

1 **Classification:** Biological Sciences; Developmental Biology

2 **Title:** Essential roles of Hdac1 and 2 in lineage development and genome-wide DNA

3 methylation during mouse preimplantation development

4 **Running title:** Overlapping roles of Hdac1 and 2 in preimplantation embryos

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16 **Keywords:** Preimplantation, Hdac1, Hdac2, Trophectoderm, Pluripotency, DNA methylation

17 **Abstract:** Epigenetic modifications, including DNA methylation and histone modifications,

18 are reprogrammed considerably following fertilization during mammalian early embryonic

19 development. Incomplete epigenetic reprogramming is a major factor leading to poor

20 developmental outcome in embryos generated by assisted reproductive technologies, such as

21 somatic cell nuclear transfer. However, the role of histone modifications in preimplantation

22 development is poorly understood. Here, we show that co-knockdown (cKD) of *Hdac1* and 2

23 (but not individually) resulted in developmental failure during the morula to blastocyst

24 transition. This outcome was also confirmed with the use of small-molecule Hdac1/2-specific

25 inhibitor FK228. We observed reduced cell proliferation and increased incidence of apoptosis

26 in cKD embryos, which were likely caused by increased acetylation of Trp53. Importantly, both

27 RNA-seq and immunostaining analysis revealed a failure of lineage specification to generate

28 trophectoderm and pluripotent cells. Among many gene expression changes, a substantial

29 decrease of *Cdx2* may be partly accounted for by the aberrant Hippo pathway occurring in cKD

30 embryos. In addition, we observed an increase in global DNA methylation, consistent with

31 increased DNA methyltransferases and Uhrf1. Interestingly, deficiency of Rbbp4 and 7 (both
32 are core components of several Hdac1/2-containing epigenetic complexes) results in similar
33 phenotypes as those of cKD embryos. Overall, Hdac1 and 2 play redundant functions required
34 for lineage specification, cell viability and accurate global DNA methylation, each contributing
35 to critical developmental programs safeguarding a successful preimplantation development.

36

37 **Significance**

38 Substantial changes to epigenetic modifications occur during preimplantation development and
39 can be detrimental when reprogrammed incompletely. However, little is known about the role
40 of histone modifications in early development. Co-knockdown of Hdac1 and 2, but not
41 individually, resulted in developmental arrest during morula to blastocyst transition, which was
42 accompanied by reduced cell number per embryo and increased incidence of apoptosis.
43 Additionally, we observed a failure of first lineage specification to generate trophectoderm and
44 pluripotent cells, which were associated with reduced expression of key lineage-specific genes
45 and aberrant Hippo pathway. Moreover, an increase in global DNA methylation was found with
46 upregulated Dnmts and Uhrf1. Thus, Hdac1 and 2 play overlapping roles in lineage
47 development, apoptosis, and global methylation during preimplantation development.

48

49 **Introduction**

50 A distinguishing feature of preimplantation development is a remarkable reprogramming of the
51 epigenome, including DNA modifications and post-translational histone modifications (1, 2).
52 Aberrant epigenetic reprogramming has been associated with defects in various biological
53 processes, including DNA replication and embryonic genome activation (EGA), which
54 eventually leads to early embryonic death (3). Moreover, incomplete epigenetic
55 reprogramming is a major contributing factor to the poor developmental outcome associated
56 with the use of assisted reproductive technologies, including in vitro embryo production (IVP)
57 (4) and somatic cell nuclear transfer (SCNT) (5-10). Indeed, modulation of certain epigenetic
58 modifications has been proved a viable tool to enhance SCNT rate and obtain live cloned
59 monkeys (11). However, little is known about the epigenetic regulation of critical
60 developmental events (e.g. lineage development) and interactions between epigenetic

61 modifications in preimplantation embryos.

62 Histone deacetylase (Hdac) 1 and 2 are highly homologous enzymes present together in

63 multiprotein complexes, the most extensively characterized being NuRD (12), Sin3 (13), and

64 CoREST (14), which are conserved ranging from yeast to human (15, 16). Histone acetylation

65 is well known for its role in transcriptional activation through opening of chromatin and

66 nucleosome compaction (17). Accordingly, Hdac1/2-containing complexes are traditionally

67 thought to act as transcriptional corepressors of target genes. However, Hdac1/2-containing

68 complexes have also been shown to be tethered to actively transcribed genes, suggesting a

69 critical role in transcriptional activation in certain situations (15, 16, 18, 19).

70 Because of high homology and physical colocalization in large multiprotein complexes, it is

71 reasonable that Hdac1 and Hdac2 are functionally redundant in multiple biological systems

72 (20-23). However, specific roles of Hdac1 and 2 have also been documented. For instances,

73 *Hdac2* is specifically involved in the regulation of memory formation and synaptic plasticity

74 (24). In contrast, knockout of *Hdac1* results in early lethality at peri-implantation stage (25).

75 Furthermore, knockdown of both maternal and zygotic *Hdac1* or *Hdac2* by siRNA injection in

76 preimplantation embryos results in no difference on blastocyst formation (26). These relatively

77 mild phenotypes in preimplantation embryos may be caused by the functional redundancy of

78 *Hdac1* and 2. Therefore, the precise role of Hdac1 and 2 and the underlying molecular

79 mechanisms during preimplantation embryogenesis remain unresolved.

80 In this study, we show that double knockdown of *Hdac1* and 2, but not individually, resulted

81 in lethality during the morula to blastocyst transition. The developmental failure is

82 accompanied by a substantial perturbation of the transcriptomes and lineage development in

83 conjunction with increased incidence of apoptosis, enhanced histone acetylation and genome-

84 wide DNA methylation. We propose that Hdac1 and 2 play compensatory and essential roles

85 during preimplantation development, at least partly through modulation of lineage

86 specification, apoptosis and global DNA methylation.

87 **Results**

88 **Double knockdown of *Hdac1* and 2 results in developmental arrest during morula to**

89 **blastocyst transition.**

90 Previous RNA-seq (GSE44183, Fig. S1A) and quantitative PCR analysis revealed extensive

91 expression of *Hdac1* and 2 through preimplantation development (27, 28). As anticipated, we
92 confirmed *Hdac1* and 2 concentrated and co-localized in nucleoplasm of blastomeres from 2-
93 cell to blastocyst stage (Fig. S1B). Moreover, both proteins appear evenly distributed in
94 trophectoderm cells (TE) and inner cells mass (ICM) in the mouse blastocyst (Fig. S1B).
95 Overall, these results imply *Hdac1* and 2 may play an overlapping role during preimplantation
96 development.

97 Both *Hdac1* and 2 are maternally derived prior to the occurrence of the major wave of EGA at
98 2-cell in mice (22, 28). Conditional double knockout of *Hdac1* and 2 in oocytes results in
99 oogenesis failure (22), prohibiting us from establishing knockout models of both maternal and
100 zygotic *Hdac1* and 2. We therefore decided to employ two complementary approaches to
101 investigate the *in vivo* roles for *Hdac1* and 2 in preimplantation development: RNAi and
102 *Hdac1/2*-specific small-molecule inhibitor, FK228 (29) (Fig. 1A).

103 The effectiveness of the siRNAs and the inhibitor was verified. Analysis of qPCR revealed
104 *Hdac1* mRNA level was depleted by approximately 90% from 8-cell to blastocyst stage (n=3,
105 P<0.05) after microinjection of *Hdac1* siRNA cocktail (H1 KD) relative to control embryos
106 injected with nonspecific siRNA (NC, Fig. S2A). In accordance, *Hdac1* protein abundance was
107 also depleted (n=3, Fig. S2B). *Hdac2* protein abundance was not affected by H1 KD, suggesting
108 a robust specificity of the siRNA (Fig. S2B). Similarly, the *Hdac2* siRNA cocktail (H2 KD)
109 produced a 90% knockdown at the mRNA level (n=3, P<0.05; Fig. S2A and 2B). Co-
110 microinjection of siRNAs targeting *Hdac1* and 2 (cKD) resulted in dramatic decreases in both
111 endogenous *Hdac1* (above 90% reduction) and *Hdac2* (above 89% reduction) between the 8-
112 cell to blastocyst stage (n=3, P<0.05; Fig. 1B). Immunoblotting (n=2) and IF (n=3) analysis
113 confirmed a successful reduction of the amount of *Hdac1* and 2 protein in morula and
114 blastocysts (Fig. 1C and 1D). Because of *Hdac1* and 2's critical roles in histone de-acetylation,
115 IF was performed to determine if histone acetylation was affected. The amount of histone H3
116 lysine 14 acetylation (H3K14ac) and H4K5ac was increased by 72.9% and 64.4%, respectively,
117 in cKD embryos relative to controls (n=3, P<0.05; Fig. S3A and 3B). Similarly, treatment of
118 mouse morula with FK228 increased both H3K14ac (by 116%) and H4K5ac (by 67.0%)
119 relative to the vehicle control (DMSO; n=3, P<0.05; Fig. S3D and 3E). Taken together, these
120 results suggest the siRNAs and inhibitor are highly effective in the context of preimplantation

121 development.

122 We next monitored the developmental potential of embryos of NC, H1 KD, H2 KD, and cKD.

123 No morphological difference was observed in H1 KD and H2 KD groups compared with NC
124 throughout preimplantation development (Fig. S4A), consistent with a previous study (26).

125 Both the blastocyst rate (above 80%, Fig. S4B) and total cell number per embryo (Fig. S4C)
126 were normal in the H1 KD and H2 KD groups. By contrast, the cKD embryos appeared normal

127 up to the morula stage (Fig. 1E) but more than half of cKD embryos fail to develop into
128 blastocysts (n=5, P<0.05; Fig. 1F). Cell counting analysis revealed that total cell number per

129 embryo declined from D3 (D3: 15.6±1.1 vs 9.9±0.9; D4: 37.2±2.8 vs 23.8±2.1; P<0.05; Fig.
130 1G). To evaluate if the development of cKD embryos was delayed, the embryos were

131 continually cultured *in vitro* until D5. At D5, the majority of cKD embryos collapsed whereas
132 NC blastocysts completed hatching, ruling out the possibility of developmental delay (Fig. 1E).

133 To test the developmental competency of the blastocysts that do develop in cKD groups,
134 blastocysts were cultured individually to examine if outgrowths could be formed. Consistent

135 with our previous report (30), the potential to form embryo outgrowth is compromised in H1
136 KD group, but not H2 KD group (Fig. S4D). In contrast, none of cKD blastocysts are capable

137 to form outgrowths (0/24 vs 17/20, n=3), even after zona pellucida removal (Fig. 1E). To test
138 if the *in vivo* environment could alleviate the phenotype, 2-cell embryos of NC and cKD groups

139 were transferred into surrogates. Embryo transfer analysis revealed that cKD embryos failed to
140 generate live offspring whereas 22-50% embryos transferred in NC group developed to term
141 (n=3, Fig. 1H). In sum, these results suggest *Hdac1* and 2 play a compensatory role in

142 supporting preimplantation development.

143 Three experiments were performed to verify the initial siRNA findings. First, an alternative
144 cocktail of siRNAs targeting 5' and 3' untranslated regions (5' and 3' UTR) of *Hdac1* and 2

145 was used. Developmental arrest during morula to blastocyst transition was also observed with
146 reduced blastocyst rate (23.6% vs 95.0% in NC; n=3, P<0.05; Fig. 1I and 1J). Second, the

147 development of cKD embryos could be rescued (blastocyst rate>70%) by co-injection of
148 exogenous *Hdac1* and/or *Hdac2* mRNA transcribed *in vitro* that were not targeted by the
149 siRNAs (n=3, P<0.05; Fig. 1I, 1J, S4E, and S4F). Last, treatment of mouse morula with FK228

150 also resulted in reduced blastocyst rate (24.3% vs 77.9% in control group, n=3, P<0.05) and

151 total cell number per embryo (34 vs 21, n=3; Fig. 1K). Overall, a combination of loss of
152 function approaches (RNAi plus small-molecule inhibitor) and rescue experiments confirm the
153 specificity of our approach and the essential role of Hdac1 and 2 in preimplantation
154 development.

155 **Effect of cKD on transcriptomic profile of preimplantation embryo**

156 To delineate the molecular basis of the developmental arrest of cKD embryos, we carried out
157 RNA-seq in NC and cKD morulae, obtained prior to the emergence of morphological
158 phenotypes (to avoid bias, Fig. 2A). Hierarchical clustering revealed a separation between NC
159 and cKD morulae (n=3; Fig. S5A). We found that 991 genes were differentially expressed (Fold
160 changes (FC) >2 or <0.5, P adjusted<0.05; Table S1), 72% of which were upregulated,
161 consistent with the notable role of Hdac1/2 as transcriptional repressors. Expression of select
162 genes (Down: *Myc*, *Dab2*, *Amot*, *Fgfr2*, and *Otx2*; Up: *Arid3a* and *Sfmbt2*; No change: *Tet1*
163 and *Ctnnb1*) was confirmed by qPCR analysis (Fig. 2B).

164 Gene ontology (GO) analysis revealed that the top GO terms (biological processes) enriched
165 in differentially expressed genes (DEGs; FC>2 or <0.5) include processes involved in DNA
166 transcription, cell differentiation, cell proliferation and apoptosis (Fig. S5B). Specifically, GO
167 analysis of downregulated genes (FC<0.5) showed that enriched GO terms include
168 transcription factor activity, cell proliferation, apoptosis, NAD dependent Hdac acitivity and
169 NuRD complex (Fig. 2C). Moreover, KEGG analyses revealed top hits in signaling pathways
170 regulating pluripotency, MAPK, P53 and Hippo signaling (Fig. 2E).

171 Among the downregulated genes, we observed an over-representation of TE specific genes
172 (*Cdx2*, *Dab2*, *Fgfr2*), genes associated with Hippo signaling (*Tead4*, *Amot*, *Lats2*) and genes
173 related with pluripotency networks (*Nanog*, *Klf5*, *Sox2*, *Pou5f1*, *Myc*) (Fig. 2C). Among the
174 upregulated genes, we observed an enrichment of genes related to cell cycle progression and
175 apoptosis (*Trp53*, *Ccnd1*, *Ccnd3*, *Cdkn1b*, *Cdkn1c*, *Cdkn2a*) and genes related to chromatin
176 modification, including *Dnmt1* and *Uhrf1* (Fig. 2C). Taken together, the transcriptome profiling
177 implies that embryos lacking Hdac1 and 2 do not properly initiate early lineage differentiation,
178 cell proliferation, and genome-wide methylation in preimplantation embryos.

179 **Hdac1 and 2 reduction leads to increased apoptosis, increased Trp53 acetylation and
180 defective proliferation in preimplantation embryos**

181 Because total cell number per embryo was drastically reduced in cKD (Fig. 1G) and RNA-seq
182 analysis in cKD embryos identified genes related with apoptosis among the DEGs (Fig. 2D),
183 we performed assays to test if apoptosis was abnormal in cKD embryos. The incidence of
184 apoptosis was markedly increased in cKD blastocysts (90%, n=10) relative to controls (10%,
185 n=10) (Fig. 3A).

186 Trp53 is a critical molecule regulating apoptosis and was also upregulated in cKD morulae as
187 determined by RNA-seq. In addition, Trp53 activity has been shown to be repressed in an
188 Hdac1-dependent manner through de-acetylation (31). Our results showed that the amount of
189 Trp53 acetylation at lysine 379 (p53ac) was greater in cKD (Fig. 3B) or FK228-treated embryos
190 relative to controls (Fig. S6A). To ascertain if Hdac1's deacetylase activity is directly
191 responsible for Trp53 acetylation in the context of preimplantation development, we performed
192 mutagenesis at the deacetylase site of *Hdac1* and injected wild-type *Hdac1* (H1 WT) and
193 mutant *Hdac1* (H1 MUT) mRNA into zygotes (Fig. 3C). No difference was observed in H1
194 WT-injected embryos relative to uninjected controls (UN; Fig. 3C and 3D). In contrast, there
195 is a dramatic increase of p53ac in H1 MUT-injected embryos (Fig. 3D). Overall, these results
196 indicate Hdac1's enzymatic activity is directly responsible for deacetylation of the non-histone
197 protein, Trp53, during preimplantation stages.

198 To ascertain if cell proliferation was affected by cKD, we performed IF against histone H3
199 serine 10 phosphorylation (pH3S10), a marker for late G2/M phase. Only 16.2% of blastomeres
200 in control morulae were subject to mitosis whereas the incidence of pH3S10 positive
201 blastomeres was increased significantly in cKD embryos (27.7%; Fig. 3E), suggesting a cell
202 cycle block at G2/M phase.

203 Interphase bridges have recently been identified as a critical subcellular structure for mouse
204 preimplantation embryos (32). As anticipated, interphase bridges were detected at cell-cell
205 junctions in control embryos (Fig. S6B arrows). Number of interphase bridges in H1 KD or H2
206 KD embryos is comparable or increased relative to controls but was reduced in cKD embryos
207 (Fig. S6B), suggesting an aberrant cellular communication in the absence of Hdac1/2.

208 **Double knockdown of Hdac1 and 2 results in failed lineage specification of trophectoderm
209 and inner cell mass**

210 Transcriptome profiling revealed substantial enrichment of TE-specific and pluripotency

211 network genes among DEGs (Fig. 2C). The earliest lineage specification takes place during the
212 morula to blastocyst transition and generate TE (precursors of the majority of placental cells)
213 and ICM (precursors of the embryo proper), we thus decided to examine the cell differentiation
214 program in cKD embryos. We quantified the expression of Cdx2, a critical molecular marker
215 of TE. Abundance of *Cdx2* mRNA was unchanged through blastocyst stage in H2 KD embryos
216 and downregulated slightly in H1 KD morulae and blastocysts (Fig. S7A). IF results displayed
217 a normal distribution of Cdx2 signal in both H1 and H2 KD embryos (Fig. S7B). In contrast,
218 Cdx2 mRNA and protein were diminished in cKD embryos during the morula to blastocyst
219 transition (Fig. 4A-C), which was confirmed in FK228-treated embryos (Fig. 4D and 4E). The
220 expression of *Cdx2* in cKD embryos could be successfully rescued by injection of either *Hdac1*
221 or 2 mRNA (Fig. 4F and 4G). To further determine if reduced expression of Cdx2 was cell-
222 autonomous, we injected siRNAs into one of two blastomeres at 2-cell stage and *H2B-RFP*
223 was used as a lineage-tracing marker (Fig. 4H). Surprisingly, we found Cdx2 disappeared not
224 only in blastomeres derived from siRNA-injected but un-injected cells, suggesting Hdac1/2 is
225 involved in regulation of signaling molecules upstream of *Cdx2* expression (Fig. 4H).
226 We next examined if the molecular signature of ICM was disrupted in embryos lacking *Hdac1*
227 and 2. Expression of Oct4, Nanog and Sox2 at both mRNA and protein level was unchanged
228 in H1 or H2 KD embryos (Fig. S8A and 8B). However, mRNA level of *Oct4*, *Nanog* and *Sox2*
229 was reduced in cKD groups during the morula to blastocyst transition (Fig. 5A). Similarly,
230 FK228 treatment led to a decrease in mRNA abundance of *Oct4* and *Nanog* (Fig. S8C). IF
231 results indicated no significant change of Oct4 signal, however, Nanog and Sox2 levels were
232 dramatically decreased in cKD blastocysts (Fig. 5B-C and Fig. S8E), which was also confirmed
233 using FK228 (Fig. S8D). Collectively, these data demonstrate a failure of the first cell fate
234 decision that normally gives rise to TE and ICM cells.
235 The second lineage specification occurs in the late blastocysts when the ICM differentiates into
236 epiblast (Epi) and primitive endoderm (PrE). We examined Gata6, a marker of PrE, and Nanog,
237 a marker of Epi, to determine if the second lineage specification failed as well. Results showed
238 Gata6 and Nanog are mutually exclusively distributed in ICM in control blastocysts, however,
239 no Gata6 and Nanog positive cells were visible in cKD embryos (Fig. S8F), confirming a
240 failure of the earliest two lineage specification programs in mouse preimplantation embryos.

241 **Abnormal Hippo pathway in embryos deficient of both Hdac1 and 2**

242 Hippo pathway components were enriched in GO analysis of DEGs between cKD and control
243 morulae (Fig. 2E). Hippo pathway plays a critical role in defining TE specification program
244 during mouse preimplantation development (33). Starting from the morula stage, Tead4 and
245 Yap1 act as upstream regulators of Cdx2 and localize in the nucleus of TE cells (33). IF results
246 showed that no visible difference was detected in Tead4 and Yap1 in H1 or H2 KD embryos
247 (Fig S9A-C). However, *Tead4* mRNA was reduced in cKD from 8-cell to blastocyst stage while
248 *Yap1* mRNA was slightly reduced (Fig 6A). IF analysis revealed that the number of Tead4
249 positive blastomeres was reduced by 50% in cKD group (Fig 6B and 6C). Immunoblotting
250 analysis further confirmed that the protein abundance of Tead4 was diminished in cKD morulae
251 (Fig 6D). Additionally, the percent Yap positive cells declined by 75% in cKD groups relative
252 to controls (Fig 6E and 6F). Lats1 and Lats2 are upstream molecules that modulate the activity
253 of Yap (34). Our qPCR results documented that mRNA of both genes was reduced significantly
254 in cKD morulae (Fig S9A), suggesting their abnormal expression could account for defective
255 Hippo signaling that we observed in the absence of Hdac1/2 activity.

256 **Genome-wide DNA methylation was enhanced in blastocysts deficient of both Hdac1 and
257 2**

258 A wave of genome-wide DNA demethylation occurs after fertilization through preimplantation
259 development, the molecular mechanism of which remains unclear (1). Changes in the
260 expression of *Dnmt1* and *Uhrf1* were notable in our RNA-seq analysis given their central role
261 in DNA methylation (Fig 2E). Thus, we sought to determine the global DNA methylation by
262 examining 5-cytosine methylation (5mc) and 5-cytosine hydroxymethylation (5hmc), a newly
263 defined DNA modification. Amounts of both DNA modifications are increased in cKD, but not
264 in individual KD groups at blastocyst stage (n=3, P<0.05; Fig 7A), which was also seen in
265 FK228-treated embryos (n=3, Fig S10C). However, little effect was observed on histone H3
266 lysine 4 trimethylation (H3K4me3), a marker for transcriptional activation, and histone H3
267 lysine 9 dimethylation (H3K9me2), a marker for transcriptional repression (Fig S10A and
268 S10B).

269 Previous studies report Hdac1 physically interacts with DNA methyltransferases (Dnmts) and
270 regulates the stability of Dnmt1 (35-38). There are three Dnmts present in preimplantation

271 embryos: Dnmt1, 3a and 3b. Uhrf1 is a Dnmt1-interacting protein involved in the recruitment
272 of Dnmt1 to maintain DNA methylation (39). The amount of Uhrf1 was increased not only in
273 the nuclear but also in the cytoplasm in cKD blastocysts relative to control (n=3; Fig 7B). We
274 have not found Dnmt1 antibody available for IF. However, immunoblotting analysis revealed
275 an increase in Dnmt1 abundance when Hdac1/2 were inhibited (Fig S10E). In addition, Dnmt3a
276 and 3b were barely detected in control mouse blastocysts whereas their signal intensity was
277 significantly improved in cKD or FK228-treated embryos (Fig 7C, 7D and S10D). In summary,
278 we conclude Hdac1 and 2 are critical for maintaining global DNA methylation properly through
279 modulating the amount of Dnmts in preimplantation embryos.

280 **Double knockdown of Rbbp4 and 7 results in similar phenotypes as Hdac1/2 cKD
281 embryos**

282 Rbbp4 and 7 (also known as RbAp48 and 46) are two homologous chromatin-binding proteins
283 that interact with Hdac1/2 to form the core components of multiple transcriptional corepressors,
284 including Sin3a, NuRD, and CoREST (15)(Fig 8A). Both proteins have direct interactions with
285 histone tails and are potentially responsible for recruitment of Hdac1/2-containing complexes
286 to target sites. We next performed RNAi experiment to examine the functional consequences
287 after knocking down Rbbp4 and 7. Effectiveness of siRNAs targeting *Rbbp4* and 7 was verified
288 by IF analysis (Fig S11A and B). Analysis of embryogenesis *in vitro* showed that individual
289 knockdown of Rbbp4 or 7 has no effect on preimplantation development, however, co-
290 knockdown of Rbbp4 and 7 results in poor blastocyst rate and reduced total cell number per
291 embryo at D4 (Fig 8B and C). The phenotype similarity between Rbbp4/7 cKD and Hdac1/2
292 cKD embryos prompted us to determine if defects in lineage specification and genome-wide
293 methylation were also found. Both Cdx2 and Nanog were diminished in Rbbp4/7 cKD embryos
294 (Fig 8D and E). An increase in global 5mc but not 5hmc was found in Rbbp4/7 cKD groups
295 relative to controls (Fig 8F).

296 **DISCUSSION**

297 This report demonstrates that there is a functional redundancy for Hdac1 and Hdac2 in
298 supporting preimplantation development. Depletion of both Hdac1 and 2 results in embryonic
299 arrest during the morula to blastocyst transition with greatly disrupted transcriptome-wide
300 expression profiles. Importantly, we document defects in three critical molecular events. First,

301 Trp53 acetylation was induced and may contribute to increased apoptosis and cell cycle arrest.
302 Second, lineage specification that generates TE and ICM was dramatically perturbed with
303 defects including suppressed *Cdx2* expression and aberrant Hippo pathway. And third, a global
304 increase of DNA methylation. Taken together, the combination of these effects contributes to
305 the developmental failure of cKD embryos.

306 Double knockdown of *Hdac1* and 2 in mouse preimplantation embryos results in
307 developmental failure to pass blastocyst stage (Fig 1E). Previous studies and our present
308 results indicate that independent knockdown of *Hdac1* or *Hdac2* does not affect blastocyst
309 formation, suggesting a dispensable role during preimplantation development (22, 30).
310 However, the developmental failure of cKD embryos suggests the viability of *Hdac1* or *Hdac2*-
311 depleted embryos is due to functional redundancy of these closely related genes. In particular,
312 we found compensatory roles of *Hdac1* and *Hdac2* in regulation of lineage specification,
313 genome-wide methylation, and expression of critical genes, such as *Cdx2* and *Nanog*. Overall,
314 these two enzymes function redundantly during preimplantation development.

315 Transcriptome profiles were disturbed in embryos deficient of *Hdac1* and 2. *Hdac1/2* cannot
316 bind to DNA directly. However, they can be tethered to DNA by many distinct transcription
317 factors including YY1 (40), p130 (41), and Trp53 (42). Moreover, *Hdac1/2* are recruited to
318 DNA as components of multiprotein complexes, including Sin3a, NuRD, and the CoREST,
319 which are well known for their transcriptional repressor activity. These facts could be the
320 reason that the majority of DEGs are upregulated genes after double knockdown of *Hdac1* and
321 2.

322 Lysine acetylation occurs not only to histones but various non-histone proteins, such as
323 mitochondrial and cytosolic proteins (43). *Hdac1/2* could also act as a “eraser” of these non-
324 histone acetylation events (43). Trp53 is one of these non-histone proteins that is subject to
325 acetylation. Trp53 plays a central role in a variety of biological processes including cell cycle
326 arrest, DNA damage repair, apoptosis and metabolic changes (44). Our results clearly revealed
327 the direct role of *Hdac1* in acetylation of Trp53. Recently, Ma et al. also demonstrated that
328 double knockout of *Hdac1* and 2 leads to increased Trp53 acetylation. Overall, these results
329 suggest it is a conserved mechanism on the direct regulation of *Hdac1* and 2 on Trp53
330 acetylation (31).

331 Total cell counting and IF analysis suggest a cell cycle arrest in G2/M phase in cKD embryos.
332 Previous studies demonstrated Hdac1 and 2 are associated with cell cycle progression across
333 different cell types or tissues (20, 25, 45, 46). For instance, loss of Hdac1 and 2 in dividing
334 cells results in a cell cycle block at G1 phase, which is partly attributed to the rise of the CDK-
335 inhibitors, including p21 and p57 (21). However, we found no difference in p21 and p57
336 expression in cKD embryos, suggesting a different cell cycle block mechanism.
337 During the morula to blastocyst transition, the first lineage specification occurs with the
338 regulation of contractility and critical signaling pathways, including Hippo and Notch (33).
339 Core lineage-specific transcription factors, including *Cdx2* (TE-specific), and *Oct4* and *Nanog*
340 (ICM-specific), are initially stochastically expressed and are gradually confined to specific
341 lineages. However, it remains poorly understood how the expression of these factors
342 themselves is controlled. Deficiency of Hdac1 and 2 results in failure of both the TE and ICM
343 differentiation program. A dramatic reduction in blastocyst rate was observed and those cKD
344 blastocyst that do form fail to outgrow, suggesting a lacking of functional ICM and TE. At a
345 molecular level, expression of key marker genes, *Cdx2*, *Nanog* and *Oct4* were suppressed. In
346 particular, *Nanog* and *Cdx2* signal was barely seen in cKD embryos. Although the intensity of
347 *Oct4* was normal, its localization was not restricted to a subset of cells. These results
348 collectively suggest Hdac1 and 2 are master regulators of the first lineage specification. Both
349 RNA-seq and qPCR results suggest Hdac1 and 2 are involved the transcription of these key
350 lineage-specific genes (Fig 2C and 4A-E). Indeed, ChIP-seq analysis shows that Hdac1 is
351 enriched in active genes in ES and TS cells, such as TE-specific genes *Cdx2*, *Elf5* and *Eomes*
352 and pluripotency network genes *Oct4*, *Nanog* and *Sox2* (18). These results may warrant further
353 investigation through low input Chip-seq to determine if Hdac1 and Hdac2 colocalize at these
354 critical genes during preimplantation stages (47).
355 The Hippo signaling pathway plays a crucial role in the first lineage specification, in particular
356 for TE-specific program (33). Loss of Tead4 leads to lethality with a failure to generate
357 functional TE and triggers downregulation of *Cdx2* (48). As a central component of Hippo
358 pathway, Yap could switch between nucleus and cytoplasm, which is phosphorylation-
359 dependent. Yap acts as a transcriptional activator of Tead4 to induce TE-specific genes (34).
360 Both Tead4 and Yap are disrupted in cKD embryos. Lats1 and Lats2 are both upstream

361 regulators of Yap1 (34). Their expression was also reduced in cKD embryos. Interestingly,
362 RNA-seq results also displayed dysregulation of genes in the Hippo pathway (Fig 2E). Thus,
363 the downregulation of Cdx2 in cKD embryos may be partly due to aberrant Tead4 and Yap1
364 expression.

365 Our results suggest Hdac1 and 2 are critical for maintaining correct DNA methylation pattern
366 during preimplantation development. Genome-wide removal of DNA methylation (5mc)
367 occurs during preimplantation development, contrasting with stable DNA methylation pattern
368 in somatic cells. There are two types of DNA demethylation: passive DNA demethylation that
369 is DNA-replication-dependent and active demethylation that is achieved by enzymatically
370 driven reactions. Our results show that Hdac1 and 2 affect the global active demethylation
371 through regulating Dnmts and Uhrf1, a critical protein for recruiting Dnmt1 to specific DNA
372 sequences. Increased Dnmt3a, 3b, and Uhrf1 protein abundance could be explained by
373 increased transcripts in cKD embryos. However, we cannot rule out the possibility that loss of
374 Hdac1 and 2 may affect the stability of Dnmts in preimplantation embryos. In contrast, Ma et
375 al. found conditional knockout of Hdac1 and 2 in mouse oocytes resulted in a global decrease
376 in DNA methylation and particularly reduced nuclear associated Dnmt3a (38). The discrepancy
377 suggests a developmental context-dependent role of Hdac1/2 in regulation of Dnmts.

378 Functional analysis of Rbbp4 and 7 suggest these two proteins are critical components for
379 ensuring functionality of Hdac1/2-containing chromatin complexes. Hdac1/2 and Rbbp4/7 are
380 shared among several critical transcriptional corepressor, including Sin3a, NuRD and CoREST
381 (15). Rbbp4/7 interacts directly with histone tails (H3 and H4) and are promising candidates
382 for recruiting these epigenetic complexes. Interestingly, our results indicate Rbbp4 and 7 play
383 a redundant function essential for preimplantation development, similar with Hdac1/2 cKD.
384 We propose that Hdac1/2-Rbbp4/7-containing complexes are critically required for
385 preimplantation development. Indeed, our previous studies documented an essential role of
386 Suds3, a component of Sin3a complex, during preimplantation development with critical
387 functions in lineage specification (30).

388 In summary, we documented a compensatory role of Hdac1 and 2 during preimplantation
389 development. Hdac1 and 2 are essential for the regulation of cell cycle progression and
390 apoptosis, which is probably mediated through acetylation of Trp53. Additionally, Hdac1 and

391 2 are required for the first cell differentiation program with a critical role in controlling
392 expression of key TE and pluripotency-specific genes. In the context of chromatin regulation,
393 Hdac1/2 are involved in maintaining proper genome-wide DNA methylation with dramatic
394 effects on the protein abundance of Dnmts. Last but not least, deletion of Rbbp4 and 7, both
395 structural partners of Hdac1/2 in several epigenetic complexes, results in similar phenotypes
396 as Hdac1/2 cKD. Further understanding the mechanisms of epigenetic control during these
397 early key molecular events will help the development of tools to reduce early embryonic
398 lethality and improve the success of reproductive cloning in mammals.

399

400 MATERIALS AND METHODS

401 Ethics Statement

402 All experiments involving lab animals were conducted according to the guidelines for the care
403 and use of lab animals and approved by Zhejiang University.

404 Mouse embryo culture

405 Superovulation in B6D2F1 (C57BL/6 × DBA2, Charles River) female mice (8-10 weeks old)
406 was performed by injecting 10 IU PMSG (San-Sheng pharmaceutical Co. Ltd., Ningbo, China)
407 followed by 10 IU hCG (San-Sheng pharmaceutical Co. Ltd., Ningbo, China) 46-48 h later. At
408 20-22 h post-hCG treatment, zygotes were collected from B6D2 F1 female mice mated to
409 B6D2 F1 males. Hyaluronidase (Sigma, St Louis, MO, USA) was used to remove cumulus
410 cells. Zygotes were cultured in KSOM at 37°C/5% CO₂. For FK228 treatment experiment,
411 mouse morula were treated with Romidepsin (FK228, Depsipeptide, Selleck, 50 nM) for 12
412 hours. For embryo transfer experiment, 2-cell stage control or cKD embryos were transferred
413 into the oviduct of pseudo-pregnant female mice (ICR).

414 Outgrowth

415 For outgrowth formation experiment, individual blastocyst was collected on D4, removed of
416 zona pellucida or kept intact, and incubated in DMEM (Gibco) containing 10% FBS (Gibco)
417 on 48-well plates coated with 0.1% Gelatin (Gibco).

418 Microinjection:

419 siRNAs and mRNAs were microinjected into the cytoplasm of zygote using a Piezo-drill
420 (Eppendorf, Germany) and Eppendorf transferman micromanipulators. siRNA (20 μM;

421 GenePharma, Shanghai) and/or synthetic mRNA (500 ng/μl) were loaded into microinjection
422 pipette and constant flow was adjusted to allow successful microinjection. Approximately 10
423 pl of siRNA and/or mRNA was delivered into the cytoplasm of zygotes or 2 cell blastomere by
424 microinjection. Sense and antisense sequences of siRNAs used in the present study was listed
425 in Table S3.

426 ***In vitro* RNA synthesis**

427 Wildtype cDNA for *Hdac1*, *Hdac2* and *H2B-RFP* were cloned into T7-driven vectors. *Hdac1*
428 mutants (H141A) were constructed as described previously (31). All sequences were confirmed
429 by Sanger sequencing prior to use. To prepare mRNAs for microinjection, expression vectors
430 were linearized and then were *in vitro* transcribed, capped and poly(A) tailed using T7
431 mMESSAGE mMACHINE Ultra Kit (Life Technologies, Grand Island, NY, USA) based on
432 the manual. mRNA was recovered and purified by MEGAclear Kit (Life Technologies, Grand
433 Island, NY, USA) and the integrity validated by electrophoresis.

434 **TUNEL**

435 The embryos were washed in 0.1% PVP/PBS, fixed in 4% PFA for 10 min and permeabilized
436 in PBS containing 0.5% Triton X-100 and 0.1% sodium citrate for 30 min. Then, the samples
437 were incubated in a buffer solution of TDT 10X, CoCl₂, 2mM dATP, 0.5 units/μl terminal
438 deoxynucleotidyl transferase enzyme and 0.5 mM FITC-dUTP for 1 h at 37°C in humidity.
439 DNA was stained with DAPI. Samples were mounted onto slides and imaged with confocal
440 microscope system (Zeiss LSM780).

441 **Immunofluorescence**

442 Preimplantation embryos were fixed with 4% paraformaldehyde in PBS for 10 min at room
443 temperature, permeabilized with 0.5% Triton X-100 for 30 min, then blocked in 10% FBS/0.1%
444 Triton X-100/PBS for 1 h after 3 times washing in 0.1% Triton X-100 PBS, and incubated with
445 antibodies (Table S3) 1 h at room temperature or overnight at 4°C followed by incubation with
446 Alexa Flour secondary antibodies 488, 595 (Invitrogen) at 37°C for 1 h. DNA was stained with
447 DAPI and samples were mounted and observed with a Zeiss LSM780 confocal microscope
448 (Zeiss).

449 **Reverse transcription and real time PCR**

450 Total RNA from embryos was extracted using the Arcturus Picopure RNA isolation kit (Life

451 Technologies, Grand Island, NY, USA). cDNA synthesis was performed using a reverse
452 transcription system (Invitrogen). To quantify gene expression differences between KD and
453 control groups, real-time PCR was performed on a StepOneTM system using FastStart
454 Universal SYBR Green Master (Roche). *H2a* was used as an endogenous control.

455 **Western blotting**

456 Embryos were lysed on ice in RIPA lysis buffer (Beyotime) supplemented with 1 mM
457 phenylmethylsulfonyl fluoride (Beyotime). Equal numbers of embryos were used in each group.
458 Protein were separated by 8% SDS-PAGE and transferred to a polyvinylidene fluoride
459 membrane (Millipore). Then, membrane was blocked with 5% non-fat milk and incubated with
460 primary antibodies overnight at 4°C and secondary antibodies for 1.5 h at room temperature.
461 Signals were detected with WESTAR NOVA 2.0 (Cyanagen).

462 **RNA-seq and bioinformatic analysis**

463 At E2.75, embryos were collected from NC and cKD groups (60 embryos per sample, n=3).
464 Total RNA was isolated from embryos using Picopure RNA isolation kit (Life Technologies,
465 Grand Island, NY, USA) according to the manufacturer's instruction. Before RNA extraction,
466 2 × 10⁶ copies of RFP and GFP mRNA was added. mRNAs were separated with oligo(dT)25
467 beads, and was used to prepare sequencing libraries with NEB Next Ultra RNA Library Prep
468 Kit for Illumina (New England Biolabs). Briefly, mRNA was fragmented and reverse
469 transcribed. The cDNA library was subject to end repair, poly(A)-tailing, adaptor ligation, and
470 PCR amplification of 12–15 cycles for sequencing library construction. The library was
471 sequenced by Illumina Hiseq X Ten and RNA-seq reads were assigned directly to transcripts
472 and counted with Salmon (<https://combine-lab.github.io/salmon/>)(49, 50). Differential
473 expression analysis was performed by DESeq2 package with P adjusted <0.05 and fold
474 change >2 or <0.5). GO and KEGG analysis for enrichment of differentially expressed genes
475 was determined using the Database for Annotation, Visualization and Integrated Discovery
476 (DAVID).

477 **Statistical Analysis**

478 Differences between two groups were determined by two-tailed unpaired Student's t tests. All
479 experiments were repeated at least three times unless otherwise stated. For quantification of IF
480 results, nuclear areas were outlined based on DAPI signal and mean intensity measured using

481 NIH ImageJ. Signal intensities were normalized to control embryos. A value of $P < 0.05$ was
482 considered to be statistically significant. RNA-seq results were analyzed with R
483 (<http://www.r-project.org>). Results are stated as mean \pm S.E.M.

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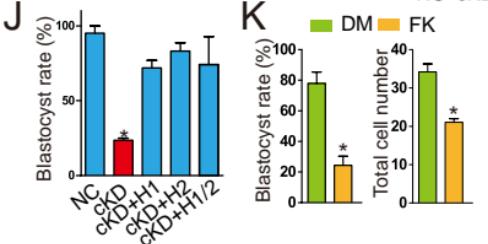
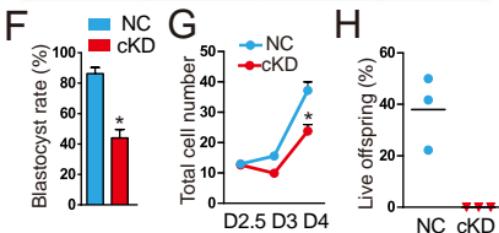
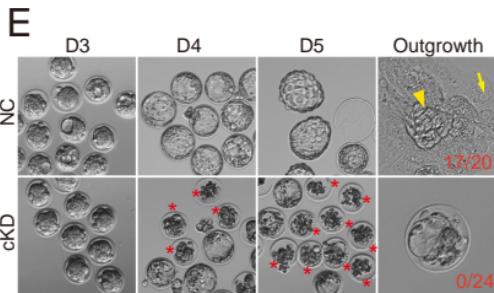
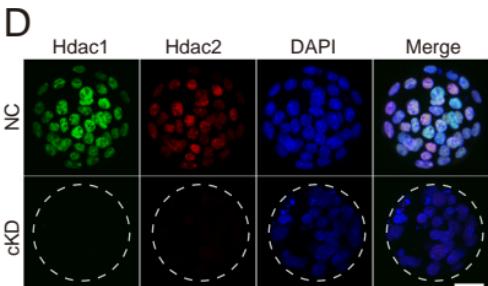
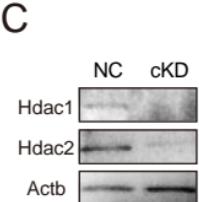
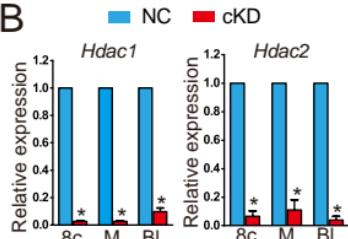
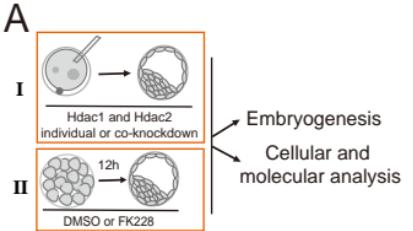
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602 **Fig. 1. Double knockdown of Hdac1 and 2 results in embryonic lethality during the**
603 **morula to blastocyst transition.** (A) Schematic overview of two approaches (I: RNAi; II:

604 small-molecule inhibitor, FK228) used to investigate *in vivo* roles of Hdac1 and 2 during

605 preimplantation development. (B) qPCR analysis of knockdown efficiency of siRNA

606 cocktails targeting Hdac1 and 2 from 8-cell to blastocyst stage. Mouse zygotes derived *in vivo*

607 were microinjected with Hdac1/2 siRNA cocktails (20 μ M, 10 pl, cKD) or negative control

608 siRNAs (NC). Embryos were collected at 8-cell (8c), morula (M) and blastocyst (Bl) stage

609 (n=3 pools of 5-10 embryos each per treatment). Data were stated as mean \pm SEM normalized

610 to endogenous control (*H2afz*; *P<0.05). (C) Immunoblot analysis of Hdac1 and 2 in NC and

611 cKD morulae (30 embryos per group, 2 replicates were performed with similar results). β -actin

612 (Actb) was used as a loading control. (D) Immunocytochemical detection of dramatic reduction

613 of Hdac1 and 2 protein in cKD blastocysts. Three replicates were conducted and at least 10

614 embryos analyzed in each group (Scale bar: 25 μ m). (E) Representative photos of NC and cKD

615 embryos from Day 3 after mating (D3) to D5. Arrow head: ICM outgrowth; Arrow: trophoblast

616 giant cell. Asterisk: Degenerated embryos. (F) Blastocyst rate in NC and cKD groups at D4

617 (n=5; 16-33 embryos per group per replicate). Data are shown as mean \pm SEM (*P<0.05). (G)

618 Cell counting analysis of NC and cKD embryos from D2.5 to D4 (n=3). (H) Percent live

619 offspring out of embryos transferred (n=3; 15-20 embryos were transferred per group). (I and

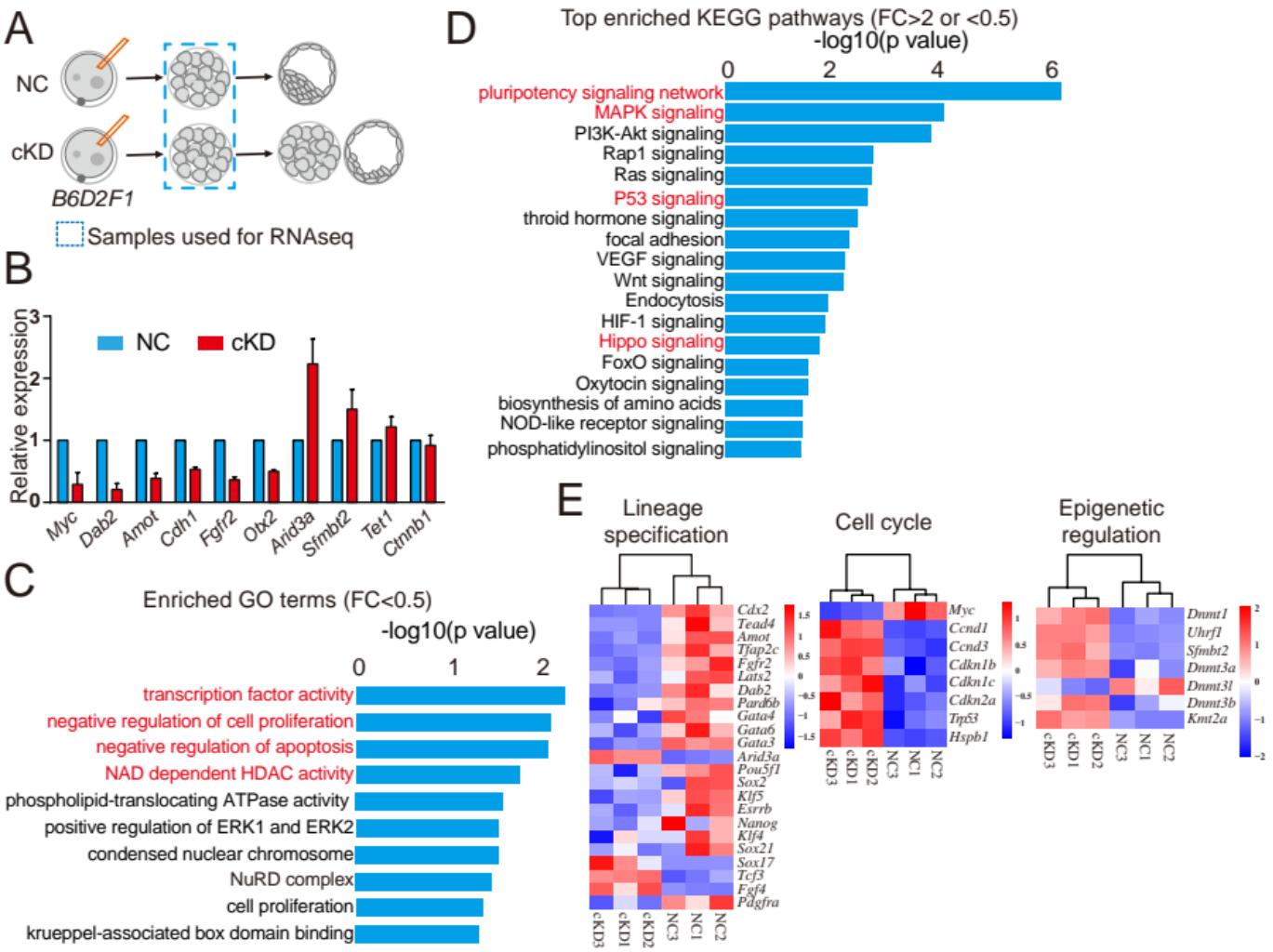
620 J) Rescue of cKD embryos by microinjection of exogenous *Hdac1* and/or *Hdac2* mRNA (n=3;

621 15-20 embryos per group; *P<0.05). (K) Blastocyst rate and total cell number per embryo in

622 embryos treated with Hdac1/2 specific inhibitor, FK228 (n=3; 15-20 embryos per group). Data

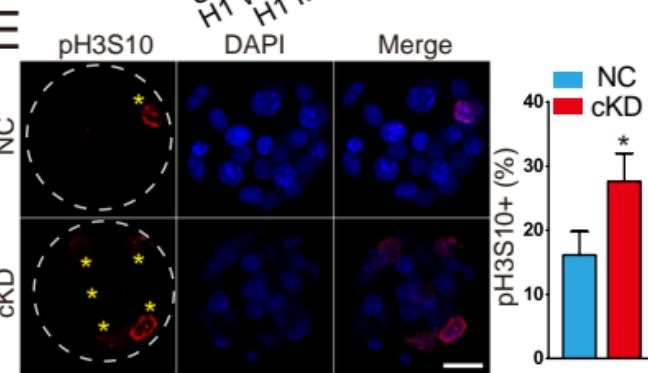
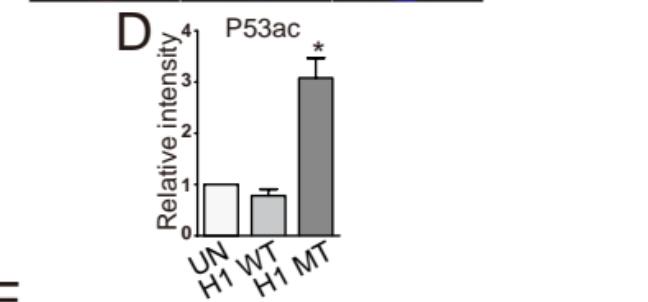
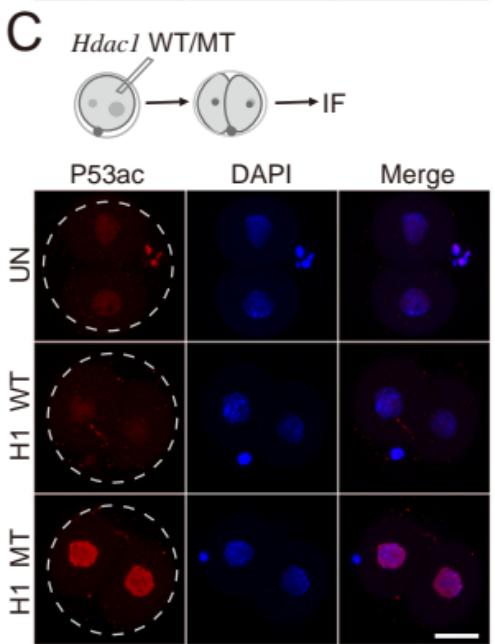
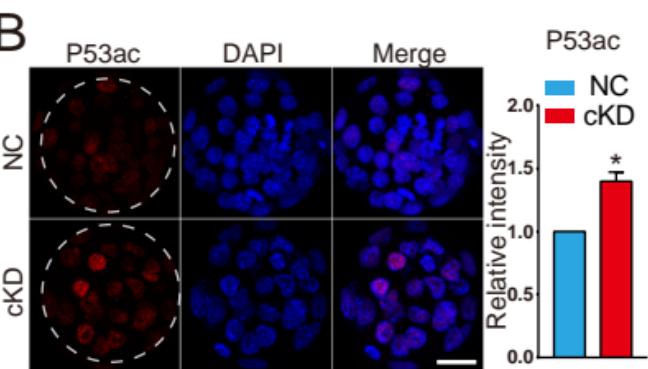
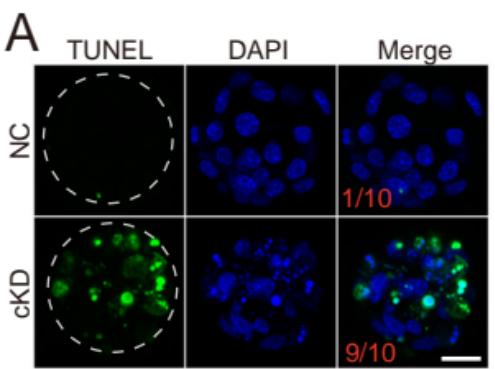
623 are expressed as mean \pm SEM. Different superscripts indicate significant differences (P < 0.05).

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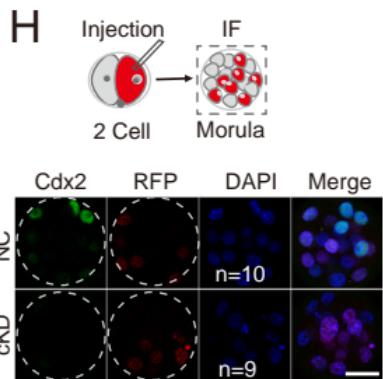
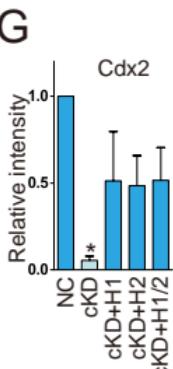
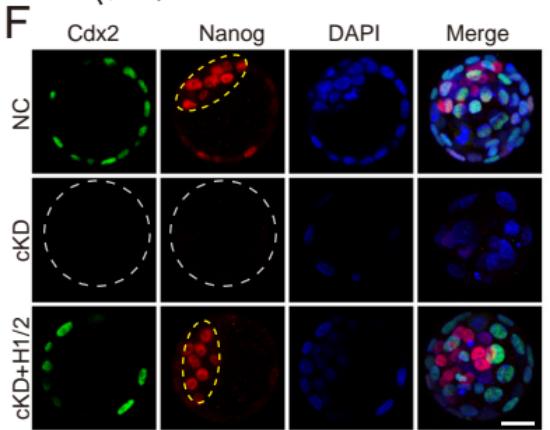
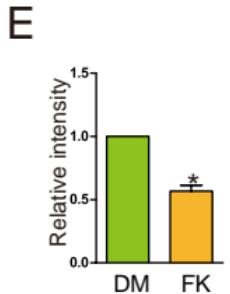
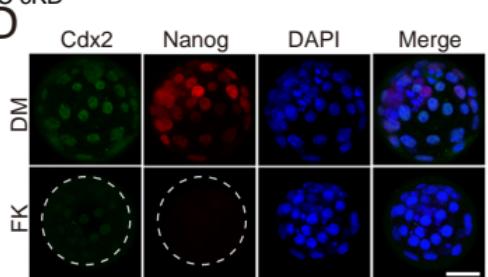
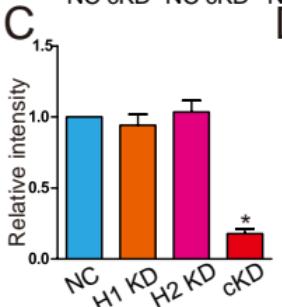
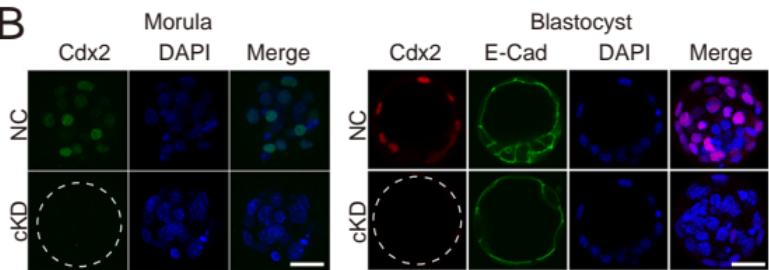
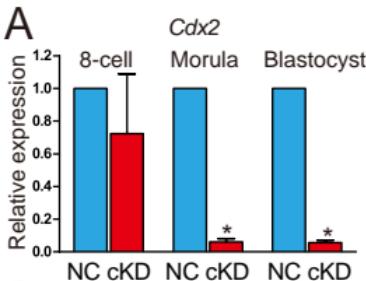
625 **Figure 2. RNA-seq analysis of embryos deficient of Hdac1 and 2.** (A) Schematic overview
626 of the samples collected for RNA-seq analysis (n=3; 60 embryos/group/replicate). (B)
627 Validation of RNA-seq results on expression levels of selected genes (Downregulated:*Myc*,
628 *Dab2*, *Amot*, *Cdh1*, *Fgfr2*, *Otx2*; Upregulated: *Arid3a*, *Sfmbt2*; No change: *Tet1*, *Ctnnb1*).
629 Three biological replicates were performed with 5-10 morula collected for each group
630 (*P<0.05). (C) GO analysis of downregulated genes in cKD morulae. The data indicate
631 enriched GO terms related to epigenetic regulation, cell proliferation and apoptosis. (D)
632 KEGG analysis of differentially expressed genes (DEGs) between NC and cKD morulae. The
633 data indicate cKD leads to abnormal signaling pathway of pluripotency network, P53 and
634 Hippo. (E) Overrepresentation of genes related to lineage specification, cell cycle and
635 epigenetic regulation among DEGs.

636



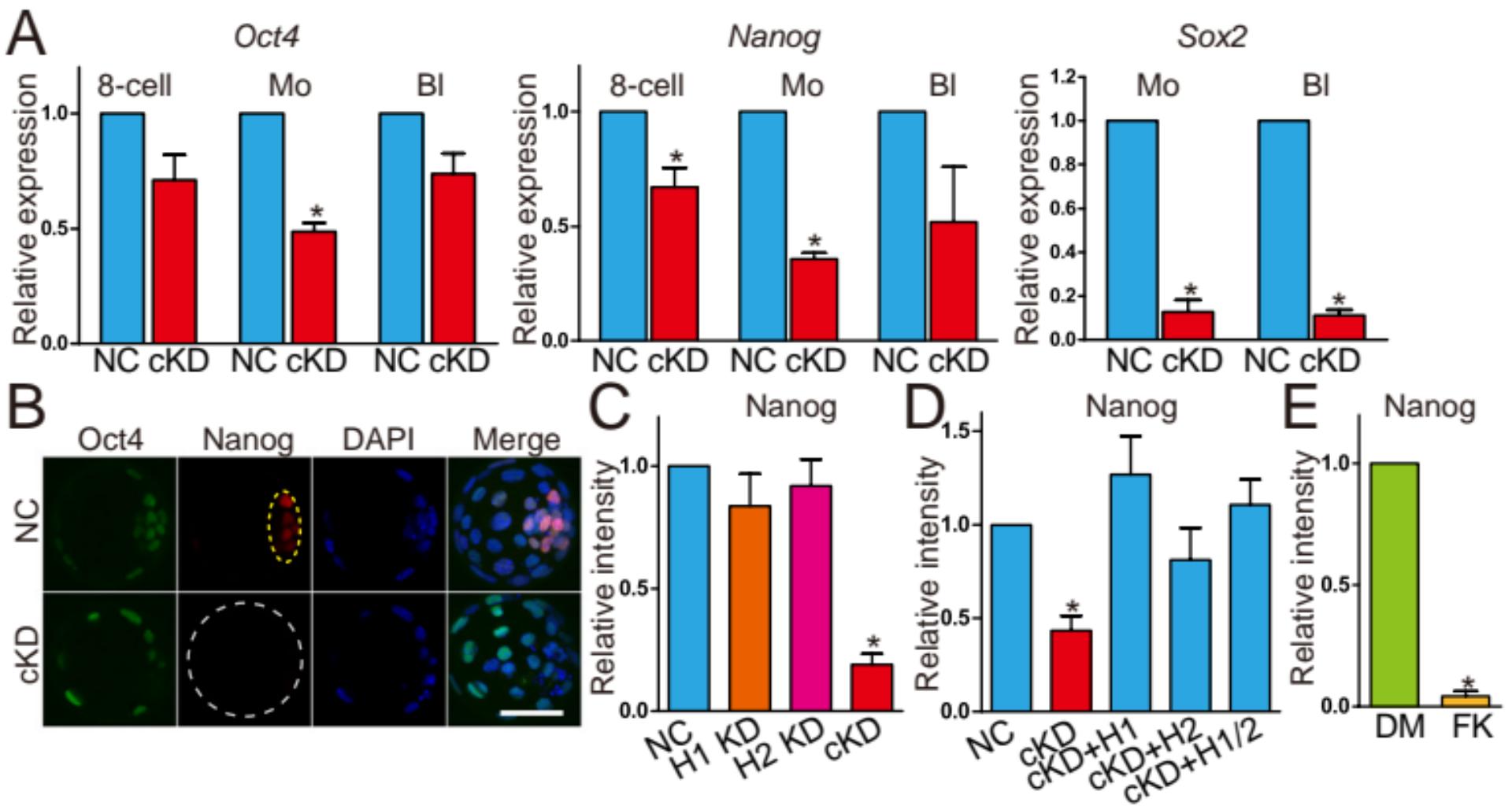
637 **Figure 3. Hdac1 and 2 deficiency leads to increased incidence of apoptosis, increased**
638 **Trp53 acetylation and cell proliferation arrest.** (A) TUNEL analysis of NC (n=10) and cKD
639 blastocysts (n=10). The data revealed a dramatic increase of incidence of apoptosis in cKD
640 blastocysts. Three biological replicates were conducted. (B) Immunocytochemical analysis of
641 Trp53 acetylated on K379 (P53ac) in blastocysts. The intensity of P53ac was improved
642 significantly (n=3; 5-10 embryos per group per replicate, Scale bar: 25 μ m). Nuclear was
643 counterstained with DAPI. (C) *Hdac1* was mutated at the deacetylase site and mRNA was *in*
644 *vitro* produced. Wildtype *Hdac1* (H1 WT) and mutant *Hdac1* (H1 MT) was introduced into
645 zygote and 2-cell embryos were collected for immunocytochemical analysis (n=3; 5-10
646 embryos per group per replicate, Scale bar: 25 μ m). (D) The intensity of P53ac was not changed
647 in H1 WT embryos but increased in H1 MT embryos (*P<0.05). (E) Immunocytochemical
648 examination of histone H3 serine 10 phosphorylation (pH3S10), a marker for late G2 and
649 mitosis, in blastocysts (n=3; 5-10 embryos per group per replicate; Asterisk: pH3S10 positive
650 blastomere; Scale bar: 25 μ m).

651



652 **Figure 4. Cdx2 was inactivated in embryos deficient of Hdac1 and 2.** (A) qPCR analysis of
653 *Cdx2* in 8-cell embryos, morula and blastocysts (n=3 pools of 5-10 embryos each per group).
654 (B-E) Immunocytochemical analysis of Cdx2 in morula and blastocysts after RNAi (B and C)
655 or FK228 treatment (D and E). Three biological replicates with 5-10 embryos analyzed per
656 group each time. The intensity of Cdx2 was diminished in cKD and FK228 treated , but not H1
657 or H2 KD embryos (C and E). E-Cad: E-Cadherin; DM: DMSO; FK: FK228. (F and G) Rescue
658 of Cdx2 in cKD embryos after injection of exogenous *Hdac1* or 2. The experiment was
659 conducted three times and 5-10 embryos analyzed per group per time. Yellow dashed oval:
660 inner cell mass. (H) Nonspecific siRNAs or siRNAs cocktail targeting *Hdac1* and 2 were
661 microinjected into one blastomere at 2-cell stage. *H2B-RFP* mRNA was co-injected as a
662 tracking marker. Blastocysts were collected for immunocytochemical analysis (n = 3; 5-10
663 embryos per group per replicate). The intensity of Cdx2 was diminished not only in cells
664 derived from the siRNA-injected blastomere but those from noninjected blastomere in cKD
665 groups.

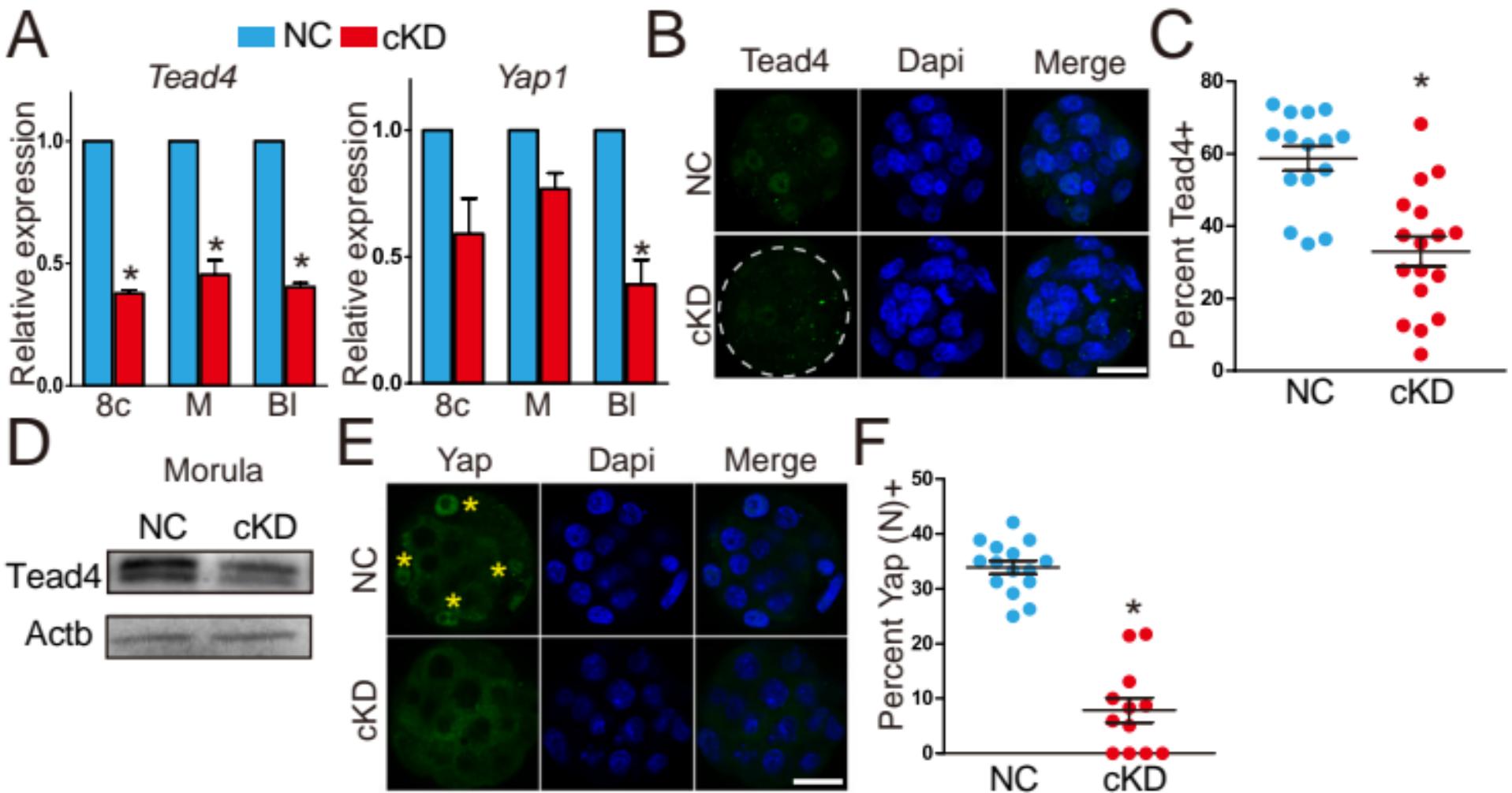
666



667 **Figure 5. Key pluripotency genes Oct4, Nanog and Sox2 were downregulated in embryos**
668 **deficient of Hdac1 and 2.** (A) qPCR analysis of *Oct4*, *Nanog* and *Sox2* in NC and cKD
669 embryos (n=3 pools of 5-10 embryos each per group). (B and C) Immunocytochemical analysis
670 of Oct4 and Nanog in blastocysts after RNAi. The intensity of Nanog, but not Oct4 was
671 diminished in cKD embryos (panel C; n=3; 5-10 embryos were analyzed per group each time,
672 *P<0.05). Yellow dashed oval: inner cell mass. (D) Rescue of Nanog in cKD embryos after
673 injection of exogenous *Hdac1* and/or 2. The experiment was conducted three times and 5-10
674 embryos analyzed per group per time. (E) Analysis of the intensity of Nanog in embryos treated
675 with either DMSO (DM, vehicle control) or FK228 (FK).

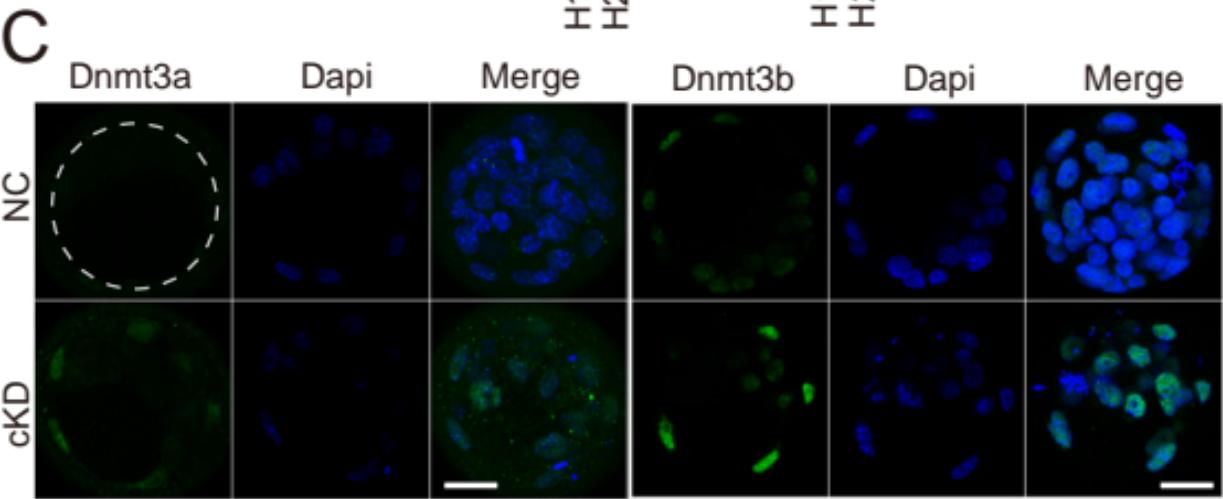
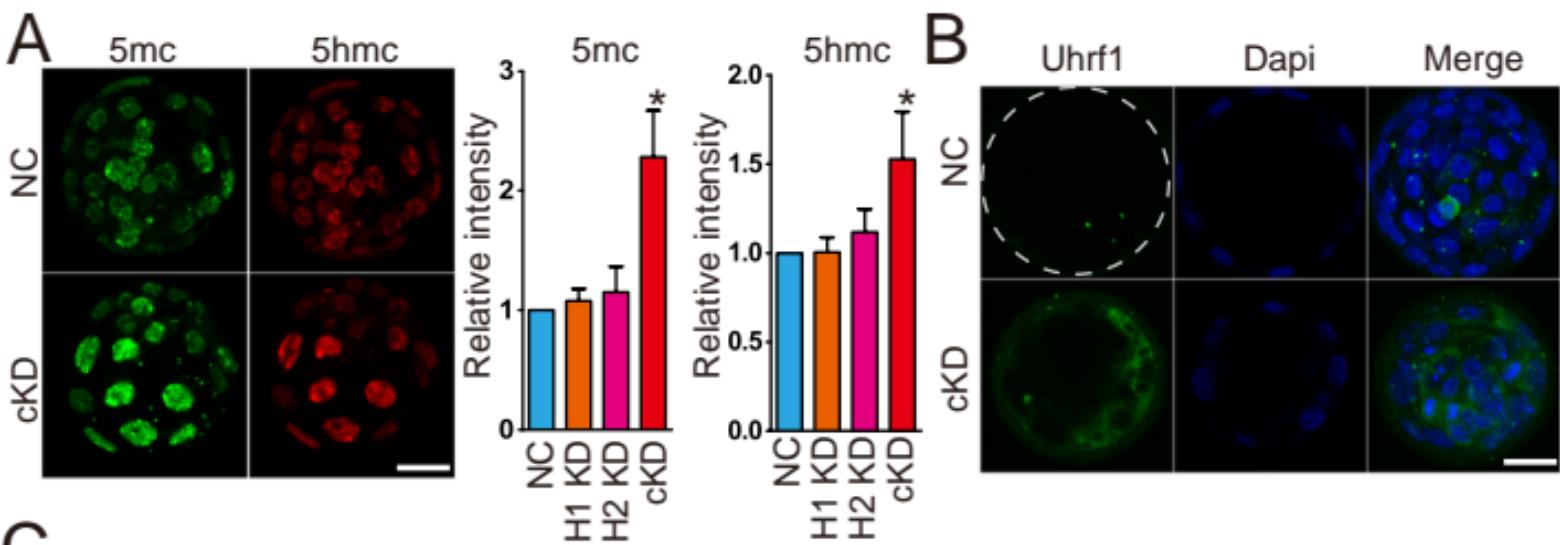
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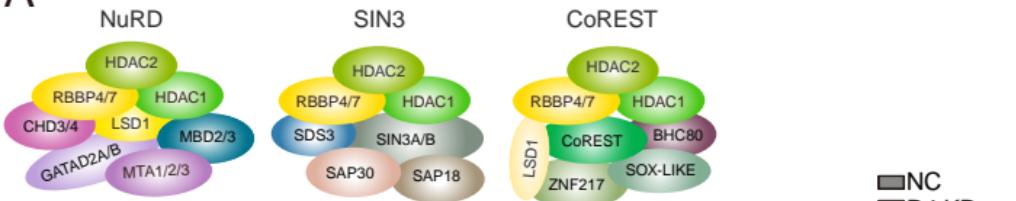
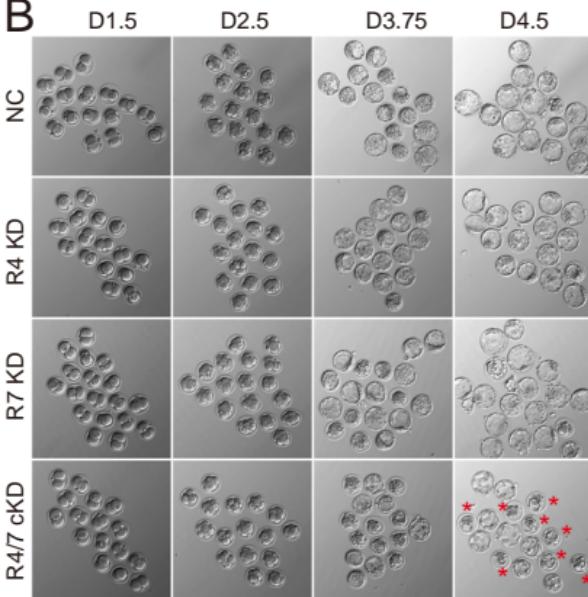
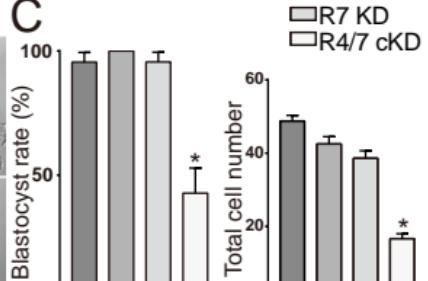
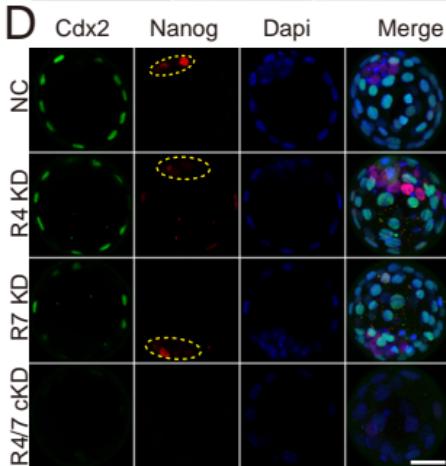
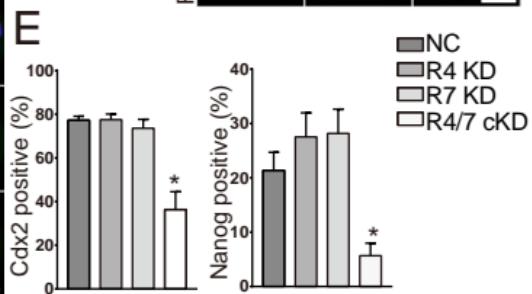
678 **Figure 6. Hda1 and 2 co-knockdown results in aberrant Hippo signaling pathway. (A)**
679 qPCR analysis of *Tead4* and *Yap1* in 8-cell embryos (8c), morula (M) and blastocysts (Bl) (n
680 =3 pools of 5-10 embryos each per group). (B-D) Immunocytochemical (n=3; 6-10 embryos
681 were analyzed per group each time,, *P<0.05) and immunoblot analysis (n=2 pools of 30
682 embryos each per group, similar effects were obtained) of *Tead4* in morula after RNAi. Both
683 percent *Tead4* positive cells (panel C) and the intensity of *Tead4* (panel D) was reduced in cKD.
684 (E and F) Immunocytochemical analysis of *Yap* in morula (n=3; 5-10 embryos were analyzed
685 per group each time). Asterisk: nuclear *Yap*.

686



687 **Figure 7. Increased global DNA methylation with upregulated DNA methyltransferases**
688 **in embryos deficient of Hdac1 and 2.** (A) Immunocytochemical analysis of 5' methylcytosine
689 (5mc) and 5 hydroxymethylcytosine (5hmc) in blastocysts. Both the intensity of 5mc and 5hmc
690 was increased in cKD embryos (C and E) (n=3; 5-10 embryos were analyzed per group each
691 time,, *P<0.05). (B-C) Hdac1 and 2 deficiency results in increased intensity of Uhrf1, Dnmt3a
692 and Dnmt3b. The experiment was conducted three times and 8-10 embryos analyzed per group
693 (*P<0.05; Scale bar: 25 μ m).

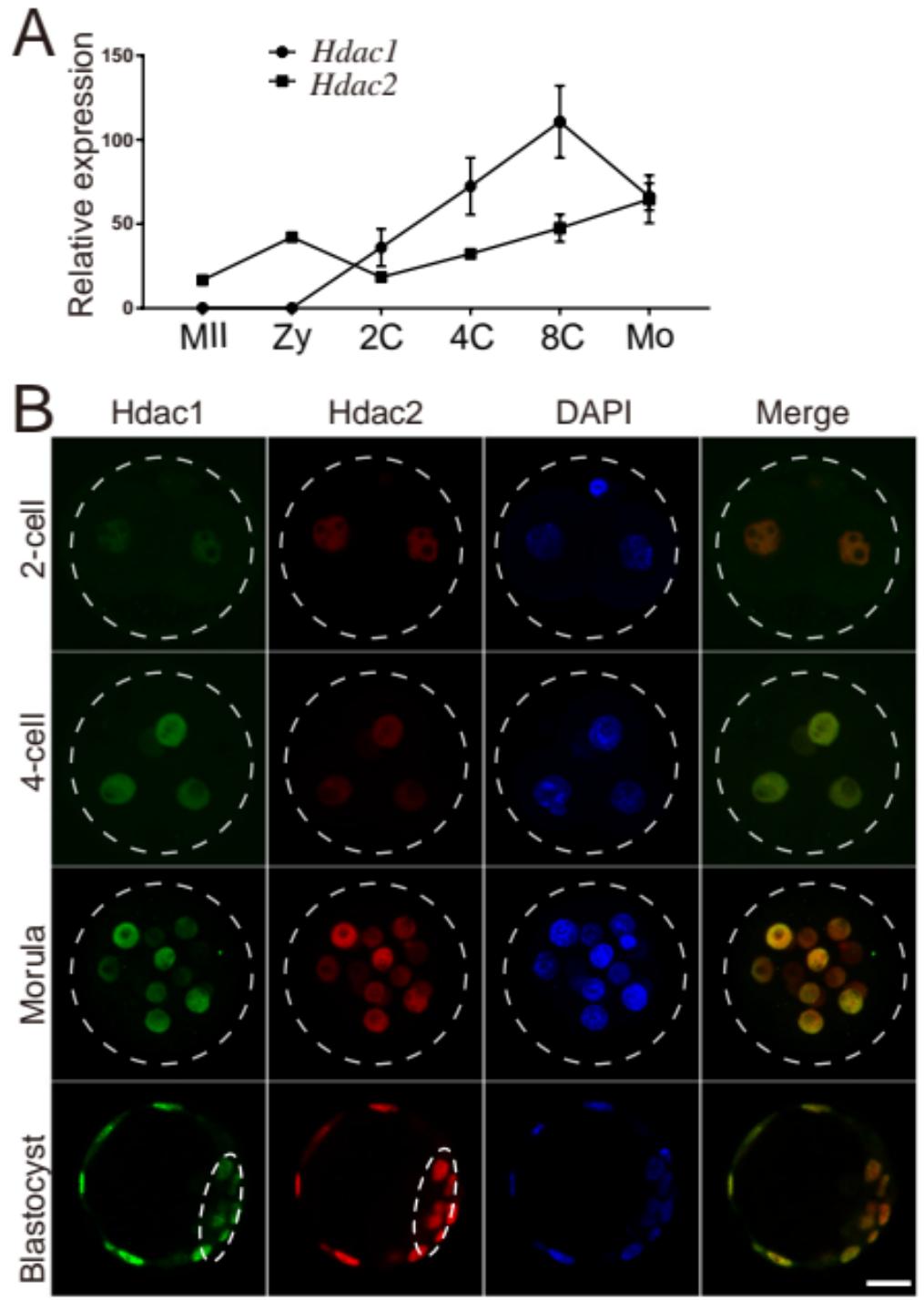
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A**B****C****D****E**

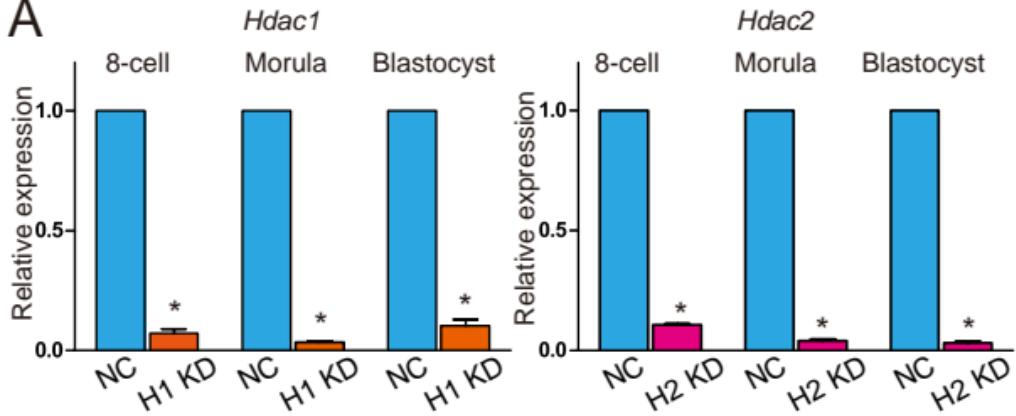
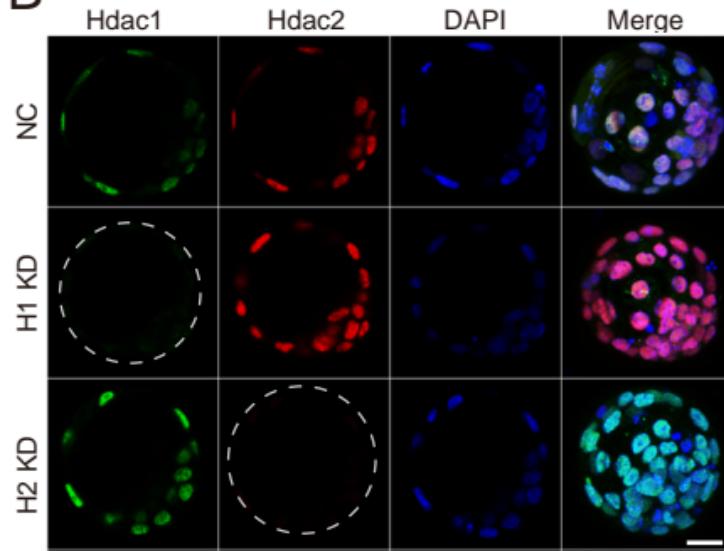
695 **Figure 8. Rbbp4 and 7 deficiency leads to similar phenotypes as in Hdac1/2 cKD embryos.**

696 (A) Hdac1, Hdac2, Rbbp4, and Rbbp7 are core components in several epigenetic complexes:
697 NuRD, Sin3, and CoREST. (B) Developmental potential of embryos lacking Rbbp4 and/or
698 Rbbp7. Three replicates were conducted with 15-20 embryos analyzed per group per replicate.
699 (C) Blastocyst rate and cell counting analysis of the experiment in panel B. (D) Both Cdx2 and
700 Nanog were diminished in Rbbp4 and 7 cKD embryos. (E) Cdx2 or Nanog positive blastomeres
701 were reduced in Rbbp4 and 7 cKD embryos. (F) Immunostaining analysis of 5mc and 5hmc.

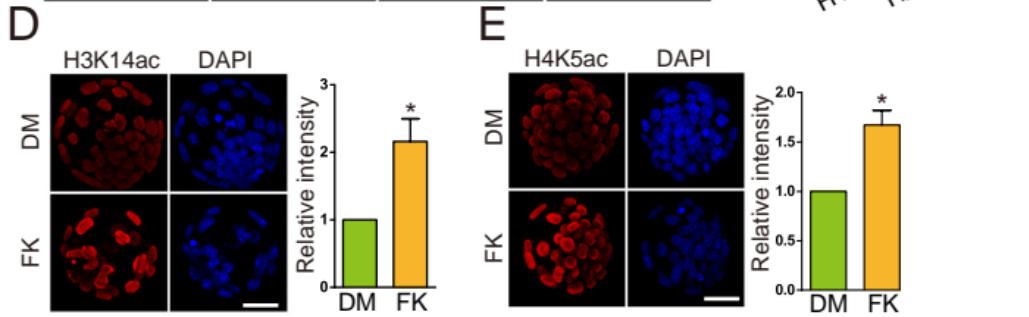
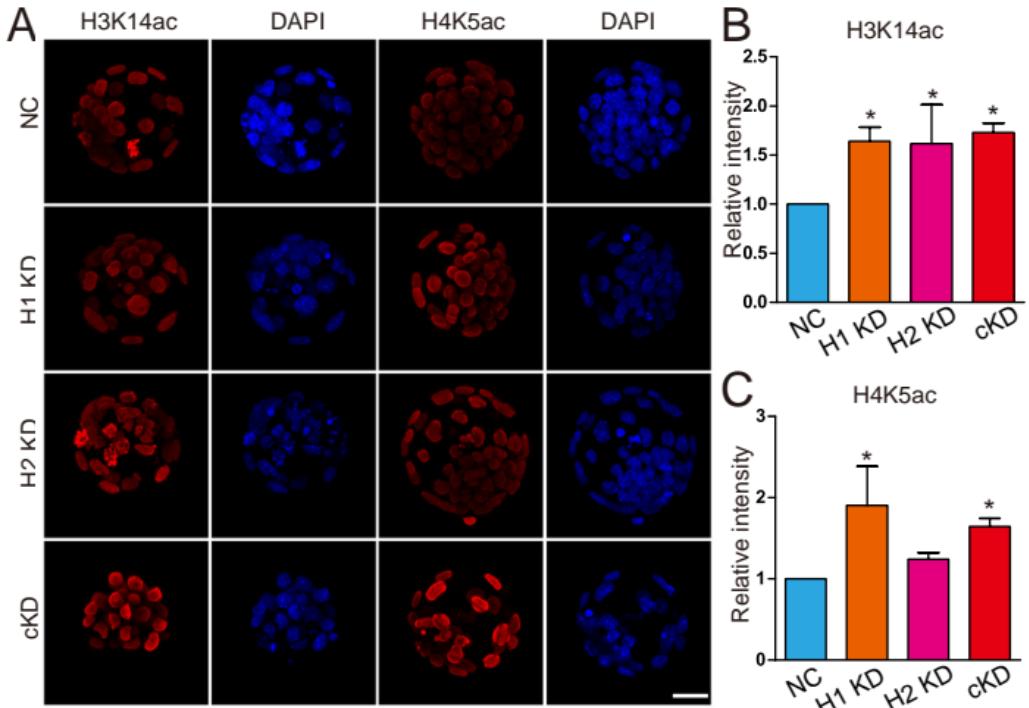
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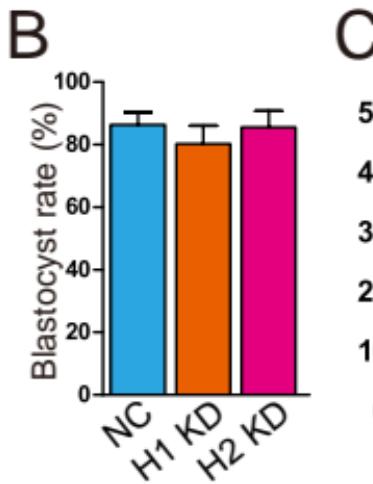
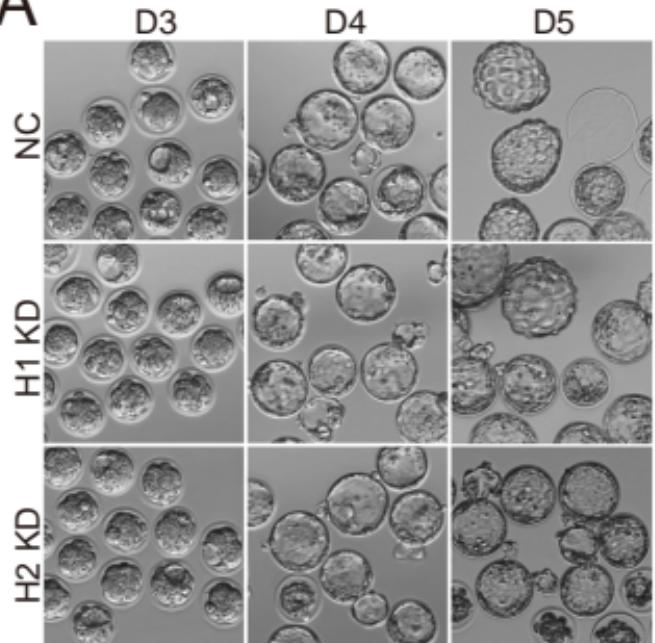
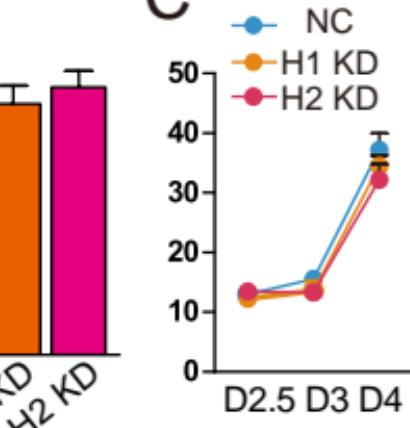
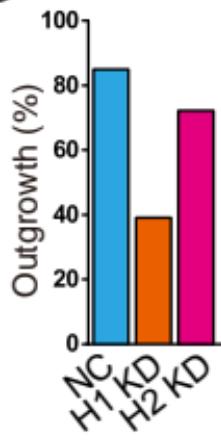
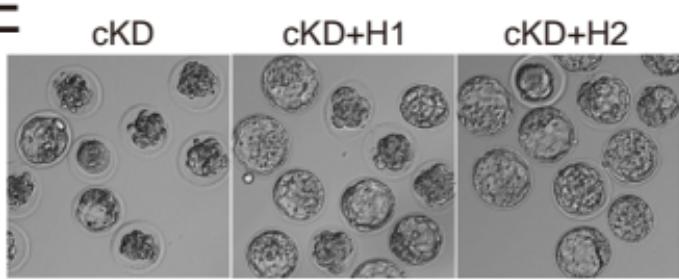
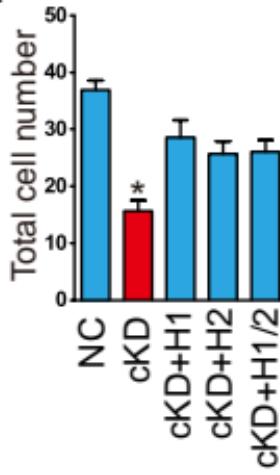
703 **Figure S1. Extensive expression and colocalization of Hdac1 and 2 through**
704 **preimplantation development.** (A) Analysis of expression level of Hdac1 and 2 from a
705 previous published single cell RNA-seq datasets (GSE44183). (B) Immunocytochemical
706 detection of Hdac1 and 2 through preimplantation development. At least 10 embryos were
707 analyzed and the experiment was performed three times. Scale bar: 25 μ m.
708

A**B**

709 **Figure S2. Validation of knockdown efficiency of siRNAs targeting *Hdac1* or 2.** (A) qPCR
710 analysis of *Hdac1* or 2 in *Hdac1* or *Hdac2* KD embryos, respectively (n=3 pools of 5-10
711 embryos each per group, *P<0.05). (B) Immunocytochemical analysis of *Hdac1* and 2 in *Hdac1*
712 or *Hdac2* KD embryos (n=3; 5-10 embryos were analyzed per group each time, *P<0.05). Scale
713 bar: 25 μ m.
714



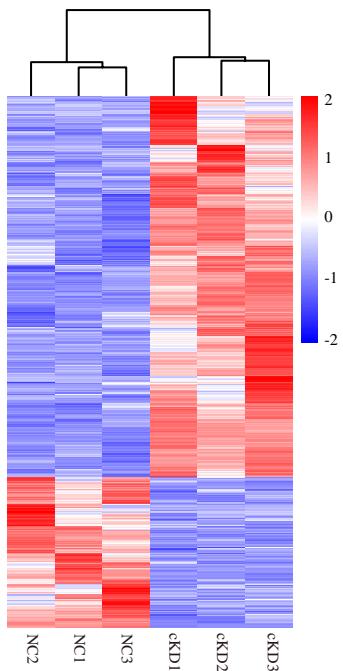
715 **Figure S3. Hdac1/2 co-knockdown or FK228 treatment results in increased intensity of**
716 **histone H3 lysine 14 acetylation and histone H4 lysine 5 acetylation. (A-E)**
717 Immunocytochemical analysis of histone H3 lysine 14 acetylation (H3K14ac) and histone H4
718 lysine 5 acetylation (H4K5ac) in embryos after RNAi (A-C) or FK228 treatment (D and E) (6-
719 10 embryos were analyzed per group, *P<0.05). Scale bar: 25 μ m.
720

A**C****D****E****F**

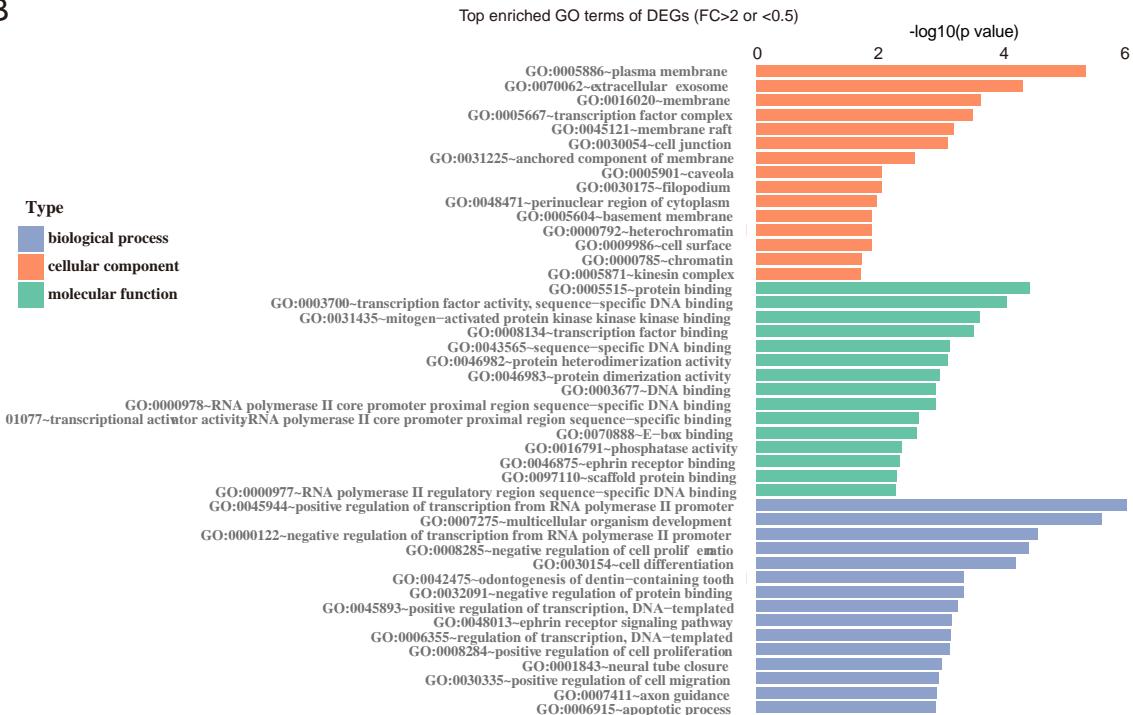
721 **Figure S4. Effects of Hdac1 or 2 individual KD on preimplantation development. (A)**
722 Representative photos of NC, Hdac1 (H1) KD, and Hdac2 (H2) KD embryos from D3 to D5.
723 No difference was noted between H1 or H2 KD and NC group through preimplantation
724 development. (B and C) Blastocyst rate (B) at D4 and total cell number per embryo (C) was
725 similar among NC, H1 KD, and H2 KD groups (n=3; 15-20 embryos analyzed per group). (D)
726 Incidence of outgrowth formation for blastocysts derived from NC, H1 KD, and H2 KD
727 embryos (20-25 embryos analyzed per group). (E and F) Rescue of cKD embryos by injection
728 of exogenous Hdac1 or Hdac2 mRNA (n=3).

729

A

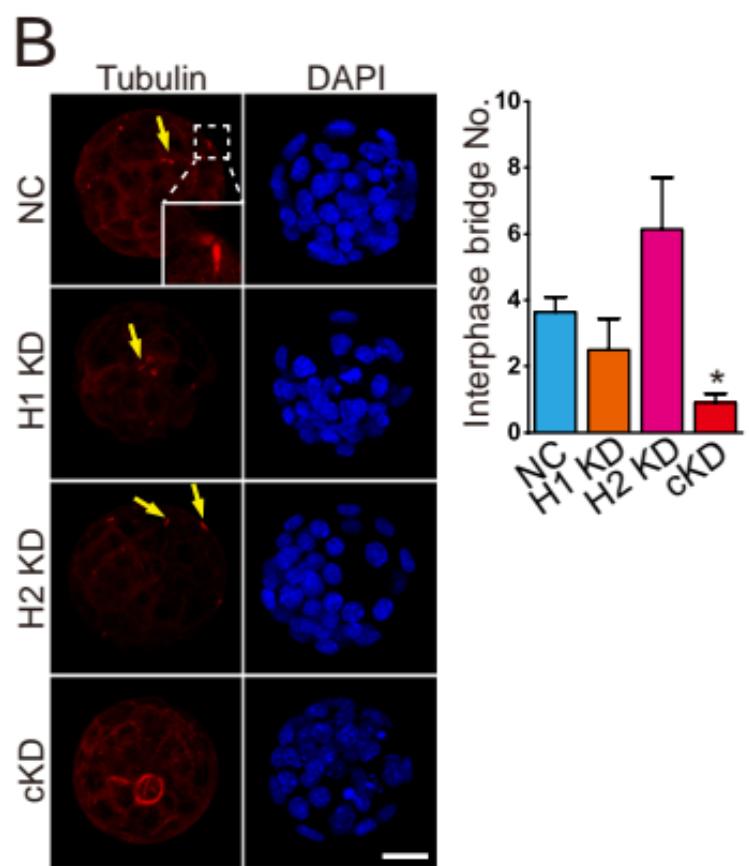
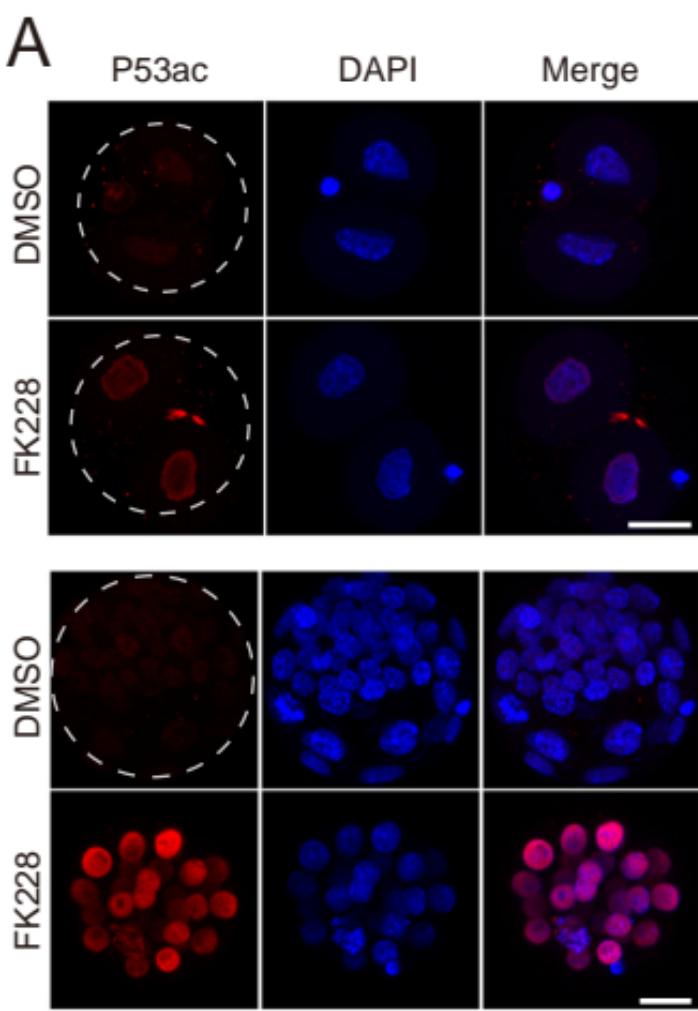


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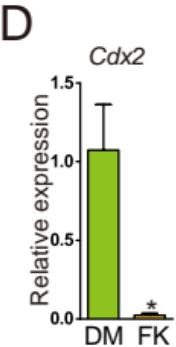
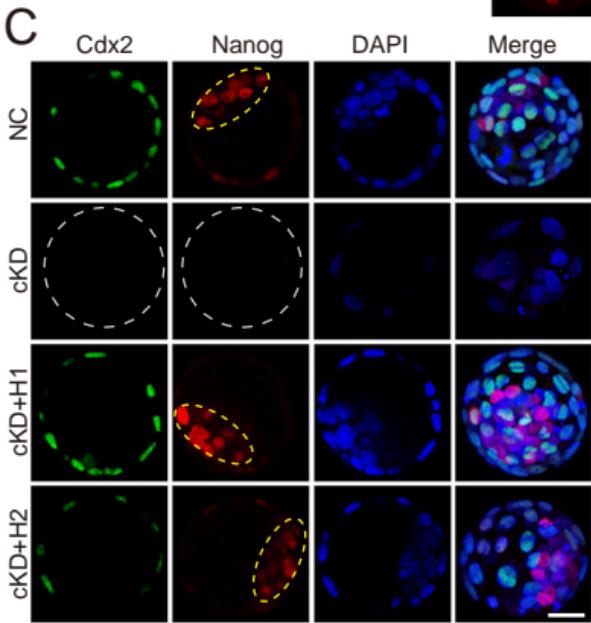
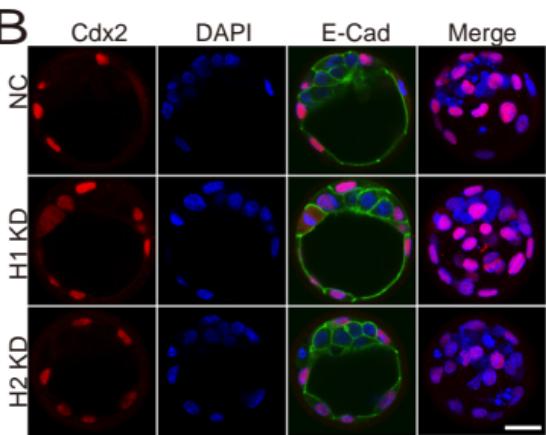
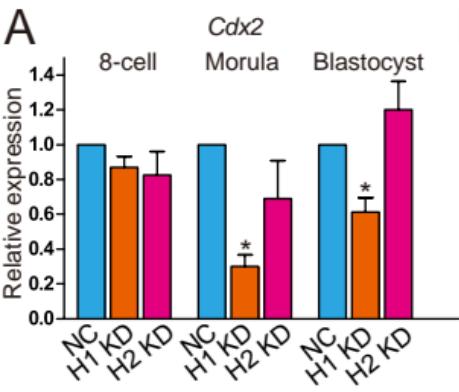
730 **Figure S5. Transcriptomic analysis of embryos deficient of Hdac1 and 2.** (A) Heatmap
731 showing differentially expressed genes (DEG) between cKD and NC embryos. (B) GO analysis
732 of all DEGs in cKD embryos related to NC groups.

733



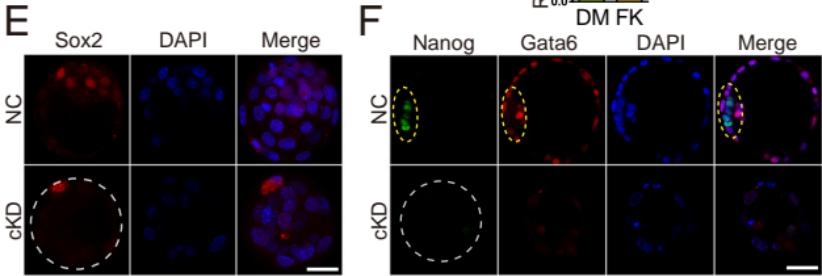
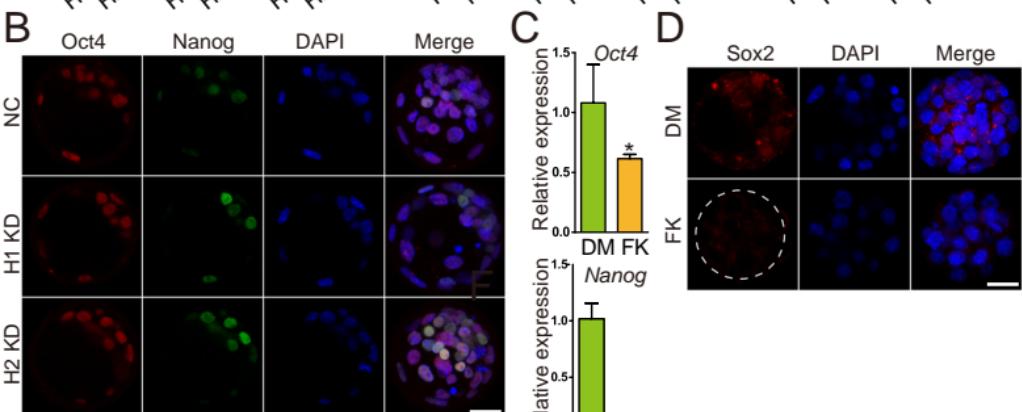
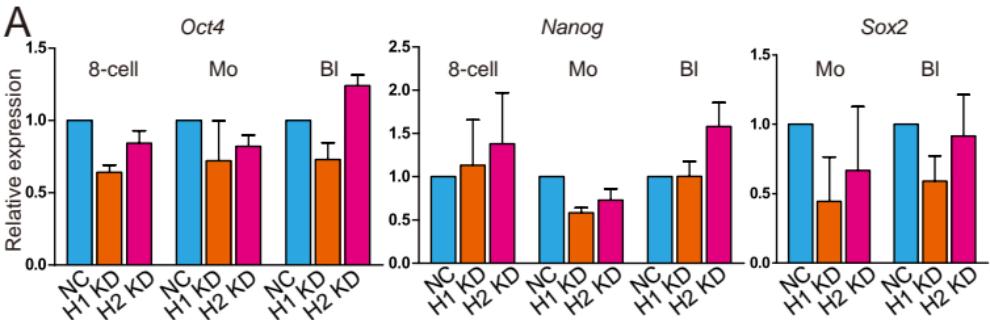
734 **Figure S6. Blockage of Hdac1 and 2 results in increased Trp53 acetylation and aberrant**
735 **interphase bridge.** (A) Immunocytochemical analysis of Trp53K379 acetylation (P53ac) in 2-
736 cell embryos and morula. Mouse zygotes or morula were treated with FK228 for 12 h. The
737 intensity of P53ac was increased dramatically in FK228-treated embryos. (E)
738 Immunocytochemical examination of α -tubulin, a marker for interphase bridge, in blastocysts
739 (Arrows: interphase bridge; n=3; 5-10 embryos were analyzed per group each time, Scale bar:
740 25 μ m).

741



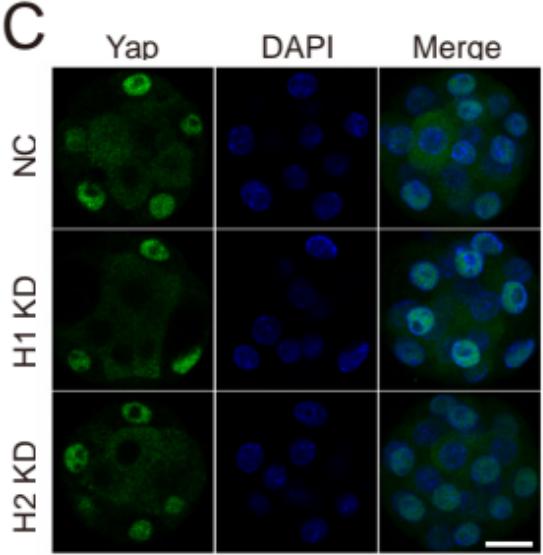
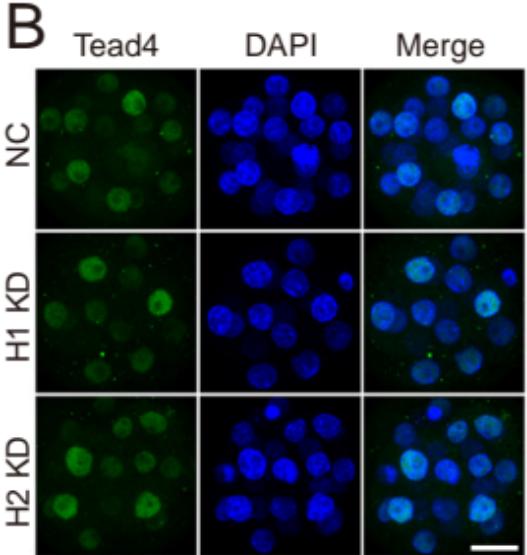
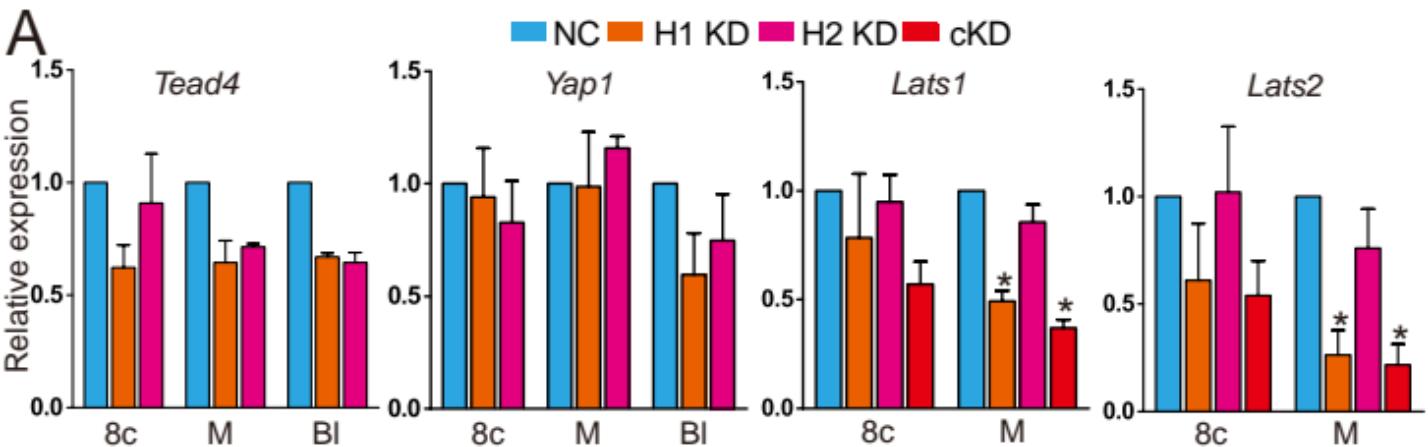
742 **Figure S7. Effects of Hdac1 KD or Hdac2 individual KD on Cdx2 expression.** (A) qPCR
743 analysis of *Cdx2* in 8-cell embryos, morula and blastocysts (n=3 pools of 5-10 embryos each
744 per group; *P<0.05). (B) Immunocytochemical analysis of Cdx2 in morula and blastocysts.
745 The intensity of Cdx2 was not changed in H1 or H2 KD embryos (n=3; 5-10 embryos were
746 analyzed per group each time). (C) Rescue of Cdx2 in cKD embryos after injection of
747 exogenous *Hdac1* and/or 2. The experiment was conducted three times and 5-10 embryos
748 analyzed per group per time. (D) FK228 treatment results in significant decrease in Cdx2
749 expression level (n=3; 5-10 embryos were analyzed per group each time, *P<0.05). Scale bar:
750 25 μ m.

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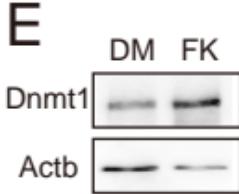
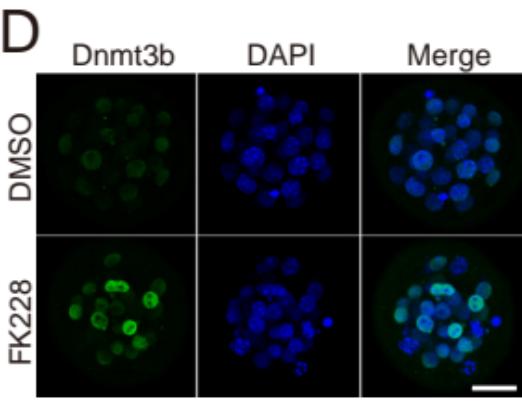
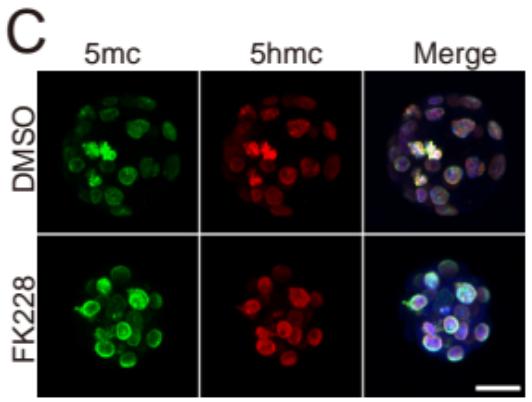
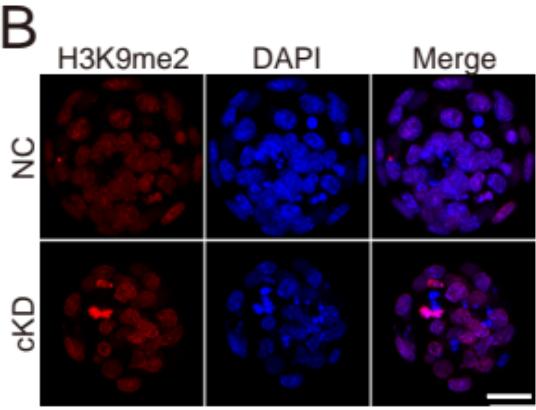
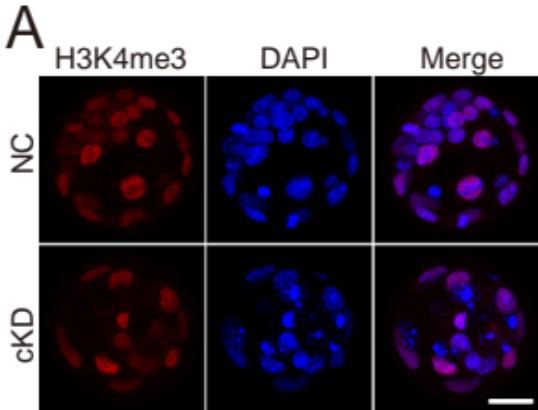


752 **Figure S8. Effects of Hdac1 KD or Hdac2 individual KD on Oct4, Nanog and Sox2.** (A)
753 qPCR analysis of *Oct4*, *Nanog* and *Sox2* in NC, Hdac1 KD, and Hdac2 KD embryos. (B)
754 Immunocytochemical analysis of Oct4 and Nanog in blastocysts. (C) qPCR analysis of *Oct4*
755 and *Nanog* in FK228-treated embryos. (D and E) Immunocytochemical analysis of *Sox2* in
756 cKD or FK228 treated embryos. (F) Immunostaining detection of Nanog and Gata6 in late
757 blastocysts.

758

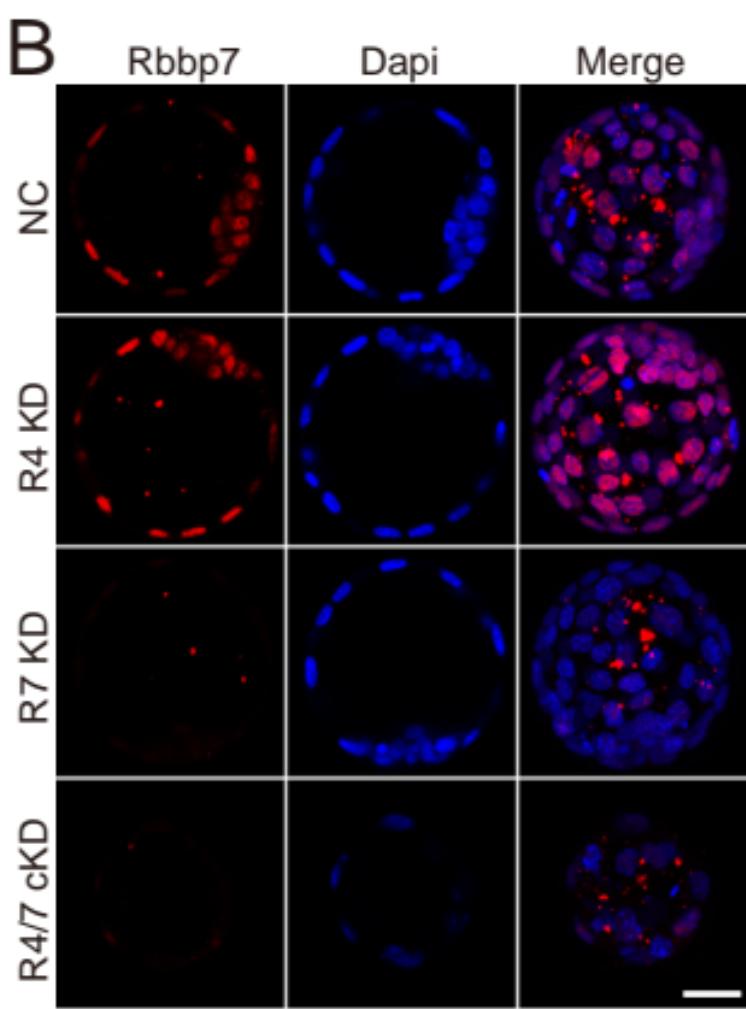
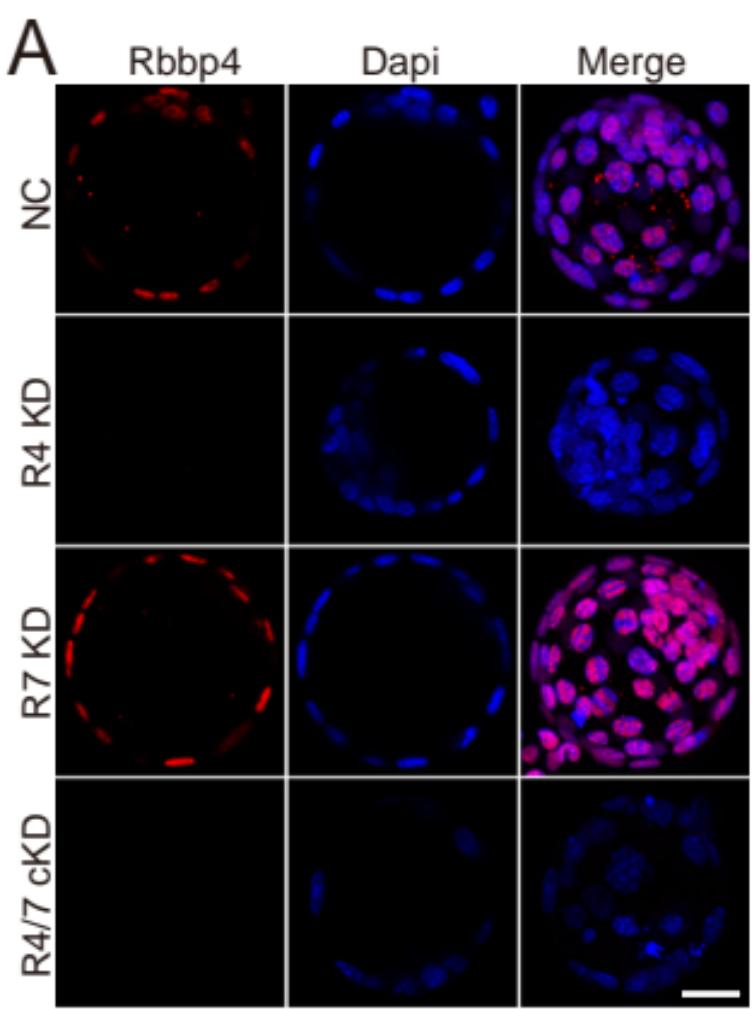


759 **Figure S9. Effects of Hdac1/2 individual KD on Hippo signaling pathway.** (A) qPCR
760 analysis of *Tead4*, *Yap1*, *Lats1*, and *Lats2* in NC, Hdac1 KD, and Hdac2 KD embryos. (B and
761 C) Immunocytochemical analysis of Tead4 and Yap in morula deficient of Hdac1 or Hdac2.
762



763 **Figure S10. Effects of Hdac1 and 2 cKD on epigenetic modifications.** (A and B)
764 Immunocytochemical analysis of H3K4me3 and H3K9me2 in morula deficient of Hdac1 and
765 Hdac2. (C and D) FK treatment results in an increase of DNA methylation (5mc) and Dnmt3b
766 intensity. (E) Immunoblotting analysis of Dnmt1 in morula/blastocysts treated with DMSO
767 (DM) or FK228 (FK). Two replicates were performed with 40 embryos used per group and
768 similar results obtained.

769



770 **Figure S11. Validation of knockdown efficiency of siRNAs targeting *Rbbp4* or 7. (A-B)**

771 Immunocytochemical analysis of Rbbp4 and 7 in Rbbp4 or 7 KD embryos. Scale bar: 25 μ m.

772

773 **Table S1.**

774 List of differentially expressed genes between control and Hdac1 and 2 cKD embryos
775 (Supporting Excel file).

776

777 **Table S2.**

778 List of siRNAs and oligos used for qPCR and *in vitro* transcription

Gene	Primer sequences (5'-3')	References
<i>Hdac1 siRNA1</i>	Sense-GCUUCUGUUACGUCAAUGATT Antisense-UCAUUGACGUACAGAAGCTT	
<i>Hdac1 siRNA2</i>	Sense-GAGUUCGUGAAGAGUUUCATT Antisense-UGAACACUCUUCACGAACUCTT	
<i>Hdac1 siRNA3</i>	Sense-GACCGGAUUUCAAGCUUCATT Antisense-UGAACGUUGAAAUCCCGGUCTT	
<i>Hdac1 UTR siRNA1</i>	Sense-GCUUGGGUAAUAGCAGCCATT Antisense-UGGCUGCUAUUACCCAAGCTT	
<i>Hdac1 UTR siRNA2</i>	Sense-GUGGAGGUUGAUAGCCUAGTT Antisense-CUAGGCUAUCAACCUCCACTT	
<i>Hdac2 siRNA1</i>	Sense-CUCAUAACUUGCUGCUAAATT Antisense-UUUAGCAGCAAGUUAUGAGTT	
<i>Hdac2 siRNA2</i>	Sense-GUCCGGUGUUUGAUGGACUTT Antisense-AGUCCAUCAAACACCGGACTT	
<i>Hdac2 siRNA3</i>	Sense-GACCGUCUCAUCCAUAATT Antisense-UUUAUGGAAUGAGACGGUCTT	
<i>Hdac2 UTR siRNA1</i>	Sense-GACUCUCCAACUUUAGGAATT Antisense-UUCCUAAAGUUGGAGAGUCTT	

<i>Hdac2 UTR</i>	Sense-UGGCAUGGACUGUAUUUAUTT
<i>siRNA2</i>	Antisense-AUAAAUAACAGUCCAUGCCATT
<i>Hdac2 UTR</i>	Sense-GCCGGAUCUAUUAAAGAAATT
<i>siRNA3</i>	Antisense-UUUCUUUAUAGAUCCGGCTT
<i>Hdac1</i>	F-TGAAGCCTCACCGAATCCG R-GGGCGAATAGAACCGCAGGA
<i>Hdac2</i>	F-GGAGGAGGCTACACAATCCG R-TCTGGAGTGTCTGGTTGTCA
<i>H2afz</i>	F-TCCAGTGGACTGTATCTCTGTGA R-GACTCGAATGCAGAAATTGG (51)
<i>Lats1</i>	F-TTTGCAGGCTGCTGGCTTG R-AGACATCTGCTCTCGACGAG (49)
<i>Lats2</i>	F-TGCGAGTCATCAAGCAGACC R-ACTTGGCTCTACTGCTGTGC (49)
<i>Yap1</i>	F-GTCCTCCTTGAGATCCCTGA R-TGTTGTTGCTGATCGTTGTGAT (49)
<i>Tead4</i>	F-TGATGCAGAGGGTGTATGGA R-GATCAGCTCATTCCGACCAT
<i>Meg3</i>	F-CGAGGACTTCACGCACAAC R-TTACAGTTGGAGGGCCTGG (52)
<i>Oct4</i>	F-CTCCCGAGGGAGTCCCAGGACAT R-GATGGTGGTCTGGCTAACACCT (53)

<i>Nanog</i>	F-CAAGGGTCTGCTACTGAGATGCTCTG R-TTTGTTGGGACTGGTAGAAGAACAG	(53)
<i>Cdx2</i>	F-CAAGGACGTGAGCATGTATCC R-GTAACCACCGTAGTCCGGGTA	
<i>Fgfr2</i>	F-GCCTCTCGAACAGTATTCTCCT R-ACAGGGTTCATAAGGCATGGG	
<i>Sox2</i>	F-GCGGAGTGGAAACTTTGTCC R-CGGGAAGCGTGTACTTATCCTT	
<i>Amot</i>	F-CCGCCAGAACATCCCTTCAAG R-CTCATCAGTTGCCCTCTGT	
<i>Myc</i>	F-ATGCCCTCAACGTGAACCTTC R-CGCAACATAGGATGGAGAGCA	
<i>Dab2</i>	F-CCCCTGAACGGTGATACTGAT R-AAGTCCTGCTTACGCCATTG	
<i>Otx2</i>	F-TATCTAAAGCAACCGCCTACG R-AAGTCCATACCGAAGTGGTC	
<i>Arid3a</i>	F-GCTTGGGACATCCGTCCTC R-CAAATGCCTATCTCCCTCAGC	
<i>Sfmbt2</i>	F-AAGATAACCGGCTCAGCAAATG R-TCTCTTCCAAATAGTCTCCCCAG	
<i>Ctnnb1</i>	F-ATGGAGCCGGACAGAAAAGC R-TGGGAGGTGTCAACATCTTCTT	

<i>Tet1</i>	F-CGGGTTACAATGGCTTCG R-GGTTGGGTGTGACTACTGGG
<i>Cdh1</i>	F-CAGGTCTCCTCATGGCTTG R-CTTCCGAAAAGAAGGCTGTCC
	T7-F-TAATACGACTCACTATAAGGAGAatgcc
<i>H2B-RFP CDS</i>	agagccagcg aagtct R-TTAGGCGCCG GTGGAGTGGC
	T7-F-TAATACGACTCACTATAAGGAGAatg gcgagactc
<i>Hdac1 CDS</i>	agggcac R-TCAGGCCAAC TTGACCTCTT CT
	T7-TAATACGACTCACTATAAGGAGAat ggcgtacagt
<i>Hdac2 CDS</i>	caaggaggc R-TCAAGGGTTG CTGAGTTGTT CT

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781 **Table S3.**

782 List of antibodies used for immunofluorescence and Western Blot

Target	Source	Dilution	Company	Catalogue
Nanog	Rabbit	1:200	Cell Signaling Technology	8822
H3K4me3	Rabbit	1:200	Cell Signaling Technology	9751
H3K9me2	Rabbit	1:200	Cell Signaling Technology	9753
E-Cadherin	Rat	1:1000	Sigma	U3254
DNMT3A	Mouse	1:200	Novus Biologicals	NB120-13888SS
DNMT3B	Mouse	1:200	Novus Biologicals	52A1018
TEAD4	Mouse	1:200	Abcam	ab58310
H4K5ac	Rabbit	1:200	Abcam	ab51997
H3K14ac	Rabbit	1:200	Abcam	ab52946
P-H3S10	Rabbit	1:1000	Cell Signaling Technology	3377

5mc	Mouse	1:200	Eurogentec	BI-MECY-1000
5-hmC	Rabbit	1:200	Active Motif	39791
HDAC2	Rabbit	1:200	Abcam	ab32117
α -Tubulin	Rabbit	1:100	Cell Signaling Technology	2125
HDAC1	Mouse	1:200	Cell Signaling Technology	5356
CDX2	Mouse	1:200	BioGenex	CDX2-88
OCT3/4	Mouse	1:200	Santa Cruz	sc-5279
SOX2	Rabbit	1:50	Millipore	AB5603
YAP	Mouse	1:200	Santa Cruz	sc-101200
GATA6	Goat	1:200	R&D Systems	AF1700
SOX17	Goat	1:200	R&D Systems	AF1924
Uhrf1	Mouse	1:200	Santa Cruz	sc-373750
P53-acetyl-lys379	Rabbit	1:200	GeneTex	GTX88013
β -actin	Mouse	1:1000 (WB)	Beyotime	AF0003
Donkey anti-Rabbit 594		1:1000	Invitrogen	A21207
Goat anti-Mouse 488		1:1000	Invitrogen	A11001
Donkey anti-Mouse 594		1:1000	Invitrogen	A11032
Donkey anti-Goat 594		1:1000	Invitrogen	A11058
Goat anti-Rat 488		1:1000	Invitrogen	A11006
Goat anti-Rabbit 488		1:1000	Invitrogen	A11008
Donkey anti-Mouse 488		1:1000	Invitrogen	A21206
