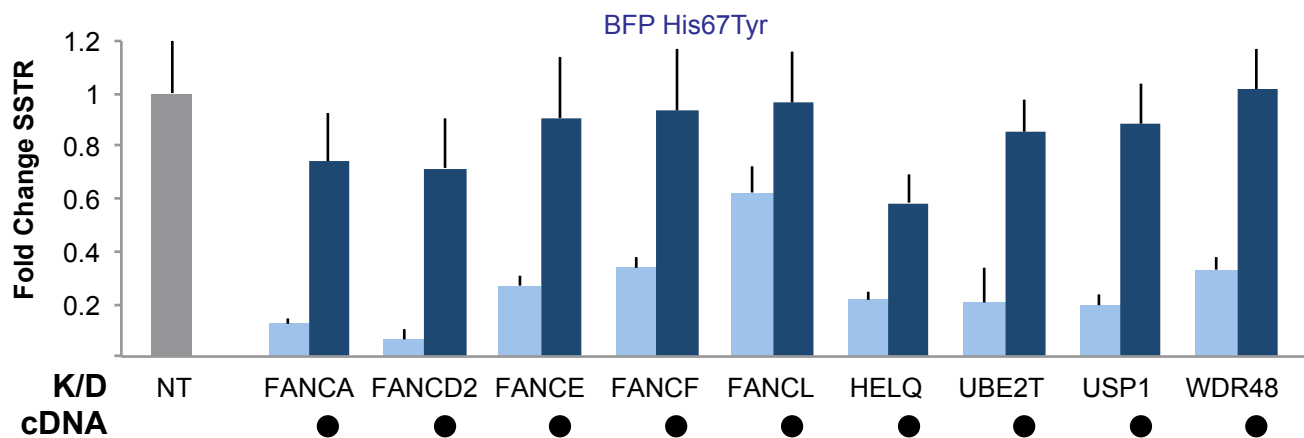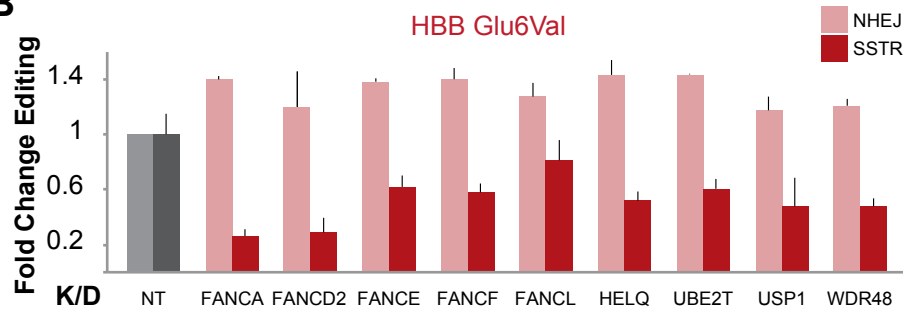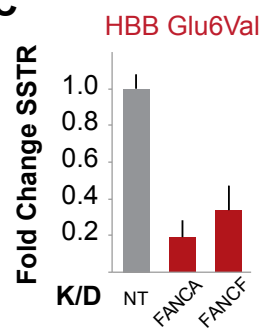
Figure 3

A

B
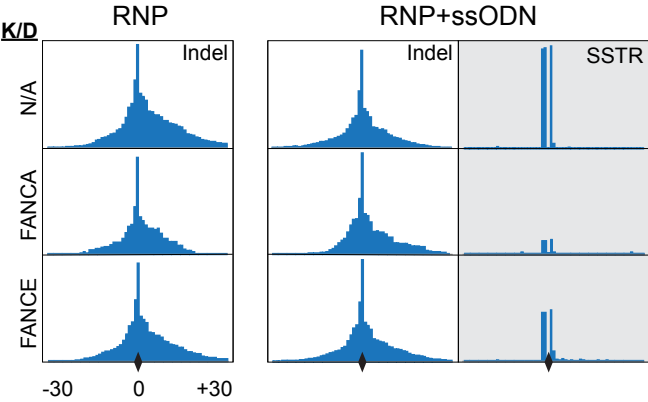
BFP His67Tyr

		Freq.	Type
WT	CCACCCTGAC<u>CCCA</u>TG-GCGTGCAGTGCTTCAGCCGC		
	................GT.C......................	26.3	SSTR
NT	.........................-................	16.6	Indel
	.................-----....................	4.4	Indel
	....	3.2	WT
	.................---......................	3.1	Indel
FANCA K/D	....	25.6	WT
	................A.........................	12.0	Indel
	...............--A........................	6.8	Indel
	...............TA.........................	6.7	Indel
	...............-----......................	6.6	Indel

HBB Glu6Val
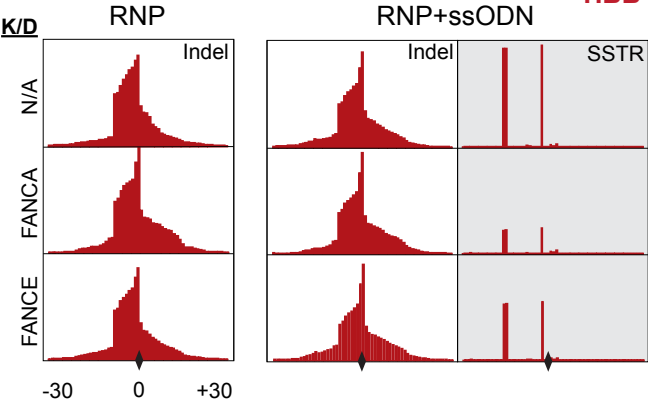
		Freq.	Type
WT	TGAGGAGAAGTCTGC<u>C</u>CGTT--ACTGCCCTGTGGGGCA		
	..TA...........G..........................	29.9	SSTR
NT	...............G..........................	7.8	SSTR
	....	7.4	WT
	...........-----------....................	4.4	Indel
	.....................----.................	2.2	Indel
FANCA K/D	....	22.5	WT
	..TA...........G..........................	5.8	SSTR
	...........-----------....................	5.4	Indel
	.....................A....................	3.0	Indel
	....................--....................	2.8	Indel

Figure 4
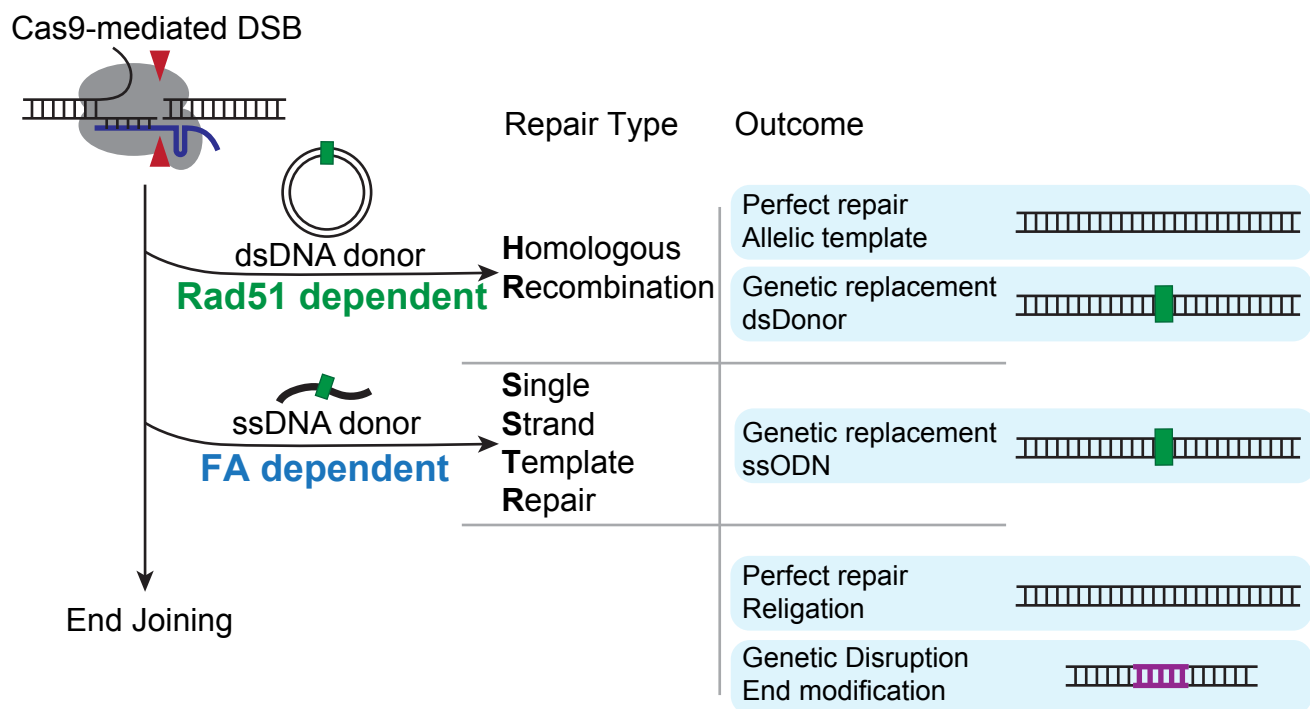
Extended Data Figure 1
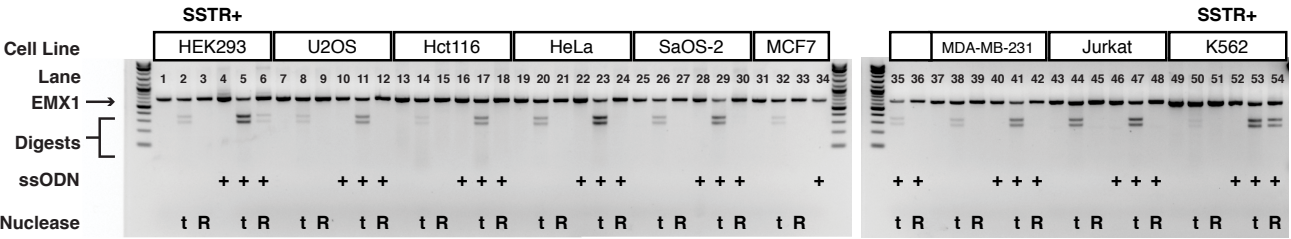
Extended Data Figure 2

A
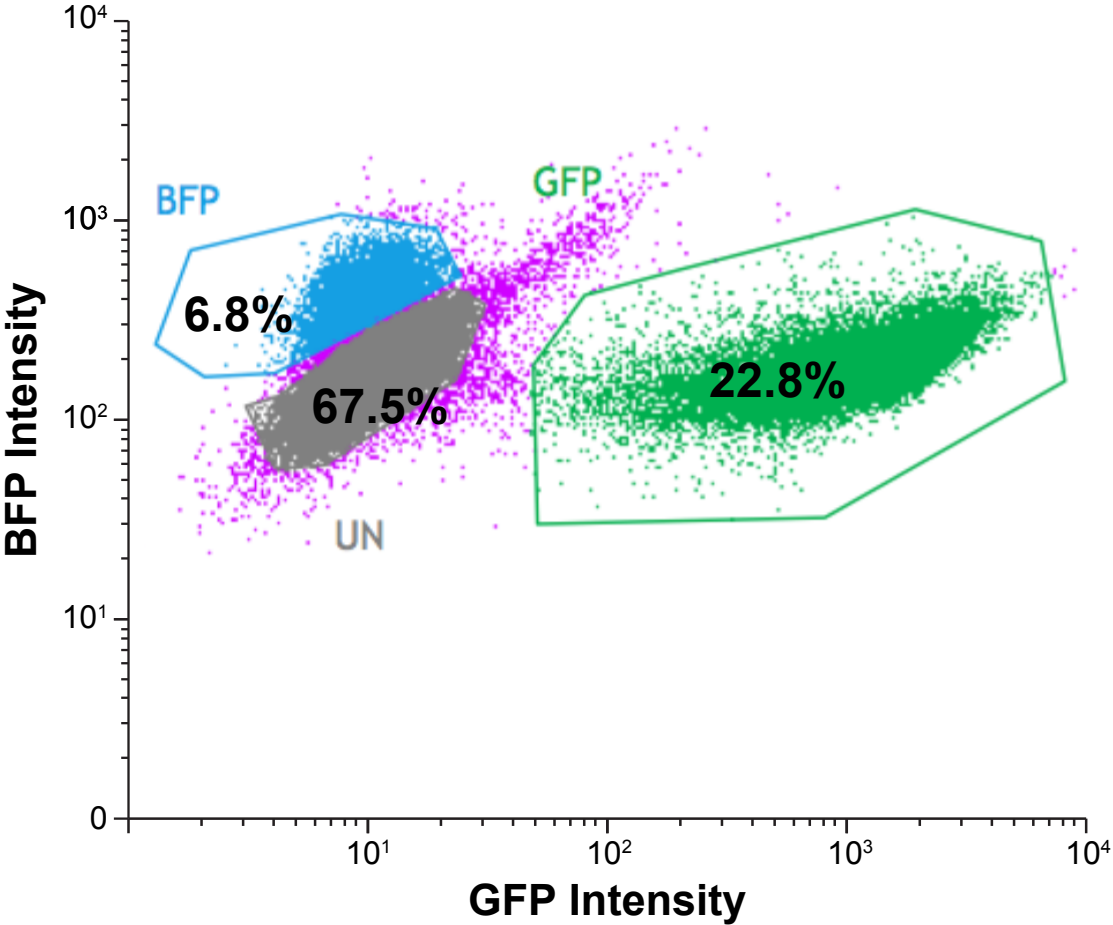
B

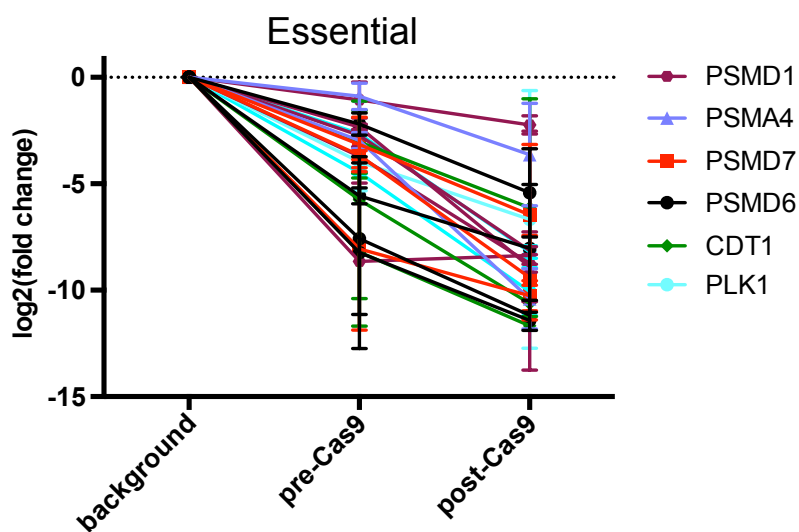| Sample	Sorted Cells
dCas9-KRAB Replicate 1	7,001,595
dCas9-KRAB Replicate 2	7,002,159
K562	7,043,117

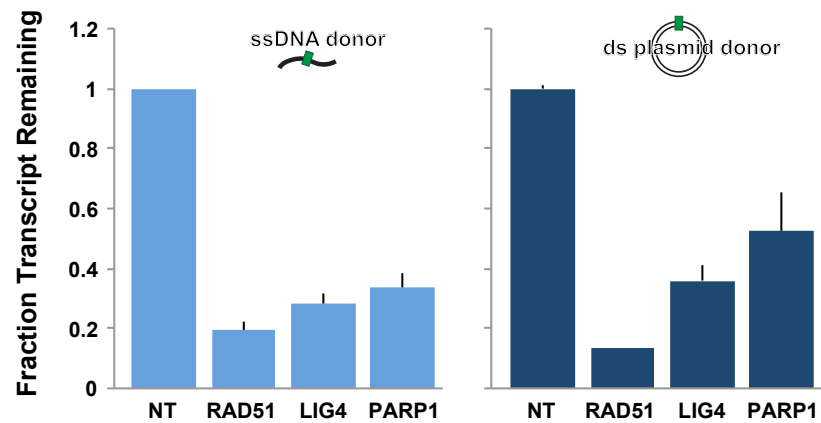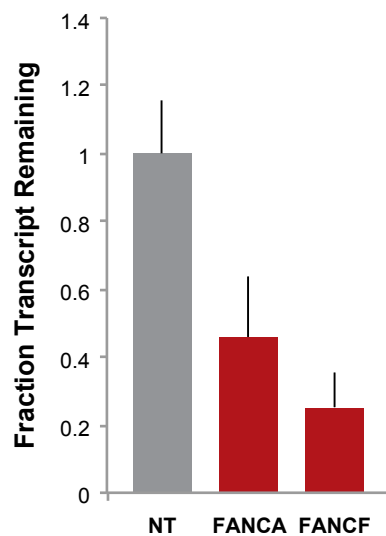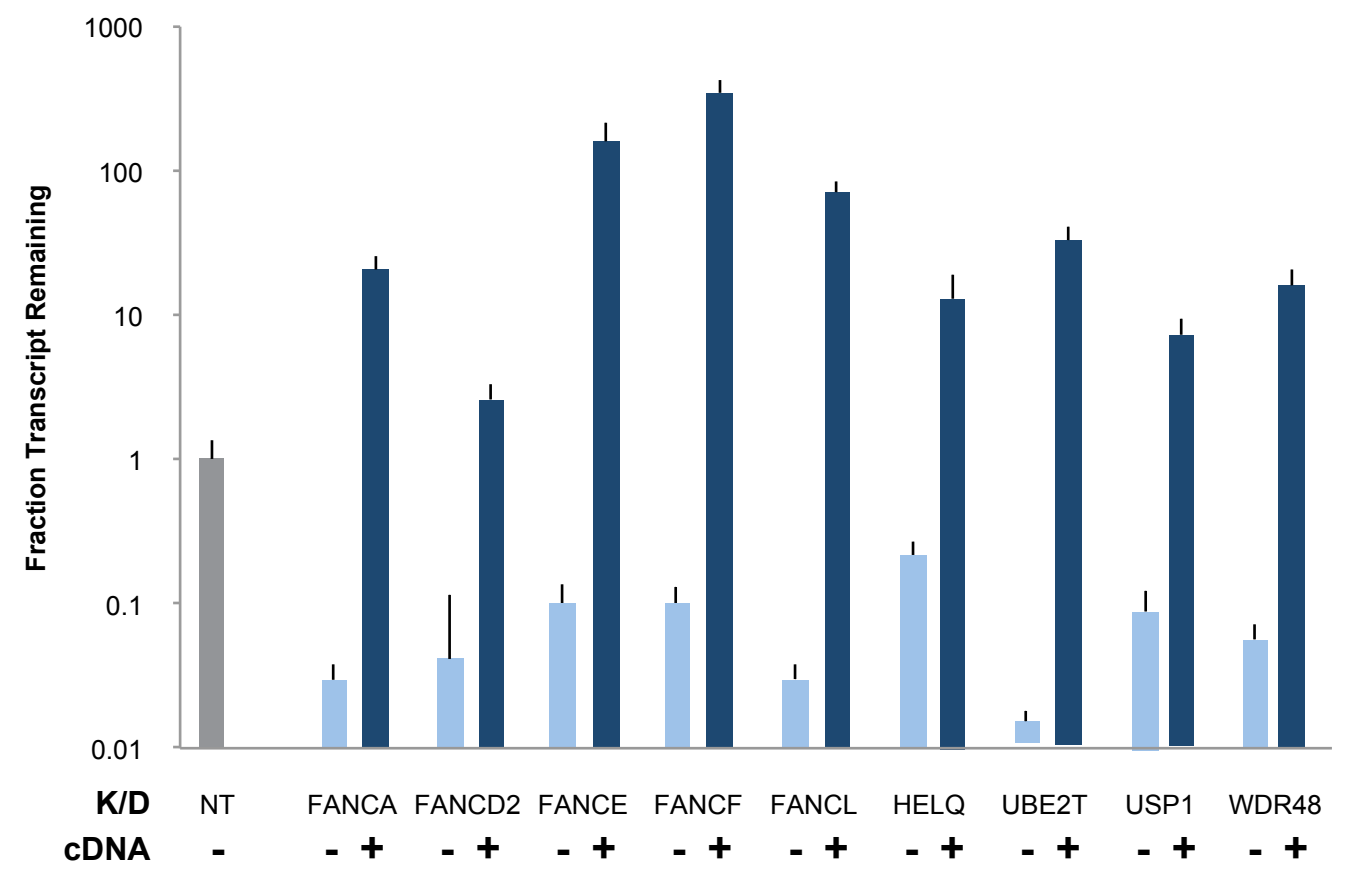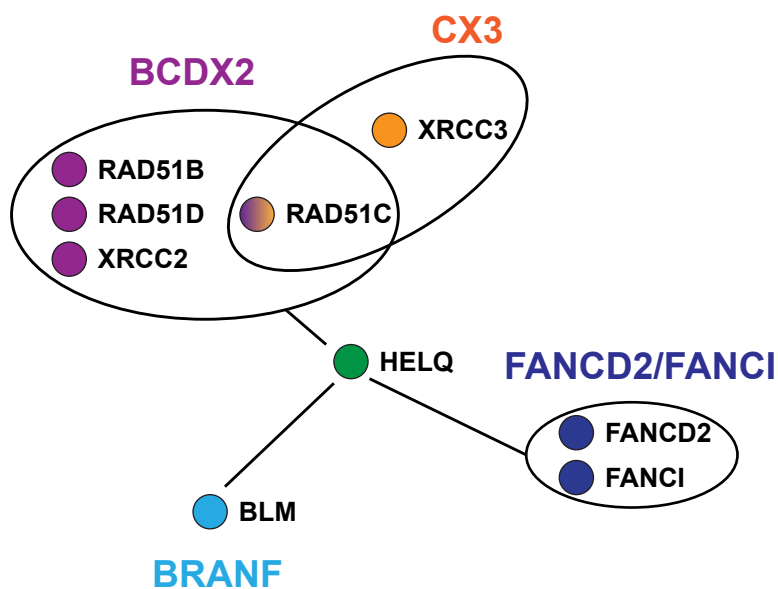# Extended Data Figure 3

A

lentiviral gRNA library

Illumina Sequencing — CRISPRi +guide, guide only

Essential

-log(p-value)

PSMD1
PSMA4
PSMD7
PSMD6
CDT1
PLK1

non-targeting guides
targeting guides

log2(fold change vs no CRISPRi)

B

lentiviral gRNA library

Cas9 RNP + GFP ssDNA donor

Illumina Sequencing — Cell Library, Edited Unsorted

Synthetic lethal with Cas9 double strand break

-log(p-value)

INTS3
RBX1
MYBBP1
POLD3
RPP30
SSBP1

non-targeting guides
targeting guides

log2(fold change vs no DSB)

Extended Data Figure 4

A

Replicate 1 | Horlbeck 2016

71

14

126

29

97

10

80

Replicate 2

B

Essential

log2(fold change)

background — pre-Cas9 — post-Cas9

- PSMD1
- PSMA4
- PSMD7
- PSMD6
- CDT1
- PLK1

C

Synthetic lethal with Cas9 double strand break

log2(fold change)

background — pre-Cas9 — post-Cas9

- INTS3
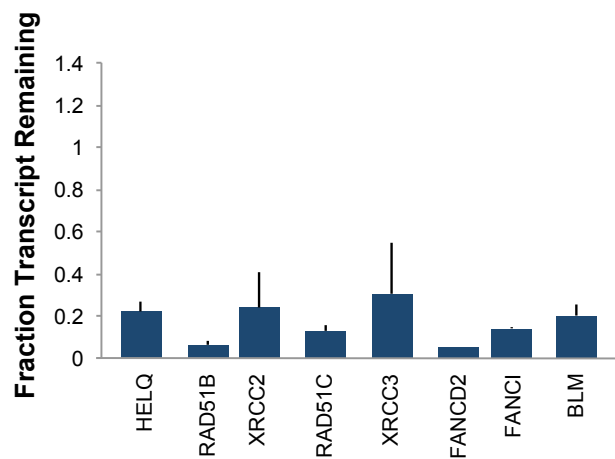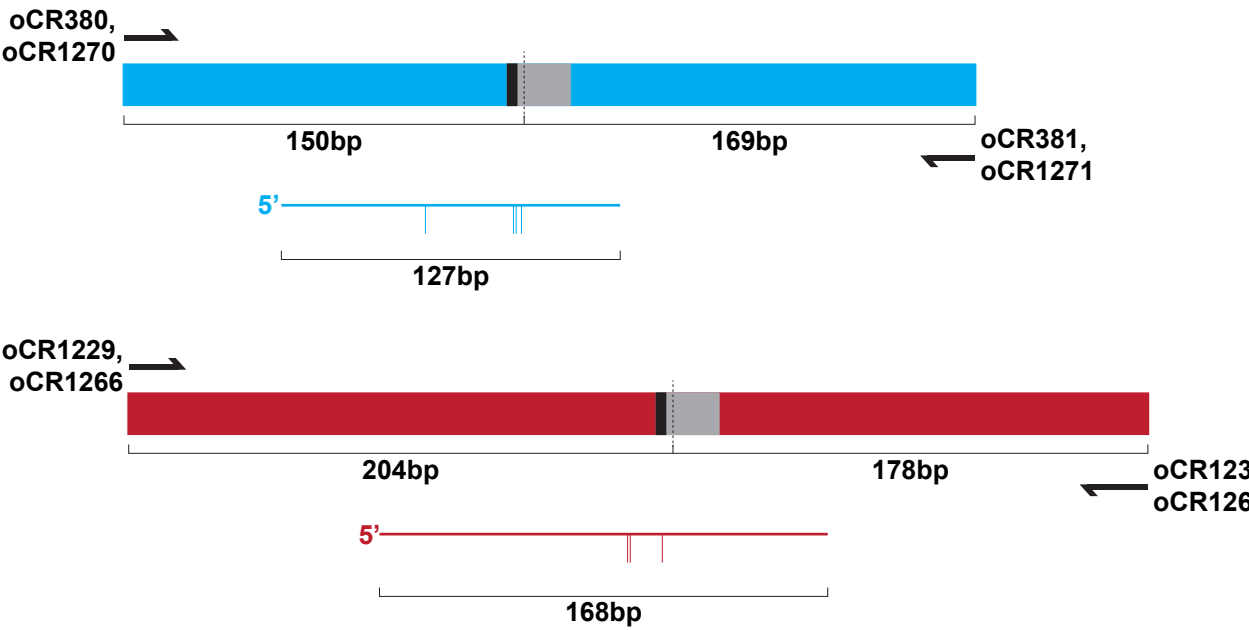- RBX1
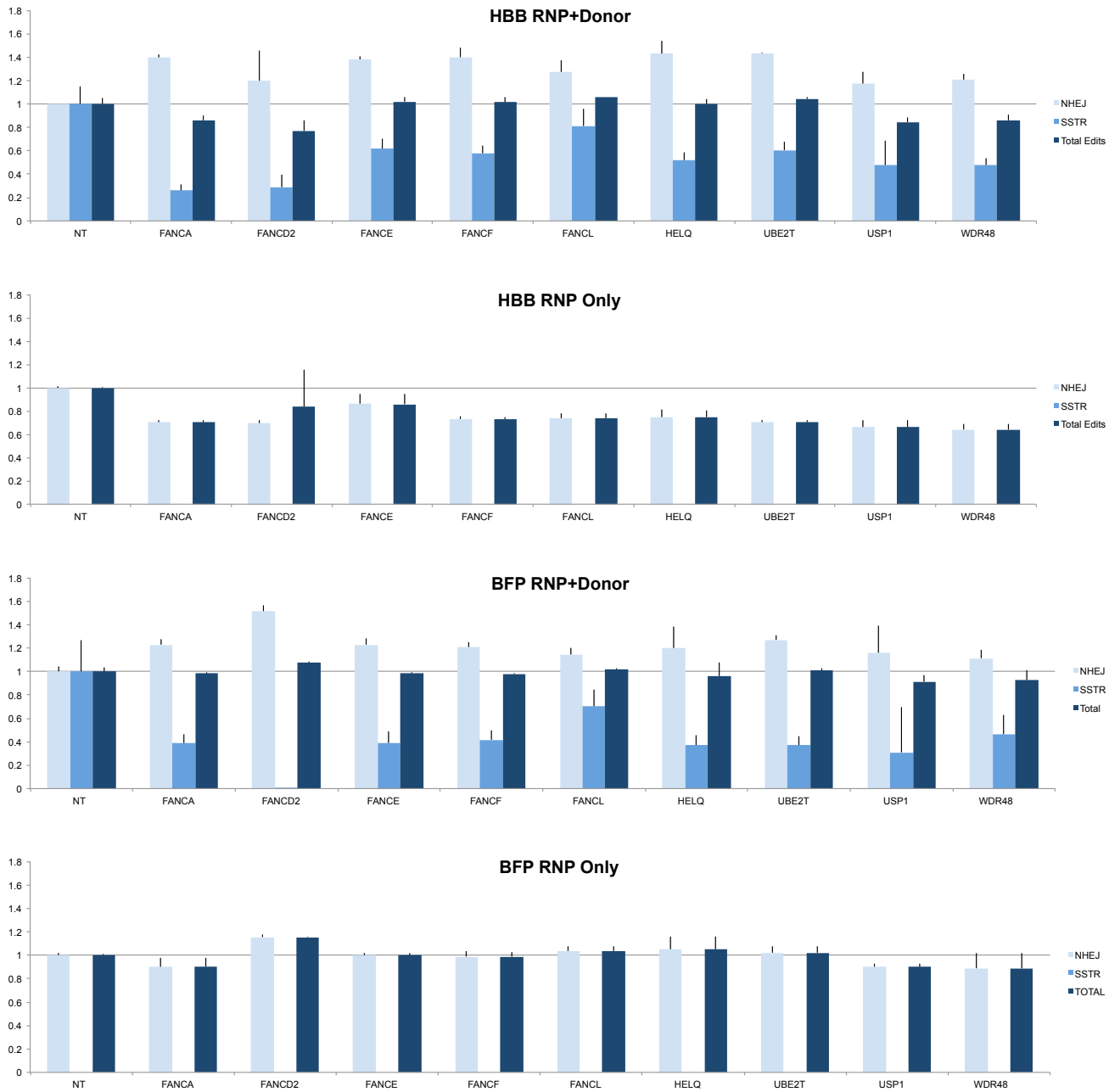- MYBBP1
- POLD3
- RPP30
- SSBP1
- INTS3

Extended Data Figure 5

Extended Data Figure 6

Extended Data Figure 7

A

B
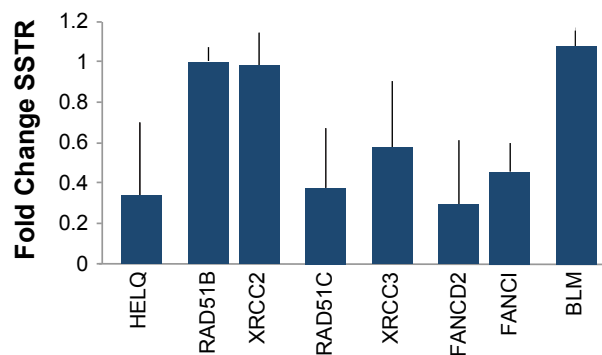
C

Extended Data Figure 8

Extended Data Figure 9

HBB RNP+Donor

HBB RNP Only

BFP RNP+Donor

BFP RNP Only

Extended Data Figure 10

Flow vs Amplify-on BFP Editing