



ELSEVIER

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Short Communication

A role for peroxiredoxins in H₂O₂- and MEKK-dependent activation of the p38 signaling pathway

Ana G. Barata, Tobias P. Dick*

Division of Redox Regulation, DKFZ-ZMBH Alliance, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

ARTICLE INFO

Keywords:

Hydrogen peroxide
H₂O₂ signaling
p38
MEKK4
Peroxiredoxins
Redox relays
Mitogen activated protein kinase

ABSTRACT

The p38 mitogen-activated protein kinase (MAPK) signaling pathway plays an important role in the cellular response to various stresses and its deregulation accompanies pathological conditions such as cancer and chronic inflammation. Hydrogen peroxide (H₂O₂) is a well-established activator of the p38 MAPK signaling pathway. However, the mechanisms of H₂O₂-induced p38 activation are not yet fully understood. In *Drosophila* cells, we find that H₂O₂-induced activation of p38 depends on the MAPK kinase kinase (MAP3K) Mekk1. In line with the emerging role of peroxiredoxins as H₂O₂ sensors and signal transmitters we observe an H₂O₂-dependent interaction between Mekk1 and the cytosolic peroxiredoxin of *Drosophila*, Jafrac1. In human cells, MEKK4 (the homologue of Mekk1) and peroxiredoxin-2 (Prx2) interact in a similar manner, suggesting an evolutionarily conserved mechanism. In both organisms, H₂O₂ induces transient disulfide-linked conjugates between the MAP3K and a typical 2-Cys peroxiredoxin. We propose that these conjugates represent the relaying of oxidative equivalents from H₂O₂ to the MAP3K and that the oxidation of Mekk1/MEKK4 leads to the downstream activation of p38 MAPK. Indeed, the depletion of cytosolic 2-Cys peroxiredoxins in human cells diminished H₂O₂-induced activation of p38 MAPK.

1. Introduction

The p38 signaling pathway is centered on a three-kinase cascade. The cascade is initiated by activation of a mitogen activated protein kinase kinase kinase (MAP3K) which phosphorylates and activates a MAP kinase kinase (MAP2K) which in turn phosphorylates and activates p38 MAP kinase (MAPK) [1]. The p38 MAPK signaling pathway can be triggered by diverse cellular stressors (including UV radiation, osmotic imbalance, heat and oxidants) as well as growth factors and cytokines [2]. Activation of the pathway results in the up-regulation of adaptive mechanisms and/or the making of cell fate decisions.

Hydrogen peroxide (H₂O₂) is a well-established activator of the p38 MAPK signaling pathway [3]. Major sources of H₂O₂ in eukaryotic cells are the mitochondrial electron transport chain and NADPH oxidases (NOXes) [4,5]. NOXes generate superoxide and consequently H₂O₂ downstream of the activation of cell membrane receptors by growth factors and cytokines or during immune responses in phagocytes [5]. H₂O₂ signaling involves the oxidative modification of particular cysteines localized at the surface of redox-regulated proteins [6]. Cysteine oxidation in redox-regulated proteins typically results in the alteration of protein function, e.g. phosphatase inactivation or kinase activation [7,8].

The question of how H₂O₂ can act as a signaling molecule and trigger specific responses has been a long-standing subject of discussion [9,10]. Evidence suggests that in most cases the direct reaction between H₂O₂ and thiols is too slow to be of biological signaling significance. It now emerges that H₂O₂ sensing and signaling can be facilitated by peroxiredoxins (Prxs) [11,12]. These thiol peroxidases are highly abundant and extraordinarily efficient in reacting with H₂O₂. Indeed, they are amongst the most H₂O₂ sensitive proteins in the cell and likely to outcompete other potential protein targets [13]. Mammalian peroxiredoxins have already been observed to relay H₂O₂-derived oxidizing equivalents to a kinase and a transcription factor [14,15]. The involvement of Prxs potentially explains the specificity of redox signaling, because the oxidation of target proteins is based on protein-protein interactions with Prxs rather than diffusional collisions with H₂O₂ molecules.

Previously, it has been reported that human Prx1 contributes to H₂O₂-induced activation of p38 by facilitating the formation of disulfide-linked oligomers of the MAP3K ASK1¹⁵. ASK1 oligomerization is necessary for the initiation of the MAPK signaling cascade [16]. Interestingly, other MAP3Ks, MEKK4 and TAK1, have also been proposed to be activated by H₂O₂ [17,18]. This suggests the possibility that multiple MAP3Ks are redox-regulated and subject to peroxiredoxin-mediated

* Corresponding author.

E-mail address: t.dick@dkfz.de (T.P. Dick).<https://doi.org/10.1016/j.redox.2019.101340>

Received 10 July 2019; Received in revised form 1 October 2019; Accepted 7 October 2019

Available online 08 October 2019

2213-2317/ © 2019 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license

<http://creativecommons.org/licenses/by-nc-nd/4.0/>.

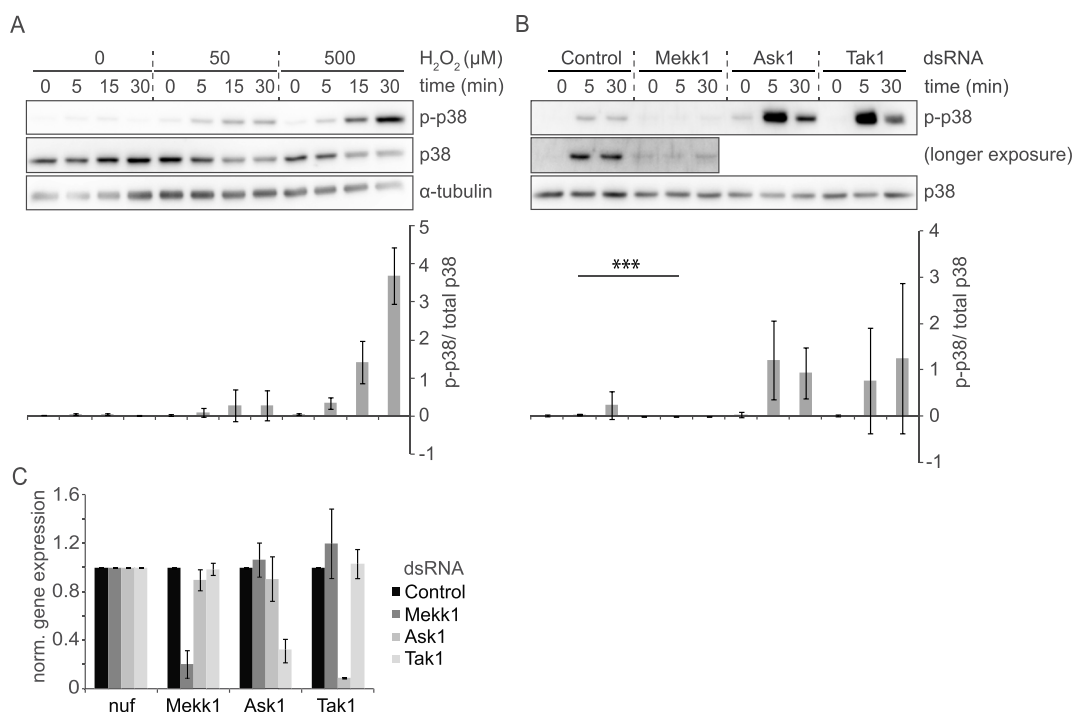


Fig. 1. The MAP3K Meck1 mediates the activation of the p38 signaling pathway in response to H₂O₂ in *Drosophila* S2R+ cells.

(A) S2R+ cells were incubated with increasing concentrations of H₂O₂ and lysed at different time points. Phosphorylation of p38 was visualized by immunoblotting (upper panel). The immunoblot is representative of 3 independent experiments (n = 3). The bar chart (lower panel) presents the mean (+/- SD) ratio of phosphorylated p38 to total p38 from 3 independent experiments. (B) S2R+ cells were treated for 5 days with dsRNAs against the MAP3Ks Meck1, Ask1 and Tak1, and against an unrelated protein (control). Cells were treated with 500 μM H₂O₂ and p38 phosphorylation was analyzed by immunoblotting at different time points (upper panel). The immunoblot is representative of 3 independent experiments (n = 3). The bar chart (lower panel) presents the mean (+/- SD) ratio of phosphorylated p38 to total p38 from 3 independent experiments. (C) Efficacy of the dsRNAs used to deplete the MAP3Ks was analyzed by qPCR. The bar chart presents the mean (+/- SD) normalized gene expression level from 3 independent experiments (n = 3).

oxidation. Here we show that Meck1, the *Drosophila* homologue of mammalian MEKK4, is the main MAP3K responsible for initiating H₂O₂-induced activation of p38 in S2R+ cells. In the presence of H₂O₂, Meck1 interacts with the single cytosolic typical 2-Cys peroxiredoxin of *Drosophila*, Jafrac1, and forms transient mixed disulfide conjugates with it. We also demonstrate a corresponding interaction between MEKK4 and peroxiredoxin-2 (Prx2) in human cells, suggesting that the interaction is evolutionarily conserved between insect and human cells. Finally, we show that combined depletion of human peroxiredoxins Prx1 and Prx2 interferes with H₂O₂-induced p38 phosphorylation, supporting the notion that peroxiredoxin-mediated oxidation activates MEKK4 and hence p38.

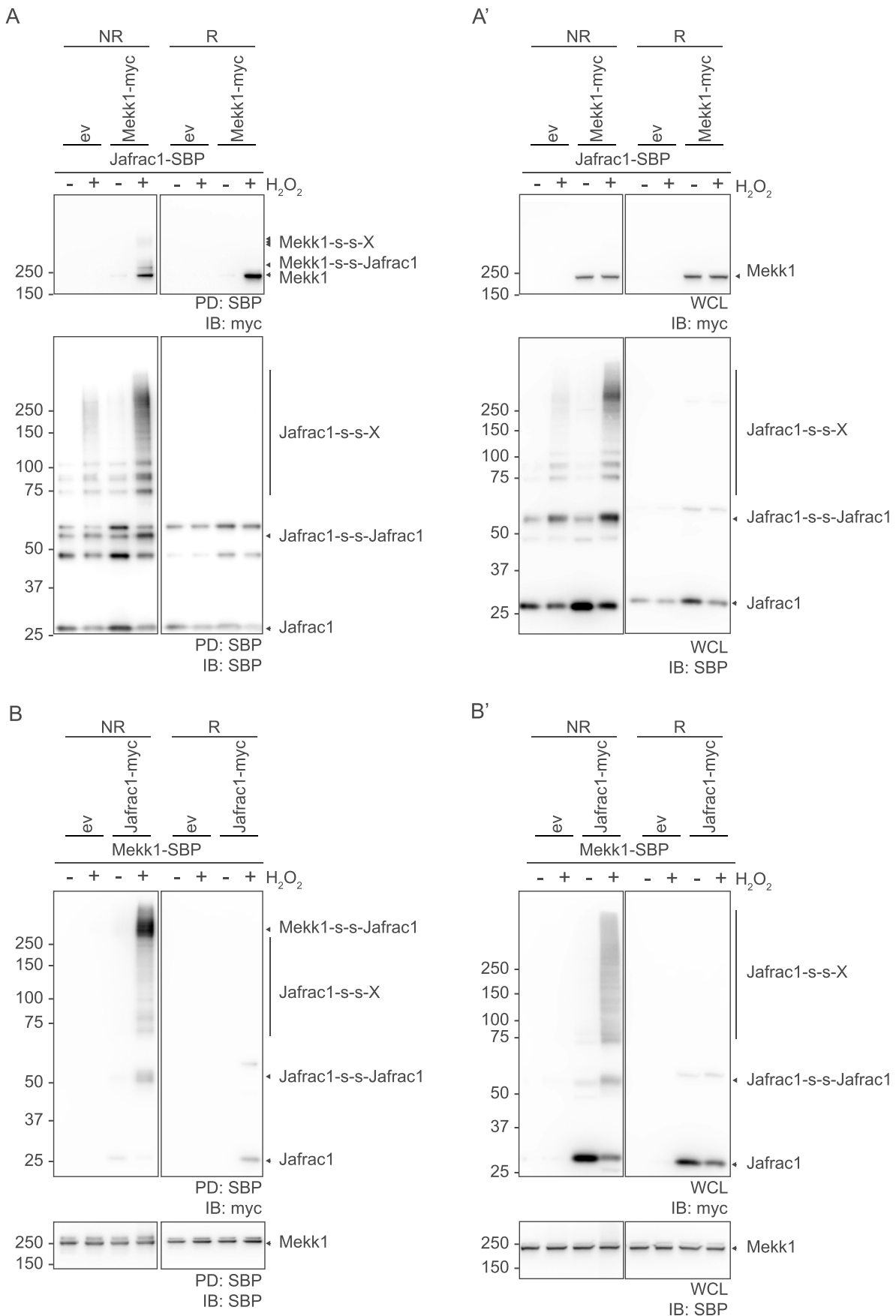
2. Results

2.1. *Drosophila* Meck1 mediates H₂O₂-dependent activation of p38

Using *Drosophila* S2R+ cells, we first confirmed that treatment with H₂O₂ triggers the phosphorylation (and hence activation) of p38 (Fig. 1A). Next, we asked which of the MAP3Ks is responsible for H₂O₂-induced p38 activation. To this end, we depleted individual MAP3Ks previously reported to be activated by oxidative stress [17–19], i.e. Meck1, Ask1 and Tak1, and then analyzed H₂O₂-induced phosphorylation of p38. The depletion of Meck1, but not of Ask1 and Tak1, prevented phosphorylation of p38 (Fig. 1B). In contrast, depletion of either Ask1 or Tak1 further increased H₂O₂-dependent phosphorylation of p38. The efficiency and specificity of mRNA depletion was confirmed by qPCR (Fig. 1C). This result suggested that Meck1 is the MAP3K primarily responsible for H₂O₂-induced p38 activation in S2R+ cells.

2.2. *Drosophila* Meck1 is a redox-sensitive protein and covalently interacts with the peroxiredoxin Jafrac1 in response to H₂O₂

Given the emergent role of peroxiredoxins as highly sensitive H₂O₂ sensors and redox signal transmitters [20], we asked if Meck1 is a target of peroxiredoxin-mediated thiol oxidation. This mechanism predicts a direct interaction between Meck1 and Jafrac1, the only cytosolic typical 2-Cys peroxiredoxin in *Drosophila*, and the formation of transient disulfide exchange intermediates between the two proteins. To test this notion, we co-expressed tagged versions of Jafrac1 and Meck1 and performed co-precipitation experiments in both directions, in the absence or presence of H₂O₂. In response to H₂O₂, Jafrac1 formed numerous mixed disulfide conjugates with other proteins (Fig. 2A, lower left panel), as previously shown for human peroxiredoxins [14]. At least three Meck1-containing species co-precipitated with Jafrac1 (Fig. 2A, upper left panel, lane 4), one of which corresponding in size to monomeric Meck1, indicating a non-covalent interaction between Meck1 and Jafrac1. Bands of higher molecular weight match the expected gel mobility of a Jafrac1-Meck1 and/or (Jafrac1)₂-Meck1 disulfide-linked conjugate. Further weak bands above 250 kDa either represent disulfide conjugates with different stoichiometry or containing other proteins. Consistently, the reverse experiment also demonstrated Jafrac1-Meck1 disulfide conjugates: Jafrac1 co-precipitated by Meck1 appeared at a molecular weight corresponding to a Jafrac1-Meck1 and/or (Jafrac1)₂-Meck1 disulfide-linked conjugate (Fig. 2B, upper left panel, lane 4). In summary, these results indicate that Meck1 and Jafrac1 interact directly and form disulfide-linked conjugates with each other in an H₂O₂-dependent manner. This finding suggests that Jafrac1 is oxidatively modifying Meck1 in response to elevated H₂O₂ levels.



(caption on next page)

Fig. 2. *Drosophila* Mekk1 is redox-sensitive and interacts with cytosolic peroxiredoxin Jafrac1 in response to H₂O₂. (A, A') Tagged versions of Jafrac1 (Jafrac1-SBP) and Mekk1 (Mekk1-myc) were expressed in S2R+ cells. Cells were treated with 500 μM H₂O₂ for 5 min and Jafrac1-SBP was affinity-purified with streptavidin beads. Precipitates (A) and whole cell lysates (WCL) (A') were analyzed by SDS-PAGE under reducing (R) and non-reducing (NR) conditions followed by immunoblotting (IB). The immunoblots are representative of 3 independent experiments (n = 3). (B, B') Complementary affinity purification experiment: Reverse-tagged versions of Jafrac1 (Jafrac1-myc) and Mekk1 (Mekk1-SBP) were expressed in S2R+ cells. Cells were treated with 500 μM H₂O₂ for 5 min and Mekk1-SBP was affinity-purified with streptavidin beads. Precipitates (B) and WCL (B') were analyzed by SDS-PAGE under R and NR conditions followed by IB. e.v.: empty vector, PD: pull down, X: unknown protein. The immunoblots are representative of 3 independent experiments (n = 3).

2.3. The Mekk1-Jafrac1 interaction is conserved in human cells

Next, we asked if the *Drosophila* Mekk1-Jafrac1 interaction is conserved in mammalian cells. The mammalian homologue of *Drosophila* Mekk1 is MEKK4 and the mammalian homologues of Jafrac1 are the closely related cytosolic peroxiredoxins Prx1 and Prx2. We expressed either Prx2-SBP or MEKK4-SBP in HEK293T cells and performed corresponding co-precipitation experiments. Upon exposure to H₂O₂, Prx2 formed disulfide conjugates with other proteins (Fig. 3A, lower left panel), as previously shown [14]. Endogenous MEKK4 co-precipitated with Prx2-SBP in an H₂O₂- and time-dependent manner (Fig. 3A, upper panels). Co-precipitated MEKK4 was part of five distinct species (Fig. 3A, upper left panel). The species with the lowest molecular weight matches the size of the MEKK4 monomer (182 kDa). Higher conjugate bands match the size of a MEKK4-Prx2 1:1 conjugate and still higher bands may correspond to MEKK4 disulfide-linked conjugates with different stoichiometry. These results show that MEKK4, like its *Drosophila* counterpart Mekk1, is an oxidation-sensitive protein. However, the pool of MEKK4 molecules covalently linked to Prx2 is very small (as expected for a reaction intermediate) and can only be detected if enriched by co-precipitation with Prx2. The reverse experiment correspondingly revealed endogenous Prx2 to co-precipitate with MEKK4-SBP in an H₂O₂- and time-dependent manner (Fig. 3B). Interestingly, Prx2 co-precipitated with MEKK4 as part of four distinct disulfide-linked conjugates. By size these conjugates match four of the MEKK4 conjugates detected in the reverse experiment (Fig. 3A, upper left panel), suggesting that all four conjugates contain both MEKK4 and Prx2, differing in stoichiometry and perhaps involving additional proteins. In summary, these results show that the interaction between Mekk1/MEKK4 and cytosolic peroxiredoxins is conserved between *Drosophila* and mammalian cells. The observation of disulfide exchange intermediates in both organisms suggests that peroxiredoxins mediate the oxidation of Mekk1/MEKK4.

2.4. Cytosolic typical 2-Cys peroxiredoxins are required for the activation of the p38 signaling pathway in response to H₂O₂ and EGF

As above results indicated a role for cytosolic peroxiredoxins in H₂O₂-dependent activation of p38, we asked if cytosolic Prxs are required for H₂O₂-induced phosphorylation of p38. Since Prx1 and Prx2 are highly homologous and may compensate for each other, we studied p38 activation in Prx1/Prx2 double-deletion (HAP1ΔPrx1+2) and double-depletion (HEK293TΔPrx1+2) backgrounds, as described previously [21]. In wild type (WT) cells, 100 μM H₂O₂ induced an increase in p38 phosphorylation which was reversed after 15 min (Fig. 4A–B). In Prx1+2-deleted or -depleted cells H₂O₂-induced phosphorylation of p38 was diminished (Fig. 4A–B). Next, we asked if Prxs are also involved in the activation of p38 under conditions that involve the endogenous generation of H₂O₂. The epidermal growth factor (EGF) is known to induce NOXes to generate H₂O₂, leading to p38 phosphorylation [22]. We treated serum-starved WT and Prx1+2-depleted cells with 20 ng/mL EGF and followed p38 phosphorylation over time. In WT cells, EGF stimulation led to an increase in p38 phosphorylation peaking after 15 min. However, EGF caused less p38 phosphorylation in Prx1+2-depleted cells (Fig. 4C). In summary, these results further supported the notion that cytosolic 2-Cys Prxs are required for the complete activation of p38 in response to increased levels of H₂O₂, from

either exogenous or endogenous sources.

3. Discussion

MAP3Ks are known to be activated by H₂O₂. Using *Drosophila* S2R+ cells, we found that depletion of the MAP3K Mekk1 prevented H₂O₂-induced p38 phosphorylation. Thus, Mekk1 appeared to be responsible for H₂O₂-induced p38 activation in this system (Fig. 1). In contrast, depletion of two other MAP3Ks, Tak1 and Ask1, enhanced H₂O₂-induced activation of p38 (Fig. 1), potentially suggesting that these MAP3Ks have an inhibitory influence on p38 activation in S2R+ cells. Alternatively, and perhaps more likely, the depletion of Ask1 and Tak1 may lead to cellular adaptations that sensitize the Mekk1-specific branch of H₂O₂-dependent p38 activation. Ask1 and Tak1 have been reported to be activated by H₂O₂ in other cellular systems [18,19], highlighting the flexibility and diversity of the MAPK signaling pathway in different cell types and organisms.

H₂O₂-based regulation of signaling proteins involves the oxidation of cysteine residues. It is becoming clear that protein thiol oxidation in many cases requires peroxiredoxins to relay oxidative equivalents from H₂O₂ to target proteins. The hallmark of this mechanism is the formation of a reaction intermediate in which peroxiredoxin and target protein are transiently linked by a disulfide bond. Indeed, we observed that Mekk1 formed a disulfide-linked conjugate with the *Drosophila* typical 2-Cys peroxiredoxin Jafrac1 in response to a H₂O₂ stimulus (Fig. 2). In human cells, MEKK4 (homologous to *Drosophila* Mekk1) covalently interacted with Prx2 (homologous to *Drosophila* Jafrac1) in a very similar manner, suggesting a conserved mechanism (Fig. 3). Both Mekk1 and MEKK4 formed additional oxidation products of higher molecular weight, indicating that Mekk1/MEKK4 is oxidized by Jafrac1/Prx2 in an H₂O₂-dependent manner. In our experiments we could only detect oxidized Mekk1/MEKK4 when it was enriched by co-precipitation with tagged Jafrac1/Prx2. This suggests that only a small proportion of the overall Mekk1/MEKK4 protein pool is associated with a peroxiredoxin and hence oxidized.

We then found that the presence of cytosolic 2-Cys peroxiredoxins (Prx1 and Prx2) is needed for complete activation of p38 MAPK as induced by either endogenous or exogenous sources of H₂O₂ in mammalian cells (Fig. 4). This finding is compatible with the notion that peroxiredoxin-dependent oxidation of Mekk1/MEKK4 is necessary for the downstream activation of p38. These observations are also compatible with the previous finding that Prx1 (a close homologue of Prx2) is necessary for full H₂O₂-induced activation of p38 in mammalian cells [15]. In this report ASK1 oxidation was found to facilitate p38 activation while the contribution of other MAP3Ks was not investigated. Our results suggest that more than one MAP3K can be oxidized in a peroxiredoxin (Prx1 and/or Prx2)-dependent manner, potentially activating parallel branches of p38 signaling. The individual contributions of the two peroxiredoxins (Prx1 and Prx2) and of the different MAP3Ks in H₂O₂-induced p38 activation remain to be investigated.

Overall, our observations are consistent with the following model of Mekk1/MEKK4 activation: First, Jafrac1/Prx2 is rapidly and sensitively oxidized by H₂O₂. Then, oxidized Jafrac1/Prx2 (either the sulfenic acid or the disulfide form) reacts with a cysteine residue exposed on the surface of Mekk1/MEKK4, generating a 1:1 disulfide-linked conjugate between Jafrac1/Prx2 and Mekk1/MEKK4. The disulfide bridge between Jafrac1/Prx2 and Mekk1/MEKK4 is then resolved by to another

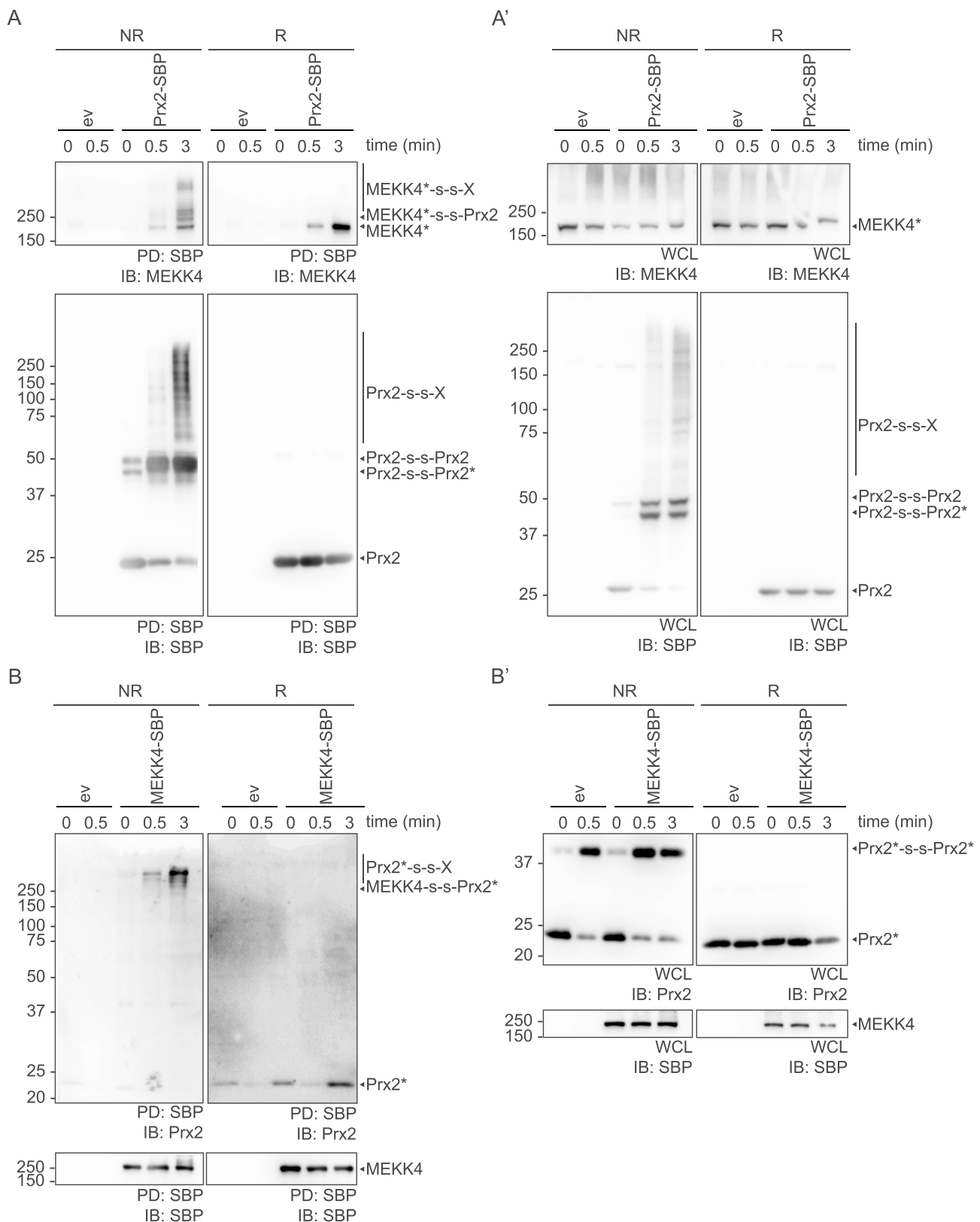


Fig. 3. Mammalian MEKK4 is redox-sensitive and interacts with cytosolic peroxiredoxin Prx2 in response to H_2O_2 .

(A, A') A tagged version of Prx2 (Prx2-SBP) was expressed in HEK293T cells. Cells were treated with H_2O_2 (100 μM) and lysed at indicated time points. Prx2-SBP was affinity-purified using streptavidin beads. Precipitates (A) and whole cell lysates (WCL) (A') were analyzed by SDS-PAGE under reducing (R) and non-reducing (NR) conditions followed by immunoblotting (IB). The immunoblots are representative of 3 independent experiments ($n = 3$). (B, B') Complementary affinity purification experiment: A tagged version of MEKK4 (MEKK4-SBP) was expressed in HEK293T cells. Cells were treated with H_2O_2 (100 μM) and lysed at indicated time points. MEKK4-SBP was affinity-purified using streptavidin beads. Precipitates (B) and WCL (B') were analyzed by SDS-PAGE under R and NR conditions followed by IB. e.v.: empty vector, PD: pull down, *: endogenous protein, X: unknown protein. The immunoblots are representative of 3 independent experiments ($n = 3$).

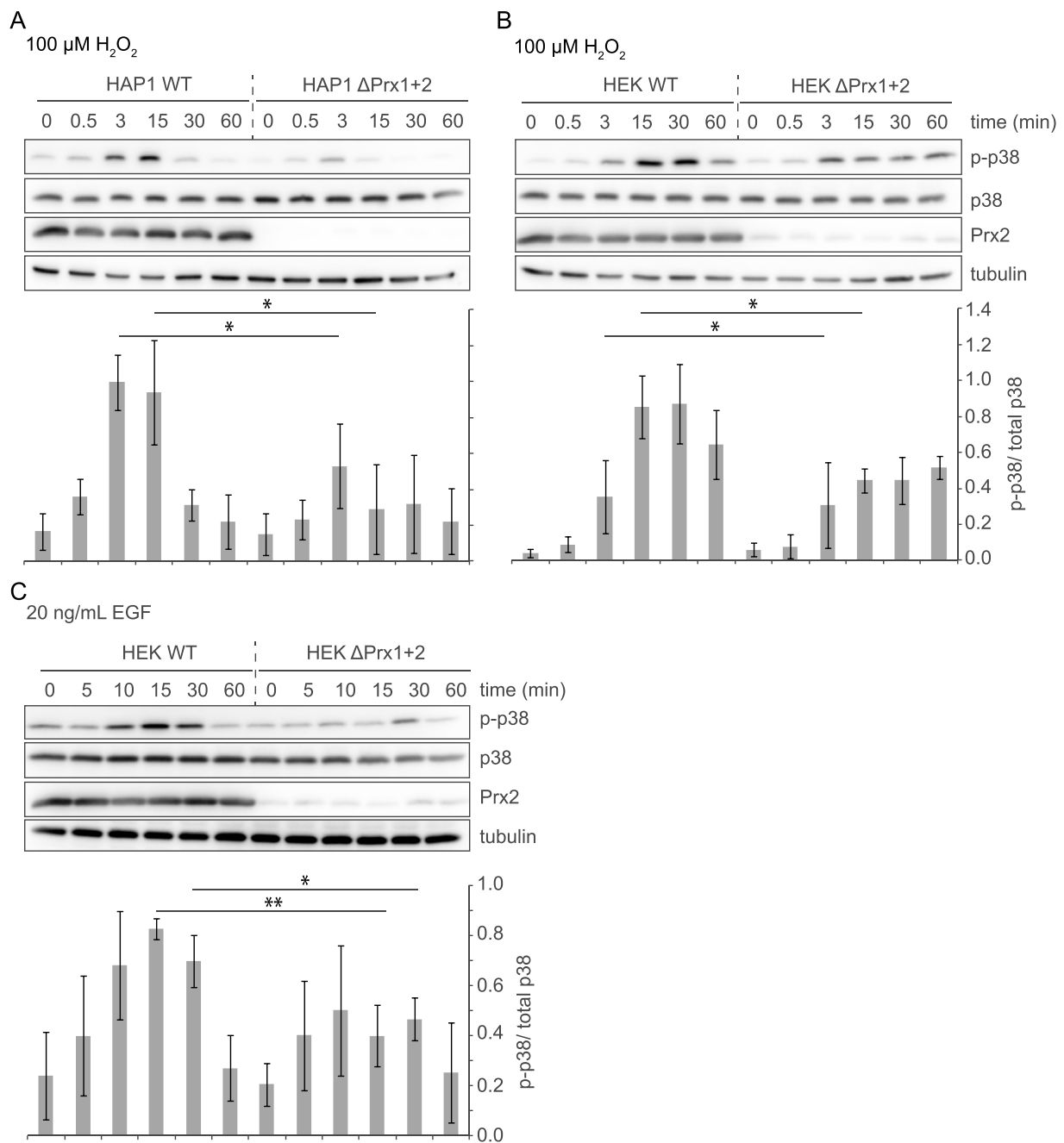


Fig. 4. 2-Cys peroxiredoxins participate in the activation of the p38 signaling pathway in response to H_2O_2 and EGF.

(A) HAP WT and HAP $\Delta\text{Prx1+2}$ cells were incubated with 100 μM H_2O_2 and lysed at indicated time points. Phosphorylation of p38 was analyzed by immunoblotting. The immunoblot is representative of 3 independent experiments ($n = 3$). The bar chart presents the mean (\pm SD) ratio of phosphorylated p38 to total p38 from 3 independent experiments. (B) HEK293T WT and HEK293T $\Delta\text{Prx1+2}$ cells were treated with doxycycline for 4 days to induce scrambled (WT) or Prx1 + 2 specific ($\Delta\text{Prx1+2}$) shRNA expression. Cells were then incubated with 100 μM H_2O_2 and lysed at the indicated time points. Phosphorylation of p38 was analyzed by immunoblotting. The immunoblot is representative of 3 independent experiments ($n = 3$). The bar chart presents the mean (\pm SD) ratio of phosphorylated p38 to total p38 from 3 independent experiments. (C) HEK293T WT and HEK293T $\Delta\text{Prx1+2}$ cells were treated with doxycycline to induce shRNA expression, serum-starved for 16 h, treated with 20 ng/mL EGF and lysed at indicated time points. Phosphorylation of p38 was analyzed by immunoblotting. The immunoblot is representative of 3 independent experiments ($n = 3$). The bar chart represents the mean (\pm SD) ratio of phosphorylated p38 to total p38 from 3 independent experiments.

cysteine residue of Mekk1/MEKK4. This will result in intra- and/or intermolecular disulfide bonds within or between Mekk1/MEKK4 monomers. Formation of disulfide-linked oligomers presumably explains the observed Mekk1/MEKK4 conjugates of higher molecular weight. However, the formation of intramolecular disulfides in Mekk1/MEKK4 monomers cannot be excluded, as those will not cause a detectable gel mobility shift, given the molecular weight of the protein

and the limited resolution of the gels.

The identification of the relevant cysteine residues will be a logical next step to obtain more insight into the mechanism of Mekk1/MEKK4 oxidation and activation. This will entail the study of Mekk1/MEKK4 cysteine mutants. However, Mekk1 and MEKK4 contain many cysteines that could potentially play a role. The *Drosophila*, human and mouse proteins contain 27, 30, and 31 cysteines, respectively. Of those, nine

cysteines are fully conserved between the three species. These are distributed along the entire protein sequence including the inhibitory and kinase domains. It may be anticipated that multiple cysteines are subject to oxidation and that cysteines can compensate for each other when single cysteines are removed by mutagenesis, as has been observed for other redox-regulated proteins with many cysteines [14,23,24]. Indeed, the formation of several different disulfide-linked Mekk1/MEKK4 conjugates resembles what has been observed for ASK1: five of its cysteines had to be mutated in combination in order to abolish all covalent ASK1 oligomerization [16]. These observations support the notion that there is some degree of redundancy and/or flexibility in the MAP3K thiol oxidation process. Extensive studies may be needed to clarify the contribution and role of Mekk1/MEKK4 cysteines in future experiments.

How Mekk1/MEKK4 oxidation leads to kinase activation also remains to be investigated. The observed high molecular weight (HMW) conjugates may represent intermediates in the activation process of Mekk1/MEKK4. Activation of MEKK4 is known to require the separation of its auto-inhibitory and kinase domains, which can be facilitated by the binding of an activating protein [25]. Accordingly, Jafrac1/Prx2 binding to Mekk1/MEKK4, and/or the conformational changes induced by thiol oxidation, may act to release the auto-inhibition of the Mekk1/MEKK4 kinase domain. De-inhibition then allows MEKK4 oligomers to auto-phosphorylate and activate downstream MAP2Ks. Oxidation of Mekk1/MEKK4 would facilitate the formation of homodimers and oligomers by covalent cross-linking of Mekk1/MEKK4 proteins, in line with the observed formation of disulfide-linked HMW complexes and similar to the mechanism described for ASK1¹⁶. For ASK1, the cysteines facilitating oligomerization were shown to be the ones required for activation. The same is likely to be true for Mekk1/MEKK4. Finally, the specific MAP2K that links Mekk1/MEKK4 oxidation to p38 phosphorylation remains to be identified. In human cells at least three MAP2Ks (MKK6, MKK4 and MKK3) are candidates [26].

4. Materials and methods

4.1. Cell lines

S2R+ cells were obtained from the Drosophila Genomics Resource Center and cultured at 25 °C in Schneider's Drosophila medium (Life Technologies, #21720-024) supplemented with 10% (v/v) Fetal Bovine Serum (FBS; Life Technologies, #10270, Lot: 41F0322K) and 1% (v/v) penicillin-streptomycin (Life Technologies, #15140). HEK293T cells were cultured at 37 °C in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, #41965039) supplemented with 10% FBS and 1% penicillin-streptomycin. HAP cells were cultured at 37 °C in Iscove's Modified Dulbecco's Medium (IMDM; Life Technologies, #21980032) supplemented with 10% FBS and 1% penicillin-streptomycin. HEK293T stable cell lines expressing doxycycline-inducible scrambled shRNAs and HAP1 WT and ΔPrx1 + 2 cells were previously described [21]. All cell lines were confirmed to be mycoplasma-free using a multiplex cell contamination test (Multiplexion).

4.2. Antibodies and reagents

Primary antibodies were diluted 1:1000 in 5% (w/v) BSA in Tris buffered saline (TBS) with 1% (v/v) Triton X-100, unless stated otherwise. Jafrac1 was detected with polyclonal goat anti-Prx2 antibody (R&D Systems #AF3489), *Drosophila* α-tubulin with a monoclonal mouse antibody (Hybridoma Bank AA4.3), *Drosophila* p-p38 (Thr180/Tyr182) with a monoclonal rabbit antibody (Cell Signaling Technology #4631), *Drosophila* p38 with a goat polyclonal antibody (Santa Cruz Biotechnology sc-15714) diluted 1:300, myc-tag with a rabbit monoclonal antibody (Cell Signaling Technology #2278), SBP-tag with a mouse monoclonal antibody (Santa Cruz Biotechnology sc-101595), human Prx2 with a rabbit monoclonal antibody (abcam #109367), MEKK4 with a mouse monoclonal antibody (sc-166196), human p-p38

with a mouse monoclonal antibody (BD #6122889) diluted 1:2000, human p38 with a rabbit polyclonal antibody (Cell Signaling Technology #9212) and human β-tubulin with a rabbit monoclonal antibody (Cell Signaling Technology #2128). The secondary antibodies were diluted 1:10 000 in 0.5% (w/v) milk powder in TBS with 1% (v/v) Triton X-100. The secondary antibodies were: goat anti-rabbit HRP (Jackson ImmunoResearch #111-035-144), donkey anti-mouse HRP (Jackson ImmunoResearch #115-035-146) and donkey anti-goat HRP (Santa Cruz Biotechnology Sc-2020). All chemicals were purchased from Sigma-Aldrich, unless stated otherwise.

4.3. dsRNA design and production

Double stranded RNAs (dsRNAs) were synthesized by *in vitro* reverse transcription using the T7 RNA polymerase (Thermo Fischer Scientific) and purified with an RNA purification column (Macherey-Nagel). The templates for dsRNA synthesis were generated in a 2-step polymerase chain reaction (PCR) first using primers targeting an exon of the gene of interest and then a general primer which introduced the T7 RNA polymerase promoter. Primers were designed using the SnapDragon online software developed by the Drosophila RNAi Screening Center (DRSC). dsRNA target exons were chosen to interfere with all transcriptional variants of a given gene. Oligos used to generate dsRNAs in this work:

Luciferase:
GCTGGGCGTTAATCAGAGAG.
TTTTCCGTCATCGTCTTTCC.
Mekk1:
ATGGATCTAGAGGACGGGCT.
TAGCTCTTGGCCACATTCT.
Ask1:
TACGCTACCATCACCACAA.
GACAACGCCTTTTCGAACTC.
Tak1:
TCGATATAAACTCGTGCCCC.
TAACTGTGCTTTGGTTCCC.

4.4. Plasmids

The Jafrac1 open reading frame (ORF) was from Genescript, the Mekk1 ORF from *D. melanogaster* cDNA, the Prx2 ORF from a Gateway vector provided by the DKFZ Genomics and Proteomics Core Facility and the MEKK4 ORF was a gift from Gary Johnson (pCMV5 MEKK4alpha WT, Addgene plasmid # 12187) [27]. pAC5-pSTABLE2-Neo plasmids were used for the expression of Jafrac1-SBP/myc or Mekk1-SBP/myc, coupled to the expression of mCherry and neomycin resistance as previously described [28]. Prx2-SBP or MEKK4-SBP was expressed from doxycycline-inducible pSF3 plasmids co-expressing mCherry from a bidirectional promoter. Cloning of tag-fused ORFs was performed using the Gibson assembly cloning kit (New England Biolabs).

4.5. Treatment and lysis of Drosophila cells

Drosophila S2R+ cells were seeded into 24-well plates at 8.75×10^5 cells per well and incubated at 25 °C overnight (ON). For experiments using dsRNAs: 4.4×10^5 S2R+ cells were seeded in 24-well plates in 250 μL of Schneider's Drosophila medium without antibiotics and FBS and treated with 3 μg dsRNA for up to 2 h. Then, 250 μL of Schneider's medium supplemented with 20% FBS and 2% penicillin-streptomycin was added to each well. Plates were incubated for 5 days at 25 °C. Before H₂O₂ treatment, cells were incubated for 4 h with fresh Schneider's medium. Cells were incubated with 50 or 500 μM H₂O₂ in PBS or PBS alone for the referred time points. Cells were lysed in 100 μL of TBS containing 1% (v/v) Triton X-100 supplemented with phosphatase and protease inhibitors and 0.5 mM EDTA (Halt, Thermo

Fischer Scientific) for 20 min. Lysates were collected in cold microfuge tubes and centrifuged at 13,000 rpm for 10 min at 4 °C. Cleared lysates were transferred to fresh tubes and incubated with 10 X Lämmli sample buffer containing 25 mM DTT for 5 min on ice. Samples were further analyzed by immunoblotting.

4.6. Treatment and lysis of HAP1 and HEK293T cells

HEK293T cells were incubated with 1.5 µg/mL of doxycycline for 3 days before seeding and then kept with doxycycline until the end of the experiment to induce the expression of the scrambled and Prx1+2 shRNAs. HAP1 and HEK293T cells were seeded in 6-well plates at 0.5×10^6 cells per well and incubated ON at 37 °C. For EGF treatment experiment, cells were incubated ON with medium without FBS. Cells were incubated with 100 µM H₂O₂ in PBS or 20 ng/mL EGF for the time indicated. Next, cells were lysed in 100 µL of TBS containing 1% (v/v) Triton X-100 supplemented with phosphatase and protease inhibitors and 0.5 mM EDTA (Halt, Thermo Fischer Scientific) for 20 min. Lysates were collected in cold microfuge tubes and centrifuged at 13,000 rpm for 10 min at 4 °C. Cleared lysates were transferred to fresh tubes and incubated with 10 X Lämmli sample buffer containing 25 mM DTT for 5 min on ice. Samples were further analyzed by immunoblotting.

4.7. Co-precipitation experiments in *Drosophila* cells

Drosophila S2R+ cells were seeded into 6-well plates at 4.5×10^6 cells per well and tagged versions of Jafrac1 and Mekk1 were transfected using Lipofectamine 2000 according to the manufacturer's instructions. Cells were incubated for 3 days at 25 °C. Cells were then incubated with 500 µM H₂O₂ in PBS or with PBS alone for 5 min. To prevent lysis-induced oxidation of proteins, cells were treated with 1 mL of 100 mM NEM in PBS for 5 min on ice. Next, the NEM solution was aspirated and cells were lysed in 500 µL TBS containing 1% (v/v) Triton X-100 supplemented with phosphatase and protease inhibitors, 0.5 mM EDTA and 20 mM NEM, for 20 min at 4 °C. Lysates were centrifuged at 13,000 rpm for 10 min at 4 °C and supernatant was transferred to a fresh microfuge tube at 4 °C. A 50 µL aliquot of each whole cell lysate (WCL) was collected, mixed with 10 µL of lysis buffer and 20 µL of non-reducing 4x LDS sample buffer. Meanwhile, 10% of Streptavidin MAG Sepharose™ bead slurry (GE Healthcare Life Sciences) was washed 2 times with 1.3 mL of TBS and incubated with TBS to a final concentration of 50% Streptavidin bead slurry. The protein lysates were incubated with 9 µL of the 50% Streptavidin bead slurry for 4 h at 4 °C, with continuous agitation. Next, the beads were washed three times with TBS, incubated with 80 µL of non-reducing 1x LDS sample buffer. Half of each sample was incubated with DTT to a final concentration of 25 mM for 5 min on ice. All samples were further analyzed by SDS-PAGE followed by immunoblotting.

4.8. Co-precipitation experiments in HEK293T cells

HEK293T cells were seeded in 15 cm cell culture dishes at 2×10^6 cells per dish in 20 mL and incubated ON at 37 °C. The next day, cells were transfected with polyethylenimine (PEI). For each dish, 25 µg DNA was mixed with 75 µg PEI in 500 µL of TBS, vortexed for 10 s and incubated for 20 min. Then, the mixture was distributed dropwise through the dish. After 6 h the medium was removed and fresh medium containing 1.5 µg/mL of doxycycline was added to the cells. Plates were further incubated at 37 °C for 2 days. Cells were treated with 100 µM H₂O₂ for different times. Cells were incubated with 100 mM NEM in PBS for 5 min on ice. After NEM was removed, cells were lysed in 3 mL of TBS with 1% (v/v) Triton X-100 containing Complete protease inhibitor cocktail tablets (Roche) for 30 min at 4 °C with constant agitation. Lysates were centrifuged at 4,000 rpm for 10 min at 4 °C. Cleared lysates were transferred to a pre-cooled tube and 80 µL WCL aliquots were set aside. Lysates were incubated with 60 µL of 50%

Streptavidin bead (Streptavidin Sepharose High Performance, GE Healthcare) slurry for 4 h at 4 °C, with continuous agitation. Next, the beads were washed three times with TBS with 0.1 and 1% (v/v) Triton X-100. Proteins were then eluted by incubating the beads with 80 µL of 4 mM Biotin in TBS for 30 min on ice. WCL and eluate samples were split in two aliquots and incubated with 10 X Lämmli sample buffer with (reducing) or without (non-reducing) 25 mM DTT. DTT-treated samples were incubated on ice for 5 min. All samples were further analyzed by SDS-PAGE followed by immunoblotting.

4.9. SDS-PAGE and immunoblotting

Samples were incubated at 95 °C for 5 min, spun down and loaded onto a 12% SDS-PAGE gel or a NuPAGE™ Bis-Tris 4–12% gel (Thermo Fischer Scientific). Proteins were transferred with a semi-dry transfer system (Bio-Rad) to a polyvinylidene (PVDF) membrane with 45 µm pores (Merck Millipore) blocked with 5% BSA in TBS with 1% (v/v) Triton X-100. Signals were detected with a Peqlab Fusion-SL system using SuperSignal West Femto chemiluminescent substrate (Thermo Scientific). Band intensities were quantified using ImageJ.

4.10. Quantitative PCR

Total RNA was isolated using the RNeasy kit (Qiagen). mRNA was reverse transcribed using the RevertAid reverse transcriptase (Thermo Fisher Scientific) following the manufacturer's instructions. For qPCR analysis, 2x Maxima SYBR Green/ROX (Thermo Fischer Scientific) was mixed with extra ROX dye to a final concentration of 0.5 µM. Per well of a 96-well plate, the cDNA samples to be analyzed were diluted 1:75 or 1:150 and mixed with 1x SYBR Green/ROX and 0.25 µM of the forward and reverse oligos.

Oligos used:

Nuf control:

AACCGAACCCTCGAAGAACAG.

CTGGGCTTGACATCTCCTC.

Mekk1:

CGTAGCGTTCATTTCCGCT.

GTCTCGACCGTACTTGACAGA.

Ask1:

GCCGGAATTGGACATAACGC.

GAGTGTTCGTCGTGGGAAGA.

Tak1:

AAGGACAAGGAGGGTCTTCA.

ATTGTGATGCGGTGGGATGA.

Statistical analysis

In all figures the error bars represent the standard deviation (SD). P values were calculated using a two-tailed unpaired Student's t-test.

Funding

A.G.B. has been supported by a PhD stipend from the Helmholtz International Graduate School for Cancer Research. T.P.D. is supported by the Deutsche Forschungsgemeinschaft (DFG), collaborative research center SFB1036, and the European Research Council, AdG742039.

Author contributions

A.G.B. and T.P.D. conceived the project and designed the experiments. A.G.B. performed the experiments and analyzed the data. A.G.B. and T.P.D. wrote the manuscript.

Declaration of competing interest

The authors declare that they have no competing interests.

Acknowledgments

We thank Gabriele Kunz for technical support and the Drosophila Genomics Resource Center, supported by NIH grant 2P40OD010949-10A1, for the supply of S2R+ cells.

References

- [1] A. Cuadrado, A.R. Nebreda, Mechanisms and functions of p38 MAPK signalling, *Biochem. J.* 429 (2010) 403–417, <https://doi.org/10.1042/bj20100323>.
- [2] T. Zarubin, J. Han, Activation and signaling of the p38 MAP kinase pathway, *Cell Res.* 15 (2005) 11–18, <https://doi.org/10.1038/sj.cr.7290257>.
- [3] Y. Son, S. Kim, H.T. Chung, H.O. Pae, Reactive oxygen species in the activation of MAP kinases, *Methods Enzymol.* 528 (2013) 27–48, <https://doi.org/10.1016/b978-0-12-405881-1.00002-1>.
- [4] M.P. Murphy, How mitochondria produce reactive oxygen species, *Biochem. J.* 417 (2009) 1–13, <https://doi.org/10.1042/BJ20081386>.
- [5] J.D. Lambeth, NOX enzymes and the biology of reactive oxygen, *Nat. Rev. Immunol.* 4 (2004) 181–189, <https://doi.org/10.1038/nri1312>.
- [6] B. Groitl, U. Jakob, Thiol-based redox switches, *Biochim. Biophys. Acta* 1844 (2014) 1335–1343, <https://doi.org/10.1016/j.bbapap.2014.03.007>.
- [7] S.R. Lee, K.S. Kwon, S.R. Kim, S.G. Rhee, Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor, *J. Biol. Chem.* 273 (1998) 15366–15372.
- [8] A. Corcoran, T.G. Cotter, Redox regulation of protein kinases, *FEBS J.* 280 (2013) 1944–1965, <https://doi.org/10.1111/febs.12224>.
- [9] C.C. Winterbourn, M.B. Hampton, Thiol chemistry and specificity in redox signaling, *Free Radic. Biol. Med.* 45 (2008) 549–561, <https://doi.org/10.1016/j.freeradbiomed.2008.05.004>.
- [10] C.C. Winterbourn, Reconciling the chemistry and biology of reactive oxygen species, *Nat. Chem. Biol.* 4 (2008) 278–286, <https://doi.org/10.1038/nchembio.85>.
- [11] L.M. Randall, G. Ferrer-Sueta, A. Denicola, Peroxiredoxins as preferential targets in H2O2-induced signaling, *Methods Enzymol.* 527 (2013) 41–63, <https://doi.org/10.1016/B978-0-12-405882-8.00003-9>.
- [12] L. Flohe, The impact of thiol peroxidases on redox regulation, *Free Radic. Res.* 50 (2016) 126–142, <https://doi.org/10.3109/10715762.2015.1046858>.
- [13] L.E. Netto, F. Antunes, The roles of peroxiredoxin and thioredoxin in hydrogen peroxide sensing and in signal transduction, *Mol. Cells* 39 (2016) 65–71, <https://doi.org/10.14348/molcells.2016.2349>.
- [14] M.C. Sobotta, et al., Peroxiredoxin-2 and STAT3 form a redox relay for H2O2 signaling, *Nat. Chem. Biol.* 11 (2015) 64–70, <https://doi.org/10.1038/nchembio.1695>.
- [15] R.M. Jarvis, S.M. Hughes, E.C. Ledgerwood, Peroxiredoxin 1 functions as a signal peroxidase to receive, transduce, and transmit peroxide signals in mammalian cells, *Free Radic. Biol. Med.* 53 (2012) 1522–1530, <https://doi.org/10.1016/j.freeradbiomed.2012.08.001>.
- [16] P.J. Nadeau, S.J. Charette, M.B. Toledano, J. Landry, Disulfide Bond-mediated multimerization of Ask1 and its reduction by thioredoxin-1 regulate H(2)O(2)-induced c-Jun NH(2)-terminal kinase activation and apoptosis, *Mol. Biol. Cell* 18 (2007) 3903–3913, <https://doi.org/10.1091/mbc.E07-05-0491>.
- [17] Y. Aissouni, G. Zapart, J.L. Iovanna, I. Dikic, P. Soubeyran, CIN85 regulates the ability of MEKK4 to activate the p38 MAP kinase pathway, *Biochem. Biophys. Res. Commun.* 338 (2005) 808–814, <https://doi.org/10.1016/j.bbrc.2005.10.032>.
- [18] Y. Onodera, T. Teramura, T. Takehara, K. Shigi, K. Fukuda, Reactive oxygen species induce Cox-2 expression via TAK1 activation in synovial fibroblast cells, *FEBS Open Bio* 5 (2015) 492–501, <https://doi.org/10.1016/j.fob.2015.06.001>.
- [19] M. Saitoh, et al., Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1, *EMBO J.* 17 (1998) 2596–2606, <https://doi.org/10.1093/emboj/17.9.2596>.
- [20] S. Stocker, K. Van Laer, A. Mijuskovic, T.P. Dick, The conundrum of hydrogen peroxide signaling and the emerging role of peroxiredoxins as redox relay hubs, *Antioxidants Redox Signal.* 28 (2018) 558–573, <https://doi.org/10.1089/ars.2017.7162>.
- [21] S. Stocker, M. Maurer, T. Ruppert, T.P. Dick, A role for 2-Cys peroxiredoxins in facilitating cytosolic protein thiol oxidation, *Nat. Chem. Biol.* 14 (2018) 148–155, <https://doi.org/10.1038/nchembio.2536>.
- [22] D.E. Heppner, A. van der Vliet, Redox-dependent regulation of epidermal growth factor receptor signaling, *Redox Biol.* 8 (2016) 24–27, <https://doi.org/10.1016/j.redox.2015.12.002>.
- [23] B. Stottmeier, T.P. Dick, Redox sensitivity of the MyD88 immune signaling adapter, *Free Radic. Biol. Med.* (2016), <https://doi.org/10.1016/j.freeradbiomed.2016.10.004>.
- [24] M. Putker, et al., Redox-dependent control of FOXO/DAF-16 by transportin-1, *Mol. Cell* 49 (2013) 730–742, <https://doi.org/10.1016/j.molcel.2012.12.014>.
- [25] Z. Miyake, M. Takekawa, Q. Ge, H. Saito, Activation of MTK1/MEKK4 by GADD45 through induced N-C dissociation and dimerization-mediated trans autophosphorylation of the MTK1 kinase domain, *Mol. Cell. Biol.* 27 (2007) 2765–2776, <https://doi.org/10.1128/mcb.01435-06>.
- [26] B.T. Bettinger, D.C. Amberg, The MEK kinases MEKK4/Ssk2p facilitate complexity in the stress signaling responses of diverse systems, *J. Cell. Biochem.* 101 (2007) 34–43, <https://doi.org/10.1002/jcb.21289>.
- [27] P. Gerwins, J.L. Blank, G.L. Johnson, Cloning of a novel mitogen-activated protein kinase kinase kinase, MEKK4, that selectively regulates the c-Jun amino terminal kinase pathway, *J. Biol. Chem.* 272 (1997) 8288–8295.
- [28] M. Gonzalez, et al., Generation of stable Drosophila cell lines using multicistronic vectors, *Sci. Rep.* 1 (2011).