Supplemental Information

ZFP560 facilitates KAP1-dependent chromatin repression to regulate exit from totipotency

The Following Files are Included:

Supplementary Figures and Legends

Supplementary Fig. 1: *Zfp560* deficiency activates totipotency programs through P53 signaling and epigenetic derepression in mouse embryonic stem cells.

Supplementary Fig. 2: Integrated analysis identifies ZFP560 as a molecular switch linking heterochromatin formation to P53-dependent cell fate decisions.

Supplementary Fig. 3: The overexpression of *Zfp560* reduces its potential for differentiation into extraembryonic tissues.

Supplementary Fig. 4: ZFP560 inhibited the expression of the three-germ-layer marker genes *in vitro*, but it still retained the ability to form teratomas.

Supplementary Fig. 5: KRAB domain-dependent KAP1 recruitment drives H3K9me3 deposition.

Supplementary Fig. 6: The analysis of *ZNF778* (*Zfp560* ortholog) reveals divergent expression dynamics during human embryonic activation and stem cell reprogramming.

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Supplementary Table 3. Zinc finger protein family expression in three cell states (Serum+Lif, EpiLC, 2i+Lif).

Supplementary Table 4. Differentially expressed genes in RNA-seq of KD-*Zfp560* and KD-Ctrl mESCs.

Supplementary Table 5. Gene sets used for GSEA, related to Figures2, 3, 5, and Supplementary Figures1, 2.

Supplementary Table 6. Differentially expressed genes in RNA-seq of OE-*Zfp560* and OE-Ctrl mESCs.

Supplementary Table 7. Mass spectrometry of ZFP560-FLAG binding proteins in OE-*Zfp560* mESCs.

Supplementary Table 8. GO analysis of the ZFP560-FLAG candidate binding protein in the mass spectrometry.

Supplementary Table 9. Zinc finger protein family expression during early human embryonic development.

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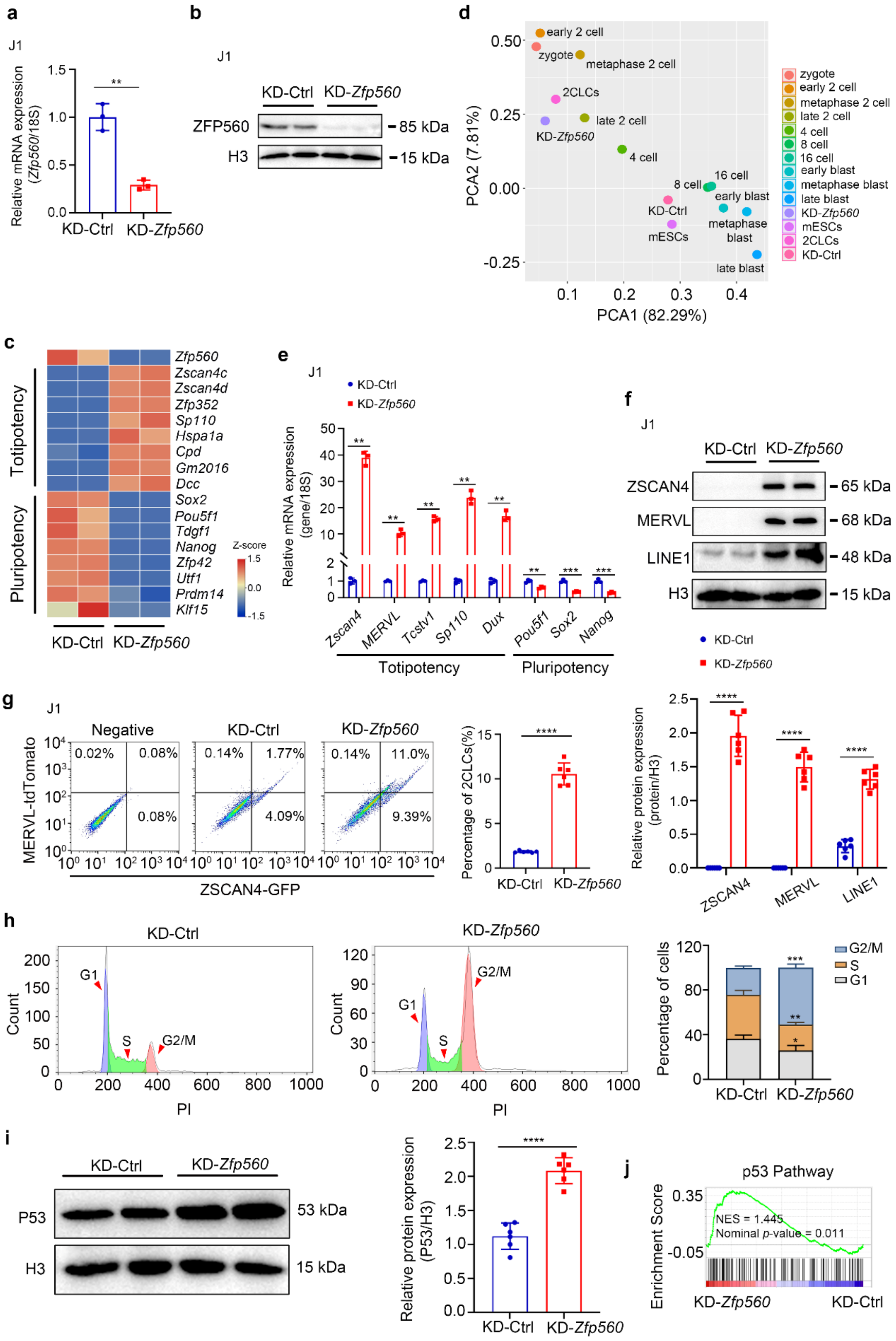
Supplementary Table 11. 8CLC ZGA genes, and gene signatures based on 8-morula embryo and hESCs.

Supplementary Table 12. Primer sequences for qPCR.

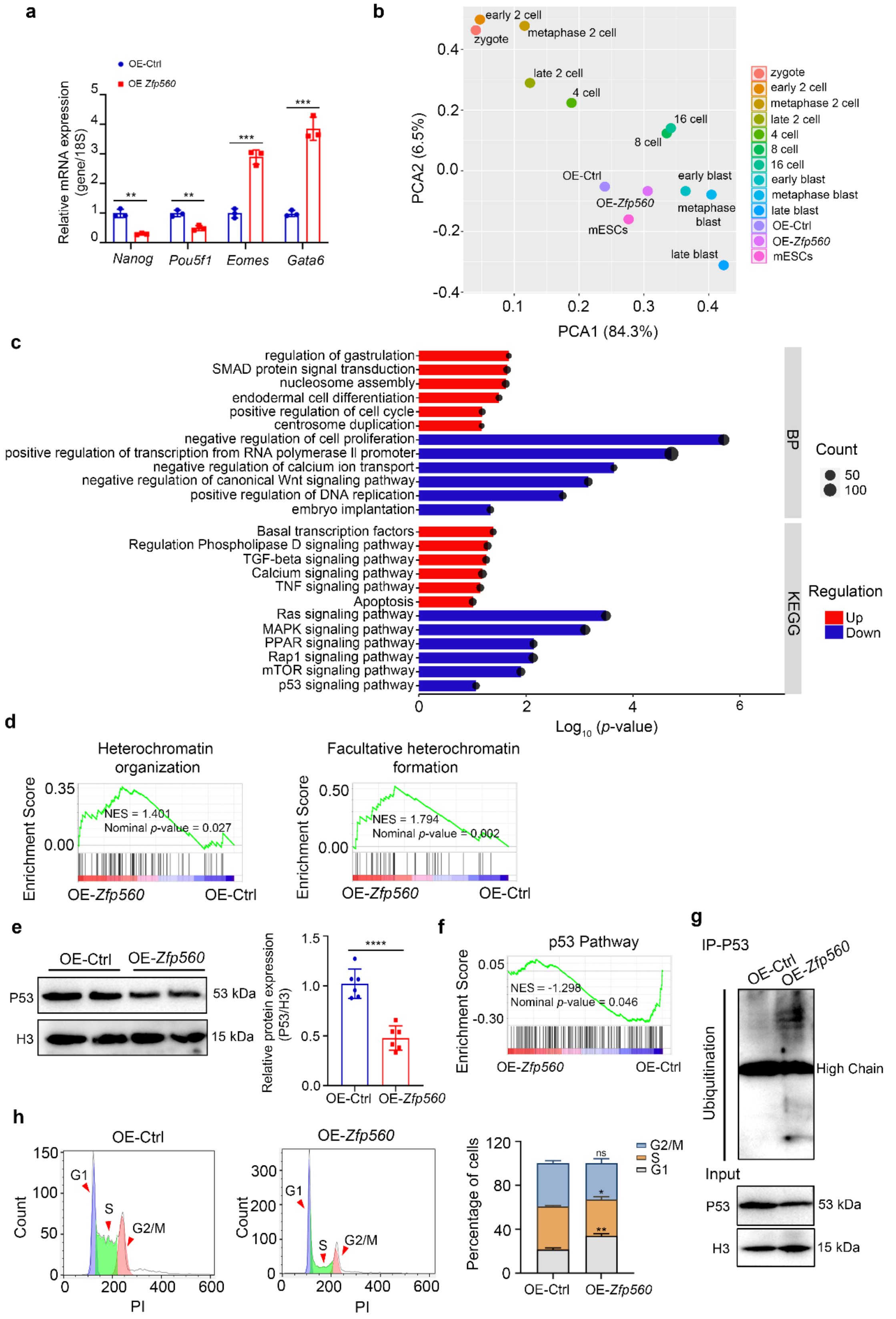
Supplementary Table 13. Primer sequences for Chip-qPCR.

Supplementary Table 14. PCR primer sequences for molecular cloning.

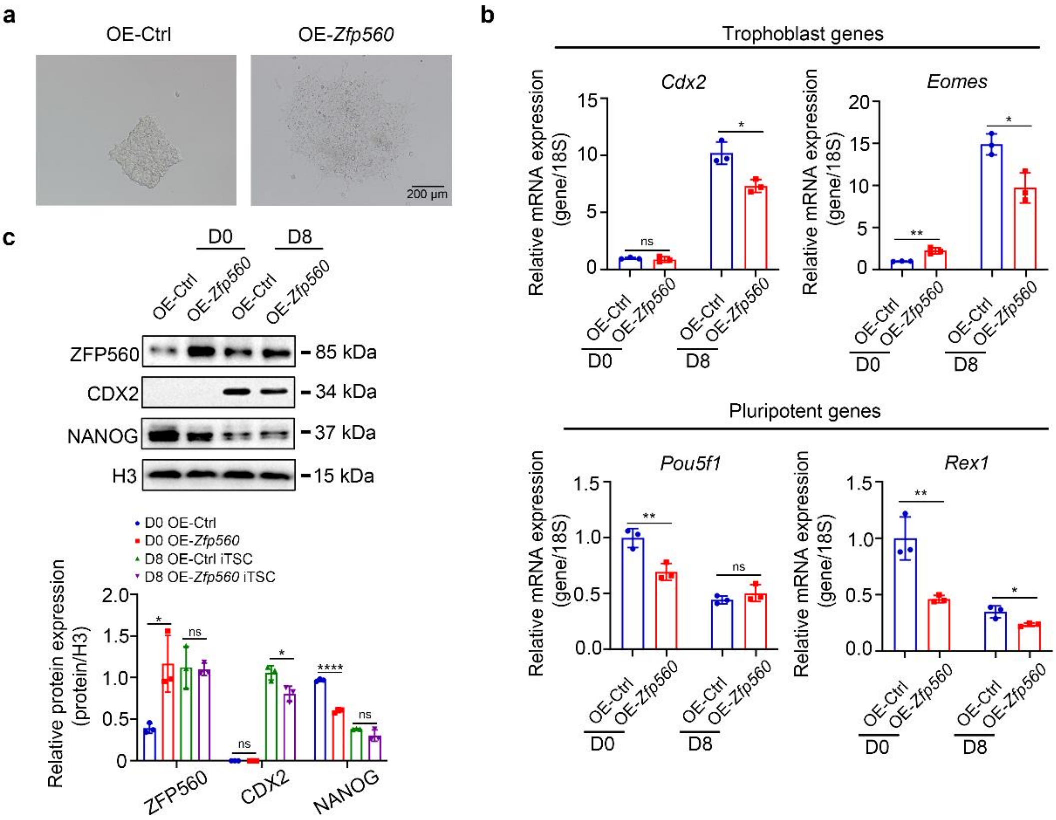
Supplementary Table 15. Antibodies used in this study.


a
J1
Relative mRNA expression (Zfp560/18S)
1.5
1.0
0.5
0.0
**
KD-Ctrl
KD-Zfp560
b
J1
KD-Ctrl
KD-Zfp560
ZFP560
H3
85 kDa
15 kDa
d
early 2 cell
zygote
metaphase 2 cell
2CLCs
late 2 cell
KD-Zfp560
4 cell
16 cell
8 cell
early blast
KD-Ctrl
metaphase blast
mESCs
late blast
0.50
0.25
0.00
-0.25
PCA2 (7.81%)
0.1
0.2
0.3
0.4
PCA1 (82.29%)
zygote
early 2 cell
metaphase 2 cell
late 2 cell
4 cell
8 cell
16 cell
early blast
metaphase blast
late blast
KD-Zfp560
mESCs
2CLCs
KD-Ctrl
c
Totipotency
Pluripotency
Zfp560
Zscan4c
Zscan4d
Zfp352
Sp110
Hspa1a
Cpd
Gm2016
Dcc
Sox2
Pou5f1
Tdgf1
Nanog
Zfp42
Utf1
Prdm14
Klf15
Z-score
1.5
0.0
-1.5
KD-Ctrl
KD-Zfp560
e
J1
KD-Ctrl
KD-Zfp560
Relative mRNA expression (gene/18S)
50
40
30
20
10
2
1
0
**
***
Zscan4
MERVL
Tcstv1
Sp110
Dux
Pou5f1
Sox2
Nanog
Totipotency
Pluripotency
f
J1
KD-Ctrl
KD-Zfp560
ZSCAN4
MERVL
LINE1
H3
65 kDa
68 kDa
48 kDa
15 kDa
KD-Ctrl
KD-Zfp560
Relative protein expression (protein/H3)
2.5
2.0
1.5
1.0
0.5
0.0
****
ZSCAN4
MERVL
LINE1
g
J1
Negative
KD-Ctrl
KD-Zfp560
MERVL-tdTomato
ZSCAN4-GFP
0.02%
0.08%
0.08%
0.14%
1.77%
4.09%
0.14%
11.0%
9.39%
10⁴
10³
10²
10¹
10⁰
Percentage of 2CLCs(%)
15
10
5
0
****
KD-Ctrl
KD-Zfp560
h
KD-Ctrl
KD-Zfp560
Count
200
150
100
50
0
120
90
60
30
G1
S
G2/M
PI
0
200
400
600
800
1000
Percentage of cells
120
80
40
0
G2/M
S
G1
***
**
*
KD-Ctrl
KD-Zfp560
i
KD-Ctrl
KD-Zfp560
P53
H3
53 kDa
15 kDa
Relative protein expression (P53/H3)
2.5
2.0
1.5
1.0
0.5
0.0
****
KD-Ctrl
KD-Zfp560
j
p53 Pathway
Enrichment Score
0.35
-0.05
NES = 1.445
Nominal p-value = 0.011
KD-Zfp560
KD-Ctrl

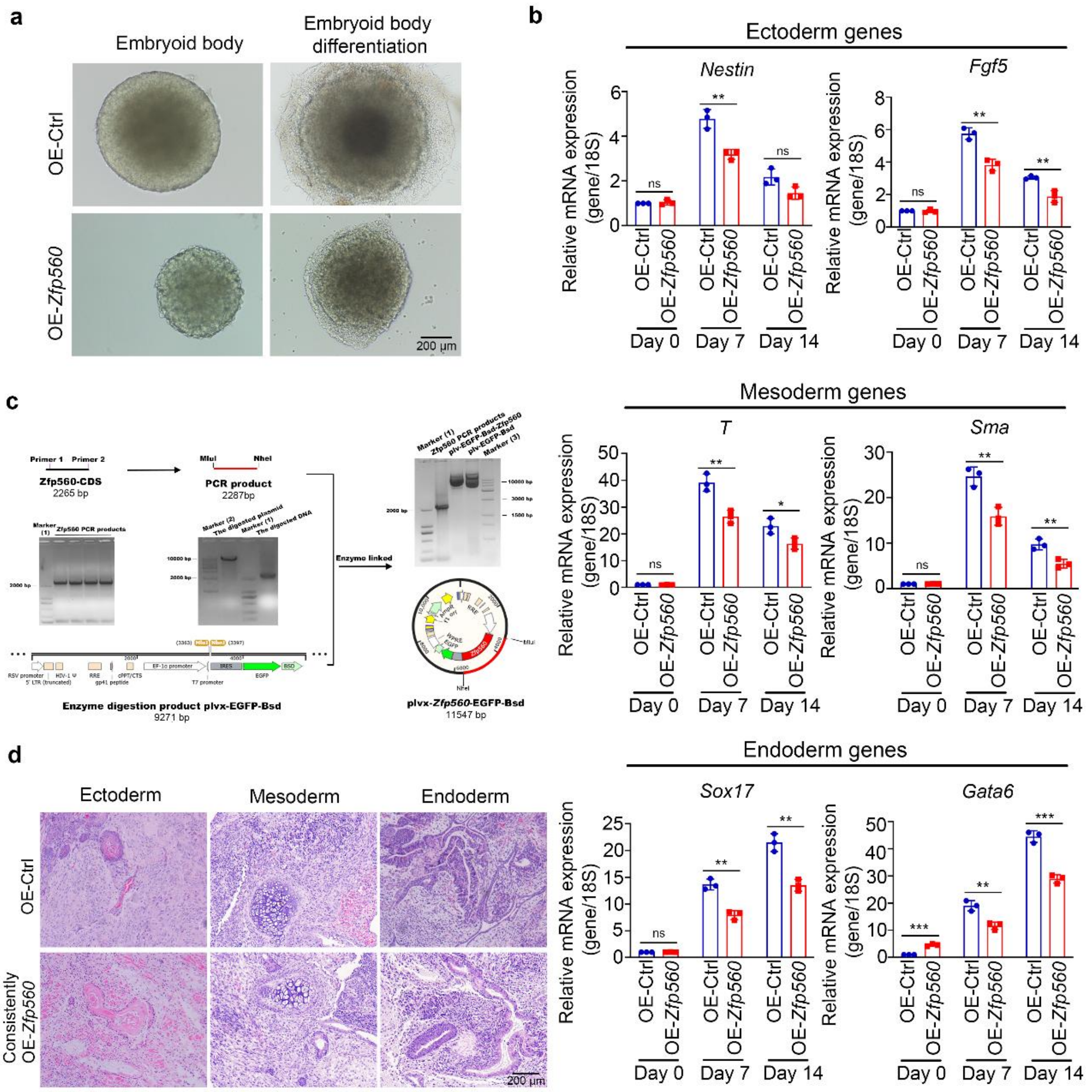
Supplementary Fig. 1: *Zfp560* deficiency activates totipotency programs through P53 signaling and epigenetic derepression in mouse embryonic stem cells. **a** RT-qPCR was used to detect the expression changes of *Zfp560* after *Zfp560* deficiency in J1 mESCs. Data are mean ± S.D. of three independent experiments. $^{**}P < 0.01$. **b** Western blot was used to detect the expression changes of ZFP560 after *Zfp560* deficiency in J1 mESCs. **c** Heat map comparing RNA-seq analysis of pluripotency- or totipotency-specific gene expression in KD-Ctrl and KD-*Zfp560* mESCs. **d** Transcriptome-based PCA of bulk RNA-seq data from KD-Ctrl, KD-*Zfp560*, mESCs and 2CLCs, and scRNA-seq data from mouse embryos from zygote to late blastocyst. **e** RT-qPCR was used to detect the expression changes of *Zscan4*, *MERVL*, *Tcstv1*, *Sp110*, *Dux*, *Pou5f1*, *Sox2* and *Nanog* after *Zfp560* deficiency in J1 mESCs. Data are mean ± S.D. of three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$. **f** Western Blot was used to detect the expression changes of ZSCAN4, MERVL and LINE1 after *Zfp560* deficiency in J1 mESCs. Gray scale statistical analysis. Data are mean ± S.D. of three independent experiments. $^{****}P < 0.0001$. **g** Changes in the proportion of totipotent 2-cell-like cells after *Zfp560* deficiency in J1 mESCs by flow cytometry and analyzed statistically. Data are mean ± S.D. of three independent experiments (n=2). $^{****}P < 0.0001$. **h** Flow cytometry analysis of cell cycle changes after *Zfp560* deficiency. Data are mean ± S.D. of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. **i** The protein level of P53 after *Zfp560* deficiency was detected by Western blot, and the gray scale statistical analysis was performed. Data are mean ± S.D. of three independent experiments (n=2). $^{****}P < 0.001$. **j** GSEA analysis showed that the transcriptome of KD-*Zfp560* vs KD-Ctrl mESCs was enriched in P53 signaling pathway.



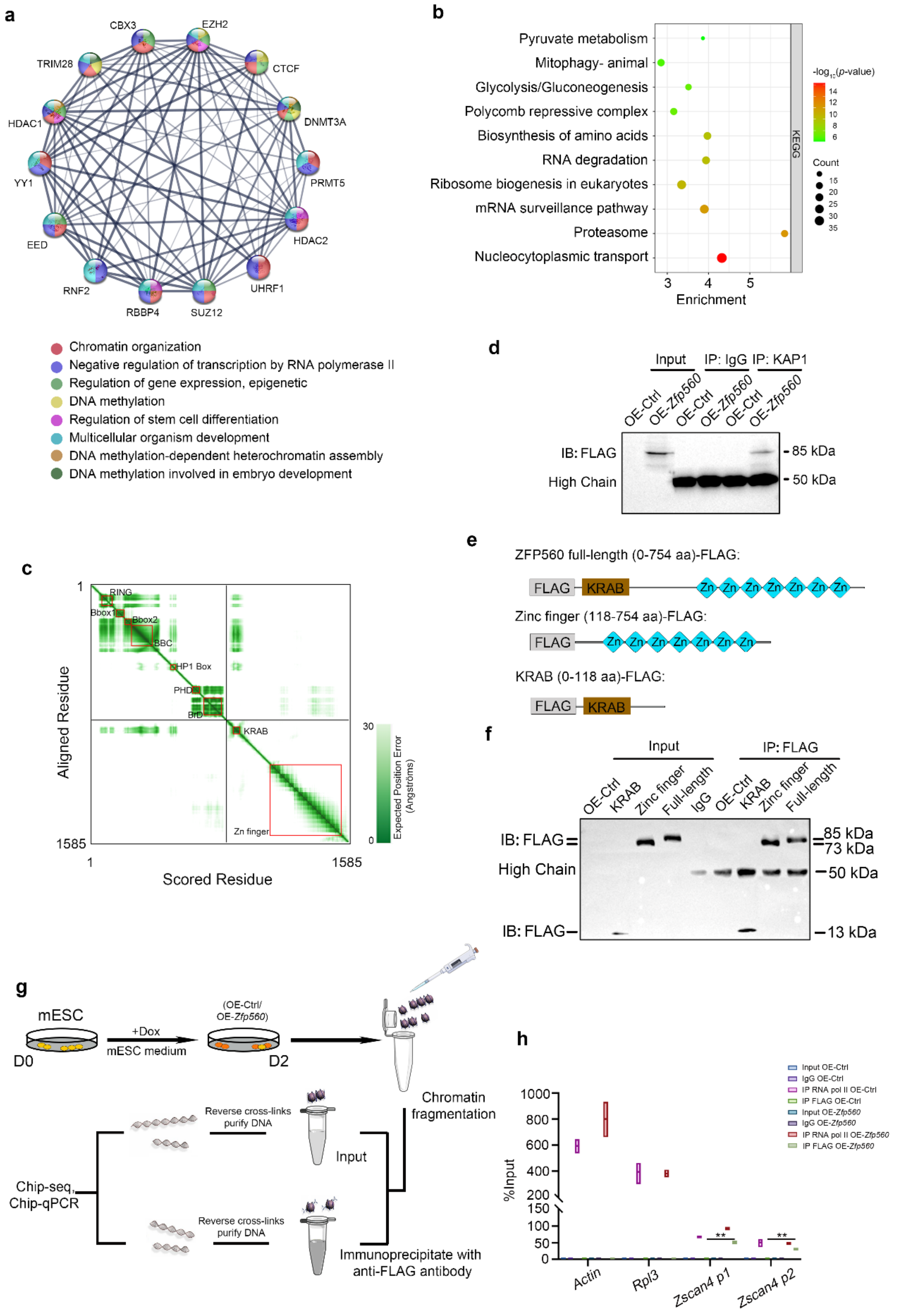
Supplementary Fig. 2: Integrated analysis identifies ZFP560 as a molecular switch linking heterochromatin formation to P53-dependent cell fate decisions. **a** RT-qPCR was performed to measure mRNA expression levels of *Nanog*, *Pou5f1*, *Eomes*, and *Gata6* after *Zfp560* overexpression. Data are mean ± S.D. of three independent experiments. $**P < 0.01$, $***P < 0.001$. **b** Transcriptome-based PCA of bulk RNA-seq data from OE-Ctrl, OE-*Zfp560*, mESCs, and scRNA-seq data from mouse embryos from zygote to late blastocyst. **c** GO analysis biological processes and KEGG analysis based on RNA-Seq data OE-*Zfp560* vs OE-Ctrl. Up-regulated and down-regulated genes are shown in red and blue, respectively. **d** Gene enrichment for heterochromatin organization and facultative heterochromatin formation in OE-Ctrl and OE-*Zfp560* mESCs was analyzed by GSEA. The signaling pathway gene sets were obtained from the GSEA official website database. **e** The protein level of P53 after *Zfp560* overexpression was detected by Western blot, and the gray scale statistical analysis was performed. Data are mean ± S.D. of three independent experiments. ****$P < 0.001$. **f** GSEA analysis showed that the transcriptome of OE-*Zfp560* vs OE-Ctrl mESCs was enriched in P53 signaling pathway. **g** Ubiquitination of P53 was detected after *Zfp560* overexpression, and P53 expression was displayed in the Input group. **h** Flow cytometry analysis of cell cycle changes after *Zfp560* overexpression. Data are mean ± S.D. of three independent experiments. *$P < 0.05$, **$P < 0.01$, ns was not statistically significant.



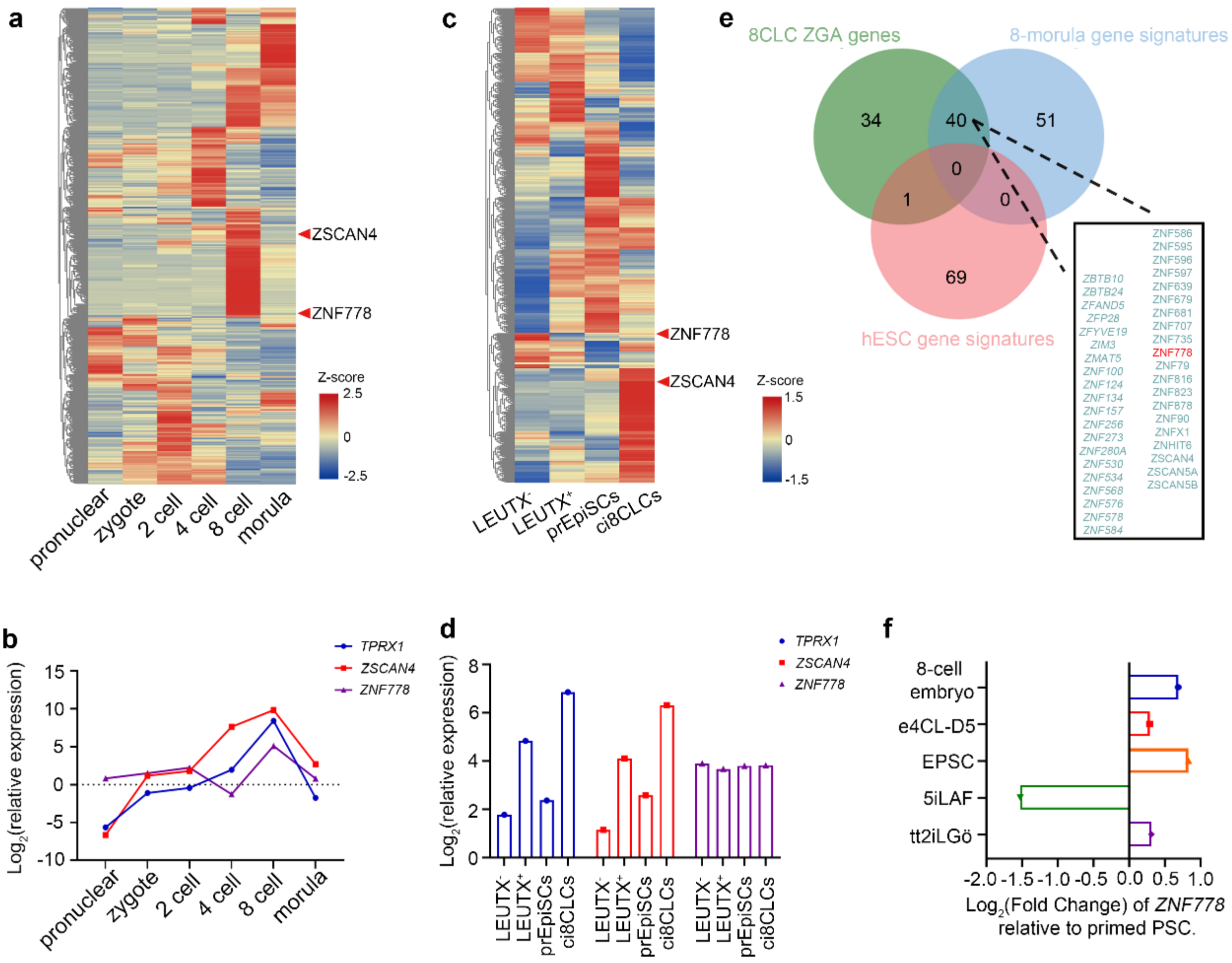
Supplementary Fig. 3: The overexpression of *Zfp560* reduces developmental potential. a Clonal morphology of induced trophoblast stem cells in OE-Ctrl and OE-*Zfp560* mESCs at day 8. Scale bar, 200 μm. **b** RT-qPCR to detect the expression levels of the trophoblast and pluripotent marker genes. Data are mean ± S.D. of three independent experiments. **P* < 0.05, ***P* < 0.01, ns was not statistically significant. **c** Western blot was used to detect the expression of ZFP560, CDX2 and Nanog in induction of trophoblast cells after *Zfp560* overexpression. Gray scale statistical analysis. Data are mean ± S.D. of three independent experiments. **P* < 0.05, *****P* < 0.0001, ns was not statistically significant.



Supplementary Fig. 4: ZFP560 inhibited the expression of the three-germ-layer marker genes *in vitro*, but it still retained the ability to form teratomas. a Morphological diagram of embryoid body formation and differentiation after *Zfp560* overexpression. Scale bar, 200 μm. **b** RT-qPCR was performed to measure mRNA levels of ectoderm genes (*Nestin* and *Fgf5*), mesoderm genes (*T* and *Sma*), and endoderm genes (*Sox17* and *Gata6*) after *Zfp560* overexpression in the EB differentiation assay. Data are mean ± S.D. of three independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, ns was not statistically significant. **c** Schematic representation of the plvx-*Zfp560*-EGFP-BSD persistent overexpression plasmid construction. **d** Haematoxylin and eosin (H&E) staining of teratomas derived from OE-Ctrl and consistently OE-*Zfp560* mESCs contain embryonic lineage cells from three germ layers. Scale bar, 200 μm.

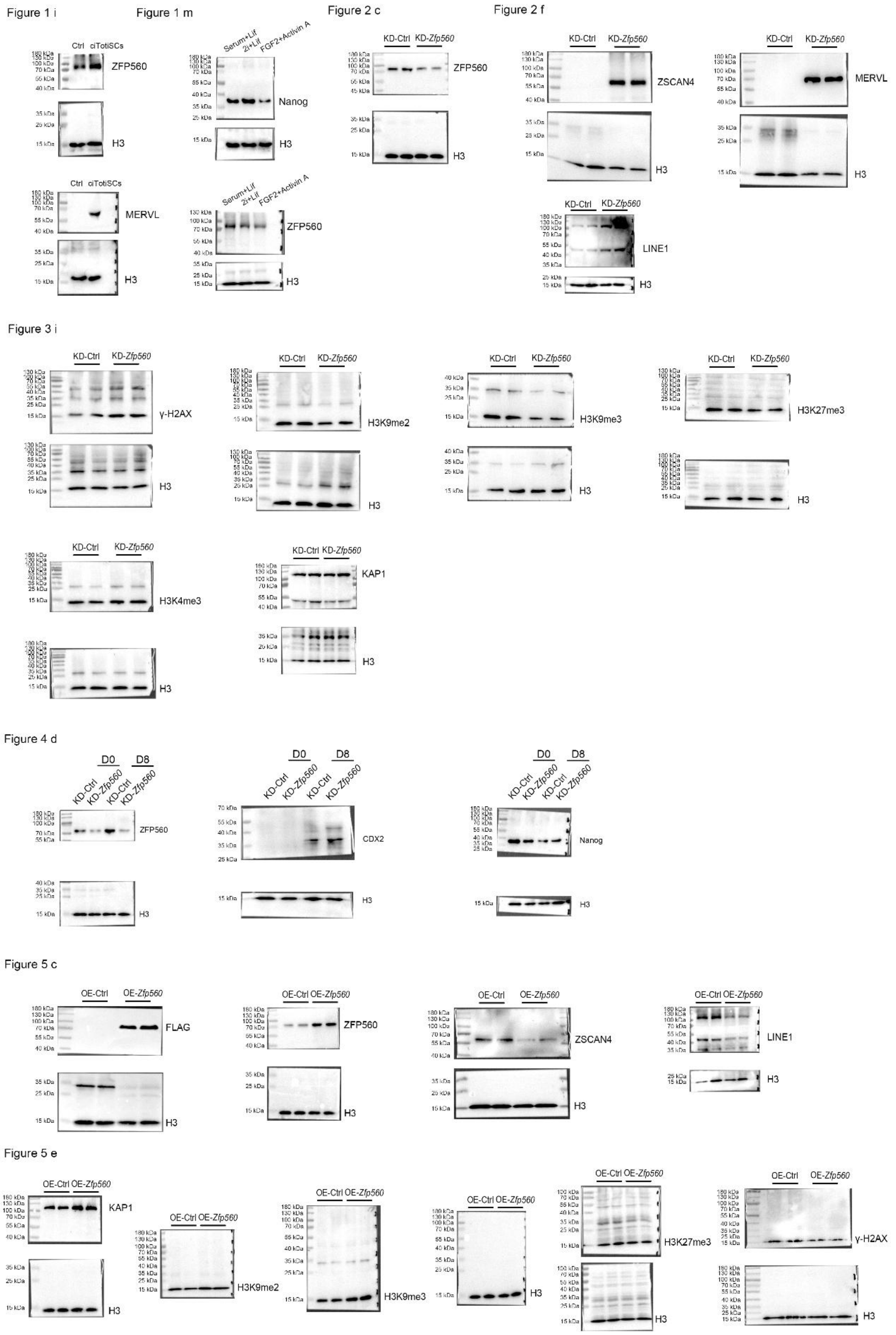


Supplementary Fig 5: KRAB domain-dependent KAP1 recruitment drives H3K9me3 deposition. a Protein-Protein Interaction Networks (PPI) analysis was performed on the core molecules in the gene pathway enrichment network analysis, and the biological processes involved in each core molecule were displayed. **b** KEGG analysis was performed based on the mass spectrometry data obtained for ZFP560-FLAG. **c** Predicted Aligned Error (PAE) plot of the best scoring model using AlphaFold 3 to predict the interaction between ZFP560 and KAP1. **d** COIP was used to detect the interaction between ZFP560 and KAP1. **e** Schematic representation of the FLAG-tagged full-length ZFP560, zinc finger, KRAB domain. **f** Expression of the KRAB domain, zinc finger domain, and full-length Flag of ZFP560 was examined by COIP. **g** Schemata of sample preparation for ChIP-qPCR. **h** ChIP-qPCR experiments were performed in OE-Ctrl, OE-*Zfp560* mESCs induced with Dox for 2 days. Chromatin was prepared from each cell line and subjected to ChIP analysis using an anti-FLAG antibody. The resulting DNA samples were subjected to RT-qPCR in triplicate. Data are mean ± S.D. of three independent experiments. $^{**}P < 0.01$.



Supplementary Fig. 6: The analysis of *ZNF778* (*Zfp560* ortholog) reveals divergent expression dynamics during human embryonic activation and stem cell reprogramming. **a** Heat map of zinc finger protein family expression during early human embryonic development. Data were normalized, and scale bars indicate Z-score. **b** The relative expression of *ZNF778* and 8C stage-specific marker genes (*TPRX1*, *ZSCAN4*) at different developmental stages in panel a was analyzed. **c** Heat map of zinc finger protein family expression in LEUTX$^-$, LEUTX$^+$ (8CLCs), prEpiSCs, chemical-induced 8CLCs (ci8CLCs). Data were normalized, and scale bars indicate Z-score. **d** The relative expression of *ZNF778* and stage 8C specific marker genes in different cell states in panel c was analyzed. **e** The Venn diagram shows the intersection of 8CLC ZGA genes and gene signatures based on human 8-morula embryos and hESCs. **f** The fold change of *ZNF778* relative to primed pluripotent stem cell (PSC) in 8-cell embryo, e4CL-D5, EPSC, 5iLAF and tt2iLGö.

Supplementary Fig. 7: Western blot - full membrane image 1



Supplementary Fig. 8: Western blot - full membrane image 2

Figure 5 j

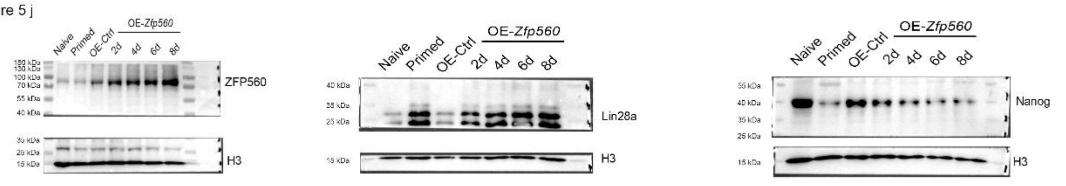


Figure 6 e

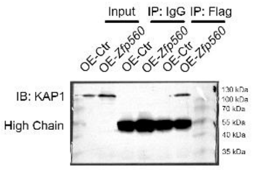


Figure 6 h

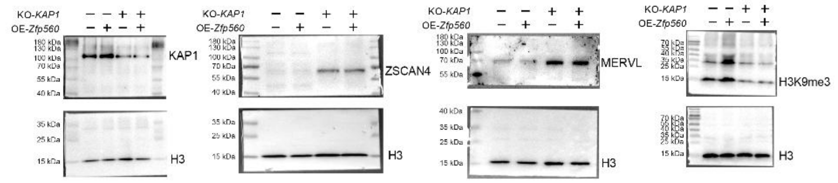


Figure 6 i

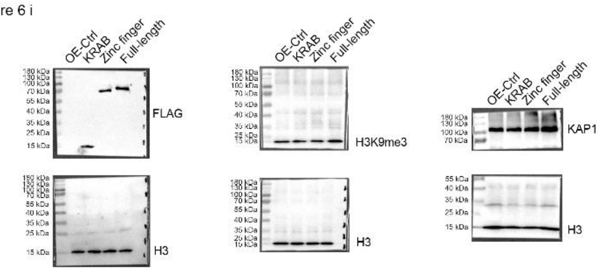


Figure 6 j

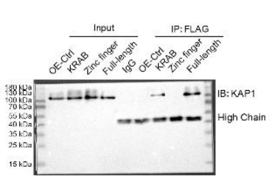


Figure 7 b

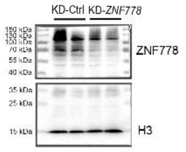
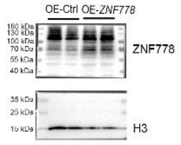
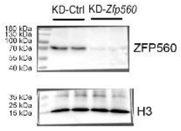


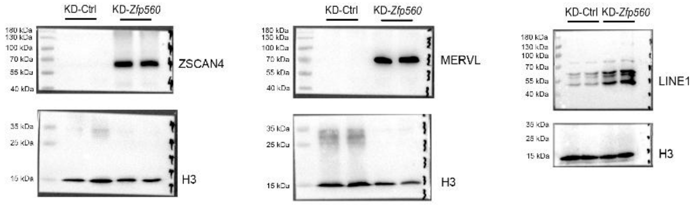
Figure 7 g



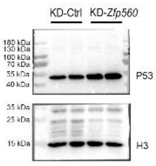
Supplementary Figure 1 d



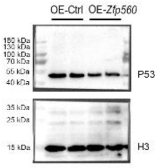
Supplementary Figure 1 f



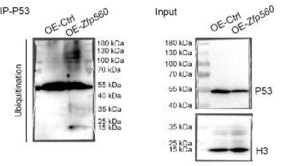
Supplementary Figure 1 i



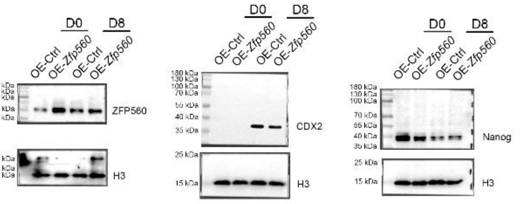
Supplementary Figure 2 f



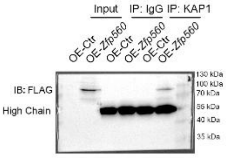
Supplementary Figure 2 h



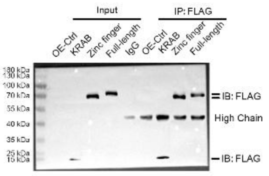
Supplementary Figure 3 c



Supplementary Figure 5 c



Supplementary Figure 5 e



Supplementary Table 12. Primer sequences for qPCR

Primer	**Species**	**Forward (5' to 3')**	**Reverse (5' to 3')**
Zscan4	Mouse	GAGATTCATGGAGAGTCTGACTG	GCTGTTGTTTCAAAAGCTTGATGA
MERVL	Mouse	CTCTACCACTTGGACCATATGAC	GAGGCTCCAAACAGCATCTCTA
Dux	Mouse	AAAGGAAGAGCATGTGCCAGC	GCAGTAAGCTGTCCTGGGAAC
Zfp560	Mouse	GGAGAACTACCAGAACCTGGCTA	CCCGCTGAAACATTGTCCCT
Nanog	Mouse	GCTCCGCTCCATAACTTCG	ACCTGGCTTTGCCCTGACT
Prdm14	Mouse	TGTGTGGTACGGAAATGGCT	GGCGTGTACTTCAGGTGCTT
Dnmt3a	Mouse	GATGCTGGGGACAAGAATGCT	CCATCTCCGAACCACATGACC
Dnmt3b	Mouse	GCTATTTGTCTTGAGGCGCT	AACTTAGAACCCAGGAGACGC
Sp110	Mouse	CCGGGACAATTCCTTCATC	ATTGTGCACCACTTTGGACA
Tcstv1	Mouse	GAACCATCCATCCTCAGGAAC	CCCTGAAGGTAAATCCTCCAC
Pou5f1	Mouse	CCCAACGAGAAGAGTATGAGG	GAGCAGTGACGGGAACAGA
Sox2	Mouse	CCAAGACTGGAGCTCACAATC	CAGGTGGAGCCTGAAAAGAAG
HP1a	Mouse	AGGAGGGTGAAAACAATAAGCC	AATCTGTTGCTCCGATGATCTTT
LINE1	Mouse	AGAACGCCACAAAGATACTCCTC	CTCTCTTCTGGCTTGTAGGGTTTC
Eomes	Mouse	GGAAGTGACAGAGGACGGTG	TTGGCGAAGGGGTTATGGTC
Cdx2	Mouse	GGACTCCGCGAGCCAA	CTCAGCCCACGGTGCTC
Rex1	Mouse	TCACTGTGCTGCCTCCAAGT	GGGCATTGATCCGCAAAC
Nestin	Mouse	GCTGGAACAGAGATTGGAAGG	CCAGGATCTGAGCGATCTGAC
Fgf5	Mouse	AGCGCGACGTTTTCTTCGT	GCCATTGACTTTGCCATCCG
T	Mouse	CACCGCTGGAAATATGTGAA	CACGATGTGAATCCGAGGTT
SMA	Mouse	CATCACCAACTGGGACGACA	TCCGTTAGCAAGGTCGGATG
Sox17	Mouse	AAAGCGGAGTCTCGCATCC	CGCTTCTCTGCCAAGGTCAA
Gata6	Mouse	GCTGAACGGAACGTACCACC	ACAGTGGCGTCTGGATGGAG
18S	Mouse	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
ZNF778	Homo	AGAGCAGTTTTCCGTGTTGGG	CTTCTCCGTTGTGACTTCCCG
TPRX1	Homo	CCTCGGGAATCCTTCCAGC	GATGCCCTTCTGGGCTCT
ZSCAN4	Homo	TGGAAATCAAGTGGCAAAAA	CTGCATGTGGACGTGGAC
POU5F1	Homo	GGGCTCTCCCATGCATTCAAAC	CACCTTCCCTCCAACCAGTTGC
SOX2	Homo	CCATGCAGGTTGACACCGTTG	TCGGCAGACTGATTCAAATAATAC
NESTIN	Homo	TGGCTCAGAGGAAGAGTCTGA	TCCCCCATTCACATGCTGTGA
FGF5	Homo	AAGGAAGTGGCTTGGAGCAG	CATCTGTGAACTTGGCTTAACAT
SMA	Homo	GATGATTCTGACATTTGGGATG	TGGCTTTTATTCTTCTTAGCAG
T	Homo	ACCCAGTTCATAGCGGTGAC	CCATTGGGAGTACCCAGGTT
SOX17	Homo	GAGCCAAGGGCGAGTCCCGTA	CCTTCCACGACTTGCCCAGCAT
GATA6	Homo	CCCACAACACAACCTACAGC	GCGAGACTGACGCCTATGTA
GAPDH	Homo	CGCTTCGCTCTCTGCTCCTCCTGT	GGTGACCAGGCGCCCAATACGA
Minor satellite RNA	Mouse	CATGGAAAATGATAAAAACC	CATCTAATATGTTCTACAGTGTGG
Major satellite RNA	Mouse	TGGAATATGGCGAGAAAACTG	AGGTCCTTCAGTGGGCATTT

Supplementary Table 13. Primer sequences for Chip-qPCR

Primer	Species	Forward (5' to 3')	Reverse (5' to 3')
Zscan4 p1	Mouse	GCATTATCTGTTCCTCTGGGTC	AACTCCTGTTCCTGGGTGGG
Zscan4 p2	Mouse	TGCTGGGTAAGGTAGTGTTT	CATCAGGCTAGGAGAAGTCA
Rpl3	Mouse	GCCTATGCGTCAACACGTTC	CGCAGATAGAGAGCGACCAG
Actin	Mouse	CGTGTGACAAAGCTAATGAGGCTG	CTAAGTTCAGTGTGTGCTGGG

Supplementary Table 14. PCR primer sequences for molecular cloning

Primer	Species	Forward (5' to 3')	Reverse (5' to 3')
shRNA-Zfp560	Mouse	CCGGGCTGGACAAGTAGTTACCAGGCTCGAGGCCTGGTAACTACTTGTCCAGCTTTTTG	AATTCAAAAAGCTGGACAAGTAGTTACCAGGCTCGAGGCCTGGTAACTACTTGTCCAGC
shRNA-ZNF778	Homo	CCGGGGACTCAGTCTGCCTTCATGACTCGAGTCATGAAGGCAGACTGAGTCCTTTTTG	AATTCAAAAAGGACTCAGTCTGCCTTCATGACTCGAGTCATGAAGGCAGACTGAGTCC
sgRNA-Zfp560-1	Mouse	CACCGTGCTGGACAAGTAGTTACC	AAACGGTAACTTGTCCAGCAC
sgRNA-Zfp560-2	Mouse	CACCGCTATACAACTTAACCCCAG	AAACCTGGGGTTAAGTTGTATAGC
sgRNA-Kap1-1	Mouse	CACCGGCCGCAGCGAATAATTCGG	AAACCCGAATTATTCGCTGCGGCC
sgRNA-Kap1-2	Mouse	CACCGGTCCTGGTACGAACTCCAC	AAACGTGGAGTTCGTACCAGGACC
Zfp560-full	Mouse	CGACGCGTCGATGGAAGCTATCAGTTTGTCC	CTAGCTAGCTAGTTACACTGATAAGCTTTCTTGCC
Zfp560-full-Flag	Mouse	GCCACCATGGATTACAAGGATGACGACGATAAGATGGAAGCTATCAGTTTGTCC	CTAGCTAGCTAGTTACACTGATAAGCTTTCTTGCC
Zfp560-ZNF	Mouse	GACTGGGCTATACAACTTAACCCCAG	CTAGCTAGCTAGTTACACTGATAAGCTTTCTTGCC
Zfp560-ZNF-Flag	Mouse	GCCACCATGGATTACAAGGATGACGACGATAAGATGACTGGGCTATACAACTT	CTAGCTAGCTAGTTACACTGATAAGCTTTCTTGCC
Zfp560-KRAB-Flag	Mouse	GCCACCATGGATTACAAGGATGACGACGATAAGATGGAAGCTATCAGTTTGTCC	TTACACTGATAAGCTTTCTTGCCATTGTG

Supplementary Table 15. Antibodies used in this study

Antibodies	host	supplier	application	Catalog number
IgG	Mouse	RD systems	COIP	MAB2566
Flag	Rabbit	ProteinTech Group, Chicago, Inc	COIP	20543-1-AP
ZFP560	Rabbit	ABclonal Biotechnology, Inc	WB, COIP	A18557
MERVL	Rabbit	Beyotime Biotechnology, Inc	WB, IF	AF0240
ZSCAN4	Rabbit	Merck Millipore, Inc	WB	AB4340
LINE1	Rabbit	Novus Biologicals, Inc	WB	NBP2-66934
NANOG	Rabbit	BethylLaboratories	WB, IF	A300-397A-T
CDX2	Rabbit	Abcam, Cambridge, MA	WB, IF	ab76541
Lin28a	Rabbit	Cell signaling technology, Inc	WB	86417
γ -H2AX	Mouse	Cell signaling technology, Inc	WB, IF	80312
H3K9me3	Rabbit	Abcam, Cambridge, MA	WB, IF	ab8898
H3K9me2	Mouse	Abcam, Cambridge, MA	WB	ab1220
H3K27me3	Rabbit	Cell signaling technology, Inc	WB	9733
H3K4me3	Rabbit	Abcam, Cambridge, MA	WB	ab8580
KAP1	Rabbit	BethylLaboratories	WB, IF	A300-274A
P53	Mouse	Santa Cruz, Dallas, TX	WB	sc-71815
Ubiquitin	Rabbit	Cell signaling technology, Inc	WB	43124
H3	Rabbit	Abcam, Cambridge, MA	WB	ab1791