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## Peroxide-dependent sulfenylation of the EGFR catalytic site enhances kinase activity

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### Abstract

Protein sulfenylation is a post-translational modification of emerging importance in higher eukaryotes. However, investigation of its diverse roles remains challenging, particularly within a native cellular environment. Herein we report the development and application of DYn-2, a new chemoselective probe for detecting sulfenylated proteins in human cells. These studies show that epidermal growth factor receptor-mediated signaling results in H<sub>2</sub>O<sub>2</sub> production and oxidation of downstream proteins. In addition, we demonstrate that DYn-2 has the ability to detect differences in sulfenylation rates within the cell, which are associated with differences in target protein localization. We also show that the direct modification of epidermal growth factor receptor by H<sub>2</sub>O<sub>2</sub> at a critical active site cysteine (Cys797) enhances its tyrosine kinase activity. Collectively, our findings reveal sulfenylation as a global signaling mechanism that is akin to phosphorylation and has regulatory implications for other receptor tyrosine kinases and irreversible inhibitors that target oxidant-sensitive cysteines in proteins.

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H<sub>2</sub>O<sub>2</sub> not only is a source of oxidative stress but also acts as an essential second messenger in signal transduction networks of normal, healthy cells, wherein growth factors, cytokines and a variety of other ligands trigger its production through the activation of their corresponding receptors<sup>1,2</sup>. Indeed, H<sub>2</sub>O<sub>2</sub> has been demonstrated to regulate many basic cellular processes including proliferation, differentiation, growth, migration and survival. For example, binding of epidermal growth factor (EGF) to the extracellular domain of the EGF receptor (EGFR) results in the assembly and activation of NADPH oxidase (Nox) complexes, which generate H<sub>2</sub>O<sub>2</sub> (refs. 3,4) (Fig. 1a). Once formed, H<sub>2</sub>O<sub>2</sub> modulates signaling cascades by reaction with specific biomolecular targets.

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#### Author contributions

C.E.P., T.H.T. and A.H. performed cell culture and immunostaining experiments. F.J.G. performed mass spectrometry experiments. V.G. and S.E.L. performed synthetic experiments. K.S.C. designed experimental strategies with help from C.E.P. and V.G. K.S.C. and C.E.P. wrote the paper with input from all coauthors.

#### Competing financial interests

The authors declare no competing financial interests.

#### Additional information

Supplementary information and chemical compound information are available online at <http://www.nature.com/naturechemicalbiology/>. Reprints and permissions information is available online at <http://www.nature.com/reprints/index.html>. Correspondence and requests for materials should be addressed to C.E.P. or K.S.C.

There is now a wealth of evidence indicating that protein cysteine residues are sensitive targets of H<sub>2</sub>O<sub>2</sub>, both by direct oxidation and through the action of thiol peroxidases<sup>5,6</sup>. The product of the reaction between H<sub>2</sub>O<sub>2</sub> and a thiolate is sulfenic acid (–SOH). Known as sulfenylation, this modification is reversible (either directly or indirectly by disulfide formation) and provides a mechanism by which changes in cellular redox state can be exploited to regulate protein function, as in phosphorylation<sup>7,8</sup>. Recent studies shed new light on the role of sulfenic acid and expand the repertoire of proteins that can undergo sulfenylation<sup>9–13</sup>, hinting at the regulatory potential and importance of these modifications. Nonetheless, the scope of sulfenylation in biological processes, particularly in eukaryotic signal transduction, remains virtually unknown.

Investigating the role of sulfenylation remains challenging, particularly in the context of the native cellular environment<sup>14</sup>. We now present the development and application of DYn-2, a chemoselective probe for detecting sulfenylated proteins directly in cells with improved sensitivity. These studies show that DYn-2 is capable of monitoring global changes in protein sulfenylation generated by Nox-mediated growth factor signaling. In addition, we demonstrate that DYn-2 has the ability to detect differences in sulfenylation rates within the cell due to differences in target protein localization. Finally, we show that modification of EGFR by H<sub>2</sub>O<sub>2</sub> at a critical cysteine (Cys797) in its catalytic site stimulates its kinase activity, thereby demonstrating that sulfenylation, as well as phosphorylation, can regulate receptor tyrosine kinase (RTK) function.

## RESULTS

### EGF modulates cell morphology and EGFR trafficking

To investigate events after the interaction of EGF with its receptor, we used the human epidermoid carcinoma A431 cell line, which naturally expresses high concentrations of EGFR. As shown by phase-contrast microscopy, EGF stimulation induces rapid changes in cell shape (Supplementary Results, Supplementary Fig. 1). Additionally, we used immunofluorescence to determine whether EGF-dependent changes in morphology coincide with receptor mobilization (Fig. 1b). EGFR localized to the plasma membrane without EGF stimulation and concentrated at sites of membrane ruffling within 2 min of mitogen treatment. By 30 min, the majority of EGFR had accumulated in punctate foci throughout the peripheral cytoplasm, and after 1 h, internalized receptors had recycled back to the cell surface. These data show that EGF stimulation markedly changes cell morphology and receptor localization, setting the stage to probe oxidant-mediated signal transduction.

### Cellular redox balance affects EGF-mediated signaling

Next, we examined the relationship between EGFR signaling and reactive oxygen species (ROS) in A431 cells. Intracellular generation of ROS was measured via the conversion of 2',7'-dihydrodichlorofluorescein diacetate (H<sub>2</sub>DCF-DA) to the fluorescent product dichlorofluorescein (DCF). Coincident with membrane ruffling, EGF-stimulated cells showed an increase in DCF fluorescence intensity (Fig. 1c). Moreover, reversible and irreversible inhibitors of EGFR (gefitinib and afatinib, respectively), Nox (apocynin), phosphatidylinositol-3-OH kinase (PI3K, wortmannin) and the antioxidant *N*-acetylcysteine (NAC) attenuated EGF-dependent ROS generation. Control experiments with an NO synthase inhibitor (L-NAME) had no significant ( $P > 0.05$ ) impact on ROS concentrations, as expected (Fig. 1c). These experiments support and extend previous observations<sup>3,15</sup> that EGF-mediated ROS production requires activation of both EGFR and Nox.

We then investigated the effect of exogenous H<sub>2</sub>O<sub>2</sub> on the phosphorylation of EGFR and the downstream kinases AKT and ERK. In the absence of EGF, treatment with H<sub>2</sub>O<sub>2</sub> was

sufficient to trigger a dose-dependent increase in phosphorylation of each kinase (Fig. 1d, and see Supplementary Fig. 2). Control experiments showed that each protein became phosphorylated in response to EGF and that EGFR or PI3K inhibitors attenuated this effect, as expected (Fig. 1d and Supplementary Fig. 3a). Subsequently, we examined the role of H<sub>2</sub>O<sub>2</sub> produced by EGF stimulation (that is, endogenous H<sub>2</sub>O<sub>2</sub>) on pathway activation. Scavenging of growth factor-induced H<sub>2</sub>O<sub>2</sub> with polyethylene glycol-catalase (PEG-catalase, Sigma) or NAC suppressed EGFR phosphorylation (Fig. 1e), global tyrosine phosphorylation and AKT and ERK activation (Supplementary Fig. 3b–e). Additionally, the Nox inhibitors apocynin and diphenyleneiodium (DPI) blunted protein phosphorylation, whereas L-NAME had no apparent effect (Fig. 1f and Supplementary Fig. 3f,g). Collectively, these data underscore the importance of endogenous H<sub>2</sub>O<sub>2</sub> for EGFR signaling as a result of Nox activation.

The requirement for protein sulfenylation in yeast H<sub>2</sub>O<sub>2</sub> sensing<sup>16</sup> and T-cell activation<sup>17</sup> has been shown through inhibition with 5,5-dimethyl-1,3-cyclohexadione (dimedone), a small molecule that reacts selectively with sulfenic acid under aqueous conditions<sup>18–20</sup> (Fig. 2a). Along these lines, treatment of cells with dimedone before EGF stimulation inhibited phosphorylation of EGFR, AKT and ERK (Supplementary Fig. 3h), consistent with an essential role for protein sulfenylation in EGFR signaling.

### Synthesis and evaluation of DYn-1 and DYn-2

Chemical probes directly conjugated to biotin or a fluorophore often have limited cell permeability owing to their bulky detection tags. Accordingly, protocols involving such reagents typically involve the homogenization of cells before labeling, which disrupts the native environment, including the redox balance. To address this issue, we have developed a strategy for detecting protein sulfenic acids directly in cells<sup>11,20,21</sup>, wherein the dimedone warhead is functionalized with a small azide chemical handle that does not impede membrane permeability (DAz-1 and DAz-2; Fig. 2b). An alkyne-functionalized detection tag is then appended after homogenization using the Staudinger ligation or click chemistry.

Recent studies demonstrate that alkynyl-chemical reporters, in combination with azide-bearing detection tags, offer superior sensitivity relative to the reverse combination of azide reporter and alkynyl detection tags<sup>22</sup>. In light of this observation, we designed and synthesized the alkyne-modified analogs DYn-1 (**1**) and DYn-2 (**2**) (Fig. 2b,c and Supplementary Methods). The synthesis began with ethyl protection of the reactive diketone. Alkylation of 3-ethoxycyclohex-2-enone with 3-bromopropyne to afford DYn-1 proceeded smoothly; however, low yields were obtained in analogous reactions for 5-iodopent-1-yne. As a result, we examined monoalkylation of the dianion of 1,3-cyclohexadione. Using this strategy, DYn-2 was prepared without protecting groups in a single step from commercially available materials in 96% yield (Fig. 2c).

With DYn-1 and DYn-2 in hand, we performed comparative studies to determine their utility for detecting protein sulfenic acid modifications alongside DAz-2. To this end, we used a recombinant thiol peroxidase from budding yeast, known as Gpx3, that has an active site cysteine (Cys36) that is readily oxidized to sulfenic acid<sup>16</sup>. Analysis of these reactions by western blotting revealed robust, H<sub>2</sub>O<sub>2</sub>-dependent labeling of Gpx3 by DYn-2, with increased intensity relative to labeling by DAz-2 (Fig. 2d). Control reactions, performed in the absence of probe, showed no detectable signal by western blotting (Fig. 2d). Conversely, DYn-1 showed a marked reduction in sulfenic acid labeling compared to DYn-2 (Supplementary Fig. 4a). Therefore, DYn-1 was not pursued further.

Next, we verified the nature of the covalent adduct formed between oxidized Gpx3 and DYn-2 by ESI-MS (Supplementary Fig. 4b,c). Analysis of the intact protein afforded a

single major species with a molecular weight of 22,916.39 Da, consistent with a single DYn-2 adduct. Detailed examination of trypsin cleavage products confirmed Cys36 as the site of modification as indicated by the doubly charged peptide ion at  $m/z$  551.52, which corresponds to H<sub>2</sub>N-C-(2)GFTPQYK-OH and the series of b- and y-type ions observed in the MS/MS spectrum. Overall, western blot and MS analyses establish that DYn-2 selectively targets protein sulfenic acid modifications.

We then evaluated DYn-2 for its ability to detect sulfenic acids in cells using the strategy outlined in Supplementary Figure 5a. Analysis of probe labeling by western blotting revealed sulfenylated proteins in both A431 and HeLa cells (Fig. 2e and Supplementary Fig. 5b,c). The qualitative profile of DYn-2 labeling was similar to that of DAz-2 labeling, suggesting that the probes reacted with the same protein targets. Notably, the total signal from DYn-2 labeling was greater than that from DAz-2 labeling under identical conditions, and the signal ratio of EGF-stimulated and unstimulated A431 cells was almost 40% greater than that for DAz-2. Detection of sulfenylated proteins by DYn-2 was also dependent on probe dose and incubation time (Supplementary Fig. 5d,e). Control reactions performed with or without catalase in lysis buffer further confirmed that DYn-2 labeling did not occur after cell homogenization (Supplementary Fig. 5f). Addition of DYn-2, before or after EGF treatment, did not affect phosphorylation of EGFR or downstream targets (Supplementary Figs. 3h and 5g), most likely because of the decrease in reactivity inherent in many dimedone analogs. Probe-treated cells showed no loss of viability and maintained redox balance (Supplementary Fig. 6). Collectively, these results validate DYn-2 as a robust chemical reporter for protein sulfenylation in cells and for our general approach of tagging oxidized proteins *in situ*.

### Dynamic, global protein sulfenylation in response to EGF

Our preceding studies reveal EGF-dependent changes in protein sulfenylation. To our knowledge, this observation is the first of its kind, and thus we investigated this discovery in greater detail. Addition of EGF to A431 cells increased intracellular ROS (Fig. 3a) and protein sulfenylation (Fig. 3b,c) in a dose-dependent manner. The maximal increase in sulfenic acid modification was apparent at 100 ng ml<sup>-1</sup> EGF, and it fell to the basal level at 500 ng ml<sup>-1</sup> EGF. ROS generation (Fig. 3d) and protein sulfenylation (Fig. 3e,f) were also dynamic temporal events that peaked 5 min after EGF stimulation (100 ng ml<sup>-1</sup>) and declined thereafter. Furthermore, pharmacological studies indicated that EGF-dependent changes in protein sulfenylation required EGFR, PI3K and Nox activation, as well as intracellular H<sub>2</sub>O<sub>2</sub> (Supplementary Fig. 7).

Fluorescence microscopy with antibodies specific for the protein-dimedone adduct<sup>21</sup> further highlighted the dynamic nature of EGF-mediated protein sulfenylation (Fig. 3g). Relative to unstimulated cells, cells treated with EGF had markedly increased signal intensity and peaked at 6 min, whereas control samples without primary antibody showed no signal (Supplementary Fig. 8). The slight difference in sulfenylation peak times observed by western blot (and ROS concentrations by DCF) and immunofluorescence analyses is most likely due to variations in sample handling inherent to each procedure.

There are seven isoforms of nonphagocytic NADPH oxidase (Nox1, Nox2, Nox3, Nox4, Nox5, Duox1 and Duox2) that show unique activation mechanisms and tissue-specific expression<sup>23</sup>. Western blot and immunofluorescence analyses revealed that Nox2 is a major isoform in A431 cells (Supplementary Fig. 9a,b). Because proteins in the vicinity of Nox are prime targets for oxidation, we wondered whether Nox2 might colocalize with sites of protein sulfenylation. Immunofluorescence analysis indicated the distribution of Nox2 at the plasma membrane and perinuclear area (Fig. 3h and Supplementary Fig. 9c). Remarkably, the merged image of Nox2 and protein sulfenylation revealed a high degree of colocalization

(Fig. 3h). Together, these data show that EGF stimulation results in dynamic changes in protein sulfenylation, which occurs in cells at sites overlapping with Nox2 localization.

### Differential oxidation of tyrosine phosphatases

We next sought to identify targets of  $H_2O_2$  within the EGFR pathway. Growth factor–induced ROS generation is commonly attributed to oxidation and inactivation of an essential active site cysteine residue in protein tyrosine phosphatases (PTPs). Though the analysis of cysteine oxidation in cell extracts indicates that PTP inhibition promotes kinase signaling<sup>17,24,25</sup>, the rates of these reactions are orders of magnitude slower than those of  $H_2O_2$ -metabolizing enzymes, making their physiological relevance uncertain<sup>26</sup>. Because direct evidence of PTP oxidation in cells has not yet been reported, we used DYn-2 to investigate sulfenylation in three signaling phosphatases, PTEN, PTP1B and SHP2. PTEN is predominantly cytoplasmic and functions reciprocally to PI3K, and PTP1B downregulates endocytosed receptors within the endoplasmic reticulum, whereas SHP2 interacts directly with autophosphorylated EGFR at the plasma membrane through its SH2 domains.

Western blot analysis of immunoprecipitated PTPs showed that each protein underwent EGF-dependent sulfenylation in A431 cells (Fig. 4a–c). Moreover, each PTP showed a distinct oxidation profile with respect to growth factor concentration (Fig. 4a–c): sulfenylation of SHP2 peaked at a relatively low concentration of EGF (20 ng ml<sup>-1</sup>), followed by PTEN (500 ng ml<sup>-1</sup>) and PTP1B (750 ng ml<sup>-1</sup>). Subsequently, we investigated PTP localization in cells before and after EGF treatment. Immunofluorescence staining shows that SHP2 underwent a dramatic change in localization, concentrating at sites of plasma membrane ruffling, whereas EGF had no apparent effect on PTEN or PTP1B (Fig. 4d–f). Overall, these data demonstrate that PTPs undergo EGF-dependent oxidation and suggest that the extent of sulfenylation in the cell may be related to differences in target protein localization.

### Identification of EGFR as a sensitive target of $H_2O_2$

The overall amount of EGFR autophosphorylation reflects the balance between kinase and phosphatase activities.  $H_2O_2$ -induced PTP inhibition would shift the balance toward phosphorylation; however, the increase in EGFR phosphorylation could similarly be accounted for by  $H_2O_2$ -mediated enhancement of intrinsic kinase activity. To examine this possibility, we first tested whether EGFR is a target of  $H_2O_2$  in cells. Remarkably, these studies revealed that EGF stimulation leads to robust sulfenic acid modification of EGFR (Fig. 5a), a result that was recapitulated with exogenous  $H_2O_2$  (Fig. 5b). EGFR sulfenylation peaked at the lowest concentration of EGF used in this study (4 ng ml<sup>-1</sup>) and at ~10  $\mu$ M exogenous  $H_2O_2$  (Supplementary Fig. 9d). Given the marked increase in EGFR oxidation at low EGF concentrations, we wondered whether the receptor might form a complex with Nox2. This proposal was confirmed by coimmunoprecipitation, which demonstrated EGF- and time-dependent association of Nox2 with EGFR and vice versa (Fig. 5c,d). In addition, we found that the EGFR–Nox complex immunoprecipitated together with SHP2, consistent with its propensity for oxidation in cells (Supplementary Fig. 9e).

### Oxidation of the EGFR active site modulates kinase activity

The kinase domain of EGFR contains six cysteine residues. Of these, a conserved cysteine within the ATP binding site (Cys797; Supplementary Fig. 9f) is a major target for irreversible inhibitors in cancer clinical trials<sup>27,28</sup>. On this basis, we hypothesized that Cys797 is the site of oxidation. This proposal was supported by studies with irreversible inhibitors, which blocked exogenous  $H_2O_2$ -mediated EGFR sulfenylation (Fig. 5e). Next, we mapped the site of EGF-induced oxidation in cells using dimedone. ESI-LC/MS/MS analysis of pepsin-digested EGFR confirmed Cys797 as the site of covalent modification on

the basis of the doubly charged peptide ion at  $m/z$  402.80, which corresponds to H<sub>2</sub>N-MPFGCL-OH, with dimedone attached to the cysteine residue, and the series of b- and y-type ions observed in the MS/MS spectrum (Supplementary Fig. 9g). The reduced EGFR peptide was also detected, and the ratio of peak areas of the dimedone-modified peptide ion relative to those in the unmodified version was approximately 6:1 (Supplementary Fig. 9h).

Given the proximity of Cys797 to the ATP binding site (Supplementary Fig. 9f), it is plausible that its oxidation modulates enzymatic activity. To test this hypothesis, we performed activity assays using the recombinant EGFR kinase domain. First, we verified that enzyme activity increased as a function of EGFR concentration and decreased with inhibitor treatment (Supplementary Fig. 9i–k). Subsequent studies revealed that tyrosine kinase activity was enhanced, relative to the untreated control, by moderate H<sub>2</sub>O<sub>2</sub> concentrations (0.05–10 μM) and was decreased at concentrations greater than 50 μM (Fig. 5f). Incubation with the reducing agent dithiothreitol mitigated inhibition by H<sub>2</sub>O<sub>2</sub> (Supplementary Fig. 9l), indicating that the decline in EGFR activity at high oxidant concentrations involves reversible thiol oxidation. Control experiments also showed that H<sub>2</sub>O<sub>2</sub> had no significant ( $P > 0.05$ ) effect on other components of the assay system (Supplementary Fig. 9m). Collectively, these data demonstrate that EGFR Cys797 is a direct target of endogenous H<sub>2</sub>O<sub>2</sub>, apparently through its association with Nox2, and that the amount of H<sub>2</sub>O<sub>2</sub> signaling enhances EGFR kinase activity.

## DISCUSSION

Historically, protein cysteine oxidation has been investigated using indirect methods of detection<sup>14</sup> (Supplementary Fig. 10a,b). As these approaches require comprehensive blocking of free thiols at the outset of the procedure, their application is restricted to the analysis of oxidation within purified proteins or cell lysates. Alternatively, oxidative cysteine modifications can be detected on the basis of their distinct chemical attributes using selective probes (Supplementary Fig. 10c), which enable the modifications to be detected directly in cells. This is not a trivial consideration as redox potentials differ markedly among subcellular compartments<sup>29</sup>, and when the redox balance of the cell is disrupted during lysis, proteins undergo a massive amount of artifactual oxidation. This fundamental but often ignored issue increases the challenge involved in detecting modifications in low-abundance proteins and in interpreting biological significance.

With the development of DYn-2, we expand the chemical toolbox used to probe protein sulfenic acid formation in cells. The discovery that protein sulfenylation is a dynamic process during EGFR-mediated signaling most likely has broader implications for other receptor-mediated processes. Consistent with this proposal, alterations in protein sulfenic acid modifications have been observed in lysates generated from HEK293 cells treated with the cytokine TNFα (ref. 30) and from CD8<sup>+</sup> T cells stimulated with CD3- and CD28-specific antibodies<sup>17</sup>. Though the changes in global protein sulfenylation observed in our study generally showed a strong positive correlation with the amount of ROS, cells treated with 500 ng ml<sup>-1</sup> EGF were an exception to this rule. Interestingly, the apparent lack of sulfenylation is consistent with the absence of global disulfide bond formation in A431 cells under these conditions<sup>31</sup> and may reflect oxidation of sulfenic to sulfinic acid, upregulation of efflux transporters, dissociation of EGFR clusters from lipid rafts, activation of alternate pathways that function independently of cysteine oxidation, or both.

Each protein analyzed in this study had a unique sulfenylation profile in cells (Fig. 6a,b). For PTPs, differential susceptibility to oxidation is particularly notable because their active site cysteines are deprotonated at physiological pH and their rates of reaction with H<sub>2</sub>O<sub>2</sub> are almost identical in biochemical studies<sup>32,33</sup>. One possible explanation for this apparent

paradox is that the proximity of target proteins to the source of ROS (such as Nox) has a considerable influence on rates of oxidation within the cell. Consistent with this model, we observed that EGFR and SHP2 form a complex with Nox2. On the other hand, oxidation of PTP1B, a phosphatase in the endoplasmic reticulum, was not observed until much higher concentrations of EGF were applied. Along these lines, a recent study in aortic endothelial cells showed that PTP1B oxidation by Nox4 requires the localization of both proteins to the endoplasmic reticulum<sup>34</sup>. Alternatively, the absence of PTP1B oxidation at lower EGF concentrations might result from sulfenyl amide formation outcompeting the DYn-2 trap (Supplementary Fig. 10d). However, this scenario seems unlikely as sulfenyl amide condensation in PTP1B is expected to be slower than intramolecular disulfide formation in PTEN and SHP2 by a factor of at least two orders of magnitude<sup>35,36</sup>.

Another central finding of this study is that EGFR becomes sulfenylated at Cys797 in EGF-stimulated cells, and this modification enhances its intrinsic tyrosine kinase activity (Fig. 6c). Interestingly, the decline in EGFR activity at higher H<sub>2</sub>O<sub>2</sub> concentrations may reflect disulfide bond formation of Cys797 with another cysteine in the kinase domain; however, future studies will be required to fully address this possibility. The biphasic response of recombinant EGFR kinase activity to H<sub>2</sub>O<sub>2</sub> paralleled that of receptor sulfenic acid modification in cells; however, the H<sub>2</sub>O<sub>2</sub> concentration required for maximal rate enhancement was lower by a factor of approximately 20 relative that needed for sulfenylation in cells. One likely explanation for this difference is that antioxidant enzymes and other biomolecular targets consume the H<sub>2</sub>O<sub>2</sub> applied to cells. Another noteworthy aspect of this biphasic behavior is that cellular sulfenylation and kinase activity decreased at concentrations of H<sub>2</sub>O<sub>2</sub> above 50 μM, whereas EGFR phosphorylation continued to increase at peroxide concentrations above 500 μM. These findings suggest a complex interplay between EGFR kinase activity and PTP inhibition at different concentrations of H<sub>2</sub>O<sub>2</sub>, wherein low concentrations stimulate catalysis, an effect that at higher doses may be lost but is compensated for by PTP inactivation. Additionally, oxidation of Cys797 could positively regulate other aspects of EGFR function, including protein-protein interactions.

It is intriguing to consider the possibility that cysteine oxidation may serve as a general mechanism to regulate RTK activity. Of the ~95 receptor and non-receptor protein tyrosine kinases (PTKs) in the human genome, nine additional members, including two additional EGFR family members, Her2 and Her4 (ref. 28), have a cysteine residue at the structural position that corresponds to Cys797. Another subfamily of PTKs, which includes cytoplasmic Src as well as FGFR1, share a cysteine residue within a conserved glycine-rich loop that interacts with the γ-phosphate of ATP. Interestingly, cellular studies implicate cysteine oxidation in Src regulation<sup>37–39</sup>, albeit with apparently contradictory results. Furthermore, biochemical analysis of Src shows that the glycine-loop cysteine is reactive and that addition of dithiothreitol to recombinant FGFR1 stimulates kinase activity<sup>40</sup>. To date, however, it has not been ascertained whether Src is a direct target of signaling-mediated H<sub>2</sub>O<sub>2</sub> in cells, nor has the reaction of peroxide with FGFR1 been reported.

The mutation or amplification of EGFR in a number of human carcinomas, including breast and lung cancers, has motivated the development of inhibitors such as analogs that covalently modify Cys797, which are currently under evaluation in clinical trials<sup>27,28</sup>. Recently, we have reported that overexpression of EGFR and Her2 in breast cancer cell lines correlates with elevated H<sub>2</sub>O<sub>2</sub> and global protein sulfenylation<sup>21</sup>. Coupled with the discovery that EGFR Cys797 undergoes sulfenic acid modification, these findings raise several fundamental questions *vis-à-vis* cysteine oxidation and thiol-targeted irreversible inhibitors. For example, how does sulfenylation affect the potency of irreversible inhibitors designed to target the thiol form of EGFR Cys797? Conversely, could the propensity for EGFR Cys797 to undergo sulfenylation be exploited to develop a new class of irreversible

inhibitors that incorporate a nucleophilic warhead? Taking this one step further, whether this strategy could be exploited to selectively target EGFR in cancer cells with abnormally high concentrations of reactive oxygen species remains to be investigated. This approach need not be restricted to kinases, however, and could be applied to other enzymes or noncatalytic proteins with redox-sensitive cysteine residues, as we have demonstrated recently for PTPs<sup>41</sup>. These topics represent new and exciting avenues for future research.

In summary, we have developed the new chemoselective probe DYn-2 for detecting sulfenylated proteins in cells. Using this reagent, we have shown that growth factor-mediated signal transduction leads to oxidation of key signaling proteins, including EGFR. From a broader perspective, our findings highlight sulfenylation as a signaling mechanism analogous to phosphorylation and allude to new redox-based strategies for therapy development. In conjunction with new tools for ROS detection<sup>2,42,43</sup> and quantitative proteomic analysis<sup>41,44–46</sup>, these results presage a bright future for the dissection of the regulatory mechanisms that underlie redox regulation of cell signaling.

## METHODS

### DYn-2 (2)

Lithium diisopropylamide (LDA) was prepared by the dropwise addition of 2.5 M solution of *n*-butyllithium (15.7 ml, 39.2 mmol) to a solution of diisopropylamine (3.97 g, 39.2 mmol) in tetrahydrofuran (THF; 40 ml), and the resulting pale-yellow mixture was stirred at  $-78\text{ }^{\circ}\text{C}$  for 30 min in a 250-ml flask equipped with a magnetic stir bar under  $\text{N}_2$  pressure. A solution of 1,3-cyclohexadione (2.0 g, 17.8 mmol) in THF (20 ml) and hexamethylphosphoramide (HMPA; 10 ml) was added dropwise to the LDA solution at  $-78\text{ }^{\circ}\text{C}$ . The resulting mixture was stirred at  $-78\text{ }^{\circ}\text{C}$  for 1.5 h. The temperature was increased to  $0\text{ }^{\circ}\text{C}$  briefly to facilitate the stirring and then cooled again to  $-78\text{ }^{\circ}\text{C}$ . To this dianion slurry, a solution of 5-iodopent-1-yne (3.81 g, 19.6 mmol) in THF (20 ml) was added dropwise at  $-78\text{ }^{\circ}\text{C}$ . The reaction was stirred and allowed to warm to  $25\text{ }^{\circ}\text{C}$  over 2 h. The mixture was then neutralized with 1.0 M HCl (22 ml) and concentrated under reduced pressure. The residue was diluted with  $\text{H}_2\text{O}$  and extracted with ethyl acetate ( $3 \times 50\text{ ml}$ ). The organic phase was then washed with brine, dried over anhydrous  $\text{MgSO}_4$  and concentrated. Purification by column chromatography (gradient: dichloromethane/methanol from 100:0 to 98:2) afforded compound **2** as a mixture of the keto and enol forms (3.0 g, 96% yield). The product was further purified by reversed-phase preparative HPLC (Varian Polaris 5 C18-A  $150 \times 21.2\text{ mm}$  column) using a gradient of water/acetonitrile from 95:5 to 5:95 over 30 min.  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  5.42 (s, 1H), 3.41 (d,  $J = 4.0\text{ Hz}$ , 2H), 2.75–1.72 (m, 16H), 1.68–1.45 (m, 6H).  $^{13}\text{C-NMR}$  (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  204.9, 204.4, 197.0, 189.2, 104.2, 84.4, 84.1, 69.1, 68.9, 58.5, 49.1, 41.8, 39.9, 30.1, 29.6, 28.5, 26.4, 26.2, 26.1, 24.7, 18.8, 18.7. ESI-MS,  $m/z$  for  $\text{C}_{11}\text{H}_{14}\text{O}_2$ : calculated, 178.23; observed, 179.1  $[\text{M} + \text{H}]^+$ .

### Cell culture

HeLa cells were cultured as previously described<sup>11</sup>. A431 cells (American Type Culture Collection) were maintained at  $37\text{ }^{\circ}\text{C}$  in a 5%  $\text{CO}_2$ , humidified atmosphere. Unless indicated otherwise, cells were cultured in high-glucose DMEM medium (Invitrogen) containing 10% FBS (Invitrogen), 1% GlutaMax (Invitrogen), 1% MEM nonessential amino acids (Invitrogen), and 1% penicillin-streptomycin (Invitrogen). For EGF treatment, cells were cultured until 80–90% confluent, rinsed with PBS and placed in high-glucose DMEM medium without serum for 16–18 h. After serum deprivation, cells were treated with the indicated concentration of EGF for the indicated time period. EGF treatment was stopped by removing the medium and washing with PBS.



### Sulfenic acid labeling in cells

HeLa cells were labeled as previously described<sup>11</sup>. A431 cells were lifted with 0.25% trypsin-EDTA, harvested by centrifugation at 1,500g for 2 min, washed and resuspended in serum-free DMEM at a density of  $3-4 \times 10^6$  cells ml<sup>-1</sup>. Intact cells in suspension were incubated with DMSO vehicle (2% v/v) or the indicated concentration of sulfenic acid probe (DYn-2, DAz-2 or dimedone) at 37 °C in a 5% CO<sub>2</sub>, humidified atmosphere with periodic gentle agitation. Following treatment for the indicated time, cells were collected and washed with PBS. The resulting cells were routinely counted using a hemocytometer and uniformly showed greater than 90% viability by trypan blue exclusion.

### Click chemistry

Cell lysate (200 µg, 1 mg ml<sup>-1</sup>) was pretreated with 75 µl NeutrAvidin-agarose (Pierce) to remove endogenously biotinylated proteins. The precleared lysate was incubated with 100 µM azide- or alkyne-biotin, 1 mM tris(2-carboxyethyl)phosphine hydrochloride, 100 µM tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine ligand and 1 mM CuSO<sub>4</sub> for 1 h at 25 °C with gentle rocking (final reaction volume of 200 µl). The reaction was quenched by 40 mM EDTA, and the proteins were subjected to methanol precipitation. The resulting protein precipitate was then resolubilized in Laemmli sample buffer containing 5% (w/v) SDS in PBS. To analyze immunoprecipitated proteins, the resin was treated with 20 µl click chemistry mix (100 µM azide-biotin, 1 mM TCEP, 100 µM TBTA, 1 mM CuSO<sub>4</sub> in PBS) as above; reactions were quenched by boiling with 20 µl Laemmli sample buffer for 10 min.

### Immunostaining and fluorescence imaging

A431 cells were seeded on collagen-coated coverslips (BD Biosciences) and cultured as described above. The cells were then fixed with 4% paraformaldehyde in PBS for 15 min, and washed three times with PBS and then blocked in 5% horse serum, 0.1% Triton X-100 (Sigma) in PBS (blocking solution) for 30 min at 25 °C. The cells were then treated with rabbit EGFR-specific antibody (1005, Santa Cruz Biotechnology), mouse PTEN-specific antibody (A2B1, Santa Cruz Biotechnology), mouse PTP1B-specific antibody (FG6, Calbiochem) or rabbit SHP2-specific antibody (Santa Cruz Biotechnology) at 2 µg ml<sup>-1</sup> in blocking solution for 1 h at 25 °C. Control cells were treated with PBS only. The cells were washed three times in PBS and incubated with Alexa 594-conjugated goat rabbit-specific (Invitrogen), Alexa488-conjugated goat rabbit-specific (Invitrogen) or Alexa 488-conjugated goat mouse-specific (Invitrogen) secondary antibodies diluted to 1:1,000 in blocking solution for 1 h at 25 °C in the dark. For experiments involving dimedone, cells were fixed in cold methanol:acetone (1:1), blocked and treated with rabbit 2-thiodimedone-specific antibody (1:3000) as previously described<sup>21</sup>. The cells were then washed three times with PBS and stained by Alexa 594-conjugated goat rabbit-specific secondary antibody (1:1,000) for 1 h at 25 °C in the dark. To visualize Nox2, cells were double stained with rabbit 2-thiodimedone-specific antibody (1:3,000) and phycoerythrin (PE)-conjugated mouse Nox2-specific antibody (7D5, MBL International, 1:1,000) followed by Alexa 488-conjugated goat rabbit-specific secondary antibody (1:1,000). Cells were then washed three times with blocking solution, counter-stained with 0.1 mg ml<sup>-1</sup> DAPI, washed with PBS and mounted with Fluoromount G (Southern Biotech). Confocal fluorescence imaging studies on A431 cells were performed with an Olympus FV1000 microscope and a 100× oil-immersion objective lens. Excitation of Alexa 488 conjugates was carried out with an argon laser and emission was collected using a 488-nm to 515-nm filter set. Excitation of Alexa 594- or PE-conjugate was carried out with a helium-neon laser, and emission was collected using a 548-nm to 644-nm filter set.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

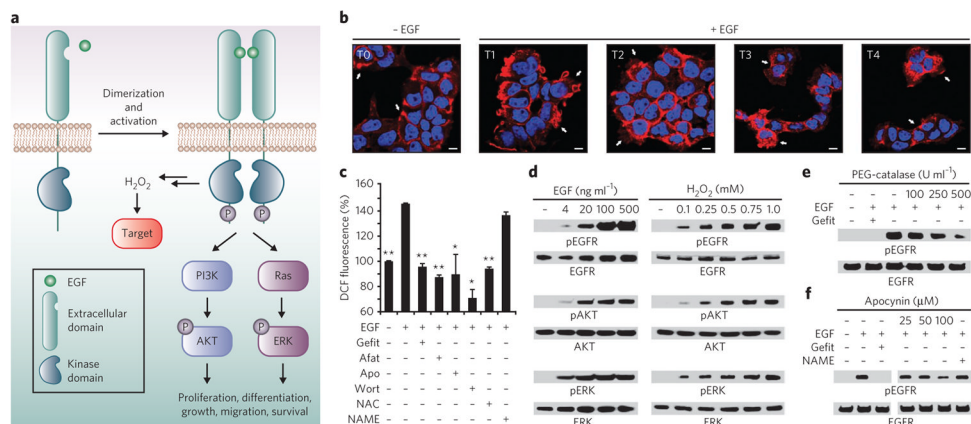
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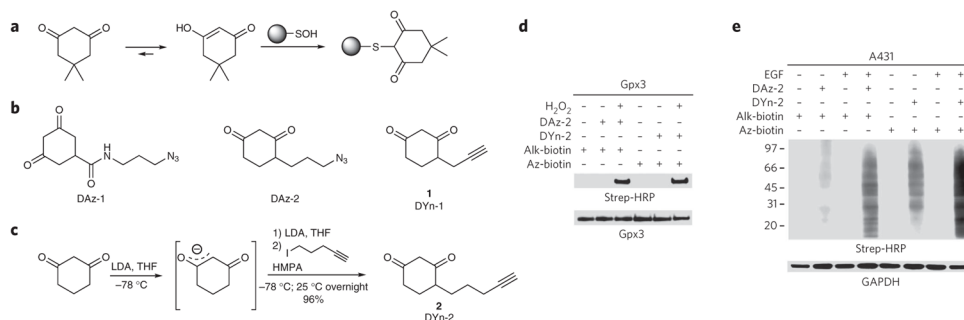
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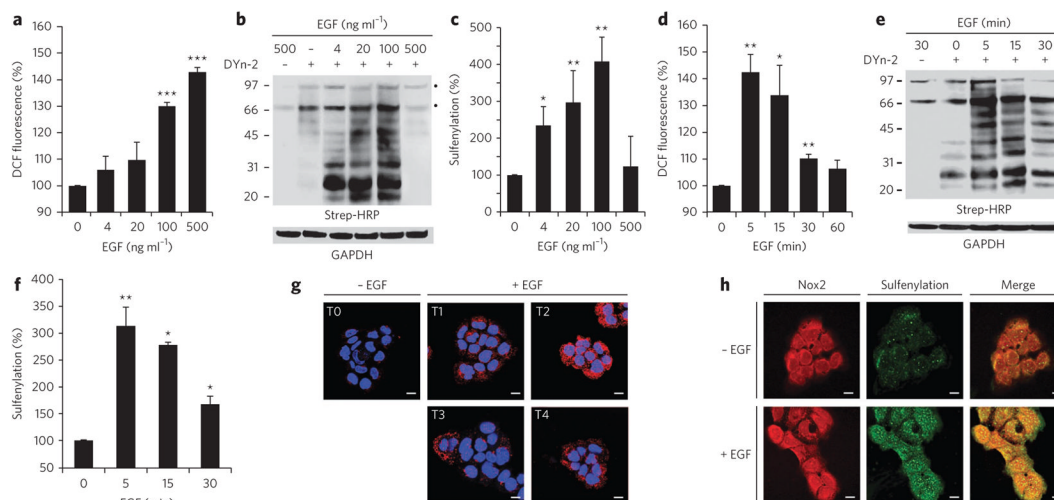
### Figure 1. Cellular redox status affects EGF-mediated signaling

(a) EGF binding to EGFR and subsequent dimerization induce receptor autophosphorylation on specific tyrosine residues within the cytoplasmic domain. The newly phosphorylated (P) sites serve as interaction platforms for proteins involved in key prosurvival pathways, such as the PI 3K-AKT and Ras-ERK pathways. Receptor-ligand interaction also stimulates the production of ROS and oxidation of intracellular biomolecules, leading to modulation of the signaling cascade. (b) Confocal fluorescence images of EGFR localization in A431 cells before (T0) and after stimulation with 100 ng ml<sup>-1</sup> EGF for 2 min, 15 min, 30 min and 60 min (T1, T2, T3 and T4, respectively). White arrows highlight changes in receptor localization (T0, plasma membrane; T1, membrane ruffles; T2, cell stretching and migration; T3, perinuclear and endosomal membranes; T4, cell surface and ruffles). Nuclei were counterstained with DAPI (blue). Scale bars, 10 μm. (c) EGF-induced ROS generation in A431 cells as revealed by DCF fluorescence. Where specified, cells were treated with gefitinib (Gefit), afatinib (Afat), apocynin (Apo), wortmannin (Wort), NAC or L-NAME before EGF stimulation. Data are representative of three independent readings and were normalized to the vehicle control. Error bars, ± s.e.m. \**P* < 0.05, \*\**P* < 0.001 when compared against cells treated with EGF only. (d–f) Western blots showing phosphorylated (p) and total EGFR, AKT and/or ERK. A431 cells were stimulated with the indicated concentrations of EGF, H<sub>2</sub>O<sub>2</sub> or vehicle for 5 min (d) or with 100 ng ml<sup>-1</sup> EGF or vehicle for 5 min (e,f). Where specified, cells were treated with the indicated concentrations of PEG-catalase (e), apocynin (f) or gefitinib (e,f) before EGF stimulation. Full western blots for all experiments are shown in Supplementary Figure 2.



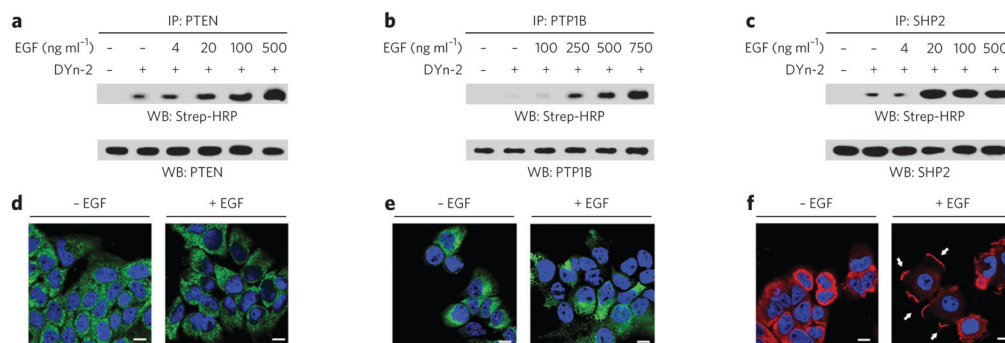
**Figure 2. Development and validation of probes for detecting sulfenic acid**

(a) Selective reaction between sulfenic acid and dione. (b) Chemical structures of chemical reporters for sulfenic acid. (c) Design and synthesis of DYN-2 (2). LD A, lithium diisopropylamide; HMPA, hexamethyl-phosphoramide. (d) Comparison of DAz-2 and DYN-2 detection of sulfenic acid in recombinant Gpx3. 50  $\mu$ M protein was untreated or exposed to 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> and incubated in the presence or absence of 1 mM probe for 15 min at 37 °C. Labeled proteins were detected by streptavidin–horseradish peroxidase (Strep-HRP) western blot. Comparable protein loading was confirmed by reprobing the blot with His tag–specific antibody. (e) Western blots showing DAz-2 and DYN-2 detection of protein sulfenic acids and total GAPDH in A431 cells. Cells were stimulated with 100 ng ml<sup>-1</sup> EGF or vehicle for 5 min and then incubated with 5 mM probe or vehicle for 1 h at 37 °C.



### Figure 3. EGF-mediated ROS production and protein sulfenylation

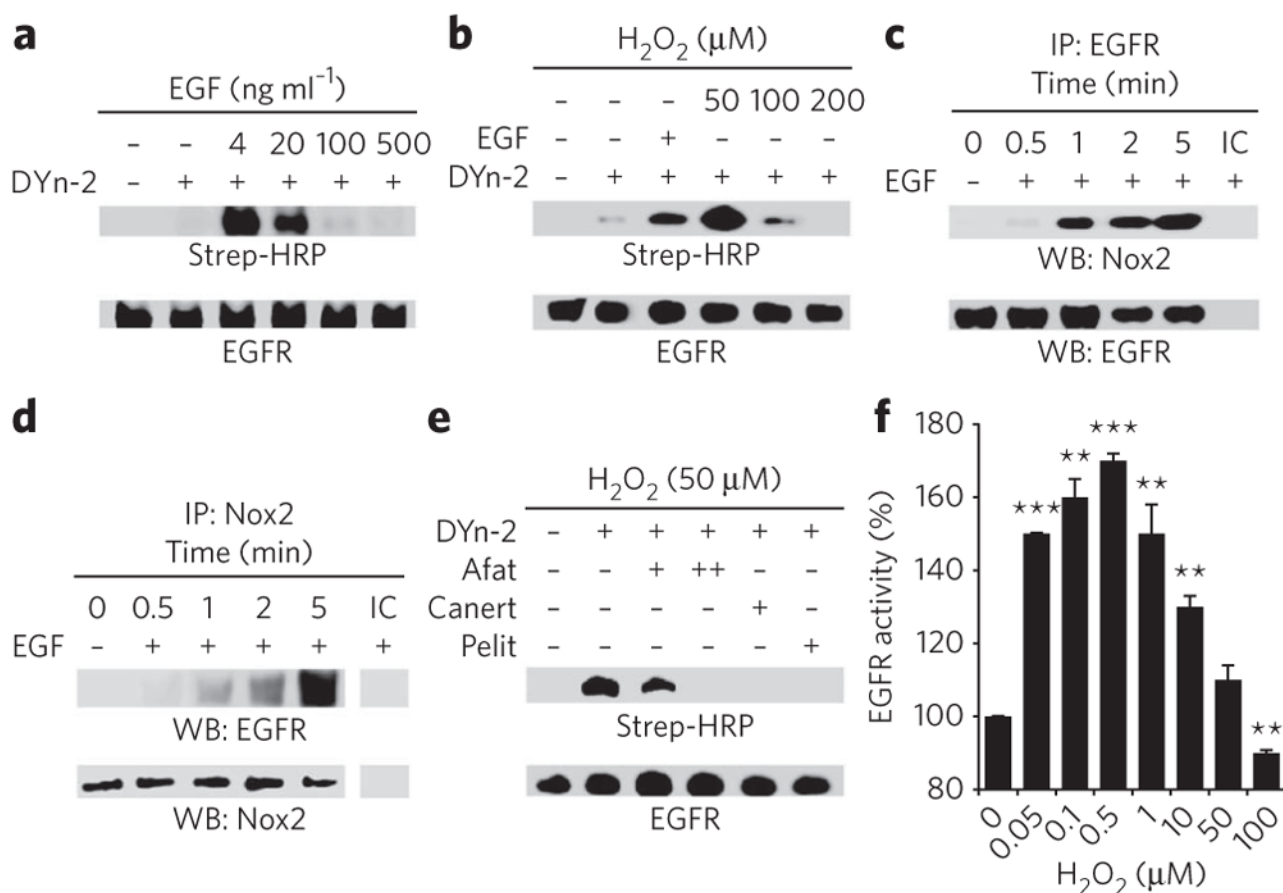
(a–f) ROS production, as indicated by DCF fluorescence (a,d) or western blotting for protein sulfenylation with Strep-HRP (b,e), in A431 cells incubated with EGF at the indicated concentrations (a,b) or for the indicated times (d,e). In d and e, all cells were stimulated with 500 (d) or 100 ng ml<sup>-1</sup> EGF (e) or vehicle. Panels c and f show densitometric quantification of b and e, respectively. Throughout, GAPDH was used as a loading control. Data are representative of three independent readings for ROS measurements and four independent experiments for western blots. Error bars, mean ± s.e.m. \**P* < 0.05, \*\**P* < 0.01 and \*\*\**P* < 0.001 compared to vehicle control. (g) Fluorescence images of sulfenylation (red) in A431 cells before (T0) and after stimulation with 100 ng ml<sup>-1</sup> EGF for 0.5 min, 1 min, 1.5 min or 2 min, followed by treatment with 5 mM dimedone for 5 min at 37 °C in EGF-containing medium; total EGF exposure was 5.5 min, 6 min, 6.5 min and 7 min (T1–4, respectively). Nuclei were counterstained with DAPI (blue). Scale bars, 10 μm. (h) A431 cells were stimulated with 100 ng ml<sup>-1</sup> EGF or vehicle for 0.5 min and treated with dimedone as in g. Cells were stained for the dimedone-protein adduct (green) and Nox2 (red). Scale bars, 10 μm.



#### Figure 4. Differential sulfenylation of PTPs in EGF-treated cells

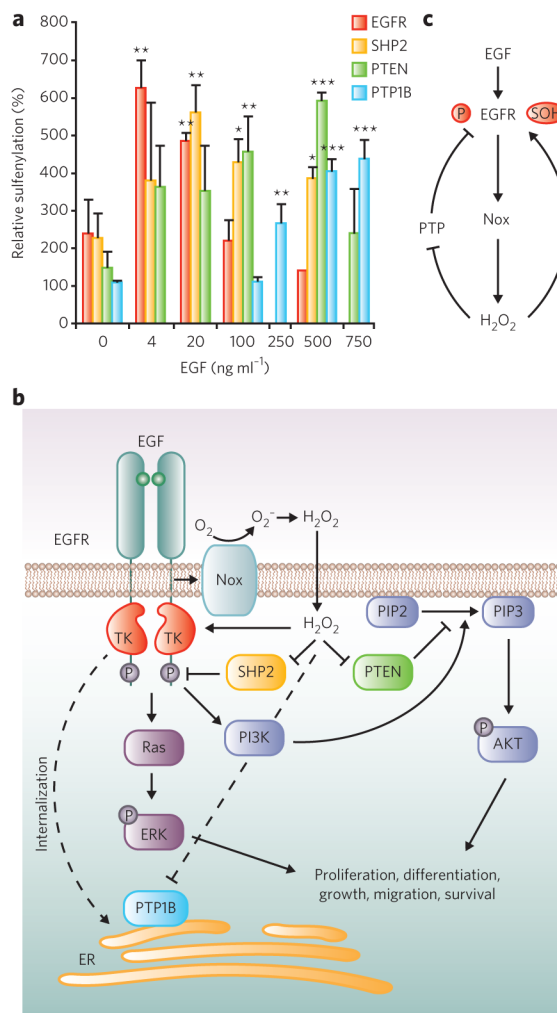
(a–c) Western blots (WB) showing sulfenylated and total immunoprecipitated PTEN, PTP1B and SHP2. A431 cells were stimulated with EGF or vehicle for 2 min at the indicated concentrations and then incubated with 5 mM DYn-2 or vehicle for 1 h at 37 °C. Lysates were immunoprecipitated with mouse PTEN- (a), mouse PTP1B- (b) or rabbit SHP2-specific antibody (c). Sulfenylation of PTPs was detected by Strep-HRP western blot. Western blots were reprobed for total PTP as indicated to verify equivalent recovery. (d–f) Confocal fluorescence images of A431 cells stimulated with vehicle or 100 ng ml<sup>-1</sup> EGF for 5 min. Cells were stained with PTEN- (d), PTP1B- (e) or SHP2-specific antibody (f). Nuclei were counterstained with DAPI (blue). Scale bars, 10 μm. The white arrows in f highlight the changes in subcellular localization of SHP2 after stimulation with EGF.





**Figure 5. EGF-mediated sulfenylation of EGFR Cys797 in cells**

(a,b) Western blots showing sulfenylated and total EGFR. A431 cells were stimulated with EGF at the indicated concentrations or vehicle for 2 min (a) or H<sub>2</sub>O<sub>2</sub> for 10 min (b), and sulfenic acids were detected by Strep-HRP western blot. (c,d) Western blots showing coimmunoprecipitation of Nox2 and EGFR. A431 cells were stimulated with 100 ng ml<sup>-1</sup> EGF or vehicle for the indicated times and immunoprecipitated using EGFR-specific (c) or Nox2-specific antibodies (d). Comparable recovery of immunoprecipitated (IP) EGFR was confirmed by probing the blot with EGFR-specific antibody in c and with Nox2-specific antibody in d. IC, isotype control. (e) Western blot showing sulfenylated and total EGFR. A431 cells were incubated with 1 μM or 5 μM afatinib (+ and ++, respectively), 10 μM canertinib (Canert), 1 μM pelitinib (Pelit) or vehicle before treatment with H<sub>2</sub>O<sub>2</sub>, and sulfenylation was detected as in b. (f) Measurement of EGFR tyrosine kinase activity *in vitro*. Data are representative of three independent readings and represent the mean ± s.e.m. \*\**P* < 0.01 and \*\*\**P* < 0.001 compared to vehicle control.



### Figure 6. Model for redox regulation of EGFR signaling

(a) Densitometric quantification of EGFR and PTP sulfenylation from blots in Figures 4a–c and 5a. Data are representative of four independent experiments and represent the mean  $\pm$  s.e.m. for each protein. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  compared to vehicle control.

(b) The mitogen EGF binds to EGFR and induces the production of ROS in A431 cells via Nox2. The proximity of target proteins to Nox2 has an impact on the rate of cysteine oxidation within the cell. Dashed lines are relevant to EGFR internalization. TK, tyrosine kinase. (c) Model for H<sub>2</sub>O<sub>2</sub>-mediated increase in EGFR kinase activity. Nox-generated H<sub>2</sub>O<sub>2</sub> directly modifies EGFR at a critical cysteine (Cys797) in the active site, which enhances its tyrosine kinase activity. Endogenous H<sub>2</sub>O<sub>2</sub> also oxidizes and deactivates PTPs, which serves to maintain EGFR phosphorylation. Collectively, these events lead to an increase in receptor autophosphorylation, which promotes signaling through downstream pathways.