Kindlin-1 protects cells from oxidative damage through activation of ERK signaling

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Abstract: Kindlin-1 is a FERM domain containing adaptor protein that is found predominantly at cell-extracellular matrix adhesions where it binds to β-integrin subunits and is required for integrin activation. Loss of function mutations in the FERMT1 gene which encodes Kindlin-1 leads to the development of Kindler Syndrome (KS) an autosomal recessive skin disorder characterized by skin blistering, photosensitivity, and predisposition to aggressive squamous cell carcinoma (SCC). Here we show that loss of Kindlin-1 sensitizes both SCC cells and keratinocytes to oxidative stress: Kindlin-1 deficient cells have higher levels of reactive oxygen species, decreased viability and increased DNA damage after treatment with either hydrogen peroxide (H2O2) or irradiation with UVA. We show that Kindlin-1 is required to fully activate ERK signalling after oxidative damage, and that activation of ERK protects cells from DNA damage following oxidative stress: inhibition of ERK activation sensitizes Kindlin-1 expressing cells, but not Kindlin-1 deficient cells to oxidative stress. Finally we demonstrate that the Kindlin-1 dependent activation of ERK and protection from DNA damage following oxidative stress depends on the ability of Kindlin-1 to bind integrins. Thus loss of Kindlin-1 leads to an imbalance in the cellular oxidative state, which renders Kindlin-1 deficient cells more prone to the effects of ROS generated in response to oxidative stress. We propose that Kindlin-1 dependent activation of ERK signaling is a key molecular mechanism that renders KS keratinocytes more sensitive to oxidative damage and contributes to the increased photosensitivity in KS patients.

Response to reviewers

Reviewer 2

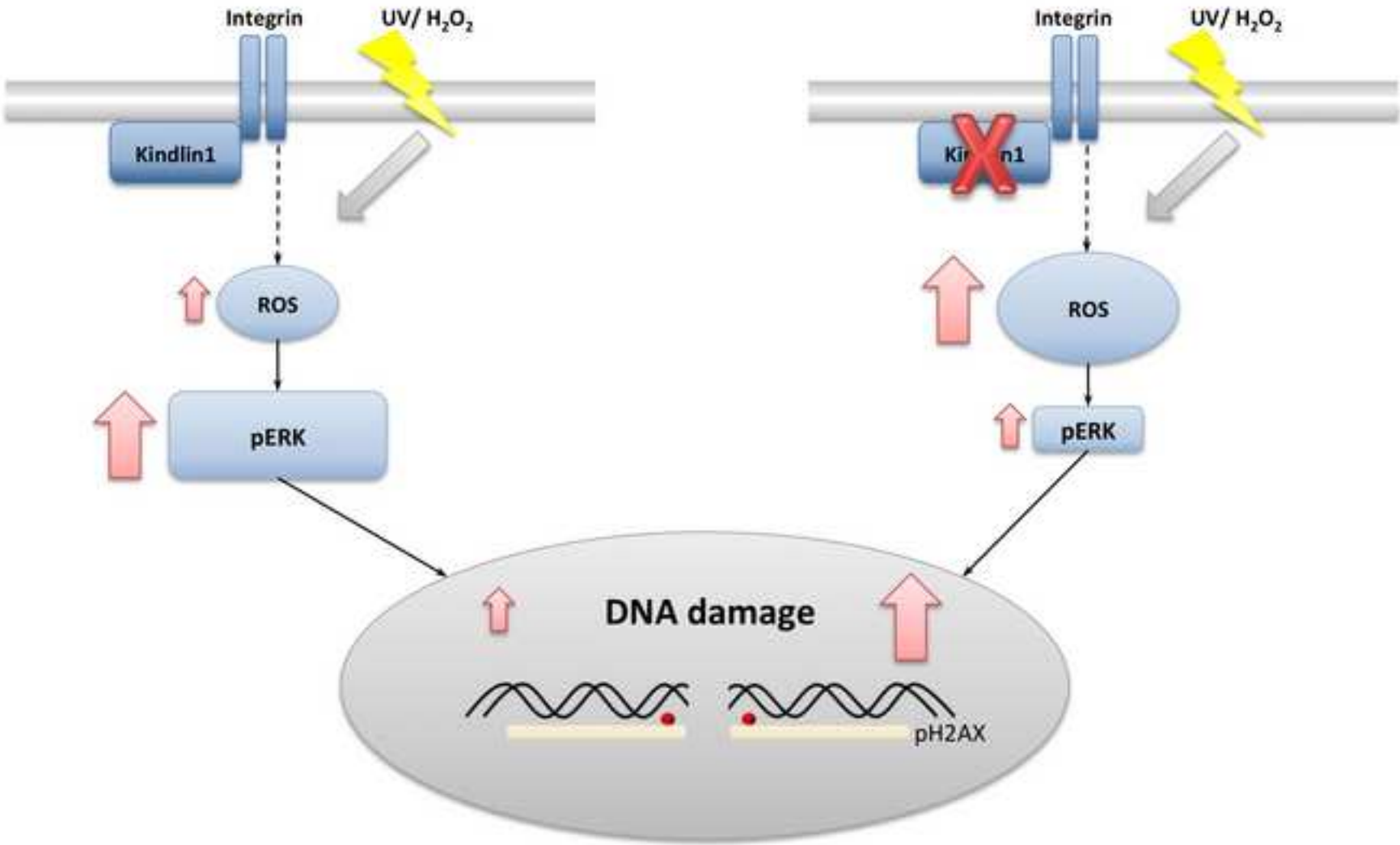
Page 3 - we have changed the wording to "The ratio of GSH over GSSG is therefore an important marker of the oxidative state of the cell. GSH/GSSG ratios are lower in KS derived keratinocytes…

This is now consistent throughout the text where GSH/GSSG ratio is always used.

Highlights

- Kindlin-1 deficiency sensitizes cells to oxidative stress
- Kindlin-1 loss leads to increased ROS and DNA damage following oxidative stress
- Kindlin-1 is required to fully activate ERK signaling following oxidative stress
- Kindlin-1 binding to integrins is required to counter oxidative stress

*Graphical Abstract



Kindlin-1 protects cells from oxidative damage through activation of ERK signaling

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Abbreviations: DCF, 2',7'-dichlorofluorescin diacetate; ERK, extracellular signal-regulated kinase; GSH, glutathione; GSSG, glutathione disulfide; H_2O_2, hydrogen peroxide; KS, Kindler Syndrome; RPPA, reverse phase protein array; ROS, reactive oxygen species; SCC, squamous cell carcinoma

Abstract

Kindlin-1 is a FERM domain containing adaptor protein that is found predominantly at cell-extracellular matrix adhesions where it binds to β-integrin subunits and is required for integrin activation. Loss of function mutations in the *FERMT1* gene which encodes Kindlin-1 leads to the development of Kindler Syndrome (KS) an autosomal recessive skin disorder characterized by skin blistering, photosensitivity, and predisposition to aggressive squamous cell carcinoma (SCC). Here we show that loss of Kindlin-1 sensitizes both SCC cells and keratinocytes to oxidative stress: Kindlin-1 deficient cells have higher levels of reactive oxygen species, decreased viability and increased DNA damage after treatment with either hydrogen peroxide (H_2O_2) or irradiation with UVA. We show that Kindlin-1 is required to fully activate ERK signalling after oxidative damage, and that activation of ERK protects cells from DNA damage following oxidative stress: inhibition of ERK activation sensitizes Kindlin-1 expressing cells, but not Kindlin-1 deficient cells to oxidative stress. Finally we demonstrate that the Kindlin-1 dependent activation of ERK and protection from DNA damage following oxidative stress depends on the ability of Kindlin-1 to bind integrins. Thus loss of Kindlin-1 leads to an imbalance in the cellular oxidative state, which renders Kindlin-1 deficient cells more prone to the effects of ROS generated in response to oxidative stress. We propose that Kindlin-1 dependent activation of ERK signaling is a key molecular mechanism that renders KS keratinocytes more sensitive to oxidative damage and contributes to the increased photosensitivity in KS patients.

1. Introduction

Kindlin-1 is one of three closely related proteins (Kindlin-1, -2 and -3) that are present in the human genome consisting primarily of a **f**our.1, **e**zrin, **r**adixin and **m**oesin (FERM) domain split by a pleckstrin homology domain (1). They play a key role in connecting dynamic actin regulation with membrane rearrangements at the leading edge of motile cells through their ability to regulate integrin activation by binding integrin β-subunits (2,3).

Loss of function mutations in the *FERMT1* gene, which encodes Kindlin-1 leads to an autosomal skin disorder called Kindler Syndrome (KS) (4-7). Patients with KS show photosensitivity and skin blistering in sun-exposed areas, especially during childhood (4,8,9). The skin is constantly exposed to UV irradiation and chemical stressors, which can provoke a toxic production of reactive oxygen species (ROS) (10-12). ROS can deplete and damage non-enzymatic and enzymatic antioxidant defence systems of the skin and therefore lead to oxidative stress and photosensitivity (13). In addition these oxidative stress conditions can lead to molecular modifications such as lipid peroxidation, protein oxidation, and DNA damage (14), and induce diverse physiological dysfunctions from tumourigenesis to ageing (15-17). To counteract oxidative stress, cells have developed various anti-oxidative mechanisms. Glutathione (GSH) is one of the most important antioxidants and scavenges ROS directly or as co-factor of the glutathione and thioredoxin systems whereby it is oxidized to glutathione disulfide (GSSG) (18,19). The ratio of GSH over GSSG is therefore an important marker of the oxidative state of the cell. GSH/GSSG ratios are lower in KS derived keratinocytes and in addition levels of ROS and malondialdehyde, which is a lipid peroxidation marker, are also elevated in KS derived keratinocytes (20,21). Together this indicates that the sensitivity observed in

KS skin towards UV results from an inability of Kindlin-1 deficient cells to effectively counteract oxidative stress.

To further understand the role of Kindlin-1 in controlling the cellular response to oxidative stress we have used two models in which we have genetic deletion of Kindlin-1. Firstly, immortalized human keratinocytes derived from a patient harbouring a known *FERMT1* mutation (c.676insC/c.676insC) (22), and secondly, a squamous cell carcinoma (SCC) model in which tumours were established in K14CreER^{T2}Kin1$^{flox/flox}$ mice following induction of a chemically induced skin carcinogenesis protocol allowing genetic deletion of *Fermt1* (23). Although many functions of Kindlin-1 are dependent on its ability to regulate integrin activation additional integrin-independent roles have been identified (24) and here we also asked whether Kindlin-1 protects cells from oxidative stress in an integrin-dependent manner.

2. Results

2.1. Kindlin-1 regulates redox homeostasis

We first set out to determine whether the loss of Kindlin-1 in the SCC model (SCC-Kin1-/-) resulted in an imbalance in redox homeostasis as reported in keratinocytes from KS patients. Wild-type Kindlin-1 was re-expressed to generate SCC-Kin1WT cells to determine Kindlin-1 dependent effects (Fig. 1A). A 2',7'-dichlorofluorescin diacetate (DCF) assay was used to determine the intracellular ROS concentrations. Basal levels of ROS were very low in both cell lines but increased following H_2O_2 treatment. ROS concentrations were significantly higher (Fig. 1B) in SCC-Kin1-/- cells compared to the SCC-Kin1WT cells. We then examined the oxidative state of SCC-Kin1-/- and SCC-Kin1WT cells by looking at the ratio of GSH

over GSSG. Measurement of GSH/GSSG ratio showed that SCC-Kin1WT cells had a higher basal GSH/GSSG ratio compared to SCC-Kin1-/- (Fig. 1C). As expected treatment with H_2O_2 led to complete depletion of reduced GSH and therefore a reduction in the GSH/GSSG ratio in both SCC-Kin1-/- and SCC-Kin1WT cells. Taken together these results demonstrate, that Kindin-1 deficient SCC cells have a higher endogenous oxidized state as characterized by their reduced GSH levels, as well as increased ROS concentrations following treatment with H_2O_2.

2.2. Kindlin-1 protects cells from oxidative stress

To determine whether Kindlin-1 plays a role in protecting cells from oxidative stress, cells were treated with H_2O_2 and cell viability measured. Treatment with H_2O_2 led to a significantly greater reduction of cell viability in Kindlin-1 deficient cells compared to SCC-Kin1WT cells (Fig. 2A) demonstrating that loss of Kindlin-1 renders cells more susceptible to H_2O_2 induced oxidative stress. Next, we sought to determine if Kindlin-1 not only affects cell viability, but is also needed for long term colony formation. Cells were treated with H_2O_2 to induce oxidative stress, and after 7 days colony formation quantified. Treatment with H_2O_2 resulted in a greater reduction of colony formation in SCC-Kin1-/- cells compared to SCC-Kin1WT cells (Fig. 2B). To determine whether these findings are specific to H_2O_2 or are due to induction of oxidative stress, we repeated the experiments using UVA instead of H_2O_2 to induce production of ROS and therefore oxidative stress. UV irradiation of cells reduced colony formation in the SCC-Kin1-/- cells to a greater extent than the SCC-Kin1WT cells (Fig. 2B).

To establish whether Kindlin-1 dependent sensitivity towards H_2O_2 treatment also occurs in keratinocytes wild-type Kindlin-1 (KS-Kin1WT) was re-expressed in

the KS patient derived keratinocytes (22). In KS keratinocytes, cell viability after H_2O_2 treatment was also reduced to a greater extent than in KS-Kin1WT keratinocytes (Fig. 2C). Furthermore analysis of colony formation in the keratinocytes also recapitulated the findings observed in the SCC cells: both treatment with H_2O_2 or irradiation with UVA led to a greater reduction of colony formation in KS keratinocytes compared to KS-Kin1WT cells (Fig. 2D). These results demonstrate that Kindlin-1 protects both SCC cells and keratinocytes from **oxidative** stress.

2.3. Kindlin-1 protects cells from DNA damage after oxidative stress

As oxidative stress can lead to molecular modifications such as lipid peroxidation, protein oxidation, and DNA damage (14), we asked whether loss of Kindlin-1 leads to accumulation of DNA damage following oxidative stress. SCC-Kin1-/- and SCC-Kin1WT cells were treated with H_2O_2 and phosphorylation of the histone H2AX, as a measure of DNA damage, was analyzed by immunofluorescence. Treatment with H_2O_2 led to an accumulation of nuclear phosphorylated H2AX (pH2AX) in both SCC-Kin1-/- and SCC-Kin1WT cells, but pH2AX levels were higher in SCC-Kin1-/- cells after treatment compared to SCC-Kin1WT cells (Fig. 3A, B). UV irradiation also led to a significantly greater increase in DNA damage in SCC-Kin1-/- cells compared to SCC-Kin1WT cells. KS keratinocytes also accumulated significantly more DNA damage after both H_2O_2 and UV irradiation than KS-Kin1WT keratinocytes (Figure 3C, D). Notably, in the SCC cells and keratinocytes, endogenous levels of DNA damage differed between the Kindlin-1 deficient cells and cells expressing Kin1WT, indicating that loss of Kindlin-1 leads to inherent differences in the levels of DNA damage.

2.4. Kindlin-1 regulates survival pathways after oxidative stress

As Kindlin-1 affects not only redox homeostasis, but also protects cells from oxidative stress, we next sought to investigate whether Kindlin-1 regulates survival signalling. We treated SCC-Kin1-/- and SCC-Kin1WT cells with H_2O_2 for 30 min, 1 h, or 4 h to induce oxidative stress and subsequently conducted a reverse phase protein array (RPPA) analysis with 169 antibodies to identify Kindlin-1-regulated pathways. After normalizing the signal intensities by global sample median normalization, we computed the fold change (FC) of treated SCC-Kin1WT cells over SCC-Kin1-/- cells for each antibody. To identify important Kindlin-1 regulated nodes we used a cut off of > 1.5 and FC < 0.75. The filtering resulted in 14 hits that we further ranked by pValue (Fig. 4A). The analysis showed that the top hit was phosphorylated extracellular signal-regulated kinase (pERK), which is up regulated after oxidative stress to a significantly higher extent in SCC-Kin1WT cells compared to SCC-Kin1-/- cells. Also up regulated in SCC-Kin1WT cells were pMSK1 and pRaf, both part of the ERK signaling cascade. These findings highlight the ERK signaling pathway as an important node in Kindlin-1 dependent signaling following oxidative stress.

To validate these results, we examined activation of ERK by western blot. In both SCC cells and KS keratinocytes, treatment with H_2O_2 led to an increased phosphorylation of ERK in Kindlin-1 expressing cells compared to Kindlin-1 deficient cells (Fig. 4B, C). Taken together, these results show that Kindlin-1 is required for optimal induction of the ERK signaling pathway following oxidative stress.

2.5. Kindlin-1 protects cells from oxidative stress by activating ERK signaling

We next wanted to establish whether the Kindlin-1-dependent activation of ERK was required to protect the cells from oxidative stress. To answer this question we used the MEK inhibitor U0126 to inhibit phosphorylation and activation of ERK. Treatment of cells with H_2O_2 led to increased phosphorylation of ERK, which was inhibited by U0126 (Fig. 5A).

Next we analyzed whether inhibition of the ERK pathway would affect colony formation (Fig. 5B, C). For this we performed a colony formation assay in SCC cells treated with H_2O_2 to induce oxidative stress and with or without U0126. H_2O_2 reduced colony formation of both SCC-Kin1-/- and SCC-Kin1WT cells. SCC-Kin1-/- cells were more sensitive to the treatment and colony formation was significantly reduced compared to SCC-Kin1WT cells (Fig. 5B). Treatment with both H_2O_2 and U0126 led to a decrease in colony formation compared to untreated cells, but showed no significant difference between SCC-Kin1-/- and SCC-Kin1WT cells. Taken together these results demonstrate that inhibiting ERK has no effect on colony formation following oxidative damage in Kindlin-1 deficient cells, but reduces this in Kin1WT expressing cells. Treatment of KS keratinocytes with both H_2O_2 and U0126 resulted in a similar reduction of colonies in both KS and KS-Kin1WT cells (Fig. 5C). This result indicates that treatment with U0126 sensitizes KS-Kin1WT cells but not SCC-Kin1-/- cells to H_2O_2 treatment. Thus defects in ERK signaling in Kindlin-1 depleted cells contribute to the increased sensitivity to oxidative damage. Finally, we wanted to confirm that the differences in colony formation reflect differences in DNA damage levels. We therefore looked at pH2AX intensity in SCC cells as well as KS keratinocytes treated with both H_2O_2 and U0126 (Fig. 5D, E). In both SCC cells and keratinocytes treatment with H_2O_2 led to a greater increase in DNA damage in

Kindlin-1 deficient cells compared to cells expressing Kin1WT. When treated with both H_2O_2 and U0126, levels of DNA damage did not differ between Kindlin-1 deficient and Kindlin-1 expressing cells, demonstrating that DNA damage is also regulated by Kindlin-1 dependent ERK activation.

2.6. Sensitivity to oxidative stress is dependent on the ability of Kindlin-1 to bind integrins

Finally we wanted to establish whether the Kindlin-1 dependent differences in sensitivity to oxidative damage is due to the ability of Kindlin-1 to bind integrins. We therefore used SCC cells expressing an integrin-binding deficient Kindlin-1 mutant (SCC-Kin1AA) (25) and performed a colony formation assay following treatment with H_2O_2 or UVA. Both H_2O_2 and UVA reduced colony formation in SCC cells, with the reduction in colony formation being greater in the Kindlin-1 depleted cells (Fig. 6A). Re-expression of the Kin1AA mutant did not restore colony formation to levels seen in the Kin1WT cells, indicating that the ability of Kindlin-1 to regulate colony formation following oxidative stress is dependent on its ability to bind integrins. Next we wanted to confirm that the integrin-dependent differences in colony formation mirrored the activation of ERK. To address this we used the SCC cells expressing the Kin1AA mutant to analyse ERK activation after oxidative stress. The results showed that only SCC-Kin1WT cells can fully activate ERK, while both SCC-Kin1-/- cells and SCC-Kin1AA cells showed reduced activation of ERK following oxidative stress (Fig. 6B). These results demonstrate that the ability of Kindlin-1 to bind integrins is crucial for activating ERK signaling and modulating colony formation after stress.

3. Discussion

Here we show that loss of Kindlin-1 leads to an imbalance in the cellular redox state with reduced levels of GSH and increased levels of ROS. This results in increased DNA damage and decreased colony formation following oxidative stress in both Kindlin-1 deficient keratinocytes and SCC cells. The increased ROS concentration in Kindlin-1 deficient cells could either be a result of increased production of ROS or a reduced ability to remove ROS. While reduction of ROS depends on ROS detoxification by antioxidative molecules or enzymes, acute ROS production is regulated by activation of NADPH oxidases (Nox) (26-30). Although there is no evidence that Kindlin-1 regulates Nox proteins our data support a role for Kindlin-1 in regulation of the GSH antioxidant pathway. Expression levels of γ-glutamyl cysteine ligase, which catalyzes the first rate limiting step for the synthesis of GSH are significantly reduced in KS keratinocytes as compared to normal keratinocytes (20) and we have found increased expression of γ-glutamyl-transferase, which catalyses the removal of γ-glutamyl groups from GSH, in SCC-Kin1-/- cells (data not shown). Furthermore, TGFβ activation can alter mitochondrial function and increase ROS levels (31) and the recent demonstration that Kindlin-1 can regulate TGFβ signaling (32,33) suggests that this may be an additional mechanism whereby Kindlin-1 can regulate the cellular redox state. Together this demonstrates that Kindlin-1 deficient cells are more prone to oxidative stress through an inability to counterbalance the effects of ROS production and indeed we show that in both SCC cells and keratinocytes loss of Kindlin-1 leads to a greater accumulation of DNA damage following treatment with H_20_2 or UV.

To counteract oxidative stress, cells have developed different anti-oxidative mechanisms, including activation of anti-oxidative signalling pathways. The

signalling pathways activated are dependent on the strength of the damage induced, and can be either pro-survival for continuous ROS detoxification and DNA damage repair or cell-death-inducing programs (34,35). It has previously been shown that Kindlin-1 is able to regulate signalling including the Wnt (1,32), TGFβ (32,33) and p38 pathways (21). Here we show for the first time that Kindlin-1 is needed to fully activate ERK signalling following both H_20_2 and UVA treatment. The ERK pathway is important for cell proliferation and survival (36-38), and we show that activation of the ERK pathway is dependent on Kindlin-1 and required for cell survival. ERK and p38 are both MAPK-activated protein kinases and are important for conveying extracellular stimuli to intracellular responses (39,40) and increased activation of p38 has been reported in KS keratinocytes following UVB treatment (21). However, we saw no significant difference in activation of p38 following H_2O_2 treatment of SCC-Kin1WT cells compared to SCC-Kin1-/- cells in the RPPA analysis. The activation of p38 by UVB is well documented and these results highlight that regulation of signalling pathways following oxidative stress by Kindlin-1 are complex and may be dependent not only on the cell type but also the type and extent of oxidative insult.

The predominant binding partners of Kindlin-1 identified to date are integrins, with loss of Kindlin-1 leading to reduced integrin activation (2,24). Perturbations of integrin signalling have been attributed to many of the pathologies observed in KS patients. For example, altered integrin-signalling plays an important role in keratinocyte migration, proliferation and adhesion (6,32,41,42). Integrins play a key role in cell-matrix attachment and the ability of cells to resist anoikis, which is a programmed cell death pathway induced upon matrix detachment. While integrin engagement is known to activate production of ROS and activate survival pathways such as PI3K and ERK thus protecting cells from anoikis, other reports show

increased ROS associated with detachment from matrix proteins (43,44). By using an integrin binding deficient Kindlin-1 mutant we could show that the viability of adherent cells following oxidative stress is dependent on the ability of Kindlin-1 to bind integrins. There are 24 known integrin heterodimers assembled from 18 α- and 8 β-subunits involved in matrix adhesion and the importance of Kindlin-1 in regulating the cellular response to oxidative stress will be dependent on the array of integrins expressed by different cell types and the relative importance of Kindlin-1 in regulating their activation. It is known that integrins can regulate MAPK-activated protein kinases (45-47) and here we show for the first time that activation of ERK signalling following oxidative stress is dependent on Kindlin-1's ability to bind integrins, and that this activation of ERK is necessary for subsequent colony formation.

The ability of Kindlin-1 to regulate the cellular redox state and activate the ERK survival pathway was seen in both keratinocytes and transformed SCC cells which demonstrates a key role for Kindlin-1 in regulating the ability of cells to respond to acute oxidative stress. In the context of cancer ROS have been reported to have both pro- and anti-survival functions (48). This most likely reflects the complex tumour environment where extrinsic factors (eg signals from the immune system) and intrinsic factors (eg hypoxia), combined with the mutational burden of tumours will determine their oxidative state and how they respond to ROS species. Whether the increased susceptibility of KS patients to develop SCC is linked to increased ROS induced mutagenesis following exposure to UV remains to be established. However, the data in the keratinocytes provides a model by which photosensitivity in KS patients results from an increased sensitivity to ROS due to impaired ERK activation following UV treatment.

4. Material and methods

4.1. Cell culture

Cells were used at passages 12-21. SCCs were induced in K14CreER^{T2} Kin1$^{flox/flox}$ mice using a two-stage chemical skin carcinogenesis protocol as previously described (8,23). Following surgical excision of carcinomas, small tissue pieces were adhered to tissue culture plates and cells allowed to grow out in DMEM supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate and 10% foetal bovine serum (FBS). To induce Kindlin-1 deletion SCC cells were cultured in the presence of 15 □M 4-hydroxytamoxifen (4OHT) for 24 h. Cells were then trypsinised and single cells seeded into each well of a 96-well plate and allowed to establish colonies. Resulting colonies were screened for genetic deletion of *Fermt1* (SCC-Kin1-/-). Wild-type Kindlin-1 (SCC-Kin1WT) and the non-integrin binding Kindlin-1 mutant (QW611/612AA) (SCC-Kin1AA) were cloned into pWZL-Hygro retroviral vector and introduced into the SCC-Kin1$^{-/-}$ cells and stable pools then selected in 0.25 mg/ml hygromycin. SCC cells were cultivated at 37 °C and 5% CO_2 in DMEM with 10 % FBS. Cells were used at passages 3-20. Immortalized human keratinocytes from a patient harbouring a *FERMT1* mutation were used for this study (a gift from Prof. Maddy Parsons, (22)). Keratinocytes were cultivated in KSF-M at 37°C and 5% CO_2.

4.2. Cell viability assay

To assess cell viability 3000 SCC cells or 4000 keratinocytes per well were seeded in 96 well plates. 8 h after plating cells were treated with 250 μM H_2O_2 for 16 h, after which media was replaced with phenol red free media containing 10% alamarBlue. Fluorescence was read after 4 h. Cell proliferation was calculated according to the manufacturers instructions (BioRad).

4.3. Colony formation assay

To assess the colony forming potential of cells after treatment, 300 cells were seeded into 10 cm culture dishes and incubated at 37°C, 5% CO_2 for 7 days. Treatment was carried out 24 h after plating. Media was changed on day 2 and 5. After 7 days, media was removed and cells were fixed in ice-cold methanol for 20 min at 4°C. Colonies were stained with 0.05% crystal violet in H_2O for 20 min, washed with H_2O three times, and counted manually.

4.4. Induction of oxidative stress

To induce oxidative stress cells were either treated with hydrogen peroxide (H_2O_2) or irradiated with UVA. For hydrogen peroxide treatment media was replaced with freshly prepared media containing 250 μM H_2O_2. For UVA irradiation, media was changed prior to irradiation to phenol red free media, and cells exposed to 5J of UVA (UVA lamp, Herbert Waldmann GmbH & Co. KG, Villingen-Schwenningen, Germany, dose 2.7 mW cm^{-2} (± 20%) (315-400nm). Control cells were treated identically but not irradiated.

4.5. GSH/GSSG ratio

To determine the change of intracellular GSH/GSSG ratio after exposure to oxidative stress, cells were seeded in a 96-well plate and cultivated for 24 h. Oxidative stress was induced by treatment of cells with 250 μM H_2O_2 for 10 min. The ratio of reduced to oxidized glutathione (GSH/GSSG) was measured using the GSH/GSSG Glo Assay Kit (Promega) according to the manufacturer's instructions.

4.6. DCF assay

Intracellular ROS levels were measured using the DCFDA assay (Abcam) according to the manufacturer's instructions. Briefly, cells were seeded in a 96-well plate and cultivated for 24 h. Cell were incubated with 10 μM 2′,7′-dichlorofluorescein (DCF) for 45 min and then washed twice. Oxidative stress was then induced by treatment of cells with 250 μM H_2O_2 for 60 min. Cells were washed twice with PBS and ROS levels were quantified by measuring fluorescence at excitation/emission of 495 nm/520 nm.

4.7. Western Blotting

For western analysis, cells were lysed in RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% TritonX-100, 0.5% sodium deoxycholate, 0.1% SDS) plus protease and phosphatase inhibitors (Roche, 04906845001 and 04693124001, respectively). Proteins were separated according to size using SDS–PAGE, transferred onto nitrocellulose membrane, and blocked for 1 h in TBS containing 0.05% Tween-20 (TBST) and 5% BSA. Membranes were incubated over night at 4°C and for 45 min at room temperature for primary and secondary antibodies, respectively. All washes and antibody incubations were carried out in TBST and TBST plus 5% BSA. Antibodies used were: Actin (Cell Signalling 3700), Kindlin-1 (Abcam, ab68041), ERK (Cell Signalling 4696), pERK (Cell Signalling 4370). Primary antibodies were used at 1:1000 and dye secondary antibodies were used as directed by the manufacturer (Licor).

4.8. H2AX Assay

Cells grown on coverslips were fixed with Fix-buffer (100 mM PIPES, 3.7% paraformaldehyde, 0.2% Triton X-100, 1 mM $MgCl_2$, and 10 mM EGTA, pH 7.4) for 10 minutes and washed three times in Wash buffer (TBS plus 0.1% Triton X-100). The incubation with primary antibodies was at 4°C overnight in Wash buffer plus 2% BSA. Cover slips were washed twice in Wash buffer and incubated with the secondary antibodies for 90 min at room temperature in Wash buffer plus 2% BSA. Cover slips were mounted with Vectashield Mounting Media containing DAPI (Vector Laboratories). Images were captured using an FV-1000 Olympus confocal microscope using a 40× objective. The primary pH2AX antibody was used 1:200 (Cell Signalling 9718) and the dye (Alexa Fluor)-conjugated secondary antibody was used at 1:400. For quantification cell nuclei were manually selected in ImageJ to create a mask. The mask was then used to measure fluorescence intensity of both the DAPI and the pH2AX channel. pH2AX intensity was normalized to the DAPI signal for each image. For each biological replicate at least 3 images and a total of at least 30 cells each per conditions were taken.

4.9. MEK inhibitor

To inhibit phosphorylation and subsequent activation of ERK (p42/ p44 MAPkinase), a MEK inhibitor was used (U0126, Cell Signalling). The inhibitor was added in a final concentration of 10 μM for 1 h to the cells. In experiments where oxidative stress was induced, the inhibitor was added together with H_2O_2 or at the beginning of irradiation, respectively.

4.10. RPPA Analysis

Cells, in biological triplicate, were washed with PBS and lysed in 1% Triton X-100, 50 mM HEPES (pH 7.4), 150 mM sodium chloride, 1.5 mM magnesium chloride, 1 mM EGTA, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, 10% glycerol, supplemented with cOmplete ULTRA protease inhibitor and PhosSTOP phosphatase inhibitor cocktails. Cleared lysates were serially diluted to produce a dilution series comprising four serial two-fold dilutions of each sample, which were spotted onto nitrocellulose-coated slides (Grace Bio-Labs) in technical triplicate under conditions of constant 70% humidity using the Aushon 2470 array platform (Aushon Biosystems). Slides were hydrated in blocking buffer (Thermo Fisher Scientific) and then incubated with validated primary antibodies Bound antibodies were detected by incubation with anti-rabbit DyLight 800-conjugated secondary antibody (New England BioLabs). An InnoScan 710-IR scanner (Innopsys) was used to read the slides, and images were acquired at the highest gain without saturation of the fluorescence signal. The relative fluorescence intensity of each sample spot was quantified using Mapix software (Innopsys).

The linear fit of the dilution series of each sample was determined for each primary antibody, from which median relative fluorescence intensities were calculated. Signal intensities were normalized by global sample median normalization. Only primary antibodies with normalized signal intensities at least 1.5 times the value of the secondary antibody alone in at least one sample were included in the analysis to exclude data derived from weak or non-specific signals. To identify important biological targets we used the fold change of SCC-Kin1WT over SCC-Kin1-/- cells with a cut off of 0.75 and 1.5. We additionally calculated pValues for each sample and filtered for samples that had pValues smaller than 0.05.

4.11. Statistical Analysis

Quantitative data were tested for normality using the Shapiro test. The null hypothesis that the samples come from a normal distribution was rejected if the p-value was smaller than 0.1. If data were normally distributed, p-values were computed using a two-tailed *t*-test for comparisons between cell lines and a one-sided *t*-test for comparisons between treated and untreated cells. If data were not normally distributed, a Mann-Whitney test was used for paired data, and a two-sided Wilcox test for unpaired data. P-values to determine statistical significance are indicated in the text.

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Conflict of interest

No conflict of interest declared.

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Figure legends

Fig. 1. Role of Kindlin-1 in cellular redox homeostasis. (A) Western blot showing re-expression of Kindlin-1 in SCC cells. (B) 2',7'-dichlorofluorescin (DCF) assay measuring reactive oxygen species in SCC cells after treatment with H_2O_2. (C) Measurement of GSH/GSSG ratio after treatment of SCC cells with H_2O_2. Results represent the means and s.e.m. from more than 3 independent experiments. *p<0.05: statistically significant difference from control value after Mann-Whitney test (B) and after paired two-sample *t*-test (C).

Fig. 2. Kindlin-1 protects cells from oxidative stress. Cell viability following treatment of SCC cells (A) and KS keratinocytes (C) with H_2O_2. Quantification of colony formation in SCC cells (B) and KS keratinocytes (D) following treatment with H_2O_2 and UV. Results represent the means and s.e.m. from more than 5 independent experiments. **p<0.01, ***p<0.001: statistically significant difference from control value after one-sample student *t*-test on fold change in (A,C) and two-sample paired student *t*-test in (B,D).

Fig. 3. Kindlin-1 protects cells from DNA damage after oxidative stress. Cells were treated with H_2O_2 or irradiated with UVA to induce oxidative stress. DNA damage was measured by analyzing levels of pH2AX via immunofluorescence staining (IF). (A, C) Quantification of nuclear pH2AX fluorescence intensity normalized to nuclear DAPI in SCC cells (A) and in KS keratinocytes (C). Representative image of nuclear pH2AX fluorescence and nuclear DAPI in SCC cells (B) and KS keratinocytes (D). Results represent the means and s.e.m. from more than 5 independent experiments. *p<0.05, **p<0.01: statistically significant difference from control value after one-sample student *t*-test.

Fig. 4. Kindlin-1 is required for full activation of ERK pathway after oxidative stress. SCC-Kin1-/- and SCC-Kin1WT cells were treated with H_2O_2 for 30 min, 1 h, or 4 h and activation of signaling pathways was measured by RPPA. After normalization, samples were filtered by a cut off FC of > 1.5 or < 0.75 of treated Kin1WT cells over Kin1-/- cells. (A) Top hits were ranked by pValue from top to bottom and color coded

by the fold change in H_20_2 treated Kin1WT cells over Kin1-/- cells with blue representing high expression in Kin1-/- cells and yellow high expression in Kin1WT cells. pValues were computed using two-sample student t-test over 3 independent biological replicates. (B, C) Western blots showing activation of the ERK pathway after H_2O_2 treatment in SCC cells (B) and KS keratinocytes (C). Results represent the means and s.e.m. from more than 3 independent experiments. *p<0.05, **p<0.01: statistically significant difference from control value after two-sample paired student *t*-test.

Fig. 5. Kindlin-1 protects cells from oxidative stress by activating ERK signaling. (A) Treatment of SCC cells with H_2O_2 leads to phosphorylation of ERK. Additional treatment with MEK inhibitor (U0126) inhibits ERK phosphorylation. (B, C) Colony formation assay in SCC cells (B) and KS keratinocytes (C) treated with H_2O_2 and U0126. (D, E) Quantification of pH2AX in SCC cells (D) and KS keratinocytes (E) after H_2O_2 and U0126 treatment. Results represent the means and s.e.m. from more than 4 independent experiments. *p<0.05: statistically significant difference from control value after two-sample paired student *t*-test.

Fig. 6. Colony formation after oxidative stress depends on Kindlin-1 ability to bind integrins. (A) Colony formation assay in SCC-Kin1-/-, SCC-Kin1WT and SCC-Kin1AA cells after H_2O_2 treatment or UV irradiation. Results represent the means and s.e.m. from more than 3 independent experiments. *p<0.05: statistically significant difference from control value after ANOVA and TukeyHSD posthoc testing. (B) SCC-Kin1-/-, SCC-Kin1WT, and SCC-Kin1AA cells were treated with H_2O_2 for 30 min, 1 h, or 4 h and activation of ERK signaling was measured by RPPA.

Figure 1

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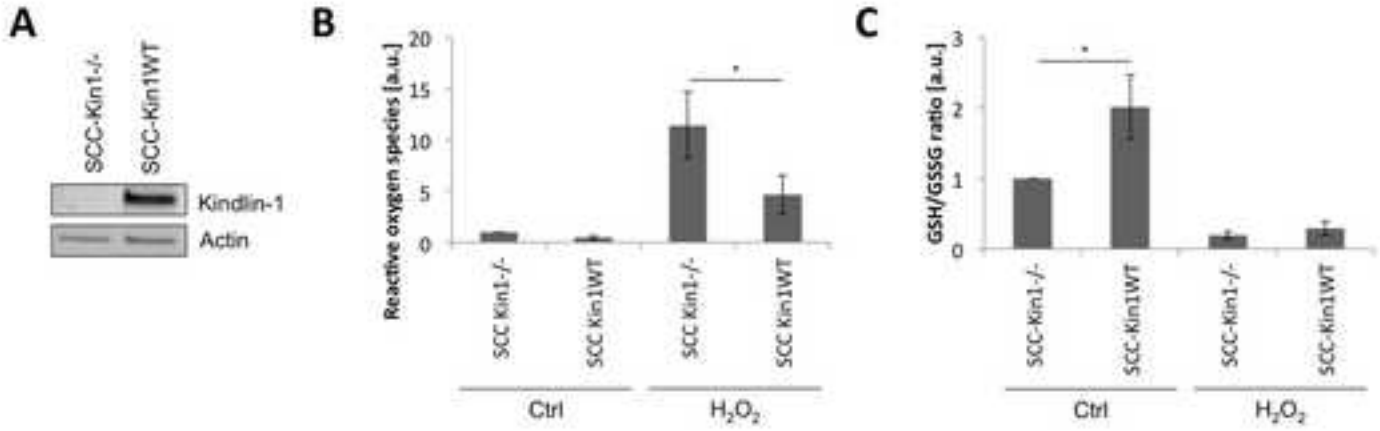

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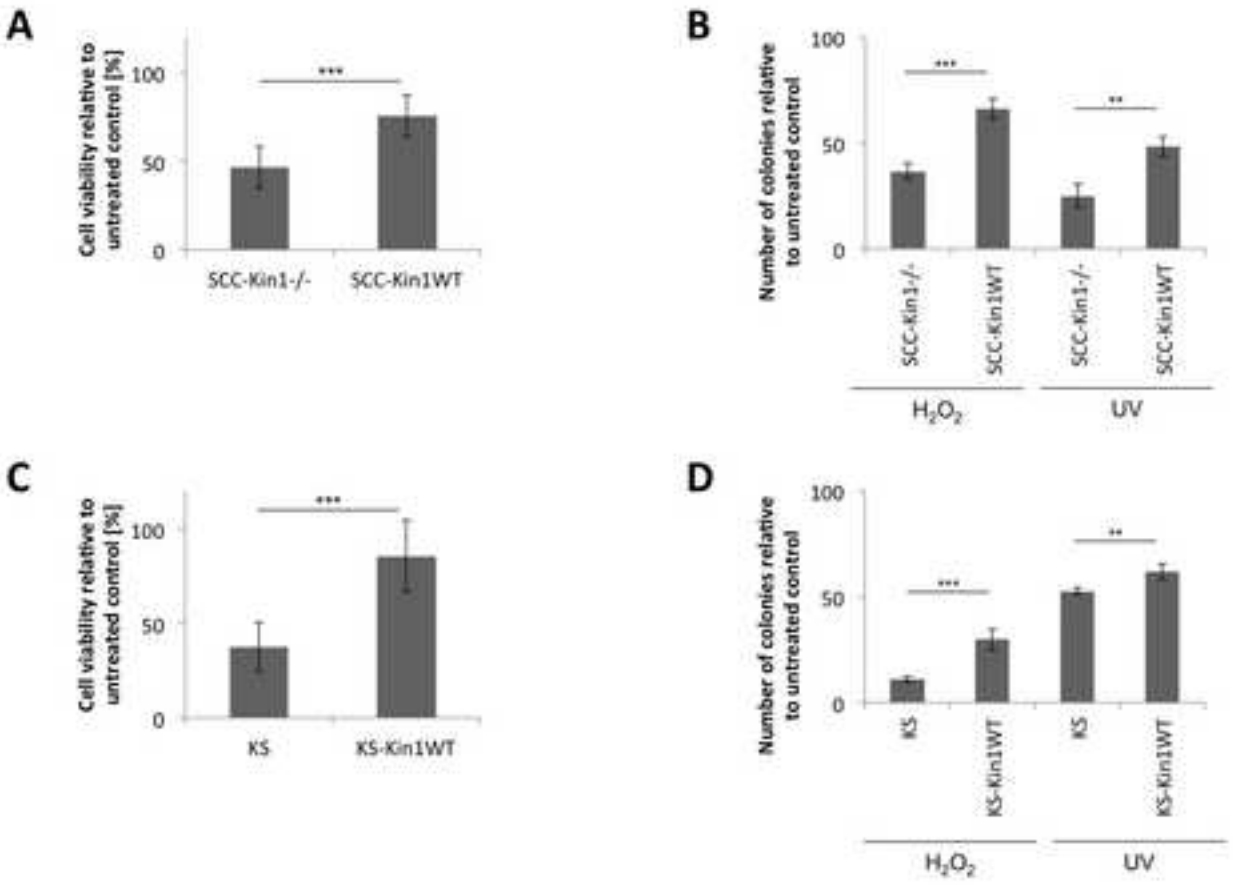

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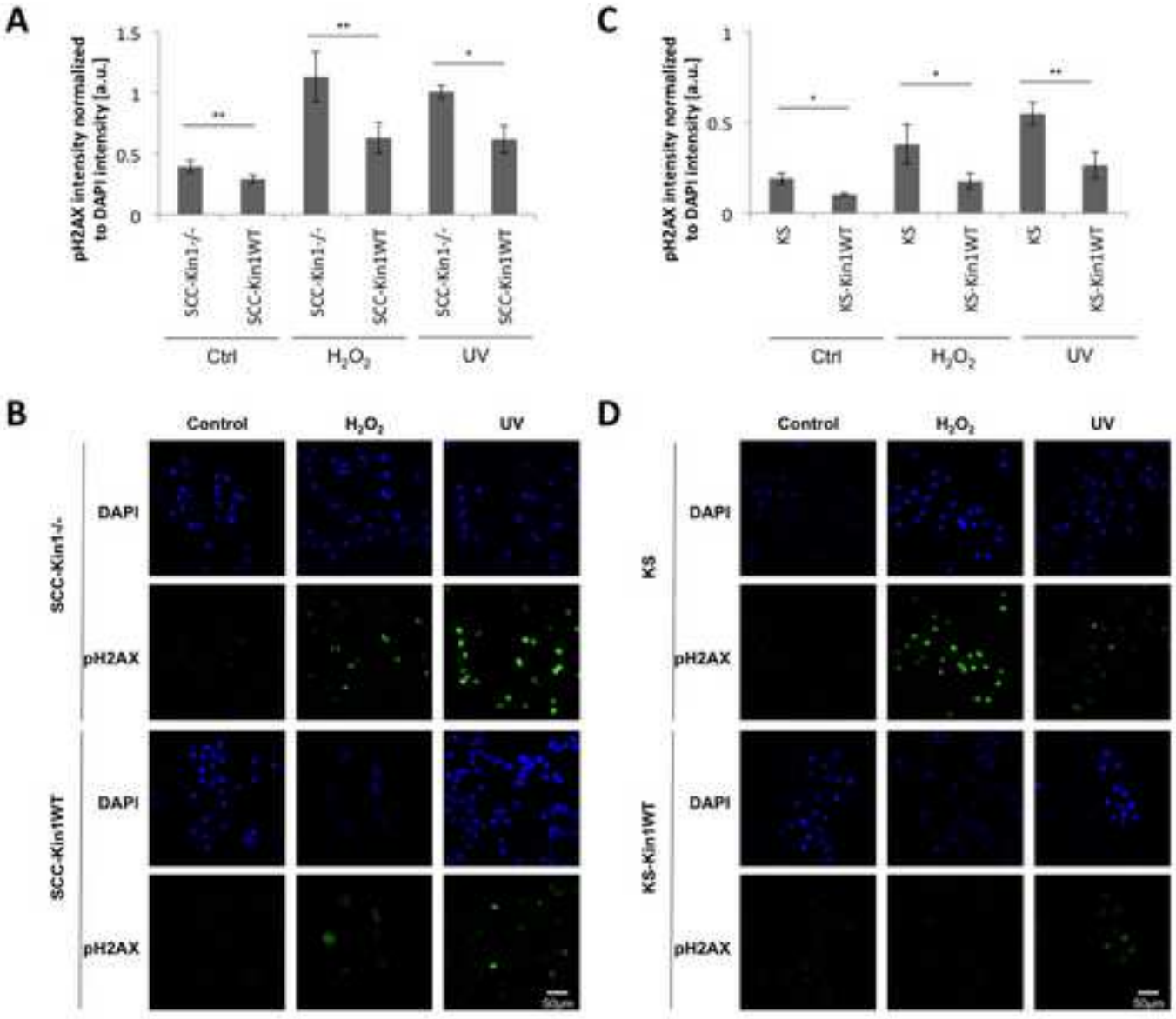

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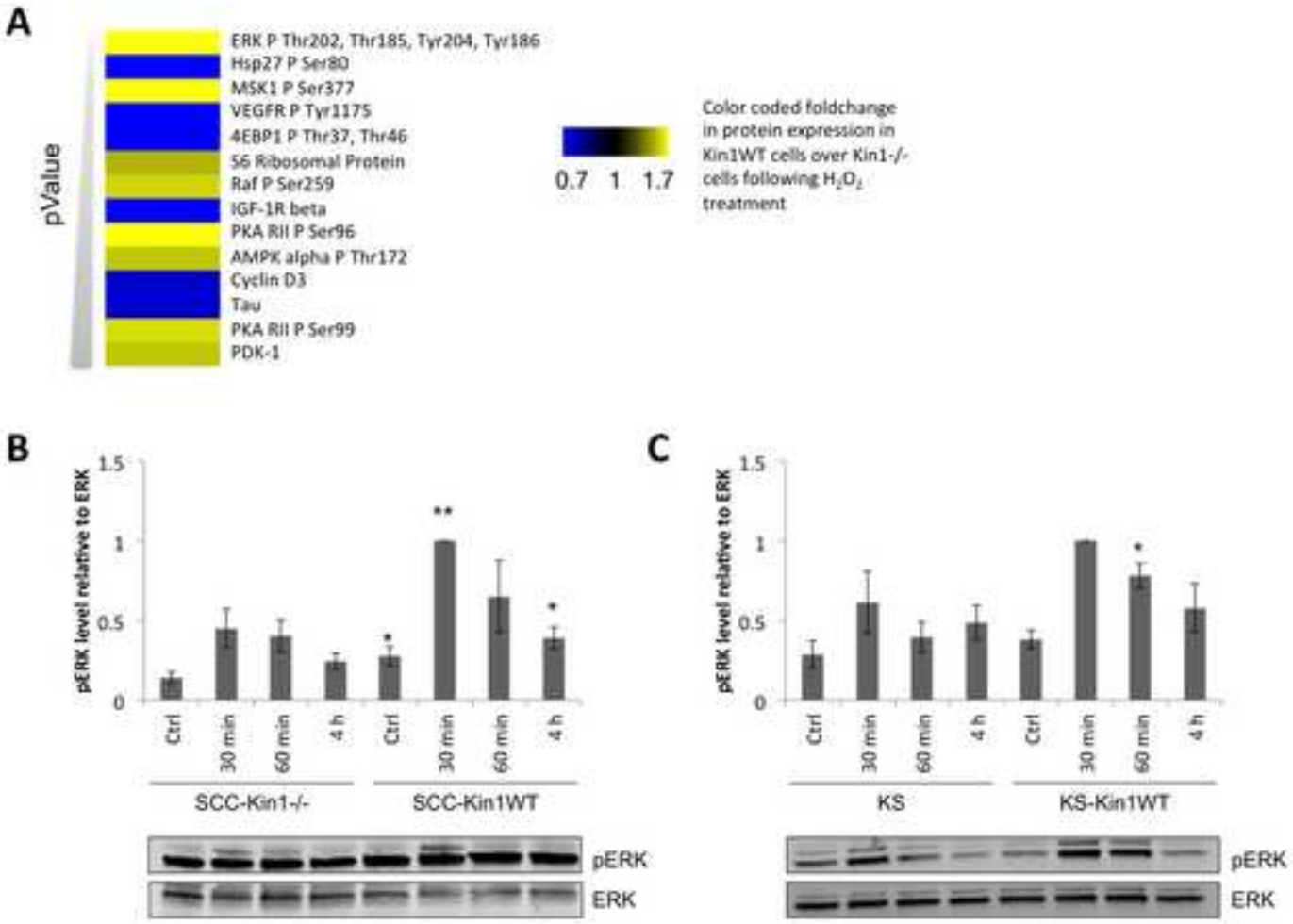

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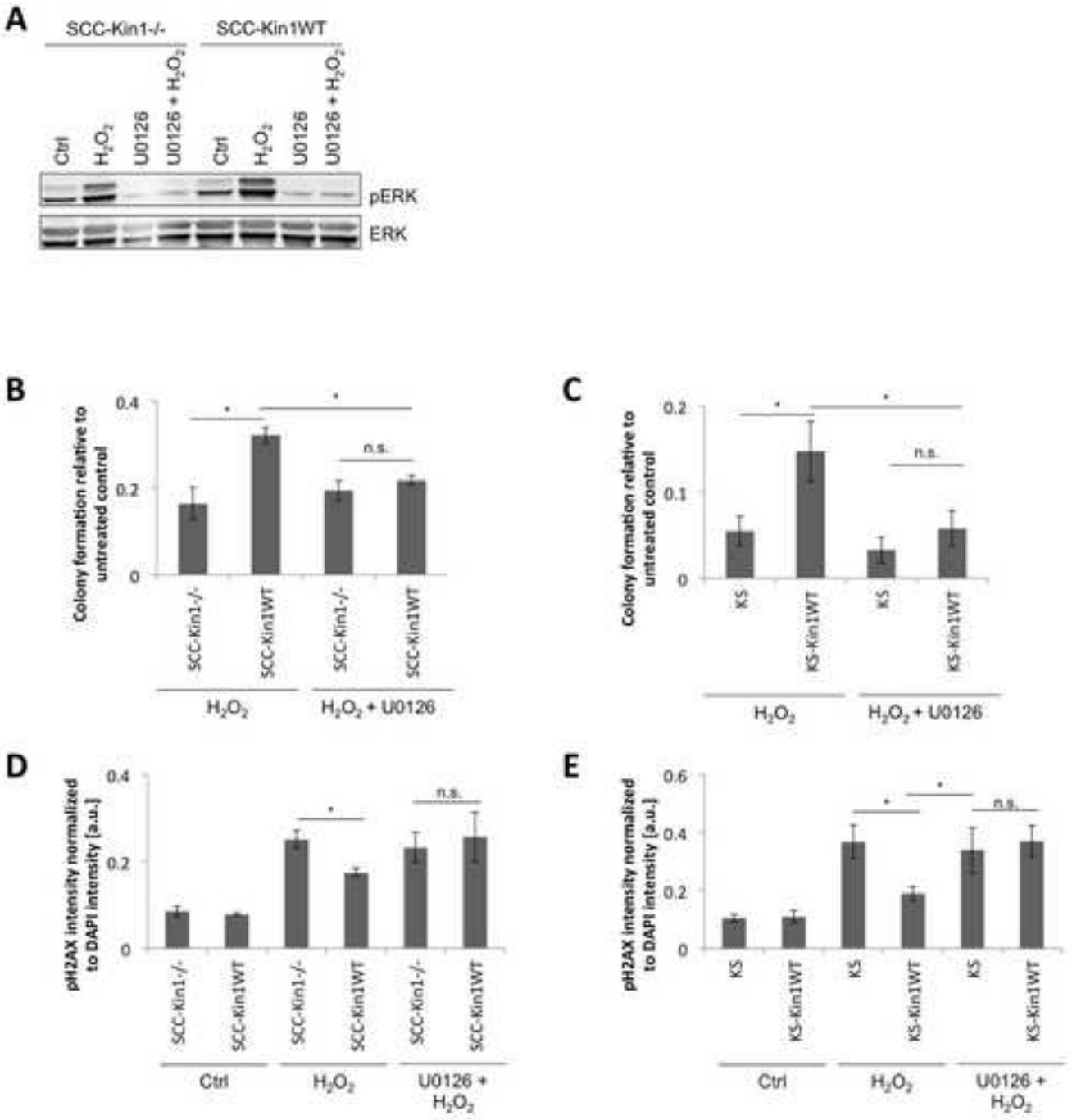

Figure 6

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