# CIBZ Regulates Mesodermal and Cardiac Differentiation of by Suppressing T and Mesp1 Expression in Mouse Embryonic Stem Cells

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## Supplementary Supporting Information

**Supplementary Methods**
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## Supplementary Methods

### siRNA and Transient Transfection

Mouse ESCs were transfected with 50-nM CIBZ-specific or scrambled negative control dicer substrate siRNA duplexes (Integrated DNA Technologies) as described previously[18]. Transfection with siRNA was performed using INTERFERin reagent (Polyplus Transfection) according to the manufacturer's instructions. Subsequently, siRNA-transfected cells were cultured for 3 days and were then cultured under rotary suspension conditions for an additional 2 days.

### Chromatin Immunoprecipitation (ChIP) Assays

ChIP assays were performed as described previously[17]. Amplification of immunoprecipitated DNA was achieved using Blend Tag plus DNA polymerase (Toyobo), after annealing primers (Table S3) for promoter regions (prom) and distal regions (dist) at 55°C. All experiments were repeated three times.

**Supplementary Figure Legends**

S1. Expression of Nanog, Oct3/4, and Sox2 in WT and CIBZ$^{-/-}$ ESCs during ESC differentiation; mRNA levels of the indicated genes were determined using semi-quantitative PCR with GAPDH as a loading control.

S2. Knockdown of CIBZ in ESCs upregulates T and Mesp1 during ESC differentiation; siRNA duplexes were used to reduce CIBZ expression in ESCs. Three days after transfection with 50-nM CIBZ siRNA or 50-nM scrambled negative siRNA (control), ESCs were cultured under rotary suspension conditions for an additional 2 days (day 5), and expression of the indicated genes was determined using semi-quantitative qPCR with GAPDH as a loading control.

S3. CIBZ overexpression in ESCs suppresses cardiac genes during ESC differentiation; mRNA levels of the indicated genes were determined using semi-quantitative qPCR with GAPDH as a loading control.

S4. CIBZ binds to the Mesp1 promoter region but fails to bind to promoter regions of Flk1, Nkx2.5, and Gata4 genes in ESCs; ChIP assays were performed using an anti-CIBZ antibody in undifferentiated ESCs. Non-specific IgG was used as a negative control. Input DNA (1%), IgG-precipitated DNA, and CIBZ-immunoprecipitated DNA were amplified using primers for the indicated promoter regions.

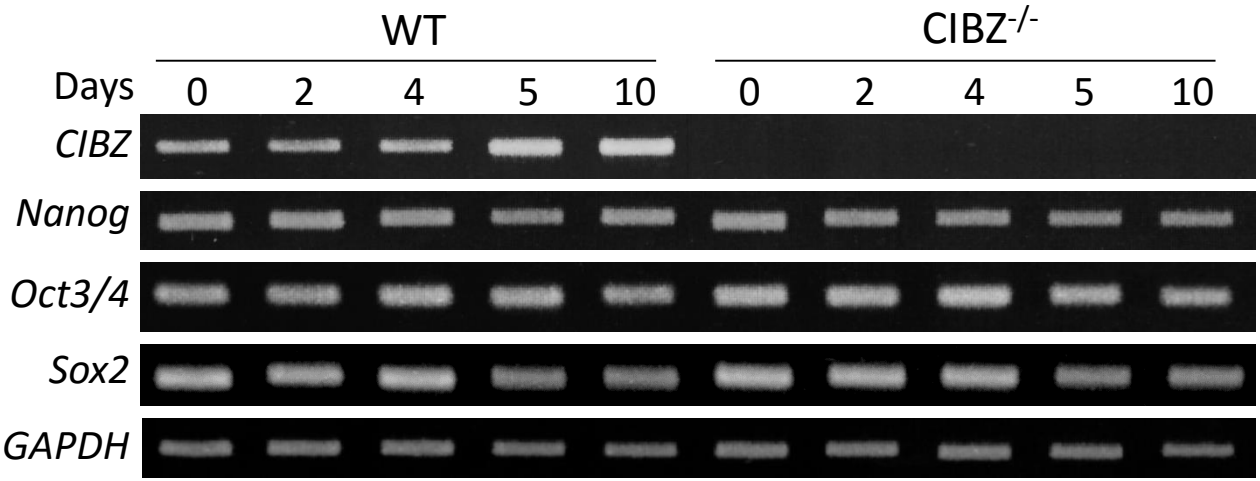
S5. Western blotting analysis of Nanog, Oct3/4, and Sox2 expression during ESC differentiation; Expression levels of the indicated proteins in WT and CIBZ$^{-/-}$ ESCs (a), and in pEF1α (control) and pEF1α-CIBZ (CIBZ OE) transfected ESCs (b); α-tubulin was used as a loading control.

S6. Treatment of ESCs with 5-aza-dC failed to abolish binding of CIBZ to T and Mesp1 promoter regions:

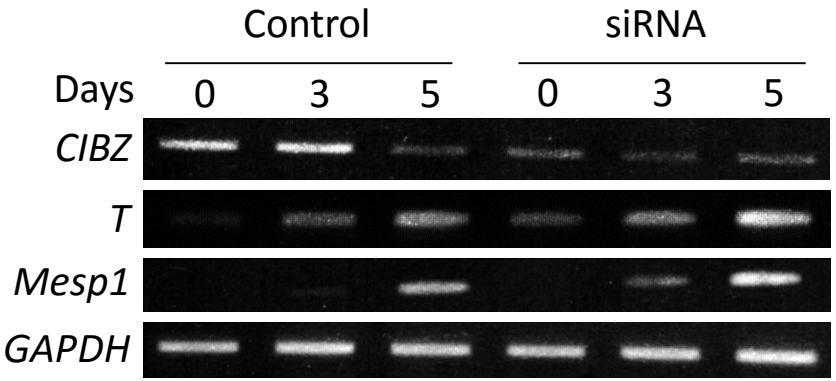
**(a),** Effects of 5-aza-dC treatment on ESCs; ESCs were treated with 5-aza-dC (0.25 μM or 0.5 μM) for 24 hours and expression of indicated genes and proteins was determined using semi-quantitative PCR (left panel) and Western blotting (right panel) with β-actin and α-tubulin as loading controls, respectively; **(b),** Schematic representation of 5′ regions of T and Mesp1 promoters (BLAT search genome, UCSC Genome Bioinformatics); Dist, distal; Prom, promoter; **(c),** ChIP assays were performed using an anti-CIBZ antibody in untreated (upper panel) and 5-aza-dC (0.25 μM) treated ESCs (lower panel). Input DNA (2.5%), IgG-precipitated DNA, and CIBZ-immunoprecipitated DNA were amplified using indicated primers for T (left panel) and Mesp1 (right panel).

S7. MG132 treatments of ESCs failed to rescue CIBZ protein expression. ESCs (denoted as EB d0) were trypsinized, were treated with MG132 (2 and 5 μM) or DMSO or vehicle ("-"), and were then cultured in ES medium on non-adherent Petri dishes on an orbital shaker for 2 days. Expression of indicated proteins was determined using Western blotting with α-tubulin as a loading control.

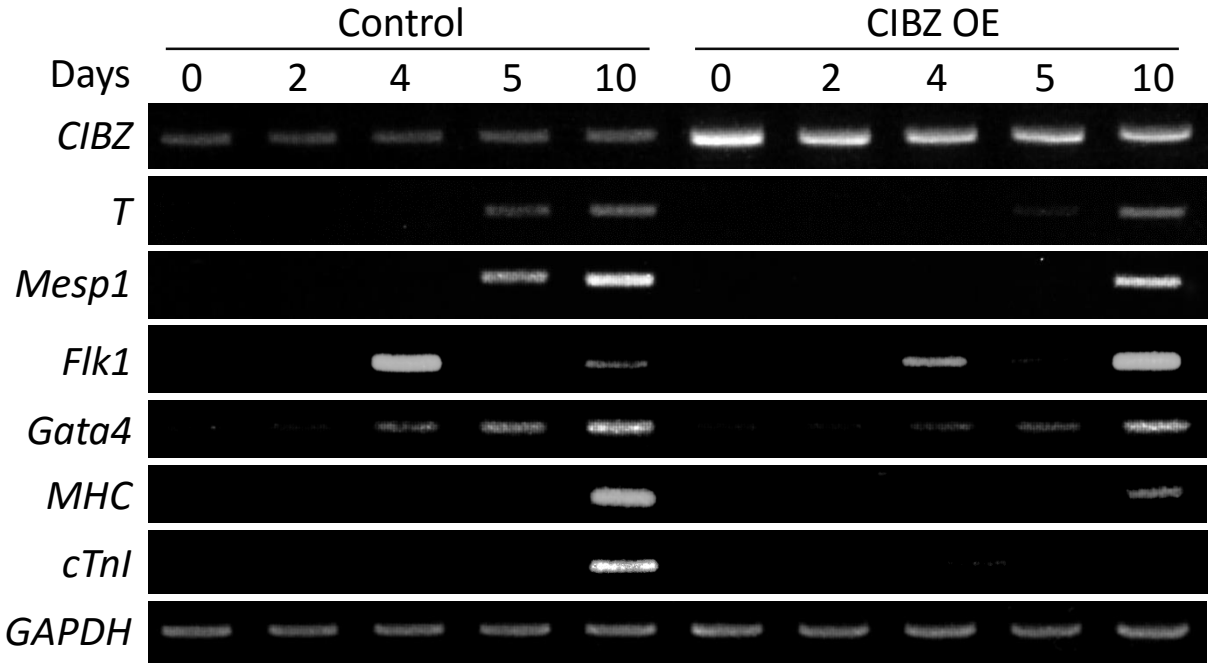
Supplementary Fig. S1



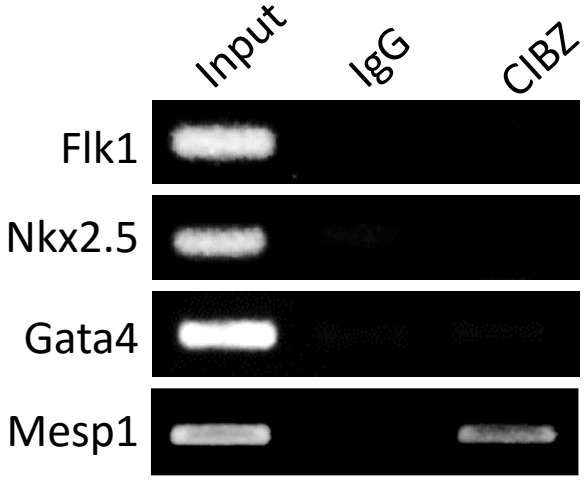
Supplementary Fig. S2



Supplementary Fig. S3

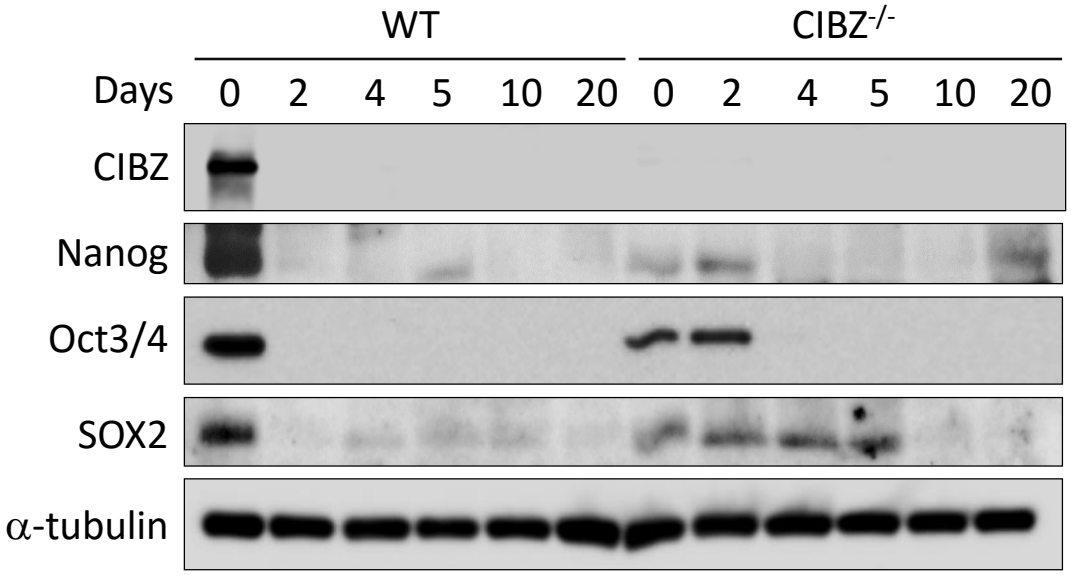


Supplementary Fig. S4



Supplementary Fig. S5

a

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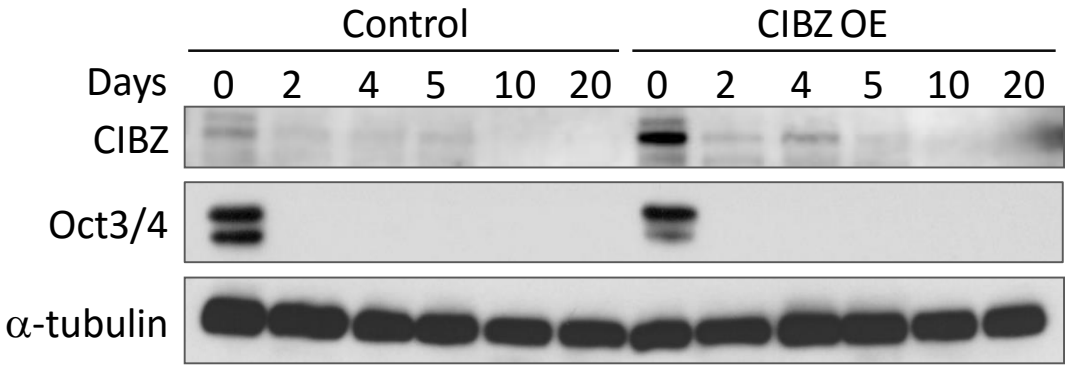
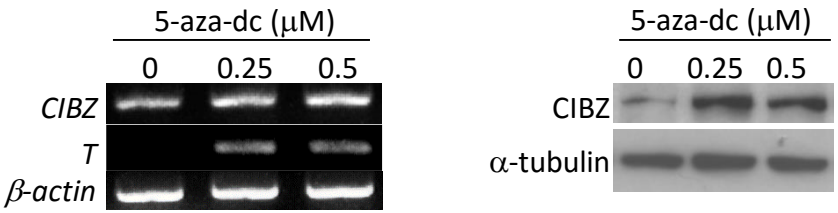


# Supplementary Fig. S6

a

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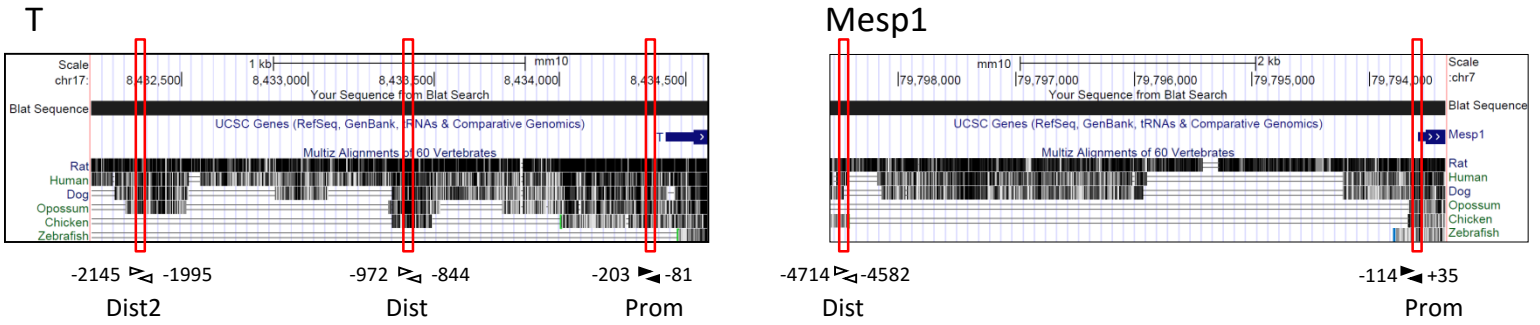


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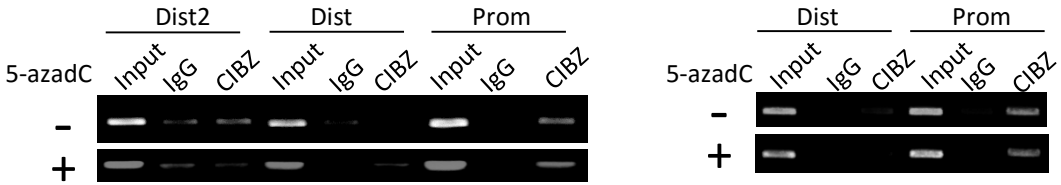

Supplementary Fig. S7

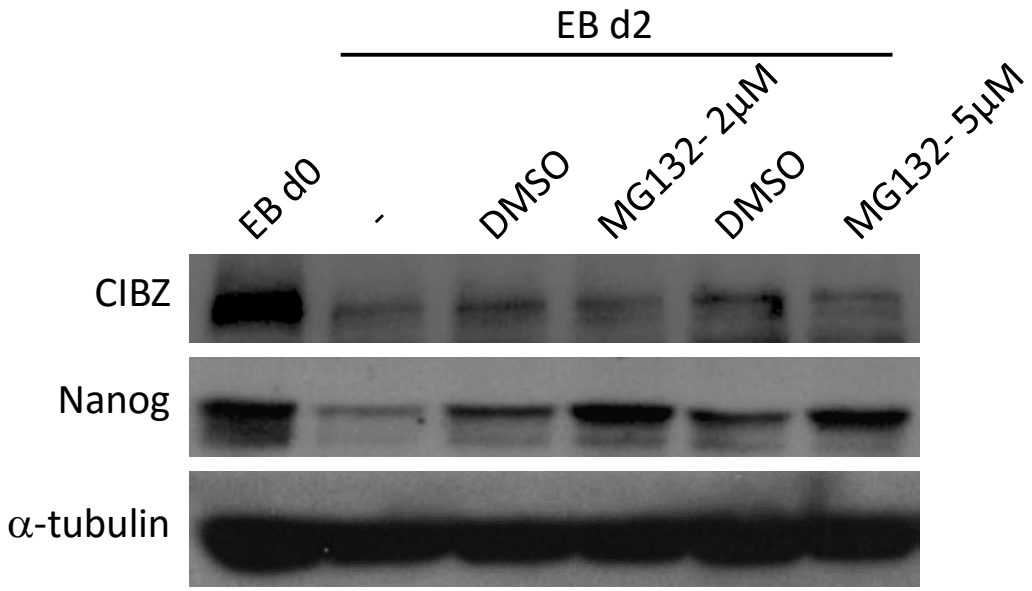

**Table S1. Primer sequences for semi-quantitative RT-PCR**

| Gene | Primer sequence (5′to 3′) | Product size |
|---|---|---|
| *Oct3/4* | F: TCACTCACATCGCCAATCAG<br>R: CCTGTAGCCTCATACTCTTCTC | 305 bp |
| *Sox2* | F: CTACAGCATGTCCTACTCGC<br>R: CCTCCCAATTCCCTTGTATCTC | 351 bp |
| *Nanog* | F: TTCAGAAATCCCTTCCCTCG<br>R: AGTAGCAGACCCTTGTAAGC | 162 bp |
| *Sox1* | F: ATACCGCAATCCCCTCTCAG<br>R: ACAACATCCGACTCCTCTTCC | 167 bp |
| *Nestin* | F: CTGGAAGTGGCTACATACAGGAC<br>R:AGTCTCAAGGGTATTAGGCAAGG | 210 bp |
| *T* | F: GAAGTGAAGGTGGCTGTTGG<br>R: ATTTACCTTCAGCACCGGGA | 298 bp |
| *Flk1* | F: TCGAGCCCTCATGTCTGAAC<br>R: CACTGAGCGATTTCTCCTCAAC | 317 bp |
| *Mesp1* | F: GTCTGCAGCGGGGTGTCGTG<br>R: CGGCGGCGTCCAGGTTTCTA | 189 bp |
| *Gata 6* | F: GGGAGAAACTGTGACAATGAC<br>R:ACGAACGCTTGTGAAATGTG | 166 bp |
| *Sox17* | F: CGAGCCAAAGCGGAGTCTC<br>R: TGCCAAGGTCAACGCCTTC | 156 bp |
| *Gata 4* | F: GTGAGCCTGTATGTAATGCC<br>R: CTGTGCCCATAGTGAGATGAC | 274 bp |
| *MEF2C* | F: GAGATGCCAGTTACCATCCC<br>R: CTTGTTCAGGTTACCAGGTGAG | 280 bp |
| *Nkx2.5* | F: CCAAGTGCTCTCCTGCTTTCC<br>R: GCCATCCGTCTCGGCTTT | 149 bp |
| *Tbx5* | F: AGGAGCACAGCCAAATTTACCAC<br>R: ATGAGCGGAGAAGTGCTGGTAG | 294 bp |
| *Isl1* | F: ATGATGGTGGTTTACAGGCTAA<br>R: TCGATGCTACTTCACTGCCAG | 174 bp |
| *cTnI* | F: TGCCAACTACCGAGCCTATG<br>R: TGGCAACGAGTCCTCAGAAC | 174 bp |
| *GAPDH* | F: CCATCACCATCTTCCAGGAG<br>R: CCTGCTTCACCACCTTCTTG | 577 bp |

**Table S2. Primer sequences for qPCR**

| Gene | Primer sequence (5′ to 3′) | Product size |
|---|---|---|
| *T* | F: GCTTCAAGGAGCTAACTAACGAG<br>R: CCAGCAAGAAAGAGTACATGGC | 117 bp |
| *Flk1* | F: CTGGAGCCTACAAGTGCTCG<br>R: GAGGTTTGAAATCGACCCTCG | 179 bp |
| *Mesp1* | F: GTCTGCAGCGGGGTGTCGTG<br>R: CGGCGGCGTCCAGGTTTCTA | 189 bp |
| *Nkx2.5* | F: GGTCTCAATGCCTATGGCTAC<br>R: GCCAAAGTTCACGAAGTTGCT | 153 bp |
| *Gata4* | F: AACGGAAGCCCAAGAACCTG<br>R: AGTGGCATTGCTGGAGTTACC | 107 bp |
| *Tbx5* | F: AATGGTCCGTAACTGGCAAAG<br>R: GGATAATGTGTCCAAACGGGTC | 159 bp |
| *Isl1* | F: ATGATGGTGGTTTACAGGCTAAC<br>R: TCGATGCTACTTCACTGCCAG | 174 bp |
| *Mef2C* | F: AGGATAATGGATGAGCGTAACAG<br>R: GTTCAATGCCTCCACAATGTC | 240 bp |
| *cTnI* | F: TGCCAACTACCGAGCCTATG<br>R: TGGCAACGAGTCCTCAGAAC | 174 bp |
| *MHC* | F: CAGAGGAGAAGGCTGGTGTC<br>R: TTGTCAGCATCTTCTGTGCC | 121 bp |
| *GAPDH* | F: CAATGTGTCCGTCGTGGATCT<br>R: GTCCTCAGTGTAGCCCAAGATG | 124 bp |

**Table S3. Primers for ChIP assay**

| | Primer sequence (5′ to 3′) |
|---|---|
| T<br>(Promoter region) | F: GCTGCTCGGTACTTCAAAGGG<br>R: GCGCGACAAGAGTAAGTCTCTG |
| T<br>(Distal region) | F: TCCTGCTCTTTGTCACCTTC<br>R: GATTGTTGGAACGCATGCTG |
| Mesp1<br>(Promoter region) | F: GTGGAGCAGACTGGACTAAG<br>R: TTATCCTGAGCCCTAGGTGTG |
| Mesp1<br>(Distal region) | F: ACTCTAGCTGCCTGTCTTGG<br>R: CCTTACTTCACATACCAGAGCCTT |
| Flk1<br>(Promoter region) | F: GACTTTCAGTGCAGCGGCGAAG<br>R: CAAATCTGGACGCAGCTCGGTTTC |
| Gata4<br>(Promoter region) | F: CGTAGATCTGAGGCTAGCAAGGC<br>R: CTCTTTCCTCCCTACTCTCAGTGGTC |
| Nkx2.5<br>(Promoter region) | F: CTGGCTGGGATTTTCAGGCTAACGAG<br>R: ACGGGCAGTTCTGCGTCACCTAAT |

**Table S4. Primers for pGL3-T and pGL-3-Mesp1 constructs**

| | Primer sequence (5′ to 3′) |
|---|---|
| T | F: AGACGACGCGTCAAAGTCGCAGGCGCCGGTGTG<br>R: GTCCCAAGCTTCCACCCTCTCCACCTTCCAG |
| Mesp1 | F: AGACGACGCGTCAAGGCTCTGGTATGTGAAGTAAGG<br>R: GTCCCAAGCTTGGCAGCGGAGGCCTGACCATTG |

**Supplementary video legends**

**Supplementary video 1.** Representative video documenting the spontaneous beating of WT EBs at day 10 of ESC differentiation.

**Supplementary video 2.** Representative video documenting the spontaneous beating of CIBZ$^{-/-}$ EBs at day 10 of ESC differentiation.