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Mingze Yao

yaomz@sxu.edu.cn

Shanxi University

Lei Zhang

Shanxi University

Wenhao Zhang

Nankai University <https://orcid.org/0000-0001-8002-3947>

Fengfeng Zhang

Shanxi University

Tinglin Ren

Shanxi University

Yiding Zhao

Nankai University

Yangyang Liu

Henan University

Yanqi Zhang

Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences

Huaisong Lin

Hong Kong Institute of Science and Innovation, Chinese Academy of Sciences

Xinquan Zeng

Southern Medical University

Yu Lei

Shanxi University

Xiaoyu Xing

Shanxi University

Shumin Li

Shanxi University

Xiaogang Cui

Shanxi University

Li Xing

Shanxi University

Zhengfeng Li

Guangzhou Institutes of Biomedicine and Health

Xiaotao Dong

Henan University

Jiajian Zhou

Dermatology Hospital, Southern Medical University <https://orcid.org/0000-0001-5206-0791>

Qingfeng Wu

Institute of Genetics and Developmental Biology, Chinese Academy of Sciences

<https://orcid.org/0000-0003-3272-6247>

Xueqing Wu

Children's Hospital of Shanxi and Women Health Center of Shanxi

Ling Shuai

Nankai University <https://orcid.org/0000-0003-0671-7478>

Yongli Shan

Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences

<https://orcid.org/0000-0001-7018-6478>

Qian Gao

Nankai University

Article

Keywords:

Posted Date: March 5th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-8625263/v1>

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Additional Declarations: There is **NO** Competing Interest.

CRB2 fine-tunes F-actin architecture to expand potential in mouse embryonic stem cells

Lei Zhang^{1,6, #}, Wenhao Zhang^{2, #}, Fengfeng Zhang^{1, #}, Tinglin Ren^{1, #}, Yiding Zhao², Yangyang Liu⁴, Yanqi Zhang³, Huaisong Lin³, Xinquan Zeng⁵, Yu Lei¹, Xiaoyu Xing¹, Shumin Li¹, Xiaogang Cui¹, Li Xing¹, Zhengfeng Li³, Xiaotao Dong⁴, Jiajian Zhou⁵, Qingfeng Wu⁷, Xueqing Wu⁶, Ling Shuai², Yongli Shan^{3, *}, Qian Gao^{2, *}, Mingze Yao^{1, *}

¹ Biomedical and Health Laboratory in Shanxi Province, Institutes of Biomedical Sciences, Shanxi Provincial Key Laboratory for Medical Molecular Cell Biology, Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education, Shanxi University, Taiyuan 030006, China.

² State Key Laboratory of Medicinal Chemical Biology, Nankai University, Tianjin 300350, China.

³ Center for Biomedical Digital Science, Guangdong Provincial Key Laboratory of Stem Cell and Regenerative Medicine, Guangdong-Hong Kong Joint Laboratory for Stem Cell and Regenerative Medicine, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou 510530, China.

⁴ Key Laboratory of Receptors-Mediated Gene Regulation and Drug Discovery, School of Basic Medical Sciences, Henan University, Kaifeng 475004, China.

⁵ Dermatology Hospital, Southern Medical University, Guangzhou 510000, China.

⁶ Center of Reproductive Medicine, Children's Hospital of Shanxi and Women Health Center of Shanxi, Taiyuan 030013, Shanxi, China.

⁷ Laboratory of Integrative Physiology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China.

E-mail: yaomz@sxu.edu.cn; gaoqian@nankai.edu.cn; shan_yongli@gibh.ac.cn.

*Corresponding author.

Abstract

Polarity proteins are involved in establishing the intracellular spatial order to define cellular identity and mediate its plasticity in development, repair, and migration. However, its precise role in pluripotency transition and totipotency remains understood. Here, we showed that Crumbs homologue 2 (CRB2), a typical member of CRUMBS protein family orchestrates cell potential extension in mouse embryonic stem cells (mESCs). CRB2 deficiency impairs formation of embryoid bodies and naive to primed transition (NPT) in mESCs. Mechanistically, CRB2 regulates the formation of F-actin branches by modulating the activity of Arp2/3 complex. Furthermore, overexpression of CRB2 confers totipotency on mESCs, enabling the generation of extra-embryonic lineages and the autonomous self-organization of blastoids. Together, these findings demonstrate that a previously unknown role of the

polarity protein CRB2 to orchestrate cell fate transition and totipotency in mouse embryonic stem cells by fine-tuning F-actin structure.

Introduction

Polarity proteins are essential for multiple physiological processes of multicellular organisms, such as cell differentiation, asymmetric cell division, cell migration, and embryonic development¹. Cell polarization is modulated by multiple polarity protein complexes, including CRUMBS complex, Scribble complex, Par complex. The CRUMBS (CRB) family members containing CRB1, CRB2, and CRB3 serve as the canonical polarity proteins which are widely involved in regulating cell polarity in mammals^{1,2}. Only CRB2 deficiency results in embryonic lethality at E12.5 due to the defect in epithelial-to-mesenchymal transition (EMT) at gastrulation³. Moreover, functional deficiencies or dysregulation of CRB proteins lead to the occurrence of multi-system diseases, such as retinitis pigmentosa, neurological disorders, kidney diseases, and tumors due to membrane homeostasis impairment or polarity change of cells⁴⁻⁶. However, the precise roles and their mechanisms of CRB proteins in embryo development and cell lineage specifications remain poorly understood.

During early embryo development, blastomere could undergo compaction to establish polarity in the 8-cell stage, which underlies first cell fate bifurcations and asymmetric cell division from totipotent cells for generating inner cell mass (ICM) and trophectoderm (TE)⁷. The developmental potency of these cells is progressively restricted to derive specific cell lineages. Thus, the phenomenon indicate that cell polarity and associated proteins play important roles in cell lineage differentiation⁸. In addition, embryonic stem cells (ESCs) derived from ICM serve as a powerful tool to decipher cell fate determination and model embryo development *in vitro*. However, mouse ESCs (mESCs) represent a naive pluripotency which are capable of generating all embryonic lineages but extra-embryonic (ExE) lineages including trophectoderm (TE) and primitive endoderm (PrE)⁹. Thus, to expand the potential of mESCs, several studies reported the limitation factors for restricting totipotency using conventional or haploid mESCs¹⁰⁻¹³. Interestingly, at peri-implantation stage, epiblast could derive epiblast stem cells (EpiSCs), representing primed pluripotency. Furthermore, naive to primed transition (NPT) serves as a good model to study cell fate decision^{14,15}. While, the detailed mechanisms underlying regulating totipotency and primed pluripotency transitions from conventional mESCs remain unknown. On the other hand, polarity proteins are indispensable in the processes of embryo polarization and the fate determination of mESCs^{8,16}. The precise roles of polarity proteins, especially CRB proteins, in totipotency expansion and pluripotency transition in mESCs remain illustrated.

Here, we show that the expression level of mouse *Crb2* not *Crb1* or *Crb3* gradually increases during embryoid body (EB) differentiation of mESCs. Notably, CRB2 loss leads to abnormal transition from naive to primed pluripotency in mESCs owing to F-actin branching formation dysregulation.

Furthermore, overexpression of CRB2 confers totipotency on mESCs, enabling the generation of extra-embryonic lineages and the autonomous self-organization of blastoids. Our findings demonstrate that a previously unknown role of the polarity protein CRB2 to fine-tune F-actin structure remodeling and orchestrate pluripotency transition and totipotency in mESCs.

Results

CRB2 is required for epiblast lineage specification of mouse embryonic stem cells

To explore the precise roles of CRB proteins in cell fate decisions, we detected the expression level of CRB members using EB formation assay, which mimics gastrulation process of early embryo development to generate three germ-layer lineages, including endoderm (EN), mesoderm (MES), ectoderm (ECT)^{17,18}. Initially, *Crb2*, not *Crb1* or *Crb3*, was significantly gradually increased during EB formation, indicating that CRB2 may play an important role in early cell fate determination (Fig. 1a, b). Thus, we generated a *Crb2* homozygous knockout mESCs line (*Crb2*^{-/-} mESCs) with typical mESC morphology, normal karyotype, cell survival, cell proliferation and pluripotency maintenance using CRISPR/Cas9 gene-editing technology (Fig. 1c and Extended Data Fig. 1a-h). Furthermore, both RNA-seq and ATAC-seq analyses revealed that *Crb2*^{-/-} mESCs exhibited similar transcriptome and chromatin accessibility compared to wild type mESCs (46C) (Extended Data Fig. 1i, j). Surprisingly, the *Crb2*^{-/-} mESCs-derived EBs displayed a significantly decreased size with the absence of the intermediate cavitation structure and down-regulation of EMT-related markers (Fig. 1d and Extended Data Fig. 2a). The EBs at Day 4-5 are mainly composed of primitive endoderm (PrE)-like and epiblast (EPI)-like lineages, and subsequently generate three germ-layer lineages at Day 6-8^{19,20}. Immunofluorescence staining showed that the GATA4-marked PrE-like structure was not significantly affected, while the SOX1-marked EPI-like structure and distribution were impaired in *Crb2*^{-/-} mESCs-derived EBs (Fig. 1e). Moreover, RNA-seq analysis and qPCR assay showed that the expression of EPI-related marker genes and three germ-layer lineages in *Crb2*^{-/-} EBs were significantly down-regulated at day 4 (D4) and day 8 (D8), respectively, suggesting that CRB2 loss blocked the EPI lineage differentiation (Fig. 1f, g). RNA-seq based principal component analysis (PCA) revealed significant difference in the whole-genome-wide transcriptome profile between *Crb2*^{-/-} and WT EBs at D4 and D8 (Fig. 1h). Additionally, differentially expressed genes (DEGs) induced by *Crb2* deletion were mainly enriched in functions related to actin filament, membranes, and extracellular matrix (ECM) organization, and classical signaling pathways including MAPK, Hippo, Wnt, PI3K-Akt, and p53 (Fig. 1i, j and Extended Data Fig. 2b-d). Together, these data demonstrate that CRB2 is essential for early epiblast lineage specification of mESCs.

CRB2 is essential for naive to primed pluripotency transition

Epiblast-derived stem cells (EpiSCs) represent primed pluripotent state^{14,15,21}. To better explore the precise roles of CRB2 in epiblast lineage specification, we further performed EpiSCs generation from WT and *Crb2*^{-/-} mESCs under defined condition for modeling NPT *in vitro*. Upon NPT medium treatment for two days, they exhibited similar morphology between WT and *Crb2*^{-/-} cells. However, compared to WT cells, *Crb2*^{-/-} cells displayed poor proliferation capability and distinct morphology (round, convex clones) from day 3 to day 5 (Fig. 2a, b and Extended Data Fig. 3a). Indeed, S-phase of cell cycle was significantly decreased and the apoptosis level was increased in *Crb2*-deficient cells at day 4 during NPT process (Fig. 2c-e). Moreover, PCA analysis based on RNA-seq data revealed that WT and *Crb2*^{-/-} cells underwent distinct fate transitions with substantial differential expressed genes during the NPT process (Fig. 2f and Extended Data Fig. 3b). At day 4 of NPT, WT cells silenced naive-related marker genes and highly expressed primed-related marker genes (Extended Data Fig. 3c). Whereas, *Crb2* loss resulted in the expression dysregulation of both naive and primed marker genes (Fig. 2g). Furthermore, on the day 4 and day 6 during NPT process, these DEGs were mainly enriched in neuronal development, actin filament, membrane, and extracellular matrix (ECM) (Fig. 2h and Extended Data Fig. 3d), in consistent with the results in EB formation process in Fig. 1j. In addition, ATAC-seq analysis also revealed that the differential open genes between WT and *Crb2*^{-/-} cells were related to the functions of actin filament, membrane, and ECM (Fig. 2i). Additionally, CRB2 deficiency significantly impaired important signaling pathways involving in cell fate decisions, such as PI3K-Akt, MAPK, p53, Wnt, and Hippo (Fig. 2j). Together, these data demonstrate that CRB2 is indispensable for naive to primed pluripotency transition might dependent on important biological functions (actin filament, membrane, and ECM) and signaling pathways (PI3K-Akt, MAPK, p53, Wnt, and Hippo).

CRB2 interacts with Arp2/3 complex to orchestrate F-actin branch distribution

During the EB differentiation and NPT, the absence of CRB2 significantly perturb biological functions related to F-actin, ECM, and the membrane (Fig. 1j and Fig. 2h). As a core component of the cytoskeleton, F-actin can influence the components of the ECM and regulate the dynamic behavior of the cell membrane through mechanical conduction^{22,23}. Next, we detected F-actin distribution by Immunofluorescence staining with phalloidin in WT and *Crb2*^{-/-} cells at the day 4 of NPT. The knockout of *Crb2* leads to a significant reduction in the distribution of F-actin branches in cells. F-actin was re-arranged in a more tightly bundled pattern to form stress fibers, generating more contractile force and causing cell clones to contract (Fig. 3a). The dynamic distribution of F-actin regulates ROCK signaling pathway^{24,25}. Thus, we hypothesize that CRB2 modulate ROCK pathway activity by orchestrating F-actin branch formation during NPT process. Interestingly, the addition of the ROCK signaling inhibitor

Y27632 can significantly restore the number and morphology of *Crb2*^{-/-} cells during the NPT process, while the addition of activators/inhibitors of neural differentiation (EGF/Afatinib), Wnt (CHIR99021/IWP2), Hippo (TRULI/Verteporfin), and MAPK (FGF2/PD0325901) signaling cannot (Extended Data Fig. 4a). We further detected the activity of ROCK signaling pathway, and found that CRB2 deficiency promoted the phosphorylation of MYPT1 (P-MYPT1), ROS level, and accumulation of active RhoA abundantly on the cell membrane. ROCK inhibitor Y27632 significantly restored phenotype defects, including cell number, morphology, cell cycle, apoptosis defects, and phosphorylated MYPT1 induced by CRB2 depletion at D4 of NPT process. In contrast, the treatment of Narciclasine, a ROCK activator, impaired cell number, morphology, as well as cell cycle and induced apoptosis level in WT cells, which was consistent with the phenotype caused by CRB2 deficiency (Fig. 3b and Extended Data Fig. 4b-g). These data suggest that CRB2 orchestrates F-actin branch formation to modulate ROCK pathway activity during NPT process.

Conventionally, F-actin branches are directly re-organized by the Arp2/3 complex^{26,27}. Therefore, we hypothesized that CRB2 fine-tunes the distribution of F-actin structure through interacting with Arp2/3 complex. Considering that the specific commercial antibody targeting CRB2 is not available, we constructed the overexpression (OE) cell line of exogenous CRB2 with a 3xFLAG-tag (OE-CRB2) (Extended Data Fig. 4h). Interestingly, CRB2 overexpression substantially enhanced the formation of F-actin branches and inhibited ROCK pathway activity during NPT process, compared to that of wild type cells (Fig. 3c and Extended data Fig. 4i, j). To illustrate the interactome of CRB2 protein, we further performed the immunoprecipitation-mass spectrometry (IP-MS) for CRB2 (Fig. 3d). Furthermore, we collected cells at day 4 of NPT in OE-empty vector (OE-EV) and OE-CRB2 cells for IP-MS assay. Among 28 potential proteins that co-precipitated with FLAG antibody, most of them were enriched in functions related to actin cytoskeleton organization, Arp2/3 complex, and actin filament (Fig. 3e). Indeed, ARP3 (~47KD) was identified as a component of CRB2 interactome using silver staining of proteins in polyacrylamide gels (Fig. 3f) and Co-IP experiments (Fig. 3g). These results demonstrate that CRB2 may affect the formation of F-actin branches by interacting with Arp2/3 complex.

The PDZ and FERM domains of CRB2 are essential to modulate F-actin branches

To further explore the mechanisms underlying regulation of CRB2 during NPT process, we performed single cell-RNA-seq (scRNA-seq) in OE-EV and OE-CRB2 mESCs. The same number (1274) of single-cell transcriptome data were randomly selected for analysis. The Uniform manifold approximation and projection (UMAP) plot showed that OE-EV and OE-CRB2 mESCs are globally similar and further divided into five clusters (cluster 0, 1, 2, 3, 4) (Fig. 4a, b). However, the proportion of cluster 3 in OE-CRB2 (18.37%) is significantly

higher than that in OE-EV (7.30%) (Fig. 4c). Furthermore, based on GO analysis, these genes in cluster 3 were enriched in functions related to actin filament and the actin cytoskeleton (Fig. 4d), suggesting that overexpression of CRB2 enhances the actin cytoskeleton, not pluripotency maintenance of mESCs. These results further validate the defective phenotype of F-actin induced by CRB2 deficiency. Moreover, the treatment of CK666, the Arp2/3 complex inhibitor, in wild type cells phenocopied the *Crb2*^{-/-} cells during the NPT process, including massive cell death and a decrease of F-actin branch distribution (Fig. 4e, f). Altogether, these results suggest that CRB2 modulates F-actin branches to mediate naive-to-primed transition.

Conventionally, CRB2 serves as a membrane protein to mediate series of cellular functions dependent on its multiple motifs, including EGF-like, Laminin G, Transmembrane, FERM, and PDZ domains. As the intracellular region of CRB2, FERM and PDZ domains play an important role in regulating intracellular related functions²⁸. Thus, we constructed 3xFLAG-labeled truncated CRB2 mutations with PDZ deletion (Δ PDZ) or PDZ/FERM deletion (Δ FERM) to validate whether these two domains are the key regions for CRB2 to regulate the distribution of F-actin branches (Fig. 4g-i). Interestingly, the full-length CRB2 rather than truncated CRB2 co-localized with ARP3 at day 4 of NPT process (Fig. 4j). Furthermore, overexpression of full-length not truncated CRB2 proteins could promote the formation of F-actin branches compared wild type cells at day 4 of NPT process (Fig. 4k). Together, these results demonstrate that CRB2 interacts with Arp2/3 complex dependent on the PDZ and FERM domains to regulate the formation of F-actin branches, thus affecting the NPT (Fig. 4i).

CRB2 overexpression expands the totipotency potential of mESCs

Given that cell polarization involves in early embryo development, we further investigated whether CRB2-Arp2/3 complex axis regulates F-actin branches in early embryo development. Indeed, we performed embryo chimerism experiments using overexpression of CRB2 or ARP3 in mESCs, and compared with GFP-labeled OE-EV cells, GFP-labeled cells overexpressing either CRB2 or ARP3 owned the more abundant distribution of F-actin branches and cell survival (Fig. 5a), indicating that CRB2-Arp2/3 complex axis promotes F-actin branches in early embryo development *in vivo*. Therefore, we hypothesized that CRB2-Arp2/3 complex axis regulates F-actin branches to expand development potential of mESCs mimicking early embryo development (Fig. 5b).

First of all, we detected the potential of mESCs towards trophectoderm. Coincidentally, OE-EV mESCs lacked the potential towards trophoblast stem cells (TSCs) at day 12 under TSCs medium¹⁰. On the contrast, CRB2 overexpression could induce TSC (OE-CRB2-iTSCs-D12) derivation from mESCs. These iTSCs were purified by performing fluorescence-activated cell sorting (FACS) using the TSC-specific antibody CDCP1 (Extended Data Fig. 5a-c). The obtained OE-CRB2-iTSCs were similar to wild type mTSCs,

evidenced by typical TSC-like morphology, higher expression of typical TSC markers, such as CDCP1, CDX2, EOMES, and silenced expression of typical mESC markers (Extended Data Fig. 5d-g). These results indicate that overexpression of CRB2 enables the formation of trophectoderm lineage from mESCs.

Secondly, we evaluated the potential of mESCs with CRB2 expression towards totipotency *in vitro* and *in vivo*. We further performed blastoid formation, a typical model to validate totipotency *in vitro* according to our previous protocol¹⁰, and found that mESCs with CRB2 overexpression were induced to self-assemble into typical blastoids after 10 days of cultivation, as shown with morphology and higher expression of three early lineage markers, CDX2 (Trophectoderm, TE), GATA6 (Primitive endoderm, PrE), and OCT4 (Epiblast, EPI), which was similar to natural mouse blastocysts at embryonic day 3.5 (E3.5) (Fig. 5c and Extended Data Fig. 5h,i). Furthermore, UMAP analysis based on single-cell RNA sequencing (scRNA-seq) in OE-CRB2 blastoids was similar to the published single-cell transcriptome data of natural mouse blastocysts²⁹, in which there were had three early lineages, EPI, PrE, and TE (Fig. 5d, e). To further validate the essential roles of the CRB2-Arp2/3-F-actin axis in blastoid formation, we induced mESCs over-expressing full-length CRB2, Δ PDZ, Δ FERM, ARP2, and ARP3 to self-assemble blastoids and found that only mESCs with overexpression of full-length CRB2, ARP2 and ARP3, not Δ PDZ, Δ FERM, could form blastoids (Fig. 5f). The results demonstrate that the activation of CRB2-Arp2/3 complex-F-actin axis enables mESCs to self-assemble into blastoids *in vitro*.

Moreover, we further evaluated the *in vivo* potential of mESCs with CRB2 overexpression through microinjecting GFP-labeled OE-EV/OE-CRB2 mESCs into 8-cell-stage mouse embryos. Then, the chimeric embryos were transferred into the oviducts of pseudopregnant mice (Fig. 5g). At E13.5, OE-CRB2 cells was clearly incorporated into the fetus, placenta, and yolk sac, while OE-EV cells only contributed to the fetus (Fig. 5h and Extended Data Fig. 5j). To examine the contribution of OE-CRB2 mESCs to extra-embryonic cell lineages at the cellular level, immunofluorescence staining was performed on placental sections containing three layers: the decidua (Dec), junctional zone (JZ), and labyrinth (Laby). GFP-labeled OE-CRB2 cells were widely distributed throughout the placental tissue and expressed PROLIFERIN, TFAP2C, and GCM1 (Fig. 5i and Extended Data Fig. 5k), indicating that OE-CRB2 mESCs can spontaneously differentiate into spongiotrophoblast cells and trophoblast giant cells *in vivo*.

Taken together, these results demonstrate that overexpression of CRB2 can expand the totipotency potential of mESCs, enabling them to develop into both intra-embryonic and extra-embryonic tissues of mice *in vitro* and *in vivo*.

Discussion

In this study, we uncover the essential roles of CRB2, a typical member of

CRUMBS protein family in fine-tuning F-actin structure and orchestrating naive to primed pluripotency and totipotency transition in mESCs. We demonstrate that CRB2 is indispensable for epiblast specification from mESCs through modulating F-actin branches formation dependent on interacting with Arp2/3 complex using EB assay and NPT systems. Furthermore, we reveal that overexpression of CRB2 could expand the totipotency potential of mESCs, enabling them to develop into both intra-embryonic and extra-embryonic tissues of mice *in vitro* and *in vivo*. Collectively, our findings offer another insight into the regulation of cell fate determination and totipotency potential in mESCs.

Cell polarization establishes intracellular spatial order to define cellular identity in multicellular organisms, which depends on polarity protein and the asymmetrical distribution of cell components¹⁶. The CRUMBS family members containing CRB1, CRB2, and CRB3 serve as the canonical polarity proteins which are widely involved in regulating cell polarity in mammals. Based on loss-of-function of CRB family, only CRB2 deficiency results in embryo lethality at E12.5 due to EMT defects during gastrulation^{3,30}. Our data also deciphered the crucial roles of CRB2 in epiblast specification evidenced as defective EB formation and NPT induced by CRB2 loss (Fig. 1-2). On the other hand, CRB2 overexpression further extended the development potential including trophectoderm, blastoid and chimera assays. Our data systematically define polarity protein CRB2 as a critical guardian ensuring the fidelity of epiblast specification and totipotency potential in mouse embryo development. In the future, the precise roles of cell polarization and other polarization proteins might be worth to investigated in embryo development and cell fate decisions.

As a core cytoskeleton component, F-actin regulates the mechanical signaling conduction and extracellular matrix composition between cells and the ECM^{22,23}. During EB differentiation and NPT, both the absence and overexpression of CRB2 significantly perturb biological functions related to F-actin, ECM, and membrane organization (Fig. 1j, Fig. 2h and Fig. 4d). The branched actin network localized near the cell cortex and membranous organelles, generates forces for cell motility, vesicular transport, and membrane remodeling^{22,23}. At the cell cortex, this pushing force balances the pulling force from myosin motors on linear bundled actin³¹. This force balance is critical for the tight junctions among epithelial cells. The deficiency of CRB2 leads to a reduction in the formation of F-actin branches by affecting the Arp2/3 complex, which blocks the inhibition of RhoA/ROCK signaling, resulting in a large accumulation of bundled actin^{32,33}. Thus, the pulling force generated by myosin between the bundled actin may far exceed the pushing force provided by the branched actin, causing a circular, protruding shape of the cell colonies during the NPT differentiation process (Fig. 2a). Additionally, CRB2 deficiency releases the inhibition of RhoA/ROCK signaling, inducing cell death. Meanwhile, ROCK activates signaling pathways such as MAPK, upregulates *Bax*, and downregulates *Bcl2*, which, together with ROS, enhances the death signal^{34,35}, and leads to a large number of *Crb2*^{-/-} cells dying during the NPT

(Fig. 2a and Extended Data Fig. 4d-g). Indeed, inhibition of ROCK pathway could rescue the cell number and clone morphology defect induced by CRB2 deletion during NPT. In this study, we identified the CRB2-Arp2/3 complex-F-actin-ROCK pathway axis. Therefore, the precise association between ROCK pathway and F-actin is a good point to explore in future studies.

Our study suggests that CRB2 plays critical roles in cell fate decisions including EB differentiation, NPT, derivation of TSCs and blastoid self-assembly, as well as chimera formation by modulating F-actin architecture. F-actin branches are regulated by the Arp2/3 complex activity, which is activated by nucleation-promoting factors (NPFs)³⁶. Here, we identified an interaction between CRB2 and Arp2/3 complex to enable its activity. However, the precise regulation of this process remains incompletely understood. CRB2 might activate or stabilize the Arp2/3 complex through cooperative interactions with NPFs. In future studies, the cryo-EM structure of CRB2-Arp2/3 complex and identification of NPFs interacting with CRB2 will be essential to resolve this question. Intriguingly, multiple studies reported that the Arp2/3 complex contributes to transcriptional regulation and cell fate determination. For example, during the transition from primed to naive pluripotency in human or mouse embryonic stem cells, it promotes the nuclear translocation of the Hippo pathway effectors YAP/TAZ³⁷ or activates the expression and localization of specific transcription factors^{38,39}. In line with this report, *Crb2* knockout in mESCs disrupted transcriptional profiles during EB differentiation and NPT, whereas CRB2 overexpression promotes TSC differentiation and supports blastoid formation. Collectively, these data support a model in which CRB2, likely as a membrane-scaffolded regulator, directly or indirectly modulates Arp2/3 complex activity to remodel the actin cytoskeleton, thereby altering transcriptional programs and ultimately directing cell fate (Fig. 6). Many details about how these processes specifically alter cell fate are indeed not fully understood yet. Whether there are similar mechanisms in the process of fate determination of human ESCs. It would be a compelling direction for future research.

Taken together, our findings indicate that the polarity protein CRB2 as a critical regulator of both pluripotency transition and the acquisition of totipotent potential in mESCs via its interaction with the Arp2/3 complex to modulate branched F-actin architecture. Collectively, our work elucidates a CRB2-Arp2/3 complex-F-actin axis which demonstrates CRB2 determines cell fate by regulating cytoskeletal remodeling. These findings provide a mechanistic framework for how polarity proteins coordinate cytoskeleton, cell fate decisions, and development potential extension in mouse embryonic stem cells.

Methods

Ethics statement

All animal-related experiments were performed according to the guidelines of the Animal Care and Use Committee of Nankai University (No. 2022-SYDWLL-

000070).

Cell culture and generation of cell lines

46C mESC line⁴⁰ was a gift from Dr. Hongjie Yao (Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences). Plasmid PGK-Puro-P2A-mCherry-SV40pA was inserted into exon 2 of the *Crb2* gene in 46C using CRISPR/Cas9 gene-editing technology to generate *Crb2*^{-/-} mESC line. Cells are seeded in cell culture plates pre-coated with 0.2% gelatin and cultured with MES medium consisted of 80% DMEM (Gibco, C11965500BT) and 20% FBS (SORFA, SX1101), supplemented with 1×NEAA (Gibco, 11140-050), 0.1mM β-mercaptoethanol (Gibco, 21985-023), 1× Glutamax (Gibco, 35050-061), 1× Sodium Pyruvate (Gibco, 11360-070), 3μM CHIR99021 (MCE, HY-10182), 1μM PD0325901 (MCE, HY-10254), 1000U/mL LIF (Sino Biological, 14890-HNAH), 1× penicillin and streptomycin (GLP BIO, EH80010). The cells are cultured in an incubator at 37°C with 5% CO₂ and the MES medium is changed every 24 hours.

Embryoid body differentiation

A single-cell suspension was prepared by digesting the cells with 0.25% trypsin (GLP BIO, EH80042). The cells were resuspended at a density of 4×10⁵ cells/mL in 8mL of EB differentiation medium (MES medium without CHIR99021, PD0325901, and LIF). A total of 15–20 mL of sterile PBS was added to a 15 cm cell culture dish, and the cell suspension was dispensed as droplets onto the lid of the dish. The cells were cultured under conditions of 37°C and 5% CO₂ for 4 days. On the Fifth day, the entire cell suspension was transferred to a 10 cm uncoated cell culture dish, supplemented with 2 mL of EB differentiation medium. The medium was replaced every other day.

Naive to primed transition

mESCs cells were acclimated and cultured for 2-3 passages in N2B27+2iL medium. Cells were digested into a single-cell suspension using Accutase (Biolegend, 423201) and resuspended in DMEM/F12 basal medium (Gibco, C11330500BT), then 2000 cells per well were seeded into a 12-well culture plate pre-coated with Fn at a concentration of 16.7μg/mL at 37°C and 5% CO₂. The pre-warmed NPT transition medium was replaced every day. N2B27 medium consisted of 50% DMEM/F12(Gibco, C11330500BT), 50% Neurobasal (Gibco, 21103049), 0.5% N2 (Gibco, 17502048), 1% B27 (Gibco,17504044), 1% Sodium Pyruvate (Gibco,11360-070), 1xGlutaMAX, 1xNEAA, 0.1mM β-mercaptoethanol, 1xpenicillin and streptomycin, 1xSodium Pyruvate. N2B27+2iL medium was composed of the N2B27 medium supplemented with 1000U/mL LIF, 3μM CHIR99021, and 1μM PD0325901. NPT conversion medium was made up of the N2B27 medium supplemented with 15 ng/mL FGF2 (PeproTech, AF-450-33-100UG), 20ng/mL Activin A (Sino Biological, 10429-HNAH), 1μM XAV939 (Selleck, S1180), and 0.5μM IWR (Selleck, S7086).

Differentiation of iTSCs

mESCs were digested into single cells using 0.25% trypsin and resuspended with mouse feeder cell medium. Approximately 50,000 cells were seeded into one well of 0.2% gelatin-precoated 6-well plates and cultured in mouse TSC medium (70CM/F4H). The medium was replaced every day. After approximately 4-5 passages, the grid-like colonies appeared. Then flow cytometry sorting was performed to select positive cells for further culture (CDCP1+). The mouse TSC basic medium consists of: 77% RPMI 1640, 20% fetal bovine serum (FBS), 0.1 mM β -mercaptoethanol, 1 $\times$ glutamine, 1 $\times$ sodium pyruvate, and 1 $\times$ penicillin-streptomycin. The mouse TSC conditioned medium was prepared as follows: mouse feeder cells are cultured in mouse TSC basic medium for 48 hours, then the medium supernatant is collected and filtered through a 0.45 μ m cell filter. The mouse TSC medium (70CM/F4H) is composed of mouse TSC conditioned medium and mouse TSC basal medium at a ratio of 7:3, with the additional supplementation of 1 $\times$ penicillin-streptomycin, 25ng/mL FGF4 (MCE, HY-P7014), and 1 μ g/mL heparin.

The differentiated cells were digested into single cells using 0.25% trypsin, resuspended with PBS containing 1% FBS after centrifugation at 250g for 5 min, then centrifuged again. After removing the supernatant, 100 μ L CDCP1 primary antibody (1:400, R&D Systems, AF4515-SP) was added to each cell sample. Then resuspension samples were incubated at 4°C in the dark for 30 minutes. Next, the PBS (1% FBS)-washed samples were incubated with the secondary antibodies at 4°C in the dark for 30 minutes, washed, and then the supernatant was removed. 300 μ L of PBS containing 0.1% FBS was added. Cells were resuspended and filtrated through a 200-mesh cell strainer. The cells were placed on ice and flow cytometry sorting was performed using a cell sorter (MoFlo Astrios EQ, Cell Sorter - Beckman). The CDCP1+ cells were sorted and seeded into dishes coated with feeder cells in mouse TSC medium (70CM/F4H). For each sample, at least 10,000 cells were used for gating, and then FlowJo software was used for data analysis. The following fluorophores and their respective gating channels were used: Alexa 488 for FITC, Alexa 647 for APC.

Self-assembly of blastoids

mESCs were digested into single cells using 0.25% trypsin. Approximately 500 cells were seeded into one well of a 96-well ultralow attachment plate in blastoids medium composed of 25% TSC basal medium (TSC medium without F4H), 25% N2B27 basal medium (N2B27 medium without 2i and LIF), and 50% K+ Simplex Optimised Medium (KSOM) (Millipore, MR-020P-5F) with 2 μ M Y-27632 (MCE, HY-10071), FGF4 (12.5 ng/ml; PeproTech, 10031), heparin (0.5 mg/ml; Millipore, 375095), 3 μ M CHIR99021 (MCE, HY-10182), BMP4 (5 ng/ml; PeproTech, 123005ET), and 0.5 μ M A83-01 (MCE, HY-10432). When the vesicle-like structures appeared around the cell aggregates, they were transferred to new 96-well plates and cultured for another 2 days, during which

self-assembled blastoids are formed.

Immunofluorescence staining of EBs/blastoids

EBs/blastoids were fixed in 4% paraformaldehyde at room temperature for 15 minutes. Then they were blocked and permeabilized in PBS containing 2% BSA (Gibco) and 0.3% Triton X-100 (VETEC, WXBC5655V) at room temperature for 2 hours. The samples were incubated with primary antibodies at 4°C overnight. The PBS-washed samples were incubated with the secondary antibodies at room temperature for 2 hours. Hoechst 33342/DAPI was used for nuclear staining at room temperature for 15 minutes in the dark. Then the samples were stored in PBS containing a fluorescent antifade agent. The samples were mounted with anti-fade mounting medium and imaged using a LSM 710 confocal microscope (Zeiss). The primary antibodies used in this study include: NANOG (Cell Signaling Technology, 4903, 1:50), OCT4 (Santa Cruz Biotechnology, Sc-5279, 1:50), SOX1 (R&D, AF3369), GATA4 (Proteintech, 19530-1-AP), FLAG (Sigma, F1804, 1: 100), CDX2 (abcam, ab76541, 1:50), GATA6 (Proteintech, 55435-1-AP, 1:50), EOMES (abcam, Ab23345, 1:50), YAP1 (Abclonal, A1002, 1:50), TEAD4 (Proteintech, 12418-1-AP, 1:50), ARP3 (Proteintech, 13822-1-AP, 1:50), RhoA (abcam, AB187027, 1:50), Phalloidin (Solarbio, CA1610). in a lysis buffer, mixed with ethanol absolute, and then

RT-qPCR

According to the manufacturer's protocol, total RNA was extracted from the cell lines using an RNA extraction kit (EZB, B0004DP). In brief, cells were first lysed in a lysis buffer, mixed with ethanol absolute, and then centrifuged. Subsequently, the liquid was combined with an adsorption column and centrifuged to precipitate the RNA. Finally, the RNA was washed with a washing buffer. The lid was uncapped, air-dried, and dissolved in RNase-free DEPC water. The purity and concentration of the RNA were measured using Nanodrop (Thermo). The RNA was reverse transcribed into cDNA using Reverse Transcription Kit (Monad) as follows: 37 °C for 2 minutes, 55 °C for 15 minutes, and 85 °C for 5 minutes. The diluted cDNA was used as a template for qPCR reactions. RT-qPCR was performed using a qPCR MIX (Monad) premix on BIO-RAD under the following conditions: 40 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 20s (with an initial denaturation step of 95°C for 10 minutes). GAPDH was used as the reference gene. The $2^{-\Delta\Delta C_t}$ method was used to calculate the relative gene expression levels. One sample was prepared in triplicate, and the experiment was repeated independently three times. The primer sequences were designed to span multiple exons, and the sequences are shown in the supplementary table.

Western blot

Total proteins were extracted by lysing cells with RIPA buffer containing a mixture of protease and phosphatase inhibitors (97% RIPA, 1% PMSF, 1%

phosphatase inhibitor A, and 1% phosphatase inhibitor B), followed by 30-minute ice-bath incubation and sonication. Denatured protein samples were obtained by adding 5× SDS loading buffer and heating at 98 °C for 10 minutes. Proteins were separated by SDS-PAGE and subsequently transferred onto PVDF membranes (Cytiva, 10600023) using a wet transfer method. The membranes were blocked with 5% skimmed milk and incubated with primary antibodies diluted in TBST at 4°C. After washing with TBST to remove unbound primary antibodies, the membranes were incubated with corresponding horseradish peroxidase-conjugated secondary antibodies at 4°C for 2 hours. Following another TBST wash to remove excess secondary antibodies, signals were detected using a high-sensitivity chemiluminescence substrate and an Amersham Imager 600 system (Amersham). Primary antibody: NANOG (Cell Signaling Technology, 4903, 1:000), OCT3/4 (Santa Cruz Biotechnology, sc-5279, 1:2000), GAPDH (Proteintech, 60004-1-Ig, 1:10000), E-cadherin (Zenbio, 340341, 1:1000), N-cadherin (Zenbio, 380671, 1:1000), Vimentin (Zenbi, R22775, 1:1000S), aspase9 (Proteintech, 66169-1-Ig, 1:2000), P53 (Proteintech, 60283-2-Ig, 1:10000), BAX (ABWAYS, CY5059, 1:2000), BCL2 (ABWAYS, CY6717, 1:2000), MYPT1 (Cell Signaling Technology, 2634, 1:1000), P-MYPT1 (Cell Signaling Technology, 4563, 1:1000), FLAG (Sigma, F1804, 1:1000), ARP3 (Proteintech, 13822-1-AP, 1:1000), HA (Proteintech, 81290-1-RR, 1:5000).

RNA-seq analysis

Use tools such as FastQC to perform quality checks on each fq file and generate detailed quality reports. Subsequently, trim galore was employed to remove low-quality bases and adapter sequences, thereby improving the accuracy and reliability of the data. Next, rsem was utilized to align the quality-controlled and cleaned reads to the reference genome, to determine the genomic origin of each read and identify the corresponding gene expression. Following alignment, the results were counted to determine the expression level of each gene. Subsequently, DESeq2 (an R package) was used to analyze the differential expression of genes/transposons under different experimental conditions. Finally, clusterProfiler was applied to perform functional annotation and pathway enrichment analysis of the differentially expressed genes.

ATAC-seq library construction and analysis

The library construction for ATAC-seq was performed using the Vazyme TD711-01 kit (Vazyme). Briefly, 50,000 cell pellets were collected and lysed by adding Lysis Buffer. After lysis, Fragmentation Mix was added, and the reaction was terminated subsequently. The fragmented products were subjected to DNA extraction using ATAC DNA Extract Beads, followed by library amplification. The amplified products were subjected to two-step fragment selection using ATAC DNA Clean Beads. Upon completion of the above steps, the quality of the constructed library was checked. For ATAC-seq analysis, tools such as FastQC

were used to perform quality control on each fq file and generate detailed quality reports. These reports include information such as read quality distribution, GC content distribution, adapter contamination status, and sequence duplication rate. Trim galore was employed to remove low-quality reads and adapter sequences. The cleaned reads were then aligned to the reference genome to determine the source location of each read to match the sequencing data with the known genome sequence, thereby identifying the gene expression status. After the alignment was completed, it was necessary to count the alignment results to determine the expression level of each gene to generate a gene expression matrix, which represents the expression level of each gene in different samples. MACS2 was used to identify the positions of peaks, and the DeepTools tool was utilized to generate heatmaps. Differential analysis was performed on the identified peaks, followed by annotation using ChIPseeker. Finally, the differential genes were identified, and subsequent overlap analysis could be conducted with RNA-seq results.

scRNA-seq analysis

Quality control was performed on the raw data obtained. Trim galore was used to filter out low-quality bases and adapter sequences to improve data accuracy and reliability. Rsem was employed to align the quality-controlled and cleaned reads to the reference genome, thereby determining the genomic origin of each read. Subsequently, the expression matrix was normalized, and dimensionality reduction was performed using methods such as principal component analysis (PCA), t-SNE, or UMAP to highlight overall differences between samples or cells. Potential cell subpopulations were identified through clustering analysis, and differential expression analysis was conducted between different groups to screen for discriminative marker genes. The FindAllMarkers function in the Seurat package was utilized to systematically identify specifically expressed genes for each cell subpopulation. Based on screening criteria such as significance and expression levels, the top 10 most differentially expressed genes from each subpopulation were selected for functional enrichment analysis to aid in functional definition of the subpopulations. Furthermore, each cell subpopulation was annotated to clarify its biological identity according to the known marker gene sets of cell types and the specific genes identified above. Finally, pseudotemporal trajectory analysis was applied to infer cellular developmental trajectories or differentiation pathways, revealing dynamic transition processes between different states.

Cell proliferation assay

Cells were seeded at an appropriate density in 6-well plates and cultured until reaching optimal confluence. The culture medium was then replaced with Edu working solution (10 μ mol/L), followed by incubation in a cell culture incubator for 30 minutes. The cells were collected by centrifugation and fixed with 4% paraformaldehyde for 15 minutes, followed by permeabilization for 15 minutes

using PBS containing 0.3% Triton X-100. A click reaction was subsequently performed to label EdU with Alexa Fluor 647 (BeyoClick™ EdU Cell Proliferation Kit, Beyotime, C0081S). Nuclei were counterstained with DAPI, and the cells were resuspended in PBS for analysis on a BD LSRFortessa™ X-20 flow cytometer. Data were processed using FlowJo software.

Cell apoptosis assay

Cells were collected and dyed with FITC Annexin V and PI according to the instructions of manufacturer (Abbkine, KTA0002). Analysis was performed using BECKMAN COULTER (BECKMAN) and data were processed using FlowJo software.

IP-WB

The cultured cells were scraped off using a cell scraper and resuspended in pre-chilled lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail). The lysate mixture was rotated at 4 °C for 40 minutes on a rocking platform, followed by brief sonication and centrifugation at 12,000×g for 10 minutes. The supernatant mixture was incubated with FLAG-beads (Millipore, Cat. No. M8823) for 3 hours. The beads were centrifuged gently, separated on a magnetic stand and washed three times (5 minutes per wash) with IP buffer of three different concentrations (0 mM NaCl IP buffer, 170 mM NaCl IP buffer, and 340 mM NaCl IP buffer, respectively). The immunoprecipitated proteins were eluted by heating the beads in 2×loading buffer at 98°C for 10 minutes. Then samples were used for western blot analysis.

Embryonic Chimerism Experiment

Healthy adult female mice were selected to inject hormones and mated with male mice. At embryonic day 1.5 (E1.5), the pregnant female mice were sacrificed. Their oviducts were isolated, and 2-cell stage embryos were flushed out. The flushed mouse embryos were washed 3 times with KSOM medium (Millipore, MR-020P-5F) and then cultured in a 37°C incubator. GFP-labeled ESCs were injected into 4-8 cell stage embryos to construct chimeric embryos. The chimeric embryos were then cultured continuously in a 37°C incubator for one day. On the next day, they were transferred into the uterus of pseudopregnant female mice, allowed to develop until E12.5, and then the embryos were harvested.

Placental Chimerism Staining

E12.5 placentas were embedded in O.C.T. compound (SAKURA, 4583) and frozen at -80°C. Tissue sections (8mm thick) were cut using a cryostat microtome and transferred onto histological slides. Samples were permeabilized and blocked with 1% Triton X-100 and 5% BSA at room temperature for 1 hour. Samples were incubated with diluted primary antibodies

at 4°C overnight. The primary antibodies used include PROLIFERIN (Santa Cruz, sc-271891), GCM1 (Santa Cruz, sc-101173), TPBPA (Abcam, ab320823), and TFAP2C (Santa Cruz, sc-12762). After washing three times in PBS, the samples were incubated with diluted secondary antibodies at room temperature for 1 hour. Following three additional washes in PBS, cell nuclei were stained with Hoechst 33342. Samples were imaged using a total internal reflection fluorescence (TIRF) microscope (Leica, DMI8).

Data availability

Source data are provided with this paper. All sequencing data generated in this study have been deposited into the Gene Expression Omnibus (GEO; <https://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE315525, 315526, 315682. RNA-seq data of mTSCs and scRNA-seq data of mouse blastoids analyzed in this study are obtained under the following accession codes from the Genome Sequence Archive (CRA005730) and GEO database (GSE135701). All other data supporting the findings of this study are available from the corresponding author on reasonable request.

Acknowledgements

We are grateful to the Shanxi Provincial Key Laboratory for Medical Molecular Cell Biology at the Institutes of Biomedical Sciences, Shanxi University, for providing us with a scientific experimental platform. We thank Jing Liu, Xichen Bao, Ping Lai and Huanhuan Li for suggestions and discussion. This work was supported by grants from the National Natural Science Foundation of China (32270843, 32070654), the Central Government Guidance on Local Science and Technology Development Fund (YDZJSX2022B002), the Four Batches Innovation Project of Invigorating Medical through Science and Technology of Shanxi Province China (2023XM058), Guangdong Special Support Program” for Young Talents in Science and Technology Innovation (2024TQ08A178 to Y.S.).

Author contributions

Lei Zhang designed and performed most of the experiments and analyzed data, and wrote the paper. Experiments related to blastoids self-assembly, iTSCs induced differentiation, and embryo chimerism were carried out by Wenhao Zhang and. Fengfeng Zhang and Tinglin Ren completed part of the molecular biology experiments. Xinquan Zeng and Yangyang Liu analyzed the sequencing data. Shuming Li, Yanqi Zhang, Huaisong Lin, Xiaoyu Xing, Yu Lei and Yiding Zhao participated in the processing of some experimental data. Ling Shuai, Qingfeng Wu, Xiaogang Cui, Li Xing, Zhengfeng Li, Jiajian Zhou, Xiaotao Dong and Xueqing Wu contributed to manuscript review and editing. Qian GAO and Yongli Shan provided conceptual advice and designed experiments. Mingze Yao conceived and supervised the study, designed experiments and wrote the paper.

Funding

Open access funding provided by Shanxi University.

Competing interests

The authors declare no competing interests.

Additional information

Extended data is available for this paper at

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Figure legend

Fig. 1 CRB2 is involved in the formation of EPI-like lineages in EBs. **a**, Expression heatmap of *Crb1/2/3* during different days of EB differentiation. **b**, Relative expression of *Crb2* during EB differentiation. *** $P < 0.001$, **** $P < 0.0001$. **c**, Schematic diagram of knocking out the *Crb2* gene in mESCs using CRISPR/Cas9 gene-editing technology. The knockout of the *Crb2* gene is achieved by inserting the selection resistance puromycin, the screening marker mCherry, and the transcription termination signal SV40pA into exon 2 of the *Crb2*. **d**, The morphology and volume of WT and *Crb2*^{-/-} EB spheres at D4/8 of EB differentiation induction. **** $P < 0.0001$. The diameter of EB spheres was counted using the software ImageJ. **e**, Immunofluorescent staining of WT and *Crb2*^{-/-} EBs at D4 of EB differentiation. Mark the EPI-like structures with SOX1 and the PrE-like structures with GATA4. **f, g**, Heatmap of representative PrE, EPI, EN, MES, ECT and BP genes in WT and *Crb2*^{-/-} EBs at D4/D8 of EB differentiation. Meanwhile, qPCR was used to validate some of the marker genes corresponding to the lineages. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. **h**, PCA of WT and *Crb2*^{-/-} mESCs and cells at D4 and D8 of EB differentiation. **i**, Heatmap showing some DEGs related to actin filament, membrane and ECM between WT and *Crb2*^{-/-} cells. **j**, GO analysis of DEGs between WT and *Crb2*^{-/-} cells at D4 of EB differentiation.

Fig. 2 CRB2 is crucial for fate transition during the NPT process. **a**, The *Crb2*^{-/-} cell colonies showed abnormal circular bulges and a large number of cells died during the NPT. **b**, Statistics of the number of WT and *Crb2*^{-/-} cells from D0 to D6 of NPT. NS $P > 0.05$, *** $P < 0.001$, **** $P < 0.0001$. **c**, Flow cytometric analysis after Edu treatment revealed that the proportion of S-phase cells in *Crb2*^{-/-} cells significantly decreased at D4 of NPT. ** $P < 0.01$. **d**, Flow cytometric analysis after Propidium Iodide and Annexin V staining showed that the deletion of CRB2 promoted apoptosis. *** $P < 0.001$. **e**, Detect the expression levels of apoptosis-promoting factor (Caspase9, P53, and BAX) and apoptosis-inhibiting factor (BCL2) in WT and *Crb2*^{-/-} cells at D4 of NPT by western blot. **f**, PCA of WT and *Crb2*^{-/-} mESCs, and cells at D4 and D6 of NPT. **g**, The heatmap shows that the expression of naive and primed marker genes in *Crb2*^{-/-} cells is disordered at D4 of NPT. **h**, GO analysis of DEGs between WT and *Crb2*^{-/-} cells at D4 of NPT. **i**, The heatmap shows the relative expression of marker genes related to actin filament, membrane and ECM in WT and *Crb2*^{-/-} mESCs, and cells at D4 and D6 of NPT. Meanwhile, a heatmap was presented to show the openness of these corresponding genes. **j**, KEGG enrichment analysis of DEGs between WT and *Crb2*^{-/-} cells at D4 and D6 of NPT.

Fig. 3 CRB2 affects NPT by regulating the formation of F-actin branches. **a**, Immunofluorescence staining of F-actin with phalloidin in WT and *Crb2*^{-/-} cells at D4 of NPT. **b**, Phase pictures of WT, WT+Y27632 (10 μ M), WT+Narciclasine (100nM), *Crb2*^{-/-} and *Crb2*^{-/-}+Y27632 (10 μ M) at D4 and D6 of NPT and

quantification of cell numbers under corresponding culture conditions. ^{NS} $P > 0.05$, $***P < 0.001$, $****P < 0.0001$. **c**, Immunofluorescence staining of F-actin with phalloidin in OE-EV and OE-CRB2 cells at D4 of NPT. **d**, A schematic diagram shows the collection of OE-EV and OE-CRB2 cells at the D4 of NPT for IP. Then the proteins bound to Flag beads are subjected to mass spectrometry analysis. **e**, GO analysis of proteins that potentially interact with CRB2 protein. **f**, Samples from OE-EV and OE-CRB2 cells after IP were subjected to Polyacrylamide Gels electrophoresis. The silver staining results showed that there were potential interacting proteins of CRB2 between 40-50KD. **g**, Co-IP of ARP3 and CRB2-FLAG in OE-CRB2 cells at D4 of NPT.

Fig. 4 CRB2 regulates the formation of F-actin branches through the PDZ and FERM domains. **a**, A UMAP plot of 2548 cells from OE-EV and OE-CRB2 mESCs after alignment using the Seurat package. **b**, The UMAP plot shows that there are five clusters in OE-EV and OE-CRB2 mESCs, namely cluster 0, cluster 1, cluster 2, cluster 3, and cluster 4. **c**, The proportion of the five cell clusters in OE-EV and OE-CRB2 mESCs. **d**, GO analysis of differentially expressed genes in cluster 3 cells. **e**, Bright field images of WT, *Crb2*^{-/-} and WT treated with CK666 (80μM) on the D3 and D4 of NPT, and count the number of cells at the D4. $***P < 0.001$. **f**, Immunofluorescence staining of F-actin with phalloidin in WT, *Crb2*^{-/-} and WT treated with CK666 at D4 of NPT. **g**, Schematic diagrams of full-length CRB2 protein, PDZ domain-deleted protein, and PDZ+FERM domain-deleted protein. qPCR (**h**) and western blot (**i**) shows the expression levels of full-length CRB2, ΔPDZ, and ΔFERM in OE-CRB2, OE-ΔPDZ and OE-ΔFERM mESCs. **j**, Immunofluorescence staining shows the localization and distribution of ARP3-HA and CRB2-FLAG, ΔPDZ-FLAG, ΔFERM-FLAG in cells at the D4 of NPT differentiation. **k**, Immunofluorescence staining of F-actin with phalloidin in OE-EV, OE-ΔPDZ, OE-ΔFERM and OE-CRB2 cells at D4 of NPT. **l**, Model of the interaction between CRB2 and ARP3 in regulating NPT differentiation.

Fig. 5 Overexpression of CRB2 endows mESCs with totipotency. **a**, Immunofluorescence staining of F-actin with phalloidin in chimeric blastocysts derived from GFP-labeled OE-EV, OE-CRB2 and OE-ARP3 mESCs. **b**, Hypothetical diagram of CRB2 expanding the potential of mESCs. **c**, Immunofluorescence staining shows that both E3.5 mouse blastocysts and OE-CRB2 self-assembled blastoids can express the EPI marker OCT4, the PrE marker GATA6, and the trophectoderm marker CDX2. **d**, UMAP plot of single-cell transcriptomes from blastocysts and blastoids. **e**, The UMAP plot shows the expression of three lineage markers (EPI, *Oct4*; PrE, *Gata6*; TE, *Cdx2*) in blastoids. **f**, The self-assembly efficiency of blastoids from OE-EV, OE-CRB2, OE-ARP2, OE-ARP3, OE-ΔPDZ and OE-ΔFERM mESCs, and the immunofluorescence localization of three lineage markers (EPI, *Oct4*; PrE, *Gata6*; TE, *Cdx2*) in the blastoids. $****P < 0.0001$. **g**, Schematic of the

developmental contribution of microinjection of OE-EV and OE-CRB2 mESCs into chimeric embryos. **h**, Images of E13.5 chimeras (including fetus, placenta, and yolk sac) derived from GFP-labeled OE-EV and OE-CRB2 mESCs. **i**, Immunohistochemistry analysis of placenta sections from E13.5 chimeras. The placenta was stained with PROLIFERIN antibody for labeling trophoblast.

Fig. 6 Schematic diagram of CRB2 expanding the potential of mESCs. CRB2 regulates the formation of F-actin branches by influencing the activity of the Arp2/3 complex, thereby causing the remodeling of the cytoskeleton. During the transition of mESCs to EpiSCs, the CRB2-Arp2/3 complex-ROCK axis plays a crucial role. Furthermore, overexpression of CRB2 promotes the formation of F-actin branches, expands the totipotent potential of mESCs, and endows them with the ability to differentiate into TSCs, self-assemble into blastoids, and have the ability to contribute to chimerism of all germ layers of mouse embryos.

Extended Data Figure legend

Extended Data Fig. 1 CRB2 is not essential for the maintenance of pluripotency and self-renewal of mESCs.

a, qPCR analysis of expression *Crb2* gene in WT and *Crb2*^{-/-} mESCs. PCR is used to identify the copy number of the *Crb2* gene in the whole genome. **b**, Karyotype analysis of *Crb2*^{-/-} mESCs after long-term culturing under the 2iL condition. **c**, *Crb2* homozygous knockout mESCs are labeled with mCherry from the inserted donor (PGK-Puro-P2A-mCherry-SV40pA). **d**, The CCK8 assay was used to generate the growth curves of WT and *Crb2*^{-/-} mESCs. ^{NS}*P* > 0.05. **e, f**, Flow cytometry analysis results show that there are no significant differences in the cell cycle ratios and apoptosis levels between WT and *Crb2*^{-/-} mESCs. ^{NS}*P* > 0.05. **g**, Immunofluorescence staining shows the distribution of NANOG and OCT4 in WT and *Crb2*^{-/-} mESCs. **h**, Western blot analysis showing the levels of NANOG and OCT4 in WT and *Crb2*^{-/-} mESCs. **i**, Analysis of pluripotency genes in RNA-seq and ATAC-seq data of WT and *Crb2*^{-/-} mESCs. **j**, Pearson correlation of RNA-seq of WT and *Crb2*^{-/-} mESCs.

Extended Data Fig. 2 CRB2 deficiency leads to abnormal EB differentiation.

a, Western blot analysis of EMT marker protein (N-Cadherin, E-Cadherin and Vimentin) in WT and *Crb2*^{-/-} EBs at D8 of EB differentiation. **b**, Heatmap showing some DEGs related to actin filament, membrane and ECM between WT and *Crb2*^{-/-} cells. **c**, GO analysis of DEGs between WT and *Crb2*^{-/-} cells at D8 of EB differentiation. **d**, KEGG enrichment analysis of DEGs between WT and *Crb2*^{-/-} cells at D4 and D8 of EB differentiation.

Extended Data Fig. 3 Homozygous knockout of *Crb2* leads to disruption of the transcriptome level during NPT.

a, Crystal violet staining showing the survival of WT and *Crb2*^{-/-} cell clones at the D4 of NPT. **b**, The differentially expressed genes between WT and *Crb2*^{-/-} cells at D4 of NPT. **c**, The relative expression of naive and primed genes in WT mESCs and cells at D4 of NPT. **d**, KEGG enrichment analysis of DEGs between WT and *Crb2*^{-/-} cells at D6 of NPT.

Extended Data Fig. 4 CRB2 regulates the ROCK signaling during the NPT process.

a, Phase pictures of WT, *Crb2*^{-/-}, *Crb2*^{-/-}+EGF (20ng/mL)/Afatinib (1μM), *Crb2*^{-/-}+ CHIR99021 (3μM)/IWP2 (2μM), *Crb2*^{-/-}+ TRULI (10μM)/Verteporfin (3μM), *Crb2*^{-/-}+ FGF2 (20ng/mL)/PD0325901 (1μM) and *Crb2*^{-/-}+ Narciclasine (100nM)/Y27632 (10μM) at D4 of NPT and quantification of cell numbers under corresponding culture conditions. ^{NS}*P* > 0.05, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. **b**, Immunofluorescence staining of WT and *Crb2*^{-/-} cells for RhoA at D4 of NPT. **c**, P-MYPT1 and MYPT1 immunoblotting of WT, *Crb2*^{-/-} and *Crb2*^{-/-} cells treated with Y27632 for 72h at D4 of NPT. **d**, Flow cytometry analysis of the cell cycle of WT, WT + Narciclasine, *Crb2*^{-/-}, and *Crb2*^{-/-} + Y27632 at D4 of NPT. *****P* < 0.0001. **e**, Western blot

analysis showing the levels of P53, BCL2 and BAX of WT, *Crb2*^{-/-} and *Crb2*^{-/-} +Y27632 at D4 of NPT. **f**, Apoptotic cell populations in WT, WT+Narciclasine, *Crb2*^{-/-}, *Crb2*^{-/-}+Y27632 were analyzed using Propidium Iodide and Annexin V staining. ****P* < 0.001. **g**, WT, WT + Narciclasine, *Crb2*^{-/-}, and *Crb2*^{-/-} + Y27632 were stained with H2DCFDA, and then the ROS levels were analyzed by flow cytometry. ***P* < 0.01, *****P* < 0.0001. **h**, Western blot analysis showing the expression level of CRB2 in OE-EV and OE-CRB2 mESCs. **i**, Western blot shows P-MYPT1 and MYPT1 protein levels in OE-EV and OE-CRB2 cells at D4 of NPT. **j**, Immunofluorescence staining shows the distribution of RhoA in OE-EV and OE-CRB2 cells at D4 of NPT.

Extended Data Fig. 5 Overexpression of CRB2 can promote the differentiation of mESCs into TSCs and the self-assembly of blastoids. a, Phase pictures of OE-EV and OE-CRB2 cells after 12 days of TSC-induced differentiation and after sorting. FACS analysis of TE-specific marker positive (CDCP1+) cells in control cells (OE-EV), OE-CRB2 cells, and mTSCs after 12 days of TSC-induced differentiation (**b**) and FACS (**c**). **d**, Immunofluorescence staining of TSC marker CDX2 and EOMES in OE-EV and OE-CRB2 cells after 12 days of TSC-induced differentiation. **e**, Heatmap showing the relative expression of representative TSC genes in OE-EV and OE-CRB2 cells after 12 days of TSC-induced differentiation. **f**, PCA of RNA-seq data from the OE-EV-mESCs, OE-CRB2-mESCs, OE-CRB2-iTSCs and mTSCs. **g**, Heatmap showing the relative expression of TSC and pluripotency genes in OE-EV and OE-CRB2 mESCs and OE-CRB2-iTSCs cells. **h**, Bright field images of *in vitro*-induced positive control (*Dyrk1a*^{-/-})¹¹ and OE-CRB2 mESCs on the D10 of blastoids self-assembly. **i**, Efficiency statistics of inducing self-assembly of blastoids from mESCs (WT, OE-EV, *Crb2*^{-/-}, OE-CRB2, positive). **j**, Images of E13.5 chimeras (including fetus, placenta, and yolk sac) derived from GFP-labeled OE-CRB2 mESCs, with un-injected embryos as a control. **k**, Immunohistochemistry analysis of placenta sections from E13.5 chimeras. The placenta was stained with TFAP2C and GCM1 antibody for labeling trophoblast.

Figure. 1

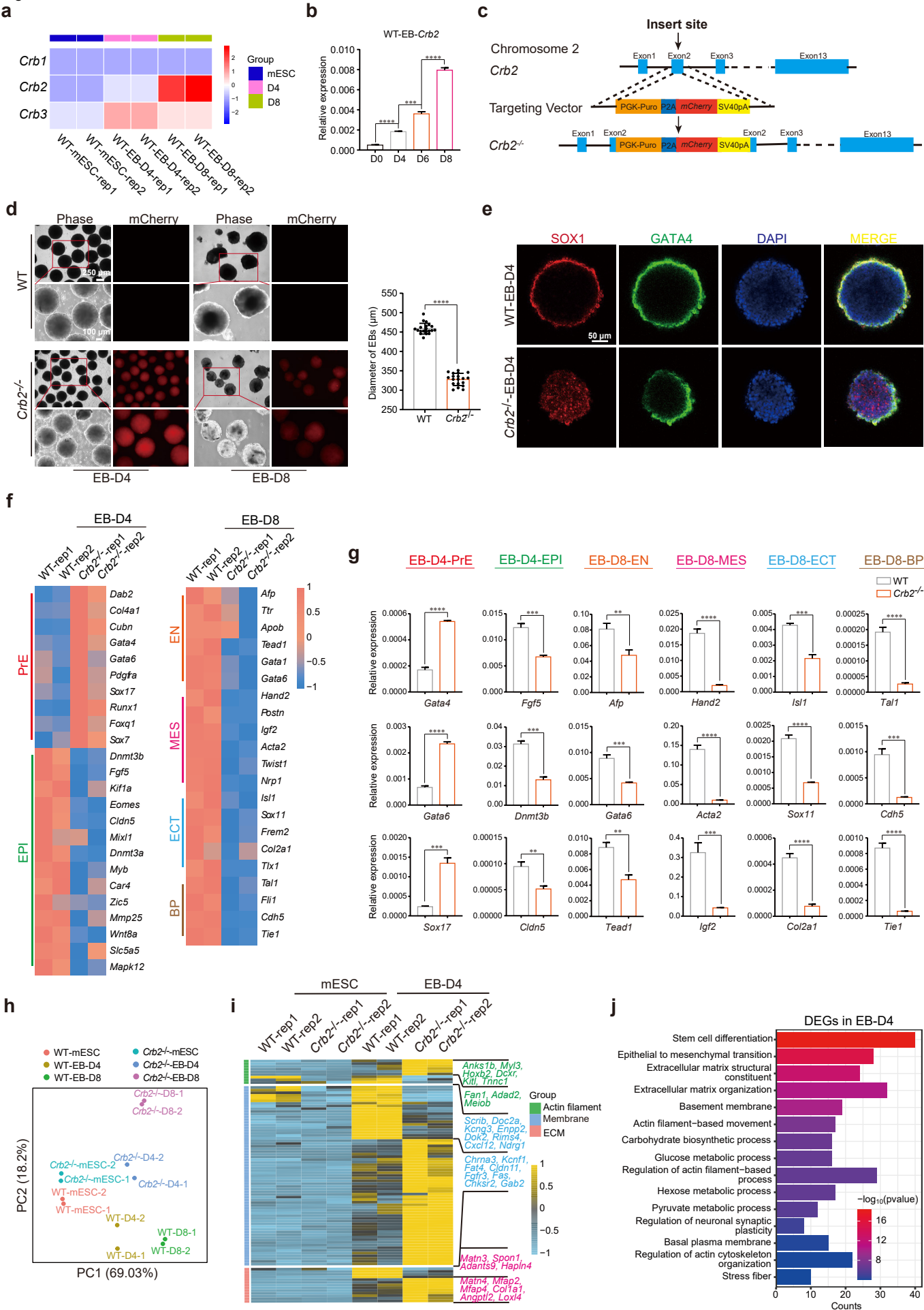


Figure. 2

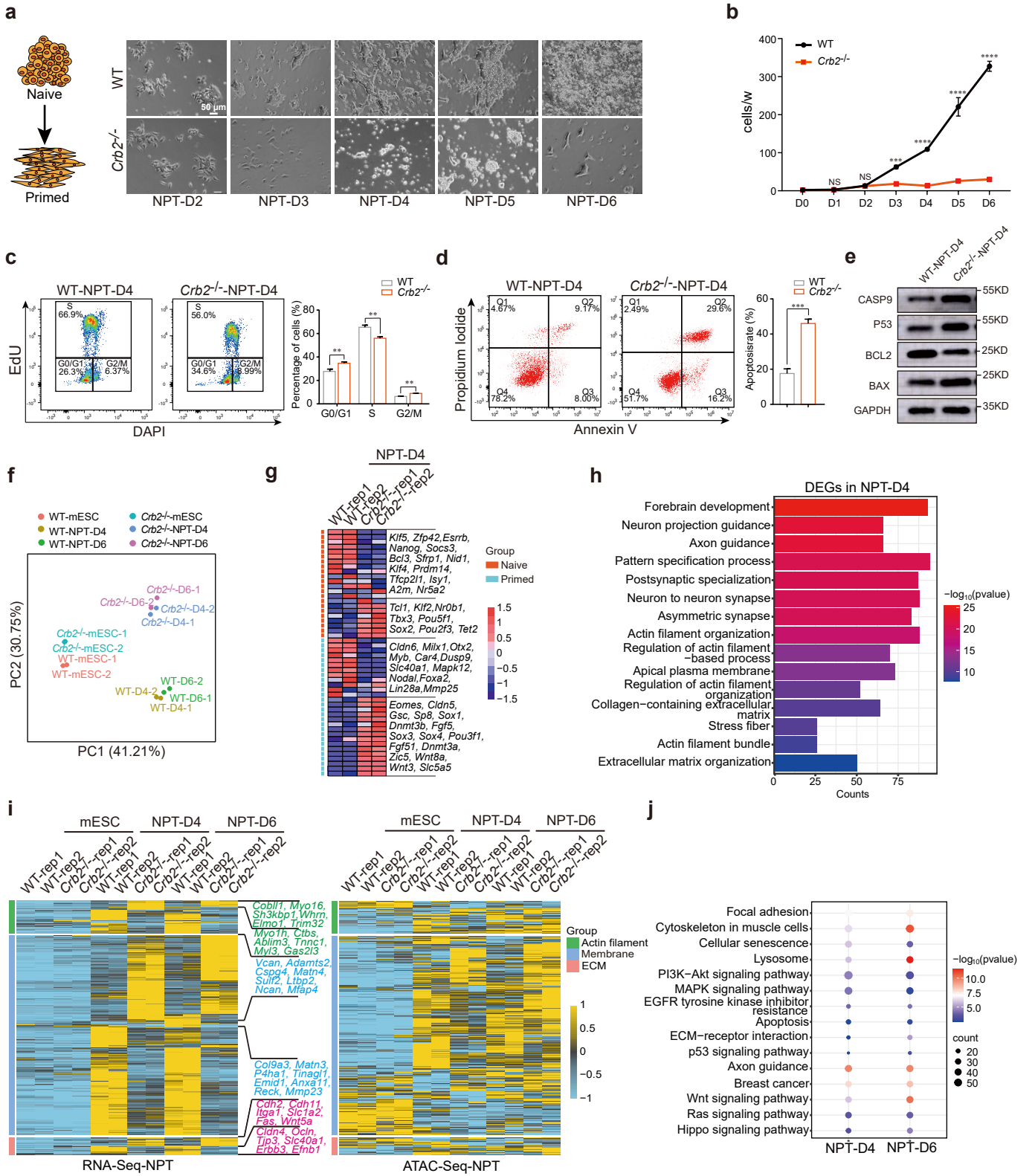


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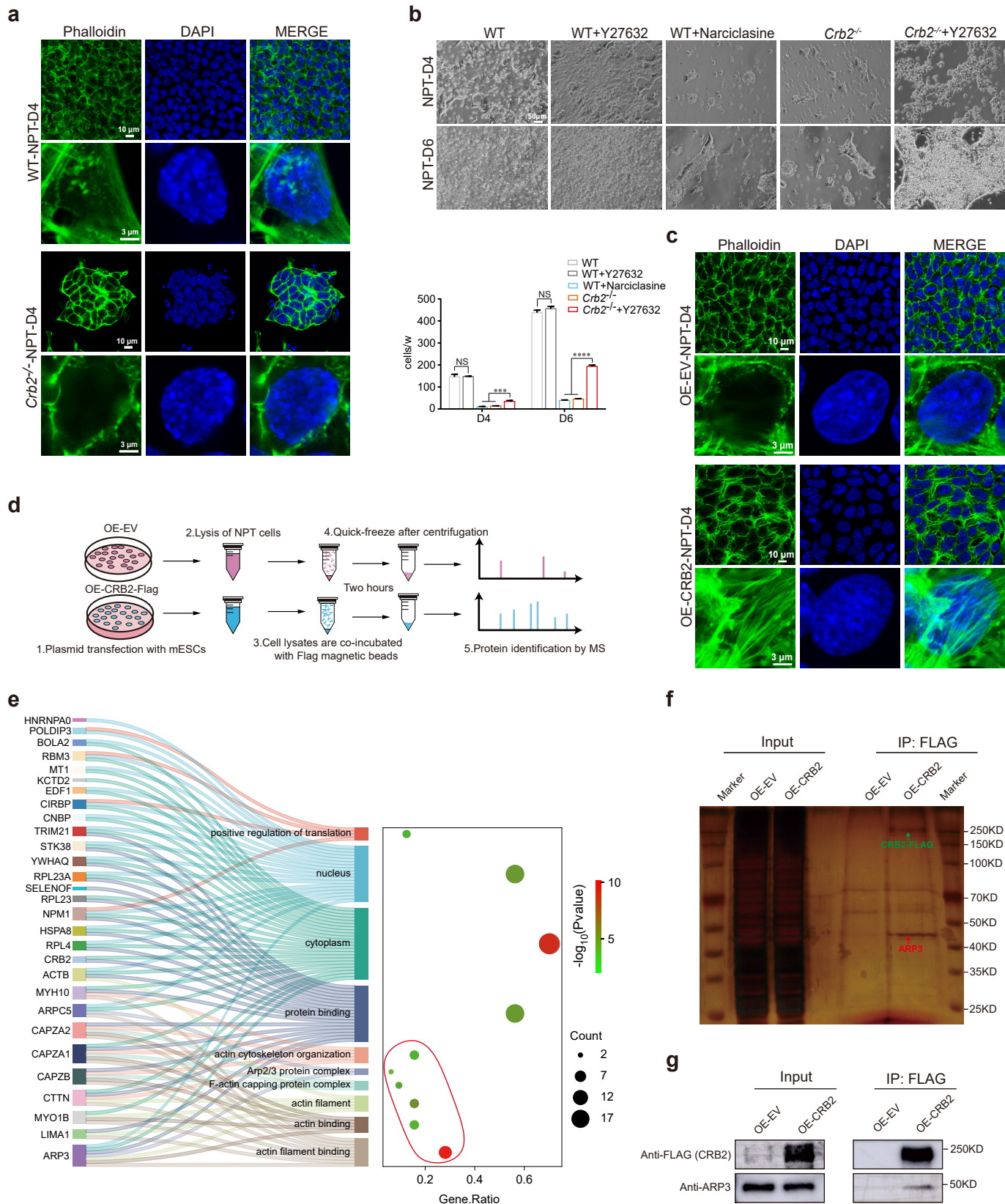


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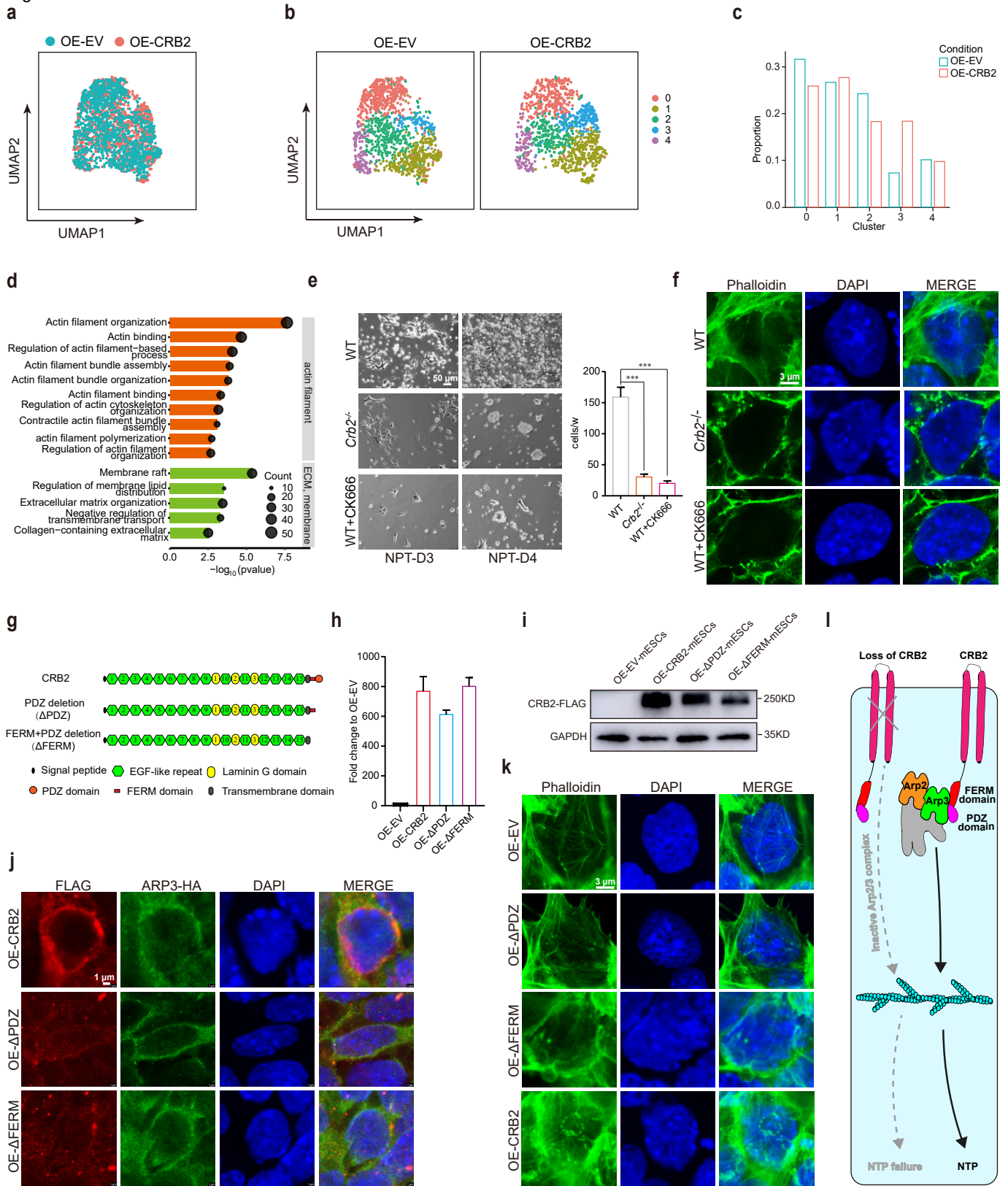


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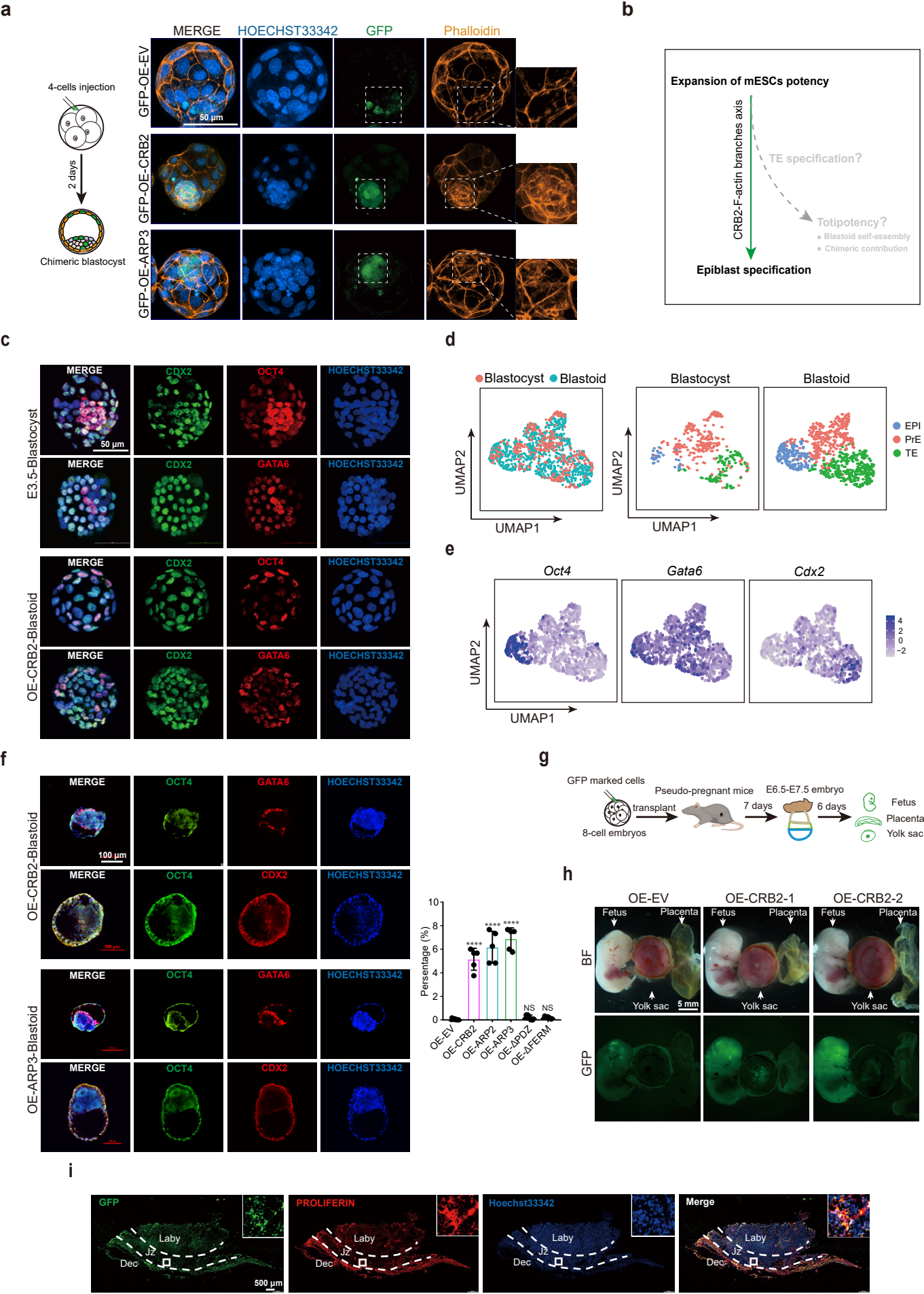
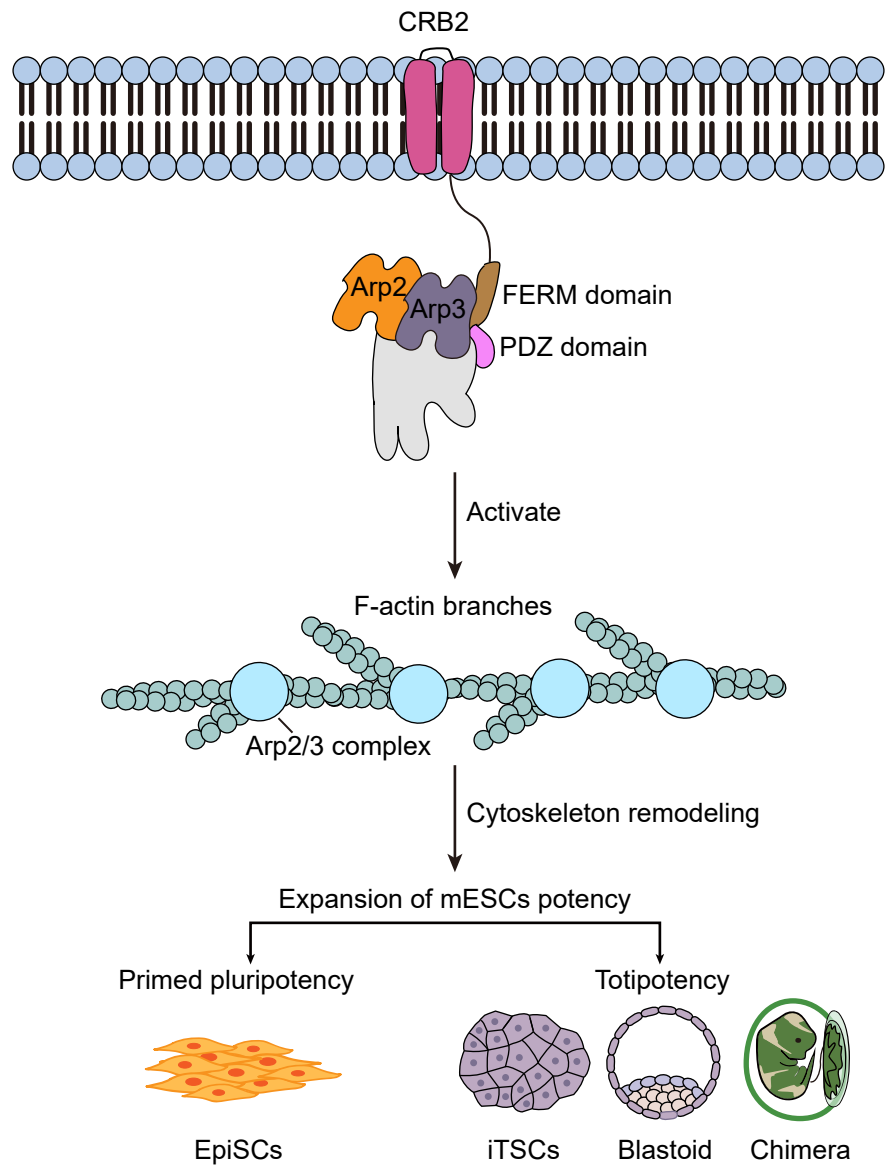
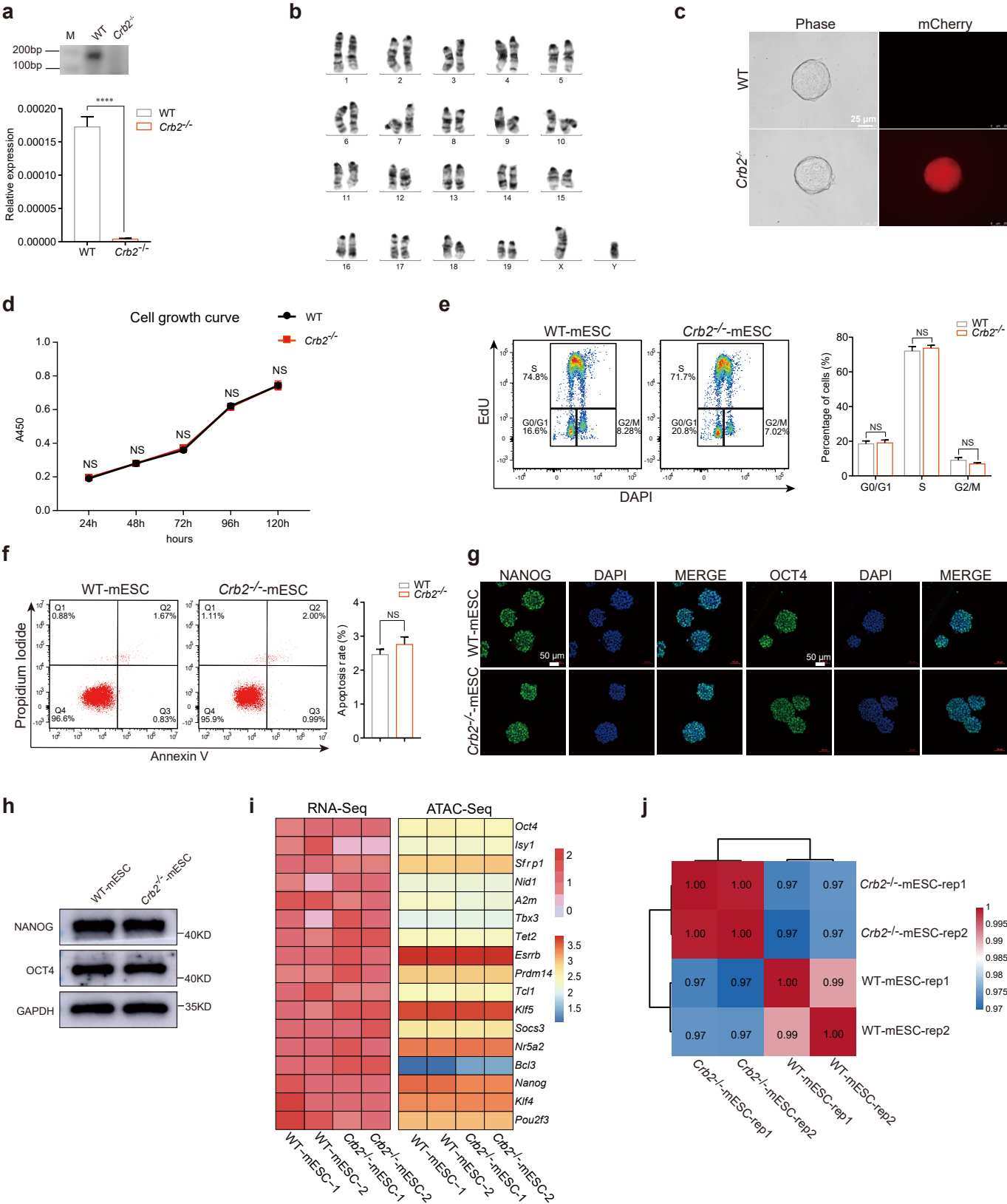


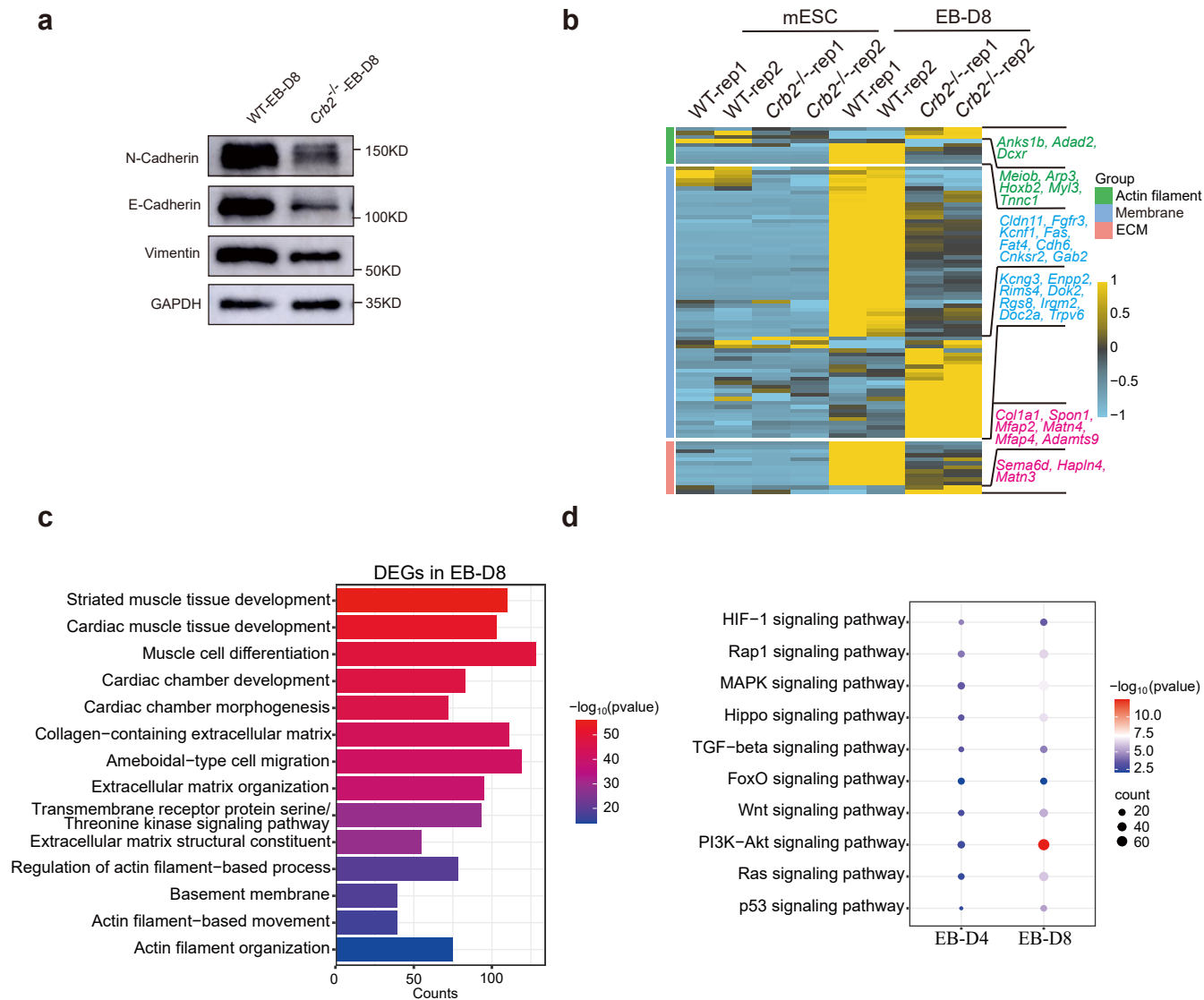
Figure. 6



Extended Data Fig. 1

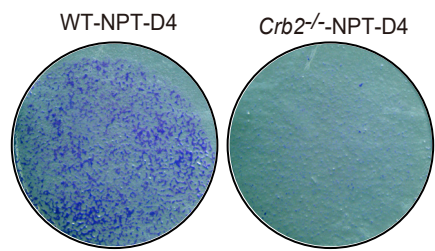


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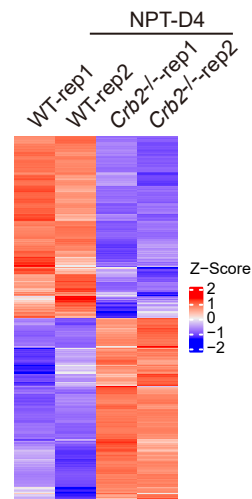


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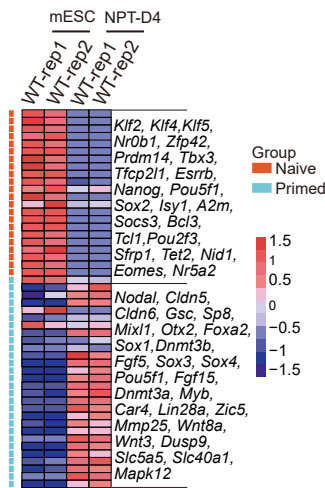
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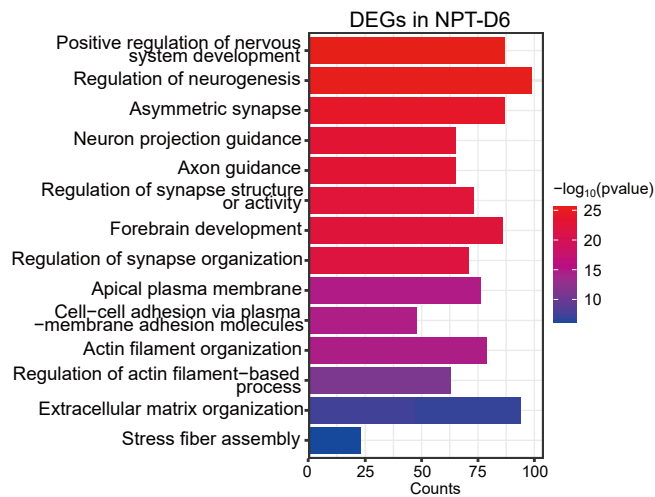
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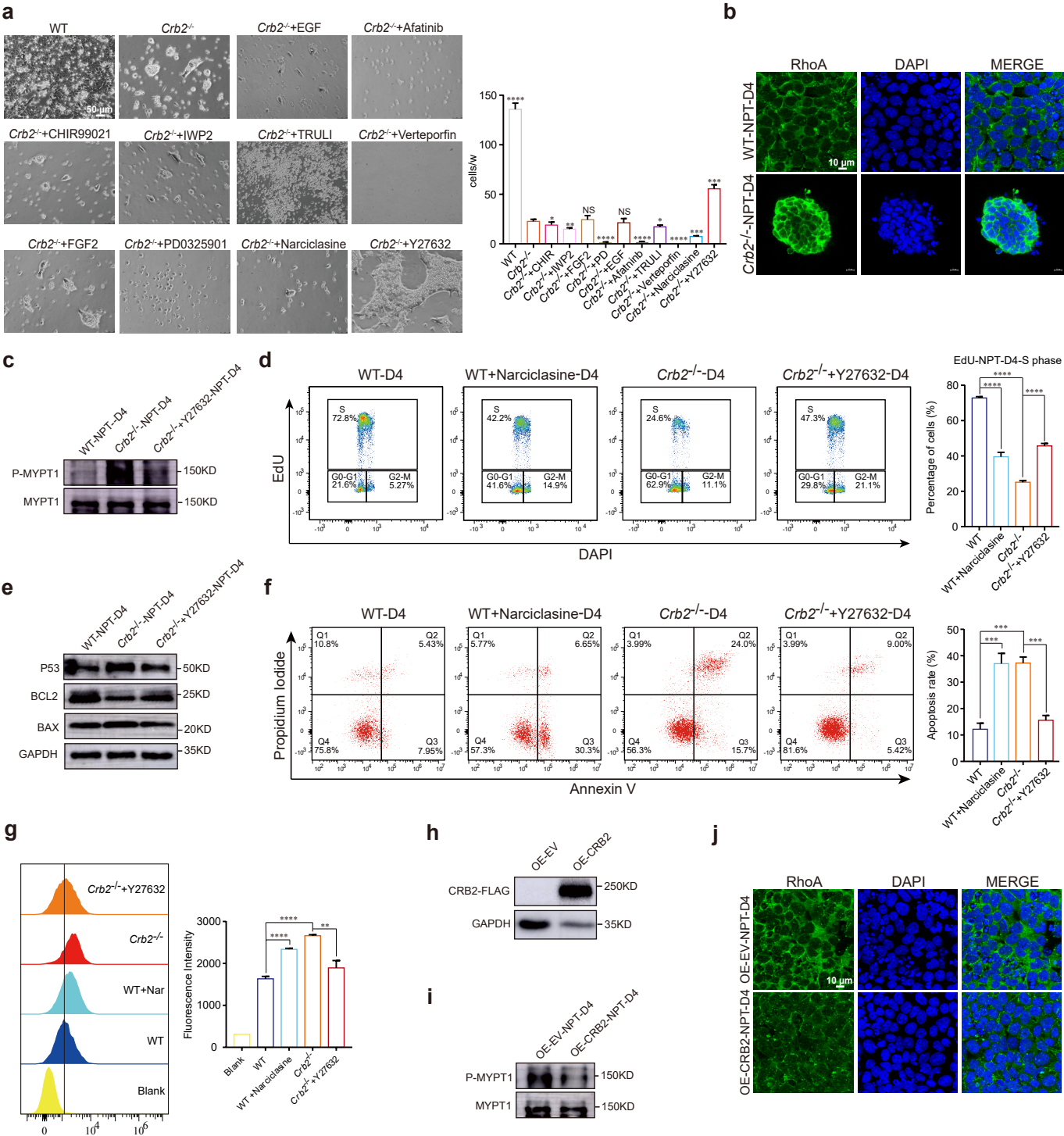
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d



Extended Data Fig. 4



Extended Data Fig. 5

