Superoxide anion radicals induce IGF-1 resistance through concomitant activation of PTP1B and PTEN

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Abstract

The evolutionarily conserved IGF-1 signalling pathway is associated with longevity, metabolism, tissue homeostasis, and cancer progression. Its regulation relies on the delicate balance between activating kinases and suppressing phosphatases and is still not very well understood. We report here that IGF-1 signalling in vitro and in a murine ageing model in vivo is suppressed in response to accumulation of superoxide anions (O²⁻) in mitochondria, either by chemical inhibition of complex I or by genetic silencing of O²⁻-dismutating mitochondrial Sod2. The O²⁻-dependent suppression of IGF-1 signalling resulted in decreased proliferation of murine dermal fibroblasts, affected translation initiation factors and suppressed the expression of α1(I), α1(III), and α2(I) collagen, the hallmarks of skin ageing. Enhanced O²⁻ led to activation of the phosphatases PTP1B and PTEN, which via dephosphorylation of the IGF-1 receptor and phosphatidylinositol 3,4,5-triphosphate dampened IGF-1 signalling. Genetic and pharmacologic inhibition of PTP1B and PTEN abrogated O²⁻-induced IGF-1 resistance and rescued the ageing skin phenotype. We thus identify previously unreported signature events with O²⁻, PTP1B, and PTEN as promising targets for drug development to prevent IGF-1 resistance-related pathologies.

Keywords ageing; IGF-1; phosphatase; reactive oxygen species; superoxide anions

Subject Category Ageing

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Introduction

Insulin-like growth factors (IGFs) play essential roles in the regulation of cell growth, proliferation, stem cell maintenance and synthesis of extracellular matrix proteins (Baker et al., 1993; Le Roith, 1997; Papaconstantinou, 2009; Pieciewicz et al., 2012) and—if dysregulated—result in connective tissue and organ atrophy with enhanced ageing or cancer progression (Baker et al., 1993; Pollak et al., 2004; Govoni et al., 2007b; Laviola et al., 2008; Anisimov & Bartke, 2013). During ageing, a gradual decline in circulating insulin-like growth factor-1 (IGF-1) levels beginning in the third and fourth decade of life occurs in humans and in other mammals (Lamberts et al., 1997; Le Roith, 1997; Tatar et al., 2003; Parekh et al., 2010). Reduced IGF-1 levels and/or impaired IGF-1 signal transduction may at least in part be responsible for enhanced muscle atrophy (sarcopenia), bone resorption (osteoporosis), and skin atrophy consistently observed in elderly individuals (Gallagher & LeRoith, 2011). Given the significant clinical implications and the current demographic development, advanced knowledge on IGF-1 signalling and its control at different steps of the signalling cascade is particularly important. Regulation of IGF-1 signalling occurs rather in integrated signalling networks, which are governed at the level of phosphorylation and dephosphorylation, catalysed by protein kinases and phosphatases, respectively (Pollak et al., 2004; Taguchi & White, 2008). Similar to other growth factors, IGF-1 signalling is initiated by the autophosphorylation of the IGF-1 receptor β subunit (IGF-1Rβ), which is followed by a series of kinase-dependent phosphorylations of downstream effectors such as phosphoinositide-3-kinase (PI3K), AKT (protein kinase B), and p70S6 ribosomal protein kinase (p70S6K)—a prerequisite for synthesis of extracellular matrix—and other proteins. This in conjunction with the IGF-1-initiated Ras-dependent upregulation of cyclin D1 ultimately promotes cell cycle progression and growth essential for overall tissue homeostasis (Pollak et al., 2004; Samani et al., 2007). In addition to the PI3K–AKT axis, IGF-1 signalling was reported to activate the mitogen-activated protein kinase (MAPK) pathway, thus exerting its prosurvival effect in many though not all cell lines (Parrizas et al., 1997; Peruzzi et al., 1999; Subramaniam et al., 2005). In fact, IGF-1 can also inhibit ERK activation in some cell types, including neurons (Subramaniam et al., 2005). Therefore,
the effect of IGF-1 on MAPK and cyclin D1 expression is not uniform for all cell types and most likely depends on the cell type, the nature, magnitude, and duration of the stimulus.

Protein tyrosine phosphatase 1B (PTP1B) was the first identified member of the classical tyrosine-specific protein phosphatase superfamily (Tonks et al., 1988), which dephosphorylates and thus inactivates the β chain of the IGF-1R (Buckley et al., 2002). PTP1B is localized at the cytoplasmic site of the endoplasmic reticulum (ER) (Frangioni et al., 1992). After translocation to the plasma membrane, it is endowed with the capacity to dephosphorylate plasma membrane-associated IGF-1R (Buckley et al., 2002; Yudushkin et al., 2007) and ligand-activated IGF-1R after endocytosis (Eden et al., 2010; Stuible et al., 2010). Similar to the activation of PTP1B, the lipid phosphatase and tensin homologue (PTEN) translocate from the cytosol to the plasma membrane, essential for its activation, and attache to the membrane through its C2 domain (Das et al., 2003; Leslie et al., 2003; Vazquez et al., 2006). Membrane-bound activated PTEN dephosphorylates membrane-anchored phosphatidylinositol 3,4,5-trisphosphate (PIP₃), thus opposing PI3K action with subsequent attenuation of IGF-1 signalling (Maehama & Dixon, 1998).

Several lines of evidence suggest that reactive oxygen species (ROS), in particular H₂O₂ interfere with insulin/IGF-1 signalling (Leslie et al., 2003; Houstis et al., 2006; Bashan et al., 2009; Loh et al., 2009; Finkel, 2011; Murphy et al., 2011). Both PTP1B and PTEN are regulated through reversible oxidation and inactivation (Leslie et al., 2003; Salmeen et al., 2003) and thus may be involved in enhanced IGF-1 signalling. PTP1B and PTEN contain a critical cysteine residue in their active site that exclusively in its reduced state is able to participate in substrate dephosphorylation. In fact, H₂O₂-mediated oxidation of cysteine inactivates these phosphatases in vitro (Gough & Cotter, 2011). In addition, it was reported that mice deficient of glutathione peroxidase (Gpx), the key enzyme responsible for H₂O₂ detoxification, were distinctly protected from high fat-induced insulin resistance (Loh et al., 2009). This study provides causal evidence for the enhancement of insulin signalling by oxidative inactivation of phosphatases (Sundaresan et al., 1995).

By contrast to H₂O₂, superoxide anion radicals (O₂⁻) physiologically occur as a metabolic by-product during oxidative phosphorylation within mitochondria. Apart from a correlative report (Hoehn et al., 2009), their biology has not been studied in great detail under physiologic and pathologic conditions. An in-depth knowledge on the role of O₂⁻ would, however, be particularly relevant as O₂⁻ accumulates in a variety of cells during ageing (Treiber et al., 2011), neurodegeneration (Wu et al., 2010), and conditions such as the metabolic syndrome and diabetes (Kim et al., 2008). In addition, O₂⁻ plays a central role in inflammatory conditions (Shishido et al., 1994; Naya et al., 1997; Cheng et al., 1999; MacArthur et al., 2000; Lu et al., 2003) and in chronic non-healing wounds (Sindrilaru et al., 2011).

Using complementary biochemical and genetic approaches to specifically dissect the effect of O₂⁻ and H₂O₂ on IGF-1 signalling, we here found that by contrast to the H₂O₂-dependent inhibition of the key phosphatases PTP1B and PTEN, accumulation of mitochondrial O₂⁻ resulted in marked activation of PTP1B and PTEN, eventually dampening the IGF-1-induced signalling cascade at distinct steps of downstream effectors. This leads to subsequent inhibition of murine dermal fibroblast (MDFs) proliferation, changed expression of factors responsible for protein translation initiation, and reduced gene expression of x1 (I), x1 (III), and x2 (I) collagen chains, key features of skin ageing. The O₂⁻-dependent IGF-1 resistance was significantly abrogated by pharmacologic and genetic inhibition of PTP1B and PTEN, suggesting a central role for these phosphatases in the O₂⁻-dependent IGF-1 resistance. This was confirmed in vivo by the rescue of the skin ageing phenotype and IGF-1 signalling in fibroblast-specific Sod2-deficient mice, where PTEN gene was heterozygously deleted in Sod2-deficient mice. Similarly, pharmacologic PTP1B inhibition in Sod2-deficient mice showed partial reversal of the impaired IGF-1 signalling. An advanced understanding of the involvement of distinct ROS species in the regulation of IGF-1 resistance holds substantial promise for prevention and therapy of age-related and other pathologies.

**Results**

**Superoxide anions and hydrogen peroxide differentially regulate the IGF-1/AKT pathway**

Murine dermal fibroblasts (MDFs) endogenously produce and release IGF-1, which via autocrine and paracrine mechanisms stimulate the IGF-1 signalling cascade (Yakar et al., 1999). To study the effect of different ROS species such as superoxide anion (O₂⁻) and hydrogen peroxide (H₂O₂) on the IGF-1/AKT axis, MDFs were treated with rotenone, an established inhibitor of complex I of the mitochondrial electron transport chain (Li et al., 2003) or with exogenous H₂O₂. Inhibition of complex I by rotenone increases electron leakage from this complex to the matrix side, finally leading to partial reduction of oxygen to O₂⁻ (Buettler et al., 2004) (Supplementary Fig S1A). As expected, rotenone concentration dependently increased mitochondrial O₂⁻ generation in MDFs assessed by MitoSOX, a specific indicator for mitochondrial O₂⁻ (Supplementary Fig S1B). We first analysed the phosphorylation of AKT as a critical major downstream signalling component following IGF-1R activation. Notably, O₂⁻ and H₂O₂ exerted opposite effects on AKT signalling in MDFs, irrespective of the rotenone and H₂O₂ concentration (Fig 1A) and the time of exposure (Fig 1B). H₂O₂ at moderate and high concentrations ranging from 10 to 1,000 μM markedly induced phosphorylation of AKT without exerting any effect on total AKT levels (Fig 1A), a finding which is in agreement with published reports (Ushio-Fukai et al., 1999). Interestingly, rotenone resulted in a concentration-dependent reduction in AKT activation as shown by reduced phosphorylation of AKT at Ser 473 (pAKT) (Fig 1A). At higher concentrations, both rotenone-induced O₂⁻ and H₂O₂ led to a marked reduction in the amount of cyclin D1 (Fig 1A and B), which at physiological concentrations is required for cell cycle progression from the G1 phase (Winston et al., 1996). Collagen type I and III synthesis is dependent on IGF-1/AKT signalling and is mainly controlled at the mRNA level (Gillery et al., 1992). These collagen types produced by MDFs constitute the major extracellular matrix proteins of the skin, which significantly decrease during ageing of...
murine and human skin (Quan et al., 2010). Interestingly, enhanced O$_2^-$ generation in MDFs at a rotenone concentration of 500 μM for 3 h inhibited the specific mRNA levels of the α1 and α2 chains of type I collagen and the α1 chain of type III collagen (Fig 1C), while H$_2$O$_2$ at a concentration of 500 μM—parallel to enhancing IGF-1 signalling—increased α chains of type I and type III collagen-specific mRNA levels (Fig 1C). Of note, more than 90% of MDFs were viable at rotenone and H$_2$O$_2$ concentrations of 500 μM, indicating that altered collagen synthesis was not related to toxicity (Supplementary Fig S1C). Collectively, these data demonstrate that O$_2^-$ and H$_2$O$_2$ exert at least partly opposing effects on IGF-1/AKT signalling in MDFs.

Figure 1.
**Figure 1.** Superoxide anion radicals and H$_2$O$_2$ differentially regulate IGF-1 signalling.

A To assess differential regulation of downstream key effector proteins of IGF-1 signalling such as activated (phosphorylated) AKT (pAKT), total AKT (AKT), and cyclin D1 (CyD1) levels in MDFs either treated with increasing rotenone concentrations (left panel) or with increasing H$_2$O$_2$ concentrations (right panel) for 3 h in serum-free DMEM, cell lysates were analysed by Western blotting equilibrated to actin expression levels. The graphs represent the densitometric analyses of cyclin D1 (CyD1) expression in the presence of different concentrations of rotenone and H$_2$O$_2$. The upper band is the hyperphosphorylated cyclin D1, and the lower band represents the hypophosphorylated cyclin D1. Comparison was made with two-tailed t-test; respective comparison was indicated with line and P-value. The results are representative for three independent experiments (n = 3).

B MDFs were treated with 500 µM rotenone (left panel) or 500 µM H$_2$O$_2$ (right panel) for the indicated time periods, and the expression of IGF-1 downstream effector proteins (as in A) was analysed by Western blotting. The graphs represent the densitometric analyses of cyclin D1 (CyD1) expression in the presence of 500 µM rotenone and 500 µM H$_2$O$_2$ at the indicated time point. The values are presented as mean ± SEM in percentage, and comparison was made with two-tailed t-test; respective comparison was indicated with line and P-value. The results are representative for three independent experiments (n = 3).

C In order to dissect the effects of different reactive oxygen species on IGF-1 target genes in MDFs, mRNA expression of different type I and type III collagen chains (Col(I)x1, Col(I)x2, and Col(III)x1) was analysed by RT-qPCR in MDFs treated with either 500 µM rotenone or 500 µM H$_2$O$_2$ for 3 h (n = 6). Values are presented as mean ± SEM of fold change, and comparison was made with two-tailed t-test; P-value indicated the significance of difference, comparing the indicated collagen chain mRNA levels of either rotenone- or H$_2$O$_2$-treated MDFs with control.

Enhanced superoxide anion concentrations induce partial IGF-1 resistance

Next, we analysed the IGF-1R sensitivity to its exogenous ligand in the presence of high O$_2^-$ concentrations (Fig 2A). Recombinant IGF-1 (IGF-1) was used at a concentration of 100 ng/ml to activate the IGF-1/AKT pathway in MDFs in the presence or absence of rotenone. Addition of IGF-1 resulted in the activation (phosphorylation) of IGF-1Rβ, AKT, ribosomal protein S6 (S6), and increased cyclin D1 levels in MDFs (Fig 2B). Notably, rotenone at concentrations of 350 and 500 µM led to a significant inhibition of IGF-1-induced activation (phosphorylation) of AKT (pAKT) (Fig 2B), without any alteration of total AKT and IGF-1Rβ levels. Rotenone treatment markedly inhibited IGF-1-mediated IGF-1Rβ phosphorylation (Fig 2B), suggesting that enhanced O$_2^-$ concentrations suppressed the IGF-1/AKT axis at the initial step of IGF-1-mediated IGF-1Rβ activation. IGF-1 has no effect on activation of ERK in MDFs at least at 60 min post-stimulation (Fig 2C), although there are reports of ERK activation by IGF-1 in other cell types (Parrizas et al, 1997; Peruzzi et al, 1999). In fact, IGF-1 induces the expression of cyclin D1 (Fig 2B), but does not induce the activation of ERK (Fig 2C). The expression of cyclin D1, which is suppressed after IGF-1 treatment in the presence of rotenone (Fig 2B) very much, suggests that cyclin D1 is not regulated by the IGF-1R-induced ERK phosphorylation, but rather by IGF-1-induced AKT phosphorylation. The observed rotenone (O$_2^-$)-mediated reduction of cyclin D1 expression is possibly due to the inhibition of other pathways or effectors downstream of IGF-1R (Winston et al, 1996). Of note, eIF4G, the protein essential for initiation of translation (Kong & Lasko, 2012), was markedly increased in the presence of high O$_2^-$ concentrations despite IGF-1 stimulation (Fig 2D). These data imply that steps critically required for translation initiation and protein synthesis might be suppressed by O$_2^-$ (Fig 2D). In addition, rotenone-induced O$_2^-$ was found to suppress IGF-1-mediated cell proliferation, as indicated by reduced Ki67 and BrdU labelling (Fig 2E and Supplementary Fig S2). Rotenone alone reduced basal cell proliferation, and additional supplementation with IGF-1 could only slightly enhance Ki67-positive cells and BrdU incorporation, indicating that O$_2^-$ at higher concentrations overwhelms the cell proliferation stimulatory effect of IGF-1 (Fig 2E and Supplementary Fig S2). H$_2$O$_2$ at a concentration of 100 µM suppressed cell proliferation (Fig 2E and Supplementary Fig S2), and IGF-1 only partly attenuated the inhibitory effect of H$_2$O$_2$ on basal cell proliferation (Fig 2E and Supplementary Fig S2). MDFs isolated from Sod2 knockout mice, due to the lack of O$_2^-$ detoxification, displayed high intracellular O$_2^-$ concentrations (Treiber et al, 2011). These MDFs revealed a marked reduction in pAKT compared to Sod2 competent murine MDFs (Fig 2F, left panel). Lentivirus-vector-based silencing of Sod2 in MDFs (Sod2 shRNA) also showed higher MitoSox fluorescence indicative of increased O$_2^-$ concentrations (Supplementary Fig S1D). Following IGF-1 treatment, Sod2-silenced MDFs showed partial IGF-1 resistance with lower pAKT levels when compared to IGF-1-treated Sod2 competent control MDFs (co) (Fig 2F, right panel). Rotenone also induced partial IGF-1 resistance in murine keratinocytes (epidermal skin cells) (Supplementary Fig S3), suggesting that O$_2^-$-induced IGF-1 resistance is not restricted to MDFs. These data suggest that enhanced O$_2^-$ concentrations impair IGF-1 signalling eventually resulting in IGF-1 resistance.

In vivo evidence for superoxide anion-induced partial IGF-1 resistance

Connective tissue-specific inducible Sod2-deficient mice were used to study whether O$_2^-$-mediated partial IGF-1 resistance also occurs in vivo. Sod2 floxed mice (Strassburger et al, 2005; Treiber et al, 2011) were crossed with transgenic mice expressing a fusion protein of Cre recombinase and a tamoxifen responsive element under the control of the fibroblast-specific collagen type I α2 promoter (Col(I)x2-CreERT mice) (Zheng et al, 2002) to generate Col(I)x2-CreERT ;Sod2f/f mice (mutant) and Col(I)x2-CreERT +; Sod2f/f mice (control with competent Sod2 gene). Deletion of Sod2 in the dermal compartment (connective tissue and fibroblast-rich part) of the skin was performed by administration of 4-OH tamoxifen (Supplementary Fig S4A and B). Both control and mutant mice were intraepidermally (i.p.) injected either with 1 mg/kg recombinant IGF-1 or with normal saline. Indeed, IGF-1 treatment significantly induced the AKT phosphorylation (Ser 473) in the skin of control mice compared to saline-treated control mice as shown by immunofluorescence staining of skin sections and Western blot analyses of skin lysates (Fig 3A and B). Strikingly, injection of
Figure 2.

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We have observed that rotenone inhibited IGF-1-induced autophosphorylation of tyrosine residues (Tyr 1135 and 1136) of IGF-1Rβ (Fig 2B), and further explored the possibility whether the activation of specific tyrosine phosphatases is responsible for the observed IGF-1Rβ dephosphorylation. To achieve this, we investigated the role of key phosphatases including PTP1B, PTEN, and PP2A (Fig 2D). The maximum stimulatory effect of IGF-1 was found 15 min after injection, which was no longer detectable at 60 min (Fig 3A). The Ras/Raf/MEK/ERK pathway regulates cyclin D1-dependent cell cycle progression, and the PI3K/AKT/p70S6K pathway regulates translation initiation, protein synthesis, and growth. Arrows (↓) indicate stepwise activation (phosphorylation) of IGF-1 downstream proteins as shown by “P” in the yellow circle. Currently, it is not resolved whether enhanced O$_2^·$ concentrations affect the Ras/MEK/ERK pathway and the PI3K/AKT pathway at the level of the IGF-1 receptor.

As we have observed that rotenone inhibited IGF-1-induced AKT axis in vivo. The Ras/Raf/MEK/ERK pathway regulates cyclin D1-dependent cell cycle progression, and the PI3K/AKT/p70S6K pathway regulates translation initiation, protein synthesis, and growth. Arrows (↓) indicate stepwise activation (phosphorylation) of IGF-1 downstream proteins as shown by “P” in the yellow circle. Currently, it is not resolved whether enhanced O$_2^·$ concentrations affect the Ras/MEK/ERK pathway and the PI3K/AKT pathway at the level of the IGF-1 receptor.

Furthermore, transient overexpression of Sod2 with increased O$_2^·$-mediated suppression of the IGF-1/AKT axis. As membrane localization of these phosphatases is an essential prerequisite for the inactivation of IGF-1Rβ and downstream effectors of the IGF-1 signalling pathway (Buckley et al., 2002; Vudushkin et al., 2007), we analysed whether upon rotenone or H$_2$O$_2$ treatment, PTP1B and PTEN translocate to the plasma membrane from the endoplasmic reticulum and cytoplasm, respectively. Notably, PTP1B and PTEN were recruited to the plasma membrane when MDFs were subjected to rotenone (Fig 5A and B) and, as earlier shown, IGF-1Rβ-bound PTP1B displayed higher phosphatase activity in rotenone-treated MDFs (Fig 4D). Furthermore, transient overexpression of Sod2 with increased O$_2^·$-scavenging capacity (Supplementary Fig S1E) significantly inhibited rotenone-induced membrane translocation of PTP1B and PTEN in MDFs (Fig 6A and B), suggesting a causal contribution of enhanced O$_2^·$ concentrations in the translocation of these two phosphatases to the plasma membrane.

This suggests an important role for PTP1B and PTEN in the O$_2^·$-mediated suppression of the IGF-1/AKT axis. As membrane localization of these phosphatases is an essential prerequisite for the inactivation of IGF-1Rβ and downstream effectors of the IGF-1 signalling pathway (Buckley et al., 2002; Vudushkin et al., 2007), we analysed whether upon rotenone or H$_2$O$_2$ treatment, PTP1B and PTEN translocate to the plasma membrane from the endoplasmic reticulum and cytoplasm, respectively. Notably, PTP1B and PTEN were recruited to the plasma membrane when MDFs were subjected to rotenone (Fig 5A and B and corresponding inserts), whereas no such translocation to the plasma membrane was observed in nontreated control and H$_2$O$_2$-treated cells (Fig 5A and B) up to fourfold more PTP1B and PTEN were translocated to the membrane of rotenone-treated cells (Fig 5A and B, right panels). Western immunoblot analyses further confirmed the enrichment of PTEN and PTP1B in the membrane fraction of rotenone-treated MDFs compared to nontreated controls and H$_2$O$_2$-treated MDFs (Fig 5C and Supplementary Fig S5A and B). Using co-immunoprecipitation (Co-IP) experiments with IGF-1Rβ antibodies, a physical interaction of PTP1B with IGF-1Rβ was found after rotenone-induced O$_2^·$ stress (Fig 5D), and, as earlier shown, IGF-1Rβ-bound PTP1B displayed higher phosphatase activity in rotenone-treated MDFs (Fig 4D).

Furthermore, transient overexpression of Sod2 with increased O$_2^·$-scavenging capacity (Supplementary Fig S1E) significantly inhibited rotenone-induced membrane translocation of PTP1B and PTEN in MDFs (Fig 6A and B), suggesting a causal contribution of enhanced O$_2^·$ concentrations in the translocation of these two phosphatases to the plasma membrane. The fact that overexpression of Sod2 also improved IGF-1 sensitivity in rotenone-treated MDFs further supports a critical role of enhanced O$_2^·$ concentrations in the activation of these phosphatases with subsequent IGF-1 suppression (Fig 6C). Stimulated by earlier reports that the phosphorylation of serine and threonine residues in the C-terminal domain of PTEN restricts it to the cytoplasm, while dephosphorylation favours its...
Figure 3. Impaired IGF-1 signalling in the skin of fibroblast-specific Sod2-deficient mice.

A To study the impact of enhanced O$_2^-$ concentrations on IGF-1 signalling in vivo, representative skin sections of either saline- or IGF-1-treated Sod2 wild-type control (with normal O$_2^-$ concentrations) and mutant mice (with enhanced O$_2^-$ concentrations in fibroblasts) were stained for pAKT (S473) (red) and DAPI (blue, nuclei). Skin samples were collected 15 min after i.p. injection of 1 mg/ml IGF-1 or saline. Scale bars, 30 lµm. E, epithelium; D, dermis; HF, hair follicle; SC, subcutaneous tissue, n = 5.

B To further confirm the suppressive effect of enhanced O$_2^-$ concentrations on IGF-1 signalling in vivo, skin lysates from control mice (with normal O$_2^-$ concentrations) and mutant mice (with enhanced O$_2^-$ concentrations in fibroblasts) were prepared 15 min or after i.p. injection of 1 mg/ml IGF-1 or saline, and the expression of pAKT (S473) and AKT was analysed by Western blotting and equilibrated to actin expression levels. A representative Western blot out of three independent experiments is shown here. The graph (right panel) depicts densitometric analyses of pAKT/AKT ratio after correction with actin (loading control) for each of the four groups. Comparison was made with two-tailed t-test; respective comparison was indicated with a line and P-value.

Source data are available online for this figure.
membrane localization and activation (Vazquez et al., 2006; Rahdar et al., 2009), we studied whether high O$_2^-$ concentrations had any impact on the phosphorylation status of PTEN. For this purpose, PTEN was immunoprecipitated from the cytoplasmic and membrane fractions of vehicle or rotenone-treated MDFs followed by immunoblotting with antibodies against phospho-serine, phosphothreonine, and phospho-tyrosine. Interestingly, a membrane translocation phosphorylation pattern was found with reduced serine phosphorylation in cytoplasmic and membrane PTEN in rotenone-treated MDFs compared to vehicle-treated MDFs, while no

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**Figure 4. Enhanced superoxide anion flux inhibits tyrosine phosphorylation of IGF-1Rβ through PTP1B and PTEN activation.**

A. Protein levels of PTP1B, PTEN, and PP2A were examined by immunoblotting of lysates derived from MDFs treated with the indicated concentrations of rotenone for 3 h. A representative Western blot is shown out of three independent experiments with similar results.

B–E. Phosphatase activity of (B) PTP1B, (C) IGF-1Rβ-associated PTP1B, (D) PTEN, and (E) PP2A were determined in lysates of MDFs treated with 500 μM rotenone for 3 h. Only PTP1B bound to IGF-1Rβ in the plasma membrane and PTEN, but not total PTP1B and PP2A, revealed significant dephosphorylating activity. Values are mean ± SEM of nmol phosphate release per mg protein, and comparison was made with two-tailed t-test (n = 6); respective comparison was indicated with a line and significance with P-value.
threonine phosphorylation was detected in the membrane fraction of PTEN in both rotenone-treated and control MDFs (Supplementary Fig S6). In the cytoplasmic PTEN fraction, rotenone-treated MDFs showed lower threonine phosphorylation (Supplementary Fig S6). Of note, enhanced tyrosine phosphorylation in the membrane PTEN pool and reduced tyrosine phosphorylation in the cytosolic PTEN were found in rotenone-treated compared to vehicle-treated MDFs (Supplementary Fig S6). These data indicate that the $O_2^-$-dependent

Figure 5.
PTEN translocation from the cytoplasm to the membrane correlates with specific post-translational modifications, the precise nature of which awaits elucidation. Taken together, the O$_2^–$-dependent PTPIB activation mediates IGF-1/AKT suppression at the step of IGF-1R dephosphorylation, while PTEN recruitment and activation at the plasma membrane enhance the dephosphorylation of its phospholipid substrate PIP3 to PIP2, thus suppressing the phosphorylation (activation) of AKT.

**Inhibition of PTPIB and PTEN rescues the superoxide anion-induced IGF-1 resistance**

To further strengthen the causal role of PTPIB and PTEN in the O$_2^–$-dependent IGF-1 resistance, we used pharmacological inhibitors and specific gene silencing and studied the phosphorylation state of AKT, a key step in IGF-1 downstream signalling. Notably, the PTPIB inhibitor (3-(3,5-Dibromo-4-hydroxy-benzoyl)-2-ethylbenzofuran-6-sulfonic acid-(4-(thiazol-2-ylsulfamyl)-phenyl)-amide) at a concentration of 50 μM in part rescued the suppression of AKT phosphorylation and reduced eIF4G levels in MDFs exposed to recombinant IGF-1 in the presence of rotenone (Fig 7A). In addition, the PTPIB inhibitor opposed rotenone-induced suppression of IGF-1Rβ phosphorylation in MDFs stimulated with IGF-1 (Supplementary Fig S7). Similarly, the suppressive action of high O$_2^–$ concentrations on IGF-1-induced AKT phosphorylation was rescued by 150 nM VO-OHpic, a vanadium-based specific PTEN inhibitor (Rosivatz et al, 2006) (Fig 7B). In addition, silencing of PTPIB or PTEN using lentivirus-mediated shRNA expression significantly alleviated the suppressed activation state (phosphorylation) of AKT in MDFs treated with IGF-1 in the presence of rotenone (Fig 7C). These data strongly suggest that enhanced activation of PTPIB and PTEN is causally involved in the O$_2^–$-dependent IGF-1 resistance. To further explore whether our in vitro observations may also have relevance in vivo for tissue homeostasis, we used a genetic approach. Deletion of Sod2 in fibroblasts of the connective tissue-rich skin resulted in skin atrophy, a key feature of ageing, with reduced thickness of all the skin layers (Fig 8A and B). Heterozygous deficiency of PTEN in the Sod2-deficient fibroblasts as achieved by crossing PTEN floxed mice with Col(1)/x2-CreERT transgenic and Sod2 floxed mice (Col(1)/x2-CreERT$^b$; Sod2$^{f/f}$) followed by activation of CreERT with tamoxifen treatment (Supplementary Fig S8A and B) rescued skin atrophy/aging in the double mutant mice (Col(1)/x2-CreERT$^b$;Sod2$^{f/f}$;PTEN$^{+/–}$) compared with Sod2 mutant mice (Col(1)/x2-CreERT$^b$;Sod2$^{f/f}$;PTEN$^{–/–}$) (Fig 8A and B). Of note, genetic heterozygous deficiency of PTEN with reduced PTEN expression or inhibition of PTPIB by specific inhibitor also attenuated the IGF-1 resistance in vivo in a Sod2-deficient murine ageing model (Fig 8C and D). Accordingly, heterozygous PTEN deletion rescued IGF-1-stimulated phosphorylation of AKT in the skin of fibroblast-specific Sod2-deficient mice (Fig 8C and D). Similarly, continuous inhibition of PTPIB with its specific inhibitor at a dose of 15 mg/kg for 5 days also rescued IGF-1-induced phosphorylation of AKT in the skin of fibroblast-specific Sod2-deficient mice (Fig 8C and D). These data show that enhanced O$_2^–$- concentrations in dermal fibroblasts in the skin in vivo via PTEN and PTPIB activation—dampen the IGF-1 signalling also in vivo and most likely contribute to skin ageing/atrophy.

**Discussion**

The major finding of this report is that accumulation of mitochondrial O$_2^–$ led to IGF-1 resistance by tilting the delicate balance of regulatory kinases and phosphatases towards enhanced activities of PTPIB and PTEN, key phosphatases of the IGF-1 signalling pathway. Enhanced activities of PTEN and PTPIB were associated with their membrane translocation. To the best of our knowledge, this finding identifies a previously unreported role of PTPIB and PTEN as molecular links mediating repression of IGF-1 signalling at high superoxide anion (O$_2^–$) concentrations in the mammalian system (Fig 9). The resulting IGF-1 resistance led to reduced fibroblast proliferation, marked suppression of specific s1 (I), s2 (I), and s1 (III) collagen chain mRNA levels, and changed expression of key factors responsible for translation initiation, collectively representing signature events of ageing and organ atrophy. Notably, genetic and pharmacological inhibition of PTPIB and PTEN alleviated...
Figure 6.

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IGF-1 resistance under conditions of enhanced O$_2^-$ concentrations underlining their causal role and making PTP1B and PTEN potential targets for drug development. The finding that Impl2, the Drosophila ortholog of insulin-like growth factor-binding protein 7 (IGFBP7), antagonizes insulin signalling in response to mitochondrial complex I perturbation (Owusu-Ansah et al., 2013) further underscores an evolutionarily conserved linkage between mitochondrial dysfunction and aberrant insulin signalling.

Enhanced O$_2^-$ concentrations have been reported in several physiological and pathological inflammatory conditions (Shishido et al., 1994; Naya et al., 1997; Cheng et al., 1999; MacArthur et al., 2000; Lu et al., 2003; Liu et al., 2004; Sindrilaru et al., 2011). In addition, enhanced O$_2^-$ concentrations were also detected in senescent dermal fibroblasts in vitro and in the fibroblast from connective tissue-specific Sod2-deficient mice (Treiber et al., 2011) and—via dampening IGF-1 signalling—may contribute to age phenotypes of connective tissue-rich organs such as skin, bone, and muscle. This notion is consistent with the finding that these organs are particularly dependent on balanced IGF-1 signalling to maintain tissue homeostasis (Govoni et al., 2007a,b). Enhanced O$_2^-$ concentrations, as shown herein, suppressed fibroblast proliferation and the synthesis of interstitial collagen proteins, the major extracellular matrix proteins of the skin. In fact, suppression of collagen synthesis and deposition as well as reduced proliferation constitute key molecular events in skin ageing (Fisher et al., 2008; Quan et al., 2010). Of note, IGF-1 was earlier reported to stimulate collagen synthesis and fibroblast growth (Gillery et al., 1992), and—if IGF-1 is suppressed by O$_2^-$-induced oxidative stress—results in skin ageing and atrophy. In addition, chronic venous leg ulcers and diabetic ulcers reveal a persistent inflammation with enhanced activation of pro-inflammatory macrophages, and an unrestrained release of O$_2^-$, which at persistent high concentrations, overruns the O$_2^-$-dissmutating capacity of non-enzymatic and enzymatic antioxidants (Sindrilaru et al., 2011). Hence, O$_2^-$ is the prevailing reactive oxygen species in chronic wounds and according to the herein reported data may dampen the signalling of IGF-1 and other growth factors and impair tissue repair processes. This notion is consistent with previous reports that many clinical trials with recombinant growth factors for the treatment of chronic wounds either failed or required extremely high concentrations of recombinant growth factors for substitution, indicating growth factor resistance of chronic wounds (Bitar & Al-Mulla, 2012). This poor outcome may at least in part be due to an O$_2^-$-dependent enhanced phosphatase activation with subsequent attenuation of growth factor signalling.

In this study, we were interested in the role of distinct ROS such as mitochondria-derived O$_2^-$ and H$_2$O$_2$ on IGF-1 sensitivity and downstream signalling. Strikingly, as opposed to the established H$_2$O$_2$-mediated enhanced insulin/IGF-1 sensitivity (Loh et al., 2009), we found that high O$_2^-$ concentrations following rotenone-mediated inhibition of mitochondrial complex I or by silencing of O$_2^-$-dismutating mitochondrial Sod2 resulted in IGF-1 resistance. Indeed, we identified enhanced activities and membrane localization of PTP1B and PTEN following high O$_2^-$ load, and interestingly, this effect was abolished by overexpression of Sod2. IGF-1 resistance was also observed in Sod2-deficient fibroblasts with significant accumulation of mitochondrial O$_2^-$, but not of H$_2$O$_2$ as proven by ROS-specific quantitative techniques (Treiber et al., 2011). Moreover, scavenging of mitochondrial O$_2^-$ by the overexpression of Sod2 in the mitochondria of rotenone-treated murine dermal fibroblasts attenuated the observed IGF-1 resistance. Rotenone may not only suppress complex I of the electron transfer chain to enhance intramitochondrial O$_2^-$ concentrations. From a previous publication, rotenone has been implicated in enhancing the NADPH oxidase in microglial cells of the brain with enhanced O$_2^-$ release into the extracellular space with subsequent neuron degeneration (Gao et al., 2003). Even though we cannot exclude this or other rotenone effects in our study, we provide consistent evidence that the rotenone-mediated inhibition of complex I with accumulation of high O$_2^-$ concentrations or O$_2^-$ accumulation by silencing of O$_2^-$-dismutating mitochondrial Sod2 resulted in IGF-1 resistance.

Our conclusion to have identified a novel route of O$_2^-$ signalling with an O$_2^-$-dependent increase of PTP1B and PTEN activity is based on several lines of evidence. First, in the case of PTP1B, we found a translocation of PTP1B from the ER to plasma membranes and co-localization with IGF-1Rβ. This translocation is indicative of PTP1B activation (Buckley et al., 2002; Yudushkin et al., 2007). Secondly, using co-immunoprecipitation, a physical interaction of PTP1B with the membrane-associated IGF-1Rβ and higher
To assess the specificity of $O_2^*$-dependent PTP1B activation with subsequently suppressed IGF-1 signalling, the levels of IGF-1 downstream effectors, such as pAKT (S473) and AKT, and eIF4G, the translation initiation protein, were determined by Western blotting of lysates from MDFs treated either individually or in combination with 100 ng/ml IGF-1, 50 µM PTP1B inhibitor, and 500 µM rotenone as indicated for 3 h. The representative experiment shown here is one of two independent experiments performed.

The identical experimental setting was used as described in (A) apart from using the specific PTEN inhibitor VO-OHpic (150 nM) instead of the PTP1B inhibitor. Similar to the PTP1B inhibitor in (A), the PTEN inhibitor almost completely alleviates the $O_2^*$-dependent suppression of the IGF-1 signalling. The representative experiment shown here is one of two independent experiments performed.

To further confirm the role of $O_2^*$-dependent PTP1B and PTEN activation on subsequent suppression of IGF-1 signalling, Western blot analyses for pAKT (S473), AKT, PTEN, and PTP1B expression in lysates from cells lentivirally transduced with the PTP1B-silencing shRNA (left panel), PTEN silencing shRNA (right panel), or with a scrambled non-targeting shRNA plasmid (control) treated with 100 ng/ml IGF-1 in the absence or presence of 500 µM rotenone for 3 h were performed with actin serving as a loading control. This representative Western blot is one of three independent experiments ($n = 3$) with similar results.

Source data are available online for this figure.
**Figure 8.**

**A**

![Images showing control and mutant tissues with double mutant comparison](image)

**B**

| Tissue          | Control | Mutant | Double Mutant |
|-----------------|---------|--------|---------------|
| Epidermis       | ![Graph](image) | ![Graph](image) | ![Graph](image) |
| Dermis          | ![Graph](image) | ![Graph](image) | ![Graph](image) |
| Subcutaneous    | ![Graph](image) | ![Graph](image) | ![Graph](image) |
| Panniculus carnosus | ![Graph](image) | ![Graph](image) | ![Graph](image) |

**C**

![Images showing saliné and IGFI-1 effects on pAKT signaling](image)

**D**

![Graphs showing pAKT and AKT expression](image)
phosphatase activity of the IGF-1Rβ-bound PTEN1B fraction was observed further supporting our hypothesis and suggesting a key role of PTEN1B in O2•−-driven repression of IGF-1 signalling. Finally, inhibition of PTEN1B either by a pharmacological inhibitor or through shRNA-mediated knockdown reversed the O2•−-induced IGF-1 resistance. The conclusion that PTEN activity was also increased at high O2•− concentrations is supported by the following results. First, translocation of PTEN from the cytosol to the plasma membrane indicating PTEN activation (Das et al., 2003; Vazquez et al., 2006; Rahdar et al., 2009) was consistently observed in fibroblasts exposed to enhanced O2•− but not to enhanced H2O2 concentrations. Second, higher activity of PTEN was found in rotenone-treated MDFs. Third, the PTEN-specific inhibitor, VO-Ohpc, significantly attenuated the O2•−-dependent suppression of IGF-1 signalling. VO-Ohpc is a highly selective inhibitor for PTEN and only inhibits other PTPs at concentrations within the high micromolar range (Rosivatz et al., 2006). Fourth, similar results were also observed upon shRNA-mediated PTEN silencing. Importantly, partial deletion of PTEN and inhibition of PTEN1B in Sod2-deficient mice also rescued the IGF-1 resistance and the atrophy/aging skin phenotype. Thus, repression of IGF-1 signalling by a high O2•− load is predominantly mediated by the active participation of PTEN1B and PTEN. Taken together, our results unify and explain earlier reports on increased IGF-1 resistance upon ROS induction (Tiganis, 2011; Johnson & Olefsky, 2013) by suggesting that enhanced O2•− concentrations are largely responsible for repression of IGF-1 signalling and that the O2•−-mediated IGF-1 resistance is mainly due to aberrant PTEN1B and PTEN translocation and activation. The precise (Vazquez et al., 2006; Rahdar et al., 2009) mechanism though of PTEN1B and PTEN translocation in response to elevated O2•− concentrations from the cytoplasm to the plasma membrane needs to be further elucidated.

As to the question how increased O2•− concentrations in the mitochondria transfer the signal to the cytoplasm, it is possible that enhanced mitochondrial O2•− concentrations lead to modifications of kinases, which shuttle between the mitochondria and the plasma membrane exerting phosphorylation and activation of PTEN1B and PTEN—the nature of these anticipated kinases remains, however, elusive. Alternatively, longer-lived lipid peroxidation intermediates may transmit the O2•−-induced signals from the mitochondria to cytosolic and/or membrane-bound PTEN1B/PTEN. Notably, O2•− is able to initiate lipid peroxidation by itself, or after reacting with nitric oxide (NO) to form peroxynitrite (ONOOC•−) (Buettner et al., 2004; Bashan et al., 2009). A recent report suggests that O2•− can escape mitochondria and enter the cytosol through mitochondrial voltage-dependent anion channel (Lustgarten et al., 2012).

Regarding the question why evolution has promoted the development of an O2•−-dependent partial IGF-1 resistance, it is important to consider that increased O2•− concentrations are extremely noxious to cells. O2•−-mediated repression of IGF-1 signalling through PTEN1B and PTEN activation might have salutary effects on cells with perturbed mitochondria as is the case in type 2 diabetes mellitus, ageing, impaired wound healing, and neurodegenerative disease. In this context, it can act as an oxidant defence mechanism that protects cells by attenuating further ROS generation from increased metabolic or anabolic activity. Also, it can help to promote clearance of damaged cells by apoptosis or by other mechanisms. In fact, lower levels of phosphorylated AKT enhance apoptosis (Franke et al., 1997). Thus, O2•−-dependent PTEN1B and PTEN activation with the induction of IGF-1 resistance may serve as a reliable guardian to protect cells and tissues from O2•−-driven oxidative stress and its deleterious consequences.

In summary, we uncovered a mechanism by which enhanced O2•− released from dysfunctional mitochondria causes IGF-1 resistance, thereby repressing IGF-1 signalling in mammalian cells. Our studies provide insight into a previously undescribed O2•−-dependent activation of PTEN1B and PTEN with subsequent repression of IGF-1 signalling. Targeting these key players may hold promise for the development of novel therapies for age-related pathologies with aberrant IGF-1 signalling such as type 2 diabetes, skin atrophy, osteoporosis, impaired wound healing, and neurodegenerative disease. Unquestionably, future studies should address detailed molecular interactions and identify additional intermediate signalling.
molecules engaged in $\text{O}_2^-$-dependent repression of IGF-1 signalling. In addition, it will be exciting to further explore whether—in addition to the IGF-1 dampening—enhanced $\text{O}_2^-$ concentrations may affect other receptor tyrosine kinases involved in tissue homeostasis.

Materials and Methods

Animal experiments

An inducible connective tissue-specific Sod2-deficient mouse model was used for in vivo experiments. This mouse line (Col(I)a2-CreERT$^+$; Sod2$^{-/-}$) was generated by crossing Col(I)a2-CreERT transgenic mice (Zheng et al., 2002) with Sod2 floxed mice (Strassburger et al., 2005; Treiber et al., 2011). The generation of Sod2 and PTEN double-mutant mice (Col(I)a2-CreERT$^+$;Sod2$^{-/-}$;PTEN$^{-/-}$) was performed by crossing Sod2-deficient mice (Col(I)a2-CreERT$^+$;Sod2$^{-/-}$) with PTEN flox mice (PTEN$^{f/+}$) (Groszer et al., 2001). All these mice were backcrossed to C57BL/6J for at least 10 generations and were maintained in the Animal facility of University of Ulm with 12 h light–dark cycle and SPF condition. All the animal experiments were approved by the animal ethical committee (Regierungspräsidium Tübingen, Germany). The genotyping of the mice was performed using standard PCR techniques. The sequences of the primers used in PCR-based genotyping are summarized in Supplementary Table S1. In brief, DNA from the tail tip of an individual mouse was purified using a commercial kit (Easy DNA kit, Invitrogen). The purified DNA was later dissolved in TE and used for PCR amplification. The PCR products were run in QIAxcel Advance system (Qiagen) using the program AM320 and then documented digitally. To initiate the activation of CreERT and deletion of Sod2 and PTEN, newborn mice (10 days old) were orally administered with 50 $\mu$l of 1 mg/ml of 4-OH tamoxifen (Sigma) suspended in 0.5% methylcellulose and 0.1% Tween-80 (Sigma) for five alternate days. When the mice (both male and female) were 21–25 days old, the mice were subcutaneously implanted with single 60-day release 4-OH tamoxifen pellets (Innovative Research of America). At the age of 65–70 days, mice of both sexes were intraperitoneally (i.p.) injected either with 100 $\mu$l of 1 mg/ml recombinant IGF-1 in normal saline or with equal volume of normal saline. Mice were sacrificed 15 or 60 min post-injection, and skin tissues were collected and processed for paraffin embedding and protein lysate preparation.

Isolation and culture of murine dermal fibroblasts

Murine dermal fibroblasts (MDFs) were isolated from ear skin of young mice and cultured as previously described (Treiber et al., 2011).

Silencing by shRNA

Silencing of Sod2, PTEN, and PTP1B was performed using lentivirus vector-based shRNA clones from the RNAi consortium (TRC, Broad Institute) (details in Supplementary Methods).

Serum starvation and IGF-1 stimulation

Murine dermal fibroblasts were washed with PBS and serum-starved for 14 h before stimulation. Stimulation with recombinant mouse IGF-1 (ProSpec) was performed at 100 ng/ml concentration for indicated time points.
**The paper explained**

**Problem**
The evolutionarily conserved IGF-1 signalling pathway is associated with longevity, metabolism, tissue homeostasis, and cancer progression. Its action is tightly regulated in multiple steps of activation and inhibition and, when dysregulated, results in organ atrophy with enhanced ageing or cancer progression. During ageing, reduced growth and regeneration occur, which lead to organ and skin atrophy characterized by wrinkle formation, reduced tensile strength of the skin, and impaired wound healing. A reduced function of IGF-1 has long been postulated to inhibit cell proliferation and synthesis of extracellular matrix proteins like collagens in ageing organs; however, the exact mechanisms of IGF-1 suppression are still poorly understood. Of note, the gradual increase in oxidative stress is now established during the ageing process and several pathologies associated with ageing and oxidative stress in elderly individuals. Here, we addressed the impact of oxidative stress on IGF-1 function, its signalling, and on cellular key features of skin ageing. The detailed understanding of the IGF-1 action during oxidative stress in skin ageing and other oxidative stress-related pathologies is of high clinical importance as it holds promise for the development of preventive and/or therapeutic strategies aiming at the delay and reversal of declining skin function.

**Results**
Of note, distinct entities of reactive oxygen species modulate IGF-1 function. Hydrogen peroxide (H2O2) activates IGF-1 signalling by inhibiting distinct phosphatases involved in IGF-1 suppression. By contrast, accumulation of mitochondrial O2- as occurs in ageing skin inactivates IGF-1 signalling. Increasing mitochondrial O2- either by chemical means (rotenone treatment) or by genetic silencing of Sod2, the enzyme which normally detoxifies O2- in murine dermal fibroblasts, leads to the activation and membrane translocation of the two phosphatases PTP1B and PTEN. PTP1B activation and membrane translocation, in fact, lead to its close association with the membrane-anchored IGF-1 receptor; this in turn promotes the dephosphorylation (inactivation) of the IGF-1 receptor (IGF-1R β subunit) with an almost complete abrogation of IGF-1 signalling and subsequent suppressed growth of skin fibroblasts. In addition, PTEN dephosphorylates PIP2 to PIP in a later step of IGF-1 signalling, leading to inhibition of Akt phosphorylation (activation), a key step essential for cell growth. Attenuation of O2--mediated inhibition of IGF-1 signalling and IGF-1 resistance by overexpression of Sod2 further supports the causal contribution of increased O2- concentrations to IGF-1 resistance. Inhibition of PTP1B and PTEN by specific chemical inhibitors as well as silencing by shRNA in vitro significantly rescues IGF-1 resistance even in the presence of higher mitochondrial O2-.

**Impact**
Our novel finding is that different entities of reactive oxygen species, such as H2O2 and O2-, exert opposite effects on the IGF-1 signalling pathway. Our studies provide insight into a previously undescribed O2--dependent activation of PTP1B and PTEN with subsequent repression of IGF-1 signalling. Targeting these key players may hold substantial promise for the development of novel therapies for age-related pathologies with aberrant IGF-1 signalling such as type 2 diabetes, skin atrophy, osteoporosis, impaired wound healing, neurodegeneration, and cancer.

**Membrane, cytosolic, and nuclear fractionation**
Murine dermal fibroblasts were harvested, and then the membrane and cytosolic extracts were prepared using the Mem-PER extraction reagents according to the manufacturer’s instructions (Thermo Scientific). Thereafter, 30 µg of cytosolic lysate or membrane extracts was subjected to Western blot analyses.

**Activity assay of phosphatases**
Phosphatases activity assays were performed in immunoprecipitated protein samples (details in Supplementary Methods).

**Immunofluorescence staining and analyses**
Immunofluorescence staining was performed as previously described (Treiber et al., 2011).

**Quantitative PCR**
Quantitative PCR was performed as previously described (Treiber et al., 2011) with slight modification (details in Supplementary Methods).

**Cloning and overexpression**
Open reading frame (ORF) of mouse Sod2 was PCR-amplified from its cDNA IMAGE clones (BC010548), and cloned in pcDNA3.1 (Invitrogen) (details in Supplementary Methods).

**Cytotoxicity assay**
The viability of murine dermal fibroblasts was measured 3, 6, 12, and 24 h after incubation with rotenone, H2O2, PTP1B inhibitor, and VO-OHpic using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) (details in Supplementary Methods).

**Statistical calculations**
All data were calculated using Graphpad Prism (GraphPad Software) or Signamap (Systat Software Inc) and presented as mean ± standard error of mean (SEM). Statistical significance (P-value) was calculated using either one-way ANOVA, followed by Bonferroni correction for comparing the difference between more than two samples or by unpaired two-tailed Student’s t-test for comparing the difference between two samples. For two-tailed t-test, the exact P-values and for ANOVA, in multiple comparison test (Bonferroni), either the exact P-value (when P > 0.001, but < 0.05) or the highest P-value (P < 0.001) were presented. The exact test which was used and the exact P-value of comparison are presented in the respective figure legends and figures, respectively.

**Supplementary information**
For this article is available online: http://embomolmed.embopress.org

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Author contributions
KS and PMA designed and carried out the experiments and wrote the initial version of the manuscript. LK, PMe, AB and NT technically supported this work. TL was involved in lentiviral works. SK, MW, and HG were involved in designing and technically supporting the experiments. KS-K designed the experiments and wrote the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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