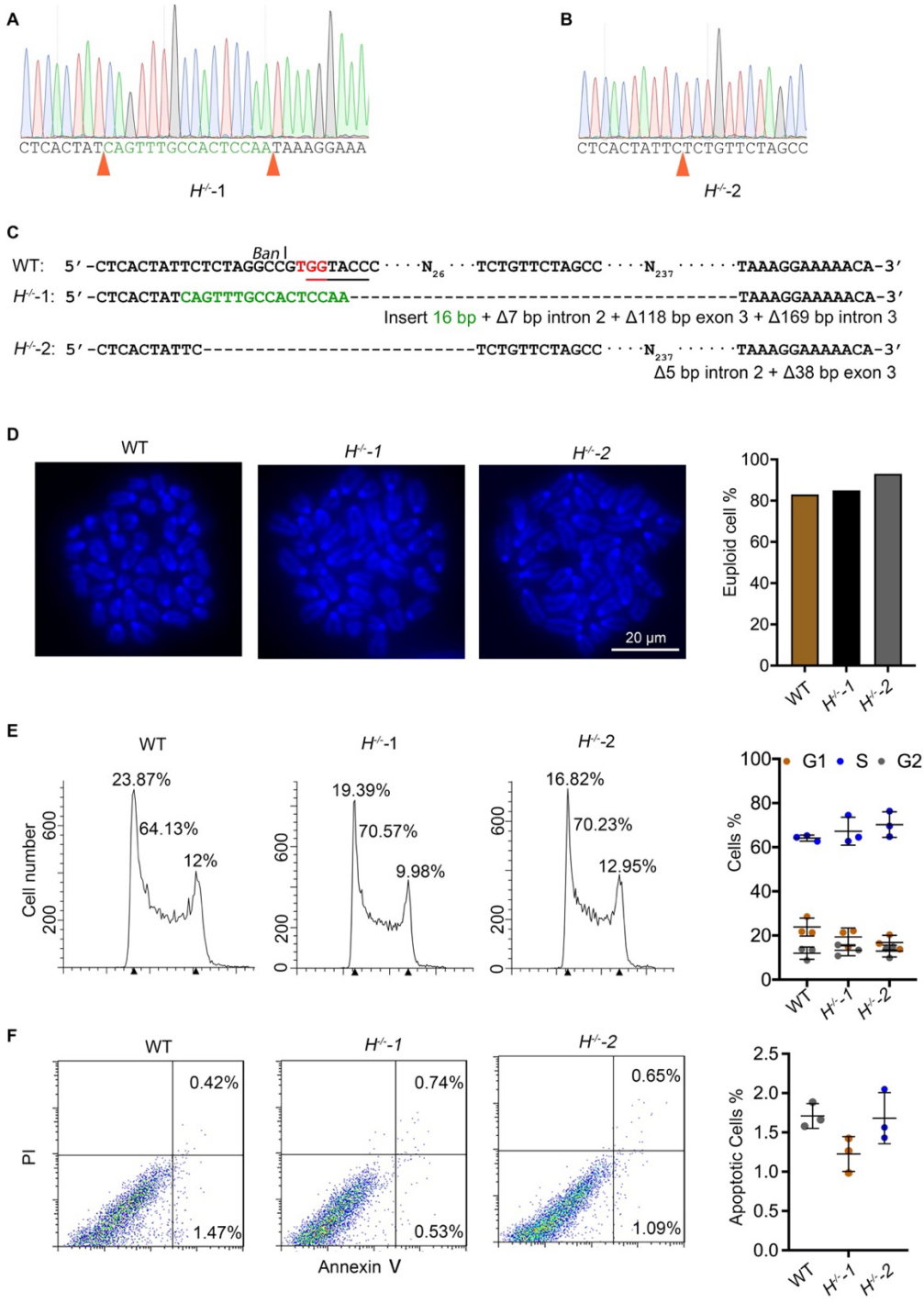

**Fig. S1. Related to Fig. 2. Validation of *Hbxip*$^{-/-}$ ESC clones.**

(A) and (B) Sequencing chromatograph of the *Hbxip* gene around the Cas9 targeting site in *H*$^{-/-}$-1 (A) and *H*$^{-/-}$-2 (B) ESCs. Orange triangles mark the indel mutations introduced by Cas9 cutting. (C) Sequence alignment of the *Hbxip* gene around the Cas9 targeting site in WT, *H*$^{-/-}$-1 and *H*$^{-/-}$-2 ESCs. (D-F) Karyotyping (D), cell cycle (E), and apoptosis (F) analyses of WT and *Hbxip*$^{-/-}$ ESCs. (D) More than 30 metaphase spreads were counted for each cell line. Scale bar: 20 μm. (E) and (F) For cell cycle and apoptosis analyses, data are shown as mean ± SD (n=3). Statistical analysis was performed with two-way ANOVA test. $p > 0.05$ was considered as statistical non-significance.

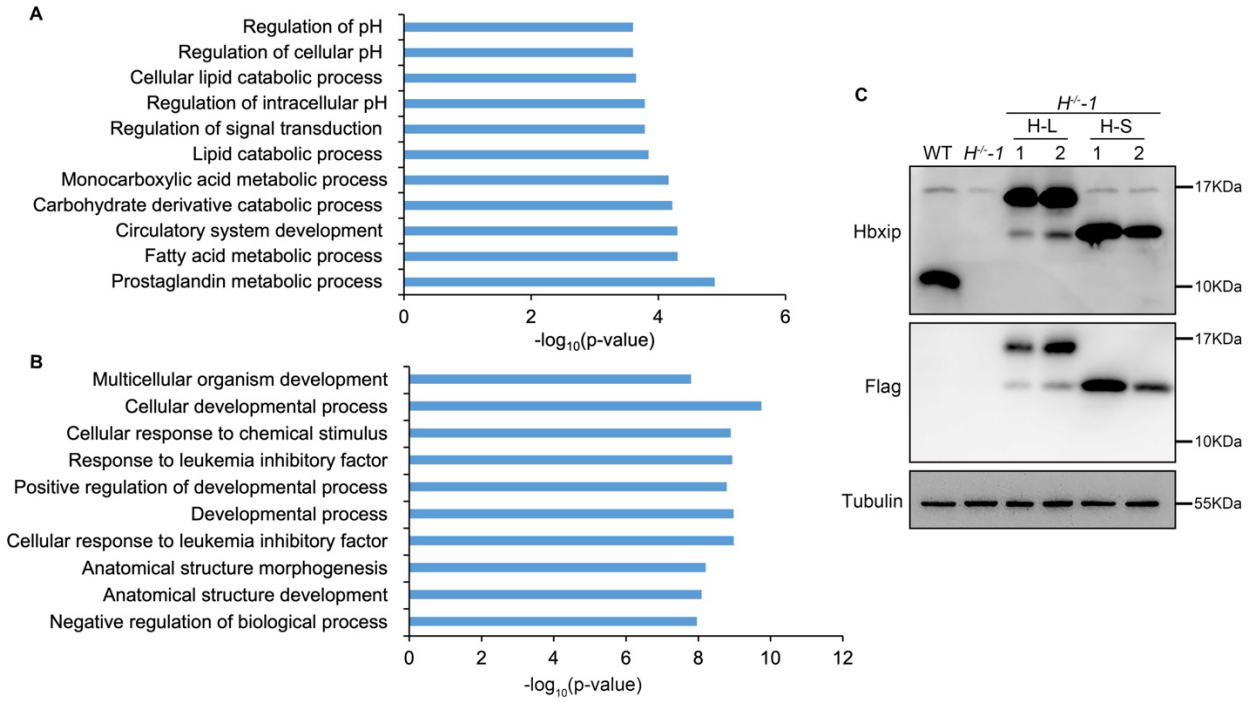


**Fig. S2. Related to Figs 2, 3. *Hbxip KO* affects gene expression and mTORC1 activity.**
(A) GO annotation of the upregulated genes in *Hbxip*$^{-/-}$ ESCs (Related to Fig. 2G, H). (B) GO annotation of the commonly upregulated genes by *Hbxip* KO (Related to Fig. 3C-F). (C) Validation of the overexpression of Flag tagged Hbxip, including H-L and H-S (Related to Fig. 3G).

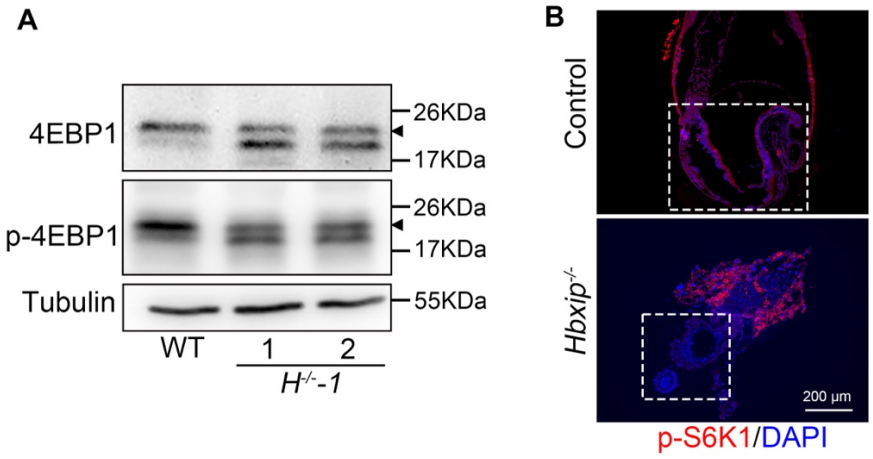


**Fig. S3. Related to Fig. 5. Reduced mTOCRC1 activity in *Hbxip*$^{-/-}$ ESCs and embryos.**
(A) Western blots of 4EBP1 and p-4EBP1 in WT and *Hbxip*$^{-/-}$ ESCs demonstrate the reduced mTORC1 activity in *Hbxip*$^{-/-}$ ESCs. Triangles mark the specific bands for 4EBP1 and p-4EBP1. (B) Immunofluorescence staining of p-S6K1 in control and *Hbxip*$^{-/-}$ E8.5 embryo sections. Dashed rectangles mark the embryo part. Control (including WT and *Hbxip*$^{+/-}$), n=4; *Hbxip*$^{-/-}$, n=4. Scale bar: 200 μm.

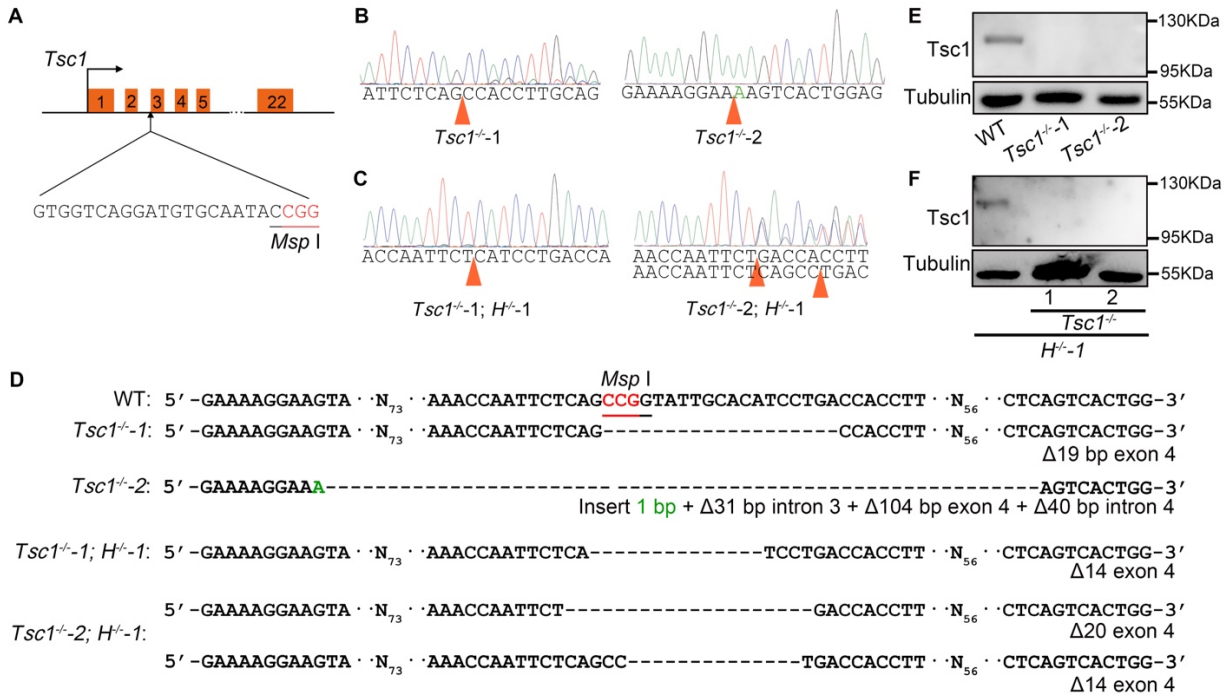


**Fig. S4. Related to Fig. 5. Validation of *Tsc1*$^{-/-}$ ESC clones.**

(A) Schematic illustration of the strategy for knocking out *Tsc1* in ESCs. The targeting sequence of sgRNA is shown. The protospacer-adjacent motif (PAM) is highlighted in red, and the *Msp* I site is underlined. (B) and (C) Sequencing chromatograph of the *Tsc1* gene around the Cas9 targeting site in *Tsc1*$^{-/-}$ (B) and *Tsc1*$^{-/-}$; *H*$^{-/-}$-1 (C) ESCs. Orange triangles mark the indel mutations introduced by Cas9 cutting. (D) Sequence alignment of the *Tsc1* gene around the Cas9 targeting site in WT, *Tsc1*$^{-/-}$ and *Tsc1*$^{-/-}$; *H*$^{-/-}$-1 ESCs. (E) and (F) Validation of Tsc1 knockout in *Tsc1*$^{-/-}$ (E) and *Tsc1*$^{-/-}$; *H*$^{-/-}$-1 (F) ESCs by Western blot.

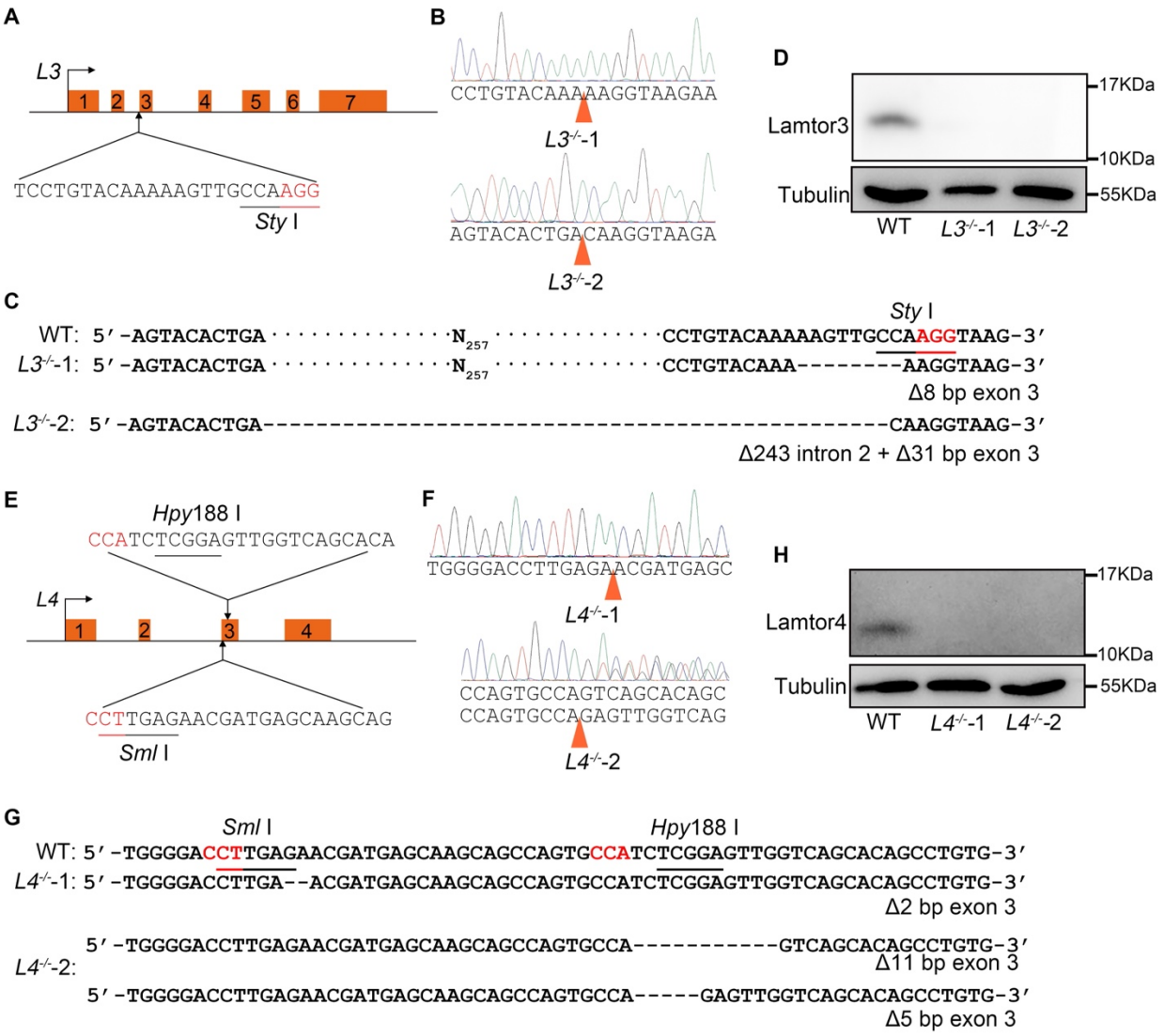


**Fig. S5. Related to Fig. 6. Validation of *Lamtor3*$^{-/-}$ and *Lamtor4*$^{-/-}$ ESC clones.**

(A) and (E) Schematic illustration of the strategy for knocking out *Lamtor3* (A) and *Lamtor4* (E) in ESCs. The targeting sequences of sgRNAs (one for *Lamtor3* and two for *Lamtor4*) are shown. The protospacer-adjacent motif (PAM) is highlighted in red, and the *Sty* I, *Sml* I and *Spy*188 I sites are underlined. (B) and (F) Sequencing chromatograph of the *Lamtor3* and *Lamtor4* gene around the Cas9 targeting site in *Lamtor3*$^{-/-}$ (B) and *Lamtor4*$^{-/-}$ (F) ESC clones. Orange triangles mark the indel mutations introduced by Cas9 cutting. (C) and (G) Sequence alignment of the *Lamtor3* (C) and *Lamtor4* (G) gene around the Cas9 targeting site in WT, *Lamtor3*$^{-/-}$ (C) and *Lamtor4*$^{-/-}$ (G) ESCs. (D) and (H) Validation of Lamtor3 (D) and Lamtor4 (H) knockout in *Lamtor3*$^{-/-}$ (D) and *Lamtor4*$^{-/-}$ (H) ESCs by Western blot.

**Table S1. Primers for quantitative RT-PCR**

| Gene | Forward | Reverse |
|---|---|---|
| *Nanog* | TACAAGGGTCTGCTACTGAGATGC | TTGGGACTGGTAGAAGAATCAGGG |
| *Oct4* | ATCAGCTTGGGCTAGAGAAGGATG | AAAGGTGTCCCTGTAGCCTCATAC |
| *Sox2* | GCGGAGTGGAAACTTTTGTCC | CGGGAAGCGTGTACTTATCCTT |
| *Nestin* | CTGGATCTGGAAGTCAACAGAGGT | ATCCTCAGTTTCCACTCCTGTAGC |
| *Gata4* | GCTATGCATCTCCTGTCACTCAGA | CCAAGTCCGAGCAGGAATTTGAAG |
| *Gata6* | CTTCTCCTTCTACACAAGCGACCA | ATACTTGAGGTCACTGTTCTCGGG |
| *T* | CATCGGAACAGCTCTCCAACCTAT | TACCATTGCTCACAGACCAGAGAC |
| *Celsr2* | CACGATGGCCTGAGGGTTT | CCTTGTGGAGAAAGGTGTCCT |
| *Dlx3* | CACTGACCTGGGCTATTACAGC | GAGATTGAACTGGTGGTGGTAG |
| *β-Actin* | CAGAAGGAGATTACTGCTCTGGCT | TACTCCTGCTTGCTGATCCACATC |

**Table S2. Differentially expressed genes (DEGs) identified in *Hbxip*$^{-/-}$ ESCs, compared to WT ESCs.**

Click here to download Table S2

**Table S3. DEGs identified in differentiated *Hbxip*$^{-/-}$ ESCs, compared to differentiated WT ESCs.**

WT, *H*$^{-/-}$-1, and *H*$^{-/-}$-2 ESCs were differentiated for 4 days by two methods, LIF withdrawal and EB. RNA purified from these differentiated cells were subjected to RNA-seq analysis.

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**Table S4. Hbxip interacting proteins identified by co-IP and mass spectrometry.**

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