**INTRODUCTION**

Definitive erythropoiesis is an essential biological process that sustains the consumption and regeneration of end-stage erythroid cells through one’s life span. During this process, erythroblasts from hematopoietic stem cells become enucleated reticulocytes at foetal liver during the embryonic stage or at bone marrow after birth. The first committed progenitor in definitive erythropoiesis is the burst forming unit-erythroid, which differentiates into colony-forming unit-erythroid (CFU-E) in response to erythropoietin (EPO). The CFU-E progenitors further undergo sequential developmental stages of proerythroblasts, basophilic erythroblasts and polychromatic erythroblasts and eventually become orthochromatic erythroblasts. Substantial changes occur during erythroid differentiation, including nuclear condensation, decreased cell size and haemoglobinization. Therefore, erythroid differentiation is typically accompanied by significant increases of haem biosynthetic enzymes and synthesis of α/β-globins, as well as changes in membrane structure and function.

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**Abstract**

The exact molecular mechanism underlying erythroblast enucleation has been a fundamental biological question for decades. In this study, we found that miR-144/451 critically regulated erythroid differentiation and enucleation. We further identified CAP1, a G-actin-binding protein, as a direct target of miR-144/451 in these processes. During terminal erythropoiesis, CAP1 expression declines along with gradually increased miR-144/451 levels. Enforced CAP1 up-regulation inhibits the formation of contractile actin rings in erythroblasts and prevents their terminal differentiation and enucleation. Our findings reveal a negative regulatory role of CAP1 in miR-144/451-mediated erythropoiesis and thus shed light on how microRNAs fine-tune terminal erythroid development through regulating actin dynamics.

**KEYWORDS**

actin filament remodelling, CAP1, enucleation, erythropoiesis, miR-144/451
components and cytoskeleton.\textsuperscript{3,8-10} Enucleation starts in orthochromatic erythroblasts, followed by an expulsion of organelles in reticulocytes.\textsuperscript{8} These events are strictly regulated to ensure the final proper production of functional erythroid cells. For example, Rac GTPases regulate the formation of contractile actin rings (CAR) on the plasma membrane of erythroblasts, which in turn contribute to their enucleation.\textsuperscript{11}

MicroRNAs (miRNAs) are critical regulators of erythropoiesis.\textsuperscript{12-14} Specially, high expression of miR-144 and miR-451 from a bicistronic miRNA locus has been observed in erythroid lineages of various species, including human\textsuperscript{15,16} and mouse.\textsuperscript{15,17,18} The miR-144/451 appears to be a positive regulator of erythroid maturation.\textsuperscript{19} Severe anaemia develops in mice with erythropoiesis deficiency, including apoptosis of erythroblasts,\textsuperscript{20} reduction in erythroid number and increased distribution width of red blood cells.\textsuperscript{17} In addition, the anaemia upon miR-144/451 deletion deteriorates under oxidative stress.\textsuperscript{21,22}

Consistent with this observation, several downstream targets of miR-144/451 have been identified, including NRF2,\textsuperscript{23} Ywhaz\textsuperscript{21,22} and Cab39.\textsuperscript{20} All these miR-144/451 targets participate in cellular reductive/oxidative processes and protect erythrocytes from oxidant damage.\textsuperscript{20-23} However, it remains elusive how abnormal morphologic phenotypes, such as the increased distribution width of red blood cells and blocked enucleation, occur in miR-144/451\textsuperscript{−/−} erythrocytes, suggesting there are undefined targets of miR-144/451 during definitive erythropoiesis.

Cyclase-associated proteins (CAPs) are highly conserved actin-binding proteins in eukaryotic organisms.\textsuperscript{24-27} Srv2, the CAP homologue, first identified from yeast, is a binding partner of adenyl cyclase and an effector of Ras during nutritional signalling.\textsuperscript{25,28} Mammals have two CAP paralogues, CAP1 and CAP2.\textsuperscript{29} In mice, CAP1 is expressed in most non-muscle cell types, whereas CAP2 is primarily restricted to certain brain regions and striated muscles.\textsuperscript{30} CAP1 was reported to be a binding protein of globular actin (G-actin, monomeric).\textsuperscript{31} In a head-to-tail fashion, G-actin polymerizes to form a helical F-actin filament with a defined polarity, in which F-actin filaments elongate at one end whereas simultaneously shrink at the other end by releasing monomeric G-actin.\textsuperscript{32} In general, proteins interacting with F-actin contribute to the assembly of actin filaments and the construction of F-actin network, whereas G-actin-binding proteins serve to sequester monomeric actin and modulate the availability of un-polymerized G-actin.\textsuperscript{24}

The actin cytoskeletal dynamics are crucial for many cellular processes including erythroblast enucleation and proper assembly of red blood cell membrane, two key steps of terminal erythropoiesis in mammals.\textsuperscript{23,24} As a G-actin-binding protein, CAP1 was reported to sequester actin monomers to prevent their polymerization\textsuperscript{31} and to stimulate nucleotide exchange of ATP onto ADP-bound G-actin, a rate-limiting step in regenerating polymerizable G-actin.\textsuperscript{35} In addition, CAP1 was found to accelerate the depolymerization of F-actin by coordinating with an actin-cofilin complex.\textsuperscript{36,37} It however remained undefined what the functions of CAP1 are in erythroid enucleation and membrane assembly. Nor is clear how CAP1 is regulated in definitive erythropoiesis. In the current study, we revealed critical roles of CAP1 in maintaining cellular actin dynamics during erythroid development. We identified CAP1 as a direct target of miR-451 and found that CAP1 down-regulation was essential for erythroid differentiation and enucleation.

## 2 | MATERIALS AND METHODS

### 2.1 | Mouse breeding and foetal liver erythroid differentiation

Female C57BL/6 mice at 12.5 and 14.5 days of gestation were sacrificed using cervical dislocation and foetal livers were collected from embryos. Purification and in vitro differentiation of foetal liver erythroblast precursors were performed according to published protocol\textsuperscript{11} with minor modifications. CD71+ Ter119− foetal cells were sorted by flow cytometry Aria II (BD Biosciences) and seeded in fibronectin-coated wells at a cell density of $1 \times 10^5$/mL. At day 1, purified cells were cultured in IMDM (Thermo Fisher Scientific) containing 1% detoxified BSA (Sigma-Aldrich), 15% FBS (Thermo Fisher Scientific), 200 g/mL holo-transferrin (Sigma-Aldrich), 2 U/mL EPO (Amgen), 2 mmol/L $\gamma$-glutamine (Thermo Fisher Scientific), 10 g/mL recombinant insulin (Sigma-Aldrich) and $10^{-4}$ mol/L $\beta$-mercaptoethanol (Sigma-Aldrich). At day 2, the medium was replaced with erythropoiesis-differentiation medium (IMDM containing 20% FBS, $10^{-4}$ mol/L $\beta$-mercaptoethanol and 2 mmol/L $\gamma$-glutamine). Foetal liver erythroblast transfection, 75 pmol siRNA was transfected into 1-2 x $10^5$ cells with Lipofectamine™ 3000 Transfection Reagent (Invitrogen) following the manufacturer's instruction. For introduction of plasmids containing transgenes, 1-5 $\mu$g of plasmid DNA per 5-10 x $10^5$ cells was electroporated into the sorted CD71+/Ter119− cells with P3 primary cell 4D nucleofector™ X Kit L (Lonza) using program CD34+ of 4D-Nucleofector™ Core Unit System (Lonza). All animal experimental procedures were conducted in accordance with the local Animal Welfare Act and Public Health Service Policy with approval from the Committee of Animal Experimental Ethics at East China Normal University (Ref #:M20170320).

### 2.2 | Cell culture and DMSO-induced erythroid differentiation in vitro

Murine erythroleukemia (MEL) cells\textsuperscript{38} were cultured in RPMI-1640 medium (Thermo Fisher Scientific) supplemented with 10% FBS and 100 U/mL of penicillin/streptomycin (Thermo Fisher Scientific). Erythroid differentiation of MEL cells was induced by adding dimethyl sulfoxide (DMSO; Sigma-Aldrich) to 2% as the final concentration in culture media.\textsuperscript{39} Differentiated cells were collected at designated time points after DMSO treatment. 293T cells were cultured in DMEM with 10% FBS.
2.3 | Benzidine staining

Benzidine staining was performed following the published protocols. Briefly, a 0.2% solution of benzidine (Sigma-Aldrich) in 3% acetic acid was freshly prepared. Prior to use, hydrogen peroxide was added to 0.3% as the final concentration. MEL cells were first smeared on glass slides, air-dried, stained in the above described solution for 10 minutes (shielded from light) and finally fixed in 100% Methanol for 10 minutes. Images were taken with a Leica microscope.

2.4 | May-Grunwald-Giemsa staining

May-Grunwald-Giemsata staining was performed following the published protocols. Briefly, cells were first smeared onto glass slides, air-dried, fixed in 100% Methanol for 3-10 minutes and then went through 2-step staining: first with 50% (v/v) May-Grunwald (Sigma, CAS NO. 63590) in phosphate-buffered saline (pH 6.8) for 3-5 minutes, then with 10% Giemsa (Sigma, CAS NO. 48900) for 10-30 minutes and washed with running water for 1-3 minutes. Images of air-dried slides were taken under a Leica microscope.

2.5 | Contractile actin ring (CAR) staining

For CAR staining, MEL cells were harvested in PBS. Cell pellets were fixed in 100 µL PBS with 0.5% acrolein for 5 minutes, and cell concentration was adjusted to approximately 5 x 10^5 cells/mL. Cells (~100 µL) were applied to poly-L-lysine-coated slides and dried at room temperature. The slides were rinsed three times in PBS to remove unbound cells. Cells were permeabilized in PBS containing 0.05% Triton X-100 for 10 seconds, followed by three washes in PBS, then incubated in blocking buffer (PBS containing 0.5 mmol/L glycine, 0.2% fish skin gelatin and 0.05% sodium azide) for 1 hour and incubated with 1 U/mL Alexa Fluor 568–phalloidin (Thermo Fisher Scientific) for 1 hour and then incubated in blocking buffer (PBS containing 0.05% Triton X-100 for 10 seconds, followed by three washes in PBS, then incubated in blocking buffer (PBS containing 0.5 mmol/L glycine, 0.2% fish skin gelatin and 0.05% sodium azide) for 1 hour and incubated with 1 U/mL Alexa Fluor 568–phalloidin (Thermo Fisher Scientific) for 1 hour and then incubated in blocking buffer for 1 hour. Slides were washed three times in blocking buffer followed by DAPI staining for 15 minutes before images were taken under a Leica microscope.

2.6 | Flow cytometry analysis

Flow cytometry analyses of mouse foetal erythroblasts with CD71 and Ter119 were performed as previously described. For enucleation analysis, cells were stained with 10 µg/mL Hoechst 33342 (Sigma–Aldrich), together with an APC-Ter119 antibody, for 30 minutes at 4°C. Flow cytometry analyses and sorting were performed respectively on Calibur or Fortessa analyzers, and an Ariall cell sorter (BD Biosciences). Cell size changes during differentiation were measured with FSC-A using flow cytometry. Anti-mouse PE-CD71, APC-Ter119 antibodies and Annexin V Early Apoptosis Detection Kit (Cat#: 553786) were purchased from BD Biosciences.

2.7 | Plasmid construction

Mouse Cap1 cDNA was cloned by PCR and inserted into a lentiviral pl3.7-EF1α-MCS-IRES-hygro vector between EcoRI/NotI sites. To overexpress pri-miR-144/451, a DNA fragment containing the pri-miR-144/451 cluster was PCR-amplified from mouse genomic DNA and then inserted into a MSCV-MCS-IRES-hygro vector between EcoRi/Xhol sites. Three Cap1-specific shRNAs and a scramble shRNA control were designed and cloned into a Plica.1-U6-shRNA-puro vector, respectively. Sequences of oligonucleotides and primers in this study are provided in the Table S1.

2.8 | Quantitative real-time PCR

Total RNAs were extracted with Trizol (Thermo Fisher Scientific), and cDNAs were synthesized using a PrimeScript® RT reagent Kit (TaKaRa) following manufacturer's protocols, as previously described. The primers used in this assay are provided in the Table S1.

2.9 | Luciferase reporter assay

The 3′-UTR fragment of mouse Cap1 was cloned into a pGL4-basic firefly luciferase reporter vector. Mutant 3′-UTR fragment of mouse Cap1 was generated by PCR-based site-directed mutagenesis. All constructs were verified by DNA sequencing. The reporter plasmids were co-transfected into 293T cells with a Renilla luciferase expressing vector, and a plx-ires-zsgreen plasmid containing the pri-miR-144/451 driven by a CMV promoter. The firefly and Renilla luciferase activities were measured with the Dual-Glo kit (Promega).

2.10 | Western blotting

Total proteins were isolated using RIPA lysis buffer, subjected to SDS-PAGE, transferred to PVDF membranes and incubated with primary antibodies. Quantification of target protein levels was performed using the ECL detection system and Quantity One software (Bio-Rad). Antibodies used in this study: CAP1 (NBP1-58320; Novus), β-ACTIN (sc-10731; Santa Cruz Biotech) and GAPDH (sc-25778; Santa Cruz Biotech). The relative protein expression levels were determined by the density (grey mean value) of the protein bands with the ImageJ software and normalized to the respective loading control GAPDH/β-ACTIN.

2.11 | Statistical analysis

Data were presented as mean ± standard error (SEM). All experiments were performed independently for more than three times unless otherwise stated. Statistical analysis between group differences
was performed with two-tail unpaired Student’s t test using Graph
Prism software (version 5.0; GraphPad). A P value <.05 was consid-
ered significant (*P < .05, **P < .01, ***P < .001).

3 | RESULTS

3.1 | CAP1 is a direct target of miR-451

To understand the molecular mechanisms of how miR-144/451 im-
acts erythropoiesis, we first predicted their targets by conducting
bioinformatic analyses through Targetscan, an online software.
We identified CAP1 as a potential target of miR-451 (Figure 1A).
Consistent with this finding, retroviral vector-mediated overexpres-
sion of pri-miR-144/451 in MEL cells reduced Cap1 mRNA and protein
levels to approximately 40% and 20%, respectively (Figure 1B,C),
suggesting that miR-144/451 negatively regulates CAP1 expression.

To further investigate whether miR-451 directly targets Cap1
mRNA, a dual-luciferase reporter assay was conducted in 293T
cells. In this assay, we constructed a firefly luciferase reporter,
downstream of which was inserted with a wild-type or mutant Cap1
3'-UTR fragment containing mutations within the miR-451 seed
targeting sequences (Figure 1D,E). We found that pri-miR-144/451
significantly inhibited the activity of firefly luciferases linked with
the wild-type Cap1 3'-UTR. By contrast, the inhibitory effect of pri-
miR-144/451 was lost in the vector containing Cap1 3'-UTR with a
mutated miR-451 seed target sequence (Figure 1F). Taken together,
these results reveal that Cap1 is a direct target gene of miR-451.

3.2 | CAP1 is down-regulated during foetal
erthroid differentiation

To study the role of CAP1 in erythroid development, we first examined
its expression level at different stages of erythroid development. We
utilized a foetal liver erythropoietic model because more than 90%
of cell population from mouse foetal liver are of erythroid lineage.11
Five distinct erythroblast populations from mouse foetal livers at
embryonic day 14.5 (E14.5) were identified with flow cytometry and designated as R1 to R5, according to their Ter119 and CD71 double-staining patterns (Figure 2A): R1 (CD71\textsuperscript{med}/Ter119\textsuperscript{low}) is mainly comprised of primitive progenitor cells and proerythroblasts; R2 (CD71\textsuperscript{high}/Ter119\textsuperscript{low}) includes proerythroblasts and early basophilic erythroblasts; R3 (CD71\textsuperscript{high}/Ter119\textsuperscript{high}) is enriched with early and late basophilic erythroblasts; R4 (CD71\textsuperscript{med}/Ter119\textsuperscript{high}) contains chromatophilic and orthochromatophilic erythroblasts; and R5 (CD71\textsuperscript{med}/Ter119\textsuperscript{med}) represents late orthochromatophilic erythroblasts and reticulocytes.\textsuperscript{11} As shown in Figure 2B,C, both β-globin (\textit{Hbb}: \textit{Hbb-β1}\textsubscript{1} & β2) and pri-miR-144/451 were significantly up-regulated all the way through R4 stage. By contrast, Cap1 mRNA was promptly down-regulated from R1 to R4 (Figure 2D). Cap1 protein showed a similar expression pattern (Figure 2E), supporting a negative reciprocal expression of Cap1 and miR-144/451 during erythroid differentiation.

We next examined Cap1 expression levels during in vitro erythroid differentiation of CD71\textsuperscript{+}/Ter119\textsuperscript{-} foetal liver cells isolated from E12.5 mice (D0).\textsuperscript{11} After induced differentiation for 2 days (D2), we clearly observed red coloured cell pellets, indicating erythroid maturation with increased levels of iron-containing haemoglobin (Figure 2F). In addition, double staining for Ter119 and a DNA dye Hoechst 33342 demonstrated a significant increase from 0.4% to 36.7% of the Ter119\textsuperscript{high}/Hoechst\textsuperscript{low} population (Figure 2G), which represents enucleated reticulocytes.\textsuperscript{45} Compared with D0 progenitors, Cap1 expression dramatically decreased in D2 differentiated erythroid cells. This is in sharp contrast to the remarkable increased transcript levels of β-globin (\textit{Hbb}: \textit{Hbb-β1}\textsubscript{1} & β2) and pri-miR-144/451 (Figure 2H). Taken together, these results illustrate that Cap1 is down-regulated both in vitro and in vivo along definitive erythroid differentiation, suggesting a vital role of Cap1 in this process.

3.3 Cap1 inhibits terminal erythroid differentiation of MEL cells

To define the function of Cap1 in erythropoiesis, three Cap1-specific shRNAs and a scramble shRNA control were introduced by lentiviral
infection into MEL cells. MEL is an erythroleukaemia line immortalized by the Friend virus complex. MEL cells are arrested before the proerythroblast stage in culture but can be induced by treatment of DMSO or other chemical agents to go through morphological and biochemical changes which mimic in vivo developmental events during erythroid differentiation. Real-time RT-PCR confirmed that Cap1 expression was significantly inhibited by shRNA knock-down in MEL cells, with shRNA #2 achieving the highest knock-down efficiency at both mRNA and protein levels (Figure 3A,B). Strikingly, knock-down of Cap1 greatly facilitated MEL differentiation upon DMSO treatment (Figure 3B,C). At day 4 after DMSO treatment, the differentiated MEL cell pellets displayed a dark red colour in Cap1 knock-down groups, in sharp contrast to the scrambled control that showed only a tinge of redness (Figure 3B, lower panel). Benzidine is a chemical

**FIGURE 3** CAP1 inhibits terminal erythroid differentiation of MEL cells. A, Cap1 mRNA levels in MEL at day 2 following the introduction of shRNAs against Cap1 were determined by real-time RT-PCR. B, Gross view of the cell pellets and Western blotting analyses of CAP1 protein levels at day 4 during DMSO-induced differentiation following shRNA knock-down of CAP1 in MEL. C, Benzidine staining at day 4 after DMSO induction following Cap1 knock-down. D, Haemoglobin mRNA levels in MEL were measured by real-time RT-PCR at day 4 during DMSO-induced differentiation upon Cap1 knock-down. E, The size distribution (FSC-A) of MEL cells was analysed by flow cytometry at day 4 after DMSO-induced differentiation following Cap1 knock-down. Three groups of cells with various sizes were detected by flow cytometry. The percentage of each group was averaged from 3 independent experiments with representative plots shown above the graph. F, Cap1 mRNA levels were determined by real-time RT-PCR at day 2 after introducing a Cap1 cDNA into MEL. G, Western blot analysis of Cap1 protein levels at day 4 during DMSO-induced differentiation upon Cap1 overexpression in MEL. A typical view of cell pellets after MEL differentiation upon Cap1 overexpression was shown. H, Benzidine staining assays of MEL at day 4 during DMSO-induced differentiation upon Cap1 overexpression. I, Haemoglobin mRNA levels in differentiated MEL were determined by real-time RT-PCR at day 4 after DMSO treatment with Cap1 overexpression, compared with an empty vector control. J, The size distribution (FSC-A) of MEL cells was analysed by flow cytometry at day 4 after DMSO-induced differentiation following Cap1 overexpression. The percentage of each group was averaged from three independent experiments with representative plots shown above the graph. A, D, E, F, I, J, The data are represented as the mean ± SEM (n ≥ 3; *P < .05; **P < .01; NS, no significance). OE, overexpression.
that forms a dark blue precipitate upon oxidation of the haem group in haemoglobin by hydrogen peroxide. It thus serves as a dye for the histochemical detection of differentiated red blood cells with high haemoglobin expression. We found that blue MEL cells were significantly increased from 52% to 87% upon Cap1 knock-down (Figure 3C and S1A). Consistent with these data, real-time RT-PCR assays showed that Cap1 down-regulation significantly promoted the expression of both α-globin (Hba: Hba-α1 & α2) and β-globin (Hbb: Hbb-β1 & β2) (Figure 3D).

Because erythroid cells become smaller during differentiation and enucleation, we next examined the size distribution of MEL cells upon DMSO induction. We were able to detect three MEL populations with different sizes by flow cytometry (Figure 3E): the large cells of erythroblasts (orange area with FSC-A >50K), terminally differentiated small-sized MEL cells (red area with FSC-A <30K) and middle-sized cells that were likely in the process of differentiation (blue area with FSC-A peak spanning between 20K and 50K). We found significantly more small-sized erythroid cells increasing from 46.2% to 90.3% upon Cap1 inhibition (red plus blue populations in Figure 3I). Interestingly, upon Cap1 overexpression, although the proportion of large-sized cells (FSC-A >50K) decreased, middle-sized MEL cells (blue area with FSC-A peak spanning between 20K and 50K) were significantly increased from 52% to 87% upon Cap1 down-regulation (Figure 3C and S1A). Consistent with these data, real-time RT-PCR assays showed that Cap1 down-regulation significantly promoted the expression of both α-globin (Hba: Hba-α1 & α2) and β-globin (Hbb: Hbb-β1 & β2) (Figure 3D).

To confirm the negative role of Cap1 in regulating erythroid differentiation, we performed siRNA interfering experiments using in vivo developed erythroblasts from foetal livers. Three siRNAs targeting against Cap1 mRNA and one scramble control siRNA were designed and transiently transfected into E12.5 foetal liver cells. As shown in Figure 4A, all three Cap1 siRNAs efficiently blocked Cap1 expression, with siRNA-2# showing the highest knock-down efficiency.

Two days after in vitro culture following siRNA-2# transfection, we observed a significant increase of late differentiated cells at the R5 stage from 8.3% to 15.6% (Figure 4B). Cell population at R4 stage was also slightly elevated. By contrast, significant declines were detected in the cells at the R3 stage upon Cap1 down-regulation (Figure 4B). Concomitantly, the proportion of large-sized cells (above FSC-A > 50K) decreased from 72.0% to 55.9% (Figure 4C), indicating increased erythroid differentiation upon Cap1 inhibition. In addition, MGG staining showed that Cap1 inhibition speeded up the process of enucleation and cells without nuclei significantly increased (Figure 4D). Consistently, double staining for Ter119 and Hoechst 33342 demonstrated a significant increase of Ter119highHoechstlow enucleated reticulocytes from 6.7% to 11.5% upon Cap1 knock-down (Figure 4E). Notably, we did not observe any obvious alterations in the death of foetal liver erythroblasts in the Cap1 down-regulated group (Figure S2) rather found a modest decrease in percentage of cells at S-phase, suggesting a reduced cell proliferation upon Cap1 knock-down (Figure S3). In conclusion, our results reveal that Cap1 inhibition promotes terminal erythroid differentiation and enucleation of foetal liver cells.

3.5 | CAP1 inhibits miR-144/451-mediated terminal erythroid differentiation

To further determine whether Cap1 inhibition is required for miR-144/451-mediated erythroid differentiation, we examined erythroid maturation of MEL cells upon co-expressing Cap1 and pri-miR-144/451 (Figure 5A, and S4). At day 4 after DMSO induction, the pellets from differentiated MEL cells exhibited a much brighter redness in the miR-144/451 overexpressing group than that in mock control (Figure 5A), confirming that miR-144/451 enhances MEL differentiation. By contrast, the cell pellets displayed a light yellow colour when Cap1 was overexpressed or co-expressed with both Cap1 and pri-miR-144/451 (Figure 5A and S4). Concomitantly, as shown in the real-time RT-PCR assay, miR-144/451 significantly promoted β-globin expression which was substantially blocked by Cap1 (Figure 5B). The percentage of benzidine-positive/dark blue-stained MEL cells was significantly elevated from 54% to 79% upon...
FIGURE 4  CAP1 inhibits terminal erythroid differentiation of foetal liver cells. A, Cap1 mRNA and protein levels at day 2 following siRNA knock-down in primarily isolated Ter119- erythroblasts from E12.5 foetal livers. B, Flow cytometry analysis of primary foetal liver erythroblasts at day 2 during differentiation following Cap1 siRNA knockdown, compared with a scrambled siRNA control. C, The size distribution (FSC-A) of foetal liver erythroid cells was analysed by flow cytometry at day 2 during differentiation following CAP1 knock-down. D, MGG staining assays of foetal liver cells at day 2 during differentiation following Cap1 siRNA knockdown, compared with scrambled siRNA controls. Red arrow indicates erythroblasts, black arrows for enucleating cells and white arrows for reticulocytes without a nucleus. E, Foetal liver cells were stained with APC-Ter119 and Hoechst 33342 at day 2 during differentiation following Cap1 siRNA knock-down. Representative plots of flow cytometry analyses were shown. A, B, C, E, The data are represented as the mean ± SEM (n ≥ 3; **P < .01; NS, no significance)
pri-miR-144/451 introduction (Figure 5C). This phenomenon disappeared in the presence of CAP1, either alone or co-overexpressed with pri-miR-144/451 (subgroup b & d, Figure 5C). We thus conclude that CAP1 overexpression directly represses the differentiation of MEL cells induced by pri-miR-144/451.

We next assessed whether CAP1 acts downstream of miR-144/451 in regulating erythroid enucleation of MEL cells by flow cytometry with double staining for Ter119 and the DNA dye Hoechst 33342. At day 4 after DMSO-induced differentiation, we found that the generation of Ter119<sup>high</sup>Hoechst<sup>low</sup> enucleated MEL cells was significantly repressed by CAP1 overexpression (Figure 5D). By contrast, exogenous pri-miR-144/451 up-regulated the formation of Ter119<sup>high</sup>Hoechst<sup>low</sup> cells, but this phenomenon largely diminished when CAP1 transgene was co-introduced (Figure 5D). Consistent with these findings, we found that miR-144/451 facilitated the decrease of cell size during MEL differentiation, analysed by flow cytometry (Figure S5A). The percentage of cells at FSC-A < 30K jumped from 9.5% in the control group to 86.2% (red populations) when miR-144/451 was up-regulated, whereas CAP1 overexpression partially blocked this function of miR-144/451.
suggesting that CAP1 acts downstream of miR-144/451 was co-overexpressed with subgroup b). In addition, few CAR staining was detected when CAP1 significantly blocked CAR formation during MEL differentiation (Figure 5E, of the CAR (red dots in subgroup c), whereas CAP1 alone signifi-
antly blocked CAR formation and stained with Alexa Fluor 568-phalloidin for CAR labelling. Remarkably, induced differentiation and stained with Alexa Fluor 568-phalloidin erythroid enucleation. MEL cells were fixed at day 4 after DMSO-
amined its functional relevance to the formation of CAR during erythroid differentiation, we performed observation, our data demonstrated CAP1 acted downstream of miR-144/451 in erythroid differentiation and enucleation.
Because CAP1 is an actin-binding protein and may play an important role in maintaining cytoplasmic actin dynamics, we examined its functional relevance to the formation of CAR during erythroid enucleation. MEL cells were fixed at day 4 after DMSO-induced differentiation and stained with Alexa Fluor 568-phalloidin for CAR labelling. Remarkably, miR-144/451 promoted the formation of the CAR (red dots in subgroup c), whereas CAP1 alone significantly blocked CAR formation during MEL differentiation (Figure 5E, subgroup b). In addition, few CAR staining was detected when CAP1 was co-overexpressed with pri-miR-144/451 (Figure 5E, subgroup d), suggesting that CAP1 acts downstream of miR-144/451 in erythroid enucleation by regulating F-actin dynamics.
To confirm the functional relationship of CAP1 and miR-144/451 in regulating physiological erythroid differentiation, we performed similar experiments using CD71+/Ter119- erythroblasts collected from foetal livers. As shown in Figure 6A, two days after in vitro differentiation, we observed a decrease of late differentiated cells at R4 and R5 stages and an elevation of cells at R3 upon CAP1 overexpression (Figure 6A). By contrast, miR-144/451 introduction promoted the formation of late differentiated cells at the R4 and R5 stages, but this elevated differentiation by miR-144/451 was blocked when CAP1 was concomitantly overexpressed (Figure 6A). In addition, flow cytometry analyses showed that the proportion of large-sized cells (above FSC-A >50K) increased from 56.2% to 72.5% upon CAP1 transgene introduction (Figure 6B), indicating a blockage in erythroid differentiation by CAP1. In addition, pri-miR-144/451 promoted the formation of erythroid cells with small size (FSC-A <30K) from 11.4% to 29.7% (Figure 6B). When CAP1 transgene was co-introduced, the percentage of these small-sized cells (below FSC-A <30K) declined (Figure 6B). Consistent with these findings, the development of Ter119<sup>−/−</sup>Hoechst<sup>low</sup> enucleated reticulocytes was significantly blocked by CAP1 when either it was overexpressed alone or co-expressed with miR-144/451 (Figure 6C), as displayed by double staining for Ter119 and Hoechst 33342 in flow cytometry analyses. In summary, our results demonstrate that CAP1 plays a negative role in erythroblast differentiation and enucleation regulated by miR-144/451.

4 | DISCUSSION

Although a few targets of miR-144/451 have been identified, the molecular mechanisms are largely unknown whereby miR-144/451 mutations result in phenotypical abnormalities of erythrocytes. Hereby, for the first time, we identified CAP1 as a direct target of miR-451 in definitive erythropoiesis. We found that miR-144/451 directly inhibited CAP1 expression, but this inhibition was lost when seeding sequences of miR-451 in Cap1<sup>−/−</sup> mice were introduced, the percentage of these small-sized cells (below FSC-A >50K) declined (Figure 6B). Consistent with this observation, our data demonstrated that miR-144/451 acted as an important regulator in the cytoskeleton dynamics and morphologic changes of red blood cells during late stages of erythroid differentiation. Therefore, our study reveals a novel molecular mechanism underlying abnormal morphologic changes of miR-144/451<sup>−/−</sup> erythrocytes and uncovered a previously unrecognized role of miR-144/451 in erythroblast enucleation.

Late stage erythroblasts undergo cell cycle exit, chromatin condensation and extrusion of the condensed nuclei via an asymmetric cell division. CAR formation is required for this enucleation process. In immature erythroblasts, F-actin distributes patchily at cell surface. However, F-actin bundles become detectable as erythroblasts mature, and finally F-actin is concentrated to form CAR between the extruding nuclei of erythroblasts and incipient reticulocytes to enable proper enucleation. We found that, as a direct target of miR-451, CAP1 played a negative role in CAR formation, erythroblast enucleation and terminal erythroid differentiation. CAP1 was gradually down-regulated along with an up-regulation of miR-144/451 during erythroid differentiation, and its overexpression significantly blocked CAR formation and enucleation driven by miR-144/451.
is plausible that CAP1 directly regulates actin dynamics as a monomeric G-actin-binding protein to inhibit polymerization of F-actin filaments during CAR formation and erythroblast enucleation. Alternatively, CAP1 may enhance coflin-mediated F-actin disassembly and stimulate recycling of coflin and actin monomers, as reported by a previous study. In either case, high level expression of CAP1 at early erythroid differentiation may serve as a gatekeeper to prevent erythroblasts from premature enucleation via regulating actin dynamics. Notably, in addition to CAP1, a few proteins were identified to regulate erythroblast enucleation through modifying actin dynamics, including RAC1 and RAC2 GTPases, dynein, Gelsolin and mDia2. It remains to be determined whether CAP1 interplays with these proteins to precisely regulate the actin dynamics of erythroblasts.

Our study also suggests that CAP1 participates in other aspects of erythroid development. We observed a modest decrease of erythroblasts at S-phase upon CAP1 inhibition (Figure S3). As terminally differentiated erythrocytes shuts down cell cycles by up-regulating of p27 and p18 CDK inhibitors, the reduced proliferation of CAP1 knock-down cells could be due to increased percentage of mature erythrocytes. CAP1 was previously reported to be a binding partner of adenyl cyclase and act as a Ras effector during nutritional changes in yeasts. Interestingly, it is known that RAS signalling plays an important role in terminal erythroid proliferation and differentiation. We thus could not exclude the possibility that CAP1 is directly involved in cell cycle regulation of erythroblasts. Nevertheless, our study undoubtedly supports a critical role of CAP1 in miR-144/451-mediated terminal erythroid differentiation and enucleation, thereby contributing to potential clinical therapy in the field of blood transfusion.

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CONFLICT OF INTEREST
The authors confirm that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS
Xiaoli Huang: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Methodology (equal); Software (equal); Validation (equal). Ruihua Chao: Data curation (equal); Investigation (equal); Methodology (equal); Validation (equal). Yanyang Zhang: Data curation (equal); Methodology (equal). Pengxiang Wang: Data curation (equal); Formal analysis (equal); Methodology (equal). Xueping Gong: Data curation (equal); Methodology (equal). Dongli Liang: Data curation (equal); Formal analysis (equal); Methodology (equal); Project administration (equal); Supervision (equal); Validation (equal); Writing-original draft (equal); Writing-review & editing (equal). Yuan Wang: Conceptualization (equal); Formal analysis (equal); Funding acquisition (equal); Investigation (equal); Methodology (equal); Project administration (equal); Resources (equal); Supervision (equal); Validation (equal); Visualization (equal); Writing-original draft (equal); Writing-review & editing (equal).

DATA AVAILABILITY STATEMENT
The data that supports the findings of this study are available in the Supporting information of this article.

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REFERENCES
1. Hattangadi SM, Wong P, Zhang LB, Flygare J, Lodish HF. From stem cell to red cell: regulation of erythropoiesis at multiple levels by multiple proteins, RNAs, and chromatin modifications. *Blood*. 2011;118:6258-6268.
2. Palis J, Robertson S, Kennedy M, Wall C, Keller G. Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. *Development*. 1999;126:5073-5084.
3. Baron MH, Isern J, Fraser ST. The embryonic origins of erythropoiesis in mammals. *Blood*. 2012;119:4828-4837.
4. Gregory CJ, Eaves AC. Human marrow cells capable of erythropoietic differentiation in vitro: definition of three erythroid colony responses. *Blood*. 1977;49:855-864.
5. Gregory CJ, Eaves AC. Three stages of erythropoietic progenitor cell differentiation distinguished by a number of physical and biologic properties. *Blood*. 1978;51:527-537.
6. Wickrema A, Boudrant MC, Krantz SB. Abundance and stability of erythropoietin receptor mRNA in mouse erythroid progenitor cells. *Blood*. 1991;78:2269-2275.
7. Malik J, Kim AR, Tyre KA, Cherukuri AR, Palis J. Erythropoietin critically regulates the terminal maturation of murine and human primitive erythroblasts. *Haematologica*. 2013;98:1778-1787.
8. Palis J. Primitive and definitive erythropoiesis in mammals. *Front Physiol*. 2014;5.3.
9. Chao MV, Mellon P, Charnay P, Maniatis T, Axel R. The regulated expression of beta-globin genes introduced into mouse erythroleukemia cells. *Cell*. 1983;32:483-493.
10. Fraser ST, Isern J, Baron MH. Maturation and enucleation of primitive erythroblasts during mouse embryogenesis is accompanied by changes in cell-surface antigen expression. *Blood*. 2007;109:343-352.
11. Zhang J, Socolovsky M, Gross AW, Lodish HF. Role of Ras signaling in erythroid differentiation of mouse fetal liver cells: functional analysis by a flow cytometry-based novel culture system. *Blood*. 2003;102:3938-3946.
12. Montagner S, Deho L, Monticelli S. MicroRNAs in hematopoietic development. *BMC Immunol*. 2014;15.14.
13. Zhang L, Flygare J, Wong P, Lim B, Lodish HF. miR-191 regulates mouse erythroblast enucleation by down-regulating Rik03 and Mxi1. *Genes Dev*. 2011;25:119-124.
14. Figueroa AA, Fasano JD, Martinez-Morilla S, Venkatesan S, Kupfer G, Hattangadi SM. miR-181a regulates erythroid enucleation via the regulation of Xpo7 expression. *Haematologica*. 2018;103:E341-E344.
15. Zhan M, Miller CP, Papayannopoulou T, Stamatoyannopoulou G, Song CZ. MicroRNA expression dynamics during murine and human erythroid differentiation. *Exp Hematol*. 2007;35:1015-1025.
16. Bruchova H, Yoon D, Agarwal AM, Mendell J, Prchal JT. Regulated expression of microRNAs in normal and polycythemia vera erythropoiesis. Exp Hematol. 2007;35:1657-1667.

17. Dore LC, Amigo JD, Dos Santos CO, et al. A GATA-1-regulated microRNA locus essential for erythropoiesis. Proc Natl Acad Sci USA. 2008;105:3333-3338.

18. Monticelli S, Ansel KM, Xiao CC, et al. MicroRNA profiling of the murine hematopoietic system. Genome Biol. 2005;6:R71.

19. Rasmussen KD, Simmini S, Abreu-Goodger C, et al. The miR-144/451 locus is required for erythroid homeostasis. J Exp Med. 2010;207:1351-1358.

20. Fang X, Shen F, Lechauve C, et al. miR-144/451 represses the LKB1/AMPK/mTOR pathway to promote red cell precursor survival during recovery from acute anemia. Haematologica. 2018:103:406-416.

21. Yu DN, dos Santos CO, Zhao GW, et al. miR-451 protects against erythroid oxidant stress by repressing 14-3-3 zeta. Genes Dev. 2010;24:1620-1633.

22. Patrick DM, Zhang CC, Tao Y, et al. Defective erythroid differentiation in miR-451 mutant mice mediated by 14-3-3zeta. Genes Dev. 2010;24:1614-1619.

23. Sangokoya C, Telen MJ, Chi JT. microRNA miR-144 modulates oxidative stress tolerance and associates with anemia severity in sickle cell disease. Blood. 2010;116:4338-4348.

24. Hubberstey AV, Mottillo EP. Cyclase-associated proteins: CAPacity for linking signal transduction and actin polymerization. FASEB J. 2002;16:487-499.

25. Field J, Vojtek A, Ballester R, et al. Cloning and characterization of Saccharomyces cerevisiae adenyl cyclase -CAP, the mechanism of mouse cyclase-associated protein. Cell. 1990;61:319-327.

26. Kawai M, Aotsuka S, Uchimya H. Isolation of a cotton CAP gene: a homologue of adenyl cyclase-associated protein highly expressed during fiber elongation. Plant Cell Physiol. 1998;39:1380-1383.

27. Benlali A, Draskovic I, Hazelett DJ, Treisman JE. act up controls cycling of cofilin and actin for rapid actin turnover. J Cell Biochem. 2016;16:487-499.

28. Fedor-Chaiken M, Deschenes RJ, Broach JR. SRV2, a gene required for gelsolin on human erythroblast maturation for erythrocyte production. J Cell Biochem. 2018;10:314-321.

29. Chaudhry F, Breitsprecher D, Little K, Sharov G, Sokolova O, Goode BL. Srv2/cyclase-associated protein forms hexameric shirikens that directly catalyze actin filament severing by cofilin. Mol Biol Cell. 2013;24:31-41.

30. Normoyle KPM, Briehler WM. Cyclase-associated Protein (CAP) acts directly on F-actin to accelerate cofilin-mediated actin severing across the range of physiological pH. J Biol Chem. 2012;287:35722-35732.

31. Bruchova H, Yoon D, Agarwal AM, Mendell J, Prchal JT. Regulated expression of microRNAs in normal and polycythemia vera erythropoiesis. Exp Hematol. 2007;35:1657-1667.

32. Konstantinidis DG, Pushkaran S, Johnson JF, et al. Signaling and microRNAs during embryonic hematopoiesis. Stem Cell Res. 2010;24:1620-1633.

33. Rivella S. Enucleate or replicate? Ask the cytoskeleton. Blood. 2008;105:3333-3338.

34. Bertling E, Hotulainen P, Matilainen T, Salminen M, Lappalainen P. Cyclase-associated protein 1 (CAP1) promotes cofillin-mediated actin severing across the range of physiological pH. J Biol Chem. 2012;287:35722-35732.

35. Ben-David Y, Giddens EB, Bernstein A. Identification and mapping of a common proviral integration site Fli-1 in erythroleukemia cells induced by Friend murine leukemia virus. Proc Natl Acad Sci USA. 1990;87:1332-1336.

36. Dore LC, Amigo JD, Dos Santos CO, et al. A GATA-1-regulated microRNA locus essential for erythropoiesis. Proc Natl Acad Sci USA. 2008;105:3333-3338.

37. Lachman HM, Skoulitchi Al. Expression of c-myc changes during differentiation of mouse erythroleukaemia cells. Nature. 1984;310:592-594.

38. Papapetrou EP, Korkola JE, Sadelain M. A Genetic strategy for single and combinatorial analysis of miRNA function in mammalian hematopoietic stem cells. Stem Cells. 2010;28:279-296.

39. Paton E, Fabre M, Goubin-Versini I, et al. Guidelines for May-Grundwald-Giemsa staining in haematology and non-gynaecological cytopathology: recommendations of the French Society of Clinical Cytology (SFCC) and of the French Association for Quality Assurance in Anatomic and Cytologic Pathology (AFAQP). Cytopathology. 2016;27:359-368.

40. Chao R, Gong X, Wang L, Wang P, Wang Y. CD71(high) population represents primitive erythroblasts derived from mouse embryonic stem cells. Stem Cell Res. 2015;14:30-38.

41. Huang X, Chao R, Lodish HF. Enucleation of cultured mouse fetal erythroblasts requires Rac GTPases and mDia2. Nat Cell Biol. 2008;10:314-321.

42. Oliff A, Russetti S, Douglass EC, Scolnick E. Isolation of transplantable erythroleukemia cells from mice infected with helper-independent Friend murine leukemia virus. Blood. 1981;58:244-254.

43. Anoniou M. Induction of erythroid-specific expression in murine erythroleukemia (MEL) cell lines. Methods Mol Biol. 1991;7:421-434.

44. Koury ST, Koury MJ, Bondurant MC. Cytoskeletal distribution and function during the maturation and enucleation of mammalian erythroblasts. J Cell Biol. 1989;109:3005-3013.

45. Jansen S, Collins A, Golden L, Sokolova O, Goode BL. Structure and mechanism of mouse cyclase-associated protein (CAP1) in regulating actin dynamics. J Biol Chem. 2014;289:30732-30742.

46. Wang C, Wu X, Shen F, Li Y, Zhang Y, Yu D. Shinc-EC6 regulates murine erythroid enucleation by Rac1-PIP5K pathway. Dev Growth Differ. 2015;57:466-473.

47. Kobayashi I, Uebukawa K, Sugawara K, et al. Erythroblast enucleation is a dynein-dependent process. Exp Hematol. 2016;44:247-256.

48. Han SY, Lee EM, Choi HS, Chun BH, Baek EJ. The effects of plasma gelsolin on human erythroblast maturation for erythrocyte production. Stem Cell Res. 2018;29:64-75.

49. Gnanapragasam MN, McGrath KE, Catherman S, Xue L, Palis J, Bieker JJ. EKLF/KLF1-regulated cell cycle exit is essential for erythroblast enucleation. Blood. 2016;128:1631-1641.

SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.