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**Enhanced homology-directed human genome engineering by controlled  
timing of CRISPR/Cas9 delivery**

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24 **Abstract**

25           The CRISPR/Cas9 system is a robust genome editing technology that works in  
26 human cells, animals and plants based on the RNA-programmed DNA cleaving activity  
27 of the Cas9 enzyme. Building on previous work (Jinek et al., 2013), we show here that  
28 new genetic information can be introduced site-specifically and with high efficiency by  
29 homology-directed repair (HDR) of Cas9-induced double-strand DNA breaks using  
30 timed delivery of Cas9-guide RNA ribonucleoprotein (RNP) complexes. Cas9 RNP-  
31 mediated HDR in HEK293T, human primary neonatal fibroblast and human embryonic  
32 stem cells was increased dramatically relative to experiments in unsynchronized cells,  
33 with rates of HDR up to 38% observed in HEK293T cells. Sequencing of on- and  
34 potential off-target sites showed that editing occurred with high fidelity, while cell  
35 mortality was minimized. This approach provides a simple and highly effective strategy  
36 for enhancing site-specific genome engineering in both transformed and primary human  
37 cells.

38

39 **Introduction**

40           The CRISPR-associated enzyme Cas9 enables site-specific genome engineering  
41 by introducing double-strand breaks (DSB) at guide RNA-specified chromosomal loci of  
42 interest (Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013a). Cells repair DSBs using  
43 the non-homologous end joining (NHEJ) or homology-directed repair (HDR) pathways.  
44 The NHEJ pathway generates variable insertions or deletions (indels) at the DSB, while  
45 HDR employs homologous donor DNA sequences from sister chromatids, homologous  
46 chromosomes or exogenous DNA molecules to produce precise insertions, deletions or

47 base substitutions at a DSB site or between two DSBs. Such precise modifications are  
48 desired for targeted genome engineering.

49 Although cells have differing abilities to repair DSBs using NHEJ or HDR, the  
50 phase of the cell cycle largely governs the choice of pathway. NHEJ dominates DNA  
51 repair during G1, S and G2 phases, whereas HDR is restricted to late S and G2 phases  
52 when DNA replication is completed and sister chromatids are available to serve as repair  
53 templates (Heyer et al., 2010). Impediments to HDR include competition with NHEJ in  
54 S and G2 phases and specific down-regulation of HDR at M phase and early G1 to  
55 prevent telomere fusion (Orthwein et al., 2014). Although chemical or genetic  
56 interruption of the NHEJ pathway can favor HDR (Shrivastav et al., 2008), such  
57 manipulations can be difficult to employ, harmful to cells or both. Consequently, high  
58 cleavage activity of programmable nucleases does not necessarily correlate with efficient  
59 HDR-induced genome editing.

60 Here we report a simple and robust approach that advances our previous findings  
61 (Jinek et al., 2013) to enhance HDR efficiency in human cells. This strategy combines  
62 well-established cell cycle synchronization techniques with direct nucleofection of pre-  
63 assembled Cas9 ribonucleoprotein (RNP) complexes to achieve controlled nuclease  
64 action at the phase of the cell cycle best for HDR (Figure 1A). HEK293T, human  
65 primary neonatal fibroblast and H9 human embryonic stem cells demonstrated robust  
66 HDR-mediated genome editing at levels up to 38% with no detected off-target editing.  
67 These results establish a superior approach to Cas9-mediated human genome engineering  
68 that enables efficient mutation, repair and tagging of endogenous loci in a rapid and  
69 predictable manner.

## 70 **Results**

71 To test whether S phase is optimal for HDR in HEK293T cells, six reversible  
72 chemical inhibitors were used in parallel experiments to synchronize HEK293T cells at  
73 G1, S and M phases of the cell cycle, followed by release prior to nucleofection with  
74 Cas9 RNP (Figure 1B, Figure 1-figure supplement 1A). Immediately after release we  
75 prepared 30- $\mu$ l nucleofection reactions containing  $2 \times 10^5$  cells, Cas9 RNP with sgRNA  
76 targeting EMX1 gene and a 183-nucleotide single-stranded oligonucleotide DNA  
77 (ssODNA) HDR template (Figure 1C). After 24 hours, cells were analyzed for HDR  
78 (specifically, exogenous donor template mediated HDR) or total editing (TE, defined as  
79 the sum of all NHEJ and HDR events that give rise to indels) at the Cas9 cleavage site  
80 within EMX1, showing that both aphidicolin and nocodazole led to pronounced increases  
81 in Cas9-mediated editing frequencies (Figure 1D, E). The enhancement is more evident  
82 at lower Cas9 RNP concentration (30  $\mu$ mol), improving HDR rates from ~9% in  
83 unsynchronized cells to ~14% with aphidicolin and ~20% with nocodazole (Figure 1E).  
84 The highest HDR frequency achieved was 31% with nocodazole synchronization and 100  
85  $\mu$ mol of Cas9 RNP. Importantly, one day after nocodazole release the synchronized cells  
86 were cycling like unsynchronized controls and appeared morphologically normal (Figure  
87 1-figure supplement 1A).

88 Next we determined systematically the dosage effect of Cas9 RNPs and HDR  
89 templates on HDR efficiency in control and nocodazole synchronized cells. At the  
90 EMX1 locus, we tested three concentrations of Cas9 RNP (10, 30 and 100  $\mu$ mol) in  
91 combination with three concentrations of HDR template (50, 100 and 200  $\mu$ mol in Figure  
92 1C). The overall frequencies of TE and HDR increased proportionally with increasing

93 Cas9 RNP concentration (Figure 2A). Synchronization increased the TE frequency two-  
94 fold at 10  $\mu$ mol and 1.5-fold at 30  $\mu$ mol Cas9 RNP, but the enhancement diminished at  
95 100  $\mu$ mol. The HDR frequency also increased dramatically with synchronization,  
96 especially at lower concentrations of Cas9 RNP, from undetectable to 9-15% at 10  $\mu$ mol  
97 of Cas9 RNP, and from 6-12% to 22-28% at 30  $\mu$ mol (Figure 2A). These results  
98 demonstrate that timed delivery of Cas9 RNP into M-phase synchronized HEK293T cells  
99 enhances HDR by several fold above the levels observed without synchronization.

100 To test these effects at other genomic loci, we programmed Cas9 RNPs to target  
101 the DYRK1 gene, which is important for brain development, autism and Downs  
102 Syndrome (Arron et al., 2006; Fotaki et al., 2002; O'Roak et al., 2012). We assayed two  
103 ssODNA HDR templates spanning the same sequence but with different orientations: one  
104 identical to the target strand sequence (+ strand) and the other its complement (- strand)  
105 (Figure 2B). Both of these templates yielded comparable levels of HDR. Strikingly,  
106 nocodazole synchronization enhanced the TE frequencies more than two-fold and HDR  
107 frequencies over six-fold at all doses of Cas9 RNP (Figure 2B). Moreover, nocodazole  
108 synchronization reduced the requirement for high Cas9 RNP concentrations, producing 5-  
109 6% HDR at 10  $\mu$ mol of Cas9; ten-fold more Cas9 RNP was required to achieve the same  
110 HDR frequency in unsynchronized cells.

111 We also programmed Cas9 RNPs to target the CXCR4 gene, a chemokine  
112 receptor implicated in HIV entry (Feng et al., 1996) and cancer metastasis (Murphy,  
113 2001). The HDR template used in these experiments was a ssODNA oriented  
114 complementary (- strand) to the target strand, containing HindIII and BamHI restriction  
115 sites flanked by 90-nt homology arms. The enhancement in TE and HDR frequencies at

116 CXCR4 was comparable to those observed for the DYRK1 target site (Figure 2C). The  
117 most significant increase was again observed at 10 and 30  $\mu$ mol of Cas9 RNP, yielding  
118 nearly five and two-fold increases, respectively. In this case, nocodazole synchronization  
119 yielded 27% HDR at 10  $\mu$ mol of Cas9 RNP. A comparable level of HDR in the  
120 unsynchronized cells would require 100  $\mu$ mol of RNP. Collectively, the results from  
121 EMX1, DYRK1 and CXCR4 loci demonstrate that nocodazole synchronization is a  
122 highly effective and broadly applicable method to enhance the TE and HDR frequencies  
123 in HEK293T cells.

124 Off-target editing increases with increasing nucleic acid-based delivery of Cas9  
125 (Fu et al., 2013; Hsu et al., 2013; Mali et al., 2013b; Pattanayak et al., 2013). We  
126 reasoned that the short-lived Cas9 RNP (Kim et al., 2014) and timed delivery would  
127 minimize off-target editing and that potential toxicity might be minimized by using lower  
128 RNP amounts. We compared the TE and HDR frequencies at the EMX1 and DYRK1  
129 target loci to those occurring at the top two predicted off-target loci (Hsu et al., 2013)  
130 respectively by deep sequencing a representative biological replicate experiment from  
131 Figure 2. Importantly, no off-target editing was detected above background levels under  
132 all conditions, and increasing RNP dosage had no effect on off-target editing. As shown  
133 in Figure 2-figure supplement 1A, the TE (indels/total reads) and HDR (HDR/total reads)  
134 frequencies at EMX1 and DYRK1 increased with RNP dosage in both cell conditions.  
135 Most importantly, there was no detectable HDR at the off-target loci. Overall, the TE  
136 and HDR frequencies detected by deep sequencing were comparable to our previous gel  
137 densitometry results (Figure 2A, B). The deep sequencing analysis also allowed us to  
138 determine the ratio of Cas9-induced DSBs being repaired by the NHEJ versus HDR

139 pathway (HDR/TE reads). Although nocodazole synchronization increased the HDR/TE  
140 ratio by two-fold at the EMX1 locus and five-fold at the DYRK1 locus, this ratio reached  
141 a maximum value of ~33% across all RNP doses, suggesting that the maximum capacity  
142 of the HDR machinery in HEK293T cells is to repair ~33% of DSBs. A panel of  
143 representative indels is shown in Figure 2-figure supplement 1B.

144 To examine the length of HDR template homology sequences required for Cas9-  
145 mediated HDR, we tested four single-stranded and two double-stranded HDR templates  
146 for EMX1 bearing homology arms ranging from 30 to 250 nt in length (template 2-7 in  
147 Figure 3A). To avoid signal saturation and better distinguish the HDR frequencies of  
148 different templates, we reduced the Cas9 RNP and HDR template concentrations to 30  
149 pmol and 50 pmol, respectively. As observed previously, nocodazole synchronization  
150 produced higher HDR frequencies than observed in the unsynchronized cells (Figure 3B).  
151 In addition to the unsynchronized and nocodazole synchronized conditions, we included a  
152 third condition in which aphidicolin, an S-phase blocker, was added to the nocodazole  
153 synchronized cells immediately after nucleofection. We hypothesized that the  
154 aphidicolin-blocked cells would show reduced HDR frequency due to the inability to  
155 enter S phase where HDR is thought to be most active. As expected, the aphidicolin  
156 block significantly reduced the HDR frequency, supporting the conclusion that cells need  
157 to proceed through S phase, and possibly G2 as well, for highly efficient HDR.

158 DNA molecules with at least 60 nt of sequence homology flanking the Cas9  
159 cleavage site were sufficient for highly efficient HDR of ~19% (Figure 3B). Further  
160 extension of the homology arms to 90 nt increased the HDR frequency only slightly  
161 (~20-23%). Both (+) and (-) template orientations were similarly effective, as also

162 observed in the DYRK1 experiment. When double-stranded templates 6 and 7 were used,  
163 HDR frequencies were reduced to 7%. Moreover, unusual banding patterns in the  
164 HindIII HDR assay (Figure 3B) implied the presence of a concatemerized HDR template  
165 or non-specific recombination products, at least with short sequences as employed here.

166 To expand our findings on the cell cycle synchronization method to other cell  
167 types, we targeted the EMX1 gene in human primary neonatal fibroblasts (neoFB) and  
168 H9 human embryonic stem (hES) cells. These cell types are challenging to transfect and  
169 typically show low levels of homologous recombination. To determine the cell cycle  
170 phase that is optimal for HDR in neoFB cells, we used the same six chemical inhibitors to  
171 synchronize neoFB cells at G1, S and M phases of the cell cycle, followed by release  
172 prior to nucleofection with Cas9 RNP (Figure 4A). Cell cycle synchronization was  
173 confirmed by FACS analysis, and cells in all conditions appeared morphologically  
174 normal. Aphidicolin-synchronized cells progressed normally through the cell cycle  
175 following release from cell cycle block (Figure 1-figure supplement 1B). In contrast to  
176 HEK293T cells, enhancement in TE and HDR frequencies was observed with aphidicolin  
177 and thymidine treatments, which synchronize the cells at S phase (Figure 4A). The TE  
178 frequencies were 17% and 13% with aphidicolin and thymidine respectively, as opposed  
179 to 5% in the unsynchronized condition. However, HDR frequency was very low across  
180 all conditions and was not detected in the unsynchronized cells. With aphidicolin  
181 synchronization, 1% and 1.3% HDR were detected at 30 and 100  $\mu$ mol Cas9 RNP  
182 respectively.

183 We then tested hES cells in a similar experimental setup. Previous reports have  
184 shown ~20% NHEJ with transfection of Cas9 RNPs but didn't analyze HDR rates (Kim



185 et al., 2014). Also, HDR frequencies are low with nucleic acid-based delivery of Cas9  
186 (Hsu et al., 2013). Screening of chemical inhibitors showed that only nocodazole  
187 synchronization enhanced TE frequencies in hES cells to 9% at 30 pmol and 28-31% at  
188 100 pmol Cas9 RNP; however, we did not detect HDR with the HDR template (-) sense  
189 strand (Figure 4B). In light of these observations, we modified a protocol from Pauklin  
190 et al. (Pauklin and Vallier, 2013), in which the cells were treated with nocodazole for 16  
191 h, washed to remove the drug, and then treated with aphidicolin for 3 h before  
192 nucleofection. Using this approach, we detected ~2% of HDR at 100 pmol Cas9 RNP  
193 (Figure 4B). Cell synchronization was confirmed by FACS analysis. Three days after  
194 release from cell cycle synchronization the hES cell cycle behavior was indistinguishable  
195 from unsynchronized control cultures, with no apparent changes in colony morphology  
196 (Figure 1-figure supplement 1C); all colonies expressed high levels of alkaline  
197 phosphatase, a marker for pluripotency (Figure 1-figure supplement 1D). ROCK  
198 apoptosis inhibitor (10  $\mu$ M) was required for survival of synchronized H9 ESCs after  
199 release when cultured at low density but not when cultured at high density. These results  
200 suggest that different cell types will have distinct requirements for synchronization to  
201 enhance Cas9 RNP-induced DNA repair. Also, it may be possible to find conditions that  
202 will enable even higher levels of HDR in neoFB and hES cells using Cas9 RNP delivery.

203

## 204 **Discussion**

205 Here we report a simple and robust system to enhance genome engineering by  
206 HDR in human cells using cell cycle synchronization and timed delivery of Cas9  
207 ribonucleoprotein complexes. Advantages of this approach include no detectable off-

208 target editing, timed introduction of pre-assembled editing complexes into cells and  
209 simultaneous transfection of multiple Cas9 RNPs and donor DNAs. In addition, Cas9  
210 RNP-mediated editing begins within 4 h of delivery and is largely completed within 24 h  
211 due to RNP degradation (Kim et al., 2014). Furthermore, higher cell viability has been  
212 observed following RNP transfection compared with DNA transfection (Kim et al., 2014;  
213 Zuris et al., 2014). These features enable robust levels of on-target editing while  
214 reducing off-target effects.

215         Using this system, we have maximized the efficiency of HDR such that ~33% of  
216 detected DSB repair events occur with homologous recombination of donor DNA. We  
217 chose the EMX1 target sequence to compare with published results using nucleic acid  
218 delivery, for which 10% HDR efficiency was reported in HEK293T cells (Ran et al.,  
219 2013). Our results are also significantly higher than reported rates of HDR in  
220 synchronized HCT116 cells using Transcription Activator-Like Effector Nucleases  
221 (TALENs) and higher than typically observed using nucleic acid-based delivery of Cas9  
222 (Rivera-Torres et al., 2014). (Hsu et al., 2013). Further increase of HDR efficiency  
223 beyond 33% will likely require manipulation of the proteins involved in the HDR or  
224 NHEJ pathways (Humbert et al., 2012).

225         It is surprising that nocodazole treatment leads to higher HDR efficiency at  
226 reduced dosage of Cas9 RNPs. Nocodazole blocks cells at M phase when the DNA is  
227 fully replicated and the nuclear membrane is broken down. One explanation may be that  
228 delivery of Cas9 RNPs into a nocodazole-synchronized cell effectively targets two cells  
229 because they divide upon release. Another possibility is that once the nuclear envelope is  
230 broken down, Cas9 RNPs can gain easy access to the DNA. The resulting high HDR

231 frequencies without off-target editing provide an important advance for generating scar-  
232 less genetic modifications, including epitope-tagged alleles, reporter genes, precise  
233 insertions and deletions and point mutations. Together these results expand the utility of  
234 CRISPR/Cas9-mediated genome engineering in human cells and provide a foundation for  
235 further advances using Cas9 RNP delivery methods.

236 **Figure Legends**

237 **Figure 1. The effect of cell cycle synchronization on total editing and homology-**  
238 **directed repair frequencies in HEK293T cells.** (A) Experimental scheme of timed  
239 delivery of Cas9-guide RNA ribonucleoprotein (RNP) into human cells for genome  
240 editing. (B) Chemical inhibitors used to arrest cells at specific phases of cell cycle  
241 included lovastatin (Lov), which blocks at early G1 and partially at G2/M phase;  
242 mimosine (Mim), aphidicolin (Aph), thymidine (Thy) and hydroxyurea (HU) which  
243 arrest cells at the G1-S border prior to onset of DNA replication; and nocodazole (Noc)  
244 which causes arrest at G2/M phase. (C) The homology-directed repair (HDR) donor  
245 DNA is a 183-nt ssODNA that is complementary to the target sequence (- strand) and  
246 contains a 9-nt insertion (HindIII and SphI restriction sequences) at the cut site and a 9-nt  
247 deletion downstream of the cut site; these modifications are flanked by 85-nt and 55-nt  
248 asymmetrical homology arms at 5' and 3' ends, respectively. (D, E) PCR-based  
249 screening of cell cycle inhibitors for enhancement of Cas9-triggered total editing (TE)  
250 (D) and HDR (E) frequencies in HEK293T cells. For each inhibitor condition (color  
251 coded), two doses of Cas9 RNP, 30 and 100 pmol, were transfected with 100 pmol of  
252 HDR DNA template; control reactions (labeled as C) contained 100 pmol of Cas9 but no  
253 sgRNA. The TE frequency was measured using a T7 endonuclease I assay and analyzed  
254 using a formula described in **Materials and Methods**. The HDR frequency was  
255 determined directly by HindIII digestion, which specifically cleaved the newly integrated  
256 HindIII sequence, and calculated as the ratio of DNA product to DNA substrate. The %  
257 TE, % HDR and standard deviation (error bars) were calculated from three experiments.  
258

259 **Figure 2. The enhancement of TE and HDR at the EMX1, DYRK1 and CXCR4 loci**  
260 **by nocodazole synchronization in HEK293T cells.** (A) The effect of nocodazole on the  
261 TE and HDR frequencies at EMX1 locus. HEK293T cells were synchronized at M phase  
262 with 200 ng/ml of nocodazole for 17 h before nucleofection. To determine the optimal  
263 dosage, three concentrations of Cas9 RNP were assayed in combination with three doses  
264 of HDR template (Figure 1C). The TE frequencies at 10 pmol of Cas9 RNP in the  
265 unsynchronized cells were too low and therefore not determined (ND). (B) The effect of  
266 nocodazole on the TE and HDR frequencies at DYRK1 locus. The directionality of  
267 ssODNA HDR templates, either identical (+ strand) or complementary (- strand) to the  
268 target sequence, was examined. The PAM is highlighted in red, the target sequence in  
269 blue and the integrated HindIII site in green. (C) The effect of nocodazole on the TE and  
270 HDR frequencies at the CXCR4 locus. The HDR template is ssODNA complementary (-  
271 strand) to the target sequence, and contains a HindIII restriction sequence flanked by 90-  
272 nt homology arms. Representative gels from two biological replicates are shown.

273

274 **Figure 3. Systematic investigation of DNA templates for efficient HDR at the EMX1**  
275 **locus in HEK293T cells.** (A) Segment of human EMX1 exon 3 shows the 20-nt target  
276 sequence (highlighted in blue), the TGG PAM region (in red) and the Cas9 cleavage site  
277 at three bases upstream from PAM. Seven HDR templates (color coded) were tested for  
278 HDR efficiency. Template 1 is as described in Figure 1C. Templates 2-7 contain HindIII  
279 and BamHI restriction sites that are flanked symmetrically by various lengths of  
280 homology arms, ranging from 30 nt to 250 nt. Templates 2-5 are ssODNA; templates 6-7  
281 are PCR amplified double-stranded DNA (see **Materials and Methods**). (B) HDR

282 efficiency was tested under three cell conditions. In addition the unsynchronized and  
283 nocodazole synchronized conditions, the cells were synchronized with nocodazole prior  
284 to nucleofection, and immediately post nucleofection, a single dose of aphidicolin (2  
285  $\mu\text{g/ml}$ ) was added to the growth media to prevent the transfected cells from proceeding  
286 into the S phase. The purpose was to test whether blocking passage through S phase  
287 reduces HDR efficiency, since the HDR pathway is thought to be most active during S  
288 phase. This one-time addition of aphidicolin is labeled as “Aph block” in the third panel,  
289 as opposed to the standard aphidicolin synchronization procedure used elsewhere in the  
290 manuscript. Thirty pmol of Cas9 RNP and 50  $\mu\text{mol}$  of HDR template were used in the  
291 nucleofection reaction; the control reaction (C) contained no HDR template. The mean  
292 % HDR and standard deviation (error bar) was determined by HindIII digestion from  
293 three experiments. Representative gels from PCR and HDR analyses are shown for each  
294 cell condition. Templates 6 and 7 produced unusual banding patterns, making  
295 quantitation of DNA bands less accurate (labeled by asterisk).

296

297 **Figure 4. The enhancement of TE and HDR frequencies at the EMX1 locus by cell**  
298 **cycle synchronization in human primary neonatal fibroblast and embryonic stem**  
299 **cells. (A)** Screening of cell cycle inhibitors for enhancement of TE and HDR frequencies  
300 in human primary neonatal fibroblast cells. For each inhibitor condition (color coded),  
301 two doses of Cas9 RNP, 30 and 100  $\mu\text{mol}$ , were transfected with 100  $\mu\text{mol}$  of HDR DNA  
302 template 4 from Figure 3A. A control reaction (labeled as C) contained 100  $\mu\text{mol}$  of  
303 Cas9 but no sgRNA. The % TE and % HDR were analyzed similarly as with HEK293T  
304 cells. **(B)** Three conditions were tested using hES cells: unsynchronized, nocodazole

305 synchronized and nocodazole-aphidicolin sequential synchronized. The cells were  
306 treated with nocodazole for 16 h, washed to remove the drug and then treated with  
307 aphidicolin for 3 h before nucleofection. Thirty or 100  $\mu\text{mol}$  of Cas9 RNP was co-  
308 transfected with 100  $\mu\text{mol}$  of HDR template 4 from Figure 3A, and cultured at high  
309 density in 96-well plates in the presence or absence of ROCK apoptosis inhibitor (10  
310  $\mu\text{M}$ ). For both experiments, representative gels from two biological replicates are shown.  
311 The contrast of the gel images was increased to show that no HDR was detected in other  
312 conditions.

313

314 **Figure 1-figure supplement 1. FACS analysis reveals cell cycle blocks and the DNA**  
315 **content in the cells that are arrested at different phases of cell cycle.** Bivariate cell  
316 cycle, BrdU (S-phase), 7-AAD (DNA content) FACS analysis reveals cells are arrested at  
317 different phases of cell cycle. Chemical inhibitors were used to arrest cells at specific  
318 phases of cell cycle. (A) Analysis of HEK293T cells with different cell cycle blocks and  
319 nocodazole released cells. (B) Analysis of human neonatal fibroblasts with different cell  
320 cycle blocks and aphidicolin released cells. (C) Analysis of H9 hES cells  
321 unsynchronized (unsync), nocodazole synchronized (Noc) and nocodazole+aphidicolin  
322 sequential synchronized (Noc+Aph) at time of cell cycle block and transfection or three  
323 days after release. Alkaline phosphatase positive and normal ES colony morphology for  
324 all three conditions. ROCK apoptosis inhibitor (10  $\mu\text{M}$ ) was required for survival of  
325 synchronized H9 ESCs after release when cultured at low density in 6-well plates but not  
326 when cultured at high density in 96-well plates.

327

328 **Figure 2-figure supplement 1. On-target NHEJ and HDR and off-target cleavage**  
329 **analyses by deep sequencing.** (A) The genomic DNAs from Figure 2A and B  
330 experiments were analyzed for NHEJ and HDR frequencies, at the on-target and off-  
331 target sites, by deep sequencing. The TE frequency (indels/total reads) was determined at  
332 the EMX1 target, DYRK1 target and selected off-target loci as a function of Cas9 RNP  
333 dosage, (n=1 representative experiment). The HDR frequency (HDR/total reads)  
334 represented specifically exogenous donor template-mediate HDR. The ratio of Cas9  
335 RNP-induced DSB repaired by the HDR pathway was determined as the percentage of  
336 HDR/TE. The controls, which included the non-transfected cells and the cells transfected  
337 with only Cas9 protein but no sgRNA, showed no evidence of on- or off-target editing.  
338 (B) Representative sequences repaired by HDR and NHEJ at the EMX1 and DYRK1  
339 loci. The Cas9 cleavage sites are marked by red triangles.

340

341 **Supplementary File 1. PCR primers for target loci amplification and indexing for**  
342 **deep sequencing analysis of on-target and off-target sites**

343

344



345 **Materials and Methods**

346 **Cell lines and cell culture**

347 DMEM media, non-essential amino acid, penicillin-streptomycin, E8 media, DPBS and  
348 0.05% trypsin were purchased from Life Technologies. HEK293T cells and human  
349 neonatal dermal fibroblasts (ScienCell, catalog # 2310) were maintained in DMEM  
350 media supplemented with 10 % fetal bovine serum, non-essential amino acid and  
351 penicillin-streptomycin. H9 human embryonic stem cells were maintained on Matrigel in  
352 E8 media plus supplement.

353

354 **Cell cycle synchronization**

355 Aphidicolin, hydroxyurea, lovastatin, mimosine, nocodazole and thymidine were  
356 purchased from Sigma-Aldrich. The synchronization protocols were modified from the  
357 following references (Adams and Lindsay, 1967; Harper, 2007; Jackman and O'Connor,  
358 2001; Pauklin and Vallier, 2013). It is important to ensure cells are maintained at <70%  
359 confluency. HEK293T cells were seeded at low density,  $3 \times 10^6$  cell density in a 10-cm  
360 culture dish and human primary neonatal fibroblasts seeded at  $1.2 \times 10^6$  in a 15-cm dish 17  
361 h before transfection. Aphidicolin and thymidine require two sequential treatments to  
362 enrich cells arrested at the entry of S phase (Jackman and O'Connor, 2001). Cells were  
363 treated with aphidicolin (2  $\mu\text{g/ml}$ ) or thymidine (5 mM) for 17 h, washed with media to  
364 remove the drugs, grown for 8 h, and treated with a second dose of drugs for 17 h. In the  
365 experiment in Figure 3B, third panel, a single dose of aphidicolin (2  $\mu\text{g/ml}$ ) was added to  
366 the nocodazole synchronized cells immediately after nucleofection. Hydroxyurea (2  
367 mM), lovastatin (40  $\mu\text{M}$ ), mimosine (200  $\mu\text{M}$ ) and nocodazole (200 ng/ml) require only

368 one treatment for 17 h. Two synchronization conditions were tested in the human ES cell  
369 experiment as shown in Figure 4B. Human ES cells were cultured in 6 well dishes, split  
370 1:10 three days before adding nocodazole. The first condition was a simple nocodazole  
371 treatment for 16 h. The second condition was modified from Pauklin et al (Pauklin and  
372 Vallier, 2013). The cells were treated with nocodazole for 16 h, washed to remove the  
373 drug, and then treated with aphidicolin for 3 h before nucleofection. We shortened the  
374 duration of aphidicolin treatment, because we noticed a substantial drop in cell viability  
375 at 10 h. After transfection cells were either seeded at high density in a 96 well plate for  
376 analysis of editing or low density, 6-well plate, for imaging and long term growth.

377

#### 378 **Cell cycle analysis**

379 The cell cycle analysis was performed using BD Biosciences BrdU-FITC FACS kit, to  
380 determine the percent of cells in each phase of the cell cycle. HEK293T and H9 ES cells  
381 were incubated with BrdU for 45 min while Fibroblasts were incubated with BrdU for 2  
382 h. To determine the percent of cells in G2/M, DNA was stained with 7-AAD (7-  
383 aminoactinomycin D) and analyzed on a BD Fortessa Flow Cytometer.

384

#### 385 **Alkaline Phosphatase Staining**

386 Followed Millipore Alkaline Phosphatase detection kit protocol, Cat. No. SCR004.

387

#### 388 **Expression and Purification of Cas9**

389 The recombinant *S. pyogenes* Cas9 used in this study carries at C-terminus an HA tag and  
390 two nuclear localization signal peptides which facilitates transport across nuclear

391 membrane. The protein was expressed with a N-terminal hexahistidine tag and maltose  
392 binding protein in *E. coli* Rosetta 2 cells (EMD Millipore) from plasmid pMJ915. The  
393 His tag and maltose binding protein were cleaved by TEV protease, and Cas9 was  
394 purified by the protocols described in Jinek et al., 2012. Cas9 was stored in 20 mM 2-[4-  
395 (2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) at pH 7.5, 150 mM KCl,  
396 10% glycerol, 1 mM tris(2-chloroethyl) phosphate (TCEP) at -80°C.

397

### 398 ***In vitro* T7 transcription of sgRNA**

399 The DNA template encoding for a T7 promoter, a 20-nt target sequence and an optimized  
400 sgRNA scaffold (Chen et al., 2013) was assembled from synthetic oligonucleotides by  
401 overlapping PCR. Briefly, for the EMX1 sgRNA template, the PCR reaction contains 20  
402 nM premix of BS16 (5'- TAA TAC GAC TCA CTA TAG GTC ACC TCC AAT GAC  
403 TAG GGG TTT AAG AGC TAT GCT GGA AAC AGC ATA GCA AGT TTA AAT  
404 AAG G -3') and BS6 (5'- AAA AAA AGC ACC GAC TCG GTG CCA CTT TTT CAA  
405 GTT GAT AAC GGA CTA GCC TTA TTT AAA CTT GCT ATG CTG TTT CCA GC -  
406 3'), 1 µM premix of T25 (5'-TAA TAC GAC TCA CTA TAG-3') and BS7 (5'- AAA  
407 AAA AGC ACC GAC TCG GTG C -3'), 200 µM dNTP and Phusion Polymerase (NEB)  
408 according to manufacturer's protocol. The thermocycler setting consisted of 30 cycles of  
409 95°C for 10 sec, 57°C for 10 sec and 72°C for 10 sec. The PCR product was extracted  
410 once with phenol:chloroform:isoamylalcohol and then once with chloroform, before  
411 isopropanol precipitation overnight at -20°C. The DNA pellet was washed three times  
412 with 70% ethanol, dried by vacuum and dissolved in DEPC-treated water. The DYRK1  
413 sgRNA template was assembled from T25, BS6, BS7 and BS14 (5'- TAA TAC GAC

414 TCA CTA TAG GTT CCT TAA ATA AGA ACT TTG TTT AAG AGC TAT GCT  
415 GGA AAC AGC ATA GCA AGT TTA AAT AAG G -3'). The CXCR4 sgRNA  
416 template was assembled from T25, SLKS3 (5'- TAA TAC GAC TCA CTA TAG GAA  
417 GCG TGA TGA CAA AGA GGG TTT TAG AGC TAT GCT GGA AAC AGC ATA  
418 GCA AGT TAA AAT AAG G -3'), SLKS1 (5'- GCA CCG ACT CGG TGC CAC TTT  
419 TTC AAG TTG ATA ACG GAC TAG CCT TAT TTT AAC TTG CTA TGC TGT TTC  
420 CAG C -3') and SLKS2 (5'- GCA CCG ACT CGG TGC CAC TTT TTC AAG -3').

421

422 An 100- $\mu$ l T7 *in vitro* transcription reaction consisted of 30 mM Tris-HCl (pH 8), 20 mM  
423 MgCl<sub>2</sub>, 0.01% Triton X-100, 2 mM spermidine, 10 mM fresh dithiothreitol, 5 mM of  
424 each ribonucleotide triphosphate, 100  $\mu$ g/ml T7 Pol and 1  $\mu$ M DNA template. The  
425 reaction was incubated at 37°C for 4 h, and 5 units of RNase-free DNaseI (Promega) was  
426 added to digest the DNA template 37°C for 1 h. The reaction was quenched with  
427 2xSTOP solution (95% deionized formamide, 0.05% bromophenol blue and 20 mM  
428 EDTA) at 60°C for 5 min. The RNA was purified by electrophoresis in 10%  
429 polyacrylamide gel containing 6 M urea. The RNA band was excised from the gel,  
430 grinded up in a 15-ml tube, and eluted with 5 volumes of 300 mM sodium acetate (pH 5)  
431 overnight at 4°C. One equivalent of isopropanol was added to precipitate the RNA at -  
432 20°C. The RNA pellet was collected by centrifugation, washed three times with 70%  
433 ethanol, and dried by vacuum. To refold the sgRNA, the RNA pellet was first dissolved  
434 in 20 mM HEPES (pH 7.5), 150 mM KCl, 10% glycerol and 1 mM TCEP. The sgRNA  
435 was heated to 70°C for 5 min and cooled to room temperature. MgCl<sub>2</sub> was added to a  
436 final concentration of 1 mM. The sgRNA was again heated to 50°C for 5 min, cooled to

437 room temperature and kept on ice. The sgRNA concentration was determined by  
438 OD<sub>260nm</sub> using Nanodrop and adjusted to 100 μM using 20 mM HEPES (pH 7.5), 150  
439 mM KCl, 10% glycerol, 1 mM TCEP and 1 mM MgCl<sub>2</sub>. The sgRNA was store at -80°C.

440

#### 441 **PCR assembly of HDR template 6 and 7**

442 Double-stranded HDR template 6 and 7 were prepared by PCR amplification. Template  
443 6 was PCR amplified from single-stranded template 5 (5'- TGG CCA GGG AGT GGC  
444 CAG AGT CCA GCT TGG GCC CAC GCA GGG GCC TGG CCA GCA GCA AGC  
445 AGC ACT CTG CCC TCG TGG GTT TGT GGT TGC GGA TCC AAG CTT TTG  
446 GAG GTG ACA TCG ATG TCC TCC CCA TTG GCC TGC TTC GTG GCA ATG  
447 CGC CAC CGG TTG ATG TGA TGG GAG CCC TTC TTC TTC TGC TCG -3') using  
448 primer set (forward 5'- CGA GCA GAA GAA GAA GGG CTC CCA TC -3' and reverse  
449 5'- TGG CCA GGG AGT GGC CAG AGT CC -3'). The PCR reaction was performed  
450 using Phusion Polymerase according to manufacturer's protocol (NEB). The  
451 thermocycler setting consisted of 30 cycles of 95°C for 20 sec, 67°C for 10 sec and 72°C  
452 for 20 sec. The PCR product was extracted once with phenol:chloroform:isoamylalcohol  
453 and then once with chloroform, before isopropanol precipitation overnight at -20°C. The  
454 DNA pellet was washed three times with 70% ethanol, dried by vacuum and dissolved in  
455 water. The concentration was determined by Nanodrop (Thermo Scientific).

456

457 Template 7 was assembled from two fragments (A and B) by overlapping PCR.  
458 Fragment A was PCR amplified from HEK293T genomic DNA using the primer set  
459 (forward 5'- GCT CAG CCT GAG TGT TGA GGC CCC AGT GGC TGC TCT GG -3'

460 and reverse 5'- GTG GTT GCG GAT CCA AGC TTT TGG AGG TGA CAT CGA TGT  
461 CCT CCC CAT TGG C -3'). Fragment B was amplified using the primer set (forward 5'-  
462 CAC CTC CAA AAG CTT GGA TCC GCA ACC ACA AAC CCA CGA GGG CAG  
463 AGT GCT GCT TGC -3' and reverse 5'- TGC GGT GGC GGG CGG GCC CGC CCA  
464 GGC AGG CAG GC -3'). Both reaction were performed using Kapa Hot start high-  
465 fidelity polymerase (Kapa Biosystems) in high GC buffer according to the  
466 manufacturer's protocol. The thermocycler setting consisted of one cycle of 95°C for 5  
467 min, 30 cycles of 98°C for 20 sec, 67°C for 10 sec and 72°C for 20 sec, and one cycle of  
468 72°C for 1 min.

469

#### 470 **Cas9 RNP assembly and nucleofection**

471 Cas9 RNP was prepared immediately before experiment by incubating with sgRNA at  
472 1:1.2 molar ratio in 20 mM HEPES (pH 7.5), 150 mM KCl, 1 mM MgCl<sub>2</sub>, 10% glycerol  
473 and 1 mM TCEP at 37°C for 10 min. HDR template was then added to the RNP mixture.  
474 Cells were dissociated by 0.05% trypsin, spun down by centrifugation at 400 g for 3 min,  
475 and washed once with DPBS. Nucleofection of HEK293T cells was performed using  
476 Lonza SF cell- kits and program CM130 in an Amaxa 96-well Shuttle system The neoFB  
477 were transfected with Lonza P2 kit and program CA137. The hES cells were transfected  
478 with P3 primary cell kit and program CB150. Each nucleofection reaction consisted of  
479 approximately 2x10<sup>5</sup> cells in 20 µl of nucleofection reagent and mixed with 10 µl of  
480 RNP:DNA. After electroporation, 100 µl of growth media was added to the well to  
481 transfer the cells to tissue culture plates. The cells were incubated at 37°C for 24 h, the  
482 media was removed by aspiration, and 100 µl of Quick Extraction solution (Epicenter)

483 was added to lyse the cells and extract the genomic DNA. The cell lysate was incubated  
484 at 65°C for 20 min and then 95°C for 20 min, and stored at -20°C. The concentration of  
485 genomic DNA was determined by NanoDrop (Thermo Scientific).

486

487 **PCR amplification of target region**

488 A 640-nt region of EMX1 and DYRK1 loci, containing the target site, were PCR  
489 amplified using the following primer sets. For EMX1: forward 5'- GCC ATC CCC TTC  
490 TGT GAA TGT TAG AC-3' and 5'-GGA GAT TGG AGA CAC GGA GAG CAG-3'.  
491 For DYRK1: forward 5'- GAG GAG CTG GTC TGT TGG AGA AGT C-3' and reverse  
492 5'- CCC AAT CCA TAA TCC CAC GTT GCA TG-3'. A 903-nt region of CXCR4  
493 locus was amplified using primer set: 5'- AGA GGA GTT AGC CAA GAT GTG ACT  
494 TTG AAA CC -3' and 5'- GGA CAG GAT GAC AAT ACC AGG CAG GAT AAG  
495 GCC -3'. These primers were designed to avoid amplifying the HDR templates by  
496 annealing outside of the homology arms. The PCR reaction was performed using 200 ng  
497 of genomic DNA and Kapa Hot start high-fidelity polymerase (Kapa Biosystems) in high  
498 GC buffer according to the manufacturer's protocol. The thermocycler setting consisted  
499 of one cycle of 95°C for 5 min, 30 cycles of 98°C for 20 sec, 62°C for 15 sec and 72°C  
500 for 30 sec, and one cycle of 72°C for 1 min. The PCR products were analyzed on 2%  
501 agarose gel containing SYBR Safe (Life Technologies). The concentration of PCR DNA  
502 was quantitated based on the band intensity relative to a DNA standard using the software  
503 Image Lab (Bio-Rad). About 200 ng of PCR DNA was used for T7 endonuclease I and  
504 HindIII analyses.

505

506 **Analysis of %TE by T7 endonuclease I assay**

507 The percentage of Cas9 induced TE was determined by T7 endonuclease I assay. T7  
508 endonuclease I recognizes and cleaves mismatched heteroduplex DNA which arises from  
509 hybridization of wild-type and mutant DNA strands. The hybridization reaction  
510 contained 200 ng of PCR DNA in KAPA high GC buffer and 50 mM KCl, and was  
511 performed on a thermocycler with the following setting: 95°C, 10 min, 95-85°C at -  
512 2°C/sec, 85°C for 1 min, 85-75°C at -2°C/sec, 75°C for 1 min, 75-65°C at -2°C/sec, 65°C  
513 for 1 min, 65-55°C at -2°C/sec, 55°C for 1 min, 55-45°C at -2°C/sec, 45°C for 1 min, 45-  
514 35°C at -2°C/sec, 35°C for 1 min, 35-25°C at -2°C/sec, 25°C for 1 min, and hold at 4°C.  
515 Buffer 2 and 5 units of T7 endonuclease I (NEB) were added to digest the re-annealed  
516 DNA. After one hour of incubation at 37°C, the reaction was quenched with one volume  
517 of gel loading dye (50 mM Tris pH 8.5, 50 mM EDTA, 1% SDS, 50% glycerol and  
518 0.01% bromophenol blue) at 70°C for 10 min. The product was resolved on 2% agarose  
519 gel containing SYBR gold (Life technologies). The DNA band intensity was quantitated  
520 using Image Lab. The TE frequency was measured using a T7 endonuclease I assay and  
521 calculated using the following equation  $(1 - (1 - (b + c / a + b + c))^{1/2}) \times 100$ , where “a”  
522 is the band intensity of DNA substrate and “b” and “c” are the cleavage products (Ran et  
523 al., 2013). Using this formula is necessary, because upon re-annealing, one duplex of  
524 mutant DNA can produce two duplexes of mutant:wild-type hybrid, doubling the actual  
525 TE frequency.

526

527 **Analysis of HDR by HindIII restriction digestion**



528 HindIII directly cleaves PCR DNA containing the newly integrated HindIII restriction  
529 sequence as the result of successful HDR. The reaction consisted of 200 ng of PCR DNA  
530 and 10 units of HindIII High Fidelity in CutSmart Buffer (NEB). After 2 h of incubation  
531 at 37°C, the reaction was quenched with one volume of gel loading dye at 70°C for 10  
532 min. The product was resolved on 2% agarose gel containing SYBR gold (Life  
533 technologies). The band intensity was quantitated using Image Lab. The percentage of  
534 HDR was calculated using the following equation  $(b + c / a + b + c) \times 100$ , where “a” is  
535 the band intensity of DNA substrate and “b” and “c” are the cleavage products.

536

### 537 **Deep sequencing analysis of on-target and off-target sites**

538 The genomic region flanking the CRISPR target site for each gene was amplified by 2-  
539 step PCR method using primers listed in Supplemental Information. First, the genomic  
540 DNA from the edited and control samples was isolated and PCR amplified 15 cycles  
541 using Kapa Hot start high-fidelity polymerase (Kapa Biosystems) according to the  
542 manufacturer’s protocol. The resulting amplicons were purified by AMPure beads to  
543 remove primers and subjected to 5 cycles of PCR to attach Illumina P5 adapters as well  
544 as unique sample-specific barcodes followed by bead purification. Berkeley Sequencing  
545 facility performed the AMPure bead cleanup. Barcoded and purified DNA samples were  
546 quantified by Qubit 2.0 Fluorometer (Life Technologies), size analyzed by BioAnalyzer,  
547 quantified by qPCR and pooled in an equimolar ratio. Sequencing libraries were  
548 sequenced with the Illumina MiSeq Personal Sequencer (Life Technologies).  
549 Amplicon sequencing data were analyzed as described below. The 300-bp paired end  
550 MiSeq raw reads were de-multiplexed using Illumina MiSeq Reporter software. This

551 generated sample specific paired end raw read files (R1 and R2 fastq files). Adapter and  
552 windowed adaptive quality trimming was performed on the raw reads (using Trim  
553 Galore). Reads containing bases with a PHRED quality score of less than 30 were  
554 removed. R1 and reverse complemented R2 reads were then merged into sample specific  
555 fasta file. Smith Waterman alignments (EMBOSS Water) were performed for each  
556 sample reads against the corresponding 53 nucleotide reference locus. These 53 nt for  
557 each locus included the 23 nt target sequence with 15 nt flanking sequences. Alignments  
558 were filtered to assess presence of indels and homologous recombination. Reads were  
559 considered to have indels if their alignments were at least 53 nt long and had any gaps.  
560 Reads were considered non-indels if their alignments were at least 53 nucleotides long  
561 without any gaps. TE frequency was calculated as  $100 \times \frac{\text{\#indel reads}}{\text{\#indel reads} + \text{\#non-indel reads}}$ .  
562 Reads were considered to have homologous recombination if  
563 alignments were at least 53 nucleotides long and had a AAGCTTGCTAGC insertion for  
564 the EMX1 loci (both on and off target) and a GCTAGCAAGCTT insertion for the  
565 DYRK1 loci (both on and off target). HDR frequency was calculated as either  $100 \times \frac{\text{\#HDR reads}}{\text{\#indel reads} + \text{\#non-indel reads}}$ . Deep sequencing data is available at the  
567 NCBI Sequence Read Archive (SRA, BioProject: 269153).

568

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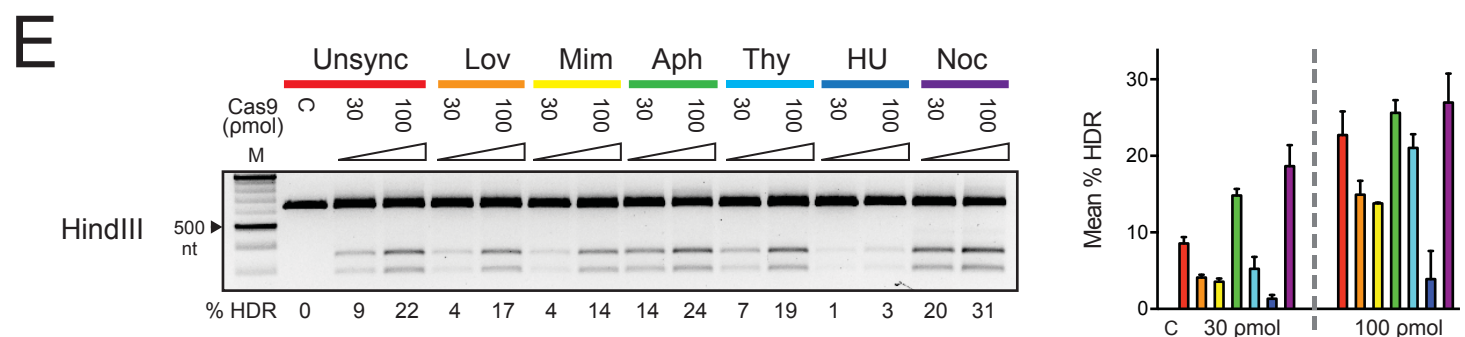
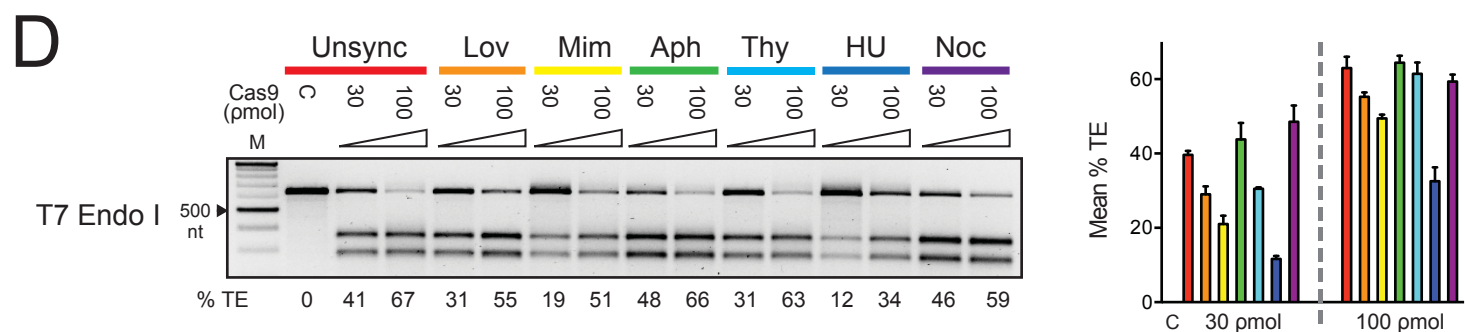
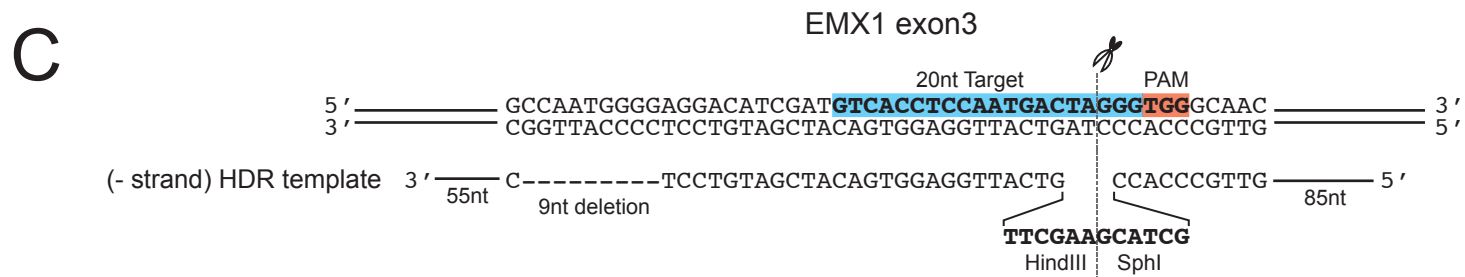
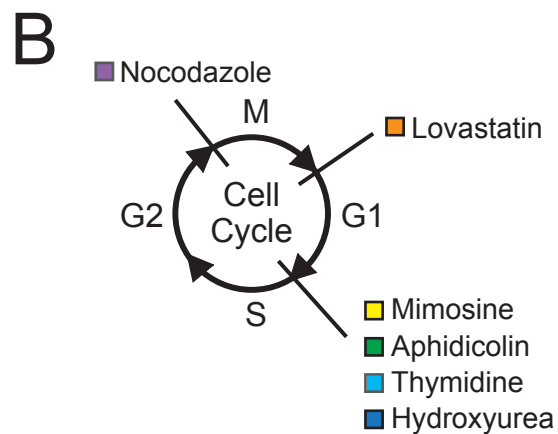
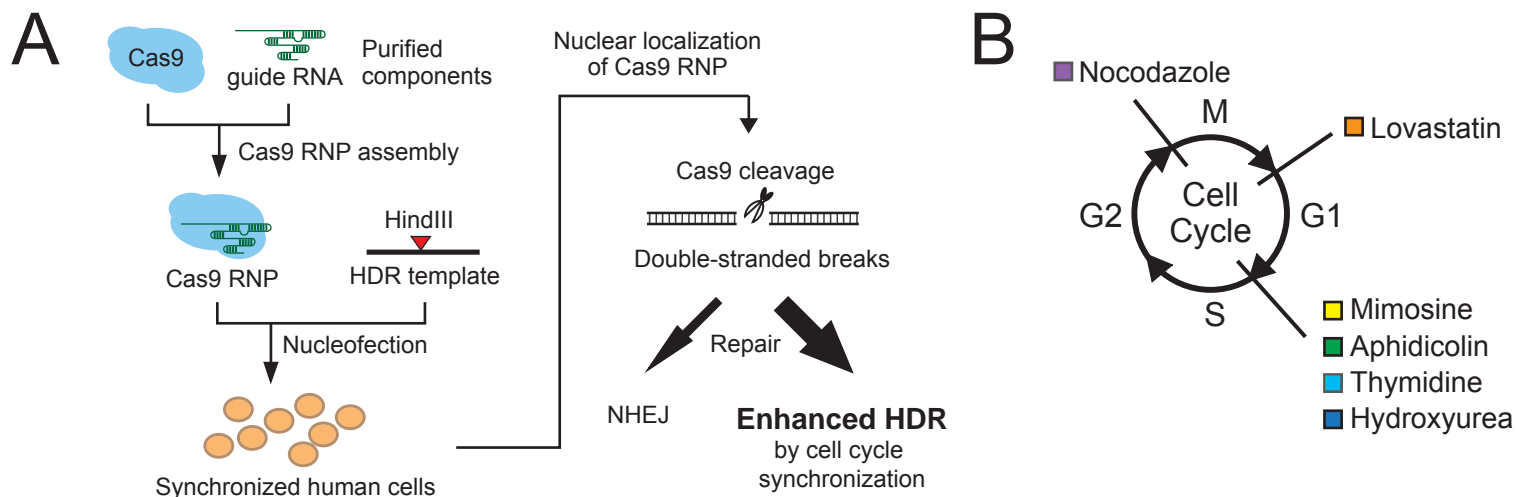
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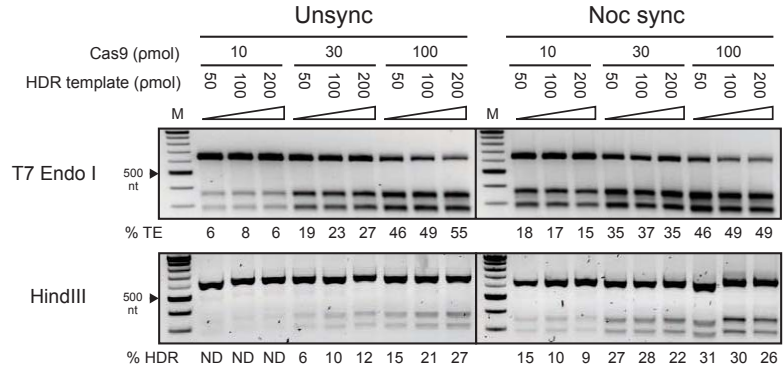
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# Figure 1

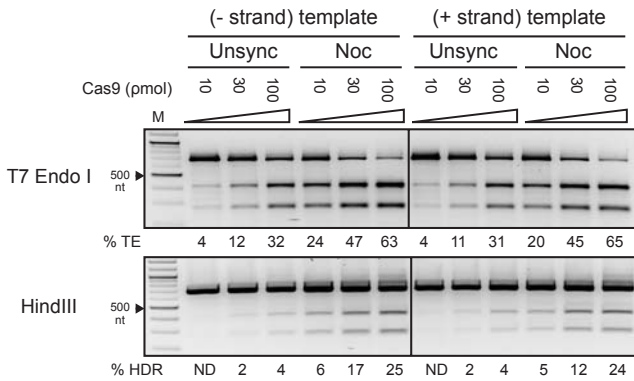
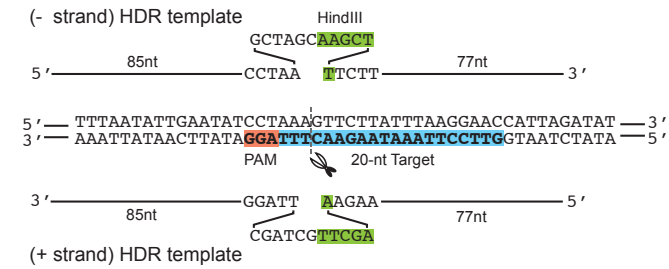


# Figure 2

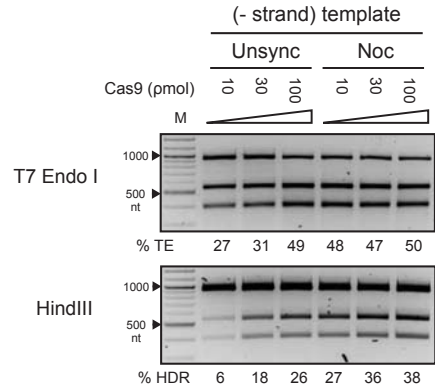
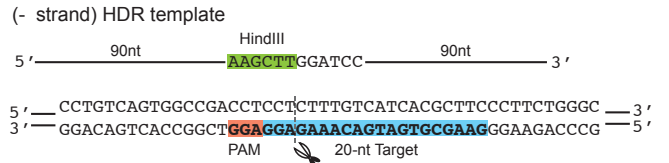
## A EMX1



## B DYRK1

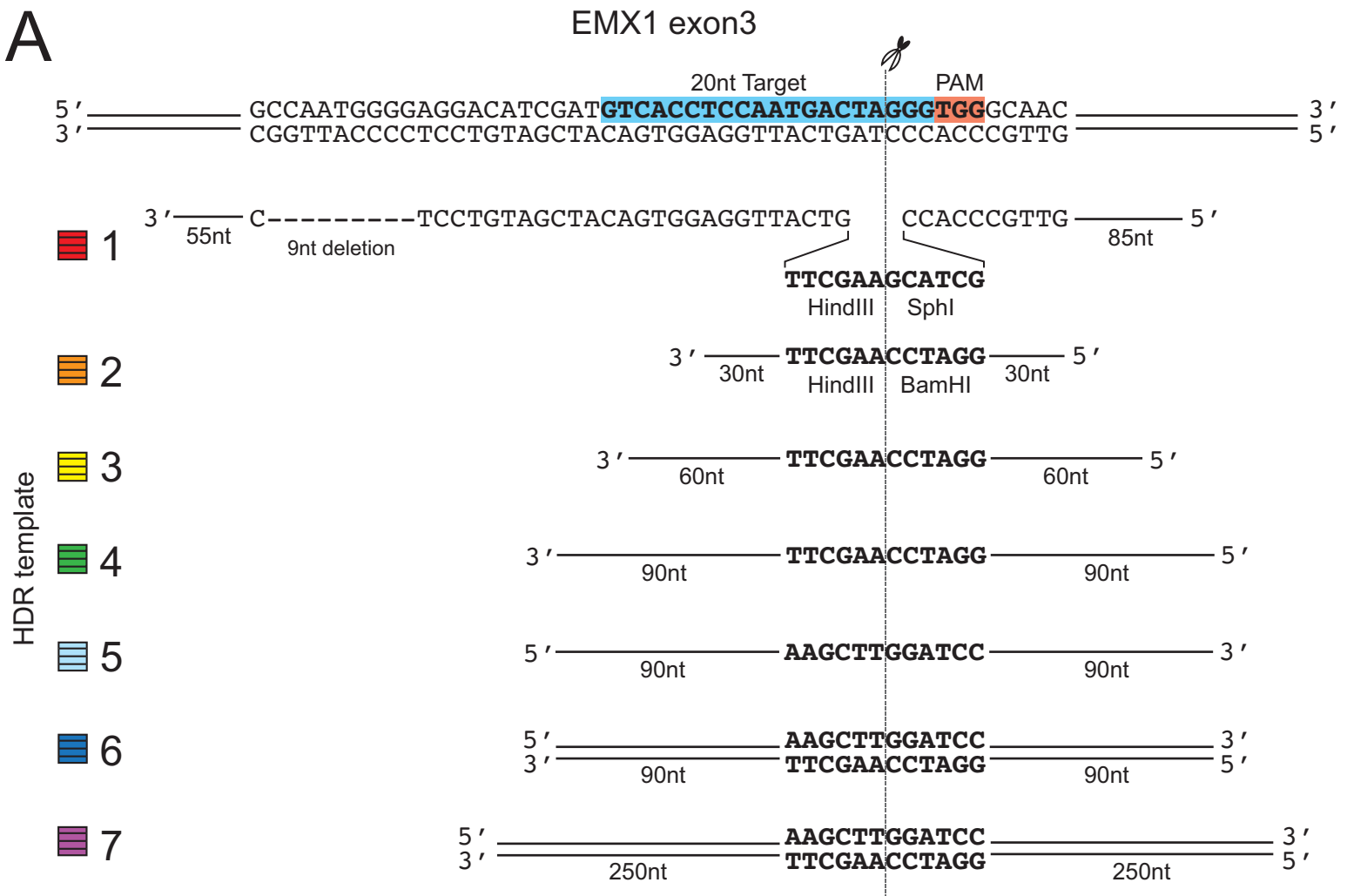


## C CXCR4

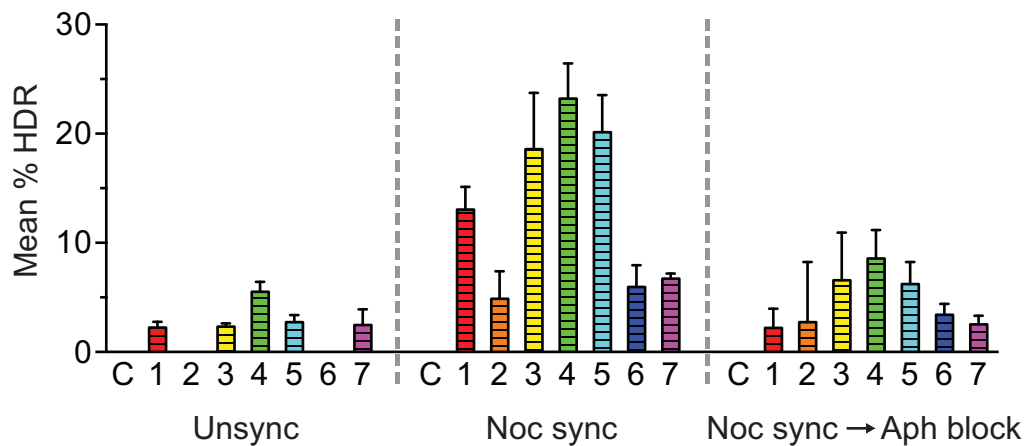
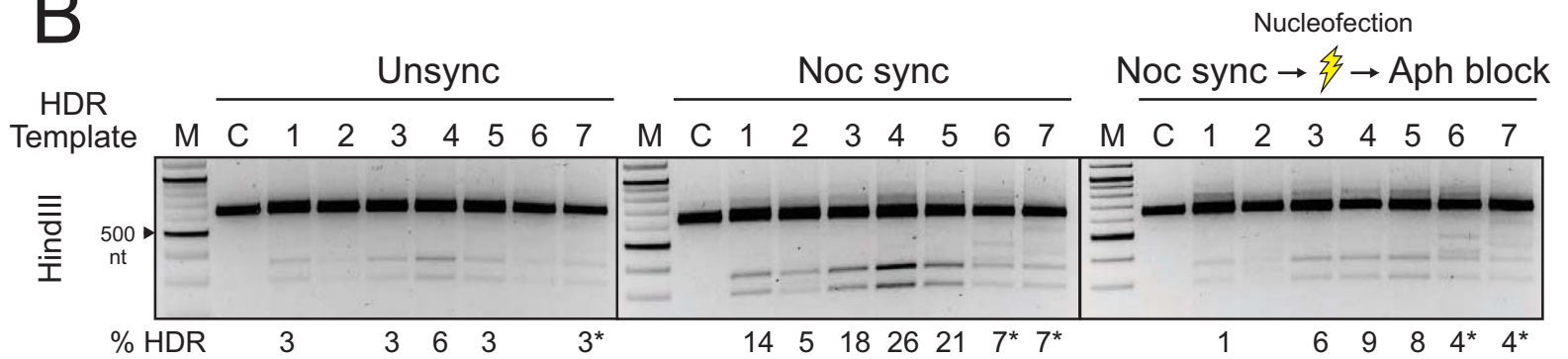


# Figure 3

## A

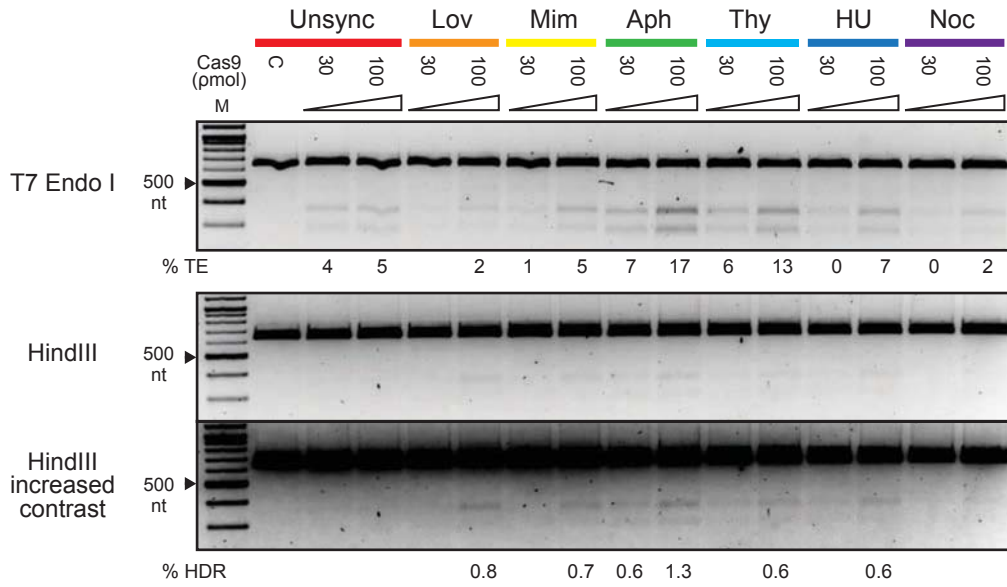


## B



# Figure 4

## A EMX1 in human primary neonatal fibroblast cells



## B EMX1 in H9 human embryonic stem cells

