

Title:**Genome editing reveals a role for OCT4 in human embryogenesis****Authors:**

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Summary:

During early human development, the totipotent zygote differentiates into a blastocyst comprised of pluripotent epiblast cells, which form the fetus, and extra-embryonic cells that contribute to the placenta and yolk sac. Despite their fundamental biological and clinical importance, the molecular mechanisms that regulate these first cell fate decisions are unclear. Here we use CRISPR/Cas9-mediated genome editing to investigate the function of the pluripotency transcription factor OCT4 during human embryogenesis. Using an inducible human embryonic stem cell (hESC)-based system we identified the most efficient OCT4-targeting single guide RNA (sgRNA). By testing homologous sgRNAs in mouse zygotes we further validated sgRNAs *in vivo* and optimised microinjection techniques. Using these refined methods, we then efficiently and specifically targeted *OCT4* in diploid human zygotes

53 and observed compromised blastocyst development. Transcriptomics analysis revealed that
54 *OCT4*-null cells downregulated the expression of not only extra-embryonic trophectoderm
55 genes, such as *CDX2* and *HAND1*, but also regulators of the pluripotent epiblast, including
56 *NANOG*. By contrast mouse embryos maintained the expression of orthologous genes, and
57 blastocyst development is established, but maintenance is compromised. Altogether, we
58 conclude that CRISPR/Cas9-mediated genome editing is a powerful method to interrogate
59 gene function in the context of human development.

60

61 **Introduction**

62 Early mammalian embryogenesis is controlled by mechanisms governing the balance between
63 pluripotency and differentiation. Expression of early lineage-specific genes varies
64 significantly between species¹⁻³ with implications for developmental control and stem cell
65 derivation. However, the mechanisms patterning the human embryo are unclear, because
66 methods to efficiently perturb gene expression of early lineage specifiers in this species have
67 been lacking.

68

69 The efficiency of genetic modification has significantly increased due to recent advances in
70 genome editing using the CRISPR (clustered regularly interspaced, short palindromic
71 repeat)/Cas (CRISPR-associated) system. The *Streptococcus pyogenes* Cas9 endonuclease is
72 guided to homologous DNA sequences via a single-guide RNA (sgRNA) whereby it induces
73 double strand breaks (DSBs) at the target site⁴. Several endogenous DNA repair mechanisms
74 function to resolve the DSBs, including error-prone non-homologous or micro-homology
75 mediated end joining, which can lead to insertions or deletions (indels) of nucleotides that can
76 result in the null mutation of the target gene. CRISPR/Cas9-mediated editing has been
77 attempted in abnormally fertilised tripronuclear and a limited number of normally fertilised
78 human zygotes with variable success⁵⁻⁸. To determine if CRISPR/Cas9 can be used to
79 understand gene function in human preimplantation development, we chose to target
80 *POU5F1*, a gene encoding the developmental regulator OCT4, as a proof-of-principle.
81 Zygotic *POU5F1* is thought to be first transcribed between the 4- to 8-cell stage and OCT4
82 protein is not detectable prior to embryo genome activation (EGA) at approximately the 8-cell
83 stage^{2,3}. OCT4 perturbation would be predicted to cause a clear developmental phenotype
84 based on studies in the mouse^{9,10} and human embryonic stem cells (hESCs)¹¹.

85

86 By employing an inducible hESC-based system and optimising mouse zygote microinjection
87 techniques, we identified conditions to efficiently and precisely target *POU5F1*. Live embryo
88 imaging revealed that while OCT4-targeted human embryos initiate blastocyst formation, the
89 inner cell mass (ICM) forms poorly, and embryos subsequently collapse. We demonstrated
90 that OCT4 has an earlier role in the progression of the human blastocyst and that mutations
91 affecting *POU5F1* are correlated with the downregulation of genes associated with all three
92 preimplantation lineages, including *NANOG* (epiblast), *GATA2* (trophectoderm) and *GATA4*
93 (primitive endoderm). By contrast, in OCT4-null mouse blastocysts, genes such as *Nanog*
94 continue to be expressed in the inner cell mass. The insights gained from these investigations
95 advance our understanding of human development and suggest that there may be distinct
96 mechanisms of lineage specification between these species.

97

98 **Results**

99

100 **Selection of a highly-efficient sgRNA targeting *POU5F1* in hESCs**

101 To target *POU5F1*, we selected 4 sgRNAs using a standard *in silico* prediction tool¹²: two
102 targeting the exon encoding the N-terminal domain of OCT4 (sgRNA1-1 and sgRNA1-2), one
103 targeting the exon encoding the conserved DNA binding POU homeodomain^{13,14} (sgRNA2b)
104 and one targeting the end of the POU domain and start of the C-terminal domain (sgRNA4)

105 (Extended Data Fig. 1a). To screen candidate sgRNAs we took advantage of hESCs as an
106 unlimited resource that reflects the cellular context of the human preimplantation embryo. We
107 engineered isogenic hESCs constitutively expressing the Cas9 gene, together with a
108 tetracycline-inducible sgRNA¹¹ (Fig. 1a), thereby allowing for comparative assessment of
109 sgRNA activities.

110
111 Cells were collected every day for 5 days for flow cytometry analysis, which revealed that
112 induction of each of the sgRNAs in hESCs imposed remarkably different temporal effects on
113 OCT4 protein expression (Extended Data Fig. 1b). sgRNA2b was most efficient at rapidly
114 causing loss of OCT4 protein expression, with only 15.6% of cells retaining detectable OCT4
115 by day 5 (d5) of induction. Immunofluorescence analysis following sgRNA2b induction
116 confirmed the efficient knockdown of OCT4 expression (Fig. 1b, Extended Data Fig. 2a).
117 Conversely, in hESCs induced to express sgRNAs 1-1, 1-2, or 4, 70.5%, 43.7% and 51.7% of
118 cells retained OCT4 expression at the equivalent time-point, respectively (Extended Data Fig.
119 1b). To determine the transcriptional consequences of OCT4 depletion, we performed qRT-
120 PCR and RNA-sequencing (RNA-seq) analysis on induced and non-induced sgRNA2b-
121 expressing hESCs (Extended Data Figs. 1c,d and 2b). sgRNA2b-induction resulted in
122 downregulation of pluripotency genes such as *NANOG*, *ETS1* and *DPPA3* consistent with
123 OCT4 depletion causing exit from self-renewal. Furthermore, *PAX6*, *SOX17*, *SIX3*, *GATA2*
124 and *SOX9* were upregulated following sgRNA2b-induction, suggesting that OCT4 normally
125 restrains differentiation (Extended Data Figs. 1c,d, Extended Data Fig. 2a,b).

126 127 **Stereotypic *POU5F1* on-target indel mutations and targeting specificity in hESCs**

128 To compare the on-target editing efficiencies and mutation spectrums induced by candidate
129 sgRNAs, we performed a time-course genotypic analysis on cells collected across 4 days
130 following sgRNA induction. Targeted deep sequencing of the on-target site revealed indels
131 from as early as 24 h post-induction of sgRNA2b, but not until 48 h post-induction of
132 sgRNAs 1-1, 1-2 or 4 (Fig. 1c). sgRNA2b-induced indels most commonly comprised a 2 bp
133 deletion upstream of the PAM site leading to a frameshift mutation and a premature stop
134 codon (Extended Data Fig. 3), consistent with the loss of OCT4 protein expression.

135
136 We evaluated putative off-target sites identified by their sequence similarity to the seed region
137 of sgRNA2b (Extended Data Fig. 4a,b). We did not observe off-target indels in sgRNA2b-
138 induced hESCs, nor any sequence alterations above background PCR error rates observed in
139 control hESC lines. In parallel we performed a genome-wide unbiased evaluation of off-target
140 events using Digenome-seq (Extended Data Fig. 4c). Targeted deep sequencing across the
141 experimentally determined putative off-target sites revealed that indels had only occurred at
142 the on-target site (Extended Data Fig. 4d). Furthermore, we used the WebLogo program to
143 determine the most frequent sequences associated with putative sites identified from
144 Digenome-seq^{15,16} (Extended Data Fig. 4e). Deep sequencing at these sites also confirmed that
145 no off-target events had occurred (Extended Data Fig. 4f). In all, due to both its efficient
146 mutagenicity and high on-target specificity, sgRNA2b appeared most promising.

147 148 **sgRNA activity during mouse preimplantation development**

149 We used published sgRNA/Cas9 mRNA zygote microinjection conditions¹⁷ to further assess
150 sgRNA activity and optimize microinjection methodologies in mouse zygotes. As it has been
151 shown that OCT4-null mouse blastocysts lack expression of the primitive endoderm marker
152 *SOX17* due to a cell-autonomous requirement for FGF4/MAPK signaling^{9,18}, we used
153 absence of both OCT4 and *SOX17* immunostaining to identify OCT4-deficient embryos (Fig.
154 1d). This OCT4-null phenotype was observed in 54% of embryos injected with Cas9 mRNA
155 and sgRNA2b, and in 0%, 10% or 3% of embryos injected with sgRNA1-1, sgRNA1-2 or
156 sgRNA4, respectively (Fig. 1e). These data confirm that sgRNA2b is superior to other tested

157 sgRNAs at inducing null mutations in both mouse embryos and hESCs. We next interrogated
158 a greater range of Cas9 mRNA and sgRNA concentrations to identify conditions that may
159 enhance rates of mutagenesis (Extended Data Fig. 5a). We confirmed that the previously
160 reported concentration of 100 ng/ μ L Cas9 mRNA together with 50 ng/ μ L sgRNA¹⁷ is optimal
161 for inducing an OCT4-null phenotype.

162
163 It has been suggested that microinjection of sgRNA/Cas9 ribonucleoprotein complexes may
164 reduce mosaicism and allelic complexity by bypassing the requirement for Cas9 translation
165 and sgRNA/Cas9 complex formation in embryos^{19,20}. To test this, we microinjected mouse
166 pronuclear zygotes with preassembled ribonucleoprotein complexes containing varying
167 concentrations of Cas9 protein (20 – 200 ng/ μ L) and sgRNA2b (20 – 100 ng/ μ L; Fig. 1f and
168 Extended Data Fig. 5b). Immunofluorescence analysis revealed that the sgRNA/Cas9 complex
169 was superior to Cas9 mRNA in causing loss of both OCT4 and SOX17, and that the optimal
170 concentration comprised 50 ng/ μ L Cas9 protein and 25 ng/ μ L sgRNA (Fig. 1f). Interestingly,
171 MiSeq analysis demonstrated that 83.3% of blastocysts derived from sgRNA2b/Cas9 complex
172 microinjections had 4 or fewer different types of indels (Fig. 1g), suggesting that editing
173 occurred prior to, or at the 2-cell stage. By contrast, only 52.6% of sgRNA2b/Cas9 mRNA
174 microinjected embryos exhibited this range of indels. Furthermore, a greater proportion of
175 blastocysts formed after sgRNA2b/Cas9 mRNA microinjection had 6 or more different types
176 of detectable indels (42.2%) compared to the sgRNA2b/Cas9 complex (8.3%). This increased
177 mutational spectrum suggests that following Cas9 mRNA injection, DNA editing occurred
178 between the 3- to 4-cell stage. Consistent with previous reports²¹, we observed a stereotypic
179 pattern in the type of indels detected in independently targeted embryos, including the
180 representative 28 bp deletion (Extended Data Fig. 5c), which was distinct from those induced
181 in hESCs.

182
183 In addition to lacking SOX17 and OCT4 expression, mouse embryos microinjected with the
184 sgRNA2b/Cas9 complex recapitulated other reported OCT4-null phenotypes such as
185 downregulation of PDGFRA, SOX7, GATA6 and GATA4 in the primitive endoderm
186 (Extended Data Fig. 5d). Consistent with the role of OCT4 in repressing TE genes⁹, the few
187 inner cell mass (ICM) cells that could be detected in sgRNA2b/Cas9 microinjected embryos
188 ectopically expressed CDX2 (Extended Data Fig. 5d). When plated in mouse ESC derivation
189 conditions, these embryos failed to generate ICM outgrowths, and instead exhibited
190 differentiation to trophoblast-like cells (Extended Data Fig. 5e). In contrast, blastocysts
191 derived from non-injected embryos formed ICM outgrowths in most instances, as did
192 blastocysts from embryos microinjected with Cas9 protein alone or an sgRNA/Cas9 complex
193 targeting *Dmcl* (a gene not essential for preimplantation development). Having thus
194 determined sgRNA2b to be an efficient and specific guide capable of generating a null
195 mutation of *POU5F1/Pou5f1* in both hESCs and mouse preimplantation embryos, we next
196 used this together with our optimized microinjection technique to target *POU5F1* in human
197 preimplantation embryos.

198
199 **Targeting *POU5F1* in human preimplantation embryos**

200 To determine the requirement for OCT4 in human embryos, we performed CRISPR editing
201 on thawed *in vitro* fertilized zygotes that were donated as surplus to infertility treatment. We
202 microinjected 37 zygotes with the sgRNA2b/Cas9 ribonucleoprotein complex (Supplementary
203 Video 1), and 17 zygotes with Cas9 protein alone, to control for the microinjection technique.
204 Of the sgRNA2b/Cas9 microinjected zygotes, 30 embryos retained both pronuclei during
205 microinjection with pronuclear fading observed approximately 6 hours later, followed by
206 cytokinesis on average 5 hours later (Supplementary Video 2). These timings are similar to
207 those previously published^{22,23} and indicate that microinjection was performed when the
208 embryos were in S-phase of the cell cycle (Fig. 2a). Genome editing via the ribonucleoprotein

209 complex has been estimated to start after approximately 3 hours *in vitro* and persist for 12-24
210 hours²⁴, therefore CRISPR/Cas9-induced DSBs are likely to be formed during late S-phase, or
211 subsequently at G2 phase. In 7 sgRNA2b/Cas9 microinjected zygotes, the pronuclei had
212 already faded after thawing, thus they had exited S-phase and were undergoing syngamy.
213 These embryos consequently underwent cell division approximately 3 hours after
214 microinjection. In these embryos editing likely occurred at the G2 or M phase, or in the G1
215 phase of the next cell cycle, at the 2-cell stage (Fig. 2a), which would promote mosaicism.

216
217 Time-lapse microscopy of the embryos showed that the timings of cleavage divisions
218 following pronuclear fading were similar between the Cas9 protein and the sgRNA2b/Cas9
219 microinjected embryos (Fig. 2b,c). By the 8-cell stage, cleavage arrest was observed in 43%
220 (16 out of 37) of sgRNA2b/Cas9 microinjected embryos compared to 41% (10 out of 17)
221 Cas9 protein control embryos (Fig. 2d). As developmental arrest at the onset of EGA at the 8-
222 cell stage strongly correlates with aneuploidy in IVF embryos²⁵, we also sought to determine
223 embryo karyotype. We performed low-pass whole genome sequencing, which has been shown
224 to accurately estimate gross chromosome anomalies²⁶. We collected blastomeres from
225 sgRNA2b/Cas9 microinjected embryos arrested up to the 8-cell stage and detected
226 chromosomal loss or gain in 83% (5 out of 6) of embryos (Extended Data Fig. 6a), which is
227 consistent with rates reported by preimplantation genetic screening^{26,27}. Trophectoderm
228 biopsies of a subset of blastocysts that developed following sgRNA2b/Cas9 microinjection
229 determined that 60% (3 out of 5) were euploid (Fig. 2e, Extended Data Fig. 6a). The other two
230 blastocysts exhibited karyotypic abnormalities including the loss of chromosome 16
231 (Extended Data Fig. 6b), an abnormality frequently observed in human preimplantation
232 embryos and thus likely to be unrelated to targeting²⁵. In the Cas9 protein control group, 57%
233 (4 out of 7) of blastocysts were euploid, and aneuploidies were observed in the remaining 3
234 blastocysts, including the loss of chromosome 14 in two sibling-matched control embryos,
235 and the gain of chromosome 15 and 18 (Fig. 2e, Extended Data Fig. 6a,b). Altogether, this
236 suggests that CRISPR/Cas9 targeting does not increase the rates of karyotypic anomalies in
237 human embryos.

238
239 47% (8 out of 17) of Cas9 protein microinjected controls developed to the blastocyst stage, a
240 rate equivalent to those of uninjected controls²⁸, suggesting that the microinjection technique
241 did not affect embryo viability (Fig. 2d). However, only 19% (7 out of 37) of sgRNA2b/Cas9
242 protein microinjected embryos developed to the blastocyst stage, significantly fewer
243 compared to Cas9 protein microinjected controls (Fig. 2d, $P < 0.05$). The blastocysts that
244 formed following sgRNA2b/Cas9 protein microinjection were of variable quality (Extended
245 Data Fig. 6c). Although all blastocysts had a discernible blastocoel cavity, only some
246 possessed a small compact ICM (Extended Data Fig. 6c), and all retained a thick zona
247 pelucida, in contrast to Cas9 microinjected controls. sgRNA2b/Cas9 microinjected human
248 embryos also went through iterative cycles of expanding and initiating blastocyst formation
249 and then collapsing until some embryos ultimately degenerated (Supplementary Video 2 and
250 3). Altogether, this suggests that targeting OCT4 in human embryos impacts both blastocyst
251 viability and quality.

252
253 To determine on-target editing efficiency, we performed targeted deep and/or Sanger
254 sequencing of all cells microdissected from the sgRNA2b/Cas9 microinjected embryos
255 arrested prior to the 8-cell stage, and found indels at the *POU5F1* on-target site in 71% (5 out
256 of 7) of embryos (Fig. 3a). The most frequently observed indels in sgRNA2b/Cas9
257 microinjected embryos were the 2 bp and 3 bp deletions that were observed in the sgRNA2b
258 induced hESCs (Fig. 3b, Extended Data Fig. 7a,b). This finding indicates that hESCs can be
259 used not only to screen sgRNA efficiency, but also to predict the *in vivo* mutation spectrum
260 induced by CRISPR/Cas9-mediated genome editing. We also detected larger *POU5F1*

261 deletions in the human embryos compared to hESCs, similar to our observations in mouse
262 embryos (Fig. 3b, Extended Data Fig. 7a,b). Furthermore, targeted deep and/or Sanger
263 sequencing in edited cells demonstrated that off-target mutations were undetectable above
264 background PCR error rates, further confirming the specificity of the sgRNA (Extended Data
265 Fig. 7c,d).

266
267 We next assessed mutational signatures in more developmentally advanced embryos, after
268 EGA. Interestingly, we confirmed that on-target editing had occurred in 8 out of 8
269 sgRNA2b/Cas9 microinjected embryos analysed from the 8-cell to the blastocyst stage.
270 However, invariably these embryos all retained wild-type copies of the *POU5F1* allele in at
271 least one cell (Fig. 3a). In sgRNA2b/Cas9 microinjected human embryos, OCT4 protein
272 expression was downregulated in most cleavage-stage cells and undetectable above
273 background in others, confirming high efficiency of editing (Fig. 3c; Extended Data Fig. 8a).
274 However, we were able to identify at least one cell that had nuclear OCT4 staining above
275 background levels (Fig. 3c; Extended Data Fig. 8a). Moreover, despite a significant reduction
276 in cell number, blastocyst-stage embryos also retained OCT4 expression in a subset of cells
277 (Fig. 3d,e Extended Data Fig. 8b,c). These findings suggest that *POU5F1* targeting efficiency
278 is high, and that only embryos with partial OCT4 expression are able to progress to the
279 blastocyst stage.

280
281 To determine if there is a high degree of editing in embryos prior to the onset of OCT4
282 expression, we microinjected 4 additional human embryos with the sgRNA2b/Cas9 complex
283 and stopped their development prior to the 8-cell stage. 100% (4 out of 4) of these embryos
284 had detectable indels, with two embryos lacking wild-type *POU5F1* alleles (Fig. 3a). In one
285 embryo editing occurred in all blastomeres, although one blastomere retained one copy of the
286 wild-type allele. In another embryo, while 4 out of 5 blastomeres had been edited, one
287 blastomere retained both copies of the wild-type allele. Together with the cleavage arrested
288 embryos above, this demonstrates that in 45% (5 out of 11) of cleavage stage embryos (either
289 stopped or developmentally arrested), all of the cells analysed from each embryo had no
290 detectable *POU5F1* wild-type alleles, indicating high rates of editing. Altogether these data
291 suggest an unexpectedly earlier function for OCT4 in humans compared to mice, prior to
292 blastocyst formation.

293 294 **Loss of OCT4 in human embryos is associated with mis-expression of genes associated** 295 **with the three lineages in the blastocyst**

296 To determine globally which genes might be affected by the loss of OCT4, we microdissected
297 single cells from microinjected embryos at the blastocyst stage. We adapted a method to
298 isolate both RNA and DNA from single cells²⁹ in order to perform RNA-seq and targeted
299 deep or Sanger sequencing of on-target and putative off-target sites. Principal component
300 analysis showed that cells from sgRNA2b/Cas9 microinjected human blastocysts clustered
301 distinctly from those derived from Cas9 protein microinjected controls (Fig. 4a). Intriguingly,
302 the cluster from sgRNA2b/Cas9 microinjected embryos contained not only cells that were
303 genotypically knockout for *POU5F1*, but also those that were wild-type or heterozygous for
304 *POU5F1*. This finding suggests that loss of *POU5F1* may impose non-cell autonomous
305 effects on gene expression in neighbouring wild-type or heterozygous cells.

306
307 Differential gene expression analysis indicated that genes most highly mis-expressed in the
308 sgRNA2b/Cas9 targeted human blastocysts compared to the Cas9 protein controls included
309 those that we previously identified as highly enriched in the epiblast, including *NANOG*,
310 *KLF17*, *DPPA5*, *ETV4*, *TDGF1*, and *VENTX* (Extended Data Fig. 9a, Supplementary Table
311 1). Immunofluorescence analysis confirmed that even in cells retaining OCT4, the expression
312 of *NANOG* was absent (Fig. 4b, Extended Data Fig. 8c). In striking contrast, OCT4-null

313 mouse blastocysts maintained Nanog expression in the ICM (Fig. 4b, Extended Data Fig.
314 8d,e), as previously reported^{9,18}.

315
316 In OCT4-null cells several trophectoderm-associated genes were also significantly
317 downregulated, including *CDX2*, *HAND1*, *DLX3*, *TEAD3*, *PLAC8* and *GATA2* (Extended
318 Data Fig. 9a, Supplementary Table 1). We confirmed loss of GATA2 protein expression in
319 human sgRNA2b/Cas9 protein injected embryos (Fig. 4c, Extended Data Fig. 8f). Coupled
320 with the failure to maintain a fully expanded blastocyst, this finding suggests that the integrity
321 of the trophectoderm may be compromised in OCT4-targeted embryos. To further
322 characterize this, we performed immunofluorescence analysis for ZO-1, which incorporates
323 into tight junctions during trophectoderm formation. In sgRNA2b/Cas9 targeted human
324 blastocysts, ZO-1 expression was interrupted, patchy and diffuse compared to the uniform
325 network-like distribution in uninjected control embryos (Fig. 4d). This is in contrast to mouse
326 OCT4-null embryos, where expression of trophectoderm markers such as *Cdx2*, *Hand1* and
327 *Gata3* are upregulated⁹.

328
329 Additionally, primitive endoderm markers such as *GATA4* were downregulated in
330 sgRNA2b/Cas9 microinjected embryos compared to Cas9 protein controls.
331 Immunofluorescence analysis suggested that SOX17 protein expression was also
332 downregulated (Fig. 3d, Extended Data Fig. 8b). Moreover, we were surprised to observe
333 ectopic expression of *PAX6* in some cells from sgRNA2b/Cas9 edited human blastocysts
334 (Extended Data Fig. 9a, Supplementary Table 1). The lack of expression of genes associated
335 with all three lineages in the blastocyst suggests that OCT4-targeted embryos either failed to
336 initiate the expression of these genes or downregulated their expression as development
337 progressed. To determine whether the gene expression patterns in OCT4-targeted cells more
338 closely resemble cells from earlier stages of human development, we integrated our data with
339 a previously published dataset comprising all stages of human preimplantation
340 development^{3,30} (Fig. 4e, Extended Data Fig. 9b). This revealed that while cells from OCT4-
341 targeted embryo were progressing towards the transcriptional state of the blastocyst, they
342 were more dispersed and heterogeneous in their gene expression. Altogether, our data
343 suggests that the integrity of the human blastocyst is compromised as a consequence of OCT4
344 downregulation. As a result, all lineages are negatively affected, pointing to a functional role
345 for OCT4 in early human development.

346
347

348 Discussion

349 CRISPR/Cas9-mediated genome editing represents a transformative method to evaluate the
350 function of putative regulators of human preimplantation development. We demonstrate the
351 importance of initially screening sgRNA efficiencies and mutagenic patterns prior to targeting
352 in human embryos, as sgRNAs were not equivalently efficient in inducing *POU5F1*-null
353 mutations despite scoring highly by *in silico* predictions. We identify different consequences
354 of OCT4 loss on human versus mouse embryos, consistent with other differences reported
355 between these species. For example, pharmacological inhibition of FGF and downstream
356 ERK signaling leads to ectopic expression of pluripotency factors in the mouse, but not the
357 human at equivalent stages^{31,32}.

358
359 Surprisingly, our data suggests OCT4 may be required earlier in human development than it is
360 in mice, for instance during the cleavage or morula stages, when OCT4 expression is initiated
361 (Fig. 4f). As the mouse maternal/zygotic *Pou5f1*-null mutation phenocopies the zygotic-null
362 mutation⁹, it is unlikely that persistence of maternal transcripts or proteins compensates for
363 the loss of OCT4 expression, and any additional compensatory mechanisms that may be
364 present in the mouse do not appear to be conserved in the regulation of human development.

365 The mis-expression of genes associated with all three blastocyst lineages further suggests that
366 OCT4 may have an essential function prior to this stage. In the future, it would be informative
367 to determine whether OCT4 mutation leads to changes in gene expression prior to the
368 blastocyst stage, which may explain the failure of blastocyst development. Alternatively,
369 inducing *POU5F1*-null mutations in human embryos slightly later in development, following
370 the onset of EGA, may bypass its earlier critical role and thereby delineate its function in the
371 fully formed blastocyst.

372
373 Significantly, CRISPR/Cas9-mediated genome editing does not appear to increase genomic
374 instability or developmental arrest prior to EGA, suggesting that this method may be used to
375 understand the function of other putative lineage specifiers. In future, a number of adaptations
376 may provide further advantages. Co-injection of the CRISPR/Cas9 components with sperm
377 during intracytoplasmic sperm injection³³ may allow more time for targeting prior to the first
378 cell division, further increasing editing efficiency. Indeed, this approach has been used
379 recently in human embryos⁸. Introducing multiple sgRNAs may also increase targeting
380 efficiency, but may also increase the risk of off-target mutations. Alternatively, introducing
381 the CRISPR/Cas9 components alongside a donor oligonucleotide complementary to the target
382 locus and harboring a premature stop codon, should favor the generation of null mutations via
383 homology directed repair. This approach may not be straightforward given recent attempts to
384 correct an abnormal paternal gene variant were reported to use the maternal allele for HDR
385 rather than an introduced template⁸. Targeting genes not essential for, or with a later or more
386 specific role in pre-implantation development will also inform our interpretation of the OCT4
387 phenotype. At present, we cannot be certain that the early developmental arrest is associated
388 with the loss of OCT4 rather than some non-specific effect of injecting both Cas9 and the
389 sgRNA, as opposed to the Cas9 alone. However, the only other study to date using genome
390 editing with human embryos that showed development beyond 8-cell stages, where a non-
391 essential gene was targeted, showed normal blastocyst formation at rates similar to controls⁸.
392 This suggests that the effects we see here are due to loss of OCT4. Altogether we developed
393 an optimized approach to target OCT4 in human embryos thus revealing a distinct function
394 compared to the mouse. This proof of principle lays out a framework for future investigations
395 that could transform our understanding of human biology, thereby leading to improvements in
396 the establishment and therapeutic use of stem cells and in IVF treatments.

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416

417 **Contributions**

418 KKN conceived the project, designed and performed experiments, microinjected embryos and
419 analysed data. NMEF performed single-cell analysis, hESC experiments, human and mouse
420 embryo phenotyping and genotyping. AM performed genotyping of hESCs, stem cell
421 derivation, mouse embryo phenotyping and generated the sgRNAs. KES generated the
422 inducible hESCs, independently performed hESC phenotyping and performed flow cytometry
423 analysis. AB designed and assisted with hESCs experiments and LV and AB supervised the
424 experiments. NK and DW performed cytogenetic analysis and independently confirmed
425 human embryo genotyping analysis. KE coordinated donation of embryos to the research
426 project. BP generated some of the sgRNAs used in the mouse and supplied sgRNA sequences.
427 PB and JK performed the RNA-seq analysis. RL and SEW assisted with phenotyping. DK,
428 and J-SK performed Digenome-seq analysis. VM assisted with genotyping. KKN, JT and
429 NMEF wrote the manuscript with help from all of the authors. All authors assisted with
430 experimental design, generated figures and/or commented on the manuscript.

431

432 **Figure Legends**

433 **Figure 1: Screening sgRNAs targeting OCT4 in optimised inducible CRISPR/Cas9**
434 **knockout human embryonic stem cells (hESCs) and mouse embryos.**

435 **a**, Schematic of the strategy used to induce sgRNA expression in hESCs. The CAG promoter
436 drives constitutive expression of the Cas9 gene as well as the tetracycline-responsive
437 repressor (tetR). The inducible H1-TO promoter drives expression of each sgRNA in the
438 presence of tetracycline (TET). The two transgenic cassettes are each targeted to one of the
439 AAVS1 genomic safe harbour loci using zinc-finger nucleases (ZFN). (TO: tetracycline-
440 responsive operator).

441

442 **b**, Immunofluorescence analysis of OCT4 (red) or PAX6 (green) and DAPI nuclear staining
443 (blue) expression in hESCs after 4 days of sgRNA2b induction (+Tet). Scale bars, 100 μ m.

444

445 **c**, Quantification of indel mutations detected at each sgRNA on-target site. One-way ANOVA
446 compared to uninduced hESCs. * P <0.05; ** P <0.01.

447

448 **d**, Immunofluorescence analysis for OCT4 (red), SOX17 (green) and DAPI nuclear staining
449 (blue) in control, OCT4-null or mosaic mouse blastocysts 4 days following zygote
450 microinjection. Scale bar, 100 μ m.

451

452 **e**, Quantification of proportions of OCT4-null, mosaic or wild-type mouse blastocysts
453 following microinjection of Cas9 mRNA plus sgRNA1-1, sgRNA1-2, sgRNA2b, or sgRNA4
454 or uninjected controls. Chi-squared test. Data are mean \pm s.d. * P <0.05; ** P <0.01;
455 **** P <0.0001.

456

457 **f**, Quantification of proportions of OCT4-null, mosaic or wild-type mouse blastocysts
458 following microinjection of the sgRNA2b/Cas9 ribonucleoprotein complex concentrations
459 indicated. Chi-squared test. Data are mean \pm s.d. **** P <0.0001.

460

461 **g**, Comparison of mutation spectrums after targeting mouse embryos with sgRNA2b plus
462 Cas9 mRNA or protein. Data are the proportion of unique indels observed. Chi-squared test.
463 **** P <0.0001

464

465 **Figure 2: The developmental potential of human embryos following CRISPR/Cas9-**
466 **mediated genome editing.**

467 **a**, Schematic of the first cell division in human embryos and time of microinjection. (PN,
468 pronuclei; PNF, pronuclear fading).

469
470 **b**, Representative human embryo at each developmental stage analysed. (SC, start of
471 cavitation; SB, start of blastocyst formation; B, blastocyst).

472
473 **c**, Morphokinetic analysis of human development after microinjection. Non-parametric two-
474 tailed Kolmogorov-Smirnov test; ns, not significant.

475
476 **d**, Kaplan–Meier survival curve of human embryos following microinjection of Cas9 protein
477 or sgRNA2b/Cas9 ribonucleoprotein complex. Zygotic *POU5F1* expression is initiated
478 between the 4- to 8-cell stage. Chi-squared test. * $P < 0.05$.

479
480 **e**, Karyotype analysis by whole genome sequencing of human blastocysts following
481 microinjection of Cas9 protein or sgRNA2b/Cas9 ribonucleoprotein complex. Representative
482 karyotypically normal embryos are shown.

483
484 **Figure 3: Genotypic characterisation of OCT4-targeted human embryos.**

485 **a**, Proportion of *POU5F1*-null, heterozygous or wild-type cells in each human embryo. The
486 number of cells analysed is indicated. Embryos 2, 5, 7 and 8 were microinjected with Cas9
487 protein as a control. All other embryos were microinjected with the sgRNA2b/Cas9
488 ribonucleoprotein complex. The development of some embryos was stopped and they were
489 removed from culture for analysis, while others were analysed following cleavage arrest.

490
491 **b**, The type and relative proportion of indel mutations observed compared to all observable
492 indel mutations within each human embryo.

493
494 **c**, Immunofluorescence analysis for OCT4 (green), and DAPI nuclear staining (blue) in an
495 uninjected control cleavage stage human embryo or an embryo that developed following
496 sgRNA2b/Cas9 ribonucleoprotein complex microinjection ($n = 5$). Confocal z-section. Arrow,
497 OCT4 expressing cell. Scale bar, 100 μm .

498
499 **d**, Immunofluorescence analysis for OCT4 (green), SOX17 (red) and DAPI nuclear staining
500 (blue) in an uninjected control human blastocyst ($n = 3$) or a blastocyst that developed
501 following sgRNA2b/Cas9 ribonucleoprotein complex microinjection ($n = 3$). Confocal z-
502 section. Scale bar, 100 μm .

503
504 **e**, Quantification of the number of DAPI or OCT4 positive nuclei in uninjected control human
505 blastocysts ($n = 3$) compared to blastocysts that developed following sgRNA2b/Cas9
506 ribonucleoprotein complex microinjection ($n = 5$). One-tailed t-test. ** $P < 0.01$; *** $P < 0.001$.

507
508 **Figure 4: Phenotypic characterisation of OCT4 targeted human embryos.**

509 **a**, Principal component analysis of single-cell RNA-seq data showing comparisons between
510 the cells from human blastocysts that developed following microinjection of the
511 sgRNA2b/Cas9 ribonucleoprotein complex (filled shapes) compared to Cas9 protein
512 microinjected controls (unfilled shapes). The genotype of each cell is distinguished by colour.
513 5 samples failed repeated genotyping but the RNA quality is good and these are listed as
514 Unknown. Each data point represents a single cell.

515
516 **b**, Immunofluorescence analysis for OCT4 (green), NANOG (red) and DAPI nuclear staining
517 (blue) in a human or a mouse uninjected control blastocyst or a blastocyst that developed
518 following sgRNA2b/Cas9 ribonucleoprotein complex microinjection (mouse: $n = 7$; human: n
519 = 3). Confocal z-section. Scale bar, 100 μm .

520

521 **c**, Immunofluorescence analysis for OCT4 (green), GATA2 (magenta) and DAPI nuclear
522 staining (blue) in an uninjected control human blastocyst ($n = 3$) or in a blastocyst that
523 developed following sgRNA2b/Cas9 ribonucleoprotein complex microinjection ($n = 3$).
524 Confocal projection. Scale bar, 100 μm .

525
526 **d**, Immunofluorescence analysis for OCT4 (green), ZO-1 (magenta) and DAPI nuclear
527 staining (blue) in an uninjected control human blastocyst ($n = 2$) or in a blastocyst that
528 developed following sgRNA2b/Cas9 ribonucleoprotein complex microinjection ($n = 2$).
529 Confocal projection. Scale bar, 100 μm .

530
531 **e**, Principal component analysis of a previously published human single-cell RNA-seq
532 dataset³⁰ integrated with the data from the Cas9 protein control and the sgRNA2b/Cas9
533 ribonucleoprotein (RNP) microinjected embryos. Each point represents a single cell.

534
535 **f**, Diagram summarising the observations made in the study and their relationship to the onset
536 of zygotic *POU5F1* expression.

537
538
539

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629

630 **Extended Data Figure Legends**

631

632 **Extended Data Figure 1: *POU5F1* targeting and comparison of sgRNAs**

633 **a**, Schematic representation of the human *POU5F1*/OCT4 locus and sgRNA targeting sites.
634 The location (not to scale) and sequences of the sgRNAs tested are shown and the
635 protospacer-adjacent motif (PAM) sequences are underlined and in red font. Sequences within
636 the exons are in uppercase and introns are in lowercase. The mouse sgRNA sequences are
637 shown below. The exons encoding the N-terminal domain (NTD), POU DNA binding domain
638 or the C-terminal domain (CTD) are indicated.
639

640 **b**, Representative flow cytometry analysis quantifying OCT4 expression in hESCs induced to
641 express each sgRNA over 5 days compared to uninduced controls. The percentage of OCT4
642 protein expression is shown.
643

644 **c**, qRT-PCR analysis after 4 days of sgRNA induction. Relative expression reflected as fold
645 difference over uninduced cells normalised to *GAPDH*. Data points and mean for all samples
646 are shown: $n = 2$ sgRNA1-1 clones; $n = 3$, sgRNA 1-2, 2b or 4 clones, representative of two
647 independent experiments and \pm s.e.m. where there are three samples. Two-way ANOVA.
648 * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.
649

650 **d**, Heat maps of selected genes showing unsupervised hierarchical clustering of uninduced
651 and sgRNA2b-induced hESCs. Normalised RNA-seq expression levels are plotted on a high-
652 to-low scale (purple-white-green).
653

654

655

655 **Extended Data Figure 2: Further characterisation of sgRNA2b-induced hESCs.**

656 **a**, hESCs induced to express sgRNA2b for 4 days (+Tet) in chemically defined media with
657 activin A and FGF2 (CDM/AF) compared to uninduced controls (No Tet).
658 Immunofluorescence analysis for pluripotency markers OCT4, NANOG and SOX2 or
659 markers associated with differentiation to early derivatives of the germ layers (SOX1-
660 expressing ectoderm cells or SOX17-expressing endoderm cells). DAPI nuclear staining
661 (blue) is shown. Scale bar, 400 μ m.
662

663 **b**, qRT-PCR analysis for selected genes associated with either pluripotency or differentiation
664 to derivatives of the germ layers in hESCs induced to express each of the sgRNA for 4 days.
665 Relative expression reflected as fold difference over wild-type hESCs and normalised to
666 *PBGD*. Data points and mean for all samples are shown: $n = 2$ sgRNA1-1 clones; $n = 3$,
667 sgRNA 1-2, 2b or 4 clones, representative of two independent experiments and \pm s.e.m. where
668 there are three samples. Two-way ANOVA. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$, ns,
669 not significant.
670

671 **Extended Data Figure 3: On-target mutation spectrum in hESCs induced to express** 672 **sgRNA1-1, sgRNA1-2, sgRNA2b or sgRNA4.**

673 Shown are frequent types of indel mutations and corresponding sequences observed in hESCs
674 induced to express sgRNA1-1, sgRNA1-2, sgRNA2b or sgRNA4. The cells were induced to
675 express each sgRNA for 4 days and the data shown is representative of the type of indel

676 mutations observed in other clonal lines ($n = 2$ sgRNA1-1 clones; $n = 3$, sgRNA 1-2, 2b or 4
677 clones) and across time (from 1 up to 4 days following induction of each sgRNA).

678

679 **Extended Data Figure 4: Off-target analysis of sgRNA2b-induced hESCs.**

680 **a**, The *POU5F1* sgRNA2b 12 bp seed sequence is highlighted in green and the NGG PAM
681 sequence in red. In black are the nucleotide sequences 5' to the sgRNA seed sequence. 7
682 putative off-target sequences and associated genes are shown including *POU5F1*
683 pseudogenes. In orange are the nucleotides that differ from the sgRNA2b sequence.

684

685 **b**, Percentage of indel mutations detected at putative off-target sites in hESCs 4 days
686 following tetracycline induction of sgRNA2b compared to uninduced controls. Data are
687 percentages of indels detected in the cell lines at each of the sites indicated. Comparisons
688 made between three clonal hESC lines induced to express sgRNA2b versus uninduced
689 controls. The percentage of indel mutations induced at the on-target site were significant
690 while all other sites were not significantly different. Two-way ANOVA. *** $P < 0.001$.

691

692 **c**, Digenome-seq results displayed as a genome-wide circos plot. The height of the peak
693 corresponds to the DNA cleavage score. The red arrow points to the *POU5F1* locus on
694 chromosome 6.

695

696 **d**, Percentage of indel mutations observed in sgRNA2b-induced hESCs and in wild-type H9
697 control cells at each locus following targeted deep sequencing of putative off-target sites
698 identified by Digenome-seq.

699

700 **e**, Off-target candidate nucleotides displayed as sequence logos using the WebLogo program.

701

702 **f**, Percentage of indel mutations observed in sgRNA2b-induced hESCs and in wild-type H9
703 control cells following targeted deep sequencing of putative off-target sites determined by
704 WebLogo sequence homology.

705

706 **Extended Data Figure 5: Assessing a range of Cas9 and sgRNA combinations for**
707 **microinjection into mouse pronuclear zygotes.**

708 Additional conditions were tested in mouse embryos microinjected with the sgRNA2b either

709 **a**, plus Cas9 mRNA or

710

711 **b**, as a complex with the Cas9 protein at the ratios indicated. Quantification was performed on
712 the proportion of mouse embryos at the blastocyst stage that are phenotypically null (loss of
713 OCT4 and SOX17 protein expression), mosaic/heterozygous (partial OCT4 and/or SOX17
714 expression) or uninjected (strong OCT4 and SOX17 expression). Data are mean \pm s.d. and
715 comparisons made between the percentage of OCT4-null embryos observed versus wild-type
716 uninjected control embryos. Chi-squared test. * $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$.

717

718 **c**, The type of indel mutations detected in mouse embryos microinjected with the
719 sgRNA2b/Cas9 ribonucleoprotein complex. The sgRNA sequence is boxed and the NGG
720 PAM site underlined. Dash, deletion position.

721

722 **d**, Further characterization of mouse embryos microinjected with sgRNA2b/Cas9
723 ribonucleoprotein complex compared to uninjected control blastocysts. Immunofluorescence
724 analysis for markers of the trophectoderm (CDX2) or primitive endoderm (GATA4, GATA6,
725 PDGFRA and SOX7) lineages together with DAPI nuclear staining. Confocal z-section. Scale
726 bar, 100 μm .

727

728 e, Quantification of blastocyst inner cell mass (ICM) or trophoblast outgrowths in mouse
729 embryonic stem cell derivation conditions. Uninjected, Cas9-injected or Cas9 plus Dmc1
730 sgRNA (targeting a gene not essential for preimplantation development) were used as
731 controls. Comparisons were made to blastocysts that developed following sgRNA2b/Cas9
732 ribonucleoprotein microinjection. Two-tailed t-test. *P<0.05.

733

734 **Extended Data Figure 6: Further assessing human embryo quality.**

735 a, Karyotype analysis following whole genome sequencing of either single blastomeres or
736 trophectoderm biopsies. Multiple biopsies were analysed from embryos C8, C12 and C16.
737 Analysis was also performed on blastocysts that developed following microinjection of Cas9.
738 The type of chromosome gains and losses are indicated.

739

740 b, Representative karyotype analysis by whole genome sequencing of human blastocysts. A
741 representative graph indicating aneuploidy in embryos following either Cas9 protein or
742 sgRNA2b/Cas9 ribonucleoprotein complex microinjection.

743

744 c, Phase-contrast images of blastocysts that developed following microinjection of the
745 sgRNA2b/Cas9 ribonucleoprotein complex compared to Cas9 protein injected controls. White
746 arrows point to the presumptive inner cell mass and a black arrow to a representative zona
747 pelucida.

748

749 **Extended Data Figure 7: Evaluating on-target and putative off-target mutations in
750 human embryo cells.**

751 a, The type and relative proportion of indel mutations observed compared to all observable
752 indel mutations within each human embryo.

753

754 b, Quantification of indels by TIDE analysis. Representative plots and Sanger sequencing
755 chromatograms are shown from *OCT4*-null, heterozygous and wild-type human cells.

756

757 c, Percentage of indel mutations detected at the sgRNA2b on-target site and putative off-target
758 sites in single cells microdissected from Cas9 protein microinjected control blastocysts or
759 blastocysts that developed following sgRNA2b/Cas9 ribonucleoprotein complex
760 microinjection. Putative off-target sites were evaluated in cells that were previously
761 determined to be *OCT4*-null (green), heterozygous (orange) or wild-type (blue) along with
762 samples from Cas9 protein microinjected embryos (red). Three representative examples are
763 shown from each group.

764

765 d, Sanger sequencing chromatograms from *OCT4*-null single cells collected from human
766 blastocysts that developed following sgRNA2b/Cas9 ribonucleoprotein complex
767 microinjections. The chromatograms exemplify the sequence detected in all of the other
768 samples analysed. Underlined is the sequence of the putative off-target site.

769

770 **Extended Data Figure 8: Phenotypic characterisation of *OCT4*-targeted embryos.**

771 a, Immunofluorescence analysis for *OCT4* (green) and DAPI nuclear staining (blue) in human
772 cleavage stage embryos following sgRNA2b/Cas9 ribonucleoprotein complex microinjection
773 ($n = 5$). Confocal z-section. Arrow, *OCT4* expressing cell. Scale bar, 100 μm .

774

775 b, Immunofluorescence analysis for *OCT4* (green), *SOX17* (red) and DAPI nuclear staining
776 (blue) in an uninjected control blastocyst ($n = 3$) or a human blastocyst that developed
777 following sgRNA2b/Cas9 ribonucleoprotein complex microinjection ($n = 3$). Confocal z-
778 section. Scale bar, 100 μm .

779

780 **c,d** Immunofluorescence analysis for OCT4 (green), NANOG (red) and DAPI nuclear
781 staining (blue) in **(c)** a human blastocyst that developed following sgRNA2b/Cas9
782 ribonucleoprotein complex microinjection ($n = 3$) or **(d)** in a mouse uninjected control
783 blastocyst or in blastocysts that developed following sgRNA2b/Cas9 ribonucleoprotein
784 complex microinjection ($n = 7$). Confocal z-section. Scale bar, 100 μm .

785
786 **g**, Quantification of NANOG and OCT4 expression in mouse uninjected control blastocysts (n
787 = 5) or in blastocysts that developed following sgRNA2b/Cas9 ribonucleoprotein complex
788 microinjection ($n = 7$). One-tailed t-test. $^{**}P < 0.01$.

789
790 **h**, Immunofluorescence analysis for GATA2 (green) and DAPI nuclear staining (blue) in a
791 human blastocyst that developed following sgRNA2b/Cas9 ribonucleoprotein complex
792 microinjection ($n = 3$). Confocal projection. Scale bar, 100 μm .

793
794 **Extended Data Figure 9:**

795 **a**, Hierarchical clustering and heat map of a selection of genes following single cell RNA-seq
796 analysis of human embryos. Embryos C8, C9, C12 and C16 (samples denoted in orange font)
797 were targeted with the sgRNA2b/Cas9 ribonucleoprotein complex. Embryos 2, 5, 7 and 8
798 were microinjected with Cas9 protein as a control. An uninjected control reference dataset
799 labelled PE (primitive endoderm cells), EPI (epiblast cells) or TE (trophectoderm cells) is
800 included³. Control cells clustered according to lineage and are indicated with the coloured
801 bars: red = PE, green = EPI and blue = TE. Grey bar highlights the samples that have low
802 expression of markers of each of the lineages shown. The genotype of the samples are noted
803 as *POU5F1* wild-type: WT, heterozygous: Het, or knockout: KO cells. 5 samples failed
804 repeated genotyping but the RNA quality is good and these are listed as X. Normalised
805 expression levels are plotted on a high-low scale (purple-white-green).

806
807 **b,c** Principal component analysis of a previously published human single-cell RNA-seq
808 dataset³⁰ integrated with the data from the Cas9 protein control and the sgRNA2b/Cas9
809 ribonucleoprotein (RNP) microinjected embryos. Each point represents a single cell. Data
810 were plotted along the **(b)** second and third or the **(c)** first and third principal components.

811
812 **Extended Data Figure 10: Reagents list**

813 **a**, Oligonucleotides used for cloning, MiSeq or qRT-PCR analysis

814
815 **b**, Antibodies used for immunofluorescence and flow cytometry analysis

816
817

818 **METHODS**

819
820 **Ethics statement**

821 This study was approved by the UK Human Fertilisation and Embryology Authority (HFEA):
822 research licence number R0162
823 (http://www.hfea.gov.uk/docs/07032016_Currently_licensed_research_projects.pdf) and the
824 Health Research Authority's Research Ethics Committee (Cambridge Central reference
825 number 16/EE/0067).

826
827 The process of approval entailed independent peer review along with approval from both the
828 HFEA Executive Licensing Panel (8 members of the Authority) and the Executive
829 Committees, which is composed of 5 members including members of the lay public. Our
830 research is compliant with the HFEA Code of Practice and has undergone independent
831 inspections by the HFEA since the licence was granted. The Research Ethics Committee is

832 comprised of 12 individuals including members of the lay public. Patient consent was
833 obtained from Bourn Hall Clinic.

834

835 Informed consent was obtained from all couples that donated spare embryos following IVF
836 treatment. Before giving consent, people donating embryos were provided with all of the
837 necessary information about the research project, an opportunity to receive counselling and
838 the conditions that apply within the licence and the HFEA Code of Practice. Specifically,
839 patients signed a consent form authorising the use of genome editing techniques including
840 CRISPR/Cas9 on donated embryos. Donors were informed that after the embryos have been
841 genetically modified their development will be stopped prior to 14 days post-fertilisation and
842 that subsequent biochemical and genetic studies would be performed. Informed consent was
843 also obtained from donors for all the results of these studies to be published in scientific
844 journals. No financial inducements are offered for donation. The patient information sheets
845 and consent document provided to patients are publicly available
846 (<https://www.crick.ac.uk/research/a-z-researchers/researchers-k-o/kathy-niakan/hfea-licence/>).
847 Embryos surplus to the patient's IVF treatment were donated cryopreserved and were
848 transferred to the Francis Crick Institute where they were thawed and used in the research
849 project.

850

851 **Power analysis**

852 The R statistical package pwr was used to determine the number of human embryos required
853 to determine the function of OCT4 compared to microinjected controls. A two-sample t-test
854 was performed to a significance level of $P < 0.05$. The effect size was 0.8 which assumes an
855 observable difference between the CRISPR injected and control embryos. The sample size
856 was estimated to be 25 CRISPR-targeted embryos.

857

858 **sgRNA design to target *POU5F1***

859 So as not to lower the targeting efficiency, we determined whether the sgRNAs targeted
860 polymorphic regions of the human genome. Most sgRNA had a single nucleotide
861 polymorphism (SNP) frequency of less than 0.1% in the human population, with the
862 exception of the sgRNA targeting exon 4, which had a SNP frequency of 32% within the
863 sgRNA target sequence as determined by the 1000 genomes project³⁴. We retained this
864 sgRNA as it had the highest *in silico* score and overlapped with a site that has been previously
865 shown in complementarity studies to be functionally required for pluripotency, suggesting
866 that even an in-frame deletion would render a loss of function in the gene¹³. We also favoured
867 the use of sgRNAs with sequence conservation of the PAM and sgRNA seed sequence
868 (approximately 12bp region proximal to the PAM sequence) that would allow us to determine
869 efficiency in mouse embryos. In the case of high-scoring sgRNAs targeting exon 2d, there is
870 no mouse equivalent sgRNA sequence that we could evaluate, and for exon 3, we could not
871 design sgRNAs where the predicted cut site would be within the exon; these options were
872 therefore excluded.

873

874 **sgRNA production and ribonucleoprotein preparation**

875 sgRNAs were prepared as previously described³⁵. The sgRNA was cloned into the bicistronic
876 expression vector px330 (Addgene; 42230³⁶) using the Bbs1 restriction site. The sgRNA
877 sequence from the correctly targeted px330 vector was amplified using the Q5 hot start high
878 fidelity DNA polymerase (NEB; M0493) and the PCR product was *in vitro* transcribed using
879 the MEGAShortscript T7 kit (ThermoFisher Scientific; AM1354) and purified using the Zymo
880 RNA Clean & Concentrator columns (Zymo Research; R1017) The sgRNA and Cas9 mRNA
881 (TriLink Biotechnologies; L61256) and recombinant Cas9 protein (Toolgen; TGEN CP1)
882 were individually re-suspended in RNase-free water, aliquoted and stored at -80°C until use.
883 Prior to injection the ribonucleoprotein complex was prepared by centrifuging the Cas9

884 protein for 1 min at 14,000 RPM at 4°C and transferring the supernatant to a fresh tube
885 containing the sgRNA. This was incubated at 37°C for 15 min, pulse spun and transferred to a
886 fresh tube for microinjection.

887

888 **Mouse zygote collection**

889 Four to eight-week-old (C57BL6 x CBA) F1 female mice were super-ovulated using injection
890 of 5 IU of pregnant mare serum gonadotrophin (PMSG; Sigma-Aldrich). 48 h post PMSG, 5
891 IU of human chorionic gonadotrophin (HCG; Sigma-Aldrich) was administered.
892 Superovulated females were set up for mating with eight-week-old or older (C57BL6 x CBA)
893 F1 males. Mice were maintained on a 12 h light/dark cycle. Mouse zygotes were isolated in
894 Global total with HEPES (LifeGlobal; LGTH-100) under mineral oil (Origio; ART-4008-5P)
895 and cumulus cells were removed with hyaluronidase (Sigma-Aldrich; H4272). All animal
896 research was performed in compliance with the UK Home Office Licence Number 70/8560.

897

898 **Human embryo thaw**

899 Human zygotes were thawed using Quinn's Advantage thaw kit (Origio; ART-8016). Briefly,
900 upon thawing the embryos were transferred to 3 ml of 0.5% sucrose thawing medium and
901 incubated for 5 min at 37°C, followed by 3 ml of 0.2% sucrose thawing medium for 10 min at
902 37°C. The embryos were then washed through 7 drops of diluent solution prior to culture.
903 Human blastocysts were thawed using the Blast thaw kit (Origio; 10542010) following the
904 manufacturer's instruction.

905

906 **Human and mouse microinjection and culture**

907 Human and mouse embryo microinjections were performed in Global Total media with
908 HEPES under mineral oil on a heated stage with a holding pipet (Research Instruments) and a
909 Femtojet 4i microinjection manipulator (Eppendorf) set at approximately 40 injection pressure
910 and 20 constant pressure. Embryos were microinjected with a mixture of Cas9
911 mRNA+sgRNA or the ribonulceoprotein complex back-filled into microfilament glass
912 capillary injection needles (World Precision Instruments; TW100F-6) pulled using a pipet
913 puller (Suter; P-97 micropipette puller). The microinjection procedure took ~15 min to
914 complete.

915

916 Human or mouse embryos were cultured in drops of pre-equilibrated Global media
917 (LifeGlobal; LGGG-20) supplemented with 5 mg/mL protein supplement (LifeGlobal; LGPS-
918 605) and overlaid with mineral oil (Origio; ART-4008-5P). Pre-implantation embryos were
919 incubated at 37°C and 5.5% CO₂ in an EmbryoScope+ time-lapse incubator (Vitrolife) for
920 either 3 – 4 d (mouse) or 5-6 d (human).

921

922 **Evaluating potential off-target sites**

923 Putative off-targets were determined using the MIT CRISPR Design tool (crispr.mit.edu)
924 which indicated top scoring off-target sites. We evaluated sequences that had mismatches of
925 less than or equal to 3 nucleotides compared to the sgRNA2b sequence. As described
926 previously¹⁷ potential off-target sites were also identified by using the following parameters:
927 12 basepairs of the sgRNA seed sequence plus an NGG PAM sequence where (N was varied
928 to include all possible nucleotides) were searched against the reference human genome
929 (hg19).

930

931 **Genomic DNA extraction**

932 hESCs were lysed using proteinase K digestion (10 µg/ml in lysis buffer [100 mM Tris buffer
933 pH 8.5, 5mM EDTA, 0.2% SDS, 200 mM NaCl]) overnight at 37°C. gDNA was extracted
934 from the lysed cells using phenol:chloroform extraction followed by ethanol precipitation.

935

936 Genomic DNA from fixed embryos (human and mouse) was isolated using the alkaline lysis
937 method; 25 μ l of 50 mM NaOH was added to the sample and incubated at 95°C for 5 min.
938 Samples were neutralized by adding 2.5 μ l of 1M Tris-HCL pH 8.0.

939
940 The Illustra Single Cell GenomiPhi DNA Amplification Kit (GE Healthcare Life Sciences;
941 29108039) was used according to manufacturer's instructions to amplify gDNA from
942 unfixed mouse blastocysts. DNA was purified by adding 30 μ l of 20 mM EDTA, 5 μ l of 3 M
943 sodium acetate and 137 μ l ice cold ethanol. Tubes were mixed by inverting and centrifuged at
944 16,000 x g for 20 min. Supernatant was removed and DNA was washed in 100 μ l ice cold
945 70% ethanol by mixing and centrifuging for 5 min. DNA was resuspended by adding 20 μ l
946 H₂O and incubating for 20 min at 4°C before mixing by gentle pipetting.

947
948 Genomic DNA from single cells microdissected from human embryos was extracted using the
949 G+T-protocol and amplified using REPLI-g Single Cell Kit (Qiagen; 150343) according to
950 manufacturer's guidelines. In preparation for PCR amplification and MiSeq analysis the
951 WGA-DNA product was diluted 1:100 in nuclease-free water, and 2 μ l of this was used as the
952 template in a reaction containing 25 μ l Phusion High Fidelity PCR Master Mix (New England
953 Biolabs), 2.5 μ l 5 μ M forward primer, 2.5 μ l reverse primer and 18 μ l nuclease-free water.
954 Thermocycling settings used were as follows: 98 °C 30 sec, 35 cycles of 98 °C 10 sec, 58 °C
955 30 sec, 72 °C 30 sec, and a final extension of 72 °C for 5 min. PCR amplicons were analysed
956 by Sanger sequencing and indels were quantified by TIDE webtool³⁷.

957
958 On- and off-target sites were amplified using primers listed in Extended Data Fig. 10a using
959 the GC rich PCR system (Sigma-Aldrich; 12140306001). Primers were designed to generate
960 amplicons of approximately 250 bp centered around the predicted cute site so as to maximize
961 the detection of a variety of mutations and ensure that each amplicon was sequenced
962 continuously from the forward and reverse barcode. We excluded primers that had SNPs
963 within their sequence so as to prevent allelic drop out. For the time-course genotypic analysis
964 bulk cells were collected every 24 h and PCR products were amplified from the extraction
965 genomic DNA. These products were used to generate multiplexed libraries for targeted
966 amplicon sequencing by MiSeq according to the manufacturer's instructions (Illumina).

967
968 For gDNA amplified through the G+T-seq protocol, amplicons for genotyping were generated
969 using Phusion High Fidelity PCR Master Mix (New England Biolabs). MiSeq library
970 preparation, quantification, pooling and denaturation were performed according to the
971 manufacturer's instructions (Illumina). For low input samples amplicons were cleaned using
972 an equal volume of AMPure XP beads according to manufacturer's instructions (Beckman
973 Coulter) Index PCR was performed using 10 μ l of cleaned amplicon, 12.5 μ l Q5 high fidelity
974 2X Master Mix (NEB; M0492S), 1.25 μ l Nextera XT Index 1 primer, 1.25 μ l Nextera XT
975 Index 2 primer (Nextera XT Index kit; FC-131-1001). The thermocycling parameters used
976 were: 98°C for 30 sec, 35 cycles of 98°C for 10 sec, optimized annealing temperature for 30
977 sec, 72°C for 30 sec, and a final extension of 72°C for 2 min. Index PCR was cleaned using
978 equal volume of AMPure XP beads as described previously. Beads were rehydrated with 20
979 μ l nuclease-free water. 5 μ l of the index PCR product was run on a gel to identify any
980 samples with over-abundance of primer dimers, which were subsequently subjected to gel size
981 selection and extraction using QIAquick gel extraction kit (Qiagen; 28704). Index PCR
982 products were quantified using QuantiFluor dsDNA system (Promega; E2670). The
983 concentration was used to determine the dilution required to obtain a 5 μ M solution of each
984 sample. 5 μ l of each sample was pooled and the library was spiked with 20% PhiX genomic
985 control (Illumina; FC-110-3001). Sequencing generated paired-end (2 x 250 bp) dual indexed
986 reads. After sequencing, reads were demultiplexed and stored as FASTQ files for downstream

987 processing and analysis. The CRISPR Genome Analyser³⁸ or CRISPR Cas Analyser³⁹ tools
988 were used to align the reads and to determine the percentage of non-wild-type reads resulting
989 from editing, as well as assessing the position and size of each indel for all of the PCR
990 amplicons evaluated.

991

992 **Digenome sequencing**

993 Digenome-seq was performed as described previously^{15,16}. Briefly, 20 µg of genomic DNA
994 was incubated with pre-incubated 100 nM recombinant Cas9 protein and 300 nM sgRNA in a
995 reaction volume of 1 ml (100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl₂, 100 µg/ml BSA,
996 pH 7.9) at 37 °C for 8 h. Digested DNA was mixed with 50 µg/ml RNase A (Qiagen) at 37 °C
997 for 30 min, and purified again with a DNeasy Tissue Kit (Qiagen). 1 µg of digested DNA was
998 fragmented using the Covaris system and ligated with adaptors using TruSeq DNA libraries.
999 DNA libraries were subjected to whole genome sequencing was performed at Macrogen using
1000 an Illumina HiSeq X Ten at a sequencing depth of 30–40X. *In vitro* DNA cleavage scores
1001 were calculated using a scoring system described previously¹⁶.

1002

1003 **Immunohistochemistry**

1004 Embryos and cells were fixed with 4% paraformaldehyde in PBS respectively for 1 h and
1005 overnight at 4°C and immunofluorescently analysed as described previously². The primary
1006 antibodies used are listed in Extended Data Fig. 10b. Embryos were placed on coverslip
1007 dishes (MatTek) for confocal imaging.

1008

1009 **Cytogenetic analysis**

1010 To determine the chromosome copy number, single or multiple blastomeres were biopsied
1011 from embryos at the cleavage stage and clumps of approximately five cells were
1012 microdissected from blastocysts. The cells were washed through 3 drops of a wash buffer
1013 (PBS/0.1% polyvinyl alcohol), which had previously been tested to confirm absence of
1014 contaminating DNA (Reprogenetics UK). The cells were transferred to 0.2 ml PCR tubes in a
1015 volume of 1.5 µL, lysed and subjected to whole genome amplification (SurePlex, Rubicon)
1016 followed by low-pass next generation sequencing (coverage depth <0.1x) (VeriSeq PGS kit,
1017 Illumina). Libraries were prepared according to the manufacturer's instructions and sequenced
1018 using the MiSeq sequencing platform. Typically, ~1 million reads were generated per sample,
1019 of which 60-70% successfully mapped to unique genomic sites. Mapped reads were
1020 interpreted using BlueFuse Multi software (Illumina) in order to generate chromosome copy
1021 number profiles. This strategy has been extensively validated and is widely used for the
1022 detection of whole chromosome losses and gains, as well as segmental aneuploidy, in human
1023 embryos undergoing preimplantation genetic diagnosis (PGD)²⁶. Analysis of single
1024 blastomeres allowed each chromosomal region of at least 5 Mb to be assigned a copy number
1025 of 0, 1, 2, 3 or 4 (corresponding to nullisomy, monosomy, disomy, trisomy or tetrasomy). In
1026 trophoctoderm samples, composed of several cells, it was also possible to detect the presence
1027 of chromosomal mosaicism, indicated when copy number values for a given chromosome had
1028 an intermediate value, between the thresholds for assigning 1 and 2 or 2 and 3 chromosome
1029 copies⁴⁰.

1030

1031 **Imaging**

1032 Confocal immunofluorescence pictures were taken with a Leica SP5 confocal microscope and
1033 3 - 5 µm thick optical section were collected. Quantification was performed manually using
1034 Fiji (ImageJ) or automated using MINS 1.3 software⁴¹.

1035

1036 Epifluorescence images were performed on an Olympus IX73 using Cell[^]F software
1037 (Olympus Corporation) or on an EVOS FL cell imaging system (AMF4300). Phase contrast

1038 images and videos were performed on an Olympus IX73 using with Cell[^]F software and RI
1039 Viewer software (Research Instruments), respectively.

1040

1041 Time-lapse imaging was performed using an EmbryoScope+ time-lapse incubator (Vitrolife)
1042 and annotated using the EmbryoViewer software.

1043

1044 **Culture conditions for hESCs and engineering inducible cell lines**

1045 Clonal H9 hESCs (WiCell) (n = 2 or 3 per sgRNA) were cultured in feeder- and serum-free
1046 conditions either in mTeSR1 (Stem Cell Technologies) on growth factor reduced Matrigel-
1047 coated dishes (BD Biosciences) or as previously described⁴². Successfully targeted cells were
1048 selected using 0.25 µg/ml puromycin (Sigma-Aldrich) and 15 µg/ml geneticin (Insight
1049 biotechnology ltd.) for 3 d prior to induction. Tetracycline hydrochloride (Sigma-Aldrich;
1050 T7660) was used at 1 µg/ml to induce guide expression. hESCs underwent routine
1051 mycoplasma screening and karyotyping.

1052

1053 **Generation of optimized inducible knockout (OPTiKO) hESC lines**

1054 The sgRNA sequences were cloned into the pAAV-Puro_siKO-TO vector as previously
1055 described¹¹. Briefly, complementary single stranded oligonucleotides (Extended Data Fig.
1056 10a) were annealed and scarlessly ligated to AarI-digested plasmids between the H1-TO
1057 tetracycline-inducible promoters and the scaffold sgRNA sequence. The Cas9 and inducible
1058 sgRNA targeting vectors were each inserted into one of the two alleles of the *AAVS1* locus by
1059 homologous-directed recombination facilitated by two obligate heterodimer Zinc Finger
1060 Nucleases (ZFN)¹¹. Cells were cultured in the presence of 10 µM ROCK inhibitor Y-27632
1061 (Sigma-Aldrich; Y0503) in media without antibiotics 24 h prior to nucleofection. Cells were
1062 washed with PBS (Life Technologies; 14190-094) and dissociated with Accutase (Life
1063 Technologies; A11105-01) for 5 min at 37°C. Colonies were mechanically triturated into
1064 clumps of 2/3 cells and counted. 2x10⁶ cells were nucleofected in 100 µl with a total of 12 µg
1065 of DNA (4 µg each for the two ZFN plasmids, and 2 µg each for the two targeting vectors)
1066 using the Lonza P3 Primary Cell 4D-Nucleofector X Kit and the cycle CA-137 on a Lonza
1067 4D-Nucleofector System. Cells were incubated for 5 min at RT, after which antibiotic-free
1068 KSR containing 10 µM ROCK inhibitor was added. After another 5 min the cell suspension
1069 was distributed on pre-plated DR4 (Applied Stem Cell; ASF-1013) drug resistant MEF
1070 feeders in antibiotic-free KSR media. Four days post nucleofection, cells underwent double
1071 antibiotic selection with 0.5 µg/ml Puromycin (Sigma-Aldrich) and 25 µg/ml Geneticin (G418
1072 Sulfate (Gibco)) for 7 days. Targeted colonies appeared after 4-8 d and were mechanically
1073 picked and clonally expanded at 10-14 d after transfection.

1074

1075 Extensive genotyping was carried out on the targeted clones to check for correct *AAVS1* gene
1076 targeting and to exclude the presence of randomly integrated plasmids, as previously
1077 described¹¹. Briefly, genomic DNA was extracted using the Wizard Genomic DNA
1078 Purification Kit (Promega; A1120). Site-specific integration was checked for both 5' and
1079 3' ends of each of the two targeting vectors (Cas9 and inducible sgRNA). Clones were also
1080 screened for the absence of the WT locus (indicating homozygous targeting) and for the
1081 absence of amplicons for both the 5' and 3' ends of the targeting vector backbones to ensure
1082 there was no random integration of the plasmid).

1083

1084 **Flow cytometry**

1085 Cells were collected every day for 5 d alongside matched control cells. Cells were dissociated
1086 into single cell suspension using TrypLE Select 1X (Gibco; 12563011) for 5 min at 37°C. The
1087 cell suspension was pelleted, washed with PBS (Life Technologies; 14190-094) then fixed
1088 and permeabilized using BD Cytotfix/Cytoperm (554714) for 20 min at 4°C. Perm/wash buffer
1089 (diluted 1:10 in embryo transfer water) was used for all subsequent antibody and wash steps

1090 unless indicated otherwise. After fixation, cells were washed once then stored at 4°C until the
1091 d5 sample had been collected, at which point all samples underwent intracellular staining.
1092 Cells were blocked for 30 min at room temperature with perm/wash buffer containing 10%
1093 donkey serum (Bio-rad; C06SB) and 0.1% Triton X-100 (ThermoFisher Scientific; 85111).
1094 Cells were stained with primary antibodies by incubating at RT for 1 h and cells were washed
1095 three times following each incubation. Negative control secondary only stained cells and
1096 unstained cells were performed on each batch of cells at a given day. Flow cytometry was
1097 performed using a Cyan ADP flow cytometer and the Summit software (Beckman Coulter),
1098 and 10,000-50,000 events were recorded. Flow cytometry result analysis was performed using
1099 FlowJo. Cells were first gated based on forward and side scatter properties, after which
1100 singlets were isolated based on the relationship between side scatter area peak area and width.
1101 A secondary only negative control was used to determine the background and OCT4 positive
1102 cells were quantified relative to cells that were OCT4 negative in the total bulk population of
1103 cells analysed.

1104

1105 **RNA isolation from hESCs for RNA-seq and qRT-PCR**

1106 qRT-PCR data presented in Extended Data Fig. 1c was generated as follows: RNA was
1107 isolated using TRI reagent (Sigma) and DNase I-treated (Ambion). cDNA was synthesized
1108 using a Maxima first strand cDNA synthesis kit (Fermentas). qRT-PCR was performed using
1109 SensiMix SYBR low-ROX kit (Bioline) on a QuantStudio 5 machine (ThermoFisher
1110 Scientific). Primers pairs used are listed in the Extended Data Fig. 10a. Each sample was run
1111 in triplicate and samples were normalized using *GAPDH* as the housekeeping gene and the
1112 results were analysed using the $\Delta\Delta C_t$ method

1113

1114 In preparation for RNA-sequencing of the hESCs induced to express sgRNA2b, samples were
1115 further cleaned using ethanol precipitation. Libraries were prepared using KAPA mRNA
1116 HyperPrep kit for Illumina platforms (Roche Sequencing Solutions Inc.)

1117

1118 qRT-PCR data presented in Extended Data Fig. 2b was generated as follows: RNA was
1119 extracted using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich; RTN350-
1120 1KT) and the On-Column DNase I Digestion kit (Sigma-Aldrich; DNASE70-1SET). 500 ng
1121 of RNA was reverse transcribed with SuperScript II (Invitrogen; 18064071). qPCR was
1122 performed using 5 ng of cDNA and SensiMix SYBR low-ROX (Bioline; QT625-20). qRT-
1123 PCR was performed on a Stratagene Mx-3005P (Agilent Technologies) and the results were
1124 analysed using the $\Delta\Delta C_t$ method. Each sample was run in duplicate and samples were
1125 normalized using *RPLP0* as the housekeeping gene.

1126

1127 **G&T-seq**

1128 Samples were processed using a previously published protocol that was adapted where
1129 indicated²⁹. Single cells from microdissected human embryos were picked using 100 μ m inner
1130 diameter Stripper pipette (Origio) and transferred to individual low bind RNase-free tubes
1131 containing 2.5 μ l RLP plus buffer (Qiagen; 79216).

1132

1133 To separate RNA and genomic DNA (gDNA) 50 μ l of Dynabeads were washed and incubated
1134 with 100 μ M biotinylated poly-dT oligonucleotide (IDT). 10 μ l of oligo-dT beads were added
1135 to each tube containing the single cell. Samples were incubated in a thermomixer for 20 min
1136 at room temperature at 2000 rpm. Tubes were put on a magnet until the beads collected into a
1137 pellet and the supernatant went clear. The supernatant containing the gDNA was transferred to
1138 a new collection tube. Beads were washed three times to collect any residual gDNA.

1139

1140 cDNA was generated from the RNA captured on the bead using the SMARTer v4 Ultra Low
1141 Input kit (Clontech; 634891) as previously described³. Reverse transcription was performed

1142 on the thermomixer using the settings 2 min at 42°C at 2,000 rpm, 60 min at 42°C at 1,500
1143 rpm, 30 min at 50°C at 1,500 rpm and 10 min at 60°C at 1,500 rpm. cDNA was amplified by
1144 adding 12.5 µl 2X SeqAmp PCR buffer, 0.5 µl PCR Primer II A (12µM), 0.5 µl SeqAmp
1145 DNA polymerase, 1.5 µl Nuclease free water. Beads were mixed on thermomixer for 60 sec at
1146 room temperature at 2,000 rpm and then were incubated on a PCR machine using the
1147 following settings: 95°C for 1 min, 24 cycles of 98°C for 10 sec, 65°C for 30 sec and 68°C for
1148 3 min, before a final extension for 10 min at 72°C. Amplified cDNA was purified by adding
1149 25 µl Ampure XP beads according to manufacturer's instructions. 12 µl of purification buffer
1150 was added to rehydrate the pellet and incubated for 2 min at room temperature. cDNA was
1151 eluted by pipetting up and down 10 times before returning the tube to the magnet. The clear
1152 supernatant containing the cDNA was removed from the immobilised beads and transferred to
1153 a new low-bind tube. cDNA was stored at -80°C until library preparation. cDNA quality was
1154 assessed by High Sensitivity DNA assay on an Agilent 2100 Bioanalyser with good quality
1155 cDNA showing a broad peak from 300 to 9,000 bp. cDNA concentration was measured using
1156 QuBit dsDNA HS kit (Life Technologies).

1157
1158 In preparation for library generation cDNA was sheared using an E220 focused-ultrasonicator
1159 (Covaris) to achieve cDNA in 200-500 bp range. 10 µl of cDNA sample and 32 µl purification
1160 buffer was added to Covaris AFA Fiber Pre-Slit Snap Cap microTUBE. cDNA was sheared
1161 using the following settings: Peak Incident power 175 W, Duty Factor 10%, 200 cycles per
1162 burst, water level 5.

1163
1164 Libraries were prepared using Low Input Library Prep Kit v2 (Clontech; 634899) according to
1165 manufacturer's instructions. Dual indexing was performed by substituting the manufacturer's
1166 provided indexing adaptors with NEBNext Multiplex Oligos for Illumina Dual Index primers
1167 set 1 (NEB; E7600S). Library quality was assessed by Bioanalyser and the concentration was
1168 measured by high sensitivity QuBit assay.

1169
1170 25 µl of AMPure beads was added to each collection tube containing the gDNA. Tubes were
1171 mixed well and incubated at room temperature for 20 min so that the DNA could be bound to
1172 the beads. Tubes were put on the magnet until the supernatant ran clear so that it could be
1173 removed and discarded. The beads were washed twice with 100 µl 80% ethanol. Any
1174 remaining ethanol was removed and beads allowed to dry.

1175
1176 **Genotyping cells from human embryos**
1177 PCR amplification of the sgRNA2b on-target site was initially performed on all samples using
1178 a primer pair generating an amplicon size of 244 bp suitable for MiSeq analysis. Any samples
1179 which failed three times to amplify using this primer pair were subjected to amplification
1180 using alternative primer pairs listed in Extended Data Fig. 10a. Putative off-target sites were
1181 evaluated using the primer pairs listed in Extended Data Fig. 10a.

1182
1183 **Single-cell RNA-seq data analysis**
1184 RNA-Seq data of single cells were obtained as paired-end reads and analysis was performed
1185 blinded to the identity of the samples. The RNA-Seq data flow was managed by a GNU make
1186 pipeline. Transcript reads were aligned to the Ensembl GRCh37 genome using Tophat2
1187 (version 2.1.1 with option no coverage search)⁴³; alignment rates were typically between 60-
1188 80%. Transcript counts were computed using the featureCounts program (version 1.5.1)⁴⁴. A
1189 quality filter was applied to the matrix, ensuring >50000 total transcript reads per cell and >5
1190 reads in at least 5 samples. The raw transcript counts were corrected for read-count depth
1191 effects using the SCnorm package⁴⁵ single-group design matrix. The RUVSeq⁴⁶ (version
1192 1.10.0) (Risso et al. 2014) was used for between-sample normalisation by applying the
1193 'betweenLaneNormalization' function with 'full' quantile regression. For PCA analysis,

1194 transcript counts were transformed using a $\text{asinh}(x/2)$ transformation with per-gene centering
1195 to obtain near-Gaussian and zero-centred count distributions. The `prcomp` function of the `stats`
1196 package in R (version 3.4.1) was applied to the count matrix and single cells were projected
1197 into the plane of the first two eigenvectors.

1198
1199 Independently, sequenced reads from all single cell samples were also aligned to the human
1200 reference genome sequence GRCh38 using TopHat2 (version 2.1.1)⁴³ and parameters were
1201 optimised for 100bp paired-end reads. Read counts per gene were calculated using the python
1202 package HTSeq (version 0.6.1)⁴⁷ and differential gene expression analysis was carried out
1203 using DESeq2 (version 1.10.1)⁴⁸. Read counts were normalised using the RPKM method⁴⁹
1204 and hierarchical clustering of samples was performed to generate a heat map using the R
1205 package `heatmap` (version 1.0.8). A previously published reference control dataset³ was
1206 integrated in the heat map and hierarchical clustering. Principal components analysis was
1207 performed using the `stats` (version 3.2.2) R package on a previously published scRNA-seq
1208 dataset covering different stages of preimplantation development³⁰ together with own OCT4-
1209 targeted samples and controls.

1210
1211 **Data availability**
1212 Source Data are provided for figures. RNA-seq and MiSeq data has been deposited into Gene
1213 Expression Omnibus under accession numbers (GSE100120). Scripts used for bioinformatics
1214 analysis can be found on the following GitHub page: [https://github.com/Genalico/RNAseq-](https://github.com/Genalico/RNAseq-BlaCy_pub)
1215 `BlaCy_pub`. Any additional information is available upon request from the corresponding
1216 author.

- 1217
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