

CRISPR Activation/Inhibition Experiments Reveal that Expression of Intronic MicroRNA *miR-335* Depends on the Promoter Activity of its Host Gene *Mest*

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ABSTRACT

MicroRNAs are small non-coding RNAs that act as rheostats to modulate gene expression during development, physiology, and disease. Approximately half of mammalian microRNAs are intronic. It is unknown whether intronic miRNA transcription depends on their host gene or a microRNA-specific promoter. Here, we show that CRISPR inhibition of host gene *Mest* downregulated hosted *miR-335* in mouse embryonic stem cells and brain organoids. Reciprocally, CRISPR transactivation of *Mest* upregulated *miR-335*. By contrast, activation of *miR-335* predicted promoter had no effect. Thus, intronic *miR-335* expression depends on the promoter activity of its host gene. This approach could serve to map microRNA promoters.

INTRODUCTION

1 microRNAs (miRNAs) are short non-coding RNAs that play a central role in regulating gene
2 expression in plants and animals (Bartel 2018; Jones-Rhoades et al. 2006). miRNAs impact on
3 development and physiology, and are dysregulated in diseases, including cancer (DeVeale et al. 2021;
4 Schanen and Li 2011; Xue et al. 2021). Stringent gene annotations suggest that there are ~500
5 miRNAs in mice (Chiang et al. 2010) and humans (Fromm et al. 2015). It is estimated that
6 approximately half of mammalian miRNAs are intronic (Meunier et al. 2013; Rodriguez et al. 2004;
7 Hinske et al. 2014).

8 miRNAs biogenesis sequentially involves transcription, cleavage of the miRNA hairpin precursor
9 out of the primary transcript, transport of intermediate forms, and loading of the mature miRNA into the
10 RNA-induced silencing complex (Bartel 2018; Westholm and Lai 2011; Ha and Kim 2014). The
11 mechanisms that regulate miRNAs transcription, a key factor of miRNA abundance and tissue-specific
12 expression, are not well defined, in particular for intronic miRNAs. Intronic miRNAs were first observed
13 as frequently co-regulated with their host genes (Baskerville and Bartel 2005; He et al. 2012; Liang et
14 al. 2007; Rodriguez et al. 2004; Seitz et al. 2004), suggesting that their transcription depends on the
15 promoter activity of the host gene. By contrast, recent work suggests that most intronic miRNAs are not
16 co-regulated with their host genes, which is supported by the fact that they have independent
17 transcription start sites (Steiman-Shimony et al. 2018). Many additional studies have tried to map
18 miRNA promoters using bioinformatics tools (Chen et al. 2019). For instance using chromatin
19 modifications (Ozsolak et al., 2008) or deepCAGE data (Marsico et al., 2013) it was estimated that
20 ~30% of intronic miRNAs have independent promoters. To our knowledge, whether the transcription of
21 an intronic miRNA depends on the promoter activity of the host gene or a miR-specific promoter has
22 not been tested experimentally.

23 *Mest* (Mesoderm-specific transcript) is a protein-coding gene that hosts *miR-335* in one of its
24 introns. *Mest* and *miR-335* are highly conserved during evolution and frequently co-regulated (Hiramuki

25 et al. 2015; Liang et al. 2007; Ronchetti et al. 2008; Tomé et al. 2011; Yang et al. 2014). This suggests
26 that *Mest* and *miR-335* are controlled via common regulatory sequences, possibly *Mest* promoter. In
27 addition, based on a luciferase assay, *miR-335* was proposed to have an independent promoter located
28 in a *Mest* intron (Zhu et al. 2014).

29 Here, to get insights into the mechanisms of transcription of an intronic miRNA, we have applied
30 CRISPR/Cas9 -Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-
31 associated protein 9 (Cas9)- technologies to *Mest* and intronic *miR-335*. More specifically, we have
32 used CRISPR activation (CRISPRa) and CRISPR inhibition (CRISPRi) where a cleavage defective
33 Cas9 (dCas9) is fused to either activators or repressors of transcription (Konermann et al. 2015; Yeo et
34 al. 2018; Gilbert et al. 2013) and directed these complexes to the endogenous promoter sequences of
35 *Mest* or to the predicted promoter of *miR-335*.

36 RESULTS & DISCUSSION

37 CRISPRi of host gene *Mest* suppresses the expression of hosted *miR-335* in embryonic stem 38 cells

39 *miR-335* is located in an intron of the protein-coding gene *Mest* (Fig. 1A, B) and is transcribed from the
40 same DNA strand as its host gene, a common feature of intronic miRNAs (Hinske et al. 2014). *Mest*
41 has one distal promoter (D) and one proximal promoter (P). *Mest* is highly expressed in mouse
42 embryonic stem cells (mESCs) and *Mest* transcripts originate predominantly from the proximal
43 promoter (P) (Fig. 1A, generated from previously published RNA-seq experiments (Bouschet et al.
44 2017)). Furthermore, *miR-335-3p* was reported to be expressed in mESCs (Kingston and Bartel 2019).

45 We reasoned that if *miR-335* expression depends on the activity of *Mest* promoters, then
46 repressing transcription at *Mest* promoters in mESCs with CRISPRi should decrease *miR-335*
47 transcripts. Using Hyper-piggyBac transposase (Yusa et al., 2011), we first generated a CRISPRi
48 mESC line that stably expressed dCas9 fused to the repressors of transcription KRAB and MeCP2.
49 dCas9-KRAB-MeCP2 was previously shown to efficiently repress a vast panel of genes in HEK293T

50 cells (Yeo et al. 2018). CRISPRi mESCs (characterized in [Supplemental Fig. S1](#)) were transduced with
51 lentiviruses that express either a control sgRNA (no match in the mouse genome) or a sgRNA targeting
52 either the distal or the proximal promoter of *Mest* ([Supplemental Fig. S2A](#)). sgRNAs targeting *Mest*
53 proximal promoter P downregulated *Mest* while targeting distal promoter D had no obvious effect
54 ([Supplemental Fig. S2B](#)). Thus, as expected, CRISPRi was efficient only when targeting the active
55 *Mest* promoter. Levels of the neighboring gene *Copg2* were unaffected ([Supplemental Fig. S2C](#)).

56 We then selected two CRISPRi mESC clones expressing the control sgRNA and two clones
57 expressing the sgRNA *Mest* P2 for further analyses ([Fig. 1B](#)). There was a >100 fold-downregulation of
58 *Mest* in CRISPRi *Mest* clones compared to CRISPRi control clones ([Fig. 1C](#)). By contrast, *Copg2*
59 expression was unaffected ([Fig. 1D](#)). We next measured the levels of miR-335-3p and miR-335-5p, the
60 final products of miR-335 biogenesis, by gene-specific RT followed by qPCR with Taqman probes. In
61 CRISPRi *Mest* clones, miR-335-3p and miR-335-5p levels were reduced to less than 1% of levels
62 measured in CRISPRi control clones ([Fig. 1E, F](#)), a massive downregulation that paralleled well that of
63 *Mest* ([Fig. 1C](#)). Thus, the transcriptional activity of *Mest* proximal promoter is required for the
64 expression of intronic *miR-335* in mESCs.

65 ***Mest* promoter activity is required for *miR-335* expression in brain organoids**

66 Next, we determined whether *miR-335* expression dependency on *Mest* promoter persists upon
67 differentiation of mESCs into brain organoids. Brain organoids were generated from mESCs according
68 to a published protocol (Eiraku et al. 2008) with slight modifications (see *Materials and Methods*). RNA-
69 seq experiments on these brain organoids show enrichment in Gene Ontology Terms such as ‘forebrain
70 generation of neurons’ after eight days of differentiation and ‘telencephalon development’ and ‘action
71 potential’ after 15 days of differentiation (Bouschet and co-workers, unpublished).

72 As expected, brain organoids contained neural progenitors of dorsal identity (NESTIN+PAX6+
73 cells) after 8 days of differentiation, and neurons (TUBB3+ cells), including some neurons that
74 expressed the cortical marker TBR1 after 15 days of differentiation ([Fig. 2A](#)).

75 *Mest* and *miR-335* transcripts were upregulated during the generation of brain organoids from
76 CRISPRi mESCs expressing the control sgRNA (Fig. 2B). By contrast, *Mest* RNA was barely
77 detectable in CRISPRi organoids expressing the *Mest* sgRNA (Fig. 2B), showing that *Mest* promoter
78 remains repressed in differentiated cells. Importantly, *miR-335* mature products were also barely
79 detectable in these organoids (Fig. 2B). Thus, the activity of *Mest* promoter is required for *miR-335*
80 expression in both undifferentiated mESCs and their neural progeny.

81 **CRISPRa on *Mest* increases the expression of hosted *miR-335***

82 We next tested whether transactivating *Mest* promoter is sufficient to increase *miR-335* levels and
83 therefore mirrors CRISPRi loss of function experiments. mESCs stably expressing the CRISPRa
84 Synergistic Activation Mediator (SAM) module (Bonev et al. 2017) -composed of three transactivators
85 (Koner mann et al. 2015)- were transduced with lentiviruses expressing either a control sgRNA or a
86 sgRNA targeting *Mest* (D) or (P) promoter (Supplemental Fig. S3A) -as described for CRISPRi-.

87 Transactivating *Mest* distal promoter efficiently increased *Mest* transcripts (Supplemental Fig.
88 S3B). By contrast, transactivating *Mest* proximal promoter with sgRNAs P1 and P2 had no major effect
89 on *Mest* transcript level (Supplemental Fig. S3B), likely because this promoter is already very active in
90 ESCs (Fig. 1A). The level of *Copg2* was not altered by any of the three *Mest* sgRNAs (Supplemental
91 Fig. S3C).

92 We selected for further analysis two CRISPRa control clones and two CRISPRa *Mest* clones
93 (expressing the D sgRNA, Fig. 3A). On average, there was a 3.2 fold increase in *Mest* transcript in
94 CRISPRa *Mest* clones compared to clones expressing the control sgRNA (Fig. 3B). As for CRISPRi,
95 *Copg2* expression was unaffected (Fig. 3C). Strikingly, the levels of both *miR-335-3p* and *miR-335-5p*
96 also increased by a ~3-fold (Fig. 3D, E). Thus, activating the distal promoter of *Mest* with
97 CRISPRa/SAM is sufficient to increase hosted *miR-335* levels in mESCs.

98

99 **CRISPRa on *miR-335* putative promoter does not affect *miR-335* levels**

100 A previous study, based on luciferase assays performed in HEK293T cells, suggests that the sequence
101 upstream of *miR-335* (situated in a *Mest* intron) has some promoter activity (Zhu et al. 2014). Thus, we
102 next tested whether we could upregulate *miR-335* by directing SAM to this genomic region.

103 Because SAM efficiency correlates with baseline expression levels of the targeted gene – the
104 fold of upregulation is inversely correlated with basal transcript level- (Konermann et al. 2015), and to
105 maximize the chance to increase *miR-335*, SAM experiments were performed on cells with lower
106 baseline levels of *miR-335* than mESCs. We observed that *miR-335*-3p and *miR-335*-5p levels were
107 respectively 13 and 47 times lower in MEFs compared to mESCs (Fig. 4A, B). *Mest* expression was
108 also ~60 times less expressed in MEFs than in mESCs (Fig. 4C), adding further support for the
109 coregulation of *Mest* and *miR-335*.

110 We designed three sgRNAs ($\mu 1$, $\mu 2$, and $\mu 3$) in the putative *miR-335* promoter –a region named
111 pro2 in (Zhu et al. 2014)- and compared their efficiency in upregulating *miR-335* to sgRNAs that target
112 *Mest* promoters (Fig. 4E). sgRNAs P1 and P2 (which target *Mest* P promoter) strongly upregulated
113 *Mest* (Fig. 4E) but also *miR-335* mature products in SAM MEFs (Fig. 4G, H). The upregulation of *Mest*
114 was much higher in MEFs than in mESCs, as expected from their relative *Mest* baseline levels (see
115 Fig. 4C). By contrast, the three sgRNAs that target the putative promoter of *miR-335* ($\mu 1$, $\mu 2$, and $\mu 3$)
116 did not affect *miR-335*-3p nor *miR-335*-5p levels (Fig. 4G, H). Thus, this genomic sequence likely does
117 not regulate *miR-335* expression in MEFs. We cannot rule out that *miR-335* has an independent
118 promoter located in another region. In this context, prediction of *miR-335* promoter location using
119 DeepCAGE data (Marsico et al. 2013) suggests that there could be several *miR-335* promoters
120 depending on the tissue. According to Marsico and coworkers, the most probable *miR-335* promoters
121 are *Mest* (D) and (P) promoters - what we confirmed experimentally here-, and less probably, a third
122 region situated in another intron of *Mest*.

123 Data obtained in MEFs also revealed that transactivating *Mest* (P) promoter resulted in a strong
124 increase in *Mest* and *miR-335* while transactivating (D) promoter had moderate effects. This contrasts

125 with results obtained in ESCs where the most potent sgRNAs were those targeting the (D) promoter
126 (Fig. 3 and Supplemental Fig. S3). Taken together, these data suggest that transcriptional activation of
127 one or the other *Mest* promoter, depending on the cell type, is sufficient to increase the levels of intronic
128 *miR-335*. This also supports the existence of primary transcripts, originating either at (D) or (P)
129 promoters, that contain both *Mest* and *miR-335* precursors.

130 To conclude, CRISPRa and CRISPRi experiments on *Mest* and *miR-335* in mouse cells reveal
131 that transcription of an intronic miRNA is regulated by the promoter of its host gene. Previous works
132 propose that evolutionarily conserved intronic miRNAs, such as *miR-335*, are more frequently co-
133 expressed with host genes than recently appeared intronic miRNAs (He et al. 2012; Steiman-Shimony
134 et al. 2018). This suggests that the transcription of conserved intronic miRNAs depends on the host
135 promoter while recently appeared intronic miRNAs tend to have independent promoters. To test these
136 predictions our CRISPRa/i approach could be used to map miRNA promoters on a genome-wide scale.

137 MATERIALS AND METHODS

138 Cell culture

139 E14Tg2a mouse ESCs and their CRISPRa and CRISPRi derivatives were cultivated on gelatine coated
140 dishes and maintained pluripotent in Serum/Lif media as described (Varrault et al. 2018). Organoids
141 were generated in 96-well (U-bottom) Ultra-Low Attachment plates (Sumitomo) by seeding 3000 ESCs
142 in corticogenesis medium 1: DMEM/F-12/GlutaMAX supplemented with 10% KSR, 0.1 mM of non-
143 essential amino acids, 1 mM of sodium pyruvate, 50U/ml penicillin/streptomycin, 0.1 mM of 2-
144 mercaptoethanol (Sigma), 1 μ M DMH1-HCl (in house synthesized, Vanderbilt University) and 240 nM
145 IWP-2 (Tocris). On day 8 of differentiation, organoids were transferred to bacterial plates (Greiner) in
146 corticogenesis medium 2: DMEM/F-12/GlutaMAX supplemented with N2 and B27 (without vitamin A)
147 supplements, 500 μ g/ml of BSA, 0.1 mM of non-essential amino acids, 1 mM of sodium pyruvate, 0.1
148 mM of 2-mercaptoethanol, and 50U/ml penicillin/streptomycin. Immortalized CRISPRa (SAM) MEFs
149 (gift from Giacomo Cavalli's lab, unpublished) were cultivated in DMEM supplemented with 10% FBS

150 and 50U/ml penicillin/streptomycin. All media components were from Life Technologies unless
151 otherwise stated. Cell lines were routinely tested for the absence of mycoplasma (Mycoalert, Lonza).

152 **Generation of constructs expressing sgRNAs**

153 sgRNA sequences targeting *Mest* promoters were designed using CRISPick
154 <https://portals.broadinstitute.org/gppx/crispick/public> (formerly GPP sgRNA Design tool) or manually.
155 sgRNAs that target the putative miR-335 promoter (mm10_dna range=chr6_30740830-30741300) were
156 designed using CHOPCHOP (Labun et al. 2019). Pairs of oligonucleotides (Eurofins) were annealed
157 and subcloned into either sgRNA(MS2) cloning backbone (Addgene Plasmid #61424) or Lenti
158 sgRNA(MS2)_zeo backbone (Konermann et al., 2015) (Addgene plasmid # 61427) that were previously
159 digested with either BbsI or BsmBI (NEB), respectively, and purified on a Chromaspin column
160 (Clontech). All constructs were verified by Sanger sequencing (Genewiz). sgRNA sequences are listed
161 in [Supplemental Table S1](#).

162 **Lentiviruses production**

163 Lentiviruses were prepared as described elsewhere (Lin et al. 2002). Briefly, lentiviral transfer vectors
164 were co-transfected with the HIV packaging plasmid psPAX2 and the plasmid pMD2G (coding for the
165 vesicular stomatitis virus envelope glycoprotein G), in HEK-293T cells by the calcium phosphate
166 method. Supernatants were collected at day 2 post-transfection and concentrated on sucrose by
167 ultracentrifugation at 95 528g for 1.5 h at 4°C.

168 **Generation of CRISPRi ESC lines using PiggyBac Transposition**

169 E14Tg2a mouse ESCs were co-transfected with pCMV-HA-HyperpiggyBase (Yusa et al., 2011) and
170 pB-dCas9-KRAB-MecP2 (Yeo et al. 2018) (Addgene plasmid # 110824) using a Neon transfection
171 system (Life Technologies). Forty-eight hours post-transfection, cells were selected using Blastidicin
172 (15 µg/ml, SIGMA). Stable pB-dCas9-KRAB-MeCP2 ESCs (CRISPRi ESCs) were then transduced with
173 lentiviruses expressing the following sgRNAs: control, *Mest* distal promoter, *Mest* proximal promoter#1,

174 or *Mest* proximal promoter#2. Seventy-two hours post-infection, cells were selected using hygromycin
175 (1 mg/ml, Life Technologies), and clones were picked and expanded in ESC media.

176 **Generation of SAM CRISPRa ESC lines targeting *Mest* promoters**

177 E14Tg2a ESCs stably expressing the SAM system (Bonev et al. 2017) –SAM ESCs- were transfected
178 with Lenti sgRNA(MS2)_zeo plasmids expressing the following sgRNAs: control, *Mest* distal promoter,
179 *Mest* proximal promoter#1, or *Mest* proximal promoter#2. ESCs were selected using Zeocin (250 µg/ml,
180 Life Technologies) and clones were picked and expanded.

181 **Transient transfection of SAM MEFs**

182 80 000 MEFs stably expressing the SAM system (SAM MEFs) were transfected using Lipofectamine
183 2000 with 300 ng of sgRNA(MS2) plasmid expressing either one control sgRNA, one *Mest* distal
184 promoter sgRNA (out of 3 different sgRNAs), one *Mest* proximal promoter sgRNA (out of 2 different
185 sgRNAs), or one *miR-335*-putative promoter sgRNA (out of 3 different sgRNAs). Forty-eight hours later,
186 RNAs were harvested.

187 **RNA extraction and RT-qPCR**

188 Total RNAs were extracted using quick-RNA miniprep kits (Zymo) and quantified on a Nanodrop. RNAs
189 were retro-transcribed with N6 primers and M-MuLV retro-transcriptase (RT). qPCR was performed
190 using validated primers and SYBR Green Mix (Roche) in 384-well plates on a LightCycler480 device
191 (Roche) as described in (Varrault et al. 2018). The level of expression of each gene was normalized to
192 the average expression levels of three housekeeping genes selected with geNorm (Vandesompele et
193 al. 2002): *Gapdh*, *Tbp*, and *Mrpl32* for ESCs and *Gapdh*, *Tbp* and *Gusb* for MEFs. qPCR primer
194 sequences are listed in [Supplemental Table S2](#).

195 miRNAs were retro-transcribed with gene-specific primers and multiscribe RT (Life Technologies). Their
196 levels of expression were measured with TaqMan probes (miRNA Taqman assays # 000546 for miR-

197 335-5p, and # 002185 for miR-335-3p). and normalized to that of U6 snoRNA (assay # 001973)
198 (ThermoFisher). We found that U6 was stably expressed across samples (not shown).

199 **Visualization of RNA-seq experiment**

200 RNA-seq reads from mESCs -GSE75486 (Bouchet et al. 2017)- were visualized using Integrative
201 Genomics Viewer (Robinson et al. 2011) -version: 2.8.13-.

202 **Immunofluorescence**

203 Immunofluorescence experiments were performed as described (Varrault et al. 2018) using antibodies
204 directed against (species; provider; catalog number): CAS9 (mouse; Cell signalling; #14697); NANOG
205 (mouse; BD Pharmingen; #560259); NESTIN (mouse; Santa Cruz; sc-33677); PAX6 (mouse; Covance;
206 PRB-278P); POU5F1 (rabbit; Cell signalling; #2840); TBR1 (rabbit, Cell signalling; #49661); TUBB3
207 (mouse; Covance; MMS-435P). Secondary antibodies were anti-mouse or anti-rabbit coupled to Alexa
208 Fluor® 488 or Cy3 (Jackson Immunoresearch Laboratories). Nuclei were labeled with DAPI and slides
209 were mounted with mowiol and observed under a fluorescence microscope (ImagerZ1, Zeiss). Images
210 of organoids were obtained by tiling and stitching, and insets were taken using the apotome mode.

211 **Statistical analysis**

212 Statistical analysis was carried out using GraphPad Prism Version 8 (GraphPad Software, San Diego,
213 USA). Mann-Whitney test was used for comparing differences between two groups. p values < 0.05
214 were considered statistically significant.

215 **COMPETING INTEREST STATEMENT**

216 The authors declare no competing interests.

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227 **AUTHOR CONTRIBUTIONS**

228 T.B. conceived the project and designed the study with inputs from A.V.; I.C., M.C., and T.B. performed
229 cell culture and qPCR experiments; A.L.D. and T.B. generated constructs; A.M. and C.L. generated
230 lentiviruses; C.H. generated DMH1-HCl; T.B. generated CRISPR cell lines; T.B. performed all of the
231 analyses and drafted the manuscript; A.V. and T.B. revised the manuscript. All authors read and
232 approved the final version of the manuscript.

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324

325 **FIGURE LEGENDS**

326 **Figure 1.** CRISPRi on *Mest* suppresses the expression of hosted *miR-335* in embryonic stem cells

327 (A) Transcription originates from the proximal promoter of *Mest* (P) in mouse embryonic stem cells.

328 Integrative Genomics Viewer tracks showing coverage plot and alignment of RNA-seq reads for mouse
329 embryonic stem cells. Reads for *Mest* (blue) are transcribed from the plus strand, while reads from
330 *Copg2* (pink) are transcribed from the minus strand. Chromosomal coordinates and gene annotation
331 are from the RefSeq mm9 build. D: *Mest* distal promoter; P: *Mest* proximal promoter.

332 (B) Schematic of mouse *Mest* gene with the CRISPRi module (dCas9-KRAB-MeCP2) targeting the
333 proximal promoter P of *Mest*.

334 (C, D) Repression of *Mest* promoter downregulates *Mest* (C) but does not affect the expression of
335 neighboring *Copg2* (D). RNAs were quantified in two CRISPRi ESC clones expressing the control
336 sgRNA (grey) and two CRISPRi ESC clones expressing *Mest* sgRNA (red). Data are mean \pm sem of
337 five independent experiments and expressed as fold change over control clone #1. **:p<0.01 (Mann-
338 Whitney test).

339 (E, F) Influence of repressing *Mest* promoter on miR-335-3p and miR-335-5p levels. Data are mean \pm
340 sem of five independent experiments and expressed as fold change over control clone #1. **:p<0.01
341 (Mann-Whitney test).

342 **Figure 2.** *miR-335* expression depends on *Mest* promoter activity in brain organoids

343 (A) Immunofluorescence staining on brain organoids derived from mESCs using antibodies for brain
344 primordium markers NESTIN/PAX6 (middle panels) and TUBB3/TBR1 (right panels) after eight and 15

345 days of differentiation. The top panels show entire organoids. The bottom panels are zoom-in insets of
346 an area in organoids. Scale bars: 200 μm for organoids (top panels) and 50 μm for insets (bottom
347 panels).

348 (B) Time course of expression of *Mest* and *miR-335* mature products during the development of brain
349 organoids from CRISPRi ESCs stably expressing either control sgRNA or *Mest* sgRNA. Heatmap
350 shows the mean of four independent experiments performed on two CRISPRa sgRNA control and two
351 CRISPRa sgRNA ESC clones. Heatmap was built using Morpheus.

352 <https://software.broadinstitute.org/morpheus/>

353 **Figure 3.** CRISPRa on *Mest* increases the expression of hosted *miR-335* in embryonic stem cells

354 (A) Schematic of mouse *Mest* gene structure with the CRISPRa SAM - synergistic activation mediator-
355 module targeting the distal promoter D of *Mest*.

356 (B, C) Transactivation of *Mest* promoter upregulates *Mest* (B) but does not affect neighboring *Copg2*
357 expression (C). Data are mean \pm sem of five independent experiments performed on two CRISPRa
358 sgRNA control (grey) and two CRISPRa sgRNA *Mest* clones (green) and expressed as fold change
359 over control clone#1. **:p<0.01 (Mann-Whitney test).

360 (D; E) Transactivation of *Mest* promoter increases miR-335-3p (D) and miR-335-5p (E) levels. Data are
361 mean \pm sem of four independent experiments and expressed as fold change over control clone#1.
362 *:p<0.05 (Mann-Whitney test).

363 **Figure 4.** CRISPRa on *miR-335* putative promoter does not affect *miR-335* levels in MEFs

364 (A-C) Endogenous expression of *miR-335* products and *Mest* is weaker in SAM MEFs than in SAM
365 ESCs. Data are mean \pm sem of qPCR experiments performed on five MEF and five ESC samples and
366 normalized to the average value obtained on ESCs. **:p<0.01 (Mann-Whitney test).

367 (D) Structure of mouse *Mest* gene with SAM targeting either the distal promoter of *Mest* D (D1-D2
368 sgRNAs), the proximal promoter of *Mest* P (P1-P3 sgRNAs), or the putative promoter of *miR-335* (μ 1-
369 μ 3 sgRNAs).

370 (E-H) Levels of expression of *Mest* (E), *Copg2* (F), *miR-335-3p* (G), and *miR-335-5p* (H) were
371 measured after transactivation of either *Mest* D or P promoters or *miR-335* putative promoter. SAM
372 MEFs were transfected with plasmids expressing sgRNAs targeting *Mest* D (sgRNAs D1, D2, and D3),
373 *Mest* P (P1 and P2), or the putative promoter of *miR-335* (μ 1, μ 2, and μ 3). Data are mean \pm sem of
374 three to four independent experiments and expressed as fold change over sgRNA control taken as 1. *:
375 $p < 0.05$ in Mann-Whitney test (comparison with sgRNA control values). None of the sgRNAs that direct
376 the CRISPRa/SAM machinery towards the *miR-335* putative promoter (μ 1, μ 2, and μ 3) altered *miR-*
377 *335* levels.

378 SUPPLEMENTAL FIGURE LEGENDS

379 **Supplemental Figure S1.** Characterization of the dCas9-KRAB-MeCP2 (CRISPRi) ESC line

380 (A) Expression of CAS9 in parental E14Tga2 ESCs and their CRISPRi derivatives. Scale bars: 50 μ m.

381 (B) Expression of pluripotency factors POU5F1 (green) and NANOG (red) in parental E14Tga2 ESCs
382 and their CRISPRi derivatives. Scale bars: 20 μ m.

383 **Supplemental Figure S2.** Efficient CRISPRi of *Mest* when targeting its proximal promoter

384 (A) Structure of mouse *Mest* gene with the CRISPRi module (dCa9-KRAB-MeCP2) directed to either
385 the distal (D) (sgRNA D, green) or proximal (P) (sgRNAs P1 -purple- and P2 -red-) promoter of *Mest*.
386 The sgRNA control (grey) has no match in the mouse genome.

387 (B, C) Repression of *Mest* proximal promoter downregulates *Mest* expression (B) and does not affect
388 neighboring *Copg2* expression (C). CRISPRi ESCs were transduced with lentiviruses expressing either
389 sgRNA control, sgRNA D, sgRNA P1, or sgRNA P2. RNAs were measured by RT-qPCR. Data are

390 mean \pm sem of seven independent experiments and expressed as fold change over control sgRNA. *:
391 $p < 0.05$, ***: $p < 0.001$ in Mann-Whitney test (comparison with sgRNA control values). Only the sgRNAs
392 targeting the proximal promoter repressed *Mest*.

393 **Supplemental Figure S3.** Efficient CRISPRa of *Mest* when targeting its distal promoter

394 (A) Structure of mouse *Mest* gene with the CRISPRa tool SAM targeting either the distal (sgRNA D,
395 green) or the proximal (sgRNAs P1 -purple- and P2 -red-) promoter of *Mest*. The sgRNA control (grey)
396 has no match in the mouse genome.

397 (B, C) Transactivation of *Mest* distal promoter upregulates *Mest* expression (B) and does not affect
398 neighboring *Copg2* expression (C). CRISPRa SAM ESCs were transduced with lentivirus expressing
399 either SgRNA control, D, P1, or P2. *Mest* (B) and *Copg2* (C) were measured by RT-qPCR. Data are
400 mean \pm sem of four independent experiments. *: $p < 0.1$ in Mann-Whitney test. Only the sgRNA targeting
401 the distal promoter upregulated *Mest* RNA.

402 **Supplemental Table S1.** Sequences of sgRNAs

403 **Supplemental Table S2.** Primers used for qPCR assays

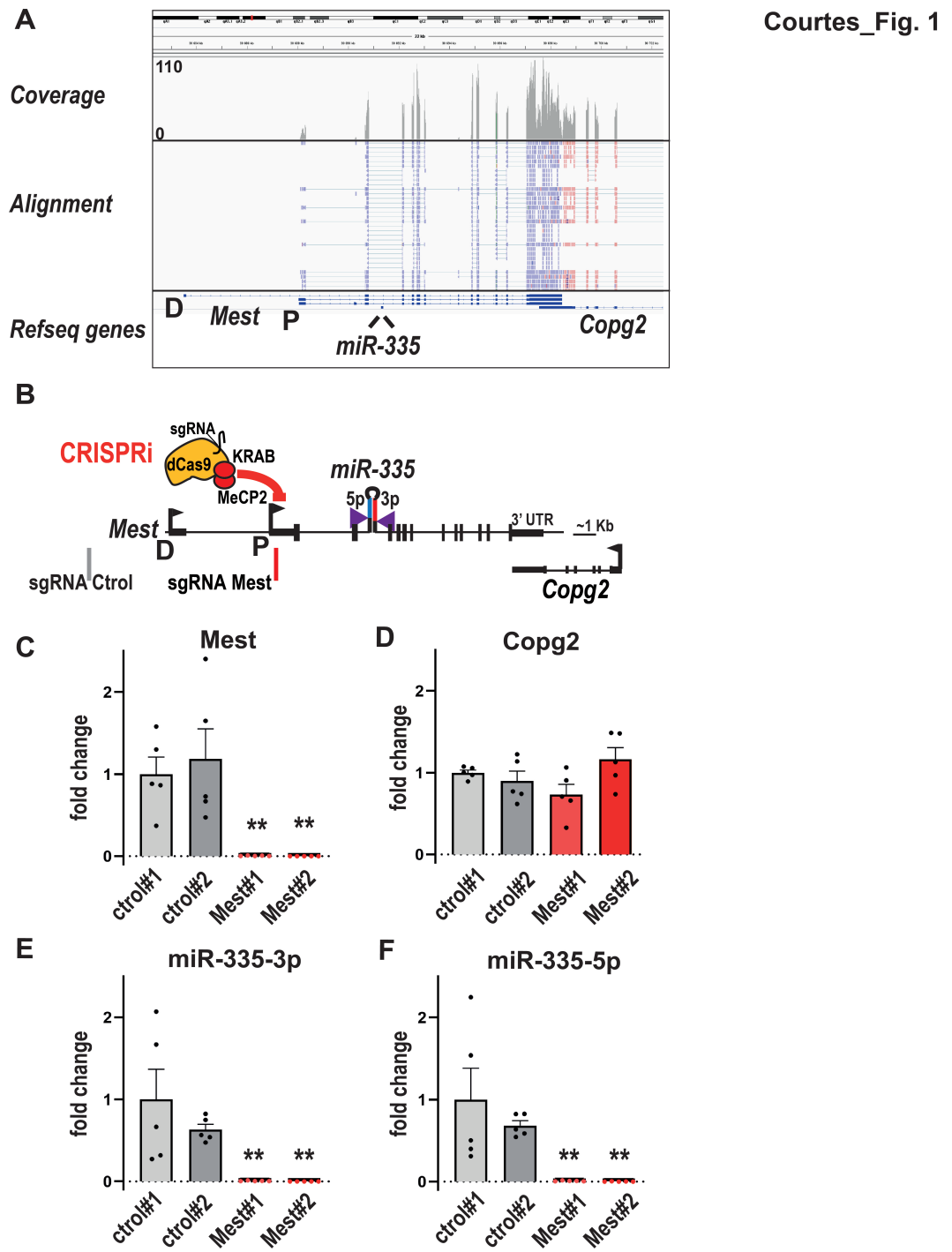


Figure 1. CRISPRi on *Mest* suppresses the expression of hosted *miR-335* in embryonic stem cells

(A) Transcription originates from the proximal promoter of *Mest* (P) in mouse embryonic stem cells. Integrative Genomics Viewer tracks showing coverage plot and alignment of RNA-seq reads for mouse embryonic stem cells. Reads for *Mest* (blue) are transcribed from the plus strand, while reads from *Copg2* (pink) are transcribed from the minus strand. Chromosomal coordinates and gene annotation are from the RefSeq mm9 build. D: *Mest* distal promoter; P: *Mest* proximal promoter.

(B) Schematic of mouse *Mest* gene with the CRISPRi module (dCas9-KRAB-MeCP2) targeting the proximal promoter of *Mest*.

(C, D) Repression of *Mest* promoter downregulates *Mest* (C) but does not affect the expression of neighboring *Copg2* (D). RNAs were quantified in two CRISPRi ESC clones expressing the control sgRNA (grey) and two CRISPRi ESC clones expressing the *Mest* sgRNA (red). Data are mean \pm sem of five independent experiments and expressed as fold change over control clone #1. **:p<0.01 (Mann-Whitney test).

(E, F) Influence of repressing *Mest* promoter activity on *miR-335-3p* and *miR-335-5p* levels. Data are mean \pm sem of five independent experiments and expressed as fold change over control clone #1. **:p<0.01 (Mann-Whitney test).

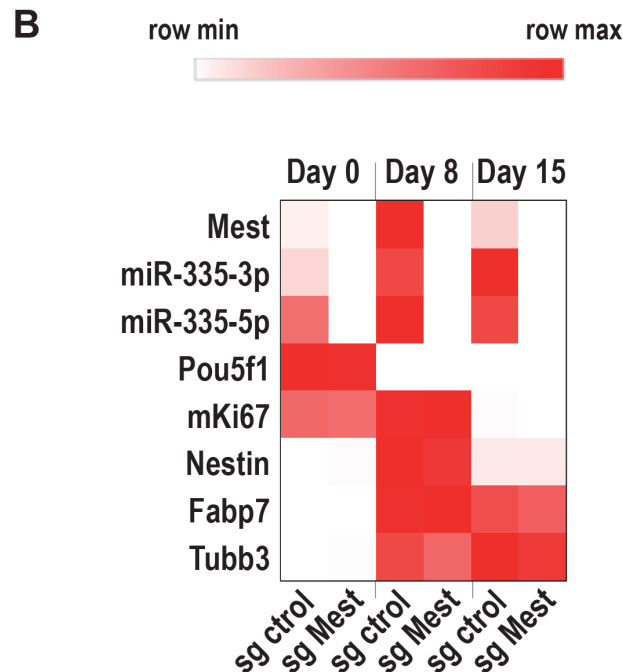
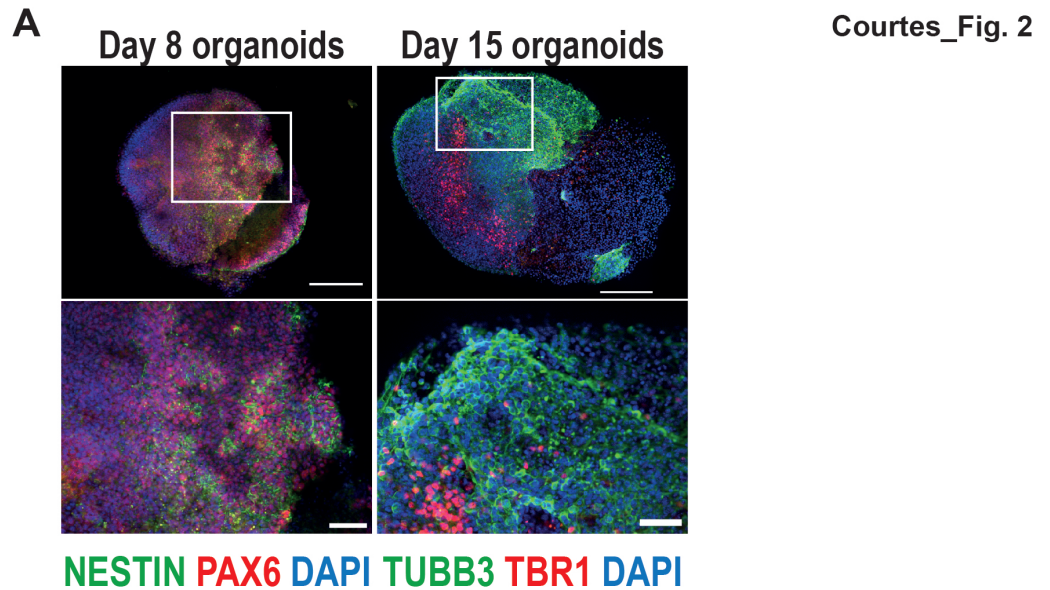


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(B) Time course of expression of *Mest* and *miR-335* mature products during brain organogenesis from CRISPRi ESCs stably expressing either control sgRNA or *Mest* sgRNA. Heatmap shows the mean of four independent experiments performed on two CRISPRa sgRNA control and two CRISPRa sgRNA ESC clones. Heatmap was built using Morpheus. <https://software.broadinstitute.org/morpheus/>

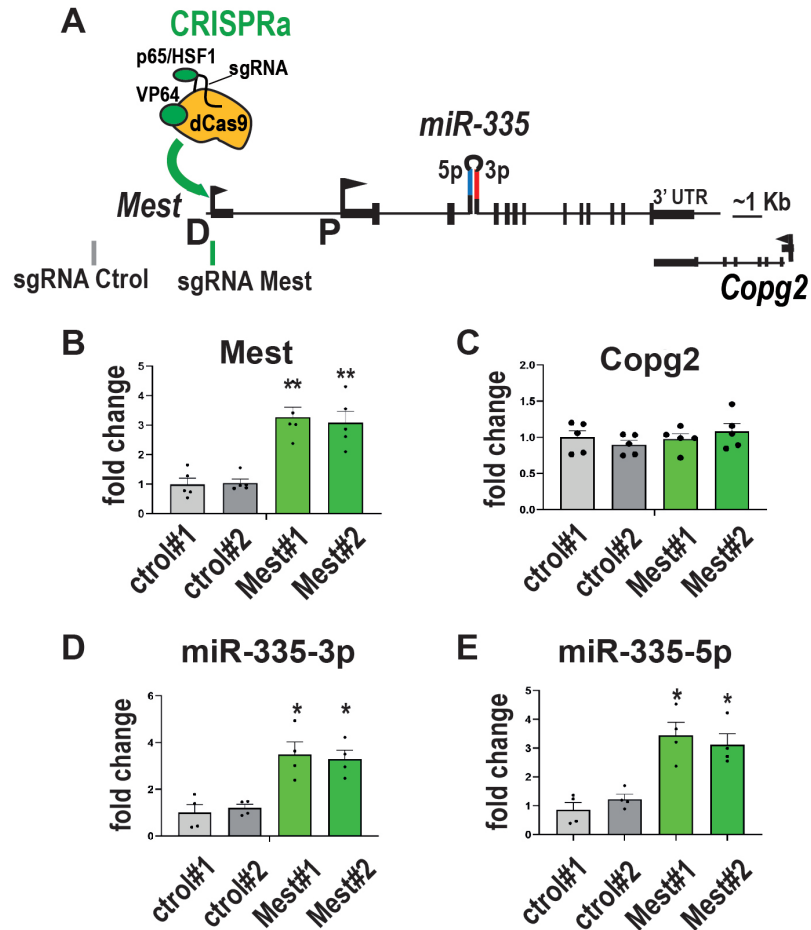


Figure 3. CRISPRa on *Mest* increases the expression of hosted *miR-335* in embryonic stem cells

(A) Schematic of mouse *Mest* gene structure with the CRISPRa SAM - synergistic activation mediator- module targeting the distal promoter D of *Mest*.

(B, C) Transactivation of *Mest* promoter upregulates *Mest* (B) but does not affect neighboring *Copg2* expression (C). Data are mean \pm sem of five independent experiments performed on two CRISPRa sgRNA control (grey) and two CRISPRa sgRNA *Mest* clones (green) and expressed as fold change over control clone#1. **:p<0.01 (Mann-Whitney test).

(D; E) Transactivation of *Mest* promoter increases *miR-335-3p* (D) and *miR-335-5p* (E) levels. Data are mean \pm sem of four independent experiments and expressed as fold change over control clone#1. *:p<0.05 (Mann-Whitney test).

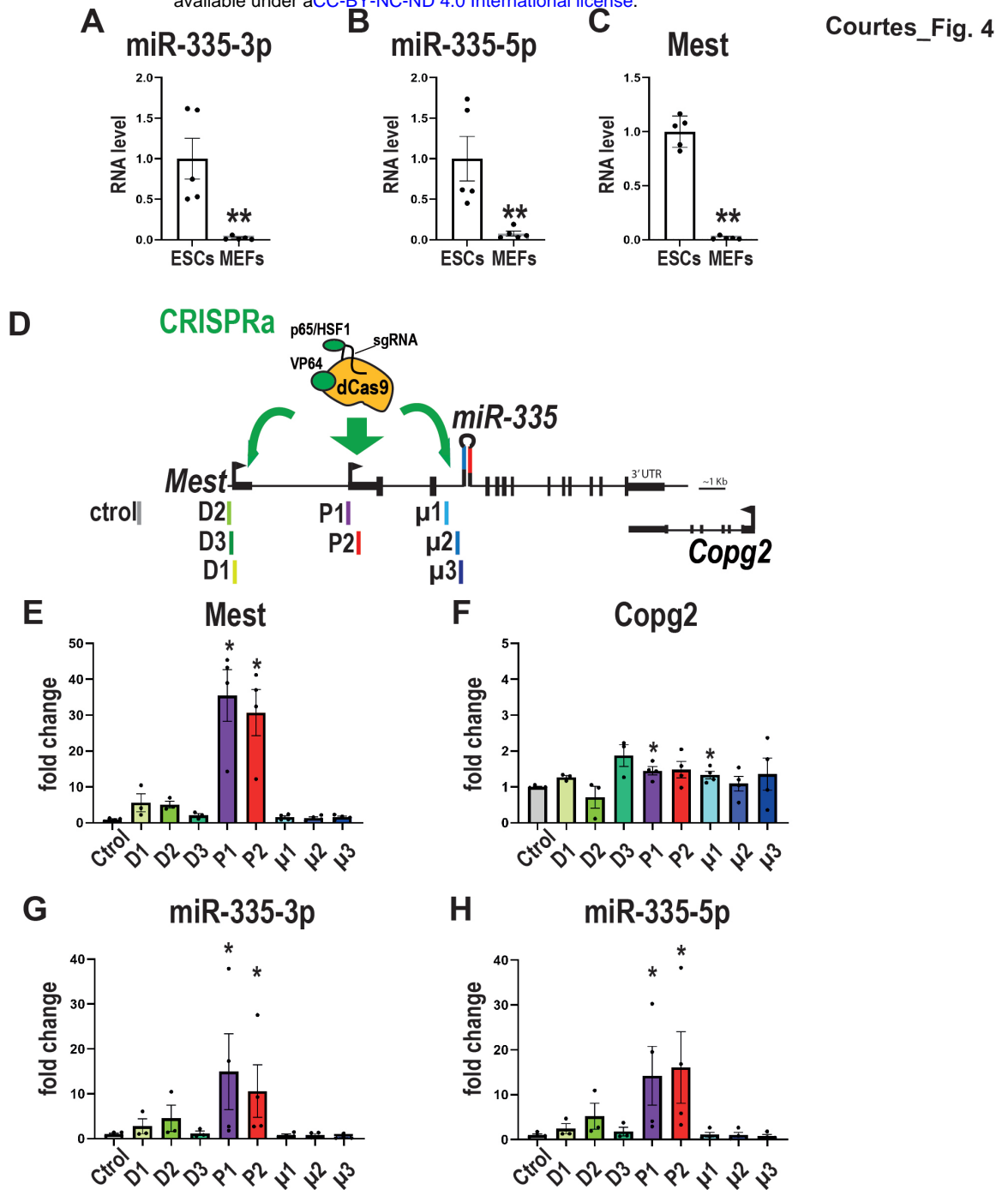
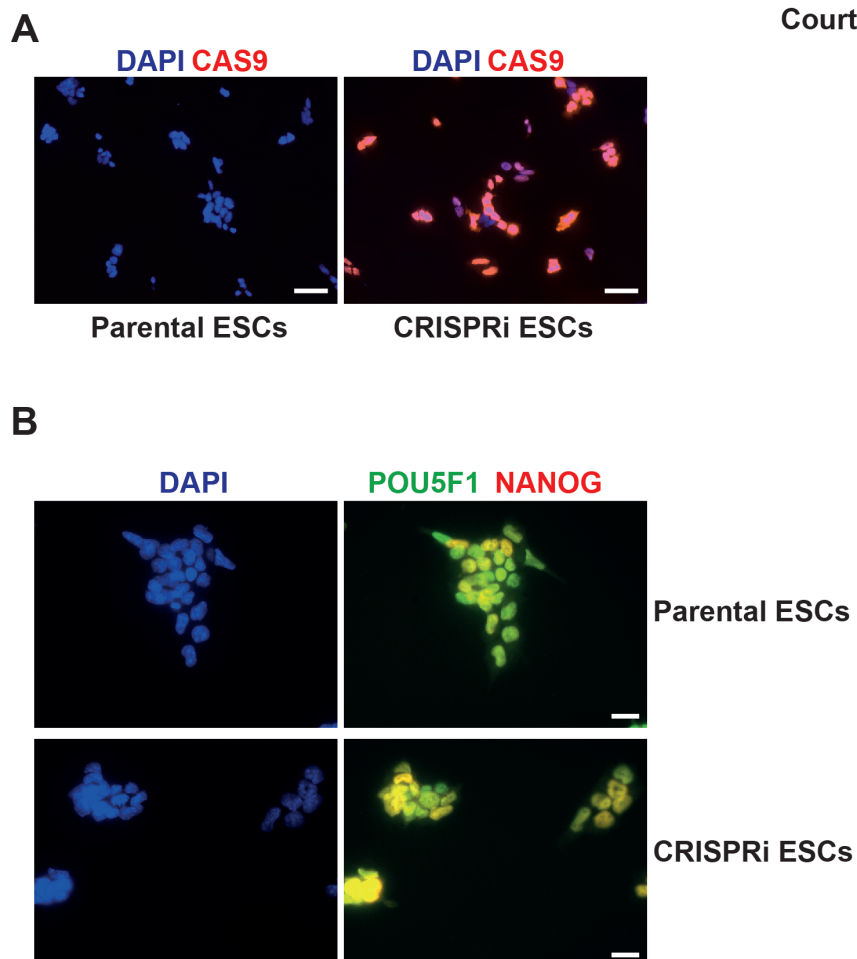


Figure 4. CRISPRa on *miR-335* putative promoter does not affect *miR-335* levels in MEFs

(A-C) Endogenous expression of *miR-335* products and *Mest* is weaker in SAM MEFs than in SAM ESCs. Data are mean \pm sem of qPCR experiments performed on five MEF and five ESC samples and normalized to the average value obtained on ESCs. **:p<0.01 (Mann-Whitney test).

(D) Structure of mouse *Mest* gene with SAM targeting either the distal promoter of *Mest* D (D1-D2 sgRNAs), the proximal promoter of *Mest* P (P1-P3 sgRNAs), or the putative promoter of *miR-335* (μ 1- μ 3 sgRNAs).

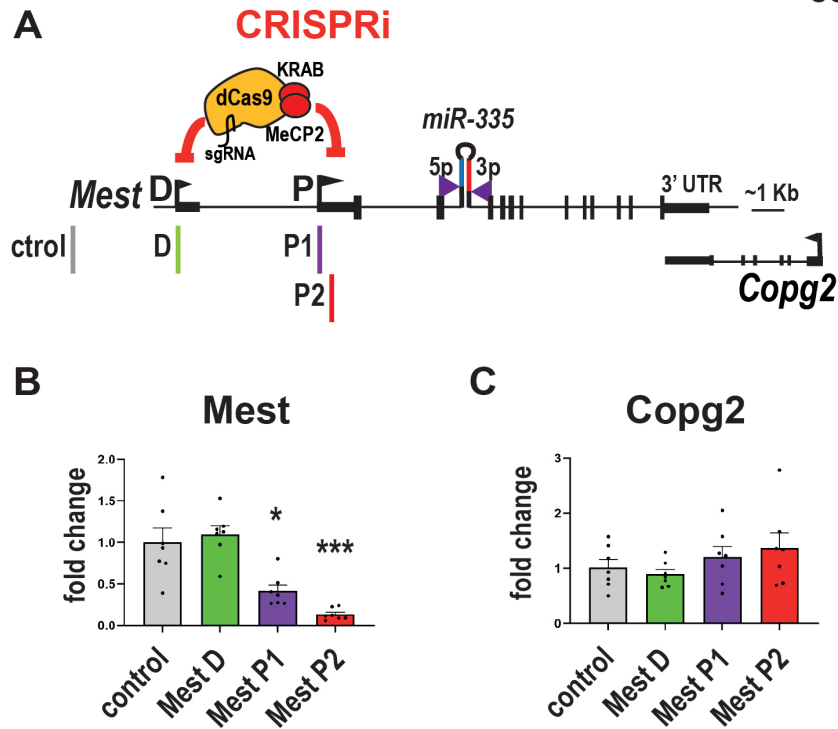
(E-H) Levels of expression of *Mest* (E), *Copg2* (F), *miR-335-3p* (G), and *miR-335-5p* (H) were measured after transactivation of either *Mest* D or P promoters or *miR-335* putative promoter. SAM MEFs were transfected with plasmids expressing sgRNAs targeting *Mest* D (sgRNAs D1, D2, and D3), *Mest* P (P1 and P2), or the putative promoter of *miR-335* (μ 1, μ 2, and μ 3). Data are mean \pm sem of three to four independent experiments and expressed as fold change over sgRNA control taken as 1. *: p<0.05 in Mann-Whitney test (comparison with sgRNA control values). Note that none of the sgRNAs that direct the CRISPRa/SAM machinery towards the *miR-335* putative promoter (μ 1, μ 2, and μ 3) altered *miR-335* levels.



Supplemental Figure S1. Characterization of the dCas9-KRAB-MeCP2 (CRISPRi) ESC line

(A) Expression of CAS9 in parental E14Tga2 ESCs and their CRISPRi derivatives. Scale bars: 50 μ m.

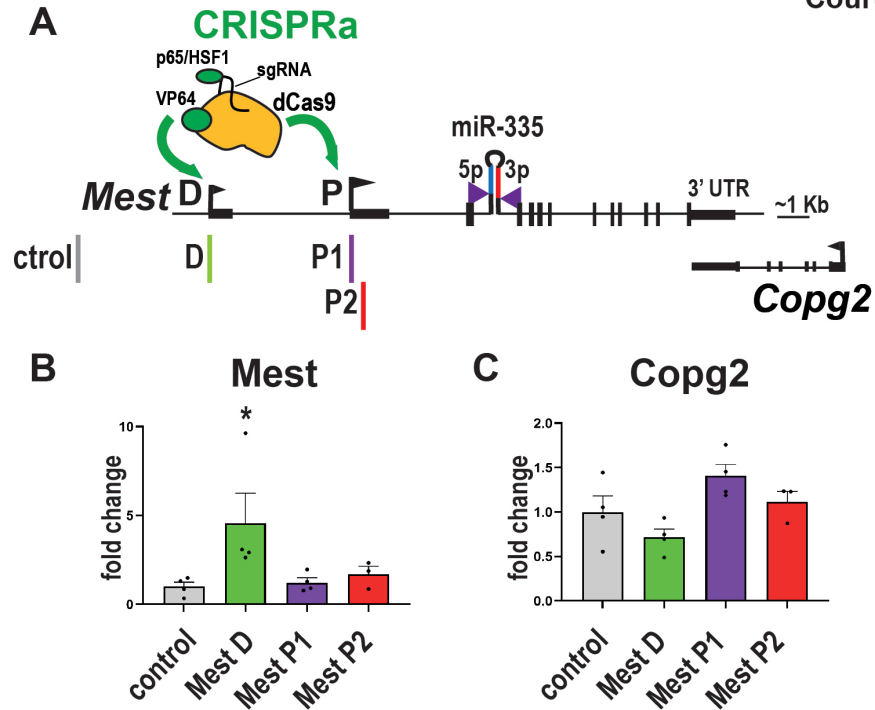
(B) Expression of pluripotency factors POU5F1 (green) and NANOG (red) in parental E14Tga2 ESCs and their CRISPRi derivatives. Scale bars: 20 μ m.



Supplemental Figure S2. Efficient CRISPRi of *Mest* when targeting its proximal promoter

(A) Structure of mouse *Mest* gene with the CRISPRi module (dCas9-KRAB-MeCP2) directed to either the distal (*D*) (sgRNA *D*, green) or proximal (*P*) (sgRNAs *P1* -purple- and *P2* -red-) promoter of *Mest*. The sgRNA control (grey) has no match in the mouse genome.

(B, C) Repression of *Mest* proximal promoter downregulates *Mest* expression (B) and does not affect neighboring *Copg2* expression (C). CRISPRi ESCs were transduced with lentiviruses expressing either sgRNA control, sgRNA *D*, sgRNA *P1*, or sgRNA *P2*. RNAs were measured by RT-qPCR. Data are mean \pm sem of seven independent experiments and expressed as fold change over control sgRNA. *: $p < 0.05$, ***: $p < 0.001$ in Mann-Whitney test (comparison with sgRNA control values). Only the sgRNAs targeting the proximal promoter repressed *Mest*.



Supplemental Figure S3. Efficient CRISPRa of *Mest* when targeting its distal promoter

(A) Structure of mouse *Mest* gene with the CRISPRa tool SAM targeting either the distal (sgRNA D, green) or the proximal (sgRNAs P1 -purple- and P2 -red-) promoter of *Mest*. The sgRNA control (grey) has no match in the mouse genome.

(B, C) Transactivation of *Mest* distal promoter upregulates *Mest* expression (B) and does not affect neighboring *Copg2* expression (C). CRISPRa SAM ESCs were transduced with lentivirus expressing either SgRNA control, D, P1, or P2. *Mest* (B) and *Copg2* (C) were measured by RT-qPCR. Data are mean \pm sem of four independent experiments. *: $p < 0.1$ in Mann-Whitney test. Only the sgRNA targeting the distal promoter upregulated *Mest* RNA.

sgRNA	sequence 5' to 3'
Control (Addgene #61424)	GGGTCTTCGAGAAGACCTGT
Control (Addgene #61427)	GGAGACGGGATACCGTCTCT
Mest p1	GCTCAGTGGGCTTTAAAAGT
Mest p2	GGCGCAGCAGCTTTCCTCTG
Mest d1	GAGGGCCCAGCGGGGCGGCG
Mest d2	AACCAGGGGAAGGACAGCTG
Mest d3	CAACCCAAATCACCTGCCCC
miR-335 μ 1	TTTTGAGCGCCCCTAGTGTC
miR-335 μ 2	TTACAACAGCATTGGAGAT
miR-335 μ 3	GAAGAAACCGAGAAACAGAT

Supplemental Table S1. Sequence of sgRNAs

Symbol	Gene ID	Forward Seq.	Reverse Seq.
<i>Mest</i>	17294	CAACAATGACGGCAACCTGGT	TCTGAATTTCTTCCTTTGATTAATGTACTGTA
<i>Copg2</i>	54160	TGATGTGGTTAAACGATGGATAAATGAAG	TGGAGACAGCAAGCCGATCAT
<i>Tubb3</i>	22152	CCAGTGCGGCAACCAGATAGG	AAAGGCGCCAGACCGAACACT
<i>Nanog</i>	71950	GCCTCTCCTCGCCCTTCCTCT	CCACCGCTTGCACTTCATCCTT
<i>Pou5f1</i>	18999	CTGTAGGGAGGGCTTCGGGCACTT	CTGAGGGCCAGGCAGGAGCAGAG
<i>Fabp7</i>	12140	TCCAGCTGGGAGAAGAGTTT	CCAACCGAACCCACAGACTTA
<i>Nes</i>	18008	CGGAGAGGGAGCAGCACCAA	GGCCTCCCCACAGCATCCT
<i>Gapdh</i>	14433	GGAGCGAGACCCCACTAACA	ACATACTCAGCACCGGCCTC
<i>Gusb</i>	110006	GATTCAGATATCCGAGGAAAGG	GCCAACGGAGCAGGTTGA
<i>Mki67</i>	17345	TCCAGACTTCCACAGAGAC	TTCACCTTCATCCAGATTCAC
<i>Mrpl32</i>	75398	AGGTGCTGGGAGCTGCTACA	AAAGCGACTCCAGCTCTGCT
<i>Tbp</i>	21374	ACTTCGTGCAAGAAATGCTGAAT	CAGTTGTCCGTGGCTCTCTTATT

Supplemental Table S2. Primers used for qPCR assays