THE EMBO JOURNAL

Secretory kinase Fam20C tunes endoplasmic reticulum redox via phosphorylation of serine145 of Ero1α

Jianchao Zhang, Qinyu Zhu, Xi'e Wang, Jiaojiao Yu, Xinxin Chen, Jifeng Wang, Xi Wang, Junyu Xiao, Chih-chen Wang and Lei Wang

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 18th December 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see from the reports, our referees all express interest in the findings reported in your manuscript although they also raise a number of concerns that you will have to address before they can support publication of the manuscript in The EMBO Journal.

For the revised manuscript I would particularly ask you to focus your efforts on the following points:

-> All three referees question the localization of Fam20C and Ero1a and the consequences for kinase activity. It will therefore be important for you to include additional data to strengthen this part of the study
-> Ref #1 makes a number of constructive suggestions that would in our view strengthen the manuscript further at both the cellular and physiological level. I realise that going into all the scenarios mentioned in point #6 may be beyond the scope of a revision but I'd encourage you to include some additional data to broaden the relevance/dynamics of Ero1a phosphorylation by Fam20C
-> In addition to these general points, Ref #2 asks for a more rigorous data quantification while ref #3 points to a number of technical clarifications that are needed in order for the reader to judge the reported findings.

Given the referees' overall positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

REFEREE REPORTS

Referee #1:

In this concise and well-written paper, Zhang et al. provide compelling evidence that Ero1α is phosphorylated by Fam20C, a secreted casein-kinase. Phosphorylation occurs in the Golgi. From here, most of phospho-Ero1α is retrieved to the ER by ERp44, and part is secreted. Interestingly, Fam20C cannot phosphorylate Ero1α extracellularly.
Several corollary observations reveal an important physiologic role for Ero1α phosphorylation. First, phosphorylation increases the efficiency of Ero1α in promoting oxidative folding. Second, the overexpression Fam20c is sufficient to improve formation of disulfide bonds in cargo proteins. Third, Fam20C (and phospho-Ero1α) increase in the mammary gland during lactation, presumably to favour abundant protein secretion.
As it is often the case with novel findings, this study raises more questions than they solve: what is the phosphatase in charge of dephosphorylating Ero1α? Does phospho-Ero1α induce oxidative stress/ redox signaling?
The data are well presented, and convincingly demonstrate the conclusions reached by the authors. There are a series of points that need be addressed before the paper can be considered for publication in the EMBO J.

Detailed criticisms
1. Like other enzymes that are mainly active in the ER, Ero1α lacks a KDEL-like motif. The authors propose that this feature may reflect the need to be phosphorylated by Fam20C. However, this secreted enzyme would transit through the ER. Is Fam20C inactive there? The experiment shown in Figure 4A indicates that Ero1α phosphorylation cannot occur extracellularly. Altogether, one would conclude that a rather unique milieu is found in the Golgi that promotes Fam20C activity.
2. When FAM20C is overexpressed, it seems that most if not all Ero1α is phosphorylated by a Golgi-resident enzyme, but still is localized in the ER. It follows that phospho-Ero1α is retrieved to the ER where it promotes oxidative folding. Does Brefeldin A induce phosphorylation by inducing relocalisation of the enzyme?
3. Figure 2D. When overexpressed, Fam20C phosphorylates endogenous Ero1α, traces of which seem to be secreted as well. The authors should attempt to determine whether phosphorylation increases the secretion of endogenous Ero1α.
4. Figure 1 panel G. There seems to be is much less endogenous Ero1α in the lysates from Fam20C over-expressing cells. Is this a reproducible finding? Does phosphorylation increases the rate of Ero1α secretion? Does this reflect an interplay between Fam20C and Ero1α?
5. Does a Fam20C inhibitor exist? If so, it would be of interest to see after the stability of phospho-Ero1α. Is it secreted? Is it dephosphorylated?
6. More endogenous Ero1α is phosphorylated in the lactating mammary gland, Figure 3 H. So, besides the artifactual over-expression of Fam20C, there are physiological conditions in which the cells decide to phosphorylate Ero1α. The authors propose that this is meant to increase oxidative protein folding. Is the system activated also in other secretory cells? Is Fam20C induced during ER stress or overload? How is expression/activity regulated? Is Ero1beta -whose transcription is increased during the UPR- phosphorylated? Or in hypoxia? What about also tissues of the gastrointestinal tract (where it is more expressed?)
7. Does ERp44 binds with higher affinity to phospho-Ero1α? Does the S145A interact with ERp44? Is its secretion regulated by ERp44 over-expression? What about the Phospho-mimetic S145E mutant?
8. The overexpressed Fam20-FLAG displays an interesting pattern. While the endogenous appears as a single band in WB, the recombinant has two additional bands, with slower migration. Importantly, these bands appear only in the cell extract and not in the medium. Since the catalytic dead mutant has also a single band pattern, it is possible that Fam20-Flag undergoes autophosphorylation. Can the authors to comment on this?
9. The Ero1αP site is conserved down to Drosophila. Do Fam20C-like kinases exist in yeast?

Referee #2:

In their study entitled Secretory kinase Fam20C tunes endoplasmic reticulum redox via phosphorylation of Serine145 of Ero1a Zhang et al. discover and analyze a new role for Fam20C as a regulator of Ero1a function, and thus ER redox homeostasis. The study is of high and general interest and very well performed. Before publication, however, the following points need to be addressed:

Major points:
• In Figure EV1, the authors should also show a blot of cell lysates for the k/o cell lines; also: an antibody against the C-terminus is maybe not ideally suited, as it would miss stable Fam20C fragments potentially generated by the INDELs
• How do the authors explain the difference in ER redox for clones 3/5 in Figure 1D? This also relates to the concern raised above.
• In Figure 1G, why do the Ero1a input levels decrease upon Fam20C expression?
• The conclusions from Figure 3B-D are not entirely clear: does only secreted Ero1a become phosphorylated by Fam20C in the absence of Fam20C / Ero1a & Fam20C overexpression? How is this reconciled with the proposed regulatory role? The same question applies to the IF data (Figure 3E&F): Can co-localization/Ero1a phosphorylation also be observed in the absence of overexpression? What effect does overexpression of a highly disulfide-bonded secreted protein in the ER/changing the ER redox conditions by small molecules have on Ero1a phosphorylation?
• In Figure 3H, upper lane: the blots show bleached out bands at the height of p-Ero1a; furthermore: how do the authors explain multiple bands observed in some samples for Ero1a (second lane)?
• The quantification in Figure 4 seems problematic, as also dimers/HMW species are oxidized. Furthermore, loading in A seems uneven between the gels (more sample each each gel, going from left to right) and unspecific bands around 35 kDa are much more pronounced in A than in C. The quantification problem becomes even more pronounced in 4C & D, where for Fam20C DA more dimers seem to be present, also representing oxidized species. In general, faster oxidation is apparent as the authors claim - a quantification should also be performed, however, including oligomeric species in EV/or the answer to this review. Furthermore, immunoblots in C/D seem indicated to show the amount of phosphorylated Ero1a.

Minor points:
• The manuscript needs some language editing, some articles are missing, some minor errors are in the text; some unclear expressions need to be taken care of, e.g. "displayed ratiometric fluorescence in the ER"
• In the abstract, the sentence "..the role of Fam20C..is largely unknown" is not clear; its role in the ER lumen? For proteins of the ER lumen?
• The expression "lumen of the secretory pathway" is awkward and should be changed to ER.
• What is the difference between Figure EV1B and the blot in Figure 1D?
• What is the difference between lane 2&3 in Figure EV3A?
• It is not always clear from the figures & legends if/how constructs were epitope-tagged.
• The role of Fam20A, being part of Figure 3G, should be explained a bit more.
• What is the difference between Figure EV6B & the blot in 6A?
• In the discussion, the authors state "phosph. of Ero1a increased the motion of the outer active site-containing loop"; where was this shown?
• The discussion about Ero1 phosphorylation/Erp44-mediated recruitment to the ER is misleading as also non-phosphorylated Ero1a appears to be recruited by Erp44, and this had been known beforehand.
• Proteomic studies do exist on plasma cell differentiation. Are Ero1a/Fam20C upregulated in these? This could be discussed.

Referee #3:

This manuscript reports the interesting observations that Fam20C phosphorylates Ero1 and that phosphorylation affects Ero1 activity and the redox state of the ER. Overall, the authors make a thorough case for these claims. Nevertheless, this reviewer raises a few points for consideration or clarification.

It is not clear from the details of the methods provided what is the basis of the Fam20C "interactome." There is no reason for an enzyme to remain stably associated with its substrates, and certainly not through an IP. On what do these interactions depend? The IPs were presumably done under oxidizing conditions? Is the Ero1 disulfide bonded to Fam20C in the IP? What detergent was used for lysis? Most importantly, the pcDNA vector used to express flag-tagged Fam20C in the ER is likely to massively over-produce the protein. Approximately by what factor is it over-expressed compared to endogenous levels? This reviewer considers it likely that IP of any protein over-expressed in the ER will pull down other abundant ER proteins due to non-specific association. How can the authors rule out this possibility? Evidence presented in the manuscript showing that Fam20C phosphorylates Ero1 is stronger than the evidence that these proteins naturally form a stable interaction.

In addition to lacking many details, there are also errors in the IP methods: "The cell extracts were incubated with anti-Flag M2 affinity gel (Sigma-Aldrich) overnight at 4 {degree sign}C for 2 h with occasional vortexing." Was the incubation overnight or for two hours?

The authors give the number of ER and Golgi proteins identified, but not the number of cytosolic and other proteins.

p. 6 "MS analysis identified only 1 phosphopeptide 137LGAVDESLpSEETQK150 (Fig2A and B) with 95.7% sequence coverage." To avoid confusion, a better presentation of these data would be: "MS analysis yielded 95.7% sequence coverage and identified only 1 phosphopeptide 137LGAVDESLpSEETQK150 (Fig2A and B)."

p. 6 "Ser145 is adjacent to the outer active site-containing flexible loop (Fig2C)..." Figure 2C does not show proximity of Ser145 to the outer active site. The reader expects to see a structure image showing proximity (such as Figure 5A or Figure EV5). Furthermore, Ser145 is about 32 Å from cysteine 94. Is this distance considered "adjacent"?

p. 6 "dependent manner, confirmed by the slowly migrating bands and by p-Ero1α blotting." Is a substantial shift in migration after 30 minutes in Fig. 3A expected for the addition of a single phosphate group? Are these gels run under oxidizing or reducing conditions?

p. 7 The point that the authors are trying to make regarding co-localization is not clear from Fig. 3E and 3F. Tagged Fam20C seems to show Golgi localization, whereas Ero1a seems to show ER localization. Indeed, later in the manuscript the authors make this same point, and this is what is shown in Fig. 4B (the authors' claim that Ero1a is also detected in the Golgi is not convincing).

p. 8 "to further restrict its traffic to the Golgi apparatus" is not clear. Do the authors mean that they intended to enhance ER localization of Ero1a and to decrease the amount that resides in the Golgi at steady state?

p. 8 "The Ero1α phosphorylation mimic S145E displayed approximately 3 times the oxidase activity of Ero1α WT in the presence of either the small molecule reducing agent DTT..." The authors show in Figure 5B that DTT bypasses the outer active site and directly reduces the inner active site. However, the authors state on page 9, "Elevation of activity caused by Ser145 phosphorylation still depended on the presence of the Ero1α outer active site (Fig EV5A), implying that phosphorylation does not alter the electron transfer pathway." There is an inconsistency here.

p. 11 It is true that yeast Ero1 does not have a clear analog of Ser145. However, this serine in the mammalian enzyme is present in the same region of space relative to the active site as the set of cysteines that affect the catalytic activity of yeast Ero1 when mutated. Perhaps this fact is worth mentioning.

p. 11 "Phosphorylation of Ero1α increased the motion of the outer active site-containing loop" is presented as a fact, but it is actually a speculation. The data supporting this speculation appears to be the change in tryptophan fluorescence, but no experiments are presented to localize the change in conformation/dynamics that causes the change in tryptophan fluorescence.

Response to the referees' comments

Referee #1:

In this concise and well-written paper, Zhang et al. provide compelling evidence that Ero1α is phosphorylated by Fam20C, a secreted casein-kinase. Phosphorylation occurs in the Golgi. From here, most of phospho-Ero1α is retrieved to the ER by ERp44, and part is secreted. Interestingly, Fam20C cannot phosphorylate Ero1α extracellularly.

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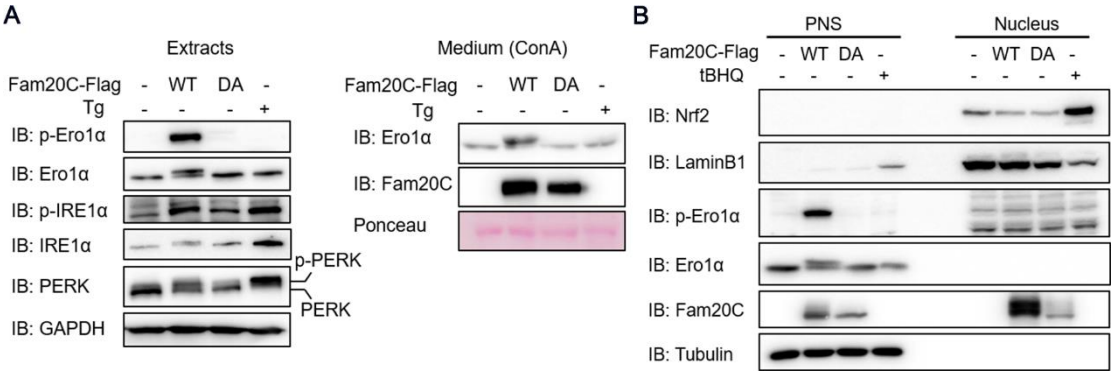
As it is often the case with novel findings, this study raises more questions than they solve: what is the phosphatase in charge of dephosphorylating Ero1α? Does phospho-Ero1α induce oxidative stress/ redox signaling?

The data are well presented, and convincingly demonstrate the conclusions reached by the authors. There are a series of points that need be addressed before the paper can be considered for publication in the EMBO J.

We appreciate these positive comments, particularly the word of "novel findings". Currently, to our knowledge no phosphatase has been reported to localize in the luminal side of the ER. Therefore, p-Ero1α and other potential phosphorylated proteins in the ER should be dephosphorylated by unidentified phosphatase; alternatively, they could be degraded by cellular degradation systems. The mechanisms underlying the dephosphorylation process in the secretory pathway definitely deserve future investigation. In Discussion, we already mentioned "…, and the identity of the protein phosphatase for dephosphorylating Ero1α remain open questions."

We expressed Fam20C in HeLa cells and examined the Nrf2-mediated antioxidant signaling and UPR signaling. It is observed that overexpression of Fam20C activated UPR pathways marked by PERK and IRE1α phosphorylation but showed no effect on nuclear translocalization of Nrf2 (Response Figure 1). Considering that Fam20C overexpression leads to Ero1α phosphorylation and further activation, these results are consistent with previous report that hyperactivity of the Ero1α oxidase elicits ER stress but no broad antioxidant response (Hansen et al, 2012).



Response Figure 1. The effects of overexpressed Fam20C on the UPR signaling and Nrf2-mediated antioxidant pathway.

A Protein immunoblotting of cell extracts (*Left*) and ConA precipitates from the conditioned medium (*Right*) of HeLa cells transfected with Fam20C for 24 h or treated with 5 μM thapsigargin (Tg) for 6 h.

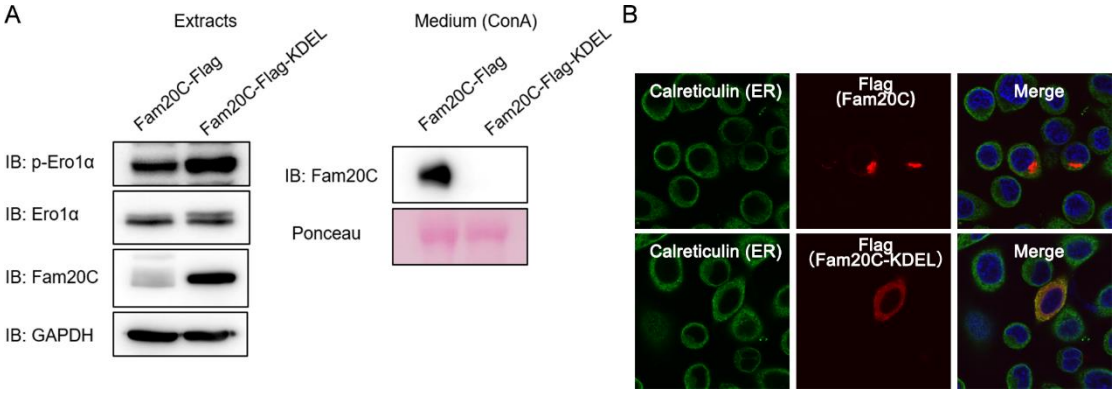
B Protein immunoblotting of post-nuclear supernatant (PNS) and nucleus from HeLa cells transfected with Fam20C for 24 h or treated with 200 μM tertiary butylhydroquinone (tBHQ, an Nrf2 activator) for 6 h.

Detailed criticisms

1. Like other enzymes that are mainly active in the ER, Ero1α lacks a KDEL-like motif. The authors propose that this feature may reflect the need to be phosphorylated by Fam20C. However, this secreted enzyme would transit through the ER. Is Fam20C inactive there? The experiment shown in Figure 4A indicates that Ero1α phosphorylation cannot occur extracellularly. Altogether, one would conclude that a rather unique milieu is found in the Golgi that promotes Fam20C activity.

Previous study showed that Fam20C containing a C-terminal KDEL sequence could still phosphorylate its substrate osteopontin in U2OS cells (Tagliabracci et al, 2015). Our experiments also showed that Fam20C-KDEL phosphorylated Ero1α in HeLa cells (Response Figure 2). Based on these data, we cannot exclude the possibility that Fam20C is active on the route to transit through the ER. However, it should be noted that the secretion of Fam20C-KDEL is abolished and the cellular amount of Fam20C-KDEL (in the ER) is much larger than that of Fam20C WT (in the Golgi), while the phosphorylation levels of substrates were comparable (Response Figure 2A; Fig 2K in Tagliabracci's paper). Therefore, the phosphorylation of substrates by ER-localized Fam20C could be also due to its abnormal expression there. Instead, our experiment showed that the phosphorylation of Ero1α-KDEL is markedly decreased compared to Ero1α WT by endogenous Fam20C (Fig 4C). Also, Brefeldin A (BFA) treatment did not promote the phosphorylation of Ero1α (see point 2 below). All these results indicate that the phosphorylation of Ero1α is favored by Golgi-located Fam20C. Overall, at this stage, we agree with the referee and believe it is pertinent to say that a rather unique milieu in the Golgi is appropriate for Fam20C activity.

We add in the Discussion section, "Although it is possible that Fam20C is active during its transit route across the ER, we propose that a rather unique milieu in the Golgi is appropriate for Fam20C activity."

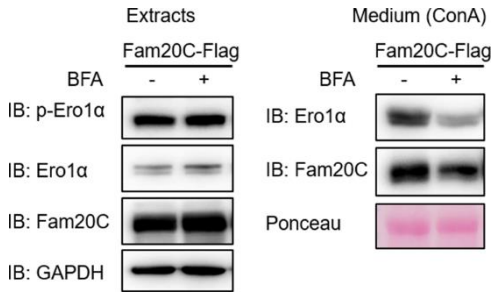


Response Figure 2. Fam20C-KDEL phosphorylates Ero1α in HeLa cells.

A Protein immunoblotting of cell extracts (*Left*) and ConA precipitates from the conditioned

medium (*Right*) of HeLa cells transfected with Fam20C-Flag or Fam20C-Flag-KDEL for 24 h.
B Immunofluorescence analysis of cellular localization of Fam20C-Flag and Fam20C-Flag-KDEL.

2. When FAM20C is overexpressed, it seems that most if not all Ero1α is phosphorylated by a Golgi-resident enzyme, but still is localized in the ER. It follows that phospho-Ero1α is retrieved to the ER where it promotes oxidative folding. Does Brefeldin A induce phosphorylation by inducing relocalisation of the enzyme?
As shown in Response Figure 3, we treated cells with BFA and found that BFA decreased the secretion of Fam20C and Ero1α, while did not increase the phosphorylation of Ero1α. As BFA disrupts the Golgi apparatus, this result is consistent with the notion that a rather unique milieu in the Golgi is appropriate for Fam20C activity.



Response Figure 3. Ero1α phosphorylation is not induced by BFA treatment.
Protein immunoblotting of cell extracts (*Left*) and ConA precipitates from the conditioned medium (*Right*) of HeLa cells transfected with Fam20C for 24 h treated with or without 5 μg/mL Brefeldin A (BFA) for 2 h.

3. Figure 2D. When overexpressed, Fam20C phosphorylates endogenous Ero1α, traces of which seem to be secreted as well. The authors should attempt to determine whether phosphorylation increases the secretion of endogenous Ero1α.
We found that overexpression of Fam20C did not substantially promote the secretion of endogenous Ero1α (Response Figure 1A). Moreover, the secretion of ectopic expressed Ero1α WT, S145E and S145A were similar (see point 7 below). Thus, it seems that phosphorylation has little effect on the secretion of Ero1α.

4. Figure 1 panel G. There seems to be is much less endogenous Ero1α in the lysates from Fam20C over-expressing cells. Is this a reproducible finding? Does phosphorylation increases the rate of Ero1α secretion? Does this reflect an interplay between Fam20C and Ero1α?
We carried out this experiment for four times. As shown in a more representative blot (new Figure 1G), overexpression of Fam20C didn't decrease the endogenous Ero1α level.

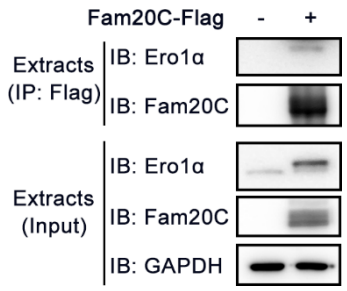
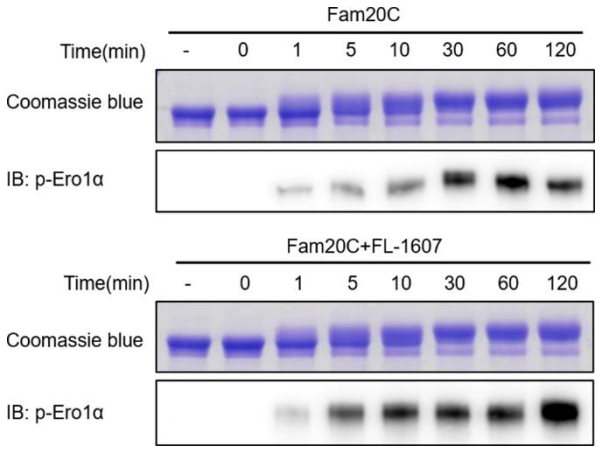


Figure 1G. Co-immunoprecipitation of endogenous Ero1α and Flag-tagged Fam20C in HeLa cells.

5. Does a Fam20C inhibitor exist? If so, it would be of interest to see after the stability of phospho-Ero1α. Is it secreted? Is it dephosphorylated?

To our knowledge only one potential Fam20C inhibitor was reported based on the systems-biology network, molecular modeling and molecular dynamics simulations (Qin et al, 2016). However, in our determinations this chemical compound at >300-fold molar excess did not inhibit Fam20C kinase activity (Response Figure 4). Thus, currently it seems unable to do the experiments suggested by using a Fam20C inhibitor.



Response Figure 4. FL-1607 didn't inhibit Fam20C kinase activity.

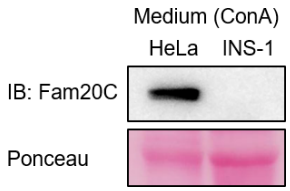
Time-dependent incorporation of phosphate group into Ero1α catalyzed by recombinant Fam20C (0.3 μM) pretreated without or with 100 μM FL-1607 for 5 min at 30 ℃. Reaction products were analyzed by Coomassie Blue staining and p-Ero1α immunoblotting.

6. More endogenous Ero1α is phosphorylated in the lactating mammary gland, Figure 3 H. So, besides the artifactual over-expression of Fam20C, there are physiological conditions in which the cells decide to phosphorylate Ero1α. The authors propose that this is meant to increase oxidative protein folding. Is the system activated also in other secretory cells? Is Fam20C induced during ER stress or overload? How is expression/activity regulated? Is Ero1beta -whose transcription is increased during the UPR- phosphorylated? Or in hypoxia? What about also tissues of the gastrointestinal tract (where it is more expressed?)

We thank the referee for recognizing physiological significance of Ero1α phosphorylation and providing these insightful suggestions.

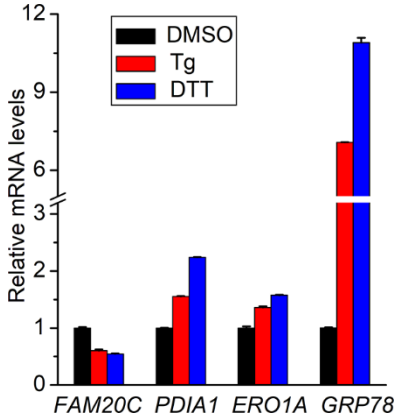
1) We checked the case in rat insulinoma INS-1 cells which produce insulin. However, there is no sufficient Fam20C expression in INS-1 cells (Response Figure 5). Please also see point

4 below.



Response Figure 5. Detection of Fam20C in the medium of HeLa and INS-1 cells.

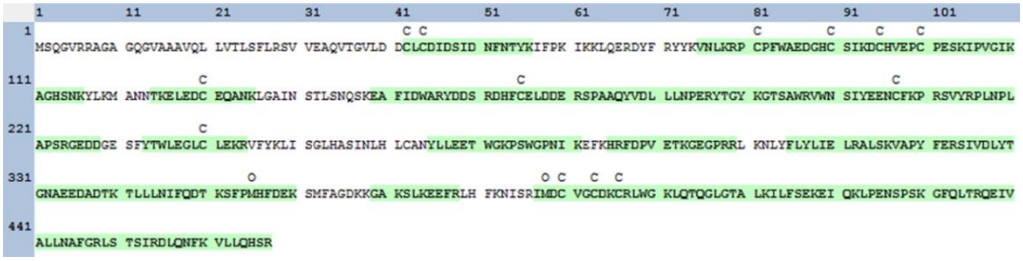
2) As shown in Response Figure 6, we found that ER-stress inducer thapsigargin (Tg) and dithiothreitol (DTT) did not induce the mRNA level of *FAM20C* after 6 h treatment, though the mRNA levels of *GRP78*, *PDIA1* and *ERO1A* were all induced. Interestingly, the *FAM20C* transcription seemed somehow decreased after 6 h treatment. We speculate that it could be one of the mechanism to turn down the activity of Fam20C for cells to recover from ER stress. However, it is too preliminary to make any conclusion and we decide not to make further discussion on this matter in the paper.



Response Figure 6. *FAM20C* was not induced during UPR.
The mRNA was isolated from HeLa cells treated with 5 μM Tg or 2 mM DTT for 6 h, and relative mRNA levels of *FAM20C* and several ER folding catalysts were determined by RT-qPCR.

3) Currently, little is known about the expression/activity regulation of Fam20C. It was reported that Fam20A as a pseudokinase forms a functional complex with Fam20C, and this complex enhances protein phosphorylation within the secretory pathway (Cui et al, 2015); and sphingosine stimulates Fam20C activity *in vitro* with both decreased K_m (ATP) and higher V_{max} (Cozza et al, 2015). We are now studying on the post-translational regulation on Fam20C secretion and activity, which is beyond the scope of this study.

4) We overexpressed HA-tagged Ero1β in HeLa cells, and performed tag purification and MS analysis. However, no phosphorylated peptide was identified with 72.8% coverage (Response Figure 7). Ero1β is specific and abundant in islet β-cells (Dias-Gunasekara et al, 2005; Zito et al, 2010); but little Fam20C was detected in islet β-cells as indicated in point 6.1. Thus, evidence supporting that Ero1β is phosphorylated by Fam20C is currently lacking.



Response Figure 7. HA-tagged Ero1β was analyzed by MS with 72.8% sequence coverage.

5) As Ero1α is induced during hypoxia, it is likely that p-Ero1α could also be induced. To test this possibility, we cultured Fam20C-expressing HeLa cells in a hypoxic chamber up to 24 h, and found that phosphorylation of Ero1α Ser145 was dramatically induced along with Ero1α upregulation (new Fig 8D)

As Fam20C increases the oxidase activity of Ero1α, we therefore designed to challenge the cells with reducing agent DTT and to see if Ero1α phosphorylation is induced. As shown in new Fig 8E and F, phosphorylation of Ero1α Ser145 was quickly and strongly induced by DTT while Ero1α protein level was not altered. Thus, upon DTT treatment, Fam20C rapidly phosphorylates Ero1α and enhances its oxidase activity to counteract with the reductive stress.

We believe these new results are important to broaden the physiological relevance of Ero1α phosphorylation by Fam20C. We added in the text, “It has been known that Ero1α is induced during hypoxia and is a key adaptive response to improve protein secretion under hypoxia (May et al, 2005). Notably, when Fam20C-expressing HeLa cells were cultured in a hypoxia chamber with 0.1% oxygen concentration, phosphorylation of Ero1α was dramatically induced along with an increase of total Ero1α and hypoxia-inducible factor 1 (HIF-1α) (Fig 8D). We also investigated whether phosphorylation of Ero1α can be induced during ER stress or reductive stress by pharmacological modulation. When Fam20C-expressing cells were treated with known ER stress inducers, including Tg (an inhibitor of sarco/endoplasmic reticulum Ca2+-ATPase (SERCA) calcium pump), tunicamycin (Tm; an inhibitor of protein glycosylation) and Brefeldin A (BFA; an inhibitor of ER-Golgi traffic), both p-Ero1α and total Ero1α levels were unchanged up to 6 h, though IRE1α and PERK UPR branches were activated (Fig 8E). Interestingly, when cells were treated with 200 μM DTT, a concentration sufficient to reduce protein disulfides but not trigger UPR, p-Ero1α was rapidly and strongly induced whereas total Ero1α protein level did not change (Fig 8E and F). These results suggest that Fam20C-catalyzed phosphorylation of Ero1α occurs as a post-translational regulatory mechanism to counteract cellular reductive stress.”

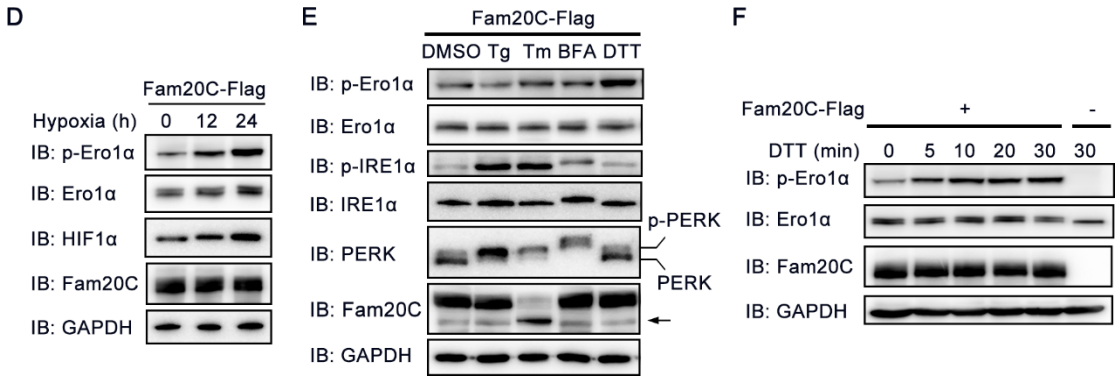


Figure 8. p-Ero1α is induced during mammalian lactation, hypoxia and reductive stress.

D Protein immunoblotting of HeLa cells expressing Fam20C-Flag following exposure to hypoxia (0.1% oxygen) for the indicated times.

E Protein immunoblotting of HeLa cells expressing Fam20C-Flag treated with 5 μM thapsigargin (Tg), 5 μg/ml tunicamycin (Tm), 5 μg/ml Brefeldin A (BFA), or 200 μM DTT for 6 h. The arrow indicates an unglycosylated form of Fam20C.

F Protein immunoblotting of HeLa cells transfected with or without Fam20C-Flag treated with 200 μM DTT for the indicated times.

6) Ero1α was reported to be highly expressed in cancers of the upper gastro-intestinal tract (Battle et al, 2013). Unfortunately, we do not have the esophageal cancer cell lines at hand. We believe it will be important to study if p-Ero1α is induced in the gastro-intestinal and other types of cancers, which has been discussed in the last paragraph in Discussion.

7. Does ERp44 binds with higher affinity to phospho-Ero1α? Does the S145A interact with ERp44? Is its secretion regulated by ERp44 over-expression? What about the Phospho-mimetic S145E mutant?

To address these questions, we expressed Ero1α WT, S145A and S145E in Hela cells. The co-IPs showed that all three constructs bind to endogenous ERp44 with similar affinities (new Fig EV4B). When ERp44 was over-expressed, the secretion of Ero1α WT, S145A and S145E were all significantly reduced (new Fig EV4C). We have included these results in the text, "We propose that relocation of p-Ero1α to the ER is also achieved by ERp44. Indeed, p-Ero1α could be co-immunoprecipitated with ERp44 and the secretion of p-Ero1α dramatically decreased with ERp44 overexpression (Fig 4D), though ERp44 bound to Ero1α WT, S145A and S145E mutants and retained them within the ER with similar affinities (Fig EV4B and C)."

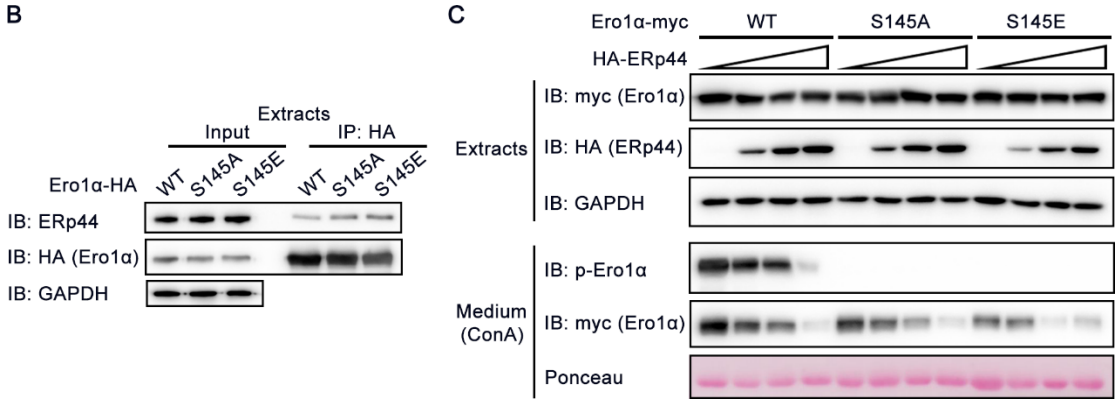


Figure EV4. The ER retention of p-Ero1α is mediated by ERp44.

B Co-immunoprecipitation of Ero1α and endogenous ERp44 in HeLa cells expressing Ero1α-HA WT/S145A/S145E.

C Protein immunoblotting of cell extracts and ConA precipitates from the conditioned medium of HeLa cells overexpressing Ero1α-myc WT/S145A/S145E and increasing amounts of HA-ERp44.

8. The overexpressed Fam20-FLAG displays an interesting pattern. While the endogenous appears as a single band in WB, the recombinant has two additional bands, with slower migration. Importantly, these bands appear only in the cell extract and not in the medium. Since the catalytic dead mutant has also a single band pattern, it is possible that Fam20-Flag undergoes autophosphorylation. Can the authors to comment on this?

Yes, Fam20C was reported to be able to undergo autophosphorylation. There are three Fam20C consensus S-x-E motifs in the molecule; however, individual mutation of each Ser to Ala did not affect Fam20C activity and had modest effects on Fam20C secretion (Tagliabracci et al, 2015).

9. The Ero1αP site is conserved down to Drosophila. Do Fam20C-like kinases exist in yeast?

There is no Fam20C-like kinase existing in yeast. Interestingly, we found that the counterpart of human Ero1α Ser145 is Cys150 in yeast Ero1p, by BLAST sequence alignment (new Fig 2D). Mutation of Cys150 resulted in elevated oxidase activity of Ero1p (Sevier et al, 2007), but reduction of the Cys150-Cys295 allosteric disulfide requires extremely reducing condition (Niu et al, 2016). By contrast, the regulatory disulfide Cys94-Cys131 in human Ero1α can be rapidly modulated by the redox states of human PDI (Zhang et al, 2014). In this paper, we report that Ero1α Ser145 can be phosphorylated under reductive stress to promote oxidative protein folding. Altogether, our findings suggest that human Ero1α is elegantly regulated to optimize the folding efficiency of large and complicated disulfide-containing secretory proteome. We have included this very interesting point in the Discussion section, and please also see our response to referee #3.

Homo sapine	137	LGAVDESLSEETQK	150
Macaca mulatta	137	LGAVDESLSEETQK	150
Bos Taurus	137	LGAVDESLSEETQK	150
Ovis aries	140	LGAVDESLSEETQK	153
Sus scrofa	137	LGAVDESLSEETQK	150
Rattus norvegicus	136	LGAVDESLSEETQK	149
Mus musculus	136	LGAVDESLSEETQK	149
Xenopus laevis	135	LSAVDESLSVETQE	148
Danio rerio	129	LGAVNGSLSDETRQ	142
Drosophila melanogaster	157	LGFLDTSISDQAHR	170
Caenorhabditis elegans	137	IDPMDRTLHDDEKR	150
Saccharomyces cerevisiae	149	L-------CQTSKK	155

Figure 2D. Amino acid sequence alignments of Ero1α homologues in several species by BLAST. Residue positions are indicated by numbers counted from the N-terminus. Human Ero1α Ser145 and its counterparts are shown in red.

Referee #2:

In their study entitled Secretory kinase Fam20C tunes endoplasmic reticulum redox via phosphorylation of Serine145 of Ero1a Zhang et al. discover and analyze a new role for Fam20C as a regulator of Ero1a function, and thus ER redox homeostasis. The study is of high and general interest and very well performed. Before publication, however, the following points need to be addressed:

We thank the referee for his/her positive notes.

Major points:

1. In Figure EV1, the authors should also show a blot of cell lysates for the k/o cell lines; also: an antibody against the C-terminus is maybe not ideally suited, as it would miss stable Fam20C fragments potentially generated by the INDELs

Fam20C is more abundant in extracellular spaces (Wang et al, 2010), thus it is difficult to detect Fam20C in the cell extracts. Nevertheless, as a glycoprotein, Fam20C could be enriched from the culture medium by Concanavalin A (ConA), a lectin which binds to mannosyl and glucosyl residues of polysaccharides and glycoproteins. Because the N-terminal is rather conserved in Fam20 family proteins, we generate the anti-Fam20C antibody against the unique C-terminal peptide.

By DNA sequencing, we were able to identify the INDELs in *FAM20C* KO cells. All 4 copies of truncated Fam20C proteins are fragments N-terminal to the kinase domain, and should have no kinase activity. Similar CRISPR/Cas9 knockout were done in HepG2 cells using the same sgRNA, though the INDELs were different with our cells (Tagliabracci et al, 2015).

2. How do the authors explain the difference in ER redox for clones 3/5 in Figure 1D? This also relates to the concern raised above.

Statistical analysis showed that the clones 3 and 5 of *FAM20C* KO cells are different in ER redox with a *p*-value of 0.036. This could be due to the heterogeneity of different cell clones.

3. In Figure 1G, why do the Ero1a input levels decrease upon Fam20C expression?

We carried out this experiment for four times, and as shown in a more representative blot (new Figure 1G), overexpression of Fam20C didn't decrease the endogenous Ero1α level. Please also see our response to referee #1, point 4.

4. The conclusions from Figure 3B-D are not entirely clear: does only secreted Ero1a become phosphorylated by Fam20C in the absence of Fam20C / Ero1a & Fam20C overexpression? How is this reconciled with the proposed regulatory role? The same question applies to the IF data (Figure 3E&F): Can co-localization/Ero1a phosphorylation also be observed in the absence of overexpression? What effect does overexpression of a highly disulfide-bonded secreted protein in the ER/changing the ER redox conditions by small molecules have on Ero1a phosphorylation?

We thank the referee for this critical point. In the absence of Fam20C / Ero1α & Fam20C overexpression but with Ero1α overexpression, p-Ero1α can be detected both in cell extracts and culture medium, by using immunoprecipitation and ConA enrichment, respectively (Fig 2E). In the mammary gland of lactating mice with abundant Fam20C expression, we also

succeeded in detecting endogenous p-Ero1α. These experiments suggest that the phosphorylation of Ero1α is *bona fide* and not due to the artificial expression of a kinase. Unfortunately, in our cultured cell lines, it was difficult to detect endogenous p-Ero1α by using our anti-p-Ero1α polyclonal antibody. Thus, to study the regulatory role of Ero1α phosphorylation, we examined the p-Ero1α levels in Fam20C-expressing HeLa cells under either hypoxic condition or DTT treatment. We observed that p-Ero1α was largely induced under these conditions (new Fig 8D-E), implying that p-Ero1α plays a role in dealing with cellular reductive stress. We believe these new results are important to broaden the physiological relevance of Ero1α phosphorylation by Fam20C. Please also see our response to referee #1, point 6.5.

5. In Figure 3H, upper lane: the blots show bleached out bands at the height of p-Ero1a; furthermore: how do the authors explain multiple bands observed in some samples for Ero1a (second lane)?

We have replaced the blots with a short exposure (new Fig 8B and C). In our hands, the 2G4 mouse monoclonal antibody for Ero1α works well and recognizes a single band in many cultured cell lines. For tissue samples from mice, we sometimes saw an additional band in the upper. However, this shortage of the antibody does not interfere with our conclusion that both Ero1α and p-Ero1α are upregulated during lactation.

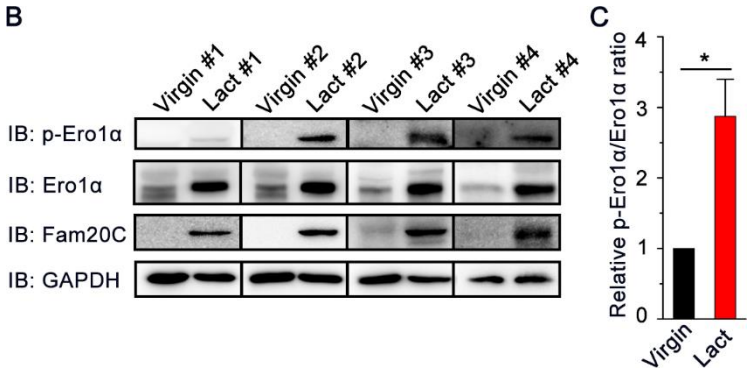


Figure 8. p-Ero1α is induced during mammalian lactation, hypoxia and reductive stress.

B Protein immunoblotting of extracts from the whole mammary glands of virgin and lactating mice.

C Quantification of relative p-Ero1α/Ero1α ratio in (B). Data are shown as mean ± SEM of four groups. * $p < 0.05$ (two-tailed, student's *t*-test).

6. The quantification in Figure 4 seems problematic, as also dimers/HMW species are oxidized. Furthermore, loading in A seems uneven between the gels (more sample each each gel, going from left to right) and unspecific bands around 35 kDa are much more pronounced in A than in C. The quantification problem becomes even more pronounced in 4C & D, where for Fam20C DA more dimers seem to be present, also representing oxidized species. In general, faster oxidation is apparent as the authors claim - a quantification should also be performed, however, including oligomeric species in EV/or the answer to this review. Furthermore, immunoblots in C/D seem indicated to show the amount of phosphorylated Ero1a.

The J chain refolding experiments have been performed for more than four times, and the

patterns of J chain oxidation are repeatable. We have now done new experiments using an inactive Ero1α mutant C99A/C104A as the negative control instead of empty vector to ensure similar expression of JcM among different groups (new Fig 7A). The amounts of Ero1α/p-Ero1α levels were included as new Fig 7C.

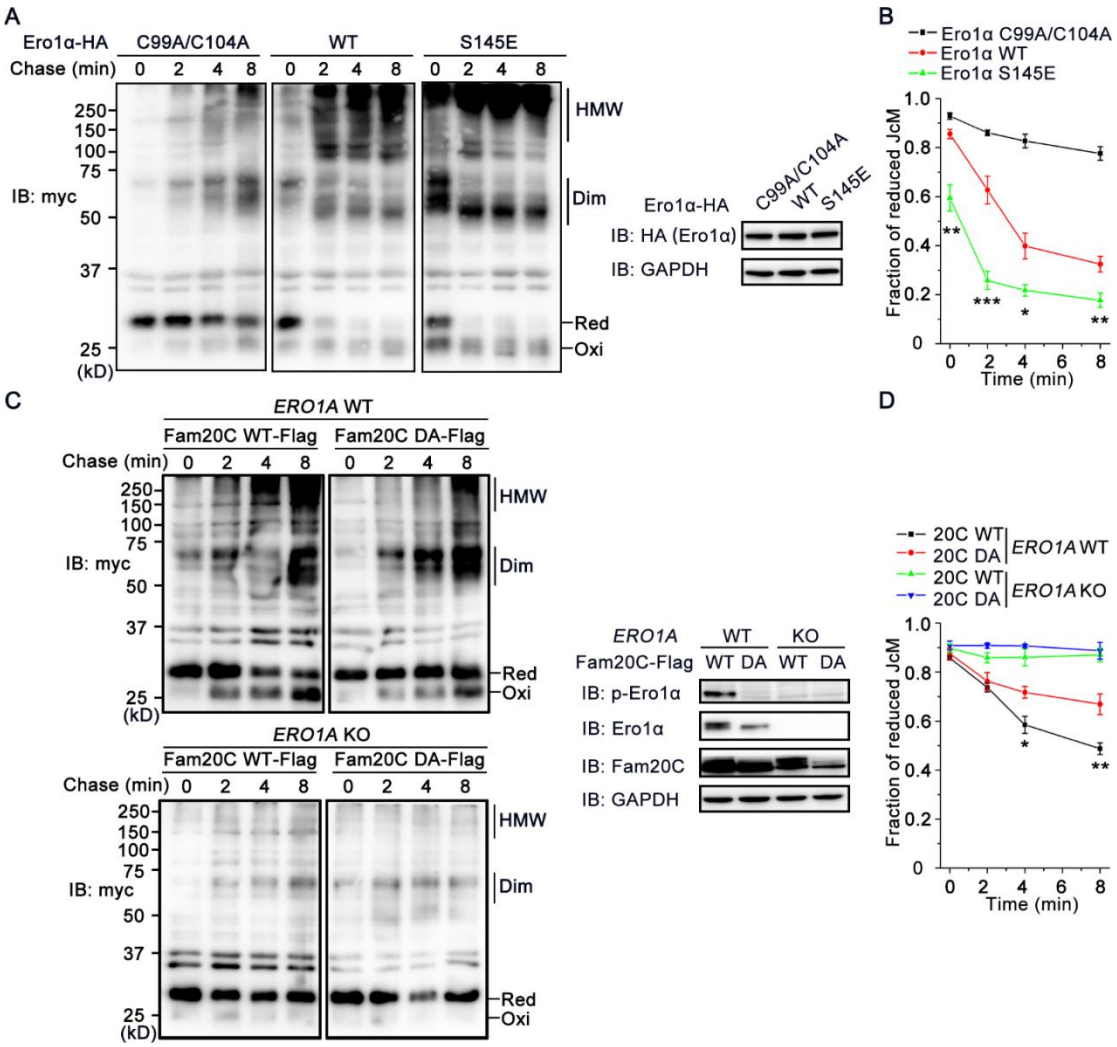


Figure 7. Phosphorylation of Ero1α promotes oxidative protein folding in cells.

A *(Left)* HeLa transfectants expressing myc-tagged JcM with Ero1α-HA C99A/C104A, WT or S145E were pulsed with DTT, washed, and chased at indicated time points by non-reducing myc blotting. The mobility of reduced JcM monomers (Red), oxidized monomers (Oxi), homodimers (Dim), and high-molecular-weight (HMW) species is indicated. (*Right*) Aliquots from cell lysates in the left panel were resolved in reducing conditions and analyzed by immunoblotting.

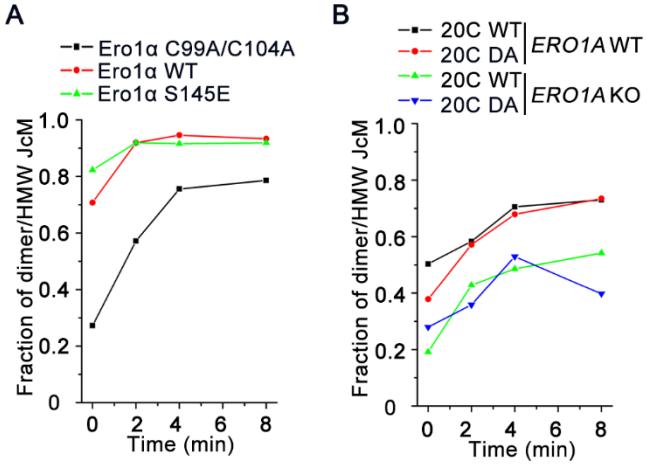
B The fraction of reduced JcM (Red/[Red + Oxi]) in (A) was quantified by densitometry. Data are shown as mean ± SEM from five independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, between Ero1α WT and S145E (two-tailed student's *t*-test).

C *(Left)* JcM re-oxidation in WT and *ERO1A* KO HeLa cells overexpressing Fam20C-Flag WT or DA was monitored as in (A). (*Right*) Aliquots from cell lysates in the left were resolved in reducing conditions and analyzed by immunoblotting.

D The fraction of reduced JcM in (C) was quantified by densitometry. Data are shown as

mean ± SEM from four independent experiments. * $p < 0.05$, ** $p < 0.01$, (two-tailed student's *t*-test).

Although the dimers/HMW species somehow varied from each experiment, the oxidation of monomer species is reproducible. Thus, usually the amounts of red/oxi monomers are quantified to represent the re-oxidation of J chain (Wang et al, 2014) (Masui et al, 2011). Here, we also made quantification of dimers/HMW species according to the referee's suggestions (Response Figure 8). This additional quantification does not change our conclusion that phosphorylation of Ero1α Ser145 by Fam20C does promote oxidative protein folding in cells.



Response Figure 8. Phosphorylation of Ero1α accelerates JcM refolding process.
The fraction of dimers/HMW species of JcM in Fig 7A and C were quantified by densitometry shown in (A) and (B), respectively.

Minor points:

7. The manuscript needs some language editing, some articles are missing, some minor errors are in the text; some unclear expressions need to be taken care of, e.g. "displayed ratiometric fluorescence in the ER"

We have made further language editing with the help from a native English speaker. We added (Mezghrani et al, 2001) (Tu & Weissman, 2002) (Gross et al, 2006) (Wang et al, 2009) (Sevier et al, 2007) in the Introduction section. We are sorry for not including many other excellent papers in this field due to the space limit.

We have revised the corresponding text as "The ratio of fluorescence intensity at 390/465 nm excitation of the superfolded-roGFP-iE$_{ER}$ increased under oxidizing conditions and decreased under reducing conditions (Fig EV2)".

8. In the abstract, the sentence "..the role of Fam20C..is largely unknown" is not clear; its role in the ER lumen? For proteins of the ER lumen?

We have made corrections, "…the role of Fam20C in regulating proteins in the endoplasmic reticulum (ER) lumen is largely unknown.".

9. The expression "lumen of the secretory pathway" is awkward and should be changed to ER.

We changed the expression as "the ER lumen".

10. What is the difference between Figure EV1B and the blot in Figure 1D?
The blot in Figure EV1B is from WT and *FAM20C* KO HeLa cells. The blot in Fig 1D is from those cells expressing the superfolded-roGFP-iE$_{ER}$ probe.

11. What is the difference between lane 2&3 in Figure EV3A?
They were duplicate loading for collecting enough Ero1α protein.

12. It is not always clear from the figures & legends if/how constructs were epitope-tagged.
Essentially, the epitopes were labelled according to their location in protein primary sequence. For the detailed description on how epitopes were tagged, please see the Methods.

13. The role of Fam20A, being part of Figure 3G, should be explained a bit more.
We have now revised the sentence to "Fig 8A shows that mRNA levels of *FAM20C* and its activator *FAM20A*, which encodes a pseudokinase that forms a functional complex with Fam20C to enhance secretory protein phosphorylation, were dramatically elevated in mammary glands of lactating mice compared to those of virgin mice, consistent with the previous report (Cui et al, 2015)."

14. What is the difference between Figure EV6B & the blot in 6A?
The blot in Figure EV6B is from WT and *ERO1A* KO HeLa cells. The blot in Fig 6A is from those cells expressing the superfolded-roGFP-iE$_{ER}$ probe.

15. In the discussion, the authors state "phosph. of Ero1a increased the motion of the outer active site-containing loop"; where was this shown?
"Phosphorylation of Ero1α increased the motion of the outer active site-containing loop" is supported by an increase in intrinsic tryptophan fluorescence in Ero1α S145E (Figure EV5E), implying that microenvironment changes occur around the aromatic residues. However, we realize that experimental evidence to localize the change in conformation that causes the change in tryptophan fluorescence is lacking. We have deleted this sentence, and made further discussion as "Instead, yeast Ero1p harbors Cys150 in the same position, and disruption of the Cys150-Cys295 allosteric disulfide is known to enhance the movement of outer active site-containing loop and increase Ero1p activity (Sevier et al, 2007), though physiological reduction of the Cys150-Cys295 disulfide requires extremely reducing condition (Niu et al, 2016). The homology between Cys150 in Ero1p and Ser145 in Ero1α further suggests that modification of Ser145 is likely to cause a similar conformational change and enzymatic enhancement." Please also see our response to referee #3.

16. The discussion about Ero1 phosphorylation/Erp44-mediated recruitment to the ER is misleading as also non-phosphorylated Ero1a appears to be recruited by Erp44, and this had been known beforehand.
In the last version, we wrote "It is known that the ER retention of Ero1α is accomplished by ERp44, a PDI family chaperone ensuring ER retrieval via KDELR in the early secretory pathway (Anelli et al, 2003; Vavassori et al, 2013; Wang et al, 2008)". To avoid any

misunderstanding, we revised in the Discussion as “Similar to non-phosphorylated Ero1α, phosphorylated Ero1α is relocated to the ER lumen, also mediated by the ERp44/KDELR system, to promote disulfide bond formation and maintain ER redox homeostasis (Fig 8G).”

17. Proteomic studies do exist on plasma cell differentiation. Are Ero1a/Fam20C upregulated in these? This could be discussed.
Thanks for this interesting point. The database on plasma cell differentiation shows that Ero1α is upregulated by about 3-fold (Bakunts et al, 2017; Romijn et al, 2005), but no Fam20C expression in those data. Whether Fam20C is involved in plasma cell differentiation is still an open question. In Discussion, we wrote “Whether Ero1α phosphorylation is also enhanced in other secretory cells, such as plasma cells secreting immunoglobulins and β-cells secreting insulin, and is important for efficient oxidative protein folding in those cells are still open questions.”

Referee #3:

This manuscript reports the interesting observations that Fam20C phosphorylates Ero1 and that phosphorylation affects Ero1 activity and the redox state of the ER. Overall, the authors make a thorough case for these claims. Nevertheless, this reviewer raises a few points for consideration or clarification.
Thanks for the positive comments.

1. It is not clear from the details of the methods provided what is the basis of the Fam20C "interactome." There is no reason for an enzyme to remain stably associated with its substrates, and certainly not through an IP. On what do these interactions depend? The IPs were presumably done under oxidizing conditions? Is the Ero1 disulfide bonded to Fam20C in the IP? What detergent was used for lysis? Most importantly, the pcDNA vector used to express flag-tagged Fam20C in the ER is likely to massively over-produce the protein. Approximately by what factor is it over-expressed compared to endogenous levels? This reviewer considers it likely that IP of any protein over-expressed in the ER will pull down other abundant ER proteins due to non-specific association. How can the authors rule out this possibility? Evidence presented in the manuscript showing that Fam20C phosphorylates Ero1 is stronger than the evidence that these proteins naturally form a stable interaction.
Thanks for these comments. We agree with the referee that evidence presented in the manuscript showing that Fam20C phosphorylates Ero1α is stronger than the evidence that these proteins naturally form a stable interaction. We did not mean that Ero1α forms a stable complex with Fam20C. However, it is possible that Ero1α physically interacted with Fam20C during the phosphorylation reaction, a similar case to the previous study (Wang et al, 2017) .

In our experiments, the cells were lysed by using RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA, Millipore) containing 1 mM phenylmethanesulfonyl fluoride (PMSF), phosphatase inhibitor cocktail (Roche) and protease inhibitor cocktail (Roche), and the IPs were done without any reducing or oxidizing agent. We did not check if Ero1 was disulfide bonded to Fam20C in the IP.

It is true that by using pcDNA vector Fam20C proteins were over-expressed largely excess to the endogenous level. Although IP of any protein over-expressed in the ER could pull down other abundant ER proteins due to non-specific association, the interaction between Ero1α and Fam20C is specific because expression of Fam20A in HeLa cells did not pull down Ero1α (data not shown). On the other hand, the result that 'protein processing in the ER' pathway is enriched in the Fam20C interactome implies that processing and maturation of Fam20C itself is supervised by the ER quality control system. We are now studying on this aspect.

2. In addition to lacking many details, there are also errors in the IP methods: "The cell extracts were incubated with anti-Flag M2 affinity gel (Sigma-Aldrich) overnight at 4 ℃ for 2 h with occasional vortexing." Was the incubation overnight or for two hours?
We are sorry for this carelessness. The incubation was overnight. We have changed this sentence to "The cell extracts were incubated with anti-Flag M2 affinity gel (Sigma-Aldrich) overnight at 4 ℃ with occasional vortexing."

3. The authors give the number of ER and Golgi proteins identified, but not the number of cytosolic and other proteins.
A total of 1876 proteins were identified to be co-IPed with Fam20C, and 349 of which were located in the ER and Golgi. We have now added these information in the Methods.

4. p. 6 "MS analysis identified only 1 phosphopeptide 137LGAVDESLpSEETQK150 (Fig2A and B) with 95.7% sequence coverage." To avoid confusion, a better presentation of these data would be: "MS analysis yielded 95.7% sequence coverage and identified only 1 phosphopeptide 137LGAVDESLpSEETQK150 (Fig2A and B)."
Thanks. We have made this correction as "MS analysis yielded 95.7% sequence coverage and identified a single phosphopeptide ^{137}LGAVDESLpSEETQK^{150} (Fig 2A and B)."

5. p. 6 "Ser145 is adjacent to the outer active site-containing flexible loop (Fig2C)..." Figure 2C does not show proximity of Ser145 to the outer active site. The reader expects to see a structure image showing proximity (such as Figure 5A or Figure EV5). Furthermore, Ser145 is about 32 Å from cysteine 94. Is this distance considered "adjacent"?
"Ser145 is adjacent to the outer active site-containing flexible loop" does not mean "Ser145 is adjacent to Cys94". We revised this sentence as "Ser145 is C-terminal to the outer active site-containing flexible loop (Fig. 2C)".

6. p. 6 "dependent manner, confirmed by the slowly migrating bands and by p-Ero1α blotting." Is a substantial shift in migration after 30 minutes in Fig. 3A expected for the addition of a single phosphate group? Are these gels run under oxidizing or reducing conditions?
These gels were run under reducing conditions, therefore the slow migration is not due to reduction of long-range disulfides. In this *in vitro* kinase assay, we cannot exclude the possibility that additional serine residues besides Ser145 were also phosphorylated and contributed to the substantial shift in migration. As we wrote in the Discussion, "Whether

Ero1α possesses other phosphorylation sites besides Ser145, the manner by which these phosphorylation sites orchestrate to regulate ER redox, and the identity of the protein phosphatase for dephosphorylating Ero1α remain open questions."

7. p. 7 The point that the authors are trying to make regarding co-localization is not clear from Fig. 3E and 3F. Tagged Fam20C seems to show Golgi localization, whereas Ero1a seems to show ER localization. Indeed, later in the manuscript the authors make this same point, and this is what is shown in Fig. 4B (the authors' claim that Ero1a is also detected in the Golgi is not convincing).

We thank the referee for this critical point. We have now performed cell fractionation experiment to provide more evidences that Ero1α is also located in the Golgi. As shown in new Figure EV4A, Golgi and ER fractions were separated by density gradient centrifugation, and p-Ero1α was detected in both Golgi and ER fractions with a pattern similar to that of total Ero1α. The fact that endogenous Ero1α can be detected in the Golgi has also been reported previously (Gilady et al, 2010). We revised in the text, "In cells, we observed p-Ero1α in both the ER and the Golgi lumen by immunofluorescence, using PDI and GM130 as respective markers (Fig 4B), and also by subcellular fractionation (Fig EV4A).". We added in the Discussion, "Indeed, engineered Ero1α-KDEL protein is more resistant to phosphorylation by Fam20C (Fig 4C). Although it is possible that Fam20C is active during its transit route across the ER, we propose that a rather unique milieu in the Golgi is appropriate for Fam20C activity."

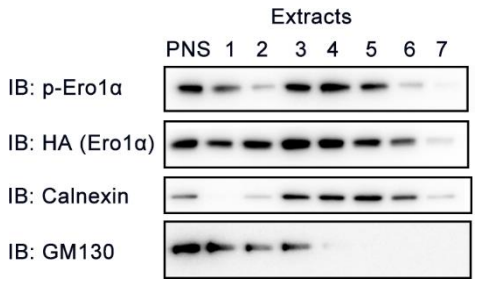


Figure EV4A Subcellular fractionation of HeLa cells. The postnuclear supernatant (PNS) of HeLa cells expressing Ero1α-HA and Fam20C-Flag was separated on a 30% Percoll gradient and the fractions were collected and subjected to protein immunoblotting. Calnexin, ER marker; GM130, Golgi marker.

8. p. 8 "to further restrict its traffic to the Golgi apparatus" is not clear. Do the authors mean that they intended to enhance ER localization of Ero1a and to decrease the amount that resides in the Golgi at steady state?

We have revised this sentence as "This engineering would enhance ER localization of Ero1α and decrease the amount that traverse the late secretory pathway."

9. p. 8 "The Ero1α phosphorylation mimic S145E displayed approximately 3 times the oxidase activity of Ero1α WT in the presence of either the small molecule reducing agent DTT..." The authors show in Figure 5B that DTT bypasses the outer active site and directly reduces the inner active site. However, the authors state on page 9, "Elevation of activity

caused by Ser145 phosphorylation still depended on the presence of the Ero1α outer active site (Fig EV5A), implying that phosphorylation does not alter the electron transfer pathway." There is an inconsistency here.

We thank the referee for this reminding. We revised this sentence as “Acceleration of PDI oxidation caused by Ser145 phosphorylation still depended on the presence of the Ero1α outer active site (Fig EV5A), implying that phosphorylation does not alter the electron transfer pathway.”

10. p. 11 It is true that yeast Ero1 does not have a clear analog of Ser145. However, this serine in the mammalian enzyme is present in the same region of space relative to the active site as the set of cysteines that affect the catalytic activity of yeast Ero1 when mutated. Perhaps this fact is worth mentioning.

We thank the referee for this insightful point. Indeed, we found that the counterpart of human Ero1α Ser145 is Cys150 in yeast Ero1p, by BLAST sequence alignment (new Fig 2D). In yeast Ero1p, Cys150 forms a disulfide bond with Cys295 at resting state, and mutation of Cys150 resulted in elevated oxidase activity of Ero1p (Gross et al, 2004; Sevier et al, 2007). The Cys150-Cys295 bond together with Cys143-Cys166 bond, constrain the outer active site-containing polypeptide to conformations different from those observed when the disulfides are removed (Heldman et al, 2010). The homology between Cys150 in Ero1p and Ser145 in Ero1α further suggests that modification of Ser145 is likely to cause similar conformational change and enzymatic enhancement. Besides previous reports on the regulatory disulfides of Ero1α, our finding that Ero1α can be phosphor-regulated via Ser145 adds an additional layer to the regulation of Ero1α activity.

We have included this very interesting point in the Discussion section, “Interestingly, Ser145 in Ero1α is highly conserved in multicellular organisms but missing in yeast Ero1p (Fig 2D), and Fam20C homologue is not present in yeast, implying that Ero1p is lack of phosphorylation regulation. Instead, yeast Ero1p harbors Cys150 in the same position, and disruption of the Cys150-Cys295 allosteric disulfide is known to enhance the movement of outer active site-containing loop and increase Ero1p activity (Sevier et al, 2007), though physiological reduction of the Cys150-Cys295 disulfide requires extremely reducing condition (Niu et al, 2016). The homology between Cys150 in Ero1p and Ser145 in Ero1α further suggests that modification of Ser145 is likely to cause a similar conformational change and enzymatic enhancement. Besides previous reports on the regulatory disulfides of Ero1α, our finding that Ero1α can be phosphor-regulated via Ser145 adds an additional layer to the regulation of Ero1α activity.” Please also see our responses to referee #1 (point 9) and referee #2 (point 15).

11. p. 11 "Phosphorylation of Ero1α increased the motion of the outer active site-containing loop" is presented as a fact, but it is actually a speculation. The data supporting this speculation appears to be the change in tryptophan fluorescence, but no experiments are presented to localize the change in conformation/dynamics that causes the change in tryptophan fluorescence.

Yes, "Phosphorylation of Ero1α increased the motion of the outer active site-containing loop" is a speculation and has been deleted in the revised version. As we mentioned above, the

homology between Cys150 in Ero1p and Ser145 in Ero1α further suggests that modification of Ser145 is likely to cause similar conformational change and enzymatic enhancement. We have included this point in the Discussion section. Please also see point 10 above.

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2nd Editorial Decision 18th April 2018

Thank you for submitting a revised version of your manuscript. It has now been seen by two of the original referees whose comments are shown below.

As you will see they both find that all criticisms have been sufficiently addressed and recommend the manuscript for publication. However, before we can go on to officially accept the manuscript there are a few editorial issues concerning text and figures that I need you to address in a final revision.

REFEREE REPORTS

Referee #1:

This well written and concise paper convincingly show s that phosphorylation of Ero1a by Fam20C promotes oxidative protein folding in the ER. In this revised version, the authors answered satisfactorily to all the criticisms of the reviewers. The paper should hence be published in the EMBO J

Referee #2:

The authors have carefully addressed all my concerns and substantiated their study with even further data. As such, this reviewer considers the manuscript now ready for publication.

EMBO PRESS

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Lei Wang
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2017-98699

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We generally do experiments for at least three times except for Figure 8 D-F, which are representitives of two independent experiments. For superfolded-roGFP-iE fluorescence measurements, six replicates were carried out in each assay.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples or animals were exluded from this study.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No blinding was used in this study.
For animal studies, include a statement about randomization even if no randomization was used.	No blinding was used in this study.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No blinding was used in this study.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was used in this study.
5. For every figure, are statistical tests justified as appropriate?	Yes, information on statistical tests is included in the figure legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Data were analysed by two-tailed student's t-test or one-way ANOVA, the post hoc Tukey's HSD test.
Is there an estimate of variation within each group of data?	Standard error of Mean (SEM) for more than three independent experiments were indicated in the figure legends.
Is the variance similar between the groups that are being statistically compared?	Yes, significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) were indicated in the figure legends.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	This information has been included in the Materials and Methods. The anti-p-Ero1 and anti-Fam20C polyclonal antibodies generated in this study have been validated as shown in Figure EV1 and Figure EV3.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All cell lines used were tested for mycoplasma contamination and confirmed as negative for experimental analysis.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	This information is included in the Materials and Methods, mouse mammary gland protein and RNA isolation.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All mice experiments were conducted in compliance with the guidelines for the care and use of laboratory animals and approved by the Institutional Biomedical Research Ethics Committee of the Institute of Biophysics, Chinese Academy of Science.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	It is compliant with the guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD009333.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No