Title

Hydrogen Peroxide regulates Cell Adhesion through the Redox Sensor RPSA

Authors

Filipe Vilas-Boas^{1δ}, Ana Bagulho^{1δ}, Rita Tenente^{1,3}, Vitor H. Teixeira¹, Gabriel Martins^{2,4},
Gonçalo da Costa¹, Ana Jerónimo¹, Carlos Cordeiro¹, Miguel Machuqueiro¹, Carla Real^{1*}

^δ These authors contributed equally to this work

¹ Centro de Química e Bioquímica, Faculdade de Ciências, Universidade de Lisboa, 1749-016 Lisboa, Portugal

² Centro de Biologia Ambiental, Faculdade de Ciências, Universidade de Lisboa, 1749-016 Lisboa, Portugal

³ Current address: Instituto de Investigação Científica Tropical, 1349-007 Lisboa, Portugal

⁴ Current address: Instituto Gulbenkian de Ciência, 2780-156 Oeiras, Portugal

* Corresponding author: e-mail address: csafonso@fc.ul.pt

Email addresses

FVB: favilas-boas@fc.ul.pt; AB: anabagulho@gmail.com; RT: rita_tenente@hotmail.com;
VHT: vhteixeira@fc.ul.pt; GM: gaby@igc.gulbenkian.pt; GC: gmcosta@fc.ul.pt; AJ:

21 ajeronimosousa@gmail.com; CC: cacordeiro@fc.ul.pt; MM: mamachuqueiro@fc.ul.pt; CR:
22 csafonso@fc.ul.pt.

23

24 **Running title**

25 H₂O₂-mediated oxidation of RPSA in cell adhesion

26

27 **Corresponding author**

28 Dr. Carla Real Afonso; Centro de Química e Bioquímica, Faculdade de Ciências,
29 Universidade de Lisboa, 1749-016 Lisboa, Portugal; Email address: csafonso@fc.ul.pt;
30 Telephone: +351 217500926

31

32 **Abbreviations¹**

33

¹37LRP – 37 kDa laminin receptor precursor; 67LR – 67 kDa Laminin Receptor; CCV – Common cardinal vein; CTCF – Corrected total cell fluorescence; ECM – Extracellular matrix; EGFP – Enhanced green fluorescent protein; FAK – Focal adhesion kinase; Hpf – Hours post fertilization; Hpi –Hours post injection; HUVEC – Human umbilical vein endothelial cells; IAM – Iodoacetamide; IAM-BODIPY – Bodipy-conjugated iodoacetamide; IRES – Internal ribosome entry site; ISV – Intersomitic vessels; M1 – Manders’ overlap coefficient M1; Mut – RPSAmut; NAC – N-acetylcysteine; NEM – N-ethylmaleimide; NLS – Nuclear localization signal; pFAK – Focal adhesion kinase phosphorylated at Tyr397; PME – Particle-Mesh Ewald; RPSA – Ribosomal Protein SA; RPSAmut – RPSA variant in which both cysteine residues were replaced by serine residues (C148S and C163S substitutions); RPSAwt – Wild-type RPSA; SEM – Standard error of mean; Wt – RPSAwt.

34 **Abstract**

35

36 **Background**

37 To become metastatic, a tumor cell must acquire new adhesion properties that allow
38 migration into the surrounding connective tissue, transmigration across endothelial cells to
39 reach the blood stream and, at the site of metastasis, adhesion to endothelial cells and
40 transmigration to colonize a new tissue. Hydrogen peroxide (H₂O₂) is a redox signaling
41 molecule produced in tumor cell microenvironment with high relevance for tumor
42 development. However, the molecular mechanisms regulated by H₂O₂ in tumor cells are still
43 poorly known. The identification of H₂O₂-target proteins in tumor cells and the understanding
44 of their role in tumor cell adhesion are essential for the development of novel redox-based
45 therapies for cancer.

46 **Results**

47 In this paper, we identified Ribosomal Protein SA (RPSA) as a target of H₂O₂ and showed
48 that RPSA in the oxidized state accumulates in clusters that contain specific adhesion
49 molecules. Furthermore, we showed that RPSA oxidation improves cell adhesion efficiency
50 to laminin *in vitro* and promotes cell extravasation *in vivo*.

51 **Conclusions**

52 Our results unravel a new mechanism for H₂O₂-dependent modulation of cell adhesion
53 properties and identify RPSA as the H₂O₂ sensor in this process. This work indicates that
54 high levels of RPSA expression might confer a selective advantage to tumor cells in an
55 oxidative environment.

56

57 **Keywords**

58 RPSA, redox signaling, hydrogen peroxide, cell adhesion, extravasation, integrin signaling

59

60

61 **Introduction**

62

63 Metastasis formation is considered a critical step in cancer outcome, since the colonization of
64 organs by tumor cells is the main cause of death among cancer patients. In order to form a
65 metastasis, tumor cells must overcome multiple challenges, including detachment from the
66 primary tumor and loss of polarity, entry into blood or lymphatic circulation (intravasation),
67 survival without attachment to the extracellular matrix (ECM), exit of circulation and
68 invasion of a new organ (extravasation) and the formation of a secondary tumor [1]. In all
69 these steps, the ability of tumor cells to regulate adhesion to different extracellular
70 components is critical for tumor progression [2].

71 H_2O_2 is an important regulator of tumor progression and metastasis [3,4]. This reactive
72 oxygen species, which was initially viewed as a damaging agent responsible for cell toxicity,
73 is now considered an important cell signaling regulator in several physiological and
74 pathological conditions [5]. H_2O_2 signaling increases tumor cell proliferation and motility,
75 giving these cells a more aggressive phenotype [6–8]. Recently, production of H_2O_2 by
76 endothelial cells at the metastatic sites was also proven to be important for tumor cell
77 adhesion during extravasation [9]. H_2O_2 can alter protein structure and function mainly by
78 reacting with susceptible sulfhydryl groups of cysteine residues (SH). When redox-sensitive
79 cysteine residues are critical for protein function, H_2O_2 -dependent oxidation can lead to
80 dramatic alterations in signaling pathways [10,11].

81 Although the relevance of H_2O_2 for cancer progression has been shown, very few thiol-
82 regulated proteins have been identified as H_2O_2 targets and the consequences of protein
83 oxidation by H_2O_2 *in vivo* are still poorly understood [12].

84 In this paper, we identified RPSA as a H₂O₂ target. RPSA (or p40) is also known as 37 kDa
85 laminin receptor precursor (37LRP), generally considered to be a precursor of the 67 kDa
86 Laminin Receptor (67LR), though the precise identity of 67LR has not been determined [13].
87 RPSA is highly expressed in tumor cells and correlates directly with metastatic potential,
88 having a role in cell adhesion as one of its extraribosomal functions [13,14]. Here, we show
89 that RPSA oxidation leads to its accumulation in clusters at the cell membrane level and that
90 these clusters contain specific integrins. Moreover, oxidized RPSA also improves cell
91 adhesion efficiency to laminin *in vitro* and promotes cell extravasation *in vivo*. Our results
92 suggest that H₂O₂, through the modulation of the oxidation state of RPSA, is able to increase
93 the metastatic potential of tumor cells.

94

95

96 **Results**

97

98 **RPSA is a H₂O₂ protein target**

99 In order to identify proteins which are H₂O₂ targets, we treated non-tumor primary cells
100 (HUVEC) for 15 minutes with H₂O₂ delivered as a steady state (using a sublethal
101 concentration of 8 μ M, see **Supplementary Figure 1**). Protein extracts from control and
102 H₂O₂-treated cells were then incubated with bodipy-conjugated iodoacetamide (IAM-
103 BODIPY) that reacts with sulfhydryl (SH) groups of cysteine residues and fluorescently
104 labels reduced proteins (**Figure 1A**). After 2D electrophoresis, the obtained pattern of spots
105 was examined. The protein spots in H₂O₂-treated cells that presented decreased fluorescence
106 compared with the ones from control cells (oxidized proteins containing disulfides (-S-S-)
107 that have not reacted with IAM-BODIPY) were selected and the corresponding proteins were
108 identified by mass spectrometry. In these experiments, RPSA was identified as a H₂O₂ target,
109 which for H₂O₂-treated cells displayed an average decrease in spot fluorescence intensity of
110 approximately 38% when compared with control cells (**Figure 1B**), for the same protein
111 levels. Thus, these results indicate that RPSA was rapidly oxidized by sublethal
112 concentrations of H₂O₂.

113

114 **RPSA oxidation results in the formation of an intramolecular disulphide**

115 Human RPSA contains two cysteine residues at positions 148 and 163 of its amino acid
116 sequence. Cys163 is conserved among all RPSA amino acid sequences of the eukaryotic
117 organisms analyzed, whereas Cys148 was only found in human, mouse, chick, frog and
118 zebrafish (**Supplementary Figure 2**). To test whether RPSA is also a H₂O₂ target in tumor

cells, HeLa cells were treated with a steady state-delivered sublethal concentration of H₂O₂ (25 μM) [15] and analyzed the oxidation state of the two cysteine residues by mass spectrometry. Reduced cysteine residues were tagged with N-ethylmaleimide (NEM) and oxidized cysteine residues were tagged with iodoacetamide (IAM) upon reduction by DTT (**Figure 1C**), and subsequently identified in the peptides resulting from trypsin hydrolysis. The only oxidized forms of sulfur which are detected by this method are disulphides and sulfenic acids since DTT is not able to reduce sulfinic or sulfenic acids. The 46% sequence coverage observed encompasses the amino acid sequences where the two cysteine residues are present. In the mass spectra from cells treated with H₂O₂, we observed the peptides containing Cys148 and Cys163 conjugated with IAM (**Figure 1D**). These results show that both cysteine residues were oxidized in a reversible manner. In non-treated samples, we found exclusively peptides containing Cys148 and Cys163 conjugated with NEM.

In organisms where the two cysteine residues are present, oxidation of RPSA might result in the formation of a disulfide, since sulfenic acids are highly unstable [16] and the sulfur atoms present in the sulfhydryl groups are close enough in the protein structure to allow a bond formation (7.3 Å for human RPSA, from crystal structure [PDB:3BCH] [17]). Alternatively, oxidation could occur via glutathione molecules. However, the average solvent accessible surface area (SASA) of both cysteine residues, calculated from the human RPSA crystal structure, shows that both Cys148 and Cys163 present values similar to the one of a buried residue (13 Å² for Cys148 and 23.3 Å² for Cys163 (**Supplementary Table 1**). Since only very few water molecules access the cysteine residues, this supports the idea that oxidation of these residues with glutathione molecules is highly unlikely due to space constraints. Therefore, the oxidation of both cysteine residues strongly indicates the formation of an intramolecular disulphide.

To determine whether the formation of a disulfide between Cys148 and Cys163 could induce alterations in the secondary structure of RPSA and potentially modulate its function, we performed molecular dynamics simulations using the crystal structure of human RPSA. Our simulations showed that the putative secondary structure of the oxidized molecule was not significantly different from the reduced one, which indicated that the overall structure was maintained (**Supplementary Table 2**). Although the main chain conformational alterations induced upon RPSA oxidation were minimal, the average solvent accessible surface area of particular amino acid residues suffered important modifications (**Supplementary Table 1**). Residues Phe32, Arg155, Pro194 and Val197 showed decreased accessibility compared to the ones of the reduced molecule (indicating an internalization), while Asn165, Ile189, Glu196 and Tyr202 showed an increased accessible surface (indicating an exposure of the residues to the solvent) (**Figure 1E**). Asn165 is present in the Peptide G, the 161-180 region of RPSA that was reported to interact directly with laminin [18] and Phe32 was also shown to be essential for laminin binding [19]. In addition, Pro194, Glu196 and Tyr202 are present in the reported RPSA ribosomal binding region [20]. These significant changes in the protein surface might change the interaction processes between RPSA and its preferred partners and, consequently, its function.

Altogether, these results indicate that the two conserved cysteine residues of human RPSA most likely form an intramolecular disulfide without compromising the structural stability of the protein, but inducing conformational changes that might account for the regulation of protein function.

RPSA oxidation increases cell adhesion to laminin

166 RPSA expression has been implicated in cell adhesion, either by direct interaction with
167 laminin [21] or by the regulation of integrin function [22]. Thus, we have tested whether
168 RPSA oxidation alters the properties of cell adhesion to laminin. To analyze RPSA function,
169 we performed overexpression studies which allow the functional study of particular amino
170 acid residues and is also more physiologically relevant in a tumor cell context, since RPSA is
171 highly expressed in a large variety of cancers with poor prognosis [13,14]. In addition, the
172 knockdown of RPSA has been shown to induce cell death in several cell types [23–26].
173 Therefore, to analyze whether RPSA oxidation alters the properties of cell adhesion to
174 laminin, we used HeLa cells to overexpress wild-type RPSA (RPSAwt, in pCIC-RPSAwt) or
175 a RPSA variant in which both cysteine residues were replaced by serine residues to prevent
176 protein oxidation (RPSAmut, with C148S and C163S substitutions, in pCIC-RPSAmut).
177 Cells expressing RPSAwt or RPSAmut had the same viability as cells transfected only with
178 the empty vector pCIC (**Supplementary Figure 3**). For the cell adhesion assay, mock,
179 RPSAwt- or RPSAmut-overexpressing cells were allowed to adhere for 40 minutes to
180 laminin-coated, fibronectin-coated or uncoated plates in the presence or absence of H₂O₂. In
181 mock cells transfected only with the empty vector pCIC and in the absence of H₂O₂, different
182 adhesion efficiencies to the different ECMs were observed. Specifically, HeLa cells adhered
183 more to ECM-coated plates, and among these, more to fibronectin than to laminin (**Figure**
184 **2A, C, E**). In the presence of H₂O₂, adhesion to fibronectin-coated or uncoated plates was not
185 affected. In clear contrast, adhesion to laminin decreased by 51%, reaching adhesion levels
186 similar to those obtained with uncoated plates (**Figure 2B**), suggesting that H₂O₂ is able to
187 specifically inhibit HeLa cell adhesion to laminin. However, in cells transfected with pCIC-
188 RPSAwt, the inhibition of cell adhesion to laminin in the presence of H₂O₂ was decreased
189 (28%) (**Figure 2B**). This was not due to decreased H₂O₂ availability caused by
190 H₂O₂ consumption by the cysteine residues present in RPSAwt, since the consumption rates

were similar for cells overexpressing RPSAwt and RPSAmut (**Supplementary Figure 4**). In contrast to the observed decreased inhibition of cell adhesion in the presence of H₂O₂ in RPSAwt-overexpressing cells, RPSAmut-overexpressing cells presented laminin adhesion properties similar to mock-transfected cells (49% and 51%, respectively) (**Figure 2B**), showing that oxidation of cysteine residues is essential in this process. In addition, both in the presence of fibronectin and in the absence of ECM, H₂O₂ had no influence in cell adhesion of either mock, RPSAwt- or RPSAmut-expressing cells (**Figure 2D, F**).

Altogether, these results clearly show that RPSA overexpression and oxidation prevents the decrease in cell adhesion to laminin in the presence of H₂O₂, while not affecting adhesion to fibronectin or to an uncoated surface.

RPSA redox state regulates its subcellular localization

The observed increase in the efficiency of cell adhesion to laminin in the presence of H₂O₂ could be due to altered expression of RPSAwt at the plasma membrane level. To test this hypothesis, we analyzed RPSA expression by immunofluorescence in non-permeabilized cells transfected with pCIC-RPSAwt or pCIC-RPSAmut in control, oxidizing (treatment with H₂O₂) and reducing conditions (treatment with N-acetylcysteine (NAC)). Our results showed that protein levels remained similar between all conditions (**Supplementary Figure 5**).

However, RPSAwt accumulated in specific regions after H₂O₂ incubation, whereas RPSAmut-expressing cells maintained a more widespread expression pattern (**Figure 3**). In the presence of NAC, RPSAwt and RPSAmut distributed similarly to control (**Figure 3**).

These results show that RPSA oxidized form accumulates in specific locations at the cell membrane level forming clusters. In addition, the oxidation state of RPSA is also important to regulate its presence in other subcellular localizations (see Ref [27]).

Altogether, our results show that the oxidation state of RPSA is important for its subcellular localization and that RPSAwt in the oxidized state associates in clusters at the cell membrane level.

RPSA oxidation promotes colocalization with the β 1 integrin subunit

The formation of RPSA clusters at the cell membrane level suggests the formation of adhesion complexes, which may account for the observed differences in cell adhesion in the presence of H_2O_2 . Since it has been proposed that the role of RPSA in cell adhesion to the ECM involves its cooperation with integrins [28–30], we investigated whether RPSA clusters in specific cell locations also contained integrins. For this, the expression of RPSA together with β 1 or β 5 integrin subunits was analyzed by performing co-immunofluorescence in cells expressing RPSAwt or RPSAmut, and incubated with or without H_2O_2 (**Supplementary Figure 6 and Figure 4A**). β 1 integrin subunit binds to various ECM molecules including laminin, while β 5 integrin subunit does not bind to laminin [31]. Our immunofluorescence data revealed that RPSA clusters overlapped completely with the expression domains of β 1 or β 5 integrin subunits in a subset of cells. In other cells the expression domains of RPSA only partially overlapped or had no overlap with integrin subunit expression domains (**Supplementary Figure 6 and Figure 4A**). β 1 integrin subunit clusters totally overlapped with RPSAwt clusters in 28% of cells. In the presence of H_2O_2 , the number of cells that presented total overlap between RPSAwt and β 1 integrin subunit subcellular distribution at the cell membrane level increased to 39% (**Figure 4B**). In clear contrast, RPSAmut expression only totally overlapped to that of β 1 integrin subunit in less than 22% of cells, both in the presence and absence of H_2O_2 (**Figure 4B**). Thus, these results show that the number of cells with totally overlapping RPSAwt and β 1 integrin subunit clusters

increases when RPSA is in its oxidized form. In clear contrast to what was observed for $\beta 1$, the $\beta 5$ integrin subunit was rarely present in either RPSAwt or RPSAmut clusters both in the presence or absence of H_2O_2 (between 4 and 19%) (**Figure 4B**). The analysis of M1 Manders' overlap coefficient in each cell confirmed that RPSAwt and $\beta 1$ integrin subunit colocalization (regions of RPSA expression that also contained integrin subunit expression) increased in the presence of H_2O_2 when comparing with the other conditions tested. As expected, no differences in colocalization were observed for RPSAmut in the presence of H_2O_2 . In contrast with these results, colocalization of RPSAwt or RPSAmut with $\beta 5$ integrin subunit did not change upon H_2O_2 addition (**Figure 4C**).

Taken together, our results clearly show that RPSA oxidation increases colocalization of RPSA and $\beta 1$ integrin subunit in RPSA clusters, but has no effect on RPSA and $\beta 5$ integrin association. These results indicate that RPSA oxidation increases the presence of particular integrins in the clusters, which may account for the observed differences in cell adhesion.

Focal adhesion kinase is phosphorylated in RPSA clusters

Integrin-mediated cell adhesion leads to integrin activation and subsequent phosphorylation of focal adhesion kinase (FAK) [32]. To determine whether integrin activation occurred in RPSA clusters, we performed co-immunofluorescence using specific antibodies for RPSA or FAK phosphorylated at Tyr397 (pFAK) in RPSAwt- or RPSAmut-expressing cells. Our results showed that RPSAwt and RPSAmut clusters contained pFAK (**Figure 5 and Supplementary Figures 7 and 8**). Interestingly, cells overexpressing high levels of RPSA, either wild-type or mutant forms, always showed high levels of pFAK compared with non-transfected cells in both control and oxidizing conditions (**Figure 5A and Supplementary Figure 8A**). Moreover, in these cells, the increased FAK phosphorylation was localized

mainly in RPSA clusters (**Figure 5B, C and Supplementary Figure 8B, C**). These results indicate that RPSA clusters present integrin activation and that increased RPSA expression in these clusters is able to increase the levels of phosphorylated FAK, independently of the oxidation state of RPSA.

RPSA oxidation increases cell extravasation *in vivo*

β 1 integrin subunit activation in tumor cells has been related to increased cell extravasation in several human cancers[33]. Since RPSA oxidation enhances β 1 integrin subunit colocalization in its clusters, we hypothesized that RPSA oxidation modulates cell extravasation. To test whether RPSA oxidation increased cell extravasation *in vivo*, RPSAwt- or RPSA^{mut}-overexpressing cells were injected into the circulation of 48 hours post fertilization (hpf) zebrafish embryos (**Figure 6A**), as previously described [34]. While, RPSA^{mut} is unable to be oxidized, RPSAwt can be oxidized by endogenous H₂O₂ produced by the HeLa cells or the zebrafish. For these experiments we used Tg(*fli*:EGFP) zebrafish embryos, which allow endothelial cell visualization by EGFP expression, and quantified mCherry-NLS positive cells inside and outside intersomitic vessels (ISV) 24 hours post injection (**Figure 6A-C**). We counted cells transfected with pCIC-RPSAwt and found that an average of 29.6% of cells extravasated from ISV (**Figure 6D**). In clear contrast, we found that an average of only 14.4% of cells transfected with pCIC-RPSA^{mut} extravasated from ISVs (**Figure 6D**). This significant difference in extravasation between cells transfected with pCIC-RPSAwt or pCIC-RPSA^{mut} clearly indicates that the redox state of RPSA is implicated in the extravasation of cells *in vivo*.

286 **Discussion**

287

288 Tumor cells present a higher level of H₂O₂ compared with normal cells, which is directly
289 related with tumor grade [35,36]. Besides tumor cells, other tumor microenvironment
290 components, such as immune and endothelial cells, produce H₂O₂. This particular
291 environment of primary tumors induces the oxidation of several proteins that function as
292 regulators, promoting tumor cell survival and aggressiveness. Although some proteins
293 containing H₂O₂-sensitive cysteine residues have been identified, the molecular players for
294 many redox-regulated cell functions are still unknown. In this study, we have identified
295 RPSA as a H₂O₂ target that, upon formation of an intramolecular disulphide, increases cell
296 adhesion to laminin *in vitro* and promotes cell extravasation *in vivo*.

297

298 **RPSA is a H₂O₂ sensor by forming an intramolecular disulphide**

299 RPSA has two conserved cysteine residues that are unlikely to contribute to the formation of
300 67LR [37] and that until now had no known function. We have now shown that although both
301 residues are slightly internalized in the crystal structure of RPSA, as shown by their low
302 average SASA (13 and 23.3 Å² for Cys148 and Cys163, respectively), a disulphide formation
303 might still occur *in vivo*. In agreement, formation of an intramolecular disulphide between
304 vicinal thiols of cysteine residues was predicted to occur with SASA values as low as 1.3
305 Å²[38]. Although the kinetics of this oxidation reaction is unknown, we have shown that
306 RPSA oxidation in endothelial cells occurred in the presence of as low as 8 μM concentration
307 of extracellular H₂O₂ in 15 minutes, in contrast to 1 mM of H₂O₂ in 10 minutes as previously
308 reported [39]. The determination of the kinetics of this oxidation reaction has to take into

account not only local steady state concentrations of H_2O_2 and signaling duration but also protein intrinsic properties, such as chemical environment that affects pKa values and nucleophilicities [11]. In addition, it is still to be determined whether the oxidation/reduction cycle of RPSA is mediated by other proteins such as peroxiredoxins and thioredoxins.

RPSA oxidation increases cell adhesion to laminin

We have found that H_2O_2 promoted RPSA clustering at the cell membrane level and also regulated its association to particular integrins within the clusters, most likely contributing to alterations in cell adhesion properties such as binding specificity. Indeed, RPSA overexpression and oxidation specifically increased the efficiency of cell adhesion to laminin while not affecting adhesion to fibronectin or to an uncoated surface. We have also shown that RPSA colocalizes with specific integrins and increases pFAK levels. Since RPSA was shown to directly interact with integrins [30] and FAK [40], it might promote their interaction in a tripartite functional complex. However, it is still to be determined how the presence of RPSA in clusters activates integrin signaling, either by promoting FAK phosphorylation or preventing pFAK dephosphorylation. Interestingly, RPSA^{mut} also colocalized with integrin subunits and promoted activation of integrin signaling within its clusters, showing that these are functional in the presence of reduced RPSA. This indicates that clusters of RPSA in either redox state are able to engage in integrin signaling activation, but oxidation of RPSA regulates the integrin content of its clusters that will most likely confer different binding specificities to various substrates. Therefore, the cellular environment might be responsible for alterations in cell adhesion specificity and signaling through the oxidation or reduction of RPSA, which would work as a switch to confer different adhesion properties to cells. These alterations in adhesion properties might regulate not only tumor cell motility, but also tumor

cell affinity for basal membranes of blood vessels, potentially increasing intravasation and extravasation to the blood stream. However, the molecular mechanisms by which RPSA oxidation regulates the different binding specificities to the ECM are still to be determined.

Role of RPSA oxidation in extravasation

During extravasation, H_2O_2 is produced locally in blood vessels of metastization sites by endothelial cells and inflammatory cells when stimulated by tumor cell-secreted factors [9], which may contribute to RPSA oxidation. $\beta 1$ integrin subunit has been described as a pro-metastatic molecule and its activation modulates tumor cell extravasation and secondary tumor formation [41]. In addition, “inside-out” activation of $\beta 1$ integrin subunit has been associated with cell extravasation [33]. Notably, we found that oxidation of RPSA induced cell extravasation *in vivo*, suggesting a crosstalk between RPSA and $\beta 1$ integrin subunit in the metastatic process. However, the mechanisms responsible for the observed increased extravasation are still to be determined. After extravasation, tumor cells encounter the pre-metastatic niche, which is a favorable microenvironment for tumor metastatic cell colonization in distant organs. This pre-metastatic niche is characterized by primary tumor-derived secreted factors, such as cytokines and growth factors that induce production of H_2O_2 by NADPH oxidases [42]. Lysil oxidase, an enzyme responsible for collagen matrix remodeling, is also present at the pre-metastatic niche and produces H_2O_2 . Interestingly, RPSA induces expression of lysil oxidase in tumor cells [43], which may potentiate tumor cell adhesion and extravasation through RPSA oxidation.

Conclusion

The role of hydrogen peroxide (H_2O_2) as a signaling molecule in the tumor development process is still poorly understood. In this paper we have identified RPSA as a molecular target of H_2O_2 and have shown that RPSA oxidation regulates its cell adhesion functions. In particular, RPSA oxidation modifies the integrin content in its clusters, improves cell adhesion efficiency to laminin *in vitro* and promotes cell extravasation *in vivo*. Elevated levels of H_2O_2 produced both in primary tumors and secondary metastatic sites, together with RPSA up-regulation, might confer a selective advantage to tumor cells in the metastatic process by contributing to alterations in cell adhesion properties such as binding specificity. The identification of redox-regulated proteins such as RPSA and the determination of their relevance in tumor development will be essential to design more specific and efficient redox-based therapies for cancer.

Materials and Methods

Cell lines

Human umbilical vein endothelial cells (HUVEC) were kindly provided by Sérgio Dias (Instituto de Medicina Molecular, Av. Prof. Egas Moniz, 1649-028, Lisbon, Portugal) and cultured in EBM2-supplemented medium (Lonza) with 5% (v/v) fetal bovine serum (FBS, Sigma-Aldrich), in 0.2% (w/v) gelatin-coated plates (gelatin was purchased from Sigma-Aldrich).

HeLa cervical carcinoma cells were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM (Hyclone, Thermo Scientific) supplemented with 10% (v/v) FBS, penicillin-streptomycin solution (Hyclone, Thermo Scientific) and 2 mM L-glutamine (Hyclone, Thermo Scientific) in uncoated or 10 µg/ml laminin-coated plates (laminin, L2020, was purchased from Sigma-Aldrich).

H₂O₂ steady state delivery method

The steady state delivery method allows the maintenance of a given H₂O₂ concentration throughout the experiment by adding the amount of glucose oxidase that counteracts H₂O₂ consumption by the cells. For this, the culture medium was replaced by RPMI 1640 (Hyclone, Thermo Scientific) supplemented with penicillin-streptomycin solution and 2 mM L-glutamine 1 hour before the assay. Then, the steady state titration method was performed as previously described [44]. The amount of glucose oxidase to be added was calculated after the determination of the consumption rates for each cell type used. H₂O₂ concentration was

verified during the assay by using catalase and measuring O₂ formation on an oxygen electrode (Hansatech).

Identification of proteins with cysteine oxidation

Reactive thiol proteome extraction and labeling with bodipy-conjugated iodoacetamide (IAM-BODIPY) was performed as previously described [45]. Immediately after the stationary state incubation period of HUVEC (8 μ M H₂O₂ during 15 min), the culture medium was removed, cells were washed twice with PBS and subsequently frozen in liquid nitrogen and stored at -80°C. Protein extraction and labeling were done simultaneously to prevent further oxidation during sample processing (see Supplementary Materials and Methods). Reactive thiol proteome was resolved by bidimensional electrophoresis. IEF was performed using Immobiline DryStrip gels pH 3-11 NL, 13 cm (GE Healthcare), and run at 18700 V-h (50 μ A) at 20°C. To achieve second dimension separation, the strips were loaded onto a 12% (w/v), 1 mm acrylamide gel and run at 25 mA at 10°C. Fluorescent images of 2D gels were acquired with a Typhoon 9210 (settings: 532 nm laser in conjunction with 526 SP emission filter, 600-700 PMT, 50 μ m pixel (GE Healthcare)). Detection of the global protein pattern was achieved through silver staining. For each set of 2D gels, we considered only the spots that displayed a difference equal or greater than 1.5 fold the normalized volume (3 independent experiments). Considering that an increase in a protein's oxidation level can be attributed to a decrease in the total amount of protein, we only considered proteins that presented concomitantly an increase in the oxidation levels and an increase, or maintenance, of their protein levels. Proteins from spots of interest were identified by mass spectrometry (see Supplementary Materials and Methods).

HeLa cells were transfected with pCIC-RPSAwt to increase the amount of RPSA in the cell and incubated with or without a sublethal concentration of 25 μM H_2O_2 for 30 min using the steady state delivery method [15]. Immediately after the stationary state incubation period, the culture medium was removed and cells were washed twice with PBS before lysis. Reduced cysteine residues were labeled with N-ethylmaleimide (NEM) and protein samples were analyzed by SDS-PAGE. Protein bands were extracted from the gel and the remaining oxidized cysteine residues were reduced with DTT and alkylated with IAM. Finally, proteins were identified by mass spectrometry (see Supplementary Materials and Methods).

Computational analysis

The starting structure for this work was the crystal structure of human RPSA [PDB:3BCH] [17]. Molecular dynamics simulations were performed with GROMACS version 4.0.7 [46,47] using the GROMOS 54A7 force field. The protein was solvated with 10044 SPC water molecules in a dodecahedron box, using periodic boundary conditions. The simulations were done using heat baths of 300 K with separate couplings for the solute and solvent with a relaxation time of 0.1 ps. A Berendsen pressure coupling was used to keep the pressure at 1 bar with a relaxation time of 1.0 ps and an isothermal compressibility of $4.5 \times 10^{-5} \text{ bar}^{-1}$. All bond lengths were constrained using the parallel version of the LINCS algorithm [47] while the SETTLE algorithm [48] was used for water. The Particle-Mesh Ewald (PME) electrostatics was applied using 1.2 Å for the maximum grid spacing for the fast Fourier transform, and a cutoff distance of 9 Å for both Lennard-Jones and Coulomb. The system total charge was +4 and 4 chloride ions were added to obtain neutrality. Five replicates of 200 ns each were done in both reduced and oxidized states.

Plasmid construction and cell transfection

To generate pCIC-RPSAwt, the coding region of human RPSA [Uniprot:P08865] was amplified by PCR from HUVEC cDNA and cloned in pCIC immediately upstream of the internal ribosome entry site (IRES). To generate pCIC-RPSAmut, the coding region of human RPSA was amplified by PCR, introducing two cysteine to serine residue substitutions at positions 148 and 163, and cloned in pCIC immediately upstream of IRES. See Supplementary Materials and Methods for more information on cloning procedures. Both pCIC-RPSAwt and pCIC-RPSAmut were screened for the correct orientation of the insert and verified by sequencing. pCIC, pCIC-RPSAwt or pCIC-RPSAmut were transfected into HeLa cervical carcinoma cells through lipofection with Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. The presence of IRES:mCherry-NLS [49] in all plasmids allowed the identification of transfected cells.

Cell adhesion assays

HeLa cells were transfected with pCIC, pCIC-RPSAwt or pCIC-RPSAmut 16 hours before the assay. Cells were detached non-enzymatically with 2 mM EDTA in PBS to avoid hydrolysis of proteins located at the cell surface. Each of these three populations of cells was split in two groups, to one of which 15 μ M H₂O₂ was added by bolus addition. All six groups of cells were then left to adhere on laminin-coated, fibronectin-coated or uncoated wells of a 96-well plate for 40 min at 37°C. After washing twice with PBS, adherent cells were fixed with 4% (w/v) paraformaldehyde (PFA) in PBS for 15 min at room temperature and stained with 0.5% (w/v) crystal violet for 10 minutes. After washing with PBS, 5 representative photos of each well were then taken under a microscope with a 10x objective and cells were manually counted with FiJi [50] (3 replicates per group, from 10-12 independent

experiments). In parallel, the six groups of cells were also left to adhere for 24 hours on uncoated wells, after which they were stained with crystal violet, washed with PBS, incubated with 2% (w/v) SDS for 20 minutes to solubilize, and absorbance was read at 550 nm. Cell counts of the 40 min incubation were then normalized by the corresponding adherent cells after 24 hours (total plated cells in Figure 2). The raw data from these experiments is supplied in a Supplementary File.

Immunofluorescence and imaging

HeLa cells were plated on laminin-coated glass coverslips and left to adhere for 24 hours, after which they were transfected with either pCIC-RPSA or pCIC-RPSA_{mut} and incubated overnight. Cells were then incubated with 30-50 μ M H₂O₂ by bolus addition or the steady state delivery method for 30 min, after which they were fixed in 2% or 4% (w/v) PFA in PBS for 15-20 min at room temperature (unless otherwise specified). Immunofluorescence was performed by standard procedures (see Supplementary Materials and Methods).

Immunofluorescence images were obtained on a Leica DMI 4000B inverted microscope using a spectral Leica TCS SPE confocal. For this, we took Z-stacks for each of at least 5 independent fields per coverslip with a Leica ACS APO 63x/1.15 water objective, a step number of 5-20 and step size of 0.5-1 μ m. Wavelength selection bandwidths were: 500-560 nm for 488 nm laser, 585-650 nm for 532 nm laser, 650-800 nm for 635 nm laser.

Quantification of RPSA protein levels and clustering

To measure RPSA sub-cellular distribution, HeLa cells were transfected with pCIC-RPSA_{wt} or pCIC-RPSA_{mut} and incubated with 30-50 μ M H₂O₂ by bolus addition, 10 mM NAC or

without any treatment, followed by immunofluorescence without permeabilization using α -RPSA (H141) antibody. Confocal images were obtained from random fields containing transfected cells (mCherry-NLS⁺) and fluorescence intensity and distribution in each transfected or non-transfected cell were analyzed with Fiji [50]. For this, the outlines of individual cells were manually delineated on bright-field single optical sections to obtain regions of interest. A small area was also drawn next to each cell to allow for background subtraction. Sum projections of Z-stacks of the green channel were then generated, and the areas, integrated densities, mean gray values and standard deviations were measured in these projections using the previously obtained regions of interest. Background signal from each cell was then subtracted to calculate the corrected total cell fluorescence (CTCF = Integrated Density - (Area of Selected cell x Mean Fluorescence of background readings). To calculate RPSA sub-cellular distribution, the fluorescence intensity standard deviation (StDev) of each cell was divided by its CTCF. We then obtained the average StDev/CTCF ratio in transfected or non-transfected cells for each of the 4 conditions analyzed. Finally, in order to correct for endogenous expression and immunofluorescence procedure variations, we normalized the average StDev/CTCF values obtained for the transfected cells with the values obtained for the non-transfected cells ($\text{StDev/CTCF}^{\text{transfected}} : \text{StDev/CTCF}^{\text{non-transfected}}$ ratio for each condition). We, therefore, obtained normalized average StDev/CTCF values for the 6 different conditions (2-6 independent experiments per condition, for a total of 40-250 cells per condition).

For quantification of protein levels of RPSA and pFAK in co-immunofluorescence experiments, CTCF was calculated as described above (2 independent experiments, for a total of 30-49 transfected cells and 6-12 non-transfected cells per condition).

Colocalization analysis

Colocalization analysis between RPSA and $\beta 1$ or $\beta 5$ integrin subunits in confocal Z-stacks was performed by determining the Manders' overlap coefficients, which reflect spatial correlations independent of intensity levels and can have values between 0 and 1, with 1 corresponding to complete spatial overlap of pixels of one channel over pixels of the other channel [51]. Because the Manders' overlap coefficients are highly sensitive to noise and background, individual cells were first cropped and deconvolved with the Huygens software v14.10 (Scientific Volume Imaging), and the remaining background removed by subtracting a median filtered image (20 pixel radius) with FiJi [50]. This strategy tends to produce a more precise co-localization analysis since it confines the co-localization analysis to spots with higher levels of staining. The M1 Manders' overlap coefficient (RPSA in channel 1, integrin subunit in channel 2) was then calculated with the Coloc 2 plugin of FiJi after applying Costes' automatic thresholding. Thus, the thresholded M1 coefficient corresponded to regions of RPSA expression (mostly clusters) that also contained integrin subunit expression.

Analysis of HeLa cell extravasation from zebrafish intersomitic vessels

HeLa cells were transfected with pCIC-RPSAwt or pCIC-RPSAmut DNA 16 hours before the assay. Cells were detached non-enzymatically with 2 mM EDTA in PBS to avoid hydrolysis of proteins located at the cell surface and injected into the circulation of 48 hours post-fertilization (hpf) Tg(*fli*:EGFP) zebrafish embryos at the common cardinal vein (CCV) as previously described [34]. Transfected cells were analyzed for their ability to extravasate from intersomitic blood vessels (ISVs) at 24 hours post injection (hpi). For this, we took Z-stacks for each zebrafish embryo on a Leica DMI 4000B inverted microscope using spectral Leica TCS SPE confocal with a 20x objective, a step number of 20 and step size of 1-5 μm .

533 Cell extravasation was analyzed on single optical sections by counting mCherry-NLS⁺ cells.
534 Number of cells analyzed per condition: pCIC-RPSAwt - total of 227 cells in the ISVs of 72
535 embryos, in 4 independent experiments; pCIC-RPSA^{mut} - total of 211 cells in the ISVs of 81
536 embryos, in 4 independent experiments. See Supplementary Materials and Methods for
537 details.

538

539 **Statistical analysis**

540 Statistical analysis was undertaken with SigmaStat (Systat Software Inc.) using a two-tailed
541 Student's *t* test or a Mann-Whitney *U* test for comparison between means of two different
542 groups. For comparison of more than two different groups, analysis was undertaken using a
543 one way analysis of variance (ANOVA), followed by a Tukey test. The results were
544 considered to be statistically significant when $p < 0.05$. *p* values were classified as * for
545 $p < 0.05$ and ** for $p < 0.01$.

546

547

548

549 **Authors' contributions**

550 AB, FVB and AJ performed cell culture experiments. AB performed protein analysis; FVB
551 performed molecular cloning, zebrafish experiments, and imaging experiments and analysis;
552 RT performed the identification of protein oxidation; MM and VHT performed molecular
553 dynamics simulations; GM provided microscopy and image analysis technical support; GC
554 and CC performed mass spectrometry experiments; CR, FVB and AB wrote the paper. CR
555 conceived and coordinated the research. All authors read and approved the final manuscript.

556

557 **Acknowledgments**

558 We thank Telmo Nunes for assistance with confocal imaging, Evguenia Bekman and
559 Domingos Henrique for pCIC, Sérgio Dias for HUVEC, and Domingos Henrique, Margarida
560 Amaral and Solveig Thorsteinsdottir for antibodies. We also thank Fernando Antunes, Susana
561 Marinho and Helena Soares for critical reading of the manuscript.

562 This work was supported by Fundação para a Ciência e a Tecnologia (FCT), grants
563 PTDC/BIA-PRO/101624/2008, PEst-OE/QUI/UI0612/2013 and UID/Multi/00612/2013.
564 Filipe Vilas-Boas and Gonçalo Costa were supported by FCT, individual fellowships
565 SFRH/BPD/74715/2010 and SFRH/BPD/73779/2010, respectively. Carla Real acknowledges
566 Ciência2008 Program (FCT). The funder had no role in study design; data collection, analysis,
567 and interpretation; decision to publish, or preparation of the manuscript.

568

References

- [1] S. Valastyan, R.A. Weinberg, Tumor Metastasis: Molecular Insights and Evolving Paradigms, *Cell*. 147 (2011) 275–292. doi:10.1016/j.cell.2011.09.024.
- [2] N. Reymond, B.B. d'Água, A.J. Ridley, Crossing the endothelial barrier during metastasis, *Nat. Rev. Cancer*. 13 (2013) 858–870. doi:10.1038/nrc3628.
- [3] K.K. Nelson, A.C. Ranganathan, J. Mansouri, A.M. Rodriguez, K.M. Providence, J.L. Rutter, et al., Elevated Sod2 Activity Augments Matrix Metalloproteinase Expression Evidence for the Involvement of Endogenous Hydrogen Peroxide in Regulating Metastasis, *Clin. Cancer Res*. 9 (2003) 424–432.
- [4] M. Nishikawa, A. Tamada, K. Hyoudou, Y. Umeyama, Y. Takahashi, Y. Kobayashi, et al., Inhibition of experimental hepatic metastasis by targeted delivery of catalase in mice, *Clin. Exp. Metastasis*. 21 (2004) 213–221.
- [5] H. Soares, H.S. Marinho, C. Real, F. Antunes, Cellular polarity in aging: role of redox regulation and nutrition, *Genes Nutr*. 9 (2014) 371. doi:10.1007/s12263-013-0371-5.

- 589 [6] R.H. Burdon, Superoxide and hydrogen peroxide in relation to mammalian cell
590 proliferation, *Free Radic. Biol. Med.* 18 (1995) 775–794.
591
- 592 [7] N. Hempel, T.R. Bartling, B. Mian, J.A. Melendez, Acquisition of the metastatic
593 phenotype is accompanied by H₂O₂-dependent activation of the p130Cas signaling
594 complex, *Mol. Cancer Res. MCR.* 11 (2013) 303–312. doi:10.1158/1541-7786.MCR-
595 12-0478.
596
- 597 [8] C. Polytarchou, M. Hatziapostolou, E. Papadimitriou, Hydrogen peroxide stimulates
598 proliferation and migration of human prostate cancer cells through activation of
599 activator protein-1 and up-regulation of the heparin affin regulatory peptide gene, *J. Biol.*
600 *Chem.* 280 (2005) 40428–40435. doi:10.1074/jbc.M505120200.
601
- 602 [9] M. Valcárcel, T. Carrascal, O. Crende, F. Vidal-Vanaclocha, IL-18 Regulates
603 Melanoma VLA-4 Integrin Activation through a Hierarchized Sequence of
604 Inflammatory Factors, *J. Invest. Dermatol.* 134 (2014) 470–480.
605 doi:10.1038/jid.2013.342.
606
- 607 [10] T. Finkel, Signal transduction by reactive oxygen species, *J. Cell Biol.* 194 (2011) 7–15.
608 doi:10.1083/jcb.201102095.
609
- 610 [11] H.S. Marinho, C. Real, L. Cyrne, H. Soares, F. Antunes, Hydrogen peroxide sensing,
611 signaling and regulation of transcription factors, *Redox Biol.* 2 (2014) 535–562.

doi:10.1016/j.redox.2014.02.006.

[12] P.J. Hogg, Targeting allosteric disulphide bonds in cancer, *Nat. Rev. Cancer.* 13 (2013) 425–431. doi:10.1038/nrc3519.

[13] J. Nelson, N.V. McFerran, G. Pivato, E. Chambers, C. Doherty, D. Steele, et al., The 67 kDa laminin receptor: structure, function and role in disease, *Biosci. Rep.* 28 (2008) 33–48. doi:10.1042/BSR20070004.

[14] S. Ménard, E. Tagliabue, M.I. Colnaghi, The 67 kDa laminin receptor as a prognostic factor in human cancer, *Breast Cancer Res. Treat.* 52 (1998) 137–145.

[15] V. de Oliveira-Marques, L. Cyrne, H.S. Marinho, F. Antunes, A Quantitative Study of NF- κ B Activation by H₂O₂: Relevance in Inflammation and Synergy with TNF- α , *J. Immunol.* 178 (2007) 3893–3902. doi:10.4049/jimmunol.178.6.3893.

[16] V. Gupta, K.S. Carroll, Sulfenic acid chemistry, detection and cellular lifetime, *Biochim. Biophys. Acta BBA - Gen. Subj.* 1840 (2014) 847–875. doi:10.1016/j.bbagen.2013.05.040.

[17] K.V. Jamieson, J. Wu, S.R. Hubbard, D. Meruelo, Crystal Structure of the Human Laminin Receptor Precursor, *J. Biol. Chem.* 283 (2008) 3002–3005.

doi:10.1074/jbc.C700206200.

[18] V. Castronovo, G. Taraboletti, M.E. Sobel, Functional domains of the 67-kDa laminin receptor precursor, *J. Biol. Chem.* 266 (1991) 20440–20446.

[19] K.V. Jamieson, S.R. Hubbard, D. Meruelo, Structure-guided identification of a laminin binding site on the laminin receptor precursor, *J. Mol. Biol.* 405 (2011) 24–32.

doi:10.1016/j.jmb.2010.10.028.

[20] A.A. Malygin, E.S. Babaylova, V.B. Loktev, G.G. Karpova, A region in the C-terminal domain of ribosomal protein SA required for binding of SA to the human 40S ribosomal subunit, *Biochimie.* 93 (2011) 612–617. doi:10.1016/j.biochi.2010.12.005.

[21] V. Romanov, M.E. Sobel, P. pinto da Silva, S. Menard, V. Castronovo, Cell localization and redistribution of the 67 kD laminin receptor and alpha 6 beta 1 integrin subunits in response to laminin stimulation: an immunogold electron microscopy study, *Cell Adhes. Commun.* 2 (1994) 201–209.

[22] A. Fatehullah, C. Doherty, G. Pivato, G. Allen, L. Devine, J. Nelson, et al., Interactions of the 67 kDa laminin receptor and its precursor with laminin, *Biosci. Rep.* 30 (2010) 73–79. doi:10.1042/BSR20090023.

- 656 [23] Y. Kaneda, K. Kinoshita, M. Sato, Y. Saeki, R. Yamada, M. Wataya-Kaneda, et al., The
657 induction of apoptosis in HeLa cells by the loss of LBP-p40, *Cell Death Differ.* 5 (1998)
658 20–28. doi:10.1038/sj.cdd.4400315.
- 659
- 660 [24] T. Susantad, D.R. Smith, siRNA-mediated silencing of the 37/67-kDa high affinity
661 laminin receptor in Hep3B cells induces apoptosis, *Cell. Mol. Biol. Lett.* 13 (2008) 452–
662 464. doi:10.2478/s11658-008-0017-6.
- 663
- 664 [25] J. Scheiman, J.-C. Tseng, Y. Zheng, D. Meruelo, Multiple functions of the 37/67-kd
665 laminin receptor make it a suitable target for novel cancer gene therapy, *Mol. Ther. J.*
666 *Am. Soc. Gene Ther.* 18 (2010) 63–74. doi:10.1038/mt.2009.199.
- 667
- 668 [26] K. Moodley, S.F.T. Weiss, Downregulation of the non-integrin laminin receptor reduces
669 cellular viability by inducing apoptosis in lung and cervical cancer cells, *PloS One.* 8
670 (2013) e57409. doi:10.1371/journal.pone.0057409.
- 671
- 672 [27] F. Vilas-Boas, A. Bagulho, R. Tenente, A. Jerónimo, C. Real, Analysis of intracellular
673 localization of RPSA upon alteration of its redox state, *Data Brief.* submitted (n.d.).
- 674
- 675 [28] S.M. Canfield, A.Y. Khakoo, The Nonintegrin Laminin Binding Protein (p67 LBP) Is
676 Expressed on a Subset of Activated Human T Lymphocytes and, Together with the
677 Integrin Very Late Activation Antigen-6, Mediates Avid Cellular Adherence to Laminin,

678 J. Immunol. 163 (1999) 3430–3440.

679

680 [29] R. Pellegrini, S. Martignone, S. Ménard, M.I. Colnaghi, Laminin receptor expression
681 and function in small-cell lung carcinoma, *Int. J. Cancer Suppl. J. Int. Cancer Suppl.* 8
682 (1994) 116–120.

683

684 [30] E. Ardini, E. Tagliabue, A. Magnifico, S. Butò, V. Castronovo, M.I. Colnaghi, et al.,
685 Co-regulation and physical association of the 67-kDa monomeric laminin receptor and
686 the $\alpha 6 \beta 4$ integrin, *J. Biol. Chem.* 272 (1997) 2342–2345.

687

688 [31] C. Margadant, H.N. Monsuur, J.C. Norman, A. Sonnenberg, Mechanisms of integrin
689 activation and trafficking, *Curr. Opin. Cell Biol.* 23 (2011) 607–614.
690 doi:10.1016/j.ceb.2011.08.005.

691

692 [32] D.S. Harburger, D.A. Calderwood, Integrin signalling at a glance, *J. Cell Sci.* 122 (2009)
693 159–163. doi:10.1242/jcs.018093.

694

695 [33] H. Kato, Z. Liao, J.V. Mitsios, H.-Y. Wang, E.I. Deryugina, J.A. Varner, et al., The
696 primacy of $\beta 1$ integrin activation in the metastatic cascade, *PloS One.* 7 (2012) e46576.
697 doi:10.1371/journal.pone.0046576.

698

699 [34] K. Stoletov, H. Kato, E. Zardouzian, J. Kelber, J. Yang, S. Shattil, et al., Visualizing
700 extravasation dynamics of metastatic tumor cells, *J. Cell Sci.* 123 (2010) 2332–2341.

doi:10.1242/jcs.069443.

[35] N. Hempel, H. Ye, B. Abessi, B. Mian, J.A. Melendez, Altered redox status accompanies progression to metastatic human bladder cancer, *Free Radic. Biol. Med.* 46 (2009) 42–50. doi:10.1016/j.freeradbiomed.2008.09.020.

[36] B. Kumar, S. Koul, L. Khandrika, R.B. Meacham, H.K. Koul, Oxidative stress is inherent in prostate cancer cells and is required for aggressive phenotype, *Cancer Res.* 68 (2008) 1777–1785. doi:10.1158/0008-5472.CAN-07-5259.

[37] Y. Fujimura, M. Sumida, K. Sugihara, S. Tsukamoto, K. Yamada, H. Tachibana, Green tea polyphenol EGCG sensing motif on the 67-kDa laminin receptor, *PloS One.* 7 (2012) e37942. doi:10.1371/journal.pone.0037942.

[38] R. Sanchez, M. Riddle, J. Woo, J. Momand, Prediction of reversibly oxidized protein cysteine thiols using protein structure properties, *Protein Sci. Publ. Protein Soc.* 17 (2008) 473–481. doi:10.1110/ps.073252408.

[39] P. Martínez-Acedo, E. Núñez, F.J.S. Gómez, M. Moreno, E. Ramos, A. Izquierdo-Álvarez, et al., A novel strategy for global analysis of the dynamic thiol redox proteome, *Mol. Cell. Proteomics MCP.* 11 (2012) 800–813. doi:10.1074/mcp.M111.016469.

- 723 [40] L. Sun, L. Liu, X. Liu, Y. Wang, M. Li, L. Yao, et al., MGr1-Ag/37LRP induces cell
724 adhesion-mediated drug-resistance via FAK/PI3K and MAPK pathway in gastric cancer,
725 Cancer Sci. (2014) n/a–n/a. doi:10.1111/cas.12414.
726
- 727 [41] D. Barkan, A.F. Chambers, β 1-Integrin: A Potential Therapeutic Target in the Battle
728 against Cancer Recurrence, Clin. Cancer Res. 17 (2011) 7219–7223. doi:10.1158/1078-
729 0432.CCR-11-0642.
730
- 731 [42] H. Peinado, S. Lavotshkin, D. Lyden, The secreted factors responsible for pre-metastatic
732 niche formation: Old sayings and new thoughts, Semin. Cancer Biol. 21 (2011) 139–146.
733 doi:10.1016/j.semcancer.2011.01.002.
734
- 735 [43] J. Xu, D. Li, X. Li, Z. Liu, T. Li, P. Jiang, et al., α 6 β 1 laminin receptor promotes the
736 malignant potential of tumour cells up-regulating lysyl oxidase-like 2 expression in
737 cholangiocarcinoma, Dig. Liver Dis. Off. J. Ital. Soc. Gastroenterol. Ital. Assoc. Study
738 Liver. 46 (2014) 750–757. doi:10.1016/j.dld.2014.03.017.
739
- 740 [44] H.S. Marinho, L. Cyrne, E. Cadenas, F. Antunes, The cellular steady-state of H₂O₂:
741 latency concepts and gradients, Methods Enzymol. 527 (2013) 3–19. doi:10.1016/B978-
742 0-12-405882-8.00001-5.
743
- 744 [45] J.R. Kim, H.W. Yoon, K.S. Kwon, S.R. Lee, S.G. Rhee, Identification of proteins
745 containing cysteine residues that are sensitive to oxidation by hydrogen peroxide at

746 neutral pH, *Anal. Biochem.* 283 (2000) 214–221. doi:10.1006/abio.2000.4623.

747

748 [46] D. Van Der Spoel, E. Lindahl, B. Hess, G. Groenhof, A.E. Mark, H.J.C. Berendsen,
 749 GROMACS: fast, flexible, and free, *J. Comput. Chem.* 26 (2005) 1701–1718.
 750 doi:10.1002/jcc.20291.

751

752 [47] B. Hess, C. Kutzner, D. van der Spoel, E. Lindahl, GROMACS 4: Algorithms for
 753 Highly Efficient, Load-Balanced, and Scalable Molecular Simulation, *J. Chem. Theory*
 754 *Comput.* 4 (2008) 435–447. doi:10.1021/ct700301q.

755

756 [48] S. Miyamoto, P.A. Kollman, Settle: An analytical version of the SHAKE and RATTLE
 757 algorithm for rigid water models, *J. Comput. Chem.* 13 (1992) 952–962.
 758 doi:10.1002/jcc.540130805.

759

760 [49] F. Vilas-Boas, R. Fior, J.R. Swedlow, K.G. Storey, D. Henrique, A novel reporter of
 761 notch signalling indicates regulated and random Notch activation during vertebrate
 762 neurogenesis, *BMC Biol.* 9 (2011) 58. doi:10.1186/1741-7007-9-58.

763

764 [50] J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, et al.,
 765 Fiji: an open-source platform for biological-image analysis, *Nat. Methods.* 9 (2012)
 766 676–682. doi:10.1038/nmeth.2019.

767

768 [51] E.M.M. Manders, F.J. Verbeek, J.A. Aten, Measurement of co-localization of objects in
769 dual-colour confocal images, *J. Microsc.* 169 (1993) 375–382. doi:10.1111/j.1365-
770 2818.1993.tb03313.x.

771

772

773

Figure legends

Figure 1. RPSA is a H₂O₂ target. (A) Bodipy-conjugated iodoacetamide (IAM-BODIPY) reacts with sulfhydryl groups (SH) of cysteine residues, fluorescently labeling reduced proteins. **(B)** Fluorescence of labeled RPSA protein diminished approximately 38% in the presence of H₂O₂ (0.175 in control vs 0.108 in H₂O₂; two-tailed Student's *t* test, **p*<0.05; N=3). **(C)** N-ethylmaleimide (NEM) reacts with SH groups of cysteine residues, labeling peptides containing reduced residues. After treatment with DTT, iodoacetamide (IAM) reacts with SH groups of cysteine residues, labeling peptides that were previously oxidized. **(D)** Representative mass spectra of peptides presenting IAM and NEM modifications in control (red) and oxidizing (with H₂O₂, black) conditions. Both cysteine residues were found to be modified with IAM in oxidizing conditions (peptides corresponding to the sequences ADHQPLTEASYVNLPTIALC(iam)NTDSPLR (m/z 2996.477) and YVDIAIPC(iam)NNK (m/z 1306.645), which represent peptides containing Cys148 and Cys163 conjugated with IAM, respectively). In non-treated samples, only peptides containing Cys148 and Cys163 conjugated with NEM were found. **(E)** Model of the RPSA structure (limited to residues 9-205) in the oxidized state, with an intramolecular disulfide. The protein structure shown is an example extracted from the full conformational ensemble of the oxidized state. Cysteine residues are shown as balls and sticks with green carbon atoms. The side chain of residues that significantly change their solvent accessible surface area upon oxidation are shown in sticks with carbons colored orange (decreased) or cyan (increased). For clarity, aliphatic hydrogen atoms were omitted.

Figure 2. RPSA oxidation prevents cell adhesion inhibition to laminin in the presence of H₂O₂. *In vitro* cell adhesion assays with HeLa cells transfected with pCIC (mock), pCIC-RPSAwt (wt) or pCIC-RPSAmut (mut) and in control or oxidizing (with H₂O₂) conditions on (A, B) laminin-coated plates, (C, D) fibronectin-coated plates or (E, F) uncoated plates (without ECM). (A), (C) and (E) show cell adhesion in the absence or presence of H₂O₂ (adherent cells normalized by total plated cells (tpc, see Materials and Methods)). (B), (C) and (F) show inhibition of cell adhesion by H₂O₂. In mock- and RPSAmut-transfected cells, cell adhesion in the presence of H₂O₂ was decreased compared to control cells (without H₂O₂) on laminin-coated plates, an effect that was not observed in cells plated on fibronectin-coated or uncoated plates. On laminin-coated plates, RPSAwt overexpression prevented the inhibition of cell adhesion in the presence of H₂O₂ compared to cells transfected with pCIC, an effect that was not observed in RPSAmut-overexpressing cells (values show the mean and SEM; two-tailed Student's *t* test in (A), Tukey test in (B), **p*<0.05, ***p*<0.01; N=10-12). The raw data from these experiments is supplied in a Supplementary File.

Figure 3. RPSA oxidation state regulates its subcellular expression. (A) Maximum intensity projections of confocal images of immunofluorescence for RPSA in non-permeabilized HeLa cells transfected with pCIC-RPSAwt (wt) or pCIC-RPSAmut (mut) and in control, oxidizing (with H₂O₂) or reducing (with NAC) conditions. Protein accumulations (clusters) are indicated with arrows. (B) Quantification of clustering level (see Materials and Methods) shows that RPSAwt had a more heterogeneous distribution in the presence of H₂O₂ than RPSAwt or RPSAmut in all other conditions analyzed (values show the mean and SEM; two-tailed Student's *t* test, **p*<0.05, ***p*<0.01; N=2-6). Scale bar = 20 μm.

Figure 4. Distinct colocalization specificities between RPSAwt or RPSAmut and β 1 or β 5 integrin subunits. (A) Single optical sections from confocal images of co-immunofluorescence for RPSA and β 1 integrin or β 5 integrin subunits in non-permeabilized HeLa cells transfected with pCIC-RPSAwt or pCIC-RPSAmutand in control or oxidizing (with H₂O₂) conditions on laminin-coated plates. The presence of integrin subunit expression in RPSA clusters was defined as total (all protein expression domains overlapping), partial (some protein expression domains overlapping) or none (no protein expression domains overlapping). Arrows and arrowheads indicate RPSA clusters with or without integrin subunits expression, respectively. (B) Quantification of the presence of integrin subunit expression in RPSA clusters, showing that RPSA oxidation increased β 1 integrin subunit expression in RPSA clusters but not that of β 5 integrin subunit (N=4, 37-123 cells per condition). (C) Analysis by the M1 Manders' overlap coefficient showed increased colocalization between β 1 integrin subunit expression and RPSA upon RPSA oxidation, whereas the same was not observed between β 5 integrin subunit and RPSA (whiskers box show the median and 25th and 75th percentiles; error bars are standard deviation and 10th and 90th percentiles are plotted as dots; Mann-Whitney *U* test, **p*<0.05, ***p*=0.002; N=4, 16-75 cells per condition). Scale bar = 20 μ m.

Figure 5. pFAK expression was increased in RPSA clusters.(A) Corrected total cell fluorescence (CTCF) intensity levels of RPSA and pFAK, from confocal images of co-immunofluorescence of non-transfected HeLa cells (black dots) and transfected (gray dots) with pCIC-RPSAwt or pCIC-RPSAmut. RPSA overexpressing cells present high pFAK expression. Confocal images were obtained from fields with transfected cells that presented high levels of RPSA expression in clusters. Each chart contains results for cells from one representative experiment. Squares indicate the cells shown in (B). (B) Sum projections of

confocal images of co-immunofluorescence for RPSA and pFAK of the cells indicated in (A), showing that high pFAK expression occurred in RPSA clusters of RPSAwt- and RPSAmut-overexpressing cells. Arrows indicate RPSA clusters. (C) Surface plots showing fluorescence intensities of RPSA and pFAK in 5 alternated single optical sections of 10-sections Z-stacks (Z-step of 0.5 μ m) of the cells shown in (B), confirming that high pFAK expression occurred in overexpressed RPSA that accumulated in clusters. Scale bar = 10 μ m.

Figure 6. RPSA oxidation regulates extravasation from zebrafish intersomitic vessels.(A)

Schematic representation of a 48 hpf Tg(*fli*:EGFP) zebrafish embryo. HeLa cells were injected in the common cardinal vein (CCV) of 48 hpf embryos and analyzed for extravasation in the posterior intersomitic vessels (ISV, rectangle area) 24 hours later. (B,C) Maximum intensity projections of confocal images showing magnifications of posterior ISVs with transfected cells inside ISV (B) or cells that extravasated (C) (indicated by arrows). (D) Analysis of the percentage of extravasation of transfected cells shows that cells overexpressing RPSAwt had a higher capacity to extravasate than the ones overexpressing RPSAmut, indicating a requirement for RPSA oxidation in this process (values show the mean and SEM; two-tailed Student's *t* test, $**p=0.003$; N=4). Scale bar = 50 μ m.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6