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Copper influx transporter 1 is required for FGF, PDGF and EGF-induced MAPK signaling

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Abstract

Copper transporter 1 (CTR1) is the major copper (Cu) influx transporter in mammalian cells. We report here that CTR1 is required for the activation of signaling to the MAPK pathway by the ligands of three major receptor tyrosine kinases (RTK) including FGF, PDGF and EGF. Induction of Erk1/2 phosphorylation was compared in isogenic wild type $CTR1^{+/+}$ and $CTR1^{-/-}$ cells. Whereas all three ligands increased pErk1/2 in the $CTR1^{+/+}$ cells, they failed to do this in $CTR1^{-/-}$ cells. While FGF did not enhance the phosphorylation of AKT in the $CTR1^{+/+}$ cells, both PDGF and EGF increased pAKT in the $CTR1^{+/+}$ but not $CTR1^{-/-}$ cells. The deficit in Erk1/2 phosphorylation in the $CTR1^{-/-}$ cells was rescued by adding Cu to the medium, and it was induced in $CTR1^{+/+}$ cells by treatment with a Cu chelator. Intracellular Cu availability was reduced in the $CTR1^{-/-}$ cells as reflected by increased expression of the Cu chaperone CCS. The failure of RTK-induced signaling to both Erk1/2 and AKT suggested the presence of a Cu-dependent step upstream of Ras. The Cu-dependent enzyme SOD1 is responsible for generating the hydrogen peroxide in response to RTK activation that serves to inhibit phosphatases that normally limit RTK signaling. SOD1 activity was reduced by a factor of 17-fold in the $CTR1^{-/-}$ cells, and addition of hydrogen peroxide restored signaling. We conclude that Cu acquired from CTR1 is required for signaling in pathways regulated by RTKs that play major roles in development and cancer.

Keywords

CTR1; SOD1; copper; receptor tyrosine kinase

1. Introduction

Superoxide is known to play central roles in receptor tyrosine kinase (RTK)[1] signaling. The activation of an RTK by its cognate ligand results in the rapid generation of superoxide by NADPH oxidase situated either in the plasma or organelle membranes. Superoxide in the cytoplasm is rapidly converted to oxygen and hydrogen peroxide (H_2O_2) by the Cu-requiring superoxide dismutase SOD1. H_2O_2 is then decomposed to water and oxygen by

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catalase. For many years, H_2O_2 was viewed as the inevitable but unwanted by-product of an aerobic existence. Given the damage potentially inflicted by H_2O_2, it was assumed that the faster the elimination of this toxic waste, the better for the cell. However, it has now been shown that mammalian cells produce H_2O_2 to regulate signaling in pathways important to cell proliferation, differentiation, and migration [1]. The H_2O_2 generated by NADPH oxidase can inactivate protein phosphatases (PTPases) by reversibly oxidizing a cysteine in the active site, preventing de-phosphorylation of substrates activated by the RTKs and thereby amplifying their signal [2–4].

In order to generate H_2O_2, SOD1 requires Cu which it must obtain from the pool of Cu available in the cell that is highly regulated by chaperones such that the concentration of free Cu within the cell is $<10^{-18}$ M [5]. In most cells Cu enters this pool on the high affinity Cu influx transporter CTR1. Cu can also enter cells on one or more lower affinity transporters that have yet to be well characterized [6]. CTR1 is a highly conserved protein as demonstrated by the fact that both human and mouse CTR1 can rescue the growth defect produced by knocking out CTR1 in *S. cerevisiae* [7–9]. Human CTR1 contains 190 amino acids organized into three transmembrane domains, an N-terminal extracellular domain rich in methionines and histidines, a large intracellular loop, and a short intracellular C-terminal tail. Conserved methionine-containing motifs and individual methionines, histidines, and cysteines essential to Cu transporter function are found within the extracellular domain, in the second and third transmembrane domains, and in the C-terminal tail. CTR1 forms a homotrimer in the membrane, and structural studies suggest that it assembles into an inverted cone-shaped pore through which Cu(I) is transmitted from one side of the membrane to the other. Recent computational studies provide support for the importance of several of conserved residues in transporter function, particularly within the second transmembrane domain [10, 11].

The first evidence that CTR1 is important to RTK signaling was reported by Haremaki *et al.* [12] who found that CTR1 routed FGFR signaling during the formation of mesodermal structures in Xenopus embryo. They demonstrated that CTR1, the FGFR docking protein FRS2α and the Src-related kinase Laloo formed a complex, and that each of these components was required for FGFR-mediated activation of the RAS-MAPK kinase cascade. Turski *et al.* [13] recently reported that CTR1 regulates signaling from the insulin and FGF receptors in murine cells. They found that reduction of intracellular Cu accumulation imposed by loss of CTR1 resulted in impaired insulin and FGF-stimulated activation of Ras/MAPK signaling as evidenced by a reduction in Erk1/2 phosphorylation. We report here studies that confirm and extend the concept that CTR1 is required for MAPK signaling in response to exposure to not only FGF and insulin, but also PDGF and EGF via RTKs that are individually important for the development of cancer. The mechanism by which CTR1 exerts this effect is through its regulation of the availability of intracellular Cu.

2. Material and methods

2.1 Drug and Reagents

Mouse FGF basic and human EGF was purchased from Cell Signaling Technologies (Danvers, MA). Human PDGF-BB was purchased from eBioscience (San Diego, CA).

2.2 Cell types and Culture

Mouse embryonic fibroblasts containing wild type alleles of CTR1 ($CTR1^{+/+}$) and a line in which both copies of CTR1 had been somatically knocked out ($CTR1^{-/-}$) were graciously provided by Dr. Dennis Thiele [14]. Cells were maintained in DMEM supplemented with 10 % FBS, non-essential amino acids, sodium pyruvate, uridine and 2- mercaptoethanol at 37 °C, 5 % CO_2.

2.3 Immunoblot Analysis

Cells were harvested and lysed in ice-cold lysis buffer containing 50 mmol/LTris, 150 mmol/L NaCl, 0.1 % SDS, 0.5 % sodium deoxycholate, 1 % Triton X-100 and supplemented with the protease and phosphatase inhibitor cocktail (Thermo Scientific; Lorgan, UT). Lysates were subjected to protein content determination using a detergent-compatible protein assay kit, *DC*™ Protein Assay (Bio-Rad; Hercules, CA). Equal amounts of total protein were loaded onto SDS-polyacrylamide gels and electrotransferred to PVDF membranes. The blots were visualized and quantified using a Li-Cor Odyssey Imager (Li-Cor Biosciences). Sources of antibodies were as follows: monoclonal antibodies against total p44/42 MAPK (Erk1/2), phospho-AKT (Ser473) (Cell Signaling, Boston, MA); β-actin (Santa Cruz Biotechnology, Santa Cruz, CA); polyclonal antibodies against phospho-p44/42 (Erk1/2) (Thr202/Tyr204), phospho-Mek1/2 (Ser217/221) and total AKT (Cell Signaling, Boston, MA). Each experiment was repeated at least three times to generate sufficient data for accurate quantification and statistical analysis.

2.4 Superoxide Dismutase Assay

An SOD assay kit-WST was purchased from Dojindo Molecular Technologies (Rockville, MD) and used according to the manufacturer's instructions. One unit of SOD is defined as the amount of the enzyme in 20 μL of sample solution that inhibits the reduction reaction of WST-1 with superoxide anion by 50%. SOD1 activity was calculated by measuring the total SOD activity in the presence and absence of 1 mmol/L diethylditiocarbamate, and then subtracting the SOD2 activity from total SOD activity.

2.5 Superoxide Detection by Electron Paramagnetic Resonance (EPR)

Cells were cultured on cover slips, starved overnight and stimulated with 1 ng/mL FGF for 15 min. Then 10 μL of 70 mmol/L 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DEPMPO, Enzo Life Sciences; Farmingdale, NY) was added to cover slips which were placed into a glass tissue cell (GZ 170–5.0×0.5, Magnet Tech) introduced into the EPR cavity of a MiniScope MS200 Benchtop spectrometer (Magnet Tech) maintained at 37°C. Mixing of the spin trap DEPMPO with the employed media in the absence of cells did not yield any EPR signals. This confirmed that the observed EPR signals arise from cellular superoxide radical and are not due to redox cycling in the studied system. EPR signals that accumulated over 5min after mixing with substrates were quantified. Assignment of the observed signals from mitochondria was confirmed through computer-assisted spectral simulation using the WinSim software (http://epr.niehs.nih.gov/pest.html) and published spin parameters[15]. EPR signal amplitudes were quantified and normalized with total cell number.

2.6 Statistic Analysis

Graphpad software was used for statistical analysis. All experiments were repeated at least 3 times. Comparison of variance and mean values was performed using Student's *t*-test ($p<0.05$). If there were three or more experimental groups, comparison of variance and mean value was performed using a one-way analysis of variance (ANOVA) with Tukey's post-test.

3. Results

3.1 Erk1/2 phosphorylation in response to FGF stimulation

The activation of the MAPK pathway was determined by quantifying phosphorylated Erk1/2 (pErk1/2) by western blot analysis using specific antibodies. The ability of FGF to activate signaling from its receptors to the downstream MAPK and signal transduction pathways was

tested in a wild type mouse embryonic fibroblast cell line ($CTR1^{+/+}$) and an isogenic line in which both alleles of CTR1 had been knocked out ($CTR1^{-/-}$). As shown in Figure 1, when serum-starved $CTR1^{-/-}$ and $CTR1^{+/+}$ cells were treated with FGF the level of pErk1/2 increased only in the $CTR1^{+/+}$ cells. Serum starvation by itself produced no change in the basal level of pErk1/2. FGF at a concentration of 1 ng/mL increased the level pERK1/2 starting at 5 min and by 15 min the increase had reached 20.4 ± 4.2 -fold (SEM) and was highly significant ($p < 0.05$). FGF did not increase the phosphorylation of AKT in either type of cell (data not shown) indicating that in these cells the primary signaling from the FGF receptor is to the MAPK pathway. To determine whether CTR1 influenced activation of kinases upstream of Erk1/2 in the RAS/Raf/Mek/Erk pathway, the effect of FGF on the phosphorylation of Mek1/2 was examined using an antibody to Mek1/2 Ser217/221. As shown in Figure 1D, while FGF induced an increase in pMek1/2 in the $CTR1^{+/+}$ cells there was a much reduced response in the $CTR1^{-/-}$ cells ($p<0.05$, Figure 1E). Thus, in the absence of CTR1 there is defective signaling from the FGFR to both Mek1/2 and Erk1/2.

3.2 Erk1/2 and AKT phosphorylation in response to PDGF and EGF

To determine whether CTR1 is required for signaling from other receptor tyrosine kinase receptors, the $CTR1^{+/+}$ and $CTR1^{-/-}$ cells were exposed to either 50 ng/mL PDGF or 5 ng/mL EGF for 15 min. As shown in Figure 2, both PDGF and EGF increased the phosphorylation of Erk1/2 only in the $CTR1^{+/+}$ cells, but unlike FGF, also increased the phosphorylation of AKT at Ser473. These results indicate that multiple different receptor tyrosine kinases can activate signaling to the MAPK pathway in the $CTR1^{+/+}$ cells and, that unlike FGF, both PDGF and EGF also activate the AKT pathway.

3.3 Regulation of FGF-induced signaling by intracellular Cu

It has previously been reported that the whole cell Cu level is low in $CTR1^{-/-}$ cells. The $CTR1^{+/+}$ cells were found to contain ~2.5 μg Cu/mg protein, and the $CTR1^{-/-}$ cells 0.6 μg Cu/mg protein [14]. The Cu chaperone CCS is a sensitive marker of the availability of intracellular Cu, increasing as the Cu level drops [16, 17]. To confirm that $CTR1^{-/-}$ cells were Cu deficient, the level of CCS was quantified by immunoblot analysis. As shown in Figures 3A and 3B there was an 8.0 ± 1.5-fold (SEM) increase of CCS in the $CTR1^{-/-}$ cells ($p < 0.05$). SOD1 requires Cu in order to reduce superoxide to H_2O_2 [18]. To determine whether the intracellular Cu deficiency was severe enough to reduce the activity of SOD1, its activity was measured in lysates from the $CTR1^{+/+}$ and $CTR1^{-/-}$ cells growing in standard tissue culture medium containing ~2 μM Cu. As shown in Figure 3C, the activity of SOD1 was reduced by a factor of 17.2 ± 8.2-fold ($p < 0.05$). Addition of 200 μM $CuSO_4$ to the culture media of the $CTR1^{-/-}$ cells for 18 h restored the ability of FGF to trigger phosphorylation of Erk1/2 (Figure 4A). Addition of the Cu chelator BCS to the culture media of the $CTR1^{+/+}$ cells at a concentration of 200 μM for 24 h inhibited the ability of FGF to phosphorylate Erk1/2 (Figure 4B). These results indicate that the $CTR1^{-/-}$ cells are deficient in intracellular Cu, and that failure of FGF-induced signaling in the $CTR1^{-/-}$ cells is due to this Cu deficiency.

3.4 Cu deficiency impedes superoxide metabolism

We predicted that the reduced activity of SOD1 in the $CTR1^{-/-}$ cells would impair the conversion of superoxide generated by the FGFR-mediated activation of NADPH oxidase to H_2O_2 and thus result in a higher concentration of superoxide in the $CTR1^{-/-}$ cells following exposure to FGF. To accurately measure intracellular superoxide production, we employed spin-trapping EPR spectroscopy which measures superoxide directly. The relatively new nitroxide spin trap DEPMPO is currently the most efficient spin trap reported for superoxide because the lifetime of its superoxide adduct is sufficiently long to allow detection and quantification of superoxide production [19]. As shown in Figure 5A, the steady-state level

of endogenous superoxide in the absence of FGF exposure was found to be 1.57 ± 0.19-fold higher in the $CTR1^{-/-}$ than in the $CTR1^{+/+}$ cells ($p<0.0001$), reflecting the lack of Cu needed for SOD1 to rapidly convert the superoxide to H_2O_2. The addition of FGF did not significantly change the level of superoxide in the $CTR1^{+/+}$ cells, presumably because the superoxide was so rapidly dismutated to H_2O_2. In contrast, there was a significant accumulation of superoxide in the $CTR1^{-/-}$ cells after FGF treatment (Figure 5A) ($p<0.0001$). This further confirmed that the function of SOD1 was compromised in the $CTR1^{-/-}$ cells. The H_2O_2 generated by the dismutation of superoxide by SOD1 inactivates the PTPases that normally antagonize signaling from RTKs by reversibly oxidizing a cysteine in the active site, preventing the de-phosphorylation of substrates phosphorylated by the RTKs [20]. To determine whether failure of FGF-induced signaling to Erk1/2 in the FGF-treated $CTR1^{-/-}$ cells was due to inadequate generation of H_2O_2 secondary to reduced SOD1 activity, $CTR1^{-/-}$ cells were exposed to 100 μmol/L H_2O_2 for 30 min prior to stimulation with FGF. As shown in Figure 5B and quantified in 5C, the addition of H_2O_2 restored the ability of FGF to increase the level of pErk1/2 by a factor of 203.9 ± 45.3 -fold ($p<0.01$) in the $CTR1^{-/-}$ cells but did not further increase the effect of FGF alone in the $CTR1^{+/+}$ cells. Thus, the failure of FGF-induced signaling in the $CTR1^{-/-}$ cells can be attributed to reduced generation of H_2O_2 that we hypothesize to be insufficient to poison the PTPases needed to sustain signaling to Erk1/2.

4. Discussion

Activation of many RTKs by their ligands triggers a phosphorylation cascade involving the sequential activation of Ras, Raf, and Mek1/2 that eventually results in the phosphorylation of Erk1/2 [21, 22]. The results reported here demonstrate that the major Cu influx transporter CTR1 is required for the ability of three different RTKs to activate the central elements of their downstream signaling pathways, and also provide an explanation of the mechanism by which this occurs. Deletion of both alleles of CTR1 was found to impair the ability of FGF, EGF and PDGF to activate the Ras/Raf/Mek/Erk cascade as evidenced by failure to trigger the phosphorylation of Erk1/2. The low intracellular Cu content of the $CTR1^{-/-}$ cells, and the increased expression of the Cu chaperone CCS, suggested that this was due to lack of Cu. This was confirmed by showing that addition of Cu to the $CTR1^{-/-}$ cells restored FGF-induced signaling whereas reduction of Cu in the $CTR1^{+/+}$ cells using the Cu chelator BCS reduced signaling.

Exposure to FGF did not trigger phosphorylation of AKT in either the $CTR1^{+/+}$ or the $CTR1^{-/-}$ cells, but AKT was phosphorylated in response to treatment with both EGF and PDGF in the $CTR1^{+/+}$ cells but not the $CTR1^{-/-}$ cells. Thus, as for Erk1/2, under circumstances where AKT was activated by an RTK, the activation was dependent on CTR1. Differences among cell lines in the ability of FGFR, EGFR and PDGFR to phosphorylate AKT have been reported previously and appear to be dependent on the cell line-specific expression of pathway modulators [23, 24].

The fact that phosphorylation of both Erk1/2 and AKT was impaired in the $CTR1^{-/-}$ cells suggests that the Cu-dependent step is upstream of the point at which the Ras/Raf/Mek/Erk and Ras/PI3K/AKT pathways diverge. As shown in the schematic presented in Figure 6, events upstream of Ras involve concurrent phosphorylation of the FGF receptor itself and activation of PTPases capable of removing these phosphates. The vigor of the phosphorylation cascade triggered by the RTK is augmented by simultaneous inactivation of PTPases that normally counteract the activity of the kinases in the pathway [1]. The RTKs accomplish this by activating NAPDH oxidases in their immediate vicinity through activation of Rac [4, 25, 26]. These oxidases generate superoxide using electrons from NADPH and couple these to molecular oxygen to produce superoxide which is then used by

superoxide dismutases to produce H_2O_2 [18, 27, 28]. H_2O_2 disables PTPases by reversibly oxidizing a cysteine in the active site, preventing the de-phosphorylation of substrates activated by the RTKs [20]. SOD1 is the key cytoplasmic enzyme in this process, and the activity of SOD1 is dependent on the availability of Cu [18]. SOD1 acquires Cu primarily from the Cu chaperone CCS which selectively delivers Cu to SOD1 [29].

Given the central role of SOD1, we sought to determine whether impairment of its activity could explain failure of RTK signaling. Measurement of SOD1 activity demonstrated a significant reduction in the $CTR1^{-/-}$ cells. To determine whether this was sufficient to affect the conversion of FGF-induced superoxide to H_2O_2, a very sensitive and specific technique was used to measure superoxide. Consistent with reduced SOD1 activity and the resulting impaired conversion of superoxide to H_2O_2, the steady-state level of superoxide was higher in the $CTR1^{-/-}$ cells. Whereas FGF triggered little increase in superoxide in the $CTR1^{+/+}$ cells consistent with its very rapid dismutation to H_2O_2, there was a clear increase in the $CTR1^{-/-}$ cells. Proof that failure of H_2O_2 generation was the basis for the loss of signaling in the $CTR1^{-/-}$ was provided by showing that addition of H_2O_2 restored FGF-induced phosphorylation of Erk1/2.

It is now apparent that superoxide is not merely a harmful byproduct of life in an aerobic environment. Various stimuli lead to the assembly of a multicomponent NADPH oxidase complex which mediates a process known as the respiratory burst. NADPH oxidase catalyses transfer of one electron from NADPH to molecular oxygen to generate superoxide anions, which in turn may yield H_2O_2 either via protonation of superoxide or through the action of SOD1. The reversible phosphorylation of tyrosyl residues in proteins is a key component of signaling pathways induced by extracellular stimuli that regulate cellular responses such as growth, proliferation, differentiation, metabolism and migration. Protein tyrosine phosphorylation is controlled through the coordinated actions of protein tyrosine kinases and phosphatases (PTPases). The PTPases are characterized by a signature motif, I/V-H-C-X-X-G-X-X-R-S/T, which forms the base of the active site cleft and contains an invariant Cys residue. Due to the unique environment of the PTPase active site, the pKα of the sulfhydryl group of this Cys is extremely low, which favors its function as a nucleophile but renders it susceptible to oxidation. The prototypic member of the PTPase family is the enzyme PTP1B. It has been shown that stimulation of A431 cells with EGF leads to the production of H_2O_2 and concomitant inhibition of PTP1B and stimulation of cells with insulin resulted in the rapid and transient oxidation and inhibition of two distinct PTPs, PTP1B and TC45, the 45-kDa spliced variant of the T cell protein-tyrosine phosphatase [30, 31]. PDGF has also been shown to induce the oxidation and inhibition of the SH2 domain-containing PTPase, SHP-2, which facilitated mitogenic signaling in response to the growth factor [32]. Our observation that addition of H_2O_2 restored FGF-induced signaling in the $CTR1^{-/-}$ cells provides further confirmation of the concept that increased production of intracellular oxidants contributes to enhance RTK signaling transiently suppressing the activity of members of the PTPase family.

The results presented here confirm and extend some of the findings recently reported by Turski *et al.* [13] who reported failure of FGF and insulin-induced phosphorylation of Erk1/2 in the same $CTR1^{-/-}$ cells. In addition to showing that Cu starvation reduced signaling in the $CTR1^{+/+}$ cells, and that Cu supplementation restored it in the $CTR1^{-/-}$ cells, they found that disabling the ability of CTR1 to transport Cu by changing MET150 to alanine or blocking the pore with Ag^+ also impaired FGF signaling. However, based on the observation that neither FGF nor insulin activated AKT, they concluded that the Ras/PI3K/AKT pathway was not sensitive to Cu depletion and that Ras was not likely affected by loss of CTR1. This led to the discovery that Mek1 is a Cu binding protein whose activity appears to be impaired in the $CTR1^{-/-}$ cells leading them to conclude that Mek1 was the Cu-

dependent step in FGF induced activation of Erk1/2. While the data supporting the concept that Mek1 activity is Cu-dependent is compelling, we find that AKT is activated in a Cu-dependent manner in response to EGF and PDGF in the $CTR1^{+/+}$ cells suggesting that there is a Cu-dependent step in the pathway upstream of the diversion of the Ras/Raf/Mek/Erk and Ras/PI3K/AKT pathways. The finding that the H_2O_2 restored FGF-induced signaling in the $CTR1^{-/-}$ cells provides strong evidence for an upstream Cu-dependent step in this pathway as well.

The requirement for CTR1, and its ability to transport Cu, suggests that extracellular rather than a pre-existing intracellular store of Cu is the source of the Cu needed for FGF-induced signaling. While Cu levels are lower in the $CTR1^{-/-}$ cells, there remain intracellular stores that one might have expected to be the proximal source of Cu for the acute needs of SOD1. In the case of SOD1 it is well documented that Cu is delivered by the Cu chaperone CCS [33, 34] which presumably receives Cu either directly or indirectly from CTR1. Our results lead to the prediction that anything that impairs the transfer of Cu from CTR1 to CCS, reduces the level of CCS, or blocks the transfer of Cu from CCS to SOD1 would interfere with FGF-induced phosphorylation of Erk1/2. Pharmacologic inhibition of any of these steps may serve to reduce RTK signaling and reduce tumor cell proliferation. We speculate that this may explain the anti-tumor activity of Cu chelating agents such as tetrathiomolybdate [18, 35].

Prior studies have documented the resistance of $CTR1^{-/-}$ cells to cisplatin and this has been ascribed to reduced Pt accumulation and adduct formation [36, 37]. The discovery that CTR1 is also required to support signaling from at least four RTKs, including FGFR, EGFR, PDGFR and the insulin receptor, suggests the alternative interpretation that cisplatin resistance may also in part be due to reduced activity in the signal transduction pathways downstream from these receptors. RTK inhibitors have now been shown to have activity in many cancers including breast, colorectal, lung, and pancreatic cancers; however, they have not worked as well in ovarian cancer. The discovery that CTR1 is essential for signaling from four important RTKs adds a new dimension to both the field of Cu homeostasis and the cellular pharmacology of the Pt-containing chemotherapeutic agents.

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Abbreviations

SOD1	superoxide dismutase 1
Cu	copper
H_2O_2	hydrogen peroxide
RTK	receptor tyrosine kinase
CCS	copper chaperone
EPR	electron paramagnetic resonance
MAPK	mitogen activated protein kinase
PTPase	protein tyrosine phosphatase
BCS	bathocuproine disulphonate

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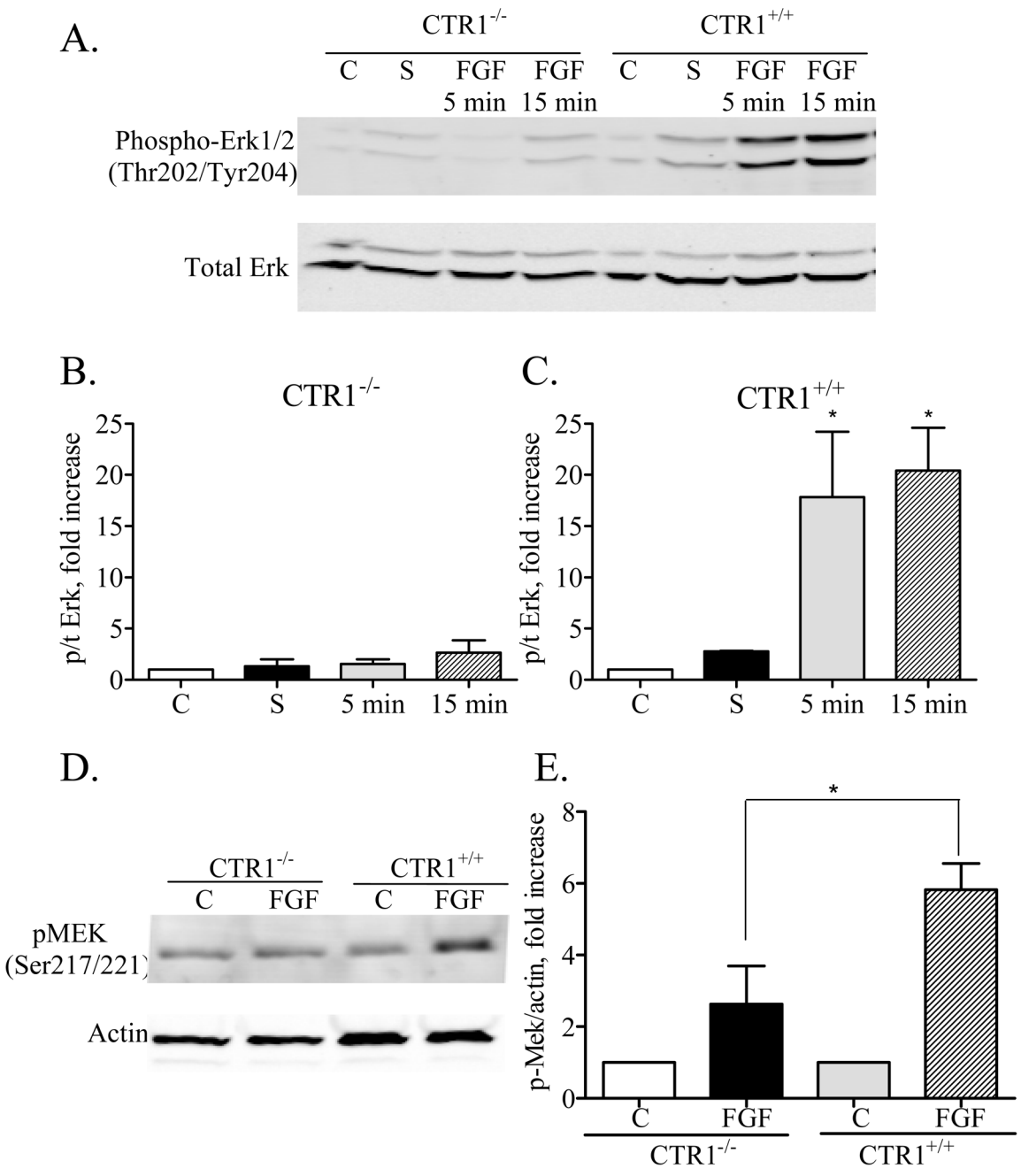

Figure 1.

Erk1/2 phosphorylation in response to FGF stimulation. A. CTR1$^{-/-}$ and CTR1$^{+/+}$ cells were treated with FGF 1 ng/mL for the indicated time points. The immunoblot shown is representative of 3 independent experiments (C, untreated control; S, cells serum starved overnight). B. Quantification of pERK1/2 protein levels in CTR1$^{-/-}$ cells. C. Quantification of pErk1/2 levels in CTR1$^{+/+}$ cells. Untreated control (white bar), serum starvation (black bar), FGF 1 ng/mL 5 min (gray bar), FGF 1 ng/mL 15 min (hatched bar). Data from immunoblots were normalized to total Erk1/2and expressed as fold increase over untreated control samples (n=3); bars, SE; *, $p<0.05$, between FGF treatment and untreated control. D. Representative western blot showing failure of FGF to enhance Mek1/2 phosphorylation in

the $CTR1^{-/-}$ cells. E. Quantification of FGF-induced phosphorylation of Mek1/2 in $CTR1^{+/+}$ versus $CTR1^{-/-}$ cells from a total of 4 western blot analyses. Mean untreated control in $CTR1^{-/-}$ (white bar); FGF 1 ng/mL 15 min in $CTR1^{-/-}$ (black bar); untreated control in $CTR1^{+/+}$ (gray bar); FGF 1 ng/mL 15 min in $CTR1^{+/+}$ (hatched bar). Vertical, bars, ± SEM; *, $p<0.05$,

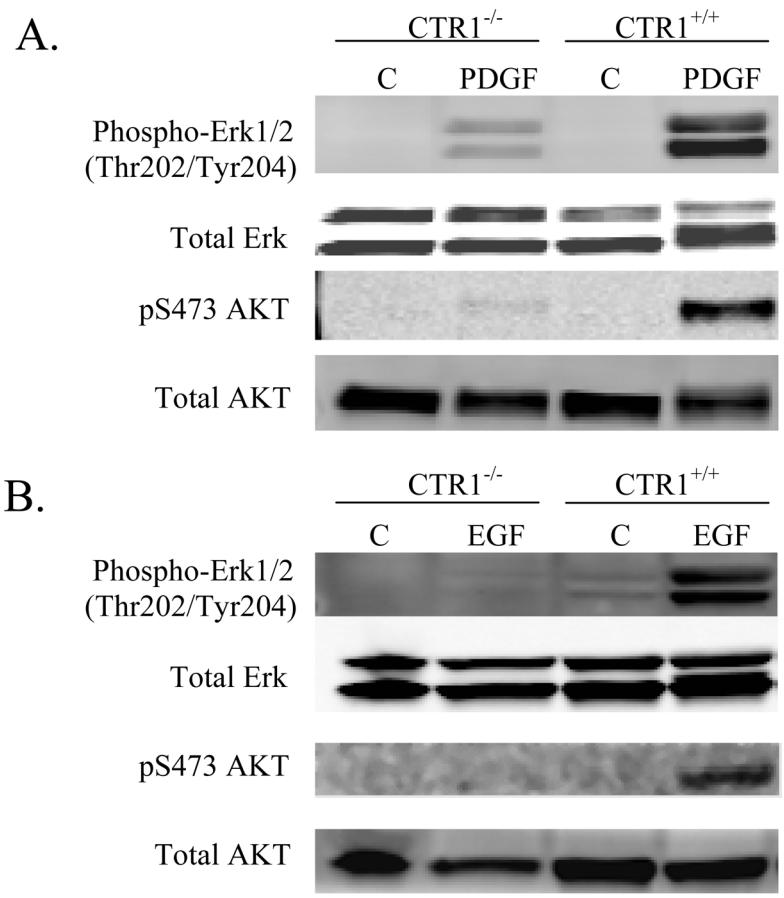

Figure 2.

Erk1/2 and AKT phosphorylation in response to PDGF and EGF. A. $CTR1^{-/-}$ and $CTR1^{+/+}$ cells were treated with PDGF 50 ng/mL for 15 min. B. $CTR1^{-/-}$ and $CTR1^{+/+}$ cells were treated with EGF 5 ng/mL for 15 min. Immunoblots shown are representative of 3 independent experiments.

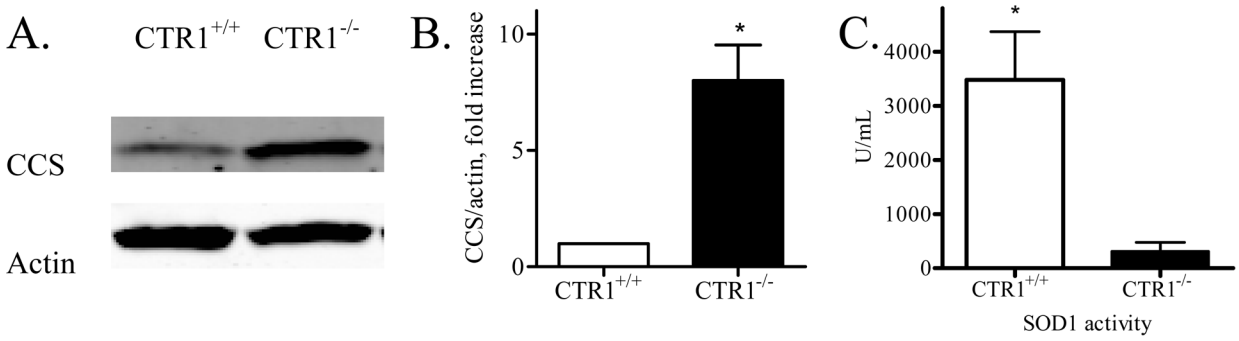

Figure 3.

Cu deficiency and SOD dysfunction in the CTR1$^{-/-}$ cells A. Western blot documenting an increase in CCS expression in the CTR1$^{-/-}$ cells. The immunoblot shown is representative of 3 independent experiments. B. Quantification of CCS protein level normalized to actin in the CTR1$^{+/+}$ cells and CTR1$^{-/-}$ cells (n=3); bars, ± SEM.*p<0.05. C. SOD1 activity in the CTR1$^{+/+}$ cells and CTR1$^{-/-}$ cells (n=3); bars, ± SEM.*p<0.05.

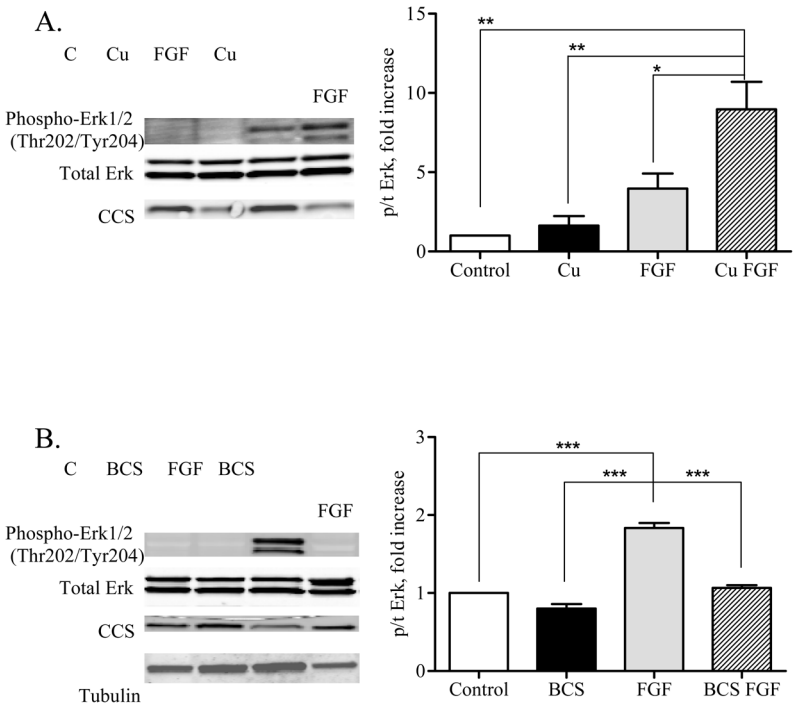

Figure 4.

Signaling to Erk1/2 is regulated by the intracellular Cu level. A. FGF-induced phosphorylation of Erk1/2 was restored when $CTR1^{-/-}$ were treated with 200 μM $CuSO_4$ 18 h prior to a 15 min exposure to FGF 1 ng/mL. Untreated control (white bar), Cu (black bar), FGF 1 ng/mL 15 min (gray bar), Cu supplement prior to FGF stimulation (hatched bar). B. Exposure to Cu chelator BCS inhibits FGF-induced phosphorylation of Erk1/2 in $CTR1^{+/+}$ cells. Untreated control (white bar), BCS (black bar), FGF 1 ng/mL 15 min (gray bar), Cu depletion prior to FGF stimulation (hatched bar). For each panel the immunoblots shown are representative of 3 independent experiments. Vertical bars, ± SEM.

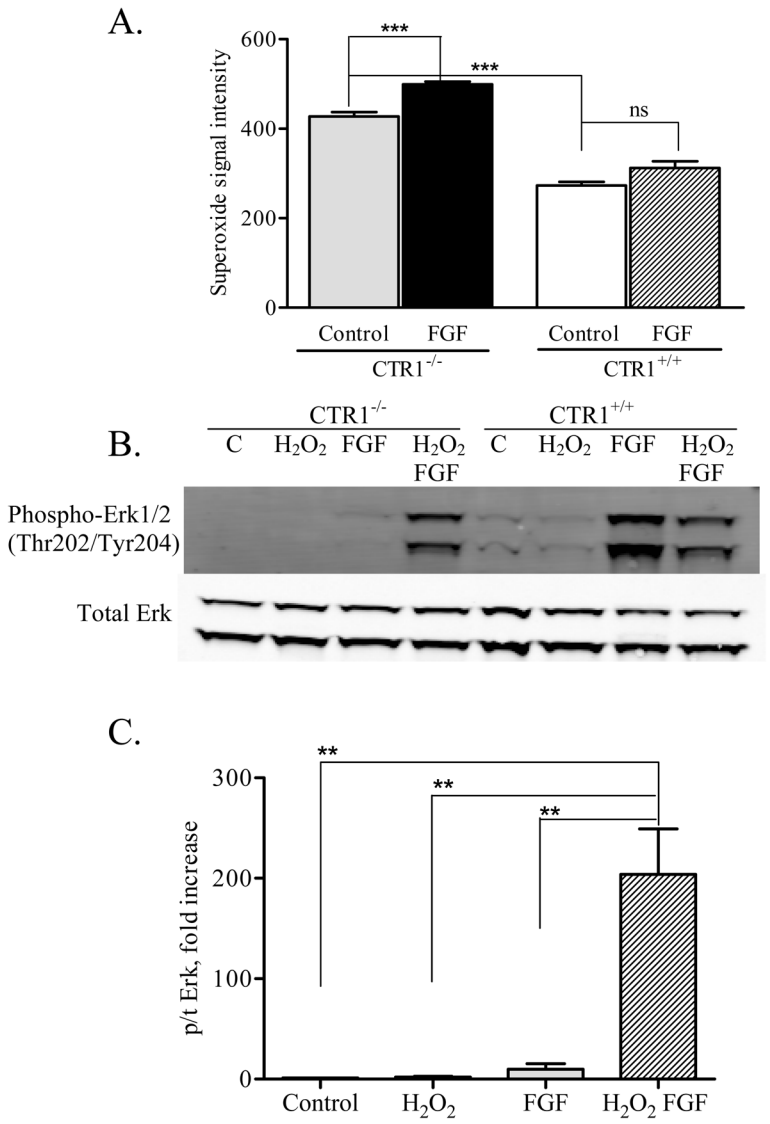

Figure 5.

Cu deficiency impedes superoxide metabolism. A. Endogenous and stimulated level of superoxide in the paired CTR1 cells. Untreated CTR1 $^{+/+}$ cells (white bar), FGF treated CTR1 $^{-/-}$ cells (hatched bar), untreated CTR1$^{-/-}$ cells (gray bar), FGF treated CTR1 $^{+/+}$ cells (black bar), (n=6). B. H_2O_2 restores FGF-induced phosphorylation of Erk1/2 in CTR1$^{-/-}$ cells. The immunoblot shown is representative of 3 independent experiments. C. Quantification of pERK1/2 levels in CTR1$^{-/-}$ cells. Mean untreated control (white bar); H_2O_2 (black bar); FGF 1 ng/mL 15 min (gray bar); 100 μmol/L H_2O_2 30 min prior to FGF stimulation (hatched bar). Bars are mean ± SEM. **p<0.01; ***p<0.001.

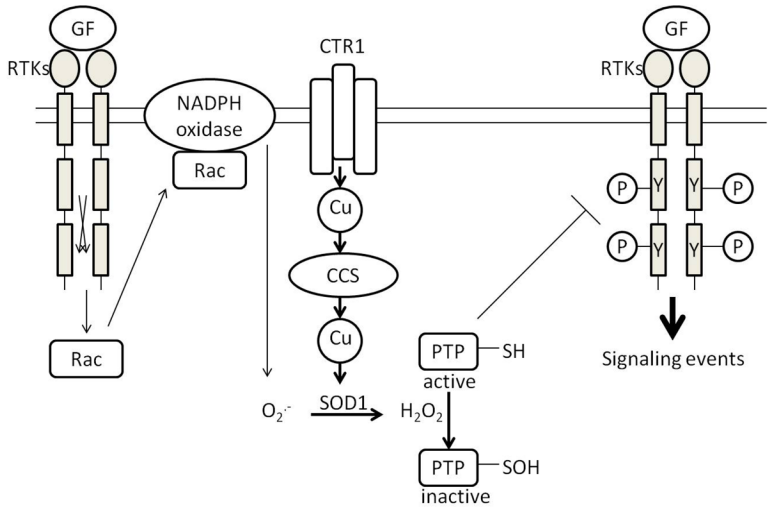

Figure 6.

Proposed Model. Binding of a growth factor to its RTK activates Rac which then causes NADPH oxidases to generate superoxide. CTR1 is required to provide Cu to SOD1 so that it can dismute superoxide into H_2O_2. H_2O_2 oxidizes and inhibits PTPases that normally counteract the phosphorylation cascade triggered by the RTK.