Oxidative stress promotes liver cancer metastasis via a PKA-activated RNF25/ECAD/YAP circuit

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Article

Keywords:

Posted Date: January 13th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1197819/v1>

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Abstract

Loss of E-cadherin (ECAD), often caused by epigenetic inactivation, is closely associated with tumor metastasis. However, how ECAD is regulated in response to oxidative stress during tumorigenesis is largely unknown. Here we identify RNF25 as a new E3 ligase of ECAD, whose activation by oxidative stress leads to ECAD protein degradation in hepatocellular carcinoma (HCC). Loss of ECAD activates YAP, which in turn promotes the transcription of RNF25, thus forming a positive feedback loop to sustain the ECAD downregulation. YAP activation mitigates oxidative stress in detached HCC cells by upregulating antioxidant genes, protecting detached HCC cells from ferroptosis, resulting in anoikis resistance. Mechanistically, we found that protein kinase A (PKA) senses oxidative stress by redox modification in its β catalytic subunit (PRKACB) at Cys200 and Cys344, which increases its kinase activity towards RNF25 phosphorylation at Ser450, facilitating RNF25-mediated degradation of ECAD. Moreover, RNF25 expression is associated with HCC metastasis and depletion of RNF25 is sufficient to diminish HCC invasion and metastasis *in vitro* and *in vivo*. Together, these results identify a dual role of RNF25 as a critical regulator of ECAD protein turnover, promoting both anoikis resistance and metastasis, and PKA is a necessary redox sensor to enable this process. Our study provides mechanistic insight into how tumor cells sense oxidative stress signals to spread while escaping cell death.

Introduction

Metastasis is frequently observed in advanced cancer cases. However, the mechanism is poorly understood, which is a major challenge for cancer therapy. ROS affect cancer metastasis in somewhat contradictory ways. A slightly elevated ROS level induces oxidative eustress to support the proliferation and invasiveness of cancer cells. In contrast, an extremely high level of ROS result in oxidative distress, leading to senescence, apoptosis, or ferroptosis of cancer cells [1]. This Janus effect emphasizes that the intracellular redox potential of cancer cells must be kept at the eustress level to achieve successful metastasis. In eustress conditions, ROS precisely controls multiple aspects of cell behaviour through thiol-based reversible redox modifications on active cysteines of certain proteins (redox sensors) to regulate the protein function, which is known as redox signaling [2]. Compelling evidence has addressed the importance of redox signaling in the antioxidant defense of metastatic cancer cells. For instance, ROS-induced oxidation of pyruvate kinase M2 (PKM2) at Cys358 causes a metabolic shift from the glucose to pentose phosphate pathway, thus providing antioxidant materials for cancer cells to withstand oxidative stress [3]. Nevertheless, how cancer cells sense oxidative stress to initiate metastatic programming in the early stage of dissemination is largely unknown.

Adherens junctions, together with other junctional complexes, comprise a significant barrier preventing cancer cells from metastasis. Loss of adherens junctions is an early event in metastasis, which has been observed in various cancers. Adherens junctions are a protein complex consisting of E-cadherin (ECAD), α -catenin, β -catenin and p120-catenin, among which ECAD has been mostly investigated. For instance, downregulation of ECAD has long been demonstrated to promote metastasis [4]. Epigenetic silencing by particular transcription factors such as ZEB, snail and twist is the well-known regulatory mechanism underlying ECAD loss, but regulating ECAD is regulated at the protein level remains elusive. Compared

with transcriptional control, the protein degradation process is relatively rapid, thus contributing to a quick adaptation for organisms in response to stress. Importantly, ROS have been shown to repress the expression of ECAD, and antioxidants are capable of maintaining ECAD levels, which at least partially explains why oxidative stress drives metastasis in certain circumstances [5].

To determine if protein degradation of ECAD holds pathological significance in the clinic, we explored the TCGA dataset to reveal that hypoxia level is negatively correlated with the protein level, but not the mRNA level, of ECAD in hepatocellular carcinoma (HCC) patients, suggesting that ECAD loss might be regulated at the protein level in HCC. We showed that the RING finger protein 25 (RNF25) is the novel E3 ligase for ECAD ubiquitination, and overexpression of RNF25 predicts poor prognosis in HCC. RNF25-mediated ECAD loss activates YAP, which in turn transcribes RNF25 to form a positive feedback loop, leading to HCC metastasis. Moreover, YAP also upregulates several antioxidant genes to withstand oxidative stress, preventing detached HCC cells from death. Mechanistic studies revealed that protein kinase A (PKA) undergoes redox modification in response to ROS, which increases its kinase activity. This effect leads to phosphorylation of RNF25, thereby facilitating RNF25-mediated ECAD degradation. These findings suggest that PKA is a critical sensor for driving HCC metastasis in response to oxidative stress by the RNF25/ECAD/YAP circuit.

Results

Oxidative stress downregulates E-cadherin at the protein level in HCC

Given that oxidative stress has been considered to play dual roles in metastasis, we explored The Cancer Genome Atlas (TCGA) dataset [6] to clarify the eventual outcomes of

HCC patients with different oxidative stress levels. As shown in Fig. 1A, hypoxia score, the indicator of oxidative stress, is elevated in HCC patients with high tumor grades. Additionally, overall survival was decreased in HCC patients with a high level of ROS (Fig. 1B-D). This evidence suggests that oxidative stress is an overall detrimental factor for HCC patients, promoting tumor progression. From the clinic perspective, oxidative stress is tightly linked to pro-inflammatory cytokines, such as TGF β and IL-6. Therefore, we treated human HCC cell lines PLC/PRF/5 (hereafter referred to as PLC) and Huh7 with recombinant TGF β 1 and IL-6 proteins. As shown, these two cytokines upregulate the ROS level in HCC cells (Fig. 1E-F and Fig. S1A-B). Moreover, an epithelial-to-mesenchymal transition (EMT) morphological change was also observed along with cytokine-induced ROS production (Fig. 1E-F). Given that the adherens junctions are critical for EMT program and detachment of cancer cells, the expression of several adherens proteins was measured. We found that treatment with TGF β 1 significantly downregulates E-cadherin (ECAD), but not other adherens proteins such as N-cadherin or ZO-1 (Fig. 1G and Fig. S1C). Interestingly, the mRNA level of ECAD was merely repressed in PLC cells, and unchanged in Huh7 cells in response to cytokine-induced ROS (Fig. 1H and Fig. S1D). These observations suggest that ROS-mediated ECAD loss is partially independent of the well-known epigenetic silencing mechanism.

To ascertain the clinical significance of this protein regulatory mechanism, we again explored the TCGA dataset to find that the hypoxia level is negatively correlated with protein, but not the mRNA levels of ECAD in HCC patients (Fig. 1I-J and Fig. S1E-H). Similarly, TGF β 1 and IL-6 are also negatively correlated with ECAD protein expression rather than mRNA level (Fig. S1I-L). However, hypoxia level appears to be positively correlated with the level of TGF β 1 but not IL-6, suggesting that from a clinic perspective the oxidative stress might be induced mainly through the upregulation of TGF β 1 instead of IL-6 (Fig. S1M-N).

Consistently, TGF β 1receptor was also found to be negatively correlated with ECAD protein but not its mRNA level (Fig. S1O-P). Together, these data indicate that oxidative stress downregulates ECAD at the protein level, which probably leads to EMT, cell detachment and consequent metastasis in HCC.

RNF25 is an E3 ligase for E-cadherin and is associated with HCC metastasis

To identify key factors mediating ECAD protein loss, we screened potential E3 ligases for ECAD using the UbiBrowser online tool [7] (Fig. 2A). The top 20 candidates were then subjected to analysis of the correlation between their mRNA levels and the protein level of ECAD using the Cancer Cell Line Encyclopedia (CCLE) database [8] (Fig. 2B). Among them, the RING finger protein 25 (RNF25) ranked as the most significant E3 (Fig. 2B). As shown in Fig. 2C and D, the expression of RNF25 is negatively correlated with the protein level of ECAD in cell lines and HCC clinical tissues. In contrast, the known E3 ligase for ECAD, c-Cbl-like protein 1 (CBLL1, also named Hakai) [9], showed no negative correlation with the ECAD protein level in HCC cells (Fig. S2A). To determine the potential E3 activity of RNF25 to ECAD, we generated stable Flag-tagged RNF25 overexpressing PLC cells and stable endogenous RNF25 knockdown Huh7 cells, followed by evaluation of their protein interaction and ubiquitination. As shown in Fig. 2E and Fig. S2B, RNF25 was found to interact with ECAD in both HCC cells, and treatment with hydrogen peroxide further enhanced this interaction. Deletion of the cytoplasmic region (731-882 aa) of ECAD abolished the interaction, suggesting RNF25 binds with the cytoplasmic fragment of ECAD (Fig. S2C). Furthermore, overexpression of RNF25 increased the ubiquitination of ECAD, whereas knockdown of RNF25 exhibited the opposite effect (Fig. 2F and Fig. S2D). Consistently, RNF25 downregulates ECAD at protein but not mRNA levels (Fig. 2G and Fig. S2E-G). Moreover, *in vitro* ubiquitination assay also demonstrated that ECAD is

ubiquitinated by RNF25 (Fig. 2H). These pieces of evidence suggest that RNF25 is a novel E3 ligase for ECAD and might be involved in loss of ECAD during HCC metastasis.

To determine the pathological significance of RNF25 in HCC, we evaluated the expression of RNF25 in 71 HCC clinical samples by immunohistochemistry (IHC). We showed that the RNF25 protein levels were upregulated in metastatic HCC tissues compared with non-metastatic samples (Fig. 2I). Additionally, RNF25 was enriched in high-grade tumors, suggesting it plays a role in tumor progression (Fig. 2J). Moreover, Kaplan-Meier analysis demonstrated a poor overall survival of HCC patients with high RNF25 expression (Fig. 2K), which is consistent with the record from the TCGA dataset (Fig. S2H). Further dataset mining [10, 11] revealed that RNF25 is overexpressed in HCC tissues compared with non-cancerous tissues (Fig. S2I-J). Furthermore, we also found a negative correlation between the protein level of RNF25 and ECAD in HCC clinical samples (Fig. 2L), and RNF25 gain together with ECAD loss demonstrated a robust prognostic power to predict unfavorable survival in HCC patients (Fig. S2K). Together, these data indicate that high expression of RNF25, a novel E3 ligase for ECAD, is associated with HCC metastasis and poor prognosis.

RNF25-induced E-cadherin loss inactivates Hippo signaling to promote cell invasion

Compelling evidence suggests that E-cadherin serves as an intercellular glue and the key signaling molecule during cell invasion. Our previous work showed that the Hippo signaling, an evolutionarily conserved pathway, plays a vital role in HCC metastasis [12]. Furthermore, YAP, as the effector of Hippo signaling, has been reported to support the survival of matrix-detached cells [13]. Therefore, we wondered if RNF25-mediated ECAD loss promotes HCC metastasis by regulating Hippo signaling. To this end, the expression of RNF25 and YAP

was detected using tissue immunofluorescence, and we found that they are colocalized at the invasive front of HCC clinical tissues (Fig. 3A). Furthermore, luciferase reporter assays showed that RNF25 upregulates the activity of the TEAD-binding promoter (Fig. 3B-C), but not the LEF/TCF-binding promoter (Fig. S3A-B), suggesting RNF25, rather than β -catenin affects the transcriptional activity of YAP. Activated YAP readily translocates to the nucleus, as was observed in RNF25-overexpressing HCC cells (Fig. S3C-D). Moreover, the YAP target genes CTGF and CYR61 were transcriptionally activated in response to RNF25 overexpression (Fig. 3D-E). These observations suggest that Hippo signaling might be inactivated by RNF25, thus leading to YAP activation. Therefore, we investigated the expression and phosphorylation of the Hippo kinase cascade. This showed that RNF25 decreases the phosphorylation of MST1 and LATS1, leading to the inactivation of Hippo signaling and consequent YAP dephosphorylation (Fig. 3F). This increases the protein stability of YAP, resulting in transcriptional activation of target genes CTGF and CYR61 (Fig. 3F).

It has been documented that ECAD maintains Hippo activation by interacting with the Hippo component, NF2 (also known as Merlin) [14]. We, therefore, postulated that RNF25-mediated ECAD degradation might inactivate NF2, thus leading to YAP activation (Fig. 3G). To test this, Myc-tagged ECAD was transiently expressed in PLC-RNF25 cells, showing that RNF25-mediated overexpression of YAP was repressed by ectopic ECAD (Fig. 3H). However, this effect was abolished by knockdown of NF2, suggesting that RNF25-induced ECAD degradation leads to loss of function of NF2, resulting in Hippo inactivation and consequent YAP activation (Fig. 3H). Moreover, knockdown of endogenous ECAD in Huh7-shRNF25 cells was shown to mimic the YAP-activating function of RNF25, which can be reversed by the expression of exogenous wild-type ECAD but not mutated ECAD lacking of the cytoplasmic region (Fig. 3I). These findings suggest that the cytoplasmic part of ECAD is required for the activation of Hippo signaling. Moreover, treatment of verteporfin (VP, a

YAP inhibitor) inhibits the migration and invasion of PLC-RNF25 cells. In contrast, ectopic overexpression of Flag-tagged YAP mimics the metastatic-promoting function of RNF25 in Huh7-shRNF25 cells (Fig. 3J-K and Fig. S3E-F). This evidence indicates that RNF25-mediated ECAD degradation inactivates Hippo signaling, leading to YAP activation required for HCC metastasis. Notably, no noticeable difference was found in the viability or colony formation rate of HCC cells, suggesting that RNF25 promotes HCC metastasis without obviously affecting proliferation (Fig. S4A-D).

YAP transcribes RNF25 to form a positive feedback loop

The high mRNA level of RNF25 in HCC tissues (Fig. S2I-J) implicates that RNF25 might be transcriptionally activated during HCC progression. To illustrate this potential upstream mechanism leading to the upregulation of RNF25, we screened the key transcription factors (TFs) that are overexpressed in HCC tissues compared with normal liver tissue according to the GSE17967 Robust Multiarray Averaging (RMA) dataset [15] (Fig. S5A). Among the top 5 TFs, the TEA domain family member 1 (TEAD1) is of particular interest due to the results we had obtained (above) showing its co-TF YAP is involved in RNF25-mediated HCC metastasis. Moreover, prediction of the binding between these 5 TFs with the RNF25 promoter using the online tool JASPAR [16] indicated TEAD1 as the candidate with the highest score, suggesting that the promoter of RNF25 may be recognized by the YAP/TEAD transcription complex (Fig. S5B). Indeed, we observed 4 TEAD-binding elements (TBE1-4) with a conserved 5'-CATTCC-3' motif in the RNF25 promoter (Fig. S5C). To verify this potential TF-promoter binding site, we employed a chromatin immunoprecipitation (ChIP) assay to demonstrate that the two distal motifs, TBE1 and TBE2, are responsible for binding with YAP in parental PLC and Huh7 cells (Fig. S5D-E). Additionally, ectopic expression of YAP increases the mRNA and protein levels of RNF25,

whereas administration of the YAP inhibitor, verteporfin, resulted in opposite effects (Fig. S5F-K). Moreover, luciferase reporter assays indicated that the activity of RNF25 promoter is significantly decreased when TBE1/2 are mutated separately or simultaneously (Fig. S5L). These data indicate that YAP binds with the promoter of RNF25 to activate its transcription, suggesting the formation of an RNF25/ECAD/YAP positive feedback loop (Fig. S5M).

Redox modification of protein kinase A regulates the phosphorylation and function of RNF25

Given that phosphorylation is a common regulatory mechanism for a number of E3 ligases including Nedd4-2, ITCH, Smurf1 [17-19], we wondered if RNF25 is also phosphorylated and thereby regulated. To this end, we explored a human phosphoproteome dataset [20], where we found that Ser450 is a potential phosphorylation site on RNF25 with a high functional score (Fig. 4A). To verify this, we generated stable PLC and Huh7 cells expressing S450A or S450D mutated RNF25, followed by analysis of their phosphorylation status. This indicated significant phosphorylation of wild-type RNF25, which was largely abolished by S450A mutation (Fig. 4B and Fig. S6A). Moreover, the expression of ECAD was partially reversed in RNF25 S450A cells but further repressed in RNF25 S450D cells, compared with RNF25 wild-type cells (Fig. 4C and Fig. S6B). These findings indicate that phosphorylation of RNF25 at S450 enhances its function of ECAD degradation. To elucidate which kinase might mediate RNF25 phosphorylation, we noticed that the amino acid sequence around S450 of RNF25 matches a typical protein kinase A (PKA)-recognized motif, suggesting PKA might be responsible for RNF25 phosphorylation (Fig. S6C).

In general, PKA consists of catalytic and regulatory subunits, the latter keeping PKA in an inactive form. Regulatory subunits are dissociated with catalytic subunits in response to

active signals (such as cAMP), therefore rendering catalytic subunits free to phosphorylate the substrates. Interestingly, it has been reported that PKA can also be activated without dissociating the regulatory and catalytic subunits [21]. This suggests that the physical separation of the regulatory subunits might not be a prerequisite for the activation of catalytic subunits. The human genome encodes three isoforms of PKA catalytic subunits, namely PRKACA, PRKACB and PRKACG. To determine which isoform plays the dominant role in RNF25 phosphorylation and subsequent ECAD degradation in HCC, we explored the CCLE dataset, finding that the ECAD protein level is negatively correlated with the mRNA level of PRKACB, but not the other two isoforms (Fig. S6D-F). Moreover, PRKACB was shown to interact with RNF25 and promote the phosphorylation of RNF25 at the S450 site in HCC cells (Fig. 4D and Fig. S6G-I). In addition, *in vitro* kinase assays showed that PRKACB induces phosphorylation of RNF25, but this phosphorylation can be largely abolished by S450A mutation (Fig. 4E-F). To determine the function of phosphorylated RNF25, we performed an *in vitro* ubiquitination assay to show an increase of ECAD ubiquitination induced by S450D mutated RNF25 compared with wild-type RNF25 (Fig. 4G). These observations indicate that PRKACB phosphorylates RNF25 at S450, thereby enhancing its potential to mediate ECAD ubiquitination and consequent degradation.

Similar to many other kinases, the activity of PKA has been shown to be regulated by oxidative stress [22]. To ascertain this, we determined the activity of PRKACB in response to H₂O₂. As shown, ROS increases the activity of PRKACB, which can be reversed by treatment with NAC (Fig. 4H). Moreover, ROS induced by H₂O₂ or TGFβ1 can phosphorylate RNF25 at S450, which is significantly abrogated by treatment with H-89 (PKA inhibitor) or NAC (Fig. S6J-K). These findings suggest that PKA might be a redox sensor that phosphorylates RNF25 in response to oxidative stress, thereby initiating the RNF25/ECAD/YAP circuit. To test this hypothesis, non-reducing SDS PAGE assays were

performed to show that treatment with H₂O₂ or TGFβ1 induces a band shift for PRKACB, which can be reversed by administration of NAC (Fig. 4I). In contrast, no obvious shift was observed on the RNF25 band (Fig. 4I). These results indicate that PRKACB, but not RNF25, is a typical redox sensor that directly senses ROS to form an intramolecular disulfide bond, resulting in the band shift in non-reducing SDS PAGE. Interestingly, there are only two cysteines within human PRKACB protein, namely C200 and C344. We therefore generated cysteine PRKACB mutants which were subjected to non-reducing SDS PAGE for the evaluation of disulfide bond formation. As a result, mutation of either cysteine or both, abolished the band shift induced by H₂O₂ (Fig. 4J). This observation demonstrates the formation of an intramolecular disulfide bond between C200 and C344 of PRKACB in response to oxidative stress. Moreover, mutation of cysteine abolishes the PRKACB kinase activation induced by ROS (Fig. 4K) and abrogates RNF25 phosphorylation (Fig. 4L). Together, these data suggest that ROS induces the formation of an intramolecular disulfide bond between C200 and C344 of PRKACB, leading to enhanced kinase activity and subsequent RNF25 phosphorylation, which facilitates the degradation of E-cadherin.

YAP triggers an antioxidant response to protect detached HCC cells from ferroptosis, leading to anoikis resistance

Tumor metastasis is an extremely inefficient process during which most circulating tumor cells (CTCs) die from oxidative stress. Therefore, powerful antioxidant machinery is required to enable CTCs to survive and eventually colonize distant organs. It has been well-established that YAP promotes the survival of matrix-detached cells, thus contributing to metastasis [13, 23, 24]. In line with these reports, we found that inhibition of YAP by verteporfin decreases the survival of detached PLC-RNF25 cells, while ectopic expression of YAP prolongs survival of detached Huh7-shRNF25 cells (Fig. 5A-B). To explore underlying

mechanisms, we treated detached HCC cells with a variety of cell death inhibitors, including 3-methyladenine (autophagy), z-VAD-FMK (apoptosis), ferrostatin-1 (ferroptosis) and necrostatin-1 (necrosis). This showed that administration of ferrostatin-1, but not other inhibitors, rescued the survival of detached HCC cells with low RNF25 expression (PLC-vector and Huh7-shRNF25) to a level comparable to those with high RNF25 expression (PLC-RNF25 and Huh7-shNC) (Fig. 5C-D). These observations suggest detached HCC cells with low RNF25 expression died from ferroptosis, a ROS-specific cell death pattern. In line with our findings, it has been reported that loss of matrix attachment confers ROS accumulation within cells [25], and metastatic cancer cells are highly susceptible to ferroptosis [26]. To elucidate the mechanism of RNF25-mediated ferroptosis resistance in detached HCC cells, the lipid ROS level was measured, and we found that RNF25 decreases lipid ROS in detached PLC cells, which can be reversed by inhibition of YAP (Fig. 5E). Besides, Huh7-shRNF25 cells were shown to increase lipid ROS levels, which can be repressed by ectopic expression of YAP (Fig. 5F). These data suggest that YAP inhibits ferroptosis of detached HCC cells by eliminating ROS. However, this finding is somewhat contradictory to a previous report describing that YAP induces ferroptosis in colorectal cancer (CRC) via upregulating of ACSL4 [14]. In an effort to explain these opposite findings, we evaluated the expression of YAP and ACSL4 in patients with CRC or HCC according to the TCGA dataset. Interestingly, a positive correlation between ACSL4 and YAP expression was observed in CRC, but not HCC patients, suggesting ACSL4 failed to be activated by YAP in HCC (Fig. 5G-H). To test this hypothesis, we measured the ACSL4 mRNA level in detached HCC cells. The mRNA level of ACSL4 remained unchanged in PLC-RNF25 cells treated with verteporfin, and was decreased in Huh7-shRNF25 cells with ectopic expression of YAP (Fig. 5I-J). In contrast, YAP was shown to transcribe a panel of antioxidant genes in these detached HCC cells (Fig. 5I-J). This is in agreement with the antioxidant function of

YAP that has been widely reported [27-29]. Taken together, our data indicate that YAP-mediated antioxidant response confers HCC cells with ferroptosis resistance during metastasis driven by the RNF25/ECAD/YAP circuit.

RNF25 promotes HCC metastasis both *in vitro* and *in vivo*

To determine the function of RNF25 in HCC metastasis, the morphology of PLC and Huh7 stable cells was investigated. We observed an EMT-like morphologic change in PLC-RNF25 cells and an opposite effect in Huh7-shRNF25 cells (Fig. 6A-B). To measure the metastatic potential of HCC cells with different expression levels of RNF25 *in vitro*, we employed Transwell and wound healing assays to determine the migration and invasion of HCC cells. Overexpression of RNF25 elevated their migration and invasive ability, while knockdown of RNF25 showed an opposite effect (Fig. 6C-F). To evaluate the function of RNF25 *in vivo*, we established a tail vein injection mouse model using the aforementioned stable cells. Overexpression of RNF25 promoted the formation of lung metastases, whereas knockdown of RNF25 decreased the metastatic capability of HCC cells (Fig. 6G-H). This *in vivo* model demonstrated the pro-survival function of RNF25 for circulating tumor cells (CTCs) in addition to degradation of adherens junctions, which is consistent with the above findings showing that HCC cells with a high level of RNF25 are more resistant to detached conditions, while RNF25 deficiency largely increases the death of detached HCC cells.

Discussion

Oxidative stress affects cancer metastasis in contradictory manners. For those tumor cells which have already entered the blood, oxidative stress inhibits their metastasis by inducing ferroptosis [30, 31]. However, how oxidative stress promotes metastasis at the

beginning of metastatic spread is largely unknown. Here, we showed that oxidative stress induces ECAD protein degradation by activating RNF25, leading to epithelial-to-mesenchymal transition (EMT) of HCC cells. Additionally, degradation of ECAD results in YAP activation, which initiates antioxidant response to protect detached HCC cells from ferroptosis. Mechanistic studies revealed that PRKACB, the catalytic subunit of PKA, undergoes redox modification between C200 and C344 in response to oxidative stress, thus leading to an increase in kinase activity. Activated PKA then phosphorylates RNF25 at S450, which facilitates RNF25-mediated ECAD degradation. Moreover, RNF25 is also transcriptionally activated by YAP, therefore forming a positive feedback loop to drive HCC metastasis (Fig. 6I). Thus, our study reveals a novel mechanism of oxidative stress-induced tumor metastasis in HCC.

Loss of ECAD has long been recognized as one of the most important early events during cancer metastasis. Though epigenetic silence of ECAD has been widely reported, the regulation of ECAD at the protein level has been largely ignored. In fact, in addition to transcriptional regulation, protein degradation of ECAD has emerged as an alternative mechanism leading to ECAD loss during cancer metastasis. For instance, the glycoprotein CD147 was shown to recruit E3 ligase CBLL1 for ECAD degradation, resulting in tumor progression [32]. These evidences suggest that ECAD loss can be induced at both the mRNA and protein level, but which pathway is preferential at a given circumstance remains to be clarified. Using bioinformatics, we found that oxidative stress is negatively correlated with the protein, but not the mRNA level of ECAD in HCC patients, suggesting oxidative stress-mediated ECAD loss in HCC is prominently attributed to protein degradation instead of epigenetic silencing from a clinical perspective. Further studies revealed that RNF25 is a novel E3 ligase for ECAD, and RNF25-induced ECAD degradation inactivates Hippo signaling, leading to YAP activation which is required for HCC metastasis. Taken together,

our findings address the importance of protein degradation pathway as an indispensable regulatory mechanism in stress management. This view is supported by the fact that the Keap1-Cul3 complex-mediated protein degradation of NRF2 plays a central role in the antioxidant machinery of organisms [33]. Hippo signaling also coordinates cellular behaviour in response to multiple stress factors through a 14-3-3-mediated protein degradation of YAP [34]. Compared with transcriptional regulation, the protein degradation pathway is rapid, thus enabling a speedy adaptation for organisms in stressful conditions.

Inactive protein kinase A (PKA) is a holoenzyme consisting of catalytic and regulatory subunits. In the presence of cAMP, the regulatory subunits are physically separated from catalytic subunits, leading to PKA activation [35]. Though not conventionally considered as an oncogene, compelling evidence suggests that PKA is involved in cancer development, which is at least partially due to its function of coping with stress. For instance, activation of PKA promotes the survival of drug-resistant cancer cells in stressful conditions including nutrient deprivation and cell detachment [36]. Besides, tumor growth can be accelerated in stressed animal models by activating PKA [37]. These findings suggest that PKA promotes the survival of tumor cells under stress. In line with this, changes in the level of cAMP, the agonist of PKA, significantly alters stress-induced behaviors in mice [38]. As one of the most common stress conditions, oxidative stress also leads to the activation of PKA [39]. Interestingly, in response to oxidative stress, PKA was found to form an intermolecular disulfide bond between its two regulatory subunits, leading to its activation independent of cAMP [40]. However, whether its catalytic subunits undergo redox modifications remains undetermined. Here, we show that PKA catalytic subunit B (PRKACB) directly senses oxidative stress by the formation of an intramolecular disulfide bond between C200 and C344, which increases its kinase activity, leading to the phosphorylation of RNF25 and consequent ECAD degradation in HCC. This finding demonstrates that PRKACB is a typical redox

sensor underlies PKA-mediated stress response. Another catalytic subunit, PRKACA, shares more than 90% similarity in amino acid sequence with PRKACB including cysteines C200 and C344, suggesting that a similar redox regulation might also exist in PRKACA. This deserves further investigation.

Signaling networks are extremely complex in cells and frequently form circuits to regulate cellular processes. Negative feedback loops restrict signaling output, maintaining the homeostasis of organisms. Positive feedback loops boost signals, leading to a significant biological effect. To some extent, tumor initiation and progression is caused by the dysregulation of signaling circuits. This study revealed that an RNF25/ECAD/YAP positive feedback loop and a ROS/YAP/antioxidant response negative feedback loop synergistically promotes tumor metastasis in HCC. In the early stages of metastasis, the ROS-induced RNF25/ECAD/YAP circuit promotes epithelial-to-mesenchymal transition (EMT) of cells, enhancing their invasive capacity, leading to detachment from the matrix. When cancer cells enter into the blood, the ROS/YAP/antioxidant response circuit is activated to avoid the ferroptosis of detached cells, eventually resulting in successful metastatic dissemination. These findings are in line with the fact that tumor metastasis is a multi-step process where metastatic cells exhibit distinct properties at different stages, and therapeutic approaches targeting cancer in a particular stage might unexpectedly favor cancer progression in another stage. Thus, our results provide mechanistic insights for the dual roles of oxidative stress in tumor metastasis, which might be attributed to different signaling circuits initiated by ROS. This view is supported by opposite findings from other clinic trials showing that antioxidant supplementation can either decrease the cancer mortality, or offer no survival benefits for cancer patients [41, 42]. Thus, informed targeting of those signaling circuits can be a rational strategy for improving the outcome of oxidant/antioxidant-based cancer therapy.

Surgery is the crucial therapeutic option for patients with most solid tumors that controls cancer progression and prolongs patient survival worldwide. However, it has been reported that surgical resection unexpectedly accelerates tumor recurrence in some cases [43, 44]. This paradox is thought to be attributed to the multifaceted inflammatory response induced by traumatic stress. Pro-inflammatory factors are required to activate the immune system during the elimination of cancer cells, but an inflammatory tumor microenvironment frequently supports the development of cancer. Surgical procedures occasionally stimulate oxidative stress, which facilitates tumor growth and metastasis [45]. In fact, surgery induces an increased level of inflammation, where proinflammatory cytokines can stimulate the production of reactive oxygen species (ROS). Thus, modulation of ROS levels in the postoperative period may improve clinical outcomes of patients undergoing surgery. In supporting our hypothesis, we showed that traumatic and psychiatric stress promotes tumor metastasis by ROS accumulation (Fig. S7). In the traumatic stress model, surgery was performed at different tumor burdens, representing different levels of traumatic stress (Fig. S7A). We observed that the level of circulating tumor cells (CTCs) was increased in resection groups (Fig. S7B). Moreover, CTC abundance and ROS levels were positively correlated with the tumor burden, indicating that more severe traumatic stress readily leads to more significant ROS accumulation and consequent cancer dissemination (Fig. S7B-C). Notably, TGF β and IL-6 were shown to increase in mice who had undergone surgery (Fig. S7D-E), suggesting oxidative stress might be attributed to inflammatory response in mice suffering from traumatic stress. Similarly, in the psychiatric stress models, the levels of CTC, ROS, TGF β and IL-6 were increased in stress groups, which could be partially reversed by the administration of NAC (Fig. S7F-O). These results suggest that traumatic and psychiatric stress stimulate an inflammatory response, which promotes ROS accumulation, leading to detachment of cancer cells from the matrix, thus contributing to metastasis. However, these

preliminary *in vivo* models are not totally able to simulate the oxidative stress-mediated tumor metastasis observed in human bodies owing to a timing issue. Tumor metastasis is a time-consuming process and most tumor recurrence cases in the clinic occur after more than three months, which largely exceeds the schedule of our mouse models. Besides, cancer dissemination is extremely complex. Other mechanisms, such as epigenetic silence of ECAD, have not been extensively investigated here. Although a long-term spontaneous mouse model and epidemiological evidence are required to validate this point further, we have clearly shown that TGF β and IL-6 play critical roles in traumatic and psychiatric stress-mediated tumor metastasis. Thus, our findings suggest that administration of neutralizing antibodies against TGF β and IL-6, or relieving stress in the postoperative period may well be beneficial for cancer patients.

In summary, our study indicates that oxidative stress mediates a redox modification on the β subunit of protein kinase A, which drives tumor metastasis via the RNF25/ECAD/YAP circuit in HCC. This finding helps explain how cancer cells sense oxidative stress to spread and survive, providing novel insights for the treatment of HCC.

Materials and methods

Antibodies and reagents. Antibodies for E-cadherin (Cell Signaling Technology, Cat # 3195), N-cadherin (Cell Signaling Technology, Cat # 13116), ZO-1 (Cell Signaling Technology, Cat # 8193), β -actin (Santa Cruz Biotechnology, Cat # sc-69879), RNF25 (Abcam, Cat # ab89281), HA-tag (Abcam, Cat # ab49969), Myc-tag (Cell Signaling Technology, Cat # 2278), Flag-tag (Cell Signaling Technology, Cat # 14793), MST1 (Cell Signaling Technology, Cat # 14946), p-MST1 (Cell Signaling Technology, Cat # 49332), LATS1 (Cell Signaling Technology, Cat # 3477), p-LATS1 (Cell Signaling Technology, Cat

9157), YAP (Thermo Fisher Scientific, Cat # PA1-46189), p-YAP (Cell Signaling Technology, Cat # 13008), CTGF (ABclonal Technology, Cat # A11943), CYR61 (ABclonal Technology, Cat # A1111), NF2 (Cell Signaling Technology, Cat # 12888), α -tubulin (Santa Cruz Biotechnology, Cat # sc-8035), Histone-H3 (Cell Signaling Technology, Cat # 4499), p-Serine (ZEN-Bioscience, Cat # 530893), and the reagents including N-acetyl cysteine (NAC) (Merck, Cat # A9165), Recombinant Human TGF- β 1 (PEPROTECH, Cat # 100-21), Recombinant Human IL-6 (PEPROTECH, Cat # 200-06), Hydrogen peroxide solution (H₂O₂) (Merck, Cat # 323381), Verteporfin (VP) (MedChemExpress, Cat # HY-B0146), H-89 (Aladdin, Cat # H129712), Kemptide (Aladdin, Cat # K118853) were purchased commercially.

Cell cultures. Liver cancer cell lines PLC/PRF/5, Huh7 and the embryonic kidney cell line HEK293T were obtained from American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing fetal bovine serum (10%, BI), penicillin (100 U/mL, Invitrogen) and streptomycin (100 μ g/mL, Invitrogen) at 37°C with 5% CO₂. To generate RNF25 stable knockdown and overexpression cells, lentivirus harboring shRNA (sh#1: 5'-GATGAACTACAGGTGATTAAA-3' and sh#2: 5'-GTTCGCTGGGAGCGCTCTAAA-3') or cDNA for RNF25 was used to infect HCC cells followed by puromycin selection. The RNF25 S450A and S450D stable cells were obtained through similar approaches.

Animal models. Animal study was approved by the Institutional Animal Care and Treatment Committee of University. Female BALB/c nude mice (6-week-old, HFK Bioscience) were raised in the SPF Laboratory Animal Center of Sichuan University. For the

surgical-induced traumatic stress mouse model, 1×10^7 Huh7 cells were subcutaneously injected into the flanks of mice. One week post injection, mice were randomly divided into three groups, namely control (n=7), early resection (n=6) and late resection (n=7). When tumor volume reached $\sim 6 \text{ mm} \times 7 \text{ mm}$, blood was collected before euthanasia. Mice in early or late resection group were subjected to surgical operation to remove xenografts when tumor burden reached $6 \text{ mm} \times 7 \text{ mm}$ or $12 \text{ mm} \times 13 \text{ mm}$, respectively. The different tumor burden represents different levels of traumatic stress. Three days post resection, blood was collected before euthanasia. The blood samples were then aliquoted for the detection of CTC, ROS, and cytokines. Briefly, genomic DNA was purified by TIANamp Genomic DNA Kit (TIANGEN, Cat # DP304-02), and the level of CTC was determined by RT-qPCR using primers targeting human-specific short interspersed elements (Forward: 5'-GGTGAAACCCCGTCTCTACT-3'; Reverse: 5'-GGTTCAAGCGATTCTCCTGC-3') according to a previous report [46]. ROS level was evaluated by DCFH-DA method. Serum TGF β 1 and IL-6 levels were measured by ELISA kits (Boster Bio, Cat # EK0515; EK0411). For the forced swimming (FS) model, tumor-bearing mice were placed in a foam box containing 15 cm-depth water to force swim for 10 minutes. This test was performed once a day for a week. Mice in FS+NAC group were also subjected to forced swimming test at the same time supplemented with 25 mg/kg NAC once a day for a week. For the rhythm disturbance (RD) model, tumor-bearing mice were subjected to an eight-hour earlier light/dark cycle every two days. For tail vein model, 1×10^6 stable cells of PLC-vector (n=6), PLC-RNF25 (n=6), Huh7-shNC (n=6), Huh7-shRNF25#1 (n=5) were injected into nude mice through tail vein. One month post injection, mice were euthanized. The lungs were collected for photographic examination and the metastases were counted. Then, the lungs were fixed in 4% formaldehyde for histological analysis.

Clinical HCC tissues and IHC. Studies using clinical samples were approved by the Biomedical Ethics Review Committee of University. Human HCC samples (n=71) were collected from university and grouped according to their pathological characteristics including metastasis status, cirrhosis status, and tumor grade as shown in the medical records. IHC assays were performed as previously described [12]. Briefly, human HCC tissues were embedded in paraffin and then sliced into 5 μ m thickness sections for preservation. To detect the protein expression, the sections were rehydrated and treated with 3% H₂O₂ for 15 minutes to block the endogenous peroxidase. Next, the slides were incubated with antigen retrieval buffer and then boiled for 4 minutes. After blocking with goat serum, sections were treated with primary antibodies at 4 °C overnight. After washing three times with phosphate buffered saline (PBS), the sections were incubated with MaxVision HRP solution (MXB Biotechnology, Cat # 5020) reagent for one hour at 25 °C. Next, the slides were washed again three times with PBS, followed by staining using DAB Peroxidase Substrate (MXB Biotechnology, Cat # 0031). Nuclei were stained with hematoxylin. Photographs were captured using a DM2500 fluorescence microscope (Leica).

Transwell assay. Polycarbonate membrane inserts with an 8 μ m pore (Corning, cat # 3422) were used in the Transwell assays, the experiment being performed as previously described [12]. Briefly, to detect the migration of cells, 600 μ L growth medium was added in 24-well plates prior to placing the insert into the well. Then, 1×10^5 cells were resuspended with 200 μ L FBS-free medium followed by seeding into the chamber. To measure the invasion of cells, the chamber was pre-coated with matrigel (BD Biosciences, Cat # 356234). Next, 2×10^5 cells were resuspended in 200 μ L FBS-free medium and seeded into the insert. The plate containing inserts were then cultured at 37 °C in a humidified chamber for 24 hours. After that, migrated or invaded cells were fixed with 4% formaldehyde for 30 minutes at room

temperature followed by staining with 0.1% crystal violet for another 30 minutes at room temperature. Photographs were captured using a DM2500 fluorescence microscope (Leica).

Co-immunoprecipitation. The co-IP assays were performed as previously described [12]. Briefly, 3×10^6 cells were lysed with NP-40 lysis buffer (0.5%, pH=7.4) containing 20 mM Tris, 100 mM NaCl and 0.5 mM EDTA supplemented with 1% protease inhibitor and phosphatase inhibitor (Bimake, Cat # B14001, B15001) for 30 minutes at 4 °C, followed by incubation with 1 µg primary antibodies at 4 °C overnight. Then, cell lysate was incubated with protein G plus protein A agarose beads (Merck, Cat # IP10-10MLCN) for 2 hours at 4 °C. Next, the beads were washed three times using NP-40 wash buffer with similar constituents as in the NP-40 lysis buffer except for a 150 mM concentration of NaCl. After that, the beads were boiled with SDS-PAGE loading buffer for 10 minutes and subjected to immunoblotting analysis.

Immunoblotting. Cells were washed with ice-cold PBS twice and then lysed with RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 0.5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, pH=7.4) supplemented with protease inhibitor and phosphatase inhibitor (as above). Next, the lysate was mixed with loading buffer and boiled for 10 minutes. For non-reduced SDS-PAGE to detect the band shift, a non-reduced loading buffer (DTT-free) was used to protect the disulfide bond. Next, the proteins were separated by SDS-PAGE followed by transfer to the PVDF membrane (Merck, Cat # ISEQ00010). Then, the membrane was blocked by TBST buffer containing 5% non-fat milk powder for 1 hour at room temperature, followed by cutting into strips. The strips were then incubated with indicated antibodies at 4 °C overnight, followed by 3 washings with TBST, and incubated

with secondary antibodies for 2 hours at room temperature. After 6 washes with TBST, the strips were incubated with Immobilon Western HRP Substrate (Merck, Cat # WBKLS0500) and the images were obtained using a ChemiScope 6000 Touch Chemiluminescence imaging system (Clinx, Shanghai).

Tissue immunofluorescence. Human or mouse tissues were handled as for the IHC methods from the beginning till the incubation with primary antibodies. Then, the slides were washed with PBS, followed by the treatment with DyLight 488/594-conjugated goat anti-rabbit/mouse IgG (Invitrogen, Cat # 35552, 35511) for 1 hour at room temperature. After 3 washes with PBS, DAPI reagent (Solarbio, Cat # C0060) was used to stain the nuclei for 5 minutes at room temperature, followed by another 3 washes with PBS. Next, the slides were sealed with neutral balsam (Solarbio, Cat # G8590) and photographed using a DM2500 fluorescence microscope (Leica).

Quantitative RT-PCR. Cells were washed with ice-cold PBS twice and the total RNA was extracted with TRIzol reagent (Thermo Fisher Scientific, Cat # 15596018). Then, the RNA was reverse transcribed by the PrimeScript RT reagent Kit (Takara, Cat # RR047A) to yield the cDNA. Next, the PCR reaction was performed using SYBR Green reagent (Bio-Rad, 1725271), and the relative cDNA levels of targeted genes was normalized to β -actin. The primer sequences are provided as follows (5' to 3'). E-cadherin: CAGCACGTACACAGCCCTAA and ACCTGAGGCTTTGGATTCCT. CTGF: AGCCCAAGGACCAAACC and CCAGGCAGTTGGCTCTAAT. CYR61: GAGTGGGTCTGTGACGAGGAT and GGTGTATAGGATGCGAGGCT. RNF25: ATCTTACAGGTGCTGGGCCA and AACCATAGAGGCAGATGACACAC. SLC7A11:

565 TCTCCAAAGGAGGTTACCTGC and AGACTCCCCTCAGTAAAGTGAC. GPX1:
 566 CCAGTCGGTGTATGCCTTCT and GATGTCAGGCTCGATGTCAA. CAT:
 567 CGCAGAAAGCTGATGTCCTG and AAAGGCCCTGCTCCTTTAG. SELENOP:
 568 CGTTGGAAGTGGTTGTGAC and CCATTGGAGTTTAGCATTGG. SOD2:
 569 AAGGGAGATGTTACAGCCCAGATA and TCCAGAAAATGCTATGATTGATATGAC.
 570 TXNRD1: GCCCTGCAAGACTCTCGAAATTA and
 571 GCCCATAAGCATTCTCATAGACGA. ACSL4: TCTGCTTCTGCTGCCCAATT and
 572 CGCCTTCTTGCCAGTCTTTT. ACTB: GACCTGACTGACTACCTCATGAAGAT and
 573 GTCACACTTCATGATGGAGTTGAAGG.

574

575 **Chromatin immunoprecipitation (ChIP)-qPCR.** About 1×10^7 cells were washed twice
 576 with ice-cold PBS and then crosslinked with 1% paraformaldehyde and quenched with
 577 glycine. Cells were then lysed with ChIP lysis buffer (140 mM NaCl, 1 mM EDTA, 1%
 578 Triton-X 100, 0.1% Na-deoxycholate, 50 mM Hepes/KOH, pH=7.5) and sonicated at high
 579 power (30 sec ON followed by 30 sec OFF, 2×15 min). Next, the lysates were incubated with
 580 either YAP antibody for immunoprecipitation, or rabbit IgG as the negative control at 4 °C
 581 overnight. Then, cell lysates were incubated with protein G plus protein A agarose beads (as
 582 above) for 2 hours at 4 °C to pull down the antibody-chromatin complexes, which were then
 583 washed using high salt buffer, Tris/LiCl buffer, and Tris/EDTA buffer. After washing, the
 584 crosslinks were reversed by the incubation in water at 65 °C for 4 hours and digested with
 585 proteinase K at 55 °C for 1 hour. Next, the DNA fragments were purified using a MinElute
 586 PCR Purification Kit (Qiagen, Cat # 28006) and quantified by qPCR. The sequences of
 587 primers were listed as follows (5' to 3'). TBE1: TGTTAGTCTCCCGCTTCCATT and
 588 AAATCTCATCTTGAGTGCTGGTC. TBE2: GCACCCAAATAATCCAACCTCAAAG
 589 and GTGCTCAGGTGTTGCACAA. TBE3: TTAGCCACCTTGCCTTTTCC and

ATGAAAGTGGGGAGGAACTACAA. TBE4: TCTCCCTCCGGTCTCTTTCG and
TGATGGCCCTGAGGCAGTT.

Cell viability assays. For MTT assays, cells were seeded in 96-well plates at a density of 1000 cells per well and cultured at 37 °C overnight. Next, at indicated time points (0, 24, 48, 72 hours), cells were treated with MTT reagent at 37 °C for 3 hours, resolved in DMSO and the absorbance detected at 570 nm using a spectrophotometer. The absorbance at 24, 48 and 72 hours were normalized to that at 0 hour. For colony formation assays, cells were seeded in 24-well plates at a density of 500 cells per well and cultured at 37 °C for 2 weeks. Next, cells were washed twice with PBS and fixed with 4% paraformaldehyde for 1 hour at room temperature. Then, cells were stained using 0.2% crystal violet for 1 hour at room temperature, followed by 3 washes with PBS. Cells were then photographed and the colonies were counted.

PKA protein kinase activity assay. Wild type, C200S mutated, C344S mutated or double mutated HA-tagged PRKACB were overexpressed in cells and immunoprecipitated by anti-HA tag antibody together with agarose beads as described above. Then, the beads were eluted with an equal volume of glycine buffer (0.2 M glycine, pH=2.6) for 10 minutes and neutralized using an equal volume of pH 8.0 Tris buffer. Next, purified proteins were subjected to an activity assay with Kemptide (Aladdin, Cat # K118853) as the exogenous substrate at 30 °C for 10 minutes. The reaction mixture consists of 10 µg Kemptide, 1 µg PRKACB, 10 mM MgCl₂, 20 µM ATP, 50 mM Tris-HCl pH=7.4 in a total volume of 100 µL. Enzymatic activity was determined by the consumption of ATP, which was measured using an ATP assay kit (Beyotime, Cat # S0026) according to the manufacturer's instructions.

614

615 ***In vitro* ubiquitination assay.** His-tagged ubiquitin, His-tagged E1 (UBA1), His-tagged E2
616 (UBE2D2), Flag-tagged E3 (RNF25-WT or RNF25-S450D), Myc-tagged substrate (E-
617 cadherin) were overexpressed in cells and purified as above. Then, the assay was performed
618 using a ubiquitination buffer (10 μ M ubiquitin, 10 nM E1, 100 nM E2, 100 nM E3, 500 nM
619 substrate, 5 mM ATP, 5 mM MgCl₂, 1 mM DTT, 50 mM Tris-HCl, pH=7.4) in a total
620 volume of 50 μ L. The reaction proceeded at 30 °C for 2 hours, followed by addition of SDS-
621 PAGE loading buffer and boiling for 10 min. The samples were then subjected to Western
622 blot for the measurement of ubiquitination.

623

624 ***In vitro* phosphorylation assay.** HA-tagged kinase (PRKACB) and Flag-tagged substrate
625 (RNF25-WT or RNF25-S450A) were overexpressed in cells and purified as above. Then, the
626 assay was performed using a kinase buffer (0.1 μ M kinase, 1 μ M substrate, 150 mM NaCl, 10
627 mM MgCl₂, 1 mM DTT, 0.1 mM ATP, 50 mM Hepes/KOH, pH=7.5) in a total volume of 50
628 μ L. The reaction was proceeded at 30 °C for 20 minutes, followed by addition of SDS-PAGE
629 loading buffer and boiling for 10 min. The samples were subjected to Western blot for the
630 measurement of phosphorylation.

631

632 **Luciferase reporter assay.** To detect the transcriptional activity of β -catenin, a TOP/FOP
633 flash assay was performed as previously described [47]. Briefly, cells were co-transfected
634 with plasmids of Top-flash (*Photinus pyralis*) plus pRL-CMV (*Renilla reniformis*). The
635 plasmids of Fop-flash plus pRL-CMV were used as negative control. After 36 hours of
636 transfection, cells were harvested and the luciferase activity was determined by a dual-
637 luciferase reporter assay system (Promega, Cat # E1910) according to the manufacturer's

instructions. To detect the transcription activity of YAP, a similar approach to that previously reported was used [12]. Briefly, cells were co-transfected with plasmids of TEAD-luc (*Photinus pyralis*) plus TK-ctr (*Renilla reniformis*), and the luciferase activity was evaluated as above. To detect the activity of RNF25 promoter, the RNF25 promoter region (-2000 to TSS) was obtained from the genome DNA of Huh7 cells using the primers (5'-TGGAGAGAGGTCGGAGGTG-3' and 5'-GCCCTTAGGGAAGTCGGAG-3'). Then, the DNA fragment was either kept as wild-type, or a mutation generated at the TBE1 or TBE2 sites. Next, the DNA fragment was cloned into firefly-luc plasmid, which was co-transfected with TK-ctr plasmid followed by measurement of the luciferase activity as above.

Statistical analysis. Statistical analysis was performed using GraphPad Prism software (version 8.3.0). Data are presented as mean $\pm$ SD obtained from at least three independent assays unless otherwise noted. Data in different groups were analyzed by two-tailed Student's t-test or one-way ANOVA. Correlation was evaluated by Pearson correlation test. Kaplan-Meier analysis for patient survival was determined by log-rank (Mantel-Cox) test. Other statistical methods are shown in figure legends. *P* values < 0.05 were considered statistically significant.

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Abbreviations

ROS, Reactive oxygen species; ECAD, E-cadherin; HCC, hepatocellular carcinoma; PKA, protein kinase A; PRKACB, protein kinase A subunit β ; TCGA, The Cancer Genome Atlas; CCLE, Cancer Cell Line Encyclopedia; PLC, PLC/PRF/5; RNF25, RING finger protein 25; CTC, circulating tumor cell; NAC, N-acetyl cysteine; TGF β , transforming growth factor- β ; IL-6, interleukin 6; EMT, epithelial-to-mesenchymal transition; CBLL1, c-Cbl-like protein 1; TF, transcription factor; TBE, TEAD-binding element; VP, verteporfin.

Conflict of interest

None of the authors have any potential conflict of interests to disclose.

Figure legends

Figure 1. Oxidative stress downregulates E-cadherin at the protein level in HCC. (A)

TCGA dataset showing the hypoxia score of HCC patients with different neoplasm histologic grades. (B-D) Kaplan-Meier analysis showing the overall survival of HCC patients with low or high hypoxia score in (A). (E-F) PLC/PRF/5 and Huh7 cells were treated with 10 ng/ml TGF β 1 or 0.5 μ M NAC for 72 hours followed by ROS measurement from fluorescence. Cell morphology was captured by microscopy. Scale bar, 50 μ m. (G-H) Western blot and RT-qPCR assays showing the protein and mRNA level of indicated genes in PLC/PRF/5 cells treated as (J). (I-J) The correlation between the protein or mRNA level of ECAD and the hypoxia score in HCC patients were determined according to the TCGA dataset. Data are mean $\pm$ SD from at least 3 independent repeats. *P < 0.05, **P < 0.01; ***P < 0.001.

Figure 2. RNF25 is an E3 ligase for E-cadherin and associated with HCC metastasis. (A)

Bioinformatic screen of putative E3 ligases for ECAD using the UbiBrowser tool (ubibrowser.ncpsb.org). (B) Top 20 hits in (A) were analyzed for the correlation between their mRNA levels and ECAD protein level according to the CCLE liver dataset. The most significant gene RNF25 is shown in red. (C-D) The correlation between RNF25 mRNA level and ECAD protein level was detected using the CCLE and TCGA datasets. (E) co-IP assays showing the interaction between ECAD and RNF25 using RNF25-overexpressed PLC stable

cells treated with or without 100 μ M H₂O₂ for 1 hour. (F) co-IP assays showing the ubiquitination of ECAD in RNF25-overexpressed and vector PLC stable cells. (G) Western blot showing the expression of indicated proteins in RNF25-overexpressed cells. (H) Ubiquitination of immunoprecipitated ECAD by purified RNF25 in vitro. (I) Immunohistochemistry (IHC) assays showing the protein level of RNF25 in HCC patients with or without metastasis. (J) HCC patients in (I) were grouped according to their tumor grade followed by evaluation of RNF25 expression. (K) HCC patients in (I) were grouped according to their RNF25 expression level followed by analysis of their overall survival. (L) The correlation of protein level of RNF25 and ECAD was determined by IHC assays using the cohort in (I). Data are mean $\pm$ SD from at least 3 independent repeats. *P < 0.05, **P < 0.01; ***P < 0.001.

Figure 3. RNF25-induced E-cadherin loss inactivates Hippo signaling to promote cell invasion. (A) HCC tissue immunofluorescence (IF) assays showing the expression of RNF25 (green) and YAP (red) at invasive front and tumor center. Scale bars: 200 μ m (zoom out) or 50 μ m (zoom in). (B-C) Luciferase reporter assays showing the activity of TEAD promoter in RNF25-overexpressed or knockdown cells. (D-E) The mRNA levels of YAP target genes were determined by RT-qPCR. (F) Western blot showing the expression and phosphorylation of several key components in Hippo signaling. (G) Schematic diagram describing possible mechanisms underlying RNF25-mediated ECAD loss and consequent Hippo inactivation. (H-I) Western blot showing the expression of indicated proteins in RNF25-overexpressed and knockdown cells. (J-K) Transwell assays showing the migration and invasion of HCC cells with or without the treatment of YAP inhibitor verteporfin (VP, 1 μ M) or overexpression of Flag-tagged YAP. Scale bars: 100 μ m. Data are mean $\pm$ SD from at least 3 independent repeats. *P < 0.05, **P < 0.01; ***P < 0.001.

830

831 **Figure 4. Redox modification of protein kinase A regulates the phosphorylation and**
832 **function of RNF25.** (A) Functional score of phosphosites in RNF25 (grey) and human
833 phosphoproteome (blue) according to a human phosphoproteome dataset. Mean, average
834 score for all human phosphosites. Regulatory, phosphosites with known regulatory functions.
835 Disease, phosphosites related with human diseases. (B) Immunoprecipitation assays showing
836 the phosphorylation of wild-type and S450A mutated RNF25 in PLC/PRF/5 stable cells. (C)
837 Western blot showing the ECAD protein levels in indicated stable cells. (D)
838 Immunoprecipitation assays showing the phosphorylation of wild-type or S450A mutated
839 RNF25 in PLC cells with or without transient overexpression of PRKACB. (E-F) In vitro
840 kinase assays showing the phosphorylation of wild-type or S450A mutated RNF25 with or
841 without 1 μ M PKA inhibitor H-89. (G) In vitro ubiquitination assay showing the
842 ubiquitination of ECAD by wild-type or S450D mutated RNF25. (H) PKA activity was
843 determined with or without the treatment of H₂O₂ (100 μ M), NAC (1 mM) or H-89 (1 μ M)
844 for 1 hour. (B) Immunoprecipitation assays showing the phosphorylation of wild-type or
845 S450A mutated RNF25 with or without the treatment of H₂O₂ (100 μ M) or H-89 (10 μ M) for
846 1 hour. (I) Non-reduced SDS-PAGE showing the band shift due to formation of intracellular
847 disulfide bond in PRKACB using HEK293T cells treated with or without H₂O₂ (100 μ M for
848 1 hour), TGF β 1 (10 ng/ml for 3 days) or NAC (1 mM for 1 hour in left 2 panels and 0.5 mM
849 for 3 days in right 2 panels). Ox., oxidized form. Re., reduced form. (J) The band shift was
850 detected using HEK293T cells expressing wild-type or cysteine mutated PRKACB. The cells
851 were treated with 100 μ M H₂O₂ for 1 hour before harvested. (K) Activity of wild-type or
852 cysteine mutated PRKACB was measured with or without the treatment of 100 μ M H₂O₂ for
853 1 hour. (L) The phosphorylation of RNF25 by wild-type or cysteine mutated PRKACB was

determined with immunoprecipitation assays using HEK293T cells. Data are mean $\pm$ SD from at least 3 independent repeats. *P < 0.05, **P < 0.01; ***P < 0.001.

Figure 5. YAP triggers antioxidant response to protect detached HCC cells from ferroptosis. (A-B) Cells were pre-treated with or without 1 μ M VP or transfected with Flag-YAP plasmid for 24 hours, followed by seeded in PolyHEMA-coated plate for 4 hours, then stained with trypan blue to determine cell death. (C-D) Cells were pre-treated with indicated cell death inhibitors (5 mM 3-methyladenine, 20 μ M z-VAD-FMK, 4 μ M ferrostatin-1 and 10 μ M necrostatin-1) for 1 hour, followed by seeding into PolyHEMA-coated plates with medium containing above inhibitors for 4 hours, followed by staining with trypan blue to determine cell death. (E-F) Lipid ROS levels in indicated cells were determined using BODIPY 581/591 C11 reagent fluorescence. (G-H) TCGA dataset showing the correlation between the mRNA level of YAP and ACSL4 in colorectal and liver cancer patients. (I-J) RT-qPCR assays showing the mRNA levels of indicated genes in detached PLC-RNF25 and Huh7-shRNF25 cells with or without the pre-treatment of VP (1 μ M) or overexpression of YAP for 24 hours. Data are mean $\pm$ SD from at least 3 independent repeats. *P < 0.05, **P < 0.01; ***P < 0.001.

Figure 6. RNF25 promotes HCC metastasis both *in vitro* and *in vivo*. (A-B) Morphology of PLC/PRF/5 stable cells expressing Flag-tagged RNF25 and Huh7 stable cells with knockdown of endogenous RNF25. Scale bars: 100 μ m (zoom out) or 50 μ m (zoom in). (C-D) Wound healing assays showing cell migration in 24 hours. (E-F) Transwell assays showing the migration and invasion of indicated cells. Scale bars: 200 μ m. (G-H) Cells were injected into BALB/c nude mice via the tail vein to establish a mouse metastasis model. Scale bars: 40

878 μm (zoom out) or 10 μm (zoom in). (I) Working model of ROS-mediated HCC metastasis
879 regulated by PKA-activated RNF25/ECAD/YAP circuit. In response to ROS, PRKACB
880 forms a disulfide bond between Cys200 and Cys344, resulting in an increase in PKA kinase
881 activity. Then, PKA phosphorylates RNF25 at Ser450, leading to the ubiquitination and
882 subsequent protein degradation of ECAD. This effect not only promotes EMT, but also
883 activates YAP to protect detached cells from ferroptosis, thereby facilitating metastasis.
884 Moreover, RNF25 can be transcribed by YAP, forming a positive feedback loop during
885 metastatic spread of HCC cells. Data are mean $\pm$ SD from at least 3 independent repeats. *P
886 < 0.05 , **P < 0.01 ; ***P < 0.001 .

Figure 1. Oxidative stress downregulates E-cadherin at the protein level in hepatocellular carcinoma

Figure 2. RNF25 is an E3 ligase for E-cadherin and associated with HCC metastasis

Figure 3. RNF25-induced E-cadherin loss inactivates Hippo signaling to promote cell invasion

Figure 4. Redox modification of protein kinase A regulates the phosphorylation and function of RNF25

Figure 5. YAP triggers antioxidant response to protect detached HCC cells from ferroptosis

Figure 6. RNF25 promotes HCC metastasis both *in vitro* and *in vivo*

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