IKKα regulates human keratinocyte migration by surveillance of the redox environment

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Summary statement

This study provides a newly identified mechanism by which H$_2$O$_2$-dependent oxidation of the inhibitor of kappa B kinase alpha and derepression of *epidermal growth factor* promoter activity stimulates keratinocyte migration.

Abstract

Although the functions of H$_2$O$_2$ in epidermal wound repair are conserved throughout evolution, the underlying signaling mechanisms are largely unknown. In this study we used human keratinocytes (HEK001) to investigate H$_2$O$_2$-dependent wound repair mechanisms. Scratch wounding led to H$_2$O$_2$ production in 2-3 cell layers at the wound margin within ~30min and subsequent cysteine modification of proteins via sulfenylation. Intriguingly, exogenous H$_2$O$_2$ treatment resulted in preferential sulfenylation of keratinocytes that adopted a migratory phenotype and detached from neighboring cells, suggesting that one of H$_2$O$_2$’s primary functions is to stimulate signaling factors involved in cell migration. Based on previous findings that revealed epidermal growth factor receptor (EGFR) involvement in H$_2$O$_2$-dependent cell migration, we analyzed oxidation of a candidate upstream target, the nuclear factor κ-B kinase alpha (IKK$\alpha$), as a mechanism of action. We show that IKK$\alpha$ is sulfenylated at a conserved cysteine residue in the kinase domain, which correlates with derepression of EGF promoter activity and increased EGF expression. Thus this indicates that IKK$\alpha$ promotes migration through dynamic interactions with the EGF promoter depending on the redox state within cells.
Introduction

During the early tissue formation stage of the cutaneous wound repair process, re-lining of wounds by basal keratinocytes restores an intact epidermal barrier (Arwert et al., 2012; Rieger et al., 2014). This step involves dynamic physiological and molecular changes to initiate migration over the provisional matrix and proliferation of keratinocytes from the surrounding epidermis (Usui et al., 2005; Watt, 2002). A number of soluble factors modulate wound repair (Gault et al., 2014; Gurtner et al., 2008; Niethammer, 2014), including the small reactive oxygen species (ROS) hydrogen peroxide (H$_2$O$_2$) (Loo et al., 2011; Niethammer et al., 2009). The H$_2$O$_2$ signal transduction cascade is evolutionarily conserved and acts via receptors, protein kinases, structural components and downstream transcription factor-dependent post-translational and genomic mechanisms (Gough and Cotter, 2011). H$_2$O$_2$ is an immediate wound attractant signal for *Drosophila* hemocytes (Moreira et al., 2010), and H$_2$O$_2$ gradients within the wounded epithelium are crucial for phagocytic cell migration (Niethammer et al., 2009; Tauzin et al., 2014) by direct oxidation of the Src family kinase member Lyn (Yoo et al., 2011). H$_2$O$_2$ also promotes the regrowth of peripheral sensory axons and their reinnervation of healing skin (Rieger and Sagasti, 2011), and it is essential for tail regeneration of *Xenopus* tadpoles (Love et al., 2013). Addition of exogenous H$_2$O$_2$ to wounds accelerates wound closure in mice (Loo et al., 2012; Roy et al., 2006) and in keratinocyte culture injury models (Loo and Halliwell, 2012; Loo et al., 2011; Pan et al., 2011). Thus H$_2$O$_2$ appears to be critical for wound repair; yet the molecular understanding of this physiological function remains elusive.

At low concentrations, H$_2$O$_2$ functions as a second messenger whereby it oxidizes cysteine thiols with low pKa in signaling proteins, which are often found in catalytic domains of signaling enzymes (Claiborne et al., 1999). Oxidation of cysteine thiols stimulates the formation of sulfenic acid (sulfenylation), a highly unstable metabolite that can rapidly convert into other metabolites, such as sulfenic and sulfonic acid, or nitrosothiol (Leonard et al., 2009). A common metabolic process in which sulfenic acid participates is the promotion of intramolecular disulfide bond formation. This alters
protein conformation and modulates the activation or inactivation of enzymes (Leonard and Carroll, 2011; Stone and Yang, 2006; Truong and Carroll, 2012a). As a consequence, H$_2$O$_2$ can modulate phosphorylation cascades within cells, often by activating kinases and deactivating phosphatases (Claiborne et al., 1999; Gough and Cotter, 2011).

The inhibitor of kappa B kinase alpha (IKK$\alpha$) (previously termed CHUK) represents a candidate for oxidative regulation of H$_2$O$_2$ during migration. First, IKK$\alpha$ is structurally related to its homolog IKK$\beta$, which has been shown to be oxidized by arsenite at a conserved cysteine residue in the kinase domain (Kapahi et al., 2000). Second, we have recently shown that IKK$\alpha$ is essential for keratinocyte migration in vitro and acts downstream of H$_2$O$_2$ (Lisse et al., 2016). Third, IKK$\alpha$ has a non-conventional role in repressing EGF and hb-EGF promoter activity to control epithelial differentiation and prevent tumor formation during skin homeostasis (Hu et al., 1999; Liu et al., 2008) (Marinari et al., 2008) (Hu et al., 2001; Sil et al., 2004). For example, IKK$\alpha$ ablation leads to increased keratinocyte proliferation and impairment of terminal differentiation (Hu et al., 2001; Sil et al., 2004). In addition, IKK$\alpha$ plays an important role in human cancers, as its dysfunction has been reported in squamous cell carcinomas of the skin (Liu et al., 2006). Studies have focused primarily on IKK$\alpha$’s role during epidermal terminal differentiation, but whether it is involved in other cellular activities, such as migration has however not been examined. In addition, it is unclear whether IKK$\alpha$’s keratinocyte-specific molecular and biological functions can be modulated by oxidation similar to IKK$\beta$. Given our previous findings implicating IKK$\alpha$ in migration, we speculated that IKK$\alpha$ might serve as an intracellular surveillant to promote either migration or differentiation depending on the redox environment. We hypothesized that oxidation of IKK$\alpha$ can promote the derepression of EGF promoter activity, which may stimulate migration via EGFR signaling.
Results

**H₂O₂ activates human keratinocyte migration**

To investigate cell-autonomous molecular mechanisms regulating H₂O₂-induced keratinocyte migration, we utilized the human epidermal keratinocyte line (HEK001) (Sugerman and Bigby, 2000). We first validated that HEK001 cellular responses were similar to primary keratinocytes. We found that these cells differentiated after treatment with high (2mM) Ca²⁺ concentrations ([Fig. S1A](#figS1A)). PCNA (proliferation marker), K14 (basal keratinocyte marker, and AXIN2, an inhibitor of the Wnt pathway, were expressed during the first 7 days *in vitro*. K10 (early differentiation marker) and INVOLCRIN (late differentiation marker) showed a lower level of expression by 24hr, which gradually increased until DIV7 (days *in vitro*). These markers seemed to increase further when treated with high Ca²⁺ concentrations. The increase in PCNA and K14 expression ≥DIV7 suggests that this cell line shows aberrant differentiation characteristics. Regardless, DIV7 cultures when treated with high calcium (2mM) showed increased stratification ([Fig. S1B](#figS1B)) and aggregation of terminally differentiated lipid-forming keratinocytes when assessed with crystal violet staining ([Fig. S1C](#figS1C)). DIV7 cultures moreover failed to close scratch wounds even after 72hr ([Fig. S1D](#figS1D)), which correlated with reduced H₂O₂ formation at the scratch wound assessed with pentafluorobenzenesulfonyl-fluorescein (HPF) ([Fig. S1E, F](#figS1E,F)). These findings show that migration is initiated in undifferentiated keratinocytes that resemble more closely a progenitor-like population (at DIV2), consistent with *in vivo* characteristics of basal keratinocytes, yet this cell line displays aberrant differentiation characteristics at later time points.

Using *in vitro* scratch-wound assays prior to DIV7, we observed rapid formation of H₂O₂ (~30min) after single-pulse labeling with HPF, with strongest signal formation in 2-3 cell layers at the scratch margin ([Fig. 1A, B](#fig1AB)). For our studies, characterization of ROS signal-to-noise ratios showed that 1µM but not 4µM was critical to detect differential H₂O₂ levels, whereas higher concentrations resulted in toxicity ([Fig. S2A-C](#figS2A-C)).
onset occurred around ~6hr and closure was typically seen between 12-18hr, which was batch-dependent (Fig. 1C). Closure coincided with downregulation of H$_2$O$_2$ in scratch margin cells. Migration was blocked by treatment with 10µM of the general reactive oxygen species (ROS) inhibitor diphenyleneiodonium (DPI) (Fig. 1D left panel), but this was not caused by cellular toxicity, as cells were viable when assessed with 6-CFDA (Fig. 1D right panel). Also the NADPH oxidase inhibitor apocynin blocked scratch repair (Fig. 1E), confirming our results. To validate that H$_2$O$_2$ levels were reduced upon inhibitor treatment we assessed cells pre-treated with DPI and HPF for 30min prior to scratching, resulting in a significant reduction of H$_2$O$_2$ at the scratch margin (Fig. 1F). Given that DPI does not decrease cell viability (Fig. 1D) but impairs migration (Fig. 1D, E). We next analyzed whether addition of exogenous H$_2$O$_2$ at concentrations ranging between 0.01-10µM enhanced scratch closure. While low concentrations increased scratch repair, high H$_2$O$_2$ levels were inhibitory (Fig. 1G). We further performed micromass assays to test whether keratinocytes at the periphery (P) of the micromass foci, which we assumed to be less differentiated than those in the center (Bedal et al., 2014; Stott et al., 1998), generated H$_2$O$_2$ similar to scratch margin cells. This showed elevated H$_2$O$_2$ levels at the outer edges compared with confluent (C) regions where less H$_2$O$_2$/HPF signal was measured (Fig. 1H, I). We further utilized human primary keratinocytes to assess H$_2$O$_2$ production upon scratch and found a similar response as in HEK001 cells (Fig. S2D). Quantification of the migration distance after H$_2$O$_2$ treatment using 0.1 and 100µM H$_2$O$_2$ showed increased migration at low levels but not high levels, which blocked migration (Fig. S2E). These findings suggest a concentration-dependent role for H$_2$O$_2$ in keratinocyte migration.

To dissect out the specific role of H$_2$O$_2$ (as opposed to other ROS) during scratch closure, we co-administered DPI and exogenous H$_2$O$_2$. We first assessed the time until scratch closure and found that DPI alone decreased closure within 18hr by 73%, whereas low H$_2$O$_2$ concentrations restored closure to near endogenous rates (Fig. 1G). To monitor bona fide migration, we utilized transwell chambers and observed a similar increase in migration with addition of low H$_2$O$_2$ concentrations (Fig. 1J, K). Low-level (0.1µM) H$_2$O$_2$ significantly promoted migration in the presence of DPI, but migration was
reduced in DPI-treated cells and those treated with 100 and 1000µM H\textsubscript{2}O\textsubscript{2}. We additionally performed transwell assays after addition of the proliferation inhibitor mitomycin C using various concentrations of H\textsubscript{2}O\textsubscript{2} to see whether proliferation accounts for the observed increase in cell number (Fig. 1L). This confirmed that the observed increase was related to \textit{bona fide} migration. Thus the presence of low H\textsubscript{2}O\textsubscript{2} concentrations specifically induces keratinocyte migration. Finally, to assess specific NADPH oxidases involved in H\textsubscript{2}O\textsubscript{2} production, we monitored \textit{NOX}1, \textit{NOX}4 and \textit{DUOX}1 transcription using quantitative PCR (qPCR). This revealed that \textit{NOX}4, but not \textit{DUOX}1 and \textit{NOX}1, was significantly upregulated after scratch wounding at 12hr post scratch while remaining low in unscratched cells (Fig. 1M). Taken together, low concentrations of H\textsubscript{2}O\textsubscript{2} promote migration of HEK001 keratinocytes, which leads to delayed upregulation of NOX4 mRNA.

\textbf{H\textsubscript{2}O\textsubscript{2}-dependent signaling factors important for keratinocyte migration}

We previously performed RNAseq and Ingenuity\textsuperscript{®} Upstream Regulator analyses to identify genome-wide transcriptional networks mediated by H\textsubscript{2}O\textsubscript{2} and showed four major upstream networks with notable cutaneous associations (i.e. H\textsubscript{2}O\textsubscript{2}, EGF, FOXO1 and IKK\textgreek{a}) following H\textsubscript{2}O\textsubscript{2} treatment of zebrafish (Lisse et al., 2016). Further subcategorization of each network to define enriched pathways within them (Fig. S3) showed genes in each of these networks that functionally cluster into overlapping categories, including cell migration, defense response and wound repair. Genes involved in migration and which were differentially regulated in our data set included FOXO1, IGFBP-1, HMOX1, HSPA1L, and ITGB4. All of those showed a dose-dependent increase in gene expression following H\textsubscript{2}O\textsubscript{2} treatment of HEK001 cells (Lisse et al., 2016).

To investigate these networks further in H\textsubscript{2}O\textsubscript{2}-dependent keratinocyte migration, we pharmacologically blocked the function of EGFR, FOXO1 and IKK\textgreek{a}/NF -\textgreek{B}. The EGFR has a demonstrated role in migration and metastasis (Jones and Rappoport, 2014) and can be modulated by either direct oxidation (Paulsen et al., 2012) or phosphorylation
As expected, EGFR inhibition completely blocked injury-induced scratch repair (Fig. 2A, B). We next analyzed FOXO1 involvement in H$_2$O$_2$–dependent migration. Consistently, we observed an impairment of injury-induced cell migration after FOXO1 inhibition (Fig. 2A) but H$_2$O$_2$ production at the scratch margin was unaffected 30 min after scratch in FOXO1-inhibited HEK001 (Fig. 2C), suggesting an upstream regulatory function of H$_2$O$_2$ consistent with our RNAseq results. We did however observe a decrease in H$_2$O$_2$ levels at the unclosed scratch margin after 12 hr inhibitor treatment compared to controls. FOXO activity is tightly controlled by post-translational modifications, which mediate FOXO nuclear localization and degradation. We therefore assessed its subcellular localization after scratch and in the presence of DPI. Immunofluorescence (IF) studies showed rapid FOXO1 nuclear localization in scratch-margin keratinocytes, which was attenuated by DPI (Fig. 2D). These results suggest that H$_2$O$_2$ regulates FOXO1 activity and nuclear localization/function during early wound repair. FOXO1 seems to modulate H$_2$O$_2$ levels at later stages, consistent with previous findings showing that it controls oxidative stress levels during wound re-epithelialization (Ponugoti et al., 2013).

We next assessed the role of IKK$\alpha$ in H$_2$O$_2$–dependent migration. Given that IKK$\alpha$ is best known for its regulation of the NF-$\kappa$B pathway, which is implicated in oxidative stress responses (Gloire et al. 2006), we first determined if NF-$\kappa$B was activated. Pharmacological inhibition of canonical NF-$\kappa$B signaling by blocking RelA (p65) nuclear translocation (Shin et al., 2004) failed to impair scratch closure (Fig. 2A, B). We did however observe transient NF-$\kappa$B p50/p105 nuclear localization at 6 hr following scratch wounding (Fig. 2E). H$_2$O$_2$ treatment however did not promote nuclear localization (Fig. 2F), suggesting alternative functions in scratch margin cells, possibly induced by the oxidant-dependent PI-3K “survival” pathway (Sonoda et al., 1999). Also NF-$\kappa$B p52/p100 staining was predominantly cytoplasmic upon scratch wounding and H$_2$O$_2$ treatment (Fig. 2G, H). Thus H$_2$O$_2$ does not appear to regulate NF-$\kappa$B signaling during scratch wound repair.
Given the absence of NF-κB involvement and our findings that IKKα is a predicted target of H₂O₂, we wanted to determine whether IKKα plays a NF-κB-independent role in scratch repair. Using the IKKα and β kinase inhibitory compound, Wedelolactone, we found that scratch wound repair was impaired only when using high inhibitor concentrations of 25 and 50µM (Fig. 2A, B). To further investigate IKKα functions, we assessed its (sub)cellular localization using immunofluorescence staining. We observed its predominant expression in keratinocytes adjacent to the scratch margin where it was localized in the cytoplasm and perinuclear regions (Fig. 3A, B). Nuclear localization was however promoted with DPI (Fig. 3A, B). In addition, nuclear IKKα was also predominant after treatment with 2mM Ca²⁺ to promote differentiation (Fig. S4A), and in cells cultured for prolonged periods (DIV7) following treatment with DPI (Fig. S4B) and after EGF withdrawal (Fig. S4C). Following H₂O₂ treatment of unscratched keratinocytes, staining was predominant in the perinuclear regions but nuclear or diffuse in untreated cells (Fig. 3C). At a high H₂O₂ concentration (500µM), we found IKKα staining to be more diffuse in the cytoplasm and occasionally nuclear (Fig. 3C), suggesting that IKKα localization is regulated by H₂O₂ in a concentration-dependent manner. To further establish that IKKα is involved in H₂O₂-dependent repair, we assessed scratch closure with and without exogenous H₂O₂ treatment using small-interference RNA against IKKα. IKKα message and protein was specifically and significantly knocked down (>60%) following siRNA transfection (Fig. S4D-F). This correlated with the absence of cell migration following scratch (Fig. S4G). In the presence of endogenous and exogenous H₂O₂, control siRNA-transfected keratinocytes showed closure rates comparable to untransfected cells whereas IKKα knockdown impaired scratch closure under both conditions (Fig. 3D). Overall, these findings indicate that IKKα is regulated by H₂O₂ and essential for scratch wound repair.
Post-translational cysteine oxidation of IKKα is involved in keratinocyte migration

Having shown the importance of IKKα during H2O2–dependent scratch repair, we next asked whether IKKα is directly oxidized by H2O2 at a conserved cysteine residue in the kinase domain, as has been shown for the structurally related kinase, IKKβ (Kapahi et al., 2000). We first determined global protein oxidation following 2hr H2O2 treatment and upon scratch wounding using the oxidation (sulfenylation)-selective compound, dimedone, followed by immunofluorescence staining for sulfenic acid (S-OH)-dimedone complexes (Paulsen et al., 2012; Truong, 2012) (Fig. 4A). First we analyzed unscratched keratinocytes treated with exogenous H2O2 (0.1µM) as control. Interestingly, this revealed cysteine oxidation specifically in cells that appeared to have adopted a migratory phenotype, as indicated by the formation of lamellipodia at the leading edge (Fig. 4B). Also the length:width ratio of sulfenylated cells (Fig. 4C) increased, indicating a migratory fate (Rieger et al., 2009) consistent with cell detachment from neighboring cells (Fig. 4D). Phalloidin staining further revealed the formation of stress fibers in cells following low H2O2 treatment, which was uncommon in untreated cells (Fig. 4E). These findings suggest that H2O2 either selectively diffuses into cells to induce protein oxidation and migration, or it is degraded in a subpopulation of keratinocytes that do not become migratory. We next assessed scratch-dependent sulfenylation, which was predominant in the cytoplasm and perinuclear regions of wound margin cells (Fig. 4F upper panel). This specific subcellular localization was not visible after treatment of unscratched cells with exogenous H2O2, possibly because of injury-specific cues, temporal differences or the potential use of higher H2O2 concentrations compared with endogenous levels. In scratched cells the localization of sulfenylated proteins was comparable to IKKα’s H2O2-dependent localization (Fig. 3A, C), indicating that IKKα may be a sulfenylation target. Intriguingly, sulfenylation was most prominent after ~2hr, indicating that this process might be highly regulated. To validate sulfenylation specificity, we further treated keratinocytes with DPI and then performed dimedone staining, which showed diminished signals at the scratch margin (Fig. 4F lower panel). To test if sulfenylation is required for scratch repair, we treated keratinocytes with dimedone, which specifically and irreversibly reacts with protein-
sulfenylated cysteines (Seo and Carroll, 2011). Addition of dimedone at various concentrations prior to scratch wounding impaired wound closure in a dose-dependent manner (Fig. 4G). We found that the highest dimedone concentration (100mM) promoted cellular toxicity assessed with 6-CFDA and apoptosis with Annexin V staining (Fig. 4H, I).

To assess IKKα sulfenylation, we utilized DYn-2, a chemically modified dimedone, which through click-chemistry allows for biotinylation and isolation via biotin-streptavidin interactions (Paulsen et al., 2012; Truong, 2012) (Fig. 4A). H$_2$O$_2$ treatment for 5min at 10µM, a concentration that promotes cell migration, increased proteome-wide sulfenylation when assessed with streptavidin-HRP in western blots (Fig. 5A, upper panel). The isolated sulfenylated fraction was further probed for IKKα, showing a 19-fold increase in sulfenylated IKKα after H$_2$O$_2$ treatment compared to basal (untreated) levels, presumably containing endogenously oxidized and unoxidized IKKα (Fig. 5A, lower panel). GAPDH served as positive control due to its known function as a oxidoreductase (Truong and Carroll, 2012b), which was oxidized under both basal and H$_2$O$_2$ conditions (the latter showing a 2-fold increase). These results demonstrate that H$_2$O$_2$ directly oxidizes IKKα.

We next asked whether IKKα cysteine oxidation is required for keratinocyte migration. We already had generated two Ikkα-tdTomato reporter constructs (wildtype and cysteine-mutated) using zebrafish Ikk1, and therefore assessed these constructs in HEK001 cells. The Ikk1 sequence is largely conserved within the kinase domain compared with human IKKα (Fig. 5B). Following transfection into HEK001, we monitored the localization of both constructs. The wildtype (full-length) form localized to the cytoplasm and became largely perinuclear following H$_2$O$_2$ treatment, whereas DPI treatment led to subnuclear staining (Fig. 5C). This is consistent with the cytoplasmic and nuclear localization of human IKKα under these treatment conditions when detected using antibody staining (Fig. 3). Intriguingly, cysteine-mutated Ikk1 localized to the nucleus with and without the presence of H$_2$O$_2$ (Fig. 5D, E), consistent with the idea that H$_2$O$_2$-dependent oxidation promotes cytoplasmic localization of IKKα, which is
abrogated when the kinase-specific cysteine residue is mutated. To further determine the role of IKKα oxidation in migration, we monitored the motility of transfected cells over time. Because transient transfections precluded the formation of confluent monolayers and thus scratch assays, we performed assays with unscratched cells at ~60-70% confluence. To validate this approach, we treated some wells with 0.1 and 100µM H2O2 and compared HEK001 migration under these conditions. This showed a migration increase after low but not high-level H2O2 treatment also seen in scratched cells (Fig. 1). Comparisons to Ikk1-transfected cells showed that the average migration distance was highest in keratinocytes transfected with FL-Ikk1-tdTomato in the presence of low H2O2 concentrations (Fig. 5F). Keratinocytes transfected with cysteine-mutated Ikk1(C179A)-tdTomato migrated however similar to keratinocytes treated with high concentrations of H2O2 (100µM), or DPI. Untreated control cells traveled intermediate distances, possibly due to effects related to reduced confluence (Fig. 5F).

These results suggest that IKKα cysteine oxidation and cytoplasmic localization is required for cell migration and it further indicates that IKKα may serve as innate surveillant of the redox environment to regulate keratinocyte migration and differentiation.

**IKKα oxidation promotes derepression of EGF promoter activity**

Evidence so far suggests an important function for ox-IKKα in keratinocyte migration. How this translates into downstream functions that promote migration is unclear. This could be mediated, in part, by IKKα’s cis-repressive transcriptional binding activity to the epidermal growth factor (EGF) promoter region (Sil et al., 2004), which regulates autocrine loops within keratinocytes (Liu et al., 2008). To assess ox-IKKα’s role in EGF promoter activity, we performed chromatin immunoprecipitation (ChIP) using H2O2 treated keratinocytes. Using ENCODE and genome assembly for human coordinates (GRCh38), we designed primers to target the predicted EGF promoter region in which IKKα may bind (Fig. 6A left panel). ChIP results showed that endogenous IKKα bound to the EGF promoter region under unstimulated (no H2O2 treatment) conditions (Fig. 6A
right panel). In contrast, binding was reduced upon treatment with 10µM H2O2 consistent with the idea that oxidation promotes EGF activity by reducing IKKα nuclear localization. RNA polymerase 2, a general marker for precursor RNA synthesis, showed reciprocal binding activity at the EGF promoter region. Consistent with these findings, siRNA knockdown of IKKα led to a >1.6 fold increase specifically in EGF transcripts whereas IKKβ and IKKγ transcripts remained unaffected (Fig. S4E), validating a functional relationship between IKKα and EGF. Similarly, we observed highly elevated EGF transcript levels upon treatment with low exogenous H2O2 (0.1µM) concentrations but intriguingly not when cells were treated with high concentrations (100µM) (Fig. 6B).

Since growth factors are regulated by rapid decay of their transcripts (Tang et al., 1997), we also investigated the potential role of H2O2 on EGF mRNA stability. Following pre-treatment with actinomycin D for 1hr, which produced steady-state EGF mRNA levels (Fig. 1C, 0min time point), we observed within 30min a transient increase and subsequent degradation of EGF mRNA at low H2O2 concentrations but not in the absence of H2O2. We also did not see this effect when assessing a known H2O2-independent control transcript, OVOL2 (Marinari et al., 2008) (Fig. 6C). This rapid effect of H2O2-dependent EGF mRNA accumulation may occur through the activation of non-genomic upstream signaling regulators such as ERK (Nagashima et al., 2014), which we also observed to be upregulated at the scratch margin (Fig. 6D).

Our findings suggest that ox-IKKα regulates EGF promoter activity, which likely regulates EGFR signaling given the activation of its downstream effector ERK at the scratch wound. However, the EGFR could also be additionally activated by other mechanisms, such as oxidation in an EGF-dependent manner, as shown in squamous carcinoma cells (Paulsen et al., 2012). We therefore investigated H2O2-dependent EGFR activation using immunofluorescence staining and western blot analysis. This showed rapid, transient EGFRY1173 phosphorylation and internalization at the keratinocyte scratch margin, which dissipated under DPI treatment (Fig. 6E). We also assessed another previously shown phosphorylation site (EGFRY1068) in squamous carcinoma cells (Paulsen et al., 2012), which did not show a difference in
phosphorylation with or without H$_2$O$_2$ treatment (not shown). Phosphorylation of EGFR$^{Y1173}$ also occurred under EGF withdrawal, suggesting wound-dependent autophosphorylation that could be mediated by endogenous EGF/H$_2$O$_2$ production (Fig. 6F). Quantitative western analyses revealed a ~3-fold increase in pEGFR$^{Y1173}$ (normalized to EGFR) after 1hr treatment with low H$_2$O$_2$ concentrations (0.1µM), and a ~28-fold decrease with DPI (Fig. S4H). These findings indicate that EGFR activity in HEK001 is regulated by H$_2$O$_2$.

Discussion

Long-term HEK001 cultures (i.e. 14DIV) resulted in deregulated proliferative (PCNA) and basal markers (K14) in the presence of high calcium as observed for other keratinocyte cell lines (Micallef et al., 2009). This hyperproliferation may be due to calcium resistance, response to hypotonic stress, or lack of differentiation factors besides calcium in the medium. Furthermore, it is possible that these differences may also reflect heterogeneous culture preparations, as it is unclear if HEK001 is a pure keratinocyte cell line. Since our studies were performed for relatively short time periods, these late changes should not have influenced our findings. We further show that similar to human lung epithelial cells, primary epithelial cells produce scratch-induced H$_2$O$_2$ and show increased migration upon H$_2$O$_2$ treatment similar to HEK001 cells. Thus, our findings are likely also relevant to other epithelial cells types and in vivo.

We assessed possible enzymes required for H$_2$O$_2$ production given various reports in the literature. For instance, Drosophila and zebrafish embryos, and human lung and neonatal foreskin epithelial cells produce H$_2$O$_2$ via DUOX (Choi et al., 2014; Niethammer et al., 2009; Razzell et al., 2013; Wesley et al., 2007). NOX1 and NOX4, and the common subunit P22/PHOX are in contrast activated in cancer cells (Ha et al., 2016; Ito et al., 2016) and have been associated with keratinocyte-specific activity (Chamulitrat et al., 2004; Nam et al., 2010; Sun et al., 2016). While DUOX1 and NOX1 expression remained unchanged during scratch wound repair, NOX4 expression was
significantly upregulated, although relatively late after 12hr. It is possible that the mRNA expression data may not fully reflect protein expression levels within cells since alternative regulatory mechanisms could be at play. For instance, regulation of subunits that stabilize Nox enzymes or increase in RNA and/or protein stability may be sufficient to generate H$_2$O$_2$ over the course of multiple hours. At present, however, our studies do not permit conclusions about NOX4 involvement given that the expression kinetics of NOX4 do not match the expected H$_2$O$_2$ production profile after scratch wounding.

Our findings show that IKK$\alpha$ is an oxidation target of H$_2$O$_2$. IKK$\alpha$ is mostly known for its kinase-dependent regulation of NF-$\kappa$B signaling, such as during cellular responses to stress (Israel, 2010). In the differentiated epidermis however, IKK$\alpha$ has additional kinase- and NF-$\kappa$B-independent nuclear repressor functions (Fukazawa et al., 2010) to maintain skin homeostasis (Descargues et al., 2008; Hu et al., 2001; Liu et al., 2008). Our results are line with these findings, suggesting NF-$\kappa$B independent mechanisms of ox-IKK$\alpha$ leading to keratinocyte migration. Previous studies showed that IKK$\alpha$’s nuclear function is associated with production of an unidentified soluble factor termed keratinocyte differentiation-inducing factor (kDIF), which can promote terminal differentiation (Descargues et al., 2008; Hu et al., 2001). In parallel, nuclear IKK$\alpha$ is also known to promote keratinocyte differentiation by cis-repression of Egf (Liu et al., 2008). Our study, for the first time, demonstrates that the EGF repressive activity can be blocked by H$_2$O$_2$ through promotion of IKK$\alpha$ cytoplasmic accumulation that stimulates keratinocyte migration. We observed increased IKK$\alpha$ at localized subcellular regions of injured keratinocytes at the scratch margin. At this point it remains to be shown how IKK$\alpha$ is regulated by H$_2$O$_2$. For instance, a steady-state flow of IKK$\alpha$ between cytoplasm and nucleus might be present under homeostatic conditions and oxidation of cytoplasmic IKK$\alpha$ may lead to cytoplasmic accumulation and reduced nuclear flow. Alternatively, nuclear IKK$\alpha$ might be oxidized, leading to active nuclear export. Indeed, it was shown that H$_2$O$_2$ can be detected in the nucleus of mammalian cells and in whole organisms, such as C. elegans (Dickinson et al., 2011). Cytoplasmic sequestration of IKK$\alpha$ suggests alternative molecular functions independent of NF-$\kappa$B signaling. It is
known that cysteine oxidation can change protein conformation and enzymatic activity. Hence, in addition to dissociation from the EGF promoter, $H_2O_2$-modified IKK$\alpha$ may regulate signaling pathways using its kinase function, which would be in line with the observation that IKK$\alpha$ enzymatic inhibition impaired scratch closure, yet endogenous NF-\(\kappa\)B inhibition did not. It is possible that ox-IKK$\alpha$ actively sequesters NF-\(\kappa\)B in the cytoplasm, which remains to be investigated. Alternatively, ox-IKK$\alpha$ could potentially regulate other migration-promoting target genes by cytoplasmic sequestration of nuclear co-repressors (Fernandez-Majada et al., 2007).

Our data shows that low but not high exogenous $H_2O_2$ concentrations induce EGF mRNA expression, correlating with the cytoplasmic accumulation of IKK$\alpha$ and the induction of migration. Our findings further suggest a concentration-related regulation of IKK$\alpha$ sulfenylation, which could be mediated by the availability of intermediate enzymes that control for the sulfenylation reaction. This would be in line with our finding that strongest sulfenylation is observed about 2hr after scratch wounding despite that $H_2O_2$ is produced as early as ~30min. Thus this suggests that initially there is IKK$\alpha$-independent EGFR signaling, which is consistent with the rapid transient $H_2O_2$-dependent activation of EGFR via phosphorylation of Tyr1173, and the temporal stabilization of EGF messenger RNA by $H_2O_2$. The stabilization could be achieved by lack of degradation of the messenger RNA. Based on these findings, we propose a model in which initially EGFR signaling is activated by direct oxidation, whereas oxidation of IKK$\alpha$ subsequently induces EGF expression through activation of the EGF promoter. This might ultimately lead to sustained EGFR signaling required for persistent migratory activity of keratinocytes (Fig. 6G). Further studies are required to determine the relationship between IKK$\alpha$ oxidation and EGFR activity, and the role of EGFR oxidation in basal keratinocyte migration. It is known that EGFR is overly active in many cancers and IKK$\alpha$ genetic/epigenetic down-regulation (Maeda et al., 2007; Park et al., 2007) and cytoplasmic sequestration (Marinari et al., 2008) can trigger oncogenic pathways, of which the regulators of sequestration remain unknown. Interestingly, to our knowledge loss of IKK$\alpha$ is associated with hyperproliferation, but not tumor metastases. On the other hand, IKK$\alpha$ expression \textit{per se} is known to promote a metastatic phenotype.
in some tumors (Luo et al., 2007). Thus, our findings may have even broader implications, as elevated ROS exist in tumors (Liou and Storz, 2010) and low but not high H$_2$O$_2$ levels may act directly on IKKα in the cytoplasm to regulate metastasis.

**Materials and Methods**

**Cell lines, differentiation assay, H$_2$O$_2$ treatment, scratch wound and inhibition assays**

HPV-16 transformed human epidermal keratinocytes (HEK001; ATTC, CRL-2404) were maintained in keratinocyte-serum free (KSF) medium (Gibco-Brl 17005-042) supplemented with 5ng/ml human recombinant EGF, low CaCl$_2$ (0.06mM) and 2mM L-glutamine (without bovine pituitary extract). Cells were incubated with 5% CO$_2$ and 92% humidified atmosphere at 34ºC, and seeded (4x10$^4$ cells/cm$^2$) in tissue culture plates pre-coated with type I collagen (Gibco-Brl, R-011-K).

For RNA and scratch analyses, cells were cultured in 12-well plates (Corning, 3513) and 8-chamber glass bottom dishes (In Vitro Scientific, C8-1.5H-N), respectively. The culture medium was changed every 2-3 days and the dishes were confluent by two days in vitro (DIV) post-seeding.

Keratinocyte differentiation was initiated with 2mM CaCl$_2$ and monitored ~ 2wks. The scratch assay was used to evaluate cell migration and wound recovery (Goetsch KP, Niesler CU, 2011). Cells were grown to confluence, replaced with EGF-minus media for 12hr, refreshed with complete media and glass Pasteur pipettes were used to make vertical scratches along the surface of the vessels. Wells were immediately washed with PBS to avoid re-plating of disassociated cells.

For western blots, after H$_2$O$_2$ treatment, cells were lysed in RIPA buffer supplemented with phosphatase and proteinase inhibitors, and analyzed using the NuPage system (Invitrogen). For RNA expression analyses, wounds were generated using a comb-like device and RNA was subsequently purified. For EGFR immunofluorescence studies (see Antibody staining section), cells were treated without EGF for >12hr and then
scratched with complete media supplementation. For some assays, cells were pre-treated for 1hr with DPI to eliminate endogenous ROS. Primary epidermal keratinocytes (ATCC; PCS-200-010) were grown in KSF media, scratch assays and H$_2$O$_2$ treatments were performed according to HEK001 cell protocols.

**Intracellular H$_2$O$_2$ detection, cell viability**

HEK001 were pretreated for 30min with H$_2$O$_2$ sensor, HPF (Pentafluorobenzensulfonyl-fluorescein (HPF) (Cayman Chemical, CAS: 728912-45-6), scratched and then detected by confocal imaging. HPF was maintained as a 1mM stock in DMSO. Initially the optimal signal:noise ratios were empirically determined using 0.1, 1, 4 and 10µM. Each condition was tested in four separate wells with four replicate images/well. Cell viability and apoptosis were monitored using the 6-carboxyfluorescein diacetate (6-CFDA, ThermoFisher) and Annexin V-Cy3 kit (Sigma, APOAC).

**Keratinocyte Boyden chamber (transwell) migration assay**

Assays were performed using Millicell with hanging transwell polyethylene terephthalate inserts (8µm pore size,Millipore, PIEP12R48) for impedance-based detection of migrated cells. 200µl of serum-free medium that contained 5x10$^4$ 12hr-unstimulated cells was added to the upper compartment, while the lower compartment was filled with 750ul KSF media with or without EGF, H$_2$O$_2$ or inhibitors. After 12-24hr, non-migratory cells remaining in the upper chamber were removed with cotton swabs. Migratory cells on the lower surface of the transwell membrane were fixed (4% PFA) and stained with Giemsa/DAPI. Cells were imaged on an FV1000-confocal microscope (Olympus) and counted using ImageJ.

For mitomycin experiments, a stock solution of mitomycin C (Sigma, M0503) was made fresh in sterile water. A working concentration of 10µg/ml mitomycin C was determined by performing bioactivity assays. For migration assays, cells were co-cultured using
Boyden transwell chambers along with mitomycin C and H$_2$O$_2$, and the number of migrating cells post 12hr were quantified using DAPI staining.

**Micromass assay**

HEK001 cells were expanded in T-25 flasks, trypsinized, and resuspended in 1ml of complete medium. Cells were transferred to 1.5ml tubes and pelleted by centrifugation at 4,500rpm. The medium was aspirated and cells resuspended in KSF-medium to perform cell counts. Cells were resuspended in a final volume (=number of wells X volume per well (in 24-well plates)) where the seeding volume was 30µl per well at 0.5x10$^6$ cells. Cells were placed at the center of each well, carefully placed into a CO$_2$ incubator and allowed to attach for 2-4hr. 1ml of KSF+EGF was carefully added to each well. The medium was replaced every other day without disturbing the centered micromass. The H$_2$O$_2$ detection assay was performed after 4d in culture.

**Quantitative PCR and EGF mRNA stability assay**

Total RNA was purified using the RNeasy mini kit (Qiagen). RNA was reversed-transcribed using Superscript III reverse transcriptase (Invitrogen) and equal amounts of poly-dT and random hexamer oligonucleotides. Gene expression was normalized with human beta-actin mRNA and analyzed using the comparative CT Livak method (Livak KJ, Schmittgen TD, 2001) using BrilliantII SYBR® Green qPCR Master Mix (Agilent) (see Table 1 for primer sequences).

For EGF mRNA stability assessment, cells were pre-treated with actinomycin D (2.5µg/ml; Sigma-Aldrich) for 1hr, followed by treatment with 0.1µM H$_2$O$_2$ before RNA extraction. Both untreated and H$_2$O$_2$-treated samples with actinomycin were compared to the starting samples on graphs.
Antibodies and chemical inhibitors

Antibodies against human Erk1 (pT202/pY204) and Erk2 (pT185/pY187) (Abcam, ab50011), EGFR (pY1068; Abcam, ab32430), EGFR (ThermoFisher, MA5-12880), EGFR (pY1173; Abcam, ab32578), sulfenic acid modified cysteine/2-thiodimethylene (Millipore, AB330), forkhead box O1 (LSBio, LS-C123562), IKKα (Abcam, ab4111), NF-κB (p52/p100) (ThermoFisher, PA5-27340) and NF-κB (p50/p105) (ThermoFisher, PA1-14284) were used at a 1:200 dilution for immunofluorescence studies. AlexaFluor®488 anti-rabbit IgG (Molecular Probes, Invitrogen) and Cy3 anti-mouse IgG (Millipore) were used as secondary antibodies at a 1:1000 dilution. Inhibitors were kept as stock solutions in DMSO for FOXO1 (Millipore, 344355; IC$_{50}$=33nM), diphenyleneiodonium chloride (DPI; Sigma-Aldrich, 2926), NF-κB (Santa Cruz Biotechnology, JSH-23; IC$_{50}$=7.1µM), IKK Wedelolactone (Millipore, 401474; IC$_{50}$=10µM), EGFR (Millipore, 324673; IC$_{50}$=20nM), and apocynin (Santa Cruz Biotech, sc-203321; IC$_{50}$=10µM). DYn-2 was a kind gift from Kate Carroll (Scripps Institute).

Antibody, Crystal violet and Phalloidin staining

HEK001 cells were fixed in 4% PFA (15min) and permeabilized in 0.25% triton-X (10min) at room temperature. Cells were blocked with 1%BSA/10% goat serum/0.1% Tween-20 in PBS (30min) and incubated with primary antibodies (1:200) in blocking buffer overnight at 4°C. Cells were washed and labeled with appropriate secondary antibody (1:1000) and DAPI/Hoechst (1:5000). To detect sulfenylated proteins, keratinocytes were incubated for 2hr in DMSO or 2mM dimedone (5,5-dimethyl-1,3-cyclohexanedione) (Cayman Chemical), scratch-wounded or treated with H$_2$O$_2$ and further incubated for 2hr. Samples were fixed and stained with a polyclonal anti-dimedone antibody (EMD Millipore, 07-2139). The cells were washed, and stained with anti-rabbit AlexaFluor488 (Life Technologies) for detection on a confocal microscope. To stain DIV7 cells that have been differentiated with 2mM Ca$^{2+}$, cells were incubated in 0.2% crystal violet/10% ethanol for 10min, followed by imaging on an inverted stereomicroscope (Zeiss, Germany). Phalloidin-647 (ThermoFisher) was used to assess
the actin cytoskeleton. Cells were grown to confluency and either left untreated or treated with 0.1 and 1µM H2O2 for 2hr. Cells were fixed in 4% PFA (15min) and washed in PBS. Phalloidin staining was performed according to the manufacturer’s protocol. The cells were washed in PBS and immediately imaged on an FV1000-confocal microscope (Olympus).

**Azide labeling and click-chemistry biotinylation of labeled oxidized proteins**

The alkyne probe DYn-2 (4-(pent-4-yn-1-yl)cyclohexane-1,3-dione; 5mM (Cayman Chemical, 11220) was added to HEK001 cells for 1hr at 37°C. The cells were lysed in modified RIPA (200U/ml catalase, EDTA-free protease/phosphatase inhibitors) and pre-cleared of endogenous biotinylated proteins using pre-equilibrated NeutrAvidin resin (Thermo, 29202). Probe-labeled proteins were detected via Click-Chemistry (Invitrogen, C10276) with solubilized 4mM biotin azide (Invitrogen, B10184). Proteins were precipitated using methanol and electrophoresed. For whole proteome detection, the membrane was blotted with streptavidin-HRP and loading antibodies (Santa Cruz Biotech, IKKα (B-8), sc-7606; GeneTex, GAPDH, 124503). For detection of specific oxidized proteins, biotin azide-labeled proteins were immunoprecipitated using NeutrAvidin agarose beads, eluted with 8M guanidine-HCL, dialyzed using a Slide-A-Lyzer® MINI dialysis device (Thermo Scientific; 88401), concentrated using Amicon Ultra(3K) centrifugal filters (Millipore, 500324), and re-probed for western analyses.

**Silencing of human IKKα expression by RNA interference assay**

A 50µM stock of Silencer® Select validated siRNA oligonucleotides for human IKKα (Locus ID: 1147) (Ambion®, Life Technologies; siRNA ID: s3077) was resuspended in nuclease-free water and stored at -80°C. For IKKα mRNA knockdown validation, HEK001 cells were grown to 60-70% confluence (1.6X10^5 cells/well (12 well-plate)) and siRNAs (10-20pmol) were transfected for 24hr using the Lipofectamin® RNAiMAX reagent and methodology (Life Technologies). Gene expression (qPCR) was investigated after 48hr transfection (see Table 1 for primers). Validation of protein knockdown was also performed at this time using IKKα (Abcam, ab4111) antibody in western blots. For scratch assays using siRNA-transfected HEK001, cells were
trypsinized and then re-plated at full confluence in 8-well chambers at 48hr post-transfection. siRNA-transfected cells were scratched 12hr after re-plating and processed accordingly (n=3 experiments performed in duplicate).

**ChIP-qPCR analyses of the hEGF promoter**

HEK001 cells were grown to 80% confluence, and cultured for 12hr without EGF. EGF was reintroduced with or without 10µM H2O2 for 30min. In vivo crosslinking of chromatin was performed with 1% formaldehyde and quenched with glycine. Digestion and isolation of chromatin-bond DNA by immunoprecipitation (chromatin IP) was performed using a magnetic ChIP kit (26157, Pierce) based on the manufacturer’s protocol. To assess physical binding of components to genomic sequences, RNA polymerase II (included in ChIP kit) and IKKα (sc-7606, Santa Cruz Biotech) ChIP-grade antibodies were used for IP. Subsequent PCR quantification was performed using SYBR™ green (600882, Agilent) with primers designating hEGF promoter and intergenic negative control DNA sequences (Table 1). The current genome assembly for human coordinates (GRCh38) was used to design primers. The negative control was used to normalize for DNA content to calculate the enrichment of regulatory hEGF region according to the Livak method.

**Generation and transient transfection of Ikka constructs**

Zebrafish *Ikka* (*Chuk/Ikka*) cDNA was purchased from Open Biosystems (Clone ID: 5914247, GE Dharmacon). Full-length (FL)-*Ikka* was amplified with Advantage 2 Taq Polymerase (Clontech) using forward primer 5’-GCTAGCTGTCAATATGGAGAAACCCCCT-3’ and reverse primer 5’-TAATGGATTGGTACCGACAAACGCGGATTATTAAAAA-3’. The amplified PCR products were cloned into pCR Blunt II-TOPO using the Zero Blunt® TOPO® PCR Cloning Kit (Life Technologies). To generate tdTomato fusion constructs, pCROTOPO-FL-*Ikka* and ptdTomato-N1 vector (Clontech) were digested with KpnI and Nhel and ligated. The *Ikka*(C179A)-tdTomato variant was generated from FL-Ikka1-tdTomato using site-directed mutagenesis (GeneArt® Site-Directed Mutagenesis PLUS Kit, LifeTechnologies). The constructs were subsequently cloned into a plasmid containing
the zebrafish tp63 promoter (pT2KXIG.tp63:AcGFP, courtesy of Gromoslav Smolen). First GFP was removed via digestion with BamHI and NotI, and both sites were Klenow blunt-ended. The ikk1-tdTomato plasmids were digested with SnaBI and SfoI and ligated into pT2KXIG.tp63 to generate tp63:FL-ikk1-tdTomato and tp63:ikk1(C179A)-tdTomato. HEK001 were transfected using Nanofectin (PAA, Q050-005) when 60% confluent in 8-chamber vessels. It was determined that 0.5µg of DNA per 0.6µl of transfection reagent was optimal.

Confocal imaging and relative comparison and data analyses
Laser scanning confocal microscopy images were obtained using an inverted Olympus FV1000 or Zeiss LSM510 unit. Z-stacks obtained from 1-3µm/slice per sample and speed of 12.5 (µs/pixel) were projected. All other parameters (e.g. pinhole diameter, gain, laser intensities) were kept constant. The series of projected “z-axis” images were used to calculate average fluorescent intensity profiles per channel using the line series and box analyses tools in the Fluoview software v. 4.1 (Olympus). A minimum of 16 evenly distributed “scratch” lines were averaged per image, or 6 evenly distributed boxes were averaged per image to generate average (background) fluorescence intensities. The fluorescence intensity was never saturated (max. 4096 intensity level) during imaging. Samples were relatively compared and background corrected between the scratch margins and averaged unscratched “margin-free” regions within the same well and vicinity. The line tool was used to measure length:width ratios for most elongated, sulfenylated cells (a total of ~60 per group). Cell-cell distances and fluorescence intensities were measured in Imaris (Bitplane) using the line tool and spots function, respectively. Migration of Ikk1-transfected HEK001 was documented with time-lapse imaging for 8hr using an incubation chamber.

Statistical analyses
Statistical comparisons were made using Prism 6 software (GraphPad). As indicated in the figures, the unpaired Student’s T-test with a 95% confidence interval was used to compare the means of two unmatched groups, assuming that the values followed a
Gaussian distribution. For multiple comparison tests of three or more groups, one-way ANOVA at an alpha=0.05 (95% confidence interval) and Tukey’s multiple comparison post-tests were utilized to compare the means of each column. Significance is denoted with asterisks: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

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Competing Interests

The authors do not declare any competing financial interests.

Author contributions

T.S.L. and S.R. conceived and designed the experiments, carried out the experiments, analyzed the data, and wrote the manuscript.

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**Figures**

**Figure 1.** Endogenous and exogenous H$_2$O$_2$ activates HEK001 migration

(A) Rapid H$_2$O$_2$ formation at the scratch (Sc) wound margin detected with HPF. (B) Quantification of H$_2$O$_2$ production in (A). (C) H$_2$O$_2$ tracking during scratch repair shows diminished HPF signal shortly before scratch closure. (D) DPI inhibition of keratinocyte migration after scratch with limited cytotoxicity, assessed using the cell viability dye 6-cfda. (E) Treatment of cells with the apocynin and DPI impairs scratch wound repair. (F) Diminished H$_2$O$_2$ levels at the scratch margin after DPI treatment. (G) Accelerated scratch closure upon treatment with exogenous H$_2$O$_2$ when compared with endogenous H$_2$O$_2$–dependent scratch closure (depicted by black dotted line). Addition of exogenous H$_2$O$_2$ to DPI-treated cells promotes scratch closure similar to endogenous rates. DPI treatment reduces closure distance over the course of 18hr (red dotted line depicts baseline for comparisons with DPI+H$_2$O$_2$). (H) HPF staining in micromass assay shows strongest fluorescence at outer edges. (I) Quantification of fluorescence intensity in (H).
(J) Transwell migration assay performed with untreated cells and after treatment with H$_2$O$_2$. Migration is absent with DPI treatment but rescued upon supplementation of DPI-treated cells with H$_2$O$_2$. High H$_2$O$_2$ levels block migration. (K) Quantification of (J). (L) Transwell migration assay in the presence of mitomycin C in the presence of 0.1 and 1µM H$_2$O$_2$ shows increased migration, independent of proliferation. (M) DUOX1, NOX1, and NOX4 qPCR in unscratched and scratched HEK001 cells. SC (scratch), HPF (hydrogen peroxide-fluorogenic probe), 6-cfda (6-carboxyfluorescein diacetate) Bars=100µm. One-way ANOVA and Tukey’s multiple comparison post-test were used. Significance: *p<0.05, **p<0.01, p***<0.001 (n≥3-5 cell culture experiments)
Figure 2. Scratch wound assay defines candidates for oxidation-dependent repair

(A) Effects of inhibitors on HEK001 scratch closure (5µM sets shown). (B) Dose response of inhibitors measured by the scratch closure distance. (C) HPF signals at the scratch margin in the absence and presence of FOXO1 inhibitor. (D) Rapid nuclear localization of FOXO1 at the scratch margin of vehicle controls. Pre-treatment with DPI decreases FOXO1 staining at the scratch. Inset shows magnification of cells marked with red arrowheads. (E) Nuclear NF-κB1 is noticeable at the wound margin at 6hr post scratching but is cytoplasmic following wound closure at ~18hr similar to unscratched cells. (F) Predominantly cytoplasmic NF-κB1 p50/p105 staining in untreated cells and following H$_2$O$_2$ treatment for 2hr. (G) Non-canonical NF-κB2 (p52/p100) immunofluorescence following scratch shows predominantly cytoplasmic staining. (H) p52/p100 immunofluorescence reveals sustained cytoplasmic localization after H$_2$O$_2$
treatment at 2hr. High $\text{H}_2\text{O}_2$ concentrations lead to increased expression. Bars (A-E, H)=100µm, (F, G)=50µm. Scratch (Sc). Two-way ANOVA and Bonferroni's multiple comparison post-tests were used. Significance: *$p<0.05$, ***$p<0.001$, ns: not significant, (n≥3-5 cell culture experiments)
Figure 3. H$_2$O$_2$-dependent regulation of IKK$\alpha$ during scratch repair

(A) Rapid IKK$\alpha$ cytoplasmic and peri-nuclear localization (white arrowheads) at the wound margin 30min post-scratch. DPI treatment increases nuclear localization within the scratch area (see higher magnification insets). Bars=100µm in the left panels and 10µm for insets. (B) Quantification of IKK$\alpha$ subcellular distribution in (A). (C) IKK$\alpha$ localization within different compartments (measured after 2hr). Right panel shows higher magnifications. White arrowheads point to IKK$\alpha$’s cytoplasmic perinuclear
accumulation at low H$_2$O$_2$. Red arrowheads point to IKK$\alpha$'s nuclear accumulation and diffuse cytoplasmic staining in untreated cells and after treatment with high H$_2$O$_2$ concentrations (500µM). Bar in left panel=50µm, Bars in insets=10µm. (D) siRNA knockdown of IKK$\alpha$ prevents H$_2$O$_2$-induced scratch closure.

Two-way ANOVA and Bonferroni's multiple comparison post-tests were utilized. Significance: *p<0.05, **p<0.01, ns: not significant, (n≥3-5 cell culture experiments)
Figure 4. Protein sulfenylation in HEK001

(A) Scheme describing dimedone and DYn-2-labeling of cysteine-sulfenic acid (S-OH)-modified proteins. (B) Low-level treatment with H$_2$O$_2$ for 2hr induces sulfenylation in a subpopulation of cells with a migratory phenotype, showing lamellipodia formation (arrows, insets show magnifications). Bars=100µm and 10µm=inset. (C) Sulfenylated
cells display increased length:width ratios compared with non-sulfenylated cells. (D)

Quantification of cell-cell distance between elongated, pro-migratory and neighboring

cells. (E) Phalloidin-647 staining shows increased stress fiber formation upon 0.1 and
1µM H₂O₂ treatment. Bars=20µm. (F) Cytoplasmic/peri-nuclear protein oxidation

predominately in scratch margin cells (arrowheads) but sulfenylation is largely absent in

scratched keratinocytes treated with DPI. Bar=100µm (G) Dimedone treatment of

impairs scratch wound repair. (H, I) Cell viability and death assessment using 6-cfda

and Annexin staining, respectively. Bar=100µm
Figure 5. IKKα sulfenylation promotes keratinocyte migration

(A) Upper blot: DYn-2 probe-loaded keratinocytes were biotinylated by “click chemistry” with a biotinylated azide probe. Sulfenylated proteins were probed with Streptavidin-HRP (upper panel). IKKα is detected in all fractions (with or without DYn-2 and H2O2).

Lower blot: H2O2 oxidizes IKKα assessed after purification of biotinylated fraction by streptavidin pull-down and probing with anti-IKKα antibody. GAPDH serves as positive control for successful pull-down of oxidized/sulfenylated proteins given its redox function (Truong, 2012).

(B) Human and zebrafish IKKα kinase domain sequence comparison shows high conservation (~70%), including zebrafish cysteine 179 (arrow).

(C) IKKα construct scheme for HEK001 in vitro transfection studies shown in lower right panel.

Full-Length (FL) Ikkα-tdTomato in cytoplasmic perinuclear regions with and without H2O2 (white arrowheads). DPI treatment (10µM) results in punctate localization within subregions of the nucleus. Bar=100µm (D, E) Point mutation (C179A) shows nuclear localization without H2O2 (D) and with H2O2 (0.1µM) (E). Bar=100µm (F) Cell movement
and directionality (arrows) tracked over time. Control (untreated) cells migrate similar distances as cells treated with high H$_2$O$_2$ (100µM) concentrations, following DPI treatment and when transfected with C179A-Ikkα. Transfection with FL-Ikkα and treatment with low H$_2$O$_2$ (0.1µM) concentrations promotes migration. Bar=40µm LZ (leucine zipper), HLH (helix-loop-helix), NBD (Nemo binding domain)
Figure 6. Ox-IKKα increases EGF expression and EGFR activity

(A) ChIP assay in HEK001 cells with or without H2O2 shows decreased IKKα binding to the EGF promoter following H2O2 treatment, whereas RNA polymerase binding increases, indicative of increased EGF expression (n=3 experiments). (B) Transcriptional activation of EGF after H2O2 treatment. (C) H2O2 treatment (0.1µM) increases EGF mRNA stability within 30min and leads to subsequent destabilization. H2O2 does not induce ovo-like zinc finger 2 (OVOL2) transcription in HEK001, which is known to be oxidation-independent. (D) Increased ERK1/2 phosphorylation in HEK001 cells adjacent to the scratch margin and quantification of ERK1/2 phosphorylation. (E) H2O2-dependent phosphorylation of EGFRY1173 after acute scratch injury of HEK001 cells. Intracellular recycling of EGFRY1173 in scratch margin keratinocytes (arrows, inset) shown by immunofluorescence studies. (F) Rapid phosphorylation of EGFRY1173 at the
scratch margin in immunofluorescence studies in the absence of exogenous EGF. Immunostaining was also prevalent at the cell boundary (arrowhead). (G) Wound keratinocyte migration model: High levels of H$_2$O$_2$ may outcompete available antioxidant complexes and lead to non-specific oxidation and apoptosis, whereas low levels stimulate cysteine sulphenylation and migration. Sulphenylation of IKKα activates keratinocyte migration through IKKα-mediated regulation of EGF transcription and stability. Low H$_2$O$_2$ also promotes EGFR phosphorylation at Tyr1173. FOXO1 might regulate H$_2$O$_2$ levels to promote migration. Bars=100µm. One-way ANOVA and Tukey’s multiple comparison post-tests were utilized. Significance: *p<0.05, **p<0.01
Figure S1. *In vitro* characterization of HEK001 keratinocytes

(A) Differentiation responses to Ca^{2+}-supplemented media. Low (0.06mM) and high (2mM) Ca^{2+} downregulates PCNA transcription over time. Long-term (>14days) cultures develop
resistance to Ca\textsuperscript{2+}-inhibition of the cell cycle (e.g. PCNA, K14). Early (K10) and late (INVOLUCRIN) keratinocyte markers increased over time influenced by calcium levels. AXIN2 (an inhibitor of the Wnt pathway) mRNA increased over time in a calcium-dependent manner. (B) High calcium increased the presence of stratifying colonies (white arrows), an indication of differentiation. (C) DIV7 cultures stained with crystal violet and maintained in high calcium developed aggregates of terminally differentiated lipid-forming keratinocytes (black arrows). (D) DIV7 cultures fail to close scratch wounds when assessed after 72 hours. (E) Decreased H\textsubscript{2}O\textsubscript{2} production at the scratch margin at DIV7 but not DIV2 cultures, indicating the loss of basal keratinocyte function upon differentiation. (F) Quantification of H\textsubscript{2}O\textsubscript{2} production at the scratch margin in (E). Bars=80µm in (B) and 100µm in (C-E).

DIV (days \textit{in vitro}), HPF (hydrogen peroxide fluorogenic probe), Sc (scratch). One-way ANOVA at an alpha=0.05 (95% confidence interval) and Tukey’s multiple comparison post-tests were utilized. Significance is denoted with asterisks: *p<0.05, **p<0.01
Figure S2. Determination of optimal H$_2$O$_2$ sensor levels in the scratch assay

(A) Example of data collected for 4µM HPF under unscratched and scratched conditions. Fluorescence intensity at the scratch margin is similar to areas distal to the scratch wound. Bar=100µm

(B) Quantification of fluorescence intensity after 1 hour of scratching following treatment with 0.1-10µM HPF (30 minutes pre-incubation prior to scratch). Measurements at both the scratch margin and unscratched sites were compared. 10µM of HPF resulted in increased toxicity as measured by 6-cfda (6-carboxyfluorescein diacetate, not shown).

(C) Optimal concentration of HPF determined to be 1µM by calculation of the signal:noise ratios 1 hour after scratch.

(D) Rapid increase in H$_2$O$_2$ production using 1µM HPF (hydrogen peroxide fluorogenic probe) at the scratch (sc) margin of primary human keratinocytes after 30min. Bottom panels represent the zoom of the upper boxed images. At 18 hours after scratching,
protruding (migrating) keratinocytes at the scratch margin (arrows) that establish contact with cells from the opposite scratch site produce less H$_2$O$_2$ compared to non-migratory cells. Bars=100 in top panel and 50µm in bottom panel. (E) Low but not high H$_2$O$_2$ levels promote keratinocyte migration after injury.

HPF (hydrogen peroxide fluorogenic probe), Sc (scratch), One-way ANOVA at an alpha=0.05 (95% confidence interval) and Tukey’s multiple comparison post-tests were utilized. Significance is denoted with asterisks: *p<0.05, **p<0.01, n=3 experiments.
Figure S3. Upstream pathway analyses of gene transcription assayed by RNAseq in H$_2$O$_2$-treated zebrafish larvae
(A) Categorization of statistically significant differentially regulated transcripts that were included in specific upstream regulator sets (H₂O₂, EGF, Chuk/ikk1, Foxo1) as determined by Ingenuity® Upstream Regulator Analysis. (B) Mechanistic gene network of enriched upstream regulators. The most common downstream effectors of all networks were mmp9, f3, hmox1, gadd45b and atf3. These genes formed three major biological clusters implicated in the negative regulation of apoptosis (mmp9, f3, hmox1, gadd45b), cell migration (mmp9, f3, hmox1), and transcriptional regulation (atf3, hmox1). Colored factors represent transcripts, which were statistically significant; color of each node indicates the level of fold change (red=elevated, green=lowered). FOXO1 (Forkhead box protein O1), IGFBP1 (insulin-like growth factor-binding protein 1), HMOX1 (Heme Oxygenase 1), HSPA1L (Heat shock 70kDa protein 1L), ITGB4 (Integrin, beta 4), IVL (involucrin)
**A** IkKa
DIV7-2mM Ca^{2+}

**B** IkKa
DIV7 + DPI

**C** IkKa
DIV7 - EGF

**D** Cy3-Cont.
>75% TE
HEK001

**E** IKKα siRNA knockdown (HEK001)

**F**

**G** 12hr post-scratch
untreated 0.1μM H2O2

**H**

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**pEGFR^{Y1173}**

134

**EGFR**

134

**GAPDH**

37

$M_w$ (kDa)
Figure S4. IKKα nuclear localization under various conditions in DIV7 HEK001 cells and siRNA knockdown

(A) HEK001 keratinocytes (DIV7) were differentiated by supplementing with 2mM CaCl2 for 24 hours followed by IKKα immunofluorescence staining, which shows nuclear IKKα localization. (B, C) HEK001 cells (DIV7) were treated with 50µM DPI (B) and without EGF (C), which also shows predominantly nuclear IKKα staining. Bar=100µm (D) Cy3-labeled control siRNA oligonucleotide revealed >75% transfection efficiency (TE) in HEK001 cells. Bar: 20µm (E) Quantitative PCR of IKKα siRNA-transfected cells shows a >60% knockdown of IKKα mRNA compared with IKKβ or IKKγ mRNA. EGF mRNA is significantly upregulated under IKKα knockdown conditions. (F) siRNA knockdown is confirmed at the protein level using IKKα western blot detection. Bars=100µm. (G) Scratch wound cells at 12 hours post scratch after control and IKKα siRNA transfection showing the absence of migrating cells at the wound edge when IKKα is knocked down. (H) H2O2-specific, injury-independent EGFRY1173 phosphorylation at 1hr, which is reduced in cells treated with DPI whereas total EGFR and GPADH expression does not change. Bars=50µm

Sc (Scratch), WB (Western blot), (small interfering RNA), One-way ANOVA at an alpha=0.05 (95% confidence interval) and Bonferroni’s multiple comparison post-tests were utilized. Significance is denoted with asterisks: *p<0.05, ***p<0.001 (n ≥ 3-5 cell culture experiments)
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