EPIDERMAL GROWTH FACTOR-INDUCED HYDROGEN PEROXIDE PRODUCTION IS MEDIATED BY DUAL OXIDASE 1

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ABSTRACT

Stimulation of mammalian cells by epidermal growth factor (EGF) elicits complex signaling events, including an increase in hydrogen peroxide (H_2O_2) production. Understanding the significance of this response is limited by the fact that the source of EGF-induced H_2O_2 production is unknown. Here we show that EGF-induced H_2O_2 production in epidermal cell lines is dependent on the agonist-induced calcium signal. We analyzed the expression of NADPH oxidase isoforms and found both A431 and HaCaT cells to express the calcium-sensitive NADPH oxidase, Dual oxidase 1 (Duox1) and its protein partner Duox activator 1 (DuoxA1). Inhibition of Duox1 expression by small interfering RNAs eliminated EGF-induced H_2O_2 production in both cell lines. We also demonstrate that H_2O_2 production by Duox1 leads to the oxidation of thioredoxin-1 and the cytosolic peroxiredoxins. Our observations provide evidence for a new signaling paradigm in which changes of intracellular calcium concentration are transformed into redox signals through the calcium-dependent activation of Duox1.

Keywords: Reactive Oxygen Species, Hydrogen Peroxide, EGF receptor, NADPH oxidase, Dual Oxidase 1

Abbreviations: Duox: Dual oxidase, DuoxA1: Duox activator 1, EGF: epidermal growth factor, LPO: lactoperoxidase, Nox: NADPH oxidase, NoxA1: NADPH oxidase activator 1, Noxo1: NADPH oxidase organizer 1, PRX1: peroxiredoxin 1, PRX2: peroxiredoxin 2, PTP: protein tyrosine phosphatase, ROS: reactive oxygen species, Trx1: thioredoxin 1

INTRODUCTION

Reactive oxygen species (ROS) are increasingly being recognized as important signaling molecules (1), (2). Regulated production of these compounds has been described in several settings and the list of their targets is continuously growing. Among ROS, hydrogen peroxide (H_2O_2) is probably the most intensively studied molecule (3). Oxidation of target proteins by H_2O_2 can occur on specific reactive cysteine residues and may introduce reversible changes in protein structure and function (4), (5). Prerequisite of being a signaling intermediate is the controlled production and elimination of the given molecule and H_2O_2 seems to fit in with these requirements. There are multiple stimuli that can trigger the formation of H_2O_2 in mammalian cells and growth factor-induced H_2O_2 production is thought to be a particularly important signaling event (6), (7). EGF-induced H_2O_2 production was first described in A431 cells and has been subsequently demonstrated in other cell types as well (8), (9). The ROS response elicited by EGF is thought to amplify tyrosine-kinase signaling through the inhibition of protein tyrosine phosphatases that normally antagonize this signaling route (10), (7). Although the signaling pathways triggered by EGF are mostly well-understood, the enzymatic source of EGF-induced H_2O_2 production is currently unknown. In this work we set out to study the possible involvement of Nox/Duox enzymes in EGF-stimulated H_2O_2 production. Using A431 and HaCaT cell models we demonstrate that EGF-induced H_2O_2 production originates from the calcium-dependent activation of Duox1.

MATERIALS AND METHODS

Materials and reagents

EGFR antibodies were from Cell Signaling Technology, the Proteome Profiler Human Phospho-Kinase Array Kit was from R&D Systems, Inc. The qPCR assays were from Applied Biosystems-Life Technologies, EGF and TGFα were from PeproTech (Rocky Hill, NJ, USA). Stealth siRNAs and Fura2-AM were from Life Technologies. Amplifu Red, thapsigargin, ATPγS, niacin, GSK1016790A were from Sigma-Aldrich. Anti-rabbit–horseradish peroxidase and anti-mouse–horseradish peroxidase were from Amersham Biosciences (Piscataway, NJ, USA). Polyclonal anti-Duox antibody was raised against the Arg618-His1044 fragment of human Duox1 (Milenkovic et al., 2007). EZ-Link™ Maleimide-PEG11-Biotin reagent was from Thermo Fisher Scientific, Life Technologies.

Cell Culture

A431 and HaCaT cells were grown in Dulbecco's Modified Eagles Medium with glutamin and 4.5 g/L glucose, supplemented with 50 U/ml penicillin and 50 µg/ml streptomycin (Lonza Group Ltd., Basel, Switzerland) and 10% fetal calf serum (Biowest SAS, France, S182P-500, French origin). Cells were grown in a humidified incubator with 5 % CO_2 in air, at 37 °C.

siRNA transfection

Stealth siRNAs were transfected at the time of cell plating in 20 nM final concentration using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturers instructions. Measurements were carried out 48 hrs after transfection. Three different Duox1 specific siRNA sequences were tested and three different control siRNAs of varying GC content were used as

control. All Duox1 specific sequences gave efficient knockdown of Duox1 expression. For DuoxA1 knockdown one specific stealth siRNA was used.

Quantitative PCR

RNA was isolated using Qiagen RNeasy Micro and Mini kit. During the RNA purification an on-column DNase I digestion step was also included. cDNA was synthesized from 1 µg of total RNA using oligo(dT)18 and RevertAid M-MuLV reverse transcriptase (Fermentas) in 20 µl reaction mix according to the manufacturer's recommendations. One µl of cDNA was used in a 10 µl qPCR reaction using the Taqman Gene Expression 20x assays and LightCycler 480 Probes Master in a LightCycler LC480 plate reader. For each cDNA sample the expression of the target gene was divided by the expression of the endogenous control, which was β-actin. The crossing point was determined by the second derivative method. The list of the used Taqman Gene Expression assays:

Nox1: Hs00246589_m1, Nox2: Hs00166163_m1, Nox3: Hs01098883_m1, Nox4: Hs00418356_m1, Nox5: Hs00225846_m1, Duox1: Hs00213694_m1, Duox2: Hs00204187_m1, DuoxA1: Hs00328806_m1, actinB: Hs00357333_g1, p22phox: Hs03044361_m1, NoxO1: Hs00376045_g1, NoxA1: Hs00736699_m1

Western blot experiments

Laemmli sample buffer was added to the cell lysate samples and these were run on 8-16% SDS polyacrylamide gels and blotted onto nitrocellulose membranes. Membranes were blocked in phosphate buffered saline containing 0,1 % Tween- 20 and 5 % dry milk or 5 % bovine serum albumin (for phospho-tyrosine specific western blots). The first antibodies were diluted in blocking buffer and used either for 2 hrs at room temperature or overnight at 4 °C. After several washing steps in PBS-Tween-20 membranes were incubated with HRP-linked secondary antibodies (Amersham Pharmaceuticals,Amersham, UK) diluted in blocking buffer. Antibody

binding was detected using enhanced chemiluminescence and Fuji Super RX medical X-ray films. Importantly samples were never boiled when processed for western blotting with the Duox1 antibody (11).

The redox state of peroxiredoxin-1, -2 and -3 was also measured by immunoblotting. HaCaT cells were treated with various stimuli as indicated in the Figure legends. After the stimuli – to prevent artefactual oxidation of peroxiredoxins - cells were alkylated by incubating with 80mM methyl-methane thiosulfonate (MMTS) in Hank's buffered salt solution. After 10 min, buffer was supplemented with 1% protease inhibitor cocktail and cells were lysed with 1% CHAPS. After 10 min incubation with CHAPS, lysates were centrifuged at 16,000xg for 5 min and supernatants subjected to non-reducing SDS-PAGE (12%). After transfer of proteins to PVDF membrane, membranes were blocked with 3% BSA in TBS-Tween-20 (500mM NaCl, 20mM Tris-HCl, pH 7,4 and 0,05% Tween 20). Detection of proteins were performed using rabbit polyclonal antibodies to PRDX-1 (1:5000), PRDX-2 (1:2000), PRDX-3 (1:5000) (Sigma, St. Louis, MO, USA) and anti-Peroxiredoxin-SO3 (1:2000, AbFrontier, Seoul, Korea) in 3% BSA, probed with anti-rabbit-IgG alkaline phosphatase conjugated secondary antibody (Sigma, St. Louis, MO, USA) (1:10,000) and BCIP/NBT substrate (Merck-Millipore, Darmstadt, Germany).

The blots were analyzed on a GBox-Chemi XX6 (Syngene, Cambridge, UK) gel doc system and relative band densities of Western blots were quantified using ImageJ software.

Ca^{2+} measurements

Cell were grown on coverslips. Before the experiment cells were washed in H-medium (containing 145 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 0.8mmol/L $CaCl_2$, 5 mmol/L glucose, and 10 mmol/L HEPES) and Fura-2-AM was loaded in a final concentration of 1 µM for 20 minutes at room temperature. Ratiometric fluorescence intensity measurements were performed on an inverted microscope (Axio Observer D1, Zeiss) equipped with a 40x, 1.4 oil immersion objective (Fluar, Zeiss) and a Cascade II camera (Photometrics). Excitation

wavelengths were set by a random access monochromator connected to a xenon arc lamp (DeltaRAM, Photon Technology International). Images were acquired every 5 s for a period of 10 to 15 min with the MetaFluor software (Molecular Devices).

AmplexRed assay

Confluent cells on 24-well plates were washed in an extracellular medium „H-medium" and background fluorescence was also measured in H-medium. For the assay an H-medium-based reaction solution was used containing horse radish peroxidase and Ampliflu Red in a final concentration of 0,2 U/ml and 50 µM respectively. Agonists and inhibitors were also added to the Amplex Red containing reaction solution immediately before pipetting it on to the cells. The measurement of fluorescence started promptly after the addition of the reaction solution (0,3 ml/well) and the cells were kept at 37 °C throughout the measurement. Background fluorescence was subtracted from the fluorescence values of each well. Each experimental condition was run in 3-6 parallels on the 24-well plate.

Thioredoxin-1 labeling and detection

HaCaT cells were stimulated for 5 min at 37 °C with the indicated stimuli then washed once quickly in icecold PBS and then lysed in ice-cold RIPA buffer (containing 150 mM NaCl, 50 mM Tris pH 8.0, 0,1 % SDS, 0,5 % Na-deoxycholate, 1 % Triton-X100 and protease and phosphatase inhibitors) in the presence of 200 µM Biotin-PEG_{11}-Maleimide. Cells were scraped on ice, collected in Eppendorf tubes and incubated at 37 °C for 25 minutes. Insoluble fraction was separated by centrifugation, the supernatant was mixed with non-reducing Laemmli sample buffer and heated at 80 °C for 5 minutes before loading onto 16 % SDS-PAGE gel.

Statistics

The statistical analysis of peroxiredoxin dimer formation experiments was carried out using paired t-test. We used Student's t test for the statistical analysis of H_2O_2 measurement results.

RESULTS

EGF induces H_2O_2 production in A431 and HaCaT cells expressing the NADPH oxidase isoform Duox1

We measured H_2O_2 production by A431 and HaCaT cells through the horseradish peroxidase-mediated oxidation of the Amplex Red reagent. As shown in Fig. 1A and B, both cell lines showed spontaneous H_2O_2 production. Stimulation by EGF increased the rate of H_2O_2 release in both cell lines. The rate of H_2O_2 production after an EGF stimulus was approximately 3 nmol/hour/1 million cell based on a simple linear regression analysis of Amplex Red fluorescence on an H_2O_2 concentration scale. This response varied between 25-40 % of the maximal response evoked by stimulating the cells with thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase, which depletes intracellular calcium stores and elicits Ca^{2+} influx from the extracellular space. Since members of the Nox/Duox family of NADPH oxidases are the primary sources of regulated ROS production in mammalian cells (12) we wanted to examine their possible involvement in the agonist-induced production of H_2O_2. First we used QPCR to analyze the expression of Nox/Duox isoforms and their regulators at the RNA level. Among the Nox/Duox isozymes, Duox1 was found to be expressed at the highest level in both cell lines, while the others were either absent or expressed only at very low level (Fig. 1C). High level of $p22^{phox}$ expression was detected in HaCaT cells, but A431 cells did not express any $p22^{phox}$ mRNA. DuoxA1, which is the activator component of the Duox1 enzyme complex (13), was also present at high levels in both cells lines, while mRNAs encoding NoxO1 and NoxA1 were barely detected. These results pointed to Duox1 as the possible source of H_2O_2 in these cell lines. To confirm the result of the QPCR experiments we checked if the Duox1 protein is

present in A431 and HaCaT cells. The result of the Western blot in Figure 1D shows the presence of the Duox1 protein in both cell lines. Since this antibody does not discriminate between the two Duox isoforms we used siRNA to further corroborate that the Duox signal is attributable to Duox1. Fig. 1D shows that siRNA against Duox1 practically erased the signal, thus confirming that it essentially originates from Duox1.

We also tested three other widely used epithelial cancer cell lines as well: A549 (adenocarcinomic alveolar epithel), A2780 (ovarian carcinoma) and HeLa (cervical cancer). None of these cell lines expressed Duox1 in a significant amount and they did not produce H_2O_2 in response to EGF or thapsigargin, although A549 and HeLa cells responded to EGF with a strong elevation in phosphotyrosine levels (Supplementary Fig. 1.).

EGF-stimulated H_2O_2 production is mediated by the calcium-dependent activation of Duox1

As Duox enzymes bear two EF-hands in their first intracellular loop which provides the structural basis for their activation by calcium (14), in subsequent experiments we studied the importance of calcium signal in EGF-induced H_2O_2 production. As shown in Supplementary Fig. 2, EGF elicited a rise in intracellular calcium level in both A431 and HaCat cells. When the cells were loaded with the intracellular calcium chelator BAPTA, the stimulatory effect of EGF was lost in both cell lines, suggesting a key role for the calcium signal in the activation process (Figs 2A and 2B). On the other hand, when distinct stimuli, with the ability to produce a calcium signal, were studied they also effectively stimulated a H_2O_2 response in A431 (thapsigargin, shown in Fig. 2C) or in HaCaT cells (thapsigargin, vitamin B3, GSK 1016790A, ATPgammaS, shown in Fig. 2D). Next we examined the specific role of Duox1 in the above described ROS responses. Duox1 siRNA treatment, which effectively reduced the expression of Duox1, inhibited the spontaneous H_2O_2 release and prevented the stimulatory effect of EGF and other agonists (Figs. 3A and 3B). The result of these experiments clearly suggested an essential role for Duox1 in

EGF-induced H_2O_2 production. Previous studies demonstrated that the activity of Duox1 is dependent on the presence of DuoxA1, which is a maturation factor for Duox1 and assists the enzyme to reach its physiological location (13), (15). As DuoxA1 was present in both A431 and HaCaT cells, we wanted to see whether the suppression of DuoxA1 results a phenotype similar to that observed in Duox1-deficient cells. Figs. 3C and D show, that siRNA-mediated reduction of DuoxA1 expression also interfered with EGF-stimulated H_2O_2 production, thus providing an additional proof for the crucial role of the Duox1-DuoxA1 complex in EGF-induced H_2O_2 production. The complete elimination of the thapsigargin-induced H_2O_2 production by Duox1 knockdown also demonstrates the central role of Duox1 in translating calcium signals of various origin into ROS signals.

Duox1-derived H_2O_2 was described to modulate calcium signaling in Jurkat cells (16), although in our previous experiments we did not observe altered calcium signaling in epithelial cells prepared from Duox1 knockout animals (17). Supplementary Figure 3. shows, that siRNA-mediated downregulation of Duox1 did not modify the calcium signal elicited by EGF.

Duox1-mediated H_2O_2 production leads to the oxidation of thioredoxin-1 and cytosolic peroxiredoxins

Since H_2O_2 was described to inhibit the enzymatic activity of PTPs (10), we wanted to see whether we can detect any change in the pattern of tyrosine phosphorylation when Duox1 activity is absent. Supplementary Figure 4. shows that EGF rapidly stimulated tyrosine phosphorylation of several proteins, however, silencing of Duox1 did not result a visible change in the overall phosphorylation pattern. To further search for an impact of Duox1-derived H_2O_2 on intracellular targets we studied the possible influence of Duox1 on thioredoxin, since calcium-dependent thioredoxin oxidation was previously described in HaCaT cells (18). To assess the ratio of the reduced/oxidized forms of thioredoxin-1 we devised a labeling method using Biotin-PEG_{11}-maleimide as labeling reagent. This maleimide reagent reacts only with the reduced thiol

groups of Trx1 and causes a shift in its molecular weight which is readily detectable on a non-reducing gel. The oxidized form of Trx1 does not bind to the Biotin-PEG_{11}-maleimide reagent and runs faster on the gel. As shown in Fig. 4A, thapsigargin, ATPgammaS and EGF increased the oxidation of thioredoxin and the effects were erased when Duox1 expression was suppressed. Since a direct reaction between Duox1-produced H_2O_2 with thioredoxin is unlikely we wanted to know whether we can detect a change in the oxidation state of peroxiredoxins. Due to a built in peroxide activating mechanism at their active site (19), (20) peroxiredoxins are primarily responsible for capturing most of the intracellular H_2O_2 and are normally reduced by thioredoxin (5). To achieve the highest possible activation of Duox1 we applied thapsigargin as a stimulus in these experiments. Treatment of HaCaT cells with thapsigargin increased the amount of peroxiredoxin 1 (PRX1) and peroxiredoxin 2 (PRX2) dimers (Figs 4B and 4D), indicating the oxidation of the protein by H_2O_2. The oxidation state of peroxiredoxin 3, which is located in the mitochondria, was not affected by thapsigargin (Fig. 4B). We occasionally observed PRX1-positive bands with higher molecular weights (Fig. 4B), reflecting oligomerization of the protein via a Prx1 specific Cys83 residue (that is not the resolving or the peroxidative Cys) at elevated levels of peroxide stimuli (21). Importantly, when Duox1 expression was suppressed by siRNA the oxidation of PRX1 and PRX2 was decreased, (Figs. 4B and 4C). The inhibition of thioredoxin reductase by DNCB (1-chloro-2,4-dinitrobenzene) increased peroxiredoxin dimer and oligomer formation in thapsigargin-treated HaCaT cells confirming the previously described dependence of the peroxiredoxin redox status on the availability of thioredoxin (Fig. 4B). These experiments clearly showed that Duox1-mediated H_2O_2 production is sensed by the thioredoxin-peroxiredoxin system.

An other intriguing question is that how Duox1-produced H_2O_2 can reach its targets. If Duox1 resides in the plasma membrane then extracellular H_2O_2 has to diffuse back to the intracellular side of the membrane. A distinct option is that Duox1 localizes to intracellular membranes and H_2O_2 appears directly in the cytosol. To test these alternatives we added

lactoperoxidase (LPO) and thiocyanate (SCN^-) to the extracellular space of cells. We assumed that the combination of a heme-peroxidase and a peroxidase substrate will efficiently consume H_2O_2 in the extracellular space. As shown in Fig. 4D, the combination of LPO and SCN^- significantly inhibited the thapsigargin-induced thioredoxin oxidation. Extracellularly applied catalase resulted in a similar inihibition of the thapsigargin-induced response (Fig. 4E). These findings support the hypothesis that H_2O_2 appears extracellularly before reaching its targets in the intracellular space.

DISCUSSION

The primary finding of our paper is that the long sought source of EGF-induced H_2O_2 production in epidermal cells is Duox1. We also demonstrated that Duox1 activation in A431 and HaCaT cells is a consequence of an increase in intracellular calcium, elicited by EGF. Our data provide the missing link between agonist-evoked calcium signals and ROS production, which were previously described to be connected in epidermal cell lines (9). EGF-induced H_2O_2 production by A431 cells was originally described by Bae *et al.* (8) and was subsequently confirmed by several other groups (22), (23). In a recent work, the phagocytic NADPH oxidase, Nox2 was detected in A431 cells, however no attempt was made to establish a correlation between ROS production and Nox2 expression (24). The involvement of Nox2 in EGF-induced H_2O_2 production seems unlikely, since we did not detect Nox2 mRNA in A431 or HaCaT cells, while Duox1 mRNA was expressed at high levels in both cell lines. It is also important to note that in contrast to Duox1, Nox2 alone is not able to produce H_2O_2 and the co-expression of four other proteins is necessary for the assembly of an active enzyme complex (25).

Dual oxidases, Duox1 and Duox2, were first identified in the thyroid gland, where the latter has an essential role in thyroid hormone synthesis (26). Subsequent studies have shown that Duox enzymes are not restricted to the thyroid, but are also present in epithelial cells of the

gastrointestinal and respiratory tract (27). The exact role of Duoxes at these sites is not completely understood but an antimicrobial function has been suggested by several studies (26). A role for Duox in host defense was also described in *Drosophila melanogaster*, where an epithelial PLCbeta-Ca^{2+}-Duox-ROS signaling pathway controls microbes in the gut of the flies (28). Although there are several examples for the cooperation between Duox enzymes and peroxidases, the functional link between these enzymes is not mandatory, since Duox enzymes are also identified in cells where peroxidases are not detected. For example Duox1 is highly expressed in the epithelium of the urinary bladder, where an antimicrobial activity seems unlikely and instead a role in mechanotransduction was proposed (17).

Duox1 was also described in primary keratinocytes, where a role in differentiation and STAT6 activation was suggested (29), (28). These studies, however, did not focus on a key feature of Duox enzymes, which is their rapid activation by intracellular calcium increase (14). In our experiments we found several agonists with different mechanisms of action to stimulate Duox1-dependent H_2O_2 production in a calcium-dependent manner, suggesting that calcium signals arising in keratinocytes ultimately result increased H_2O_2 output.

Although we measured H_2O_2 release into the extracellular space, we found evidence for H_2O_2 affecting the redox milieu of the cytosol as well, assessed by the oxidation of PRX1, PRX2 and Trx1. A possible explanation for the presence of Duox1-produced H_2O_2 in the cytosol is that extracellular H_2O_2 diffuses back to the intracellular space as it was previously suggested by Miller *et al* (30). This mechanism is supported by our observations that adding high concentrations of catalase or an H_2O_2-consuming peroxidase-peroxidase substrate system to the extracellular space significantly reduced the thapsigargin-induced oxidation of thioredoxin. When we applied H_2O_2 to the extracellular space of the cells, relatively high peroxide concentrations were required to evoke a change in Trx1 redox status (Supplementary Figure 5.). Keratinocytes reside in the epidermis in close proximity to each other without intervening extracellular matrix. Thus extracellularly released H_2O_2 can probably reach very high local

concentrations and can easily affect cells in an autocrine and paracrine manner. It would also imply that in the epidermis stimuli that elicit more subtle changes in calcium levels than thapsigargin can still result in significant increase in oxidant levels.

Previous reports suggested that EGF-induced H_2O_2 can increase tyrosine phosphorylation of proteins through the inhibition of protein tyrosine phosphatases (10), (7). It is important to note, however, that up till now this concept could not have been adequately tested, since the source of H_2O_2 was unknown. In our experiments we did not notice a major change in the overall tyrosine phosphorylation pattern, when Duox1 expression was suppressed. Thereby, our results suggest that protein tyrosine phosphatases are not targets of Duox1-produced H_2O_2, although we cannot exclude that subtle changes in phosphorylation (or phosphorylation changes of specific targets) were missed in our experiments. To analyze the possible connection of Duox1 to other growth factor signaling pathways, we studied the effects of VEGF and IGF-1. According to literature data these two ligands were both shown to have receptors on HaCaT cells(31), (32). We found that neither IGF-1 nor VEGF did induce such a significant rise in intracellular calcium as EGF did. Accordingly we could not detect significant H_2O_2 production following IGF-1 or VEGF stimuli (data not shown). In summary we think that the simultaneous presence of a calcium signal elicited by a specific growth factor and Duox1 activity is necessary for the appearance of detectable growth factor-induced H_2O_2 production.

On the other hand, we found evidence that H_2O_2 production by Duox1 leads to an oxidative shift in the redox state of the peroxiredoxin-thioredoxin system. The most plausible explanation for this finding is that H_2O_2 directly reacts with PRX1 and PRX2 in the cytosol, and Trx becomes oxidized during the regeneration of peroxiredoxins. To our knowledge our work is the first to report the transmission of an oxidative signal from an identified, regulated source of H_2O_2 to the Prx-Trx system. Trx has several protein interactions, which are determined by the oxidative state of the protein, thus the Duox1-dependent oxidation of the protein may affect signaling pathways, which are regulated by thioredoxin-interacting proteins (33). Furthermore,

recent studies have suggested that the oxidation of PRX2 is transmitted to STAT3 through a redox-relay mechanism in a manner similar to that described in the yeast Orp1-Yap1 system (34). These observations suggest that peroxiredoxins actively participate in signaling, thus the traditional view of these oxidoreductases as mere antioxidants is no longer acceptable. However, the regulated source of H_2O_2 in the PRX2-STAT3 redox relay was not identified. Since both PRX1 and PRX2 are oxidized by Duox1-produced H_2O_2, it is likely that activation of Duox1 represents a signaling step which is proximal to the formation of redox-relay systems like the PRX2-STAT3 interaction. In future experiments we would like to study if EGF-induced Duox1 activation and Duox1-mediated oxidation of thioredoxin and peroxiredoxins are also detectable in primary epidermal cell cultures or *in vivo* in epidermal layers of the skin.

Taken together, our experiments identified the enzymatic source of EGF-stimulated H_2O_2 production and also shed light on a previously unknown signaling paradigm, where calcium signals elicited by receptor agonists are transformed into oxidative protein modification through the calcium-dependent activation of Duox1.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

1. Reczek, C. R.; Chandel, N. S. (2015) ROS-dependent signal transduction. *Curr. Opin. Cell Biol.* **33**, 8-13

2. Murphy, M. P.; Holmgren, A.; Larsson, N. G.; Halliwell, B.; Chang, C. J.; Kalyanaraman, B.; Rhee, S. G.; Thornalley, P. J.; Partridge, L.; Gems, D.; Nystrom, T.; Belousov, V.; Schumacker, P. T.; Winterbourn, C. C. (2011) Unraveling the biological roles of reactive oxygen species. *Cell Metab* **13** (4), 361-366

3. Winterbourn, C. C. (2013) The biological chemistry of hydrogen peroxide. *Methods Enzymol.* **528**, 3-25

4. Brewer, T. F.; Garcia, F. J.; Onak, C. S.; Carroll, K. S.; Chang, C. J. (2015) Chemical approaches to discovery and study of sources and targets of hydrogen peroxide redox signaling through NADPH oxidase proteins. *Annu. Rev. Biochem.* **84**, 765-790

5. Winterbourn, C. C. (2008) Reconciling the chemistry and biology of reactive oxygen species. *Nat. Chem. Biol.* **4** (5), 278-286

6. Rhee, S. G.; Bae, Y. S.; Lee, S. R.; Kwon, J. (2000) Hydrogen peroxide: a key messenger that modulates protein phosphorylation through cysteine oxidation. *Sci. STKE.* **2000** (53), E1

7. Tonks, N. K. (2005) Redox redux: revisiting PTPs and the control of cell signaling. *Cell* **121** (5), 667-670

8. Bae, Y. S.; Kang, S. W.; Seo, M. S.; Baines, I. C.; Tekle, E.; Chock, P. B.; Rhee, S. G. (1997) Epidermal growth factor (EGF)-induced generation of hydrogen peroxide. Role in EGF receptor-mediated tyrosine phosphorylation. *J. Biol. Chem.* **272** (1), 217-221

9. Goldman, R.; Moshonov, S.; Zor, U. (1998) Generation of reactive oxygen species in a human keratinocyte cell line: role of calcium. *Arch. Biochem. Biophys.* **350** (1), 10-18

10. Lee, S. R.; Kwon, K. S.; Kim, S. R.; Rhee, S. G. (1998) Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor. *J. Biol. Chem.* **273** (25), 15366-15372

11. Milenkovic, M.; De, D., X; Jin, L.; De Felice, M.; Di Lauro, R.; Dumont, J. E.; Corvilain, B.; Miot, F. (2007) Duox expression and related H2O2 measurement in mouse thyroid: onset in embryonic development and regulation by TSH in adult. *J. Endocrinol.* **192** (3), 615-626

12. Lambeth, J. D.; Neish, A. S. (2014) Nox enzymes and new thinking on reactive oxygen: a double-edged sword revisited. *Annu. Rev. Pathol.* **9**, 119-145

13. Grasberger, H.; Refetoff, S. (2006) Identification of the maturation factor for dual oxidase. Evolution of an eukaryotic operon equivalent. *J. Biol. Chem.* **281** (27), 18269-18272

14. Donko, A.; Peterfi, Z.; Sum, A.; Leto, T.; Geiszt, M. (2005) Dual oxidases. *Philos. Trans. R. Soc. Lond B Biol. Sci.* **360** (1464), 2301-2308

15. Morand, S.; Ueyama, T.; Tsujibe, S.; Saito, N.; Korzeniowska, A.; Leto, T. L. (2009) Duox maturation factors form cell surface complexes with Duox affecting the specificity of reactive oxygen species generation. *FASEB J.* **23** (4), 1205-1218

16. Kwon, J.; Shatynski, K. E.; Chen, H.; Morand, S.; De, D., X; Miot, F.; Leto, T. L.; Williams, M. S. (2010) The nonphagocytic NADPH oxidase Duox1 mediates a positive feedback loop during T cell receptor signaling. *Sci. Signal* **3** (133), ra59

17. Donko, A.; Ruisanchez, E.; Orient, A.; Enyedi, B.; Kapui, R.; Peterfi, Z.; De, D., X; Benyo, Z.; Geiszt, M. (2010) Urothelial cells produce hydrogen peroxide through the activation of Duox1. *Free Radic. Biol. Med.* **49** (12), 2040-2048

18. Gitler, C.; Zarmi, B.; Kalef, E.; Meller, R.; Zor, U.; Goldman, R. (2002) Calcium-dependent oxidation of thioredoxin during cellular growth initiation. *Biochem. Biophys. Res. Commun.* **290** (2), 624-628

19. Nagy, P.; Karton, A.; Betz, A.; Peskin, A. V.; Pace, P.; O'Reilly, R. J.; Hampton, M. B.; Radom, L.; Winterbourn, C. C. (2011) Model for the exceptional reactivity of peroxiredoxins 2 and 3 with hydrogen peroxide: a kinetic and computational study. *J. Biol. Chem.* **286** (20), 18048-18055

20. Nagy, P. (2013) Kinetics and mechanisms of thiol-disulfide exchange covering direct substitution and thiol oxidation-mediated pathways. *Antioxid. Redox. Signal.* **18** (13), 1623-1641

21. Lee, W.; Choi, K. S.; Riddell, J.; Ip, C.; Ghosh, D.; Park, J. H.; Park, Y. M. (2007) Human peroxiredoxin 1 and 2 are not duplicate proteins: the unique presence of CYS83 in Prx1 underscores the structural and functional differences between Prx1 and Prx2. *J. Biol. Chem.* **282** (30), 22011-22022

22. Jin, H.; Heller, D. A.; Kalbacova, M.; Kim, J. H.; Zhang, J.; Boghossian, A. A.; Maheshri, N.; Strano, M. S. (2010) Detection of single-molecule H2O2 signalling

from epidermal growth factor receptor using fluorescent single-walled carbon nanotubes. *Nat. Nanotechnol.* **5** (4), 302-309

23. Dickinson, B. C.; Huynh, C.; Chang, C. J. (2010) A palette of fluorescent probes with varying emission colors for imaging hydrogen peroxide signaling in living cells. *J. Am. Chem. Soc.* **132** (16), 5906-5915

24. Paulsen, C. E.; Truong, T. H.; Garcia, F. J.; Homann, A.; Gupta, V.; Leonard, S. E.; Carroll, K. S. (2012) Peroxide-dependent sulfenylation of the EGFR catalytic site enhances kinase activity. *Nat. Chem. Biol.* **8** (1), 57-64

25. Sumimoto, H. (2008) Structure, regulation and evolution of Nox-family NADPH oxidases that produce reactive oxygen species. *FEBS J.* **275** (13), 3249-3277

26. De, D., X; Corvilain, B.; Dumont, J. E.; Miot, F. (2014) Roles of DUOX-mediated hydrogen peroxide in metabolism, host defense, and signaling. *Antioxid. Redox. Signal.* **20** (17), 2776-2793

27. Geiszt, M.; Witta, J.; Baffi, J.; Lekstrom, K.; Leto, T. L. (2003) Dual oxidases represent novel hydrogen peroxide sources supporting mucosal surface host defense. *FASEB J.* **17** (11), 1502-1504

28. Bae, Y. S.; Choi, M. K.; Lee, W. J. (2010) Dual oxidase in mucosal immunity and host-microbe homeostasis. *Trends Immunol.* **31** (7), 278-287

29. Hirakawa, S.; Saito, R.; Ohara, H.; Okuyama, R.; Aiba, S. (2011) Dual Oxidase 1 Induced by Th2 Cytokines Promotes STAT6 Phosphorylation via Oxidative Inactivation of Protein Tyrosine Phosphatase 1B in Human Epidermal Keratinocytes. *J. Immunol.* **186** (8), 4762-4770

30. Miller, E. W.; Dickinson, B. C.; Chang, C. J. (2010) Aquaporin-3 mediates hydrogen peroxide uptake to regulate downstream intracellular signaling. *Proc. Natl. Acad. Sci. U. S. A* **107** (36), 15681-15686

31. Yang, X. H.; Man, X. Y.; Cai, S. Q.; Yao, Y. G.; Bu, Z. Y.; Zheng, M. (2006) Expression of VEGFR-2 on HaCaT cells is regulated by VEGF and plays an active role in mediating VEGF induced effects. *Biochem. Biophys. Res. Commun.* **349** (1), 31-38

32. Wraight, C. J.; Werther, G. A. (1995) Insulin-like growth factor-I and epidermal growth factor regulate insulin-like growth factor binding protein-3 (IGFBP-3) in the human keratinocyte cell line HaCaT. *J. Invest Dermatol.* **105** (4), 602-607

33. Lu, J.; Holmgren, A. (2014) The thioredoxin antioxidant system. *Free Radic. Biol. Med.* **66**, 75-87

34. Sobotta, M. C.; Liou, W.; Stocker, S.; Talwar, D.; Oehler, M.; Ruppert, T.; Scharf, A. N.; Dick, T. P. (2015) Peroxiredoxin-2 and STAT3 form a redox relay for H2O2 signaling. *Nat. Chem. Biol.* **11** (1), 64-70

FIGURE LEGENDS

Fig.1. **Analysis of EGF induced hydrogen peroxide production and Nox/Duox expression in keratinocyte cell lines.** Time course of changes in AmplexRed fluorescence in control or EGF (500 ng/ml) stimulated, adherent A431 (A) and HaCaT (B) cells. These are representative results of at least 3 similar experiments. (C) Quantitative PCR analysis of the expression of NADPH oxidase family components in A431 and HaCaT cells. Expression values are normalized to actinB expression. Similar results were measured from 3 different A431 and HaCaT RNA samples. (D) HaCaT and A431 cells were transfected with 20 nM scrambled/Duox1 stealth siRNA and harvested 48 hours later. Cell lysates were processed for Duox1 and actin western blots. Efficient knock-down of Duox1 in A431 and HaCaT cells was verified by Duox1 western blot in more than four independent siRNA transfections.

Fig. 2. **Analysis of the role of intracellular calcium on the production of hydrogen peroxide in A431 and HaCaT cells.** A431 (A) and HaCaT (B) cells were incubated in the presence or absence of 50 μM BAPTA-AM for 10 minutes at 37 °C before stimulating with 500 ng/ml EGF for 10 minutes in the presence of AmplexRed (AR) and horseradish peroxidase (HRP). (C) Time course of AR assay following thapsigargin (1 μM) stimulus in A431 cells. (D) Time course of AR assay in HaCaT cells stimulated with thapsigargin, ATPγS, niacin (vitamin B3, 100 μM), and the TRPV4 agonist GSK1016790A (100 nM).

Fig. 3. **Effect of Duox1 as well as DuoxA1 specific siRNA treatment on the hydrogen peroxide production in EGF and thapsigargin treated cells.** HaCaT (A-C) or A431 (B-D) cells

were transfected with Duox1 (A and B) or DuoxA1 (C and D) siRNA along with control cells transfected with scrambled siRNA. 48 hours after transfection the AmplexRed fluorescence was measured in control, EGF and thapsigargin stimulated cells in the presence of extracellular horseradish peroxidase. The cells were stimulated for 20 minutes at 37 °C. In (A) the average of four independent experiments is shown +/- SEM, in (B-D) one representative experiment of two similar ones is displayed with three parallels +/- SD.

Fig. 4. **Duox1 dependent redox changes of thioredoxin-1 and peroxiredoxin-1 and -2.** (A) siRNA treated HaCaT cells were stimulated with thapsigargin (1 μM), ATPγS (10 μM) or 500 ng/ml EGF for 5 minutes at 37°C and then lysed in the presence of 200 μM of Biotin-PEG_{11}-maleimide (BPM) and processed for Trx1 western blot. The maleimide reagent reacts with the reduced form of Trx1 causing a significant weight shift. This experiment was repeated twice with identical results. (B) siRNA treated HaCaT cells were stimulated with 1 μM thapsigargin for five minutes then alkylated by incubating with 80mM methyl-methane thiosulfonate (MMTS) for 10 minutes. Cells were lysed and processed for Prx1, Prx2 and Prx3 western blots. The effect of 30 μM thioredoxin reductase inhibitor DNCB (2,4-dinitrochlorobenzene) treatment on the Prx1 dimer and oligomer formation is also displayed. (C) the statistical analysis of 5 independent experiments of peroxiredoxin dimer formation is displayed (paired t-tests). Columns indicate the average values for each condition, error bars show standard error of mean (SEM) values. (D) HaCaT cells were stimulated with thapsigargin in the presence of 20 μg/ml lactoperoxidase (LPO) and 1mM thiocyanate or thiocyanate only and then processed for Trx1 western blot as described above. This western blot is representative of three experiments with similar results. (E) HaCaT cells were stimulated with thapsigargin in the presence of 600 or 1200 U/ml catalase and then processed for Trx1 western blot as described above. This experiment was repeated twice with similar results.

Supplementary Fig. 1. **EGF-evoked tyrosine phosphorylation response and Duox expression in different cell lines. A)** A431, HaCaT, HeLa, A549 and A2780 cells were stimulated with 100 ng/ml EGF for 5 minutes at 37 °C. Cells were lysed and harvested on ice in the presence of protease and phosphatase inhibitors and processed for phosphotyrosine western blot. **B)** Quantitative PCR analysis of the expression of DuoxA1, Duox1, Duox2 in five different cell lines. Expression values are normalized to actinB expression.

Supplementary Fig. 2. **EGF- and thapsigargin-elicited calcium signals in A431 and HaCaT cells.** A431 and HaCaT cells were grown on coverslips for 48 hours. The cells were then loaded with fura2-AM and ratiometric fluorescence intensity measurements were carried out. Images were taken every 5 seconds. The black curve indicates the average of 64 (A431) and 28 (HaCaT) region of interests (ROIs) from the same field of view. The six coloured lines indicate the fluorescence of individual ROIs. EGF (500 ng/ml) and thapsigargin (1 μM) were added at the indicated times.

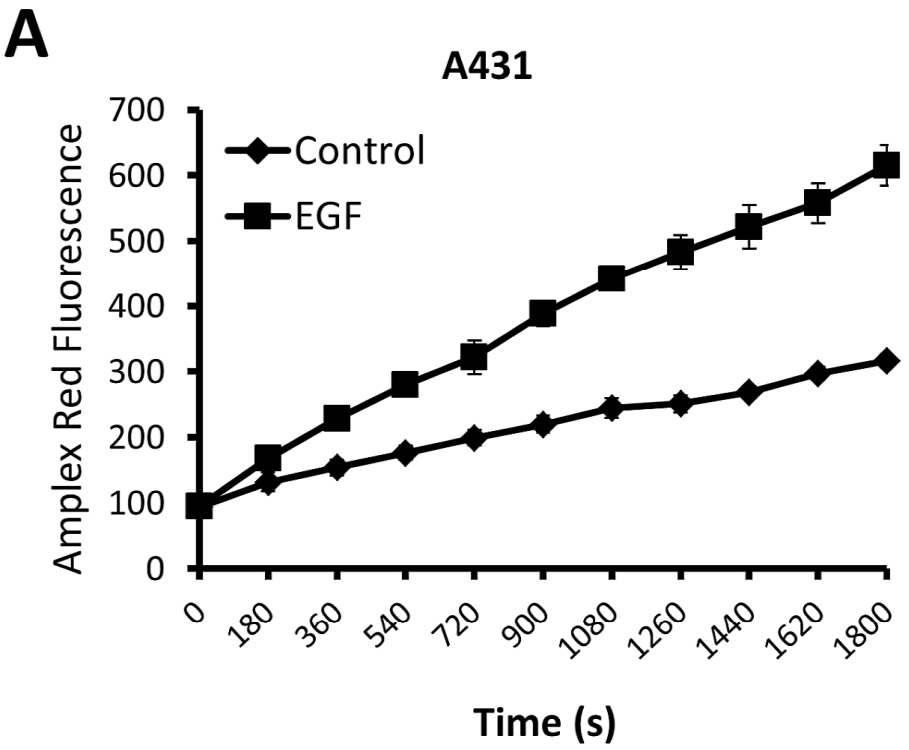
Supplementary Fig. 3. **EGF and thapsigargin elicited calcium signals in Duox1 or scrambled siRNA transfected A431 and HaCaT cells.** HaCaT (A, B) and A431 (C, D) cells were treated with scrambled (A, C) or Duox1 specific (B, D) siRNA for 48 hours. The cells were then loaded with fura2-AM and ratiometric fluorescence intensity measurements were carried out. Images were taken every 5 seconds and the average intensity of at least 30 region of interests (ROIs) is displayed at every timepoint. EGF (500 ng/ml) and thapsigargin (1 μM) were added at the indicated times.

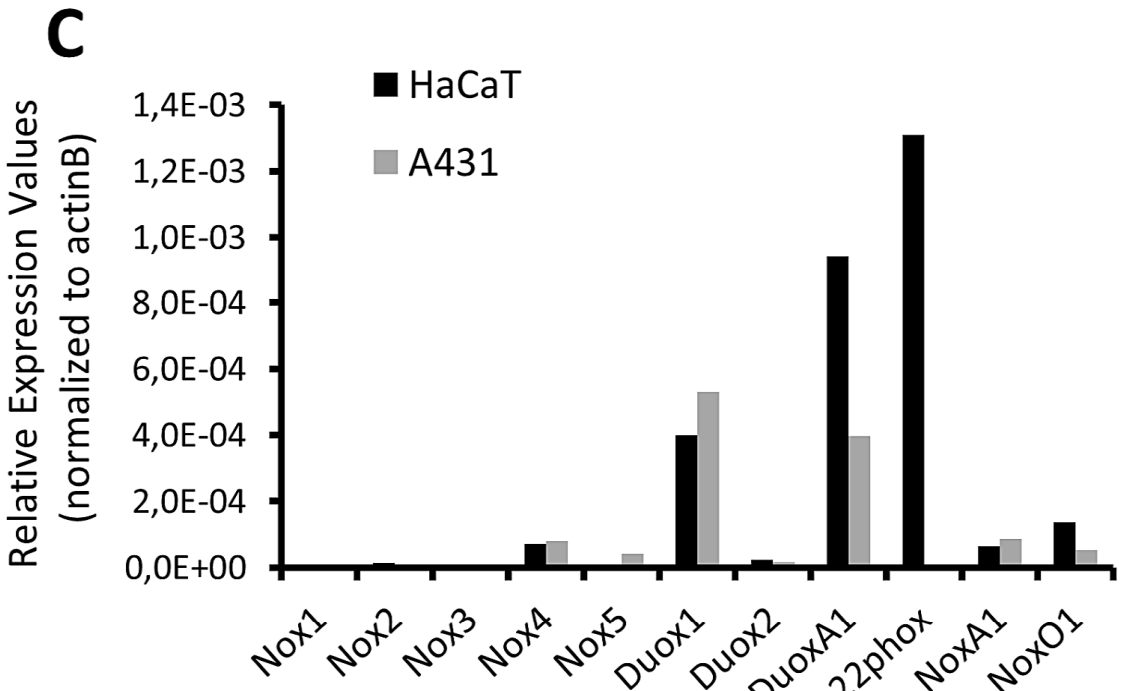
Supplementary Fig. 4. **Time course of tyrosine phosphorylation in EGF stimulated HaCaT cells following Duox1 or scrambled siRNA treatment.** 48 hrs after siRNA transfection 500

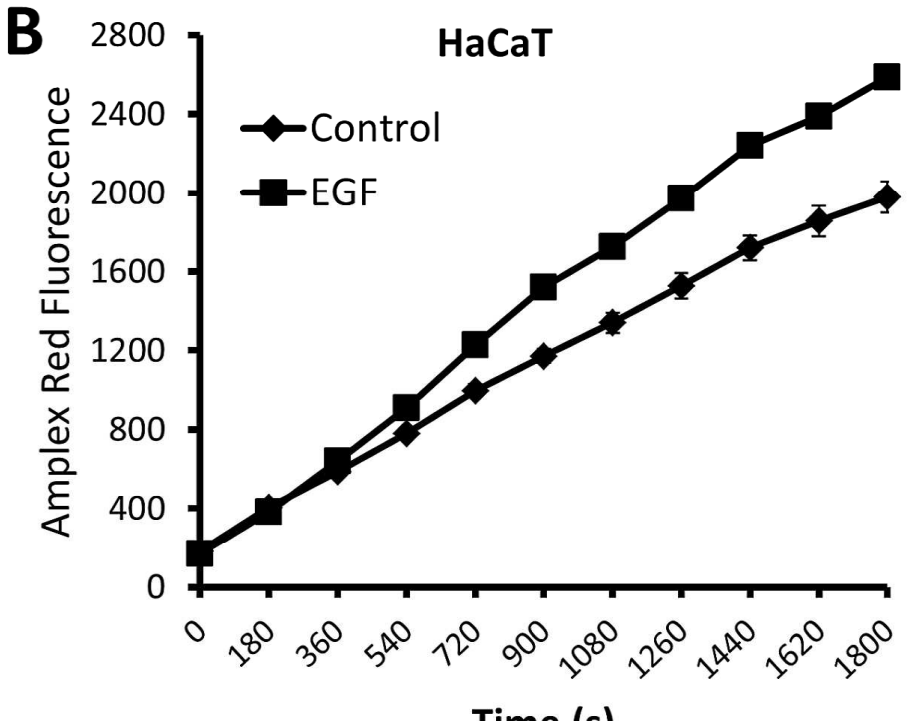
ng/ml EGF was added for the indicated times. Cells were lysed and harvested on ice in the presence of protease and phosphatase inhibitors and processed for the indicated western blots.

Supplementary Fig. 5. **Changes in thioredoxin-1 oxidation upon treatment of cells with various concentrations of extracellular hydrogen peroxide.** HaCaT cells were treated with the indicated concentrations of hydrogen peroxide for 5 minutes at 37 °C then lysed in the presence of 200 μM of Biotin-PEG_{11}-maleimide (BPM) and processed for Trx1 Western blot. This figure is a representative of three experiments with similar results.

Fig. 1.


A
A431
Amplex Red Fluorescence
Control
EGF
Time (s)


C
HaCaT
A431
Relative Expression Values (normalized to actinB)
Nox1
Nox2
Nox3
Nox4
Nox5
Duox1
Duox2
DuoxA1
p22phox
NoxA1
NoxO1


B
HaCaT
Amplex Red Fluorescence
Control
EGF

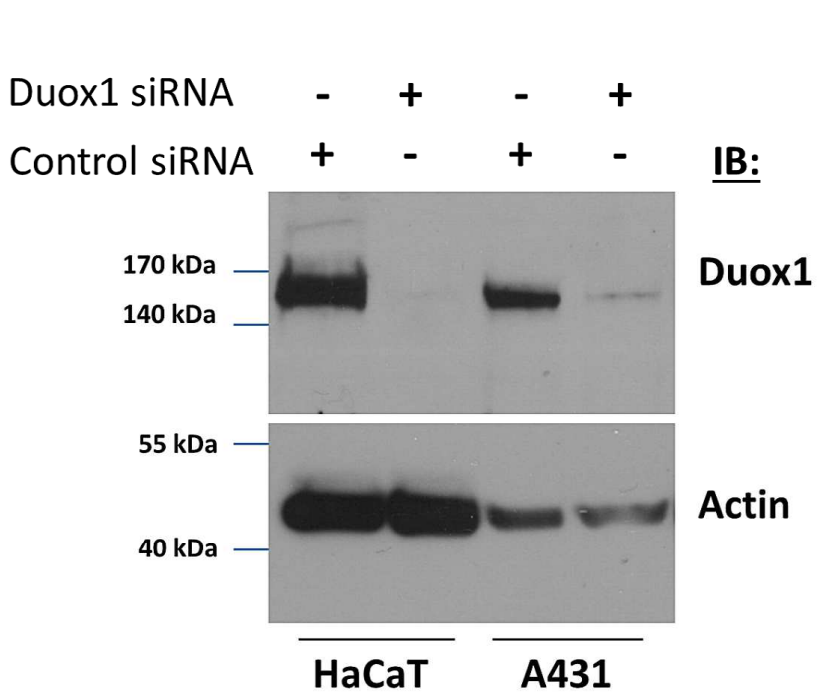
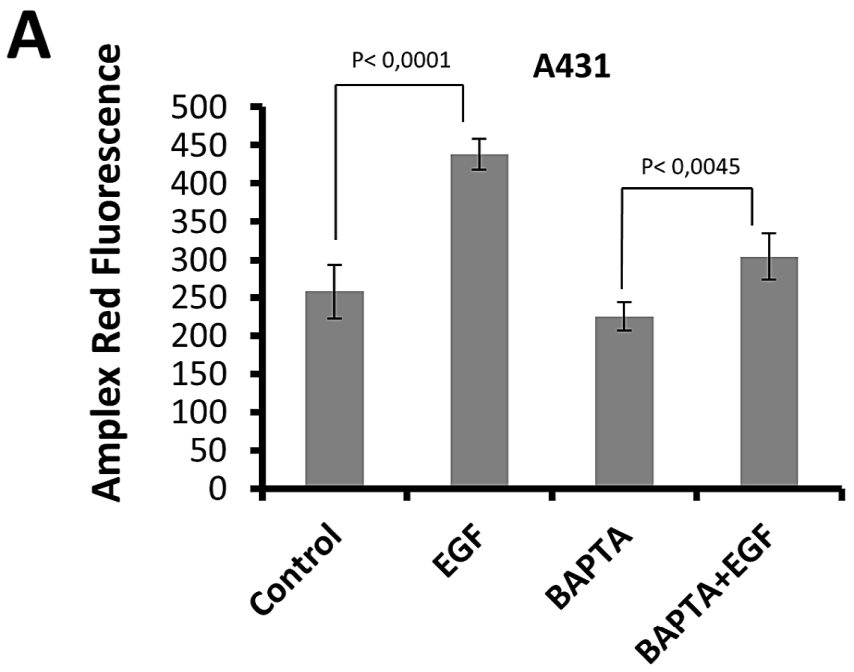
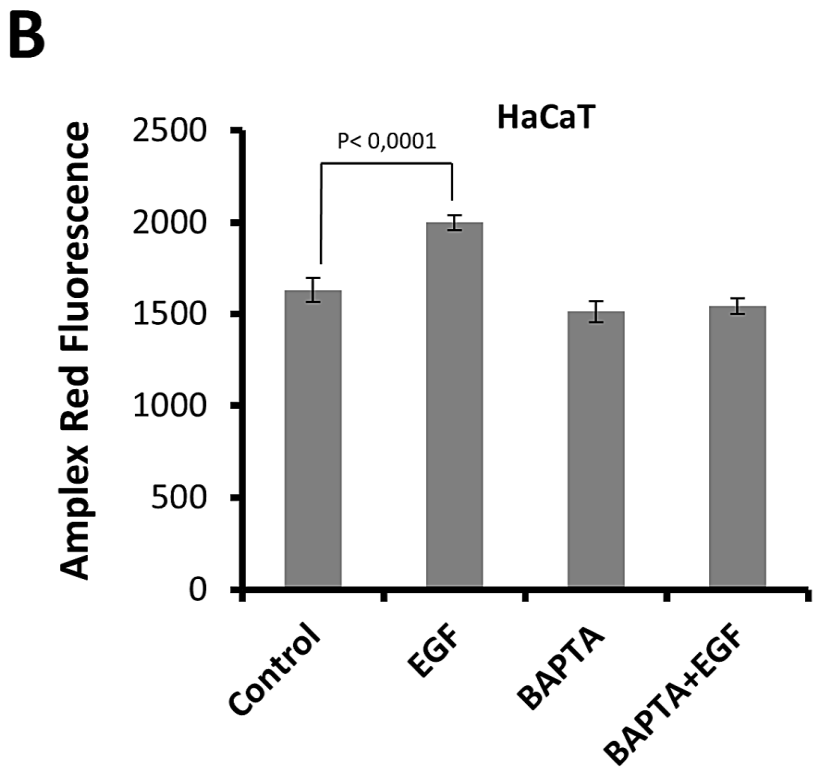
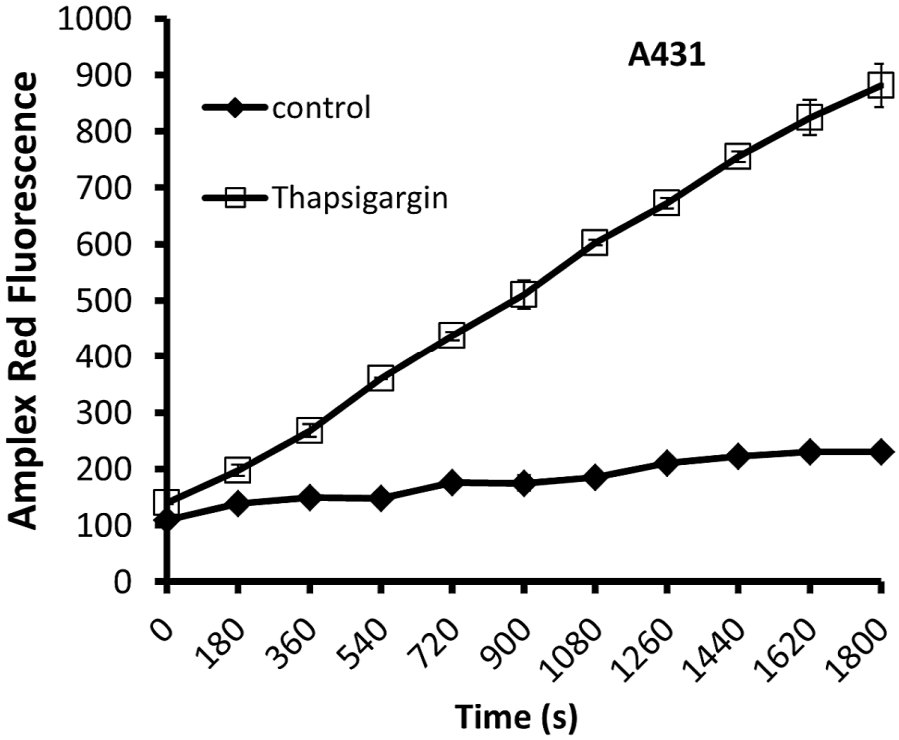

D
Duox1 siRNA
Control siRNA
IB:
170 kDa
140 kDa
Duox1
55 kDa
40 kDa
Actin
HaCaT
A431

Fig. 2.


A
A431
P< 0,0001
P< 0,0045
Amplex Red Fluorescence
500
450
400
350
300
250
200
150
100
50
0
Control
EGF
BAPTA
BAPTA+EGF


B
HaCaT
P< 0,0001
Amplex Red Fluorescence
2500
2000
1500
1000
500
0
Control
EGF
BAPTA
BAPTA+EGF


C
A431
control
Thapsigargin
Amplex Red Fluorescence
1000
900
800
700
600
500
400
300
200
100
0
0
180
360
540
720
900
1080
1260
1440
1620
1800
Time (s)

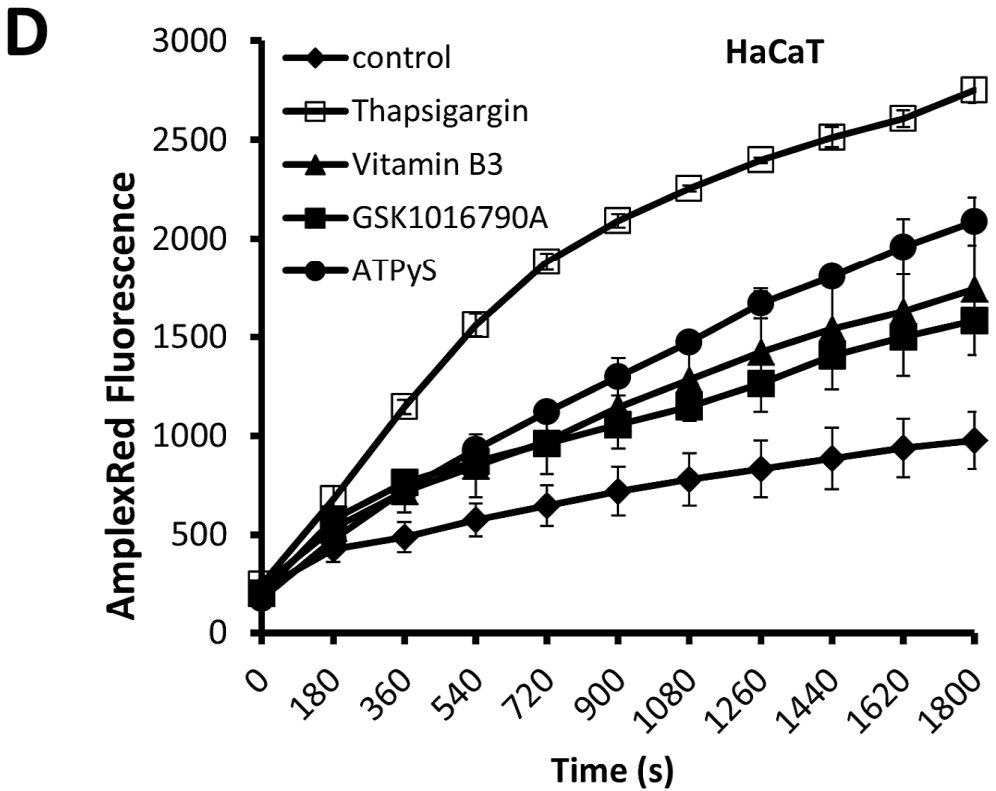

D
HaCaT
control
Thapsigargin
Vitamin B3
GSK1016790A
ATPγS
AmplexRed Fluorescence
3000
2500
2000
1500
1000
500
0
0
180
360
540
720
900
1080
1260
1440
1620
1800
Time (s)

Fig. 3.

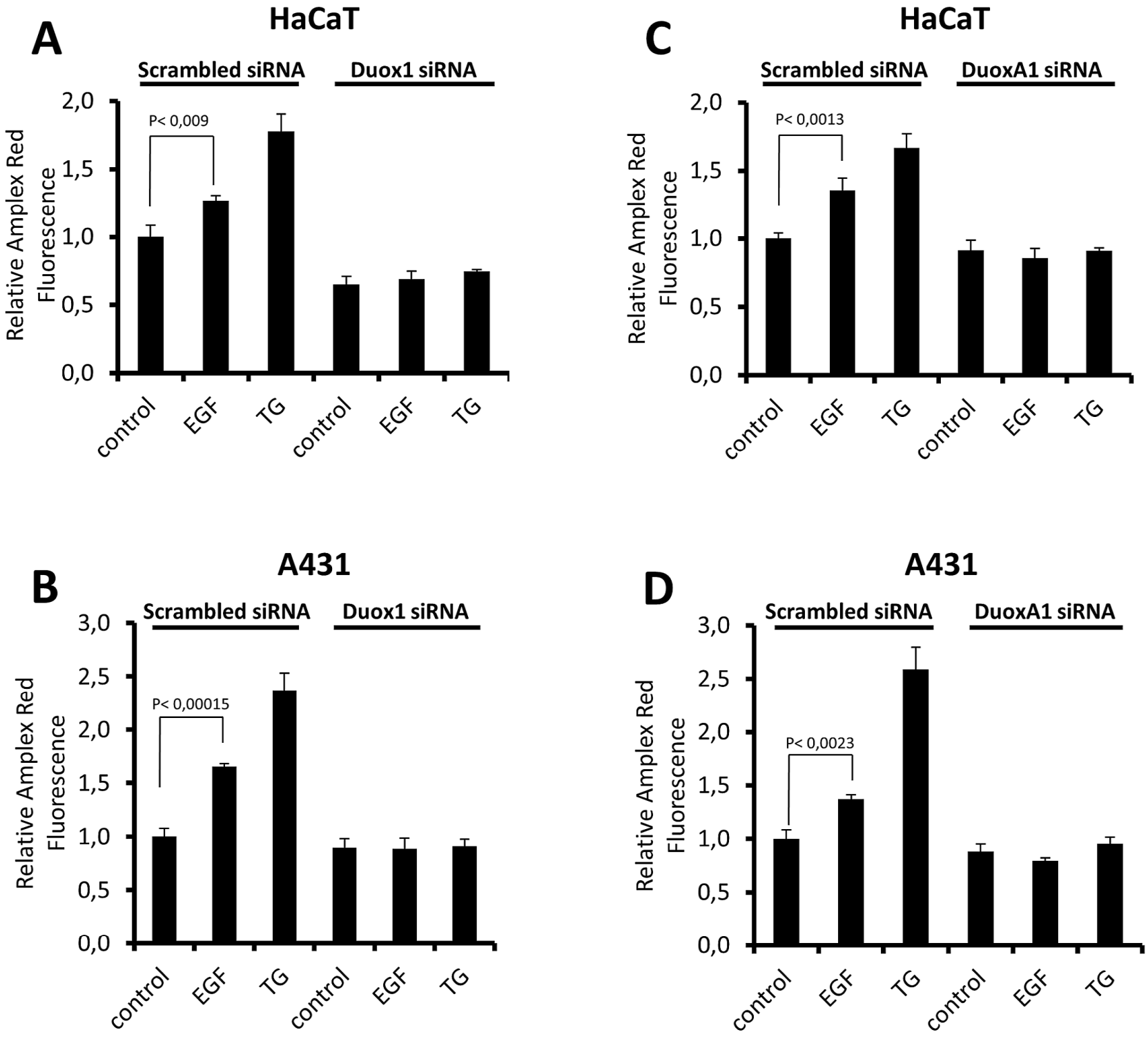
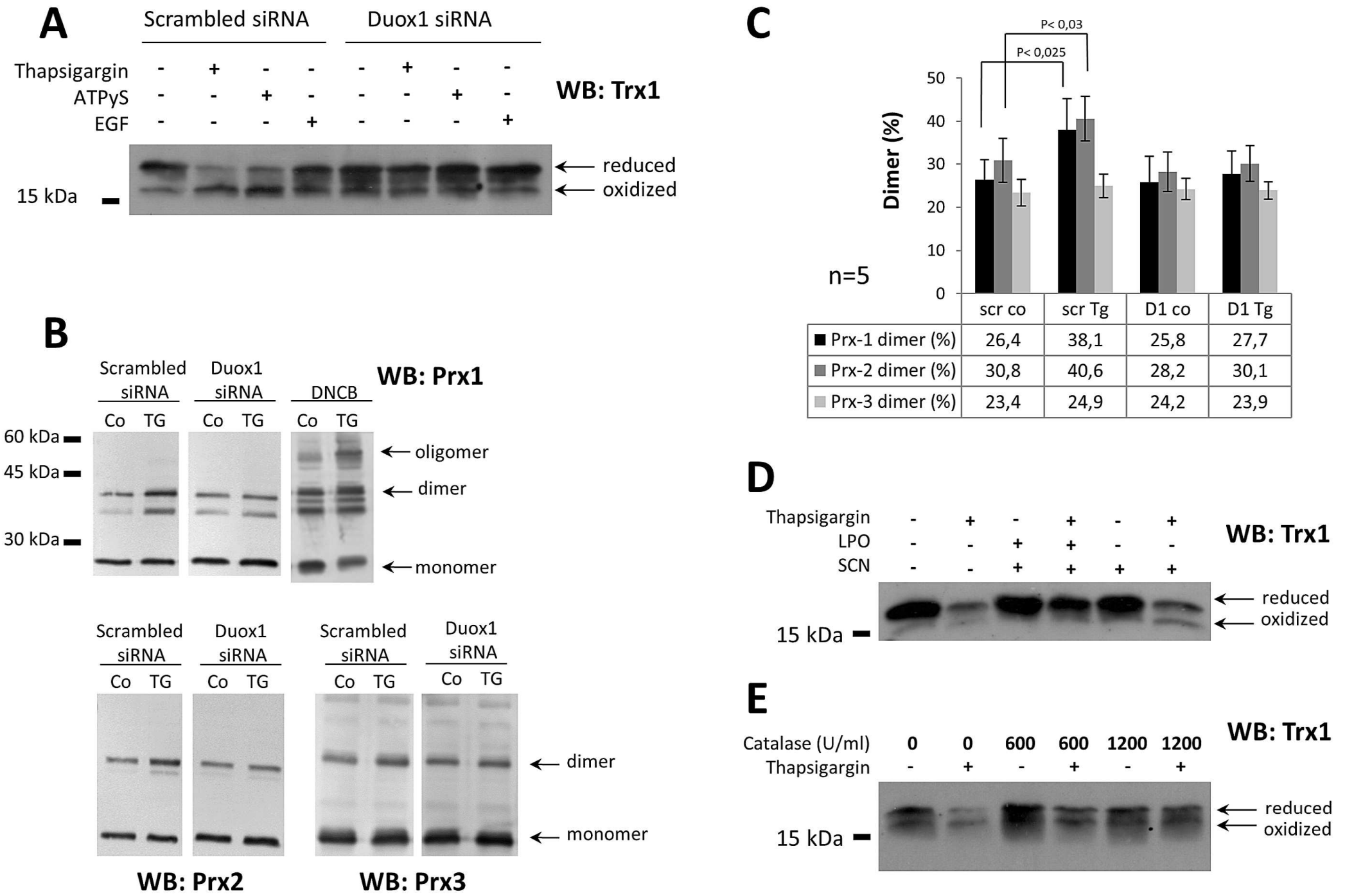
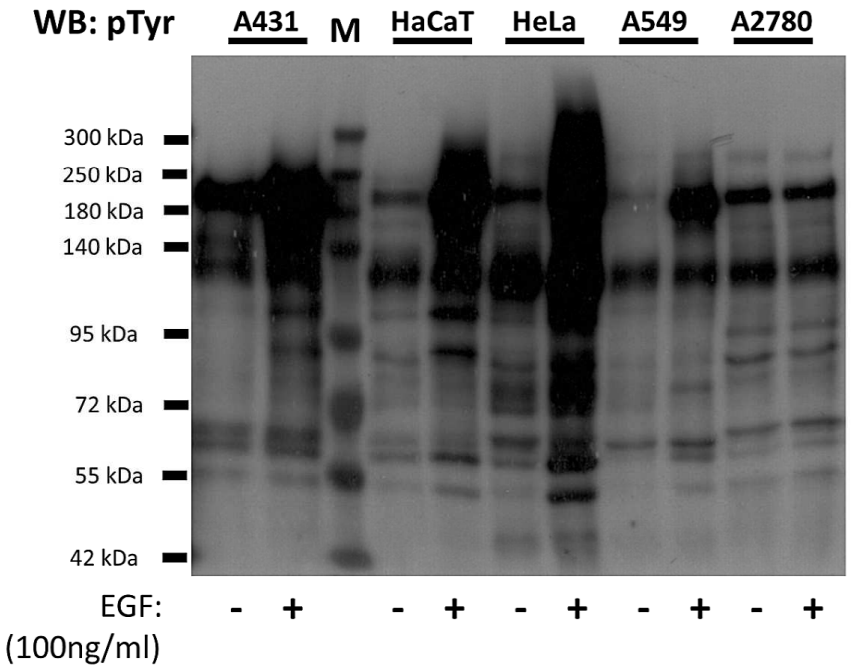

A
HaCaT
Scrambled siRNA
Duox1 siRNA
P< 0,009
Relative Amplex Red Fluorescence
2,0
1,5
1,0
0,5
0,0
control
EGF
TG
control
EGF
TG
C
HaCaT
Scrambled siRNA
DuoxA1 siRNA
P< 0,0013
Relative Amplex Red Fluorescence
2,0
1,5
1,0
0,5
0,0
control
EGF
TG
control
EGF
TG
B
A431
Scrambled siRNA
Duox1 siRNA
P< 0,00015
Relative Amplex Red Fluorescence
3,0
2,5
2,0
1,5
1,0
0,5
0,0
control
EGF
TG
control
EGF
TG
D
A431
Scrambled siRNA
DuoxA1 siRNA
P< 0,0023
Relative Amplex Red Fluorescence
3,0
2,5
2,0
1,5
1,0
0,5
0,0
control
EGF
TG
control
EGF
TG

Fig. 4.

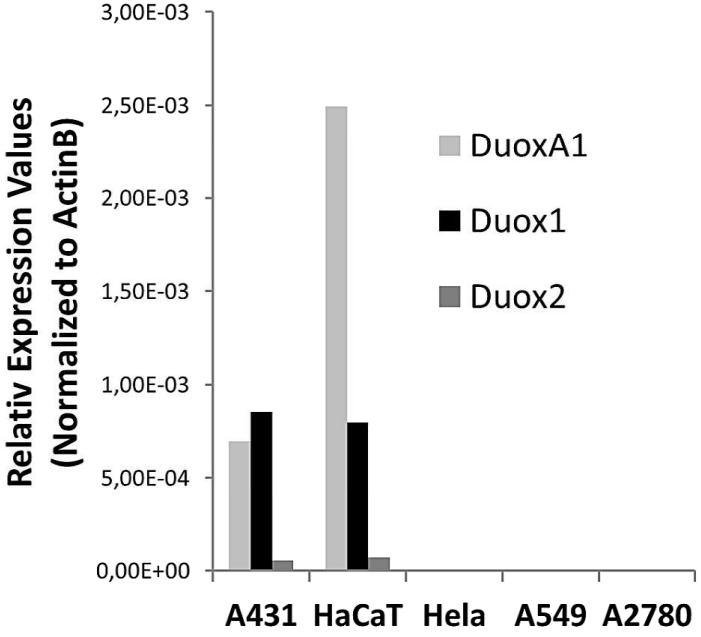

A
Scrambled siRNA
Duox1 siRNA
Thapsigargin
ATPyS
EGF
WB: Trx1
15 kDa
reduced
oxidized
B
Scrambled siRNA
Duox1 siRNA
DNCB
Co TG
WB: Prx1
60 kDa
45 kDa
30 kDa
oligomer
dimer
monomer
WB: Prx2
WB: Prx3
C
P< 0,03
P< 0,025
Dimer (%)
n=5
scr co
scr Tg
D1 co
D1 Tg
Prx-1 dimer (%) 26,4 38,1 25,8 27,7
Prx-2 dimer (%) 30,8 40,6 28,2 30,1
Prx-3 dimer (%) 23,4 24,9 24,2 23,9
D
Thapsigargin
LPO
SCN
WB: Trx1
15 kDa
reduced
oxidized
E
Catalase (U/ml) 0 0 600 600 1200 1200
Thapsigargin
WB: Trx1
15 kDa
reduced
oxidized

Suppl. Fig. 1.

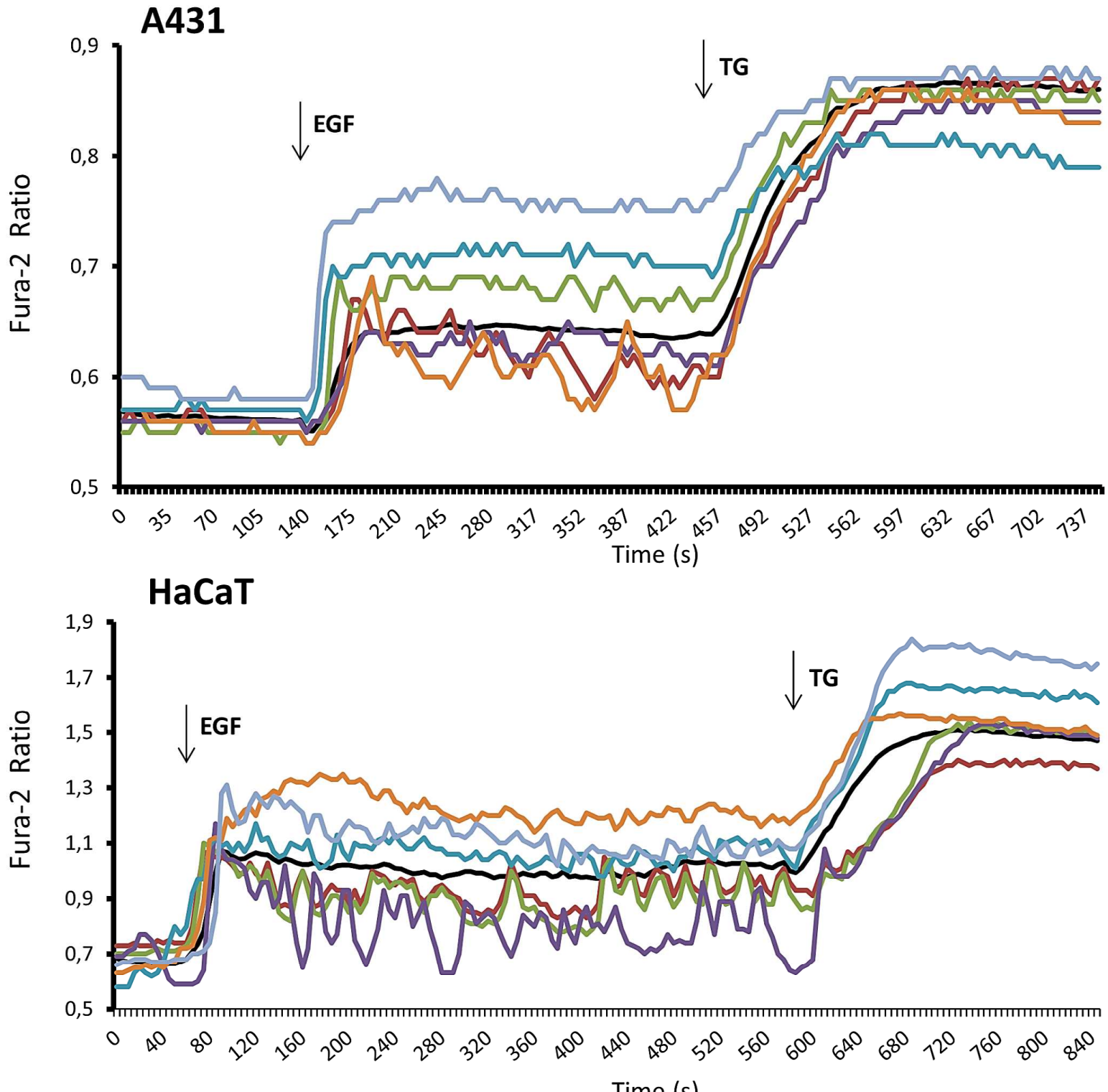
A



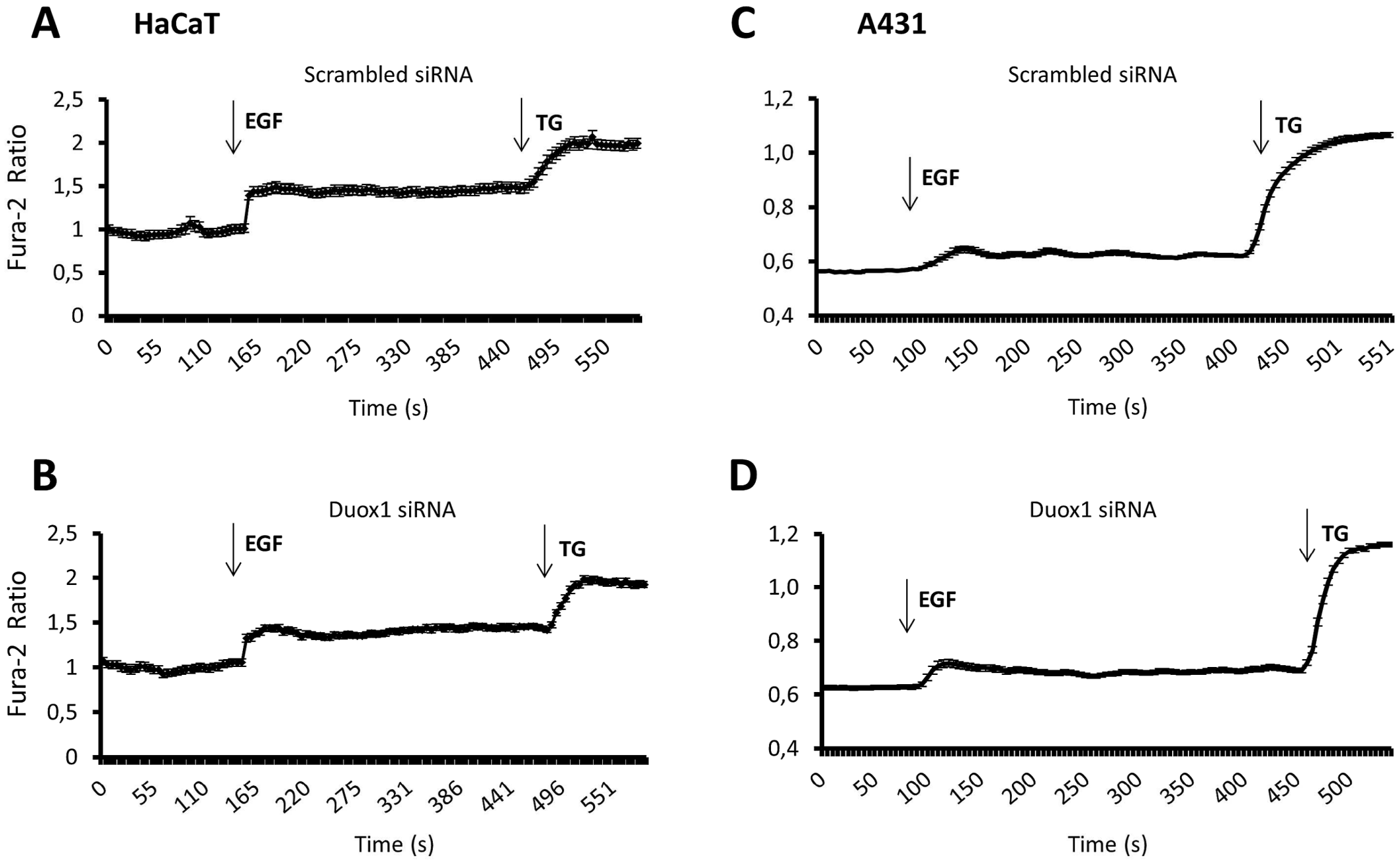
B



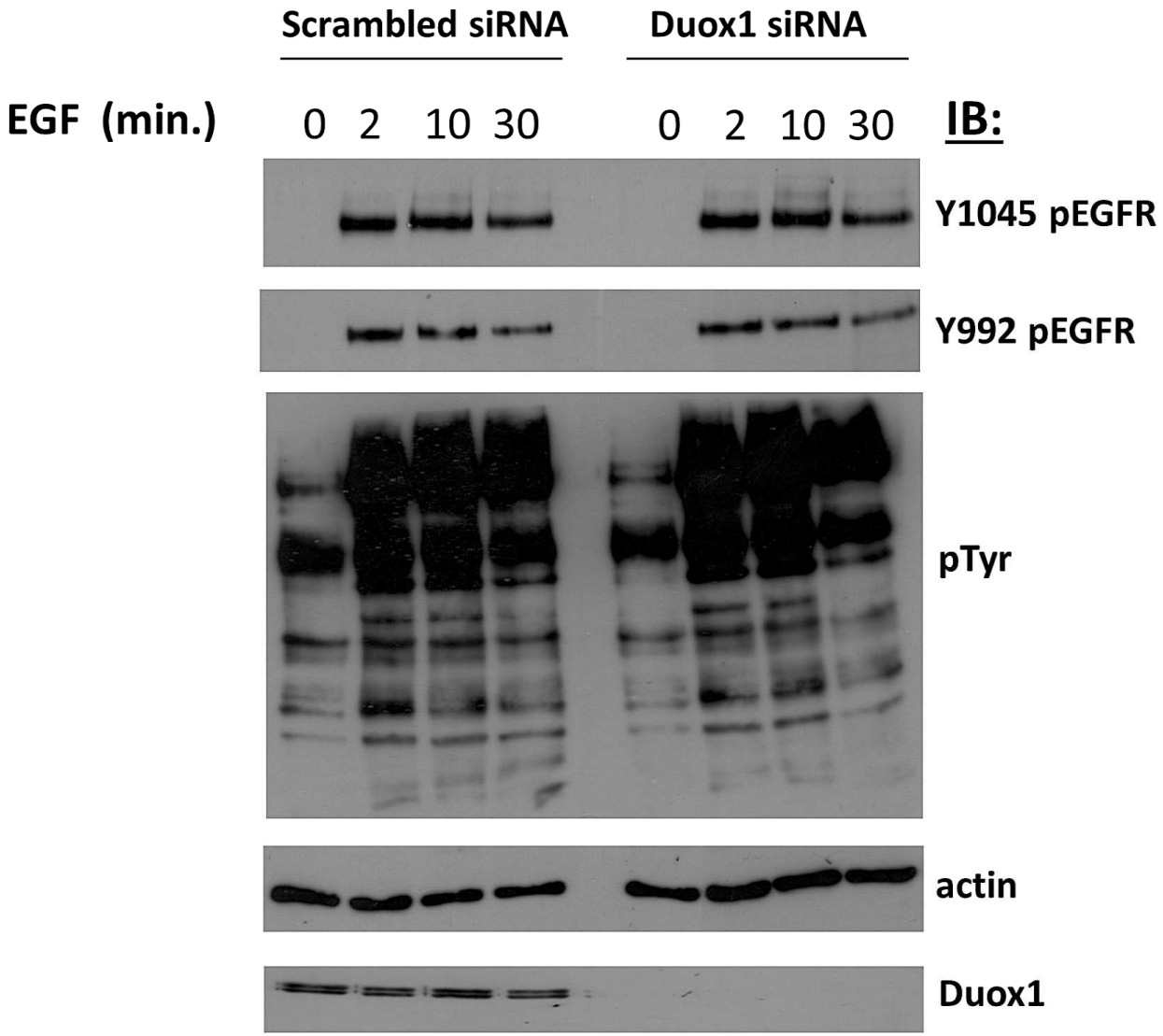
Suppl. Fig. 2.


A431
Fura-2 Ratio
0,9
0,8
0,7
0,6
0,5
EGF
TG
0 35 70 105 140 175 210 245 280 317 352 387 422 457 492 527 562 597 632 667 702 737
Time (s)
HaCaT
Fura-2 Ratio
1,9
1,7
1,5
1,3
1,1
0,9
0,7
0,5
EGF
TG
0 40 80 120 160 200 240 280 320 360 400 440 480 520 560 600 640 680 720 760 800 840
Time (s)

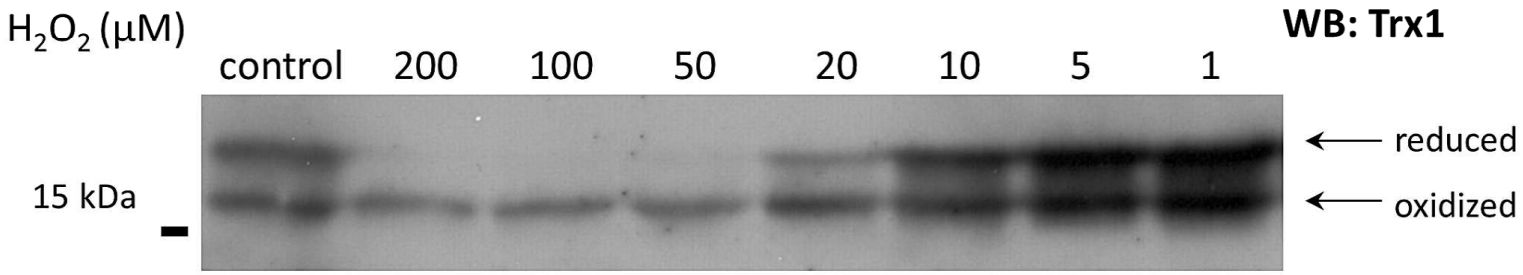
Suppl. Fig. 3.


A
HaCaT
Scrambled siRNA
EGF
TG
Fura-2 Ratio
Time (s)
B
Duox1 siRNA
C
A431
D

Suppl. Fig. 4.


Scrambled siRNA
Duox1 siRNA
EGF (min.)
0 2 10 30
0 2 10 30
IB:
Y1045 pEGFR
Y992 pEGFR
pTyr
actin
Duox1

Suppl. Fig. 5.


H2O2 (μM)
control
200
100
50
20
10
5
1
WB: Trx1
15 kDa
reduced
oxidized