RNF114 suppresses tumour metastasis through the regulation of PARP10

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Abstract

Background

ADP-ribosylation is a multifunctional post-translational modification catalysed by intracellular ADP-ribosyltransferases. Previously, we demonstrated that the mono-ADP-ribosyltransferase PARP10 suppresses tumour metastasis through the negative regulation of Aurora A kinase activity. However, the mechanisms of PARP10 regulation and modification were unclear.

Methods

Interaction of RNF114 and PARP10 was identified by exogenous and endogenous reciprocal immunoprecipitation (IP) assays and pull-down assays. Ubiquitination of PARP10 by RNF114 was analysed by *in vivo* ubiquitination assays. Potential effects of ubiquitination on PARP10's activity was detected by western blots and pull-down assays. Potential role of RNF114 in tumour metastasis were determined by migration and invasion assays and *in vivo* metastasis assay.

Results

That E3 ubiquitin ligase RNF114 is a partner of PARP10 was identified by IP assays. The auto-mono-ADP-ribosylation of PARP10 is required for the interaction of RNF114 and PARP10. RNF114 ubiquitinates PARP10 through K27-linked polyubiquitination, which enhances PARP10 enzymatic activity. Moreover, RNF114 deficiency promotes tumour cell migration and tumour metastasis through the regulation of PARP10 and its downstream signalling pathway.

Conclusions

Our findings identify RNF114 as a novel functional regulator of PARP10 and provide evidence of crosstalk between the components of K27-linked polyubiquitination and mono-ADP ribosylation.

Background

ADP-ribosylation is a post-translational modification catalysed by intracellular ADP-ribosyltransferases (ARTDs or PARPs), which transfers only one ADP-ribose unite or multiple ADP-ribose units successively from nicotinamide adenine dinucleotide (NAD^+) to substrates in a process called mono-ADP-ribosylation (MARylation) or poly-ADP-ribosylation (PARylation), respectively[1, 2]. PARylation is mainly mediated by PARP1/2 and PARP5A/B, which have been found to be involved in diverse biological processes, such as DNA repair, chromatin remodelling, telomere homeostasis, transcription, chromosome segregation, cell proliferation or cell death[1, 3–5]. Emerging evidence indicates that MARylation catalysed by mono-ADP-ribosyltransferases is also involved in many physiological and pathological processes[6], including

transcriptional regulation[7, 8], the unfolded protein response[9], DNA repair[10], insulin secretion[11], immunity[12] and tumour development[13]. While much is known about the cellular roles of PARPs that catalyse PARylation, the function of the PARPs that catalyse MARylation is substantially less understood.

PARP10, as the first-identified member of the mono-ADP-ribosyltransferase family, was originally labelled as a Myc-interacting protein, and its overexpression inhibits the c-Myc-mediated transformation of rat embryo fibroblasts[14]. Subsequently, PARP10 was found to interact with PCNA and was required to promote PCNA-dependent genomic stability and tolerance to DNA damage[15]. It was also found to be involved in the G1/S cell-cycle transition[16], NF-κB signalling[17], autophagy[18], apoptosis[19] and tumour development[20, 21]. More recently, we identified PARP10 as a tumour metastasis suppressor. We found that the expression level of PARP10 was lower in intrahepatic metastatic hepatocellular carcinoma (HCC) compared with its level in corresponding primary HCC and adjacent non-tumour tissues. We demonstrated that, mechanistically, PARP10 interacts with mono-ADP-ribosylated Aurora A and inhibits its kinase activity, thereby regulating Aurora A downstream signalling to suppress tumour cell epithelial-mesenchymal transition (EMT) processes and tumour metastasis[13].

Although PARP10 plays important roles in physiological and pathological processes, the regulation and modification mechanisms of PARP10 remain largely unclear. In this study, we show RNF114 to be a newly discovered functional partner and regulator of PARP10 and provide new insight into the regulation and modification of PARP10 in tumour metastasis.

Results

RNF114 is a newly discovered partner of PARP10

Recently, we identified Aurora A as a functional partner of PARP10 through tandem affinity purification[13]. In addition to Aurora A, RNF114, a ubiquitin E3 ligase[22], was found in the PARP10 complex, suggesting that RNF114 might also be a PARP10-interacting protein. Then, we performed exogenous and endogenous reciprocal immunoprecipitation (IP) assays to verify the interaction between PARP10 and RNF114. As expected, exogenously expressed HA-RNF114 interacted with SFB-PARP10, and vice versa (Figure 1a and 1b). Endogenous PARP10 also interacted with RNF114, as determined by a co-IP assay using an anti-PARP10 antibody (Figure 1c). Taken together, these results indicate that RNF114 is a previously unknown partner of PARP10.

Auto-mono-ADP-ribosylation of PARP10 is required for its association with RNF114

To gain further understanding of the interaction between PARP10 and RNF114, we generated serial deletion mutants of PARP10 to identify the region of PARP10 that is essential for its association with RNF114. As shown in Figure 2a, deletion of the 700-1025 aa region of PARP10, which contains the catalytic domain, abolished the interaction between PARP10 and RNF114, suggesting that the catalytic domain of PARP10 is critical for its association with RNF114. Then, we generated an enzymatically inactive PARP10 mutant to investigate whether the interaction between PARP10 and RNF114 is

dependent on the enzymatic activity of PARP10. As shown in Figure 2b, the G888W mutant, which abolished the enzymatic activity of PARP10[14], did interact with RNF114, as indicated by the co-IP assay results. Interestingly, we found that His-PARP10, which was purified from *E. coli*, did not interact with SFB-RNF114, which is expressed in 293T cells. However, after auto-mono-ADP-ribosylation *in vitro*, the mono-ADP-ribosylated His-PARP10 efficiently pulled down SFB-RNF114, suggesting that the auto-mono-ADP-ribosylation of PARP10 is required for its association with RNF114 (Figure 2c). Taken together, these results indicate that the interaction between RNF114 and PARP10 is dependent on the auto-mono-ADP-ribosylation of PARP10.

RNF114 ubiquitinates PARP10 through K27-linked ubiquitination

Both PARP10 and RNF114 are enzymes, raising the possibility that RNF114 is a substrate of PARP10 or PARP10 is a substrate of RNF114. To identify the link between modified PARP10 and RNF114, we first performed *in vitro* mono-ADP-ribosylation assays using biotin-NAD^+ as a donor to examine whether PARP10 can modify RNF114 via mono-ADP-ribosylation. As shown in Supplementary Figure 1, PARP10 was modified by auto-mono-ADP-ribosylation, as shown by western blotting with an anti-biotin antibody. However, RNF114 was not modified by PARP10 mono-ADP-ribosylation, as shown by *in vitro* assay, suggesting that RNF114 may not be a PARP10 substrate *in vitro*.

Therefore, we performed ubiquitination assays to determine whether PARP10 is an RNF114 substrate. As shown in Figure 3a, co-expression of GFP-RNF114 with Flag-PARP10 dramatically increased the ubiquitination level of Flag-PARP10 in 293T cells. Compared with wild-type RNF114, the RNF114 C29G/C32G mutant, which abolishes the E3 ligase activity of RNF114, did not promote the ubiquitination of PARP10 (Figure 3b), suggesting that RNF114 mediates the ubiquitination of PARP10 in a manner dependent on the RNF114 E3 ligase activity.

To investigate the ubiquitination of PARP10 by RNF114, we generated RNF114-knockout HeLa cells by CRISPR-Cas9 editing. We obtained two individual RNF114-knockout cell lines. The RNF114-knockout cells, which expressed no RNF114 protein, was examined by western blotting using anti-RNF114 antibody (Supplementary Figure 2a) and was found to contain several base pair deletions in genomic DNA, which caused a frame-shift mutation in RNF114, as indicated by DNA sequencing (Supplementary Figure 2b).

Then, we used the RNF114-knockout HeLa cells to examine the effect of RNF114 deficiency on PARP10 ubiquitination. As shown in Figure 3c, the ubiquitination level of PARP10 was dramatically decreased in the RNF114-deficient cells compared with the level in the wild-type cells, further suggesting that PARP10 is an RNF114 substrate. However, the total level of PARP10 protein was not obviously changed in the RNF114-deficient cells compared with the wild-type cells (Figure 3c), suggesting that the ubiquitination of PARP10 by RNF114 may not promote its degradation. Next, an *in vivo* ubiquitination assay was performed by using a panel of ubiquitin mutants with single K/R mutations at the sites of the seven lysine residues in ubiquitin. As shown in Figure 3d, compared with wild-type and other lysine mutants, the ubiquitin K27R mutant abolished the ubiquitination of PARP10 that is mediated by RNF114. We also used

the K27-only (K27O), K33-only (K33O), K48-only(K48O) and K63-only(K63O) mutants of ubiquitin to perform an *in vivo* ubiquitination assay. As shown in Figure 3e, the K33O, K48O and K63O mutants abolished the ubiquitination of PARP10 mediated by RNF114, while the K27O mutant had no effect on the ubiquitination of PARP10, compared with the effect of wild-type ubiquitin. Taken together, these results indicate that RNF114 ubiquitinates PARP10 through K27-linked ubiquitination.

RNF114 regulates PARP10 enzymatic activity

Emerging evidence suggests that K27-linked polyubiquitination plays important roles in the regulation of substrate function but not stability[23]. Therefore, we examined whether RNF114-mediated ubiquitination of PARP10 affected its function. First, we examined the mono-ADP-ribosylation levels of PARP10 and its substrate Aurora A in wild-type and RNF114-knockout cells by pull-down assay using the GST-Macro domain 2 of PARP14, which specifically associates with mono-ADP-ribosylated proteins [24, 25]. As shown in Figure 4a and 4b, PARP10 and Aurora A were pulled down by the GST-Macro2 domain from wild-type cells more efficiently than by the domain from the RNF114-knockout cells, suggesting that the mono-ADP-ribosylation levels of PARP10 and Aurora A were decreased after RNF114 depletion. Next, we examined the phosphorylation levels of Aurora A and its substrate Akt in wild-type and RNF114-knockout cells. As shown in Figure 4c, compared with those in the wild-type cells, the phosphorylation levels of Aurora A and Akt were increased in the RNF114-knockout cells, suggesting that RNF114 deficiency increased Aurora A kinase activity. Taken together, these results indicated that RNF114-mediated ubiquitination of PARP10 positively regulated PARP10 activity, which in turn suppressed Aurora A activity and downstream signalling.

RNF114 regulates the EMT process and tumour metastasis

Recently, we demonstrated that PARP10 is a tumour metastasis suppressor[13]. RNF114 regulates PARP10 activity, raising the possibility that RNF114 may also play important roles in cancer cell migration and tumour metastasis. To test this hypothesis, we performed cell migration and invasion assays using wild-type and RNF114-deficient HeLa cells. As shown in Figure 5a and 5b, compared with these processes in wild-type cells, the extent of the migration and invasion of RNF114-deficient cells was dramatically increased, suggesting that RNF114 negatively regulates the migration and invasion of HeLa cells. Then, we performed an analysis of the function of RNF114 in the distant metastasis of HeLa cells injected into the tail veins of mice. Wild-type HeLa cells and two RNF114-deficient cell lines were injected into BALB/c nude mice. Eight weeks after the injection, the mice were sacrificed, and metastatic lung tumours were analysed and properties calculated. As shown in Figure 5c and 5d, compared with the tumours established by the wild-type HeLa cells, the number and size of the lung tumours derived from the RNF114-deficient HeLa cells were significantly increased, suggesting that RNF114 deficiency promoted tumour metastasis *in vivo*.

Since the epithelial-mesenchymal transition (EMT) is implicated in cell migration and invasion[26] and because PARP10 is also involved in EMT regulation, the expression levels of EMT-associated markers were also analysed in the wild-type and RNF114-knockout cells. As shown in Figure 5e, compared with

that in the wild-type cells, the findings showed that the expression level of the epithelial marker E-cadherin was reduced and that the expression levels of the mesenchymal markers N-cadherin and Vimentin were increased in the RNF114-deficient cells, suggesting that RNF114 deficiency promotes the EMT process.

Next, we re-expressed RNF114 in the RNF114-deficient HeLa cells to validate the function of RNF114 in the EMT process and cell migration. As shown in Figure 5f, the expression levels of wild-type RNF114 and the RNF114 mutant introduced to the RNF114-deficient HeLa cells were similar to those of endogenous RNF114 in the control cells. The re-expression of wild-type RNF114 decreased the expression levels of N-cadherin and Vimentin and increased the expression level of E-cadherin, while re-expression of the enzymatic RNF114 mutant did not lead to these outcomes (Figure 5f). Consistently, the re-expression of wild-type RNF114, but not the enzymatic RNF114 mutant, dramatically suppressed the migration of RNF114-deficient HeLa cells (Figure 5g), suggesting that RNF114 regulates the EMT process and that cancer cell migration is dependent on its ubiquitin ligase activity. Taken together, these results suggest that RNF114 is a tumour suppressor and plays important roles in the EMT process and tumour metastasis.

In addition, PARP10 is overexpressed in RNF114-deficient cells, and the migration and invasion of wild-type, RNF114-deficient and PARP10-overexpressing RNF114-deficient cells were examined to confirm that RNF114 regulates the migration and invasion of HeLa cells in a PARP10-dependent manner. As shown in Supplementary Figure 3, PARP10 overexpression inhibited the migration and invasion of RNF114-deficient cells to the same level as the wild-type cells, indicating that RNF114 mediates cell migration and invasion through the regulation of PARP10.

Discussion

Although PARP10 was found to be involved in diverse physiological and pathological processes, the post-translational modification and regulation of PARP10 remained largely unclear. In a screen of PARP10-interacting proteins by tandem affinity purification and mass spectrometric analysis, we identified and confirmed that the ubiquitin E3 ligase RNF114 is a newly discovered partner of PARP10. More interesting, the interaction between RNF114 and PARP10 was found to be dependent on the auto-mono-ADP-ribosylation of PARP10 (Fig. 2). It has been reported that the E882 of PARP10 is auto-mono-ADP-ribosylation site [27]. However, we found that the E882A mutant did not affect the interaction between RNF114 and PARP10 (data not shown), suggesting that other auto-mono-ADP-ribosylation sites in PARP10 may mediate the interaction between PARP10 and RNF114. Indeed, multiple auto-mono-ADP-ribosylation sites in PARP10 have been identified by mass spectrometry analysis[27]. Moreover, two independent mass spectrometry approaches revealed that Lys916 of PARP10 is ubiquitinated in cells[28, 29], which might affect PARP10 catalytic activity, as the PARP10-K916I mutant shows a profound reduction in auto-mono-ADP-ribosylation capacity[14]. We also found that RNF114 did not promote the ubiquitination of the PARP10-K916I mutant (Supplementary Fig. 4), suggesting that Lys916 in PARP10 may be the major ubiquitination site mediated by RNF114. The determination of the precise PARP10 auto-mono-ADP-ribosylation site required for the interaction between PARP10 and RNF114 needs further

investigation. The interaction between RNF114 and PARP10 is dependent on the auto-mono-ADP-ribosylation of PARP10, which also raises the possibility that RNF114 contains a region that is specifically associated with mono-ADP-ribose. Although we found that both the UIM motif and zinc finger region, but not the ring finger of RNF114, are required for its association with PARP10 (Supplementary Fig. 5), the identification of the underlying mechanism awaits further investigation.

RNF114 belongs to the family of truly interesting new gene (RING) domain E3 ubiquitin ligases originally identified as a psoriasis susceptibility gene[30–32]. It has been reported that RNF114 is amplified and upregulated in patients with psoriasis but downregulated in the testes of azoospermic patients compared with its level in fertile adult testes[33], suggesting that it may be involved in human diseases, although the underlying molecular mechanism remains largely undefined. RNF114 substrates that have been reported include P21, which is involved in cell cycle regulation[22], TAB1 involved in maternal-to-zygotic transition[34] and A20 involved in NF-κB activity and T cell activation[35]. In this study, we demonstrated PARP10 as a newly identified RNF114 substrate. However, in contrast to P21, TAB1 and A20, which can be degraded by RNF114-mediated ubiquitination, PARP10 is not degraded by RNF114. PARP10 is ubiquitinated by RNF114 via K27-linked polyubiquitination, which enhances PARP10 enzymatic activity. More recently, porcine RNF114 was found to inhibit classical swine fever virus (CSFV) replication via the K27-linked polyubiquitination of viral NS4B[36]. These results indicate that RNF114-mediated polyubiquitination is complicated and that RNF114 may regulate substrate stability and activity.

All these findings provide new insight into the regulation of PARP10 by RNF114 and suggest novel RNF114-PARP10-Aurora A axis involvement in the regulation of tumour cell migration and invasion, which engages in crosstalk through K27-linked polyubiquitination, mono-ADP ribosylation and phosphorylation (Fig. 6).

Conculsions

Our findings identify RNF114 is a novel functional regulator of PARP10, and provide evidence of crosstalk between the components of K27-linked polyubiquitination and mono-ADP ribosylation. Both *in vitro* and *in vivo*, our studies demonstrate that RNF114 mediate tumour metastasis through RNF114-PARP10-Aurora A axis, indicating a potential target for cancer therapy.

Materials And Methods

Cell culture and transfection

293T and HeLa cell lines were obtained from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultured at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 100 μg/ml streptomycin and 100 U/ml penicillin.

Plasmids

Full-length PARP10 (gene ID: 84875) was cloned into modified pCDNA3.1 and pRIES2-EGFP vectors to generate constructs encoding HA-tagged PARP10 and S-Flag-SBP (SFB)-tagged PARP10. RNF114 (gene ID: 55905) was cloned into modified pRIES2-EGFP, pEGFP-C2 and pCDNA3.1 vectors to generate constructs encoding S-Flag-SBP (SFB)-tagged, GFP-tagged and HA-tagged RNF114, respectively. The deletion mutants of PARP10 and RNF114 were generated by using a Quick Change site-directed mutagenesis kit (Stratagene, Cedar Creek, TX, USA) according to the manufacturer's protocol. pET-28a and pGEX-4T-1 vectors were used to generate His-tagged or GST-tagged constructs, respectively.

Antibodies and reagents

Anti-RNF114 (H00055905-M03) antibody was purchased from Abnova (USA). Anti-Flag (F3165) antibody was purchased from Sigma (St louis, MO, USA). Anti-HA (ab18181), anti-GAPDH (ab8245), anti-PARP10 (ab70800), anti-Aurora A (ab1287) and anti-Aurora A (ab13824) antibodies were purchased from Abcam (Cambridge, MA, USA). Anti-p-Aurora A (Thr288) (3079S), anti-p-Akt (Thr473) (4060S), anti-Akt (4691S), anti-E-cadherin (3195S), anti-N-cadherin (14215S) and anti-vimentin (5741S) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-HRP (19534-050) antibody was purchased from Invitrogen (Carlsbad, CA, USA). Anti-ADP antibody (A01316) was purchased from GenScript (Nanjing, China). Biotin-NAD^+ was purchased from Invitrogen (Carlsbad, CA, USA); glutathione sepharose and Ni sepharose were purchased from GE Healthcare (Little Chalfont, UK, EU). High-capacity streptavidin agarose (20359) and protein A/G agarose (20422) were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Generation of RNF114-deficient cells

RNF114-deficient HeLa cells were constructed by the CRISPR/Cas9 method. sgRNA1 (GGACACGTGAAGCGTCCTAG) and sgRNA2 (GGTGTACGAGAAGCCGGTAC) sequences against human RNF114 were designed by http://crispr.mit.edu, and then, they were subcloned into a pX335-U6-Chimeric_BB-CBh-hSpCas9n Briefly, HeLa cells were transiently transfected with the vector. After 48 h, the cells were cultured for selection with 1 μg/ml puromycin for 3 days, and the surviving cells were cultured for two weeks to form single colonies, which were subsequently identified by immunoblotting and subjected to sequencing to verify the loss of RNF114 expression.

Recombinant protein expression and purification

GST (glutathione S-transferase)-tagged or His-tagged proteins were expressed in *Escherichia coli* strain BL21 (DE3) pLysS. The cells were grown at 37°C in LB medium to an optical density of 0.6–0.8 at 600 nm and then induced with a 12-h treatment of 0.2 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) at 20°C. The cells were collected and subjected to sonication and centrifugation. Then, the supernatants of the GST-tagged proteins and His-tagged proteins were incubated for 3 h with glutathione sepharose (GE Healthcare) or Ni sepharose, respectively, at 4°C according to the manufacturer's instructions, and then, the beads were washed five times with NETN buffer. Additionally, for the His-tagged proteins, the beads were incubated with washing buffer (5 mM imidazole; 20 mM Tris-HCl, pH 8; and 100 mM NaCl) for 30

min at 4°C to remove the non-specific protein. Next, the recombinant proteins were eluted for 1 h with elution buffer (200 mM imidazole; 20 mM Tris-HCl, pH 8; and 100 mM NaCl) at 4°C.

ADP-ribosylation assay

ADP-ribosylation assays were routinely carried out at 30°C for 30 min with 0.5 μg of enzyme and 1 μg of purified substrate protein in the reaction buffer (100 mM Tris-HCl, pH 7.6; 12.5 μM biotin- NAD^+; 10 mM $MgCl_2$; 50 μg of DNA oligos; and 10 mM DTT) in a total reaction volume of 30 μl. Reactions were stopped by adding SDS loading buffer and boiling at 98°C for 8 min, and subsequently subjecting the samples to SDS-PAGE and western blotting.

Ubiquitination assay

Ubiquitination assays were conducted in 293T cells. Flag-PARP10, HA-RNF114 and HA-Ub were transfected into 293T cells. After 48 h, the cells were lysed in NETN buffer. Flag-PARP10 was pulled down with streptavidin agarose and subsequently analysed by western blotting.

Lentivirus preparation and reconstitution assay

Viral packaging was performed with 293T cells co-transfected with a pCDH-CMV-HA-RNF114-EF1-copGFP vector carrying a psPAX2 packaging plasmid and a pMD2.G envelope plasmid. The viruses were harvested 72 h after transfection, and the viral titres were determined. The day before inoculation, the RNF114-deficient HeLa cells were seeded into 35 mm dishes and then infected with the viruses. pCDH-CMV-Flag-PARP10-EF1-copGFP vector was used for overexpression of PARP10.

Migration and invasion assay

Migration and invasion assays were carried out in 24-well Transwell plates (Corning, Corning City, New York, USA). For the migration assay, 50,000 HeLa cells were seeded in the upper chamber with serum-free medium, and the lower chamber was filled with complete DMEM with 10% FBS as an attractant. After 24 h, the cells that had migrated to the bottom surface were fixed with 4% paraformaldehyde and stained with crystal violet solution; the non-migrated cells in the upper chamber were gently removed with a cotton swab. Five random visual fields were recorded and analysed statistically by ImageJ software (NIH). For the invasion assays, the upper chamber was precoated with Matrigel (BD Biosciences).

Immunohistochemistry staining

The immunohistochemistry staining assay was conducted with the indicated antibodies according to a protocol we have described previously[37].

In vivo metastasis

Female nude mice (6 weeks old) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and maintained in accordance with the National Institutes of Health Guide for the Care

and Use of Laboratory Animals. All animal procedures were reviewed and approved by the Ethics Review Committee for Animal Experimentation of Fudan University. To determine the role of RNF114 in tumour cell metastasis *in vivo*, 1×10^6 HeLa cells suspended in 200 μl of PBS were injected into nude mouse tail veins. Eight weeks later, the mice were sacrificed for the examination of metastasis.

Statistical analysis

All experiments were repeated three or more times. Samples or animals were allocated to experimental groups randomly. The significance of the differences was determined by Student's *t*-test. Biostatistical analysis was carried out using GraphPad software (GraphPad Prism 8, La Jolla, California, USA).

Abbreviations

PARP10, poly (ADP-ribose) polymerase 10; RNF114, RING finger 114; EMT, epithelial-mesenchymal transition; PARP14, poly (ADP-ribose) polymerase 14; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; and HCC, hepatocellular carcinoma

Declarations

Ethics approval and consent to participate

All animal procedures were reviewed and approved by the Ethics Review Committee for Animal Experimentation of Fudan University.

Consent for publication

All authors have agreed to the publication of this manuscript.

Availability of data and materials

All data and materials used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing financial interests.

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Authors' contributions

Y.Z. designed the study, conducted most of the experiments and wrote the manuscript. X.L. and L.W. performed the experiments. Y.L., J.L., H.F., F.Z., and Y.W. contributed to the manuscript completion. J.W. and H.M. conceived the study, provided overall guidance and contributed to the manuscript completion.

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Figures

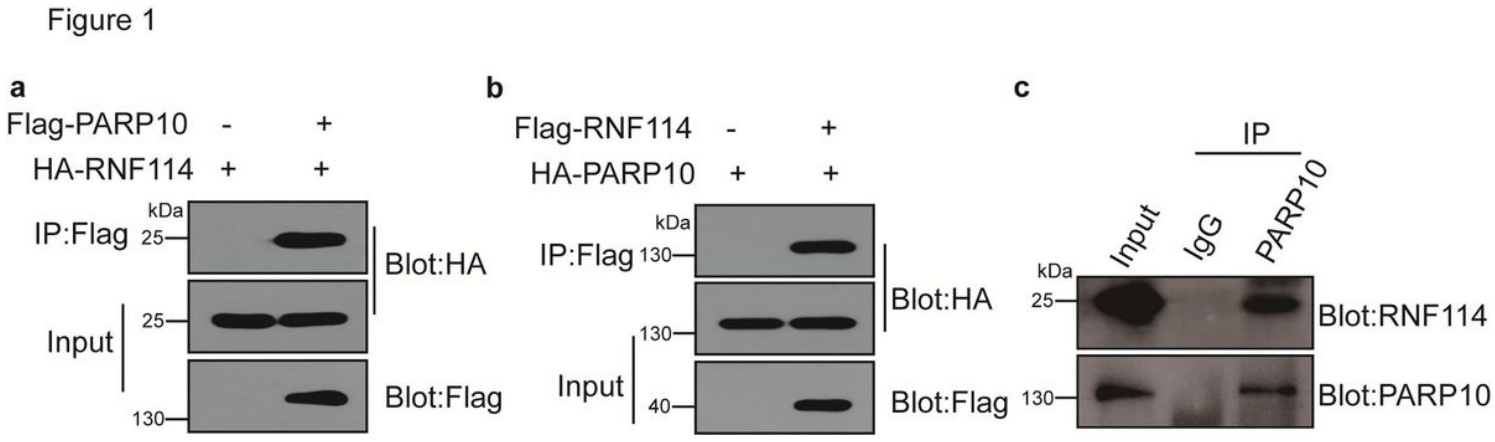

Figure 1

RNF114 is a newly discovered partner of PARP10. (a, b) PARP10 interacts with exogenous RNF114. 293T cells were transiently transfected with plasmids encoding SFB-PARP10 and HA-RNF114 or plasmids encoding SFB-RNF114 and HA-PARP10. Transfected cells were lysed, subjected to immunoprecipitation (IP) with streptavidin-conjugated beads, and subjected to western blotting. Whole-cell lysates were blotted and shown as input. (c) Endogenous PARP10 interacts with RNF114. HeLa cell lysates were subjected to immunoprecipitation (IP) using an anti-PARP10 antibody and analysed by western blotting using the indicated antibody. Irrelevant IgG was used as the IP control.

Figure 2

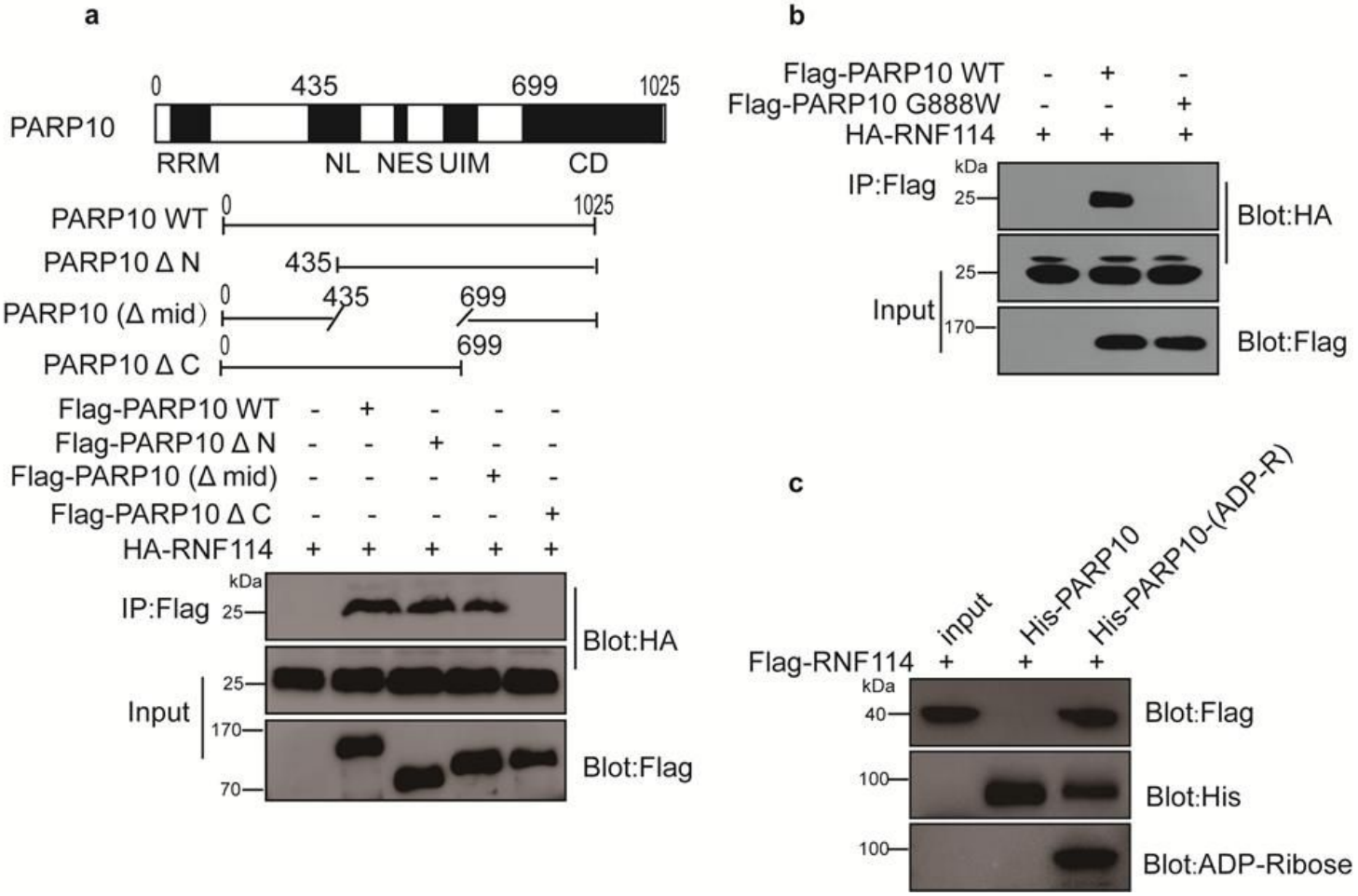

Figure 2

Auto-mono-ADP-ribosylation of PARP10 is required for its association with RNF114. (a) The C-terminus of PARP10 (700-1025 aa) is required for the interaction between PARP10 and RNF114. 293T cells were transiently co-transfected with plasmids encoding HA-RNF114 and SFB-PARP10 or deletion mutants of PARP10. The transfected cell lysates were subjected to immunoprecipitation (IP) and western blotting. (b) Enzymatic activity of PARP10 is critical for the interaction between PARP10 and RNF114. SFB-PARP10 or SFB-PARP10 G888W was transfected into 293T cells with HA-RNF114. Whole cell lysates were subjected to IP with streptavidin beads and subjected to western blotting. (c) Auto-mono-ADP-ribosylation of PARP10 is required for its association with RNF114. His-PARP10 was expressed in Escherichia coli BL21 (DE3) pLysS, purified with Ni sepharose and subjected to in vitro ADP-ribosylation assay. His-PARP10 and

His-PARP10-ADP-ribose coupled to Ni Sepharose were used to pull down SFB-RNF114 from the transfected 293T cells. Proteins were separated by SDS-PAGE and analysed by western blotting with the indicated antibodies.

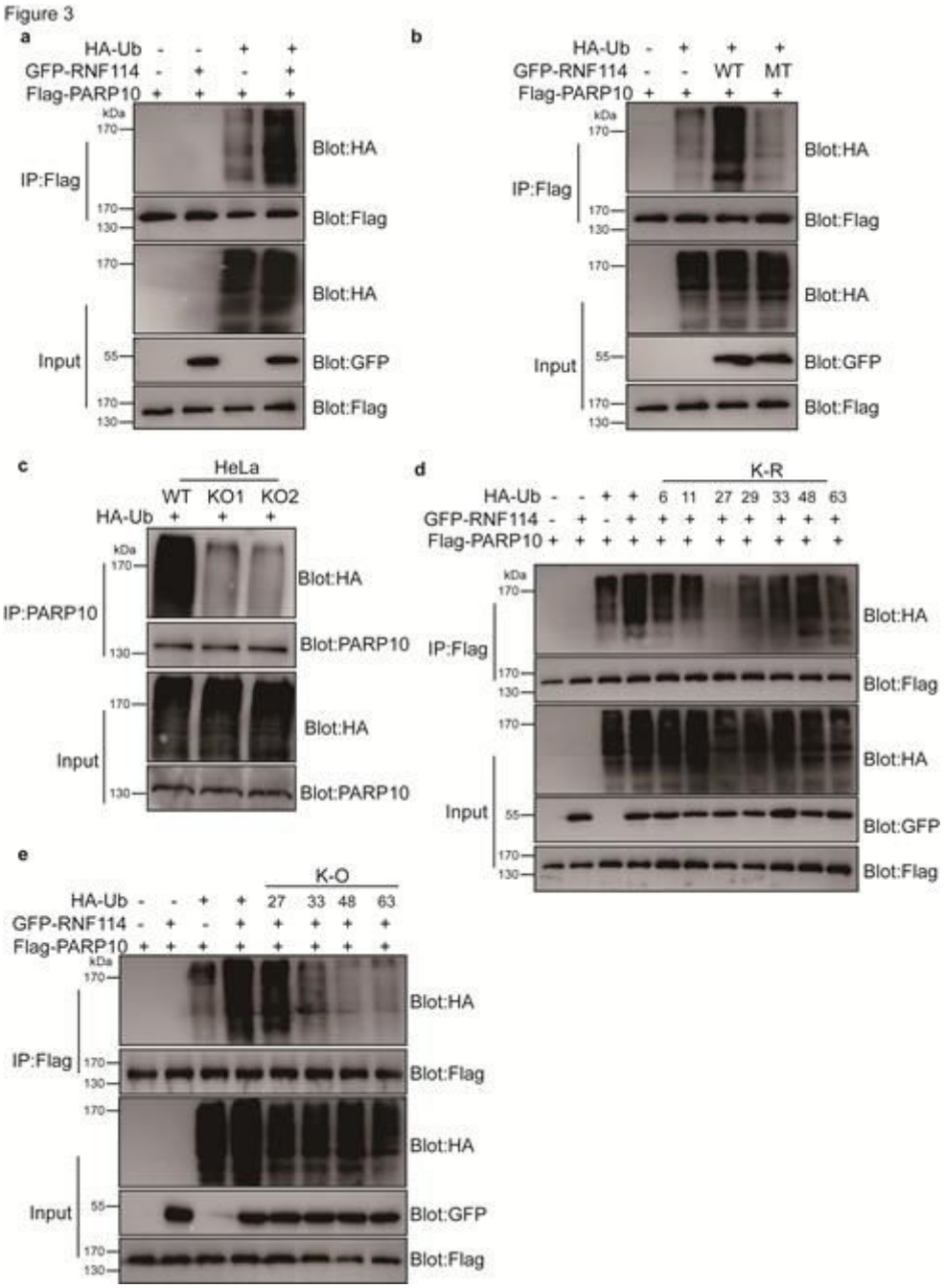

Figure 3

RNF114 ubiquitinates PARP10 through K27-linked ubiquitination. (a) RNF114 enhances the ubiquitination of PARP10. SFB-PARP10 was transfected into 293T cells in the presence or absence of GFP-RNF114 and HA-Ub. SFB-PARP10 and subjected to immunoprecipitated (IP) with streptavidin-conjugated beads and then to western blotting with the indicated antibodies. (b) RNF114 mediated the ubiquitination of PARP10, which depended on RNF114 E3 ligase activity. SFB-PARP10 was transfected into 293T cells in the presence or absence of HA-Ub and GFP-RNF114 WT or GFP-RNF114 C29/32G. SFB-PARP10 was

subjected to immunoprecipitation (IP) with streptavidin-conjugated beads and then to western blotting with the indicated antibodies. (c) RNF114 deficiency inhibits the ubiquitination of PARP10. HA-Ub was transfected into wild-type and RNF114-deficient HeLa cells. PARP10 was pulled down with an anti-PARP10 antibody and subjected to western blotting with the indicated antibodies. (d) PARP10 was ubiquitinated by RNF114 through K27-linked ubiquitination. SFB-PARP10 was transfected into 293T cells with GFP-RNF114 and wild-type HA-Ub or mutated HA-RNF114 with a single K/R substitution as indicated. SFB-PARP10 was subjected to immunoprecipitation (IP) with streptavidin-conjugated beads and then to western blotting with the indicated antibodies. (e) In vivo ubiquitination assay was performed as in (d) with the K-only mutants of HA-Ub.

Figure 4

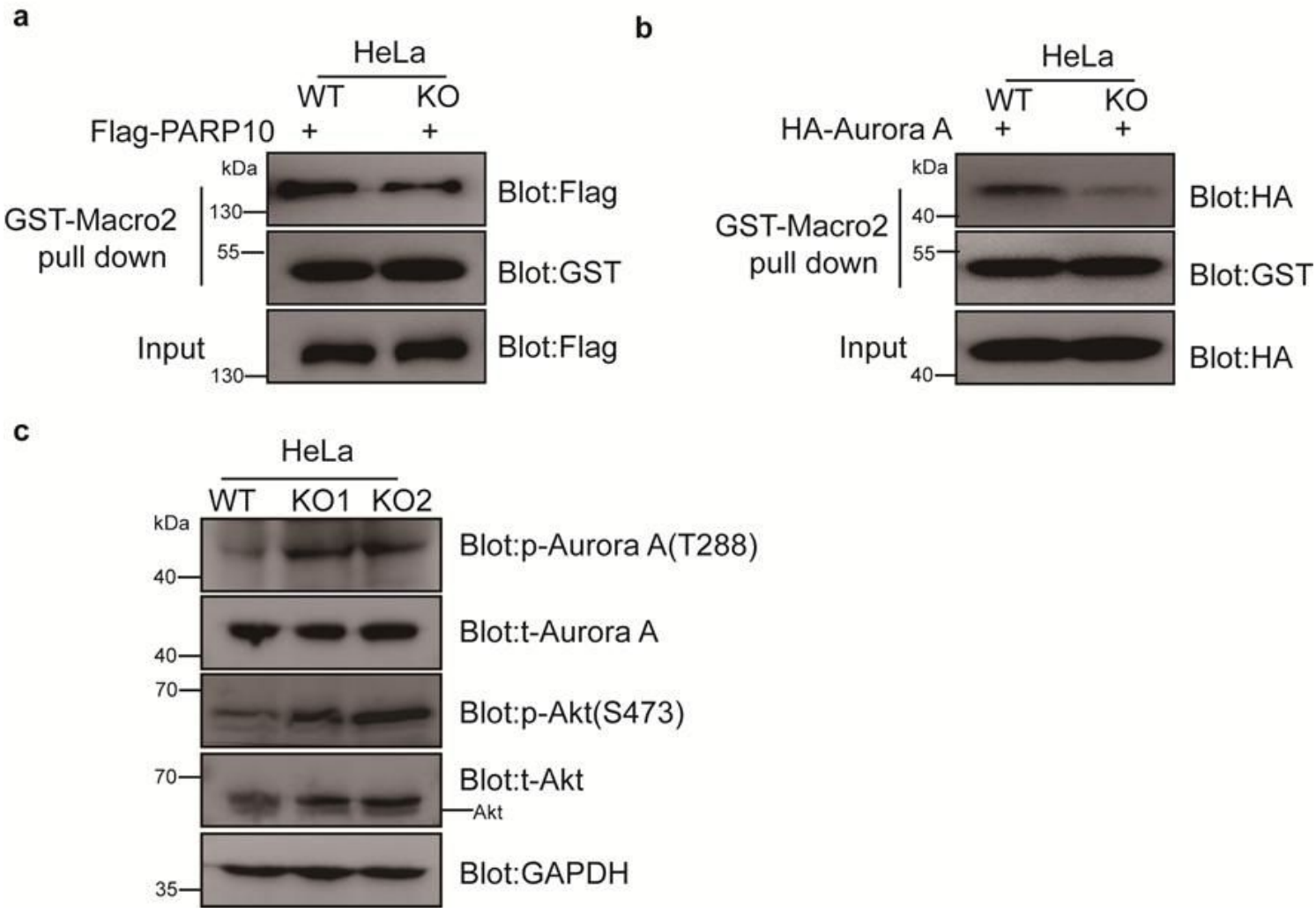

Figure 4

RNF114 positively regulates PARP10 enzymatic activity. (a) Auto-mono-ADP-ribosylation of PARP10 was inhibited in the RNF114-deficient HeLa cells. SFB-PARP10 was transfected into wild-type and RNF114-deficient HeLa cells. Whole-cell lysates were subjected to pull-down assays with GST-Macro domain 2 of PARP14 coupled to glutathione sepharose. After incubation, the proteins binding to glutathione sepharose were analysed by western blotting with the indicated antibodies. (b) Mono-ADP-ribosylation of

Aurora A was inhibited in RNF114-deficient HeLa cells. HA-Aurora A was transfected into wild-type and RNF114-deficient HeLa cells. The whole cell lysates were subjected to pull-down assays with GST-Macro domain 2 of PARP14 coupled to glutathione sepharose. The protein binding to glutathione sepharose was analysed by western blotting with the indicated antibodies. (c) Phosphorylation of Aurora A at T288 and phosphorylation of Akt at S473 were analysed in the wild-type and RNF114-deficient HeLa cells by western blotting using the indicated antibodies, where GAPDH was used as a loading control.

Figure 5

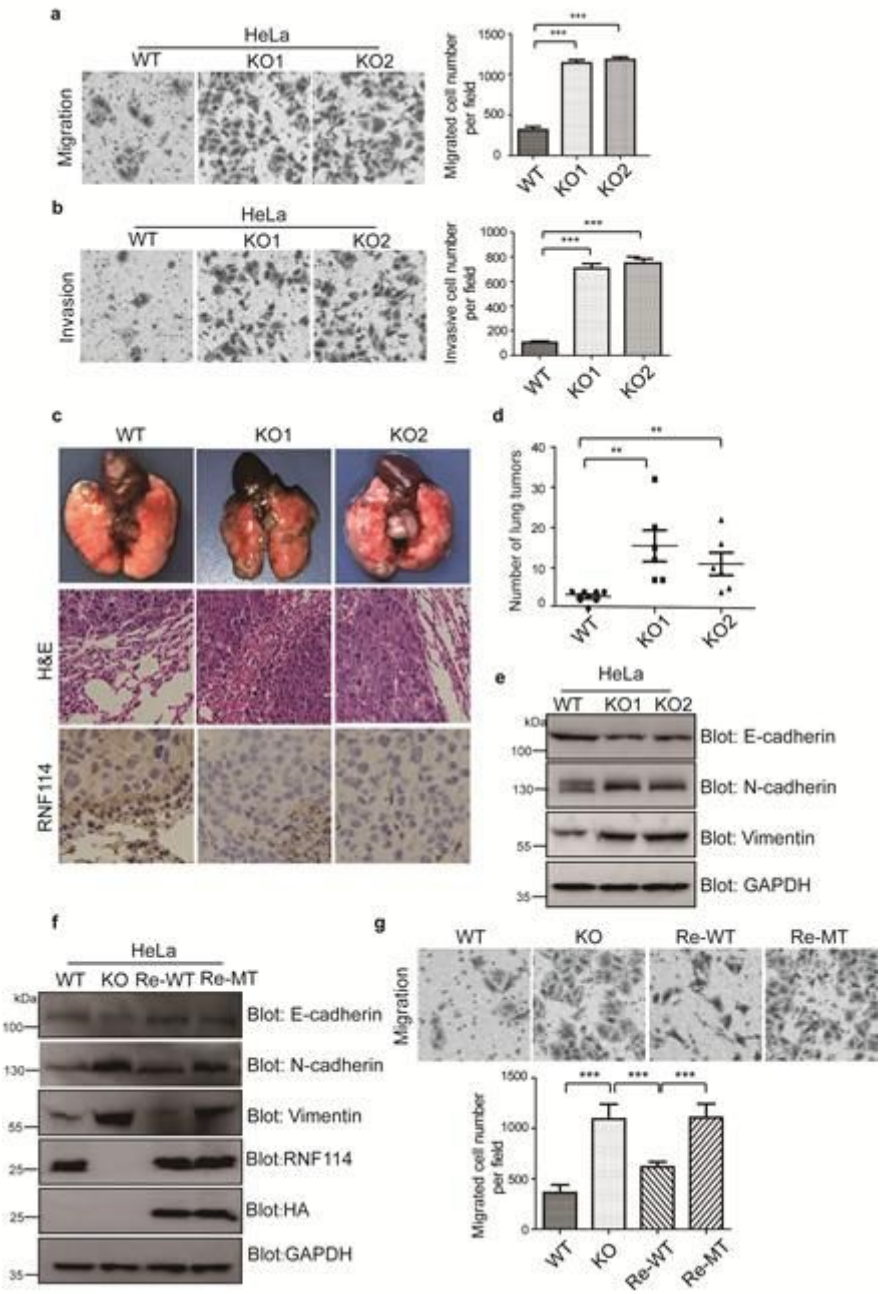

Figure 5

RNF114 regulates tumour metastasis and the EMT. (a) RNF114 deficiency promotes HeLa cell migration. Representative images of migrated cells are shown as originally magnified by 100×. The histograms show the mean number of migrated cells based on three independent tests (the means±s.d.). ***P<0.001.

(b) RNF114 deficiency promotes HeLa cell invasion. Representative images of invasive cells are shown as originally magnified by 100×. The histograms show the mean number of invasive cells based on three independent tests (the means±s.d.). ***P<0.001. (c) RNF114 inhibits tumour metastasis in vivo. Wild-type and RNF114-deficient HeLa cells were injected into the tail vein of nude mice. After 8 weeks, the mice were sacrificed, and the lung tumours were analysed. Representative images of metastatic lung tumours, H&E staining results and the immunohistochemistry staining results of RNF114 are shown. (d) The scatter gram shows the number of tumour nodules in each mouse. (e) Expression of E-cadherin, N-cadherin and Vimentin in the wild-type and RNF114-deficient HeLa cells was analysed by western blotting. GAPDH was used as a loading control. (f) Expression of E-cadherin, N-cadherin and Vimentin was analysed in the wild-type, RNF114-deficient and reconstituted HeLa cells. (g) Reconstitution of wild-type RNF114 rescued the inhibition of cancer cell migration. Representative images of migrated cells are shown as originally magnified by 100×. The histograms show the mean number of migrated cells based on three independent tests (the means±s.d.). ***P<0.001.

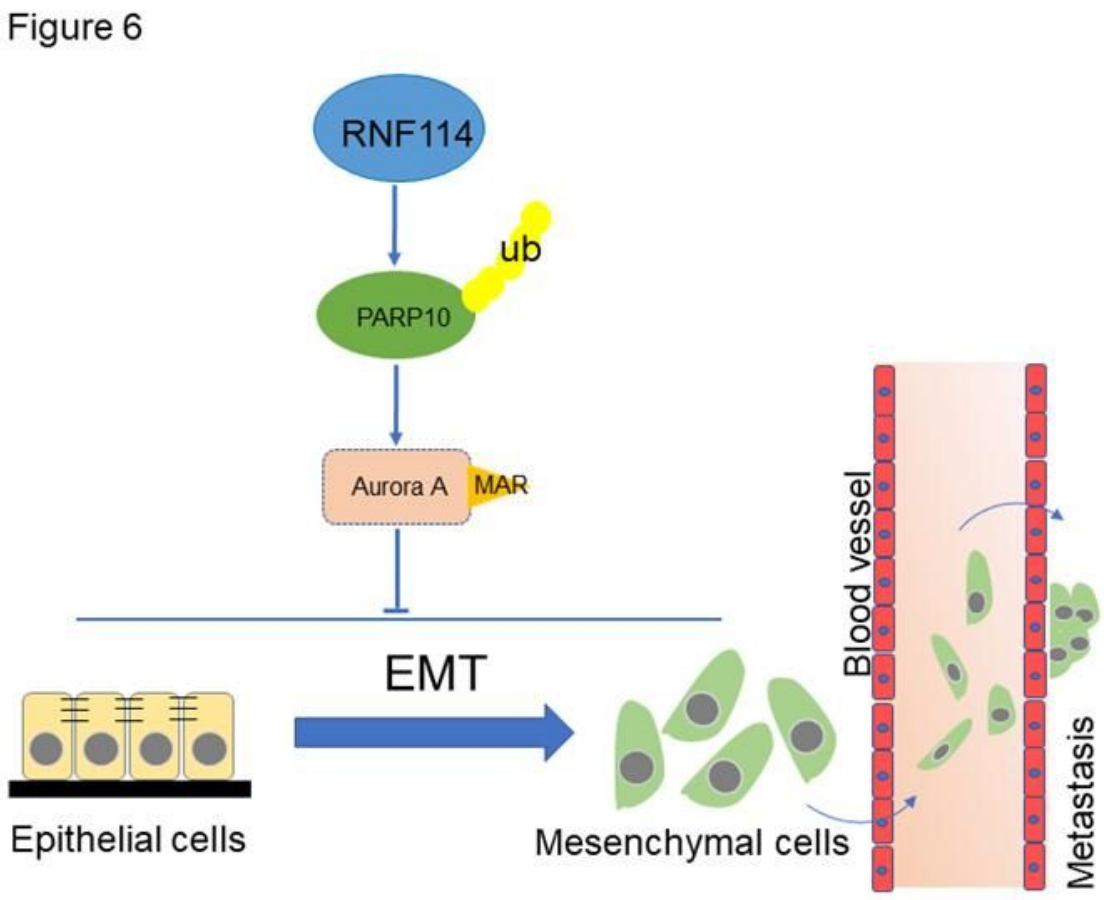

Figure 6

Model of the RNF114-PARP10-Aurora A signalling pathway during the EMT transition and tumour metastasis. Ubiquitination of PARP10 by RNF114 enhances PARP10 enzymatic activity and increases the MARylation of Aurora A, leading to the inhibition of Aurora A kinase activity and suppression of the EMT and tumour metastasis.

Supplementary Files

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