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# Induction of Prdx1 and Prdx6 in Liver Cells by Serum and TPA

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**Abstract**

Peroxiredoxins are thiol-specific antioxidants that protect cells from oxidative damage and have proliferative and anti-apoptotic activity. We investigated the effect of serum and phorbol ester treatment on expression of Prdx1 and Prdx6 in H2.35 cells, and the possible role of Sp1 on Prdx6 induction. Serum stimulation induced a 30% increase in Prdx1 mRNA and a three-fold increase in Prdx6 mRNA. We showed a similar effect of phorbol ester treatment, which led to a 30% increase in Prdx1 mRNA, and over a two-fold increase in Prdx6 expression. Analysis of the Prdx6 proximal promoter sequence revealed four consensus Sp1 sites. Inhibition of Sp1 with mithramycin A blocked Prdx6 induction by TPA and inhibited the serum-induced transcriptional activity of the Prdx6 proximal promoter. These data suggest an important role for Prdx6 in the cellular response to serum and TPA, and implicate Sp1 as a possible mediator of Prdx6 regulation.

**Keywords:** Peroxiredoxin, Prdx1, Prdx6, H2.35, Antioxidant, TPA, Serum

## 1. Introduction

Reactive oxygen species (ROS) are unstable free radical derivates of oxygen formed as byproducts of many biochemical processes in the cell. ROS serve critical functions for many types of cells (Halliwell and Gutteridge, 1999), and recently have been implicated as key regulators of signal transduction (Immenschuh and Baumgart-Vogt, 2005). However, when concentrations of ROS become too high, macromolecules can become oxidized, causing detrimental cellular effects such as cell death (Halliwell and Gutteridge, 1999). These consequences have pathophysiological relevance, as oxidative stress is linked to the development of a number of diseases including cancer and neurodegenerative disease (Cross et al., 1987; Sun and Chen, 1998; Gius and Spitz, 2006).

Peroxiredoxin proteins are thiol-specific antioxidant enzymes that reduce various cellular peroxide substrates using cysteine-containing active sites (Hofmann et al., 2002; Rhee et al., 2005). Mammals possess six members of the peroxiredoxin gene family, and the encoded proteins are classified into three groupings based on the number and position of active site cysteines: the 2-Cys type (*Prdx1-4*), the atypical 2-Cys Prdx (*Prdx5*), and the 1-cys member (*Prdx6*). In addition to their structural differences, these proteins vary in peroxide substrate specificity, cellular localization, tissue expression, and additional enzymatic activities (Hofmann et al., 2002; Rhee et al., 2005). Based on their functions,

peroxiredoxins can protect cells from ROS-induced cell damage and apoptosis, and regulate redox sensitive cell signaling involved in processes such as cell proliferation, differentiation, and transformation (Hofmann et al., 2002; Immenschuh and Baumgart-Vogt, 2005; Rhee et al., 2005). They now have been implicated in the modulation of disease states such as neurodegenerative diseases and cancer (Karihtala et al., 2003; Kinnula et al., 2004; Arner and Holmgren, 2006).

Prdx6 is the only peroxireodoxin that contains a single conserved cysteine residue, and it utilizes glutathione as a reductant (Kang et al., 1998; Hofmann et al., 2002; Rhee et al., 2005). Unlike other peroxiredoxins, Prdx6 reduces both aqueous and lipid peroxides and possesses distinct phospholipase A2 activity (Chen et al., 2000; Manevich et al., 2004; Manevich and Fisher, 2005). As a result of these functions, Prdx6 appears to be involved in phospholipid metabolism and membrane turnover (Manevich et al., 2002; Manevich and Fisher, 2005), and we and others have demonstrated that Prdx6 protects cells from ROS-mediated membrane damage and apoptosis (Manevich et al., 2002; Pak et al., 2002; Phelan et al., 2003; Wang et al., 2003; Wang et al., 2004; Wang et al., 2006; Wang et al., 2006). Prior studies from our lab have reported an abundance of Prdx6 in liver (Sparling and Phelan, 2003; Wang et al., 2003), and its transcriptional regulation by various oxidative stresses in H2.35 cells, a virally transformed mouse hepatocyte cell line (Sparling and Phelan, 2003; Simeone and Phelan, 2005; Gallagher and Phelan, 2007). A recent report demonstrated that Prdx6-knockout mice exhibit a significant increase in hepatocellular injury (Eismann et al., 2009), confirming an important antioxidant role for this protein in liver. Prdx1, the most ubiquitous peroxiredoxin, is also highly expressed in liver (Ishii et al., 1993; Ishii et al., 1995; Ishii et al., 2000), although its regulation has largely been studied in non-liver tissues. Originally named PAG (proliferation associated gene) in human (Prospéri et al., 1993), Prdx1 is overexpressed during cell proliferation and is transcriptionally induced by serum in human mammary epithelial cells (Prospéri et al., 1993). Prdx1 is also induced by the tumor promoter phorbol-12-myristate-13-acetate (TPA) in liver tissue macrophages (Hess et al., 2003). Since liver tissue is susceptible to high levels of ROS associated with normal cellular physiology and acute and chronic injury (Novo and M, 2008), we sought to compare Prdx1 and Prdx6 induction by serum and phorbol ester in H2.35 cells. Given that these proteins represent two distinct members of the peroxiredoxin family expressed in liver, understanding their regulation in hepatocytes can provide important insight into their relative roles in the cellular response to stress in this tissue.

## 2. Materials & Methods

### *2.1 Cells, Inducers & Inhibitors*

The H2.35 hepatocyte cell line (ATCC) was cultured in DMEM with 4% FCS and 0.2μM dexamethasone, and grown at 33°C in a 10% $CO_2$ atmosphere. Phorbol-12-myristate-13-acetate (TPA) (Cell Signaling Technologies) was supplied as a 200 μM stock in DMSO. Mitrhamycin A (Tocris) was dissolved in DMSO to 10 mM, and subsequently diluted to 10 μM in PBS.

### *2.2 Real-Time PCR*

For real-time PCR studies, H2.35 cells were plated into 96-well plates at a cell density of approximately 20%, and cultured under appropriate experimental conditions. At the time of harvest, media was removed and adherent cells were rinsed with cold 1X PBS. Cell lysates and reverse transcriptase reactions were performed using the Cells-to-CT Kit (Applied Biosystems). Briefly, cells were lysed in Cell Lysis solution containing DNAseI for five minutes, followed by two-minute incubation with the stop solution. Cell lysates were immediately used for RT reactions, or were stored temporarily at -20 degrees, to be used within 2 weeks. Fifty-microliter reverse transcription reactions were performed using 10 μl of each cell lysate, according to the manufacturer's suggestions. Twenty-μl PCR reactions were performed in triplicate for each target gene using 2 μl RT reaction, 7 μl water, 10 μl of 2X Taqman PCR buffer (Cells-to-CT kit), and 1 μl of the appropriate mouse TaqMan assay: Prdx1 (Mm01621996_s1), Prdx6 (Mm00725435_s1), beta-actin endogenous control (4352933E), or 18s control (4333760F). To ensure that PCR amplification was not due to genomic DNA contamination, parallel RT-reactions were performed in the absence of reverse transcriptase for each experiment. In all cases, reactions from RTs lacking reverse transcriptase amplified no product, or resulted in cycle threshold (Ct) values beyond 35 and seven CTs higher than the positive reactions, representing negligible genomic DNA contamination. Relative expression was calculated using the $C_T$ method (Livak and Schmittgen, 2001), with each target gene normalized against beta-actin levels for the same sample. An appropriate calibrator was chosen for each analysis, and all other samples were calibrated against that sample. All treatments were done in triplicate wells, and experiments were repeated.

### *2.3 Western Blotting*

H2.35 cells were cultured in 60 mm dishes to near confluence under appropriate experimental conditions. Media was discarded, and cells were washed with 2 mL of cold 1X PBS. Cells were lysed in 100 μl of M-PER reagent (Thermo Scientific) at room temperature rocking for 10 minutes, and lysates were collected, spun to remove cell debris, and supernatants transferred to tubes. Total protein was quantified using the Biorad Protein Assay (Biorad) according to manufacturer's recommendations. Twenty-five μg of protein from each sample were separated on 10% tris-HCL gel (Biorad) under denaturing conditions and transferred to PVDF membranes. Membranes were blocked for 15 minutes in

4% skim milk in TBST, and incubated overnight at 4°C with 1:1,000 dilution of Prdx6 antibody (Biosensis, R-169-100) and 1:5,000 dilution of beta-actin antibody (Sigma, A5441) diluted in 1% dry milk in TBST. Blots were rinsed 3 x15 minutes in TBST and incubated for one hour at room temperature with alkaline phosphatase-linked secondary antibodies (Sigma) diluted in 1% dry milk in TBST. Blots were again rinsed 3 x 15 minutes in TBST, followed by 5 minutes in TBS, and then BCIP/NBT substrate was added until band appearance. Reactions were stopped with distilled water.

*2.4 Synthesis of Deletion Constructs & Transfections*

Various deletions of the *Prdx6* mouse promoter had previously been cloned into the pSEAP2-Basic vector (Clontech) by our laboratory, as previously described (Gallagher and Phelan, 2007). These included constructs containing the mouse *Prdx6* promoter fragment that extends to nucleotide position -184 (relative to the +1 transcription start site). Primers used to amplify the -184 fragment were: Prom-184 forward (5'-CGCTCGAGTACAAGTCCCCGCAATTCTC-3') and PromReverse (5'- CGAAGCTTGTGGTGACGC TGAGAACAAGG-3'). The PCR products were amplified and cloned into the pSEAP2-Basic reporter vector (Clontech) as previously described. H2.35 cells were seeded into 48-well cell culture plates and grown overnight. For each transfection, 0.4 μg of the -184-SEAP2 construct and 0.1 μg of the pGluc-control plasmid were mixed with lipofectamine 2000 reagent (Invitrogen) according to manufacturer's suggestions. Cell media in each well was replaced with either normal growth media or serum free/dexamethasone free media and transfection mixes were added. For Sp1-inhibition studies, mithramycin A was added two hours after transfection. Transfected cells were incubated under normal growth conditions for five days and media collected and spun to remove debris.

*2.5 Reporter Assays*

The QUANTI-Blue Reagent (Invivogen) was used to measure SEAP levels in cell supernatants. The reagent was freshly prepared from the supplied powder by dissolving one packet in 100 ml sterile water, and filtering through a 0.2 μm membrane. For each assay, 20 μl of sample media were added to 200 μl of QUANTI-Blue reagent, and incubated at 37°C for 24 hours. The absorbance at 655 nM was measured for each sample, and blanked against reagent plus 20 μl appropriate media. For luciferase expression, the Gaussia Luciferase Assay was used (New England Biolabs). Briefly, 15 μl of each media sample was added to a separate well of a 96 well flat bottom white plate. Using the Tecan Infinite F200 microplate reader (Tecan), 50 μl of reconstituted luciferase substrate was auto-injected into each well, mixed for 2 seconds, and integrated for 5 seconds to measure luminescence. All SEAP levels were normalized against relative luciferase levels.

*2.6 Sequence Analysis*

The proximal 184 nucleotides of the mouse Prdx6 promoter were analyzed for putative transcription factor binding sites using TFSEARCH version 1.3 (Heinemeyer et al., 1998).

**3. Results**

*3.1 Induction of Prdx1 and Prdx6 mRNA by Serum and TPA*

We previously determined that Prdx6 expression is significantly inhibited in H2.35 cells upon serum deprivation (Gallagher and Phelan, 2007). To quantify this effect and compare this response to that of Prdx1, we examined the expression of both genes in the presence and absence of serum. As shown in Figure 1A, Prdx1 expression is moderately, but significantly, increased (by approximately 30%) in complete growth media as compared to serum-deprived conditions ($p<0.05$). In contrast, Prdx6 expression is elevated three-fold in the presence of serum ($p<0.001$). These data indicate that Prdx6 is more highly elevated in proliferating vs. quiescent cells as compared to Prdx1. Since Prdx1 is regulated by TPA in other cells (Hess et al., 2003), we also examined the regulation of Prdx1 and Prdx6 by TPA in H2.35 cells. As shown in Figure 1B, TPA also induced the expression of both genes, but with a much greater effect on Prdx6. Prdx1 was upregulated by TPA by approximately 30% after 8 hours ($p<0.005$). After 8 hours of TPA treatment, Prdx6 was induced approximately two-fold ($p<0.005$). These data suggest that both genes may be involved in the cellular response to these stimuli, but possibly to different degrees.

*3.2 Induction of Prdx1 and Prdx6 Protein by Serum and TPA*

To determine if the significant increase in Prdx6 mRNA in response to serum and TPA resulted in a corresponding protein induction, we used western blotting to measure Prdx6 protein levels before and after stimulation. As shown in Figure 2A, the addition of serum to serum-deprived H2.35 cells for eight hours led to an increase in Prdx6 protein expression. Likewise, eight hours of TPA stimulation also resulted in an increase in Prdx6 protein levels. This data suggests that serum and TPA result in a marked increase in the amount of Prdx6 protein in H2.35 liver cells.

*3.3 Identification of Sp1 Sites in Prdx6 Promoter*

Based on the marked upregulation of Prdx6 in growing cells and in response to TPA, we were interested in identifying potential transcriptional regulators of Prdx6 that may mediate its induction by one or both of these stimuli. We previously

synthesized several deletion constructs of the mouse Prdx6 promoter, linked to the pSEAP2-basic reporter gene (Gallagher and Phelan, 2007). We analyzed the smallest of these fragments, containing the proximal 184 nucleotides of the mouse Prdx6 promoter, for consensus transcription factor binding sites against the TransFac4.0 database (Heinemeyer et al., 1998). We found three putative Sp1 sites in this sequence, shown in Figure 3 (with the location of the consensus Sp1 sites indicated). In addition, we identified consensus E-box sequences which are putative binding sites for Upstream Stimulatory Factor (USF) and Myc.

*3.4 Effect of Mithramycin A on Prdx6 Induction*

To test whether Sp1 is involved in the regulation of Prdx6 in growing H2.35 cells, we compared SEAP reporter expression from the SEAP-184 construct in the presence and absence of Mithramycin A, a potent Sp1 inhibitor. As shown in Figure 4A, SEAP expression driven by this proximal promoter is almost completely inhibited by Mithramycin A ($p<0.05$). There is no significant difference between serum-stimulated reporter expression in the presence of mithramycin A and levels found in the absence of serum, suggesting that Sp1 sites in the proximal promoter are largely responsible for the transcriptional activity of this region in growing cells. We also wanted to test whether Sp1 is also involved in the induction of Prdx6 in response to TPA. Experiments using TPA induction of the SEAP-184 construct resulted in no induction (data not shown) suggesting that this proximal promoter is not sufficient to drive induction by TPA. Therefore, we measured Prdx6 expression by real time PCR in serum-deprived cells treated with and without mithramycin A. As shown in Figure 4B, Mithramycin A treatment partially inhibited the TPA-stimulated induction of Prdx6 ($p<0.05$). Although the level of Prdx6 expression in the presence of mithramycin A was significantly suppressed, it was not as low as serum-deprived levels (data not shown).

## 4. Discussion

*4.1 Summary*

The mammalian peroxiredoxin family of proteins includes structurally and functionally distinct members that protect different cell types from oxidative stress-induced damage. However, the precise role for these proteins in different cells, and the mechanism by which they are transcriptionally regulated in response to oxidative insult, remains unclear. In the present study, we compared the regulation of Prdx1 and Prdx6 in the H2.35 cell line using real time PCR. We showed that both genes are induced at the mRNA level in response to serum and TPA, with a much more robust effect on Prdx6. Prdx6 induction translated into a corresponding increase in Prdx6 protein expression. We further showed that treatment with mithramycin A resulted in a partial block of TPA-stimulated induction of Prdx6. Examination of the proximal Prdx6 promoter containing four Sp1 sites further showed complete inhibition of serum-stimulated promoter activity upon mithramycin A treatment. Together, these data suggest that Prdx6 is particularly responsive to serum and TPA stimulation in H2.35 cells, and implicate Sp1 as a possible mediator of Prdx6 induction.

*4.2 Regulation of Peroxiredoxins by Serum*

The cellular regulation of peroxiredoxin activity is known to occur at many levels, suggesting that tight control of this family is critical (Rhee et al., 2005). A wide variety of oxidative stress-inducing agents, including growth factors and cytokines, stimulate peroxiredoxin expression at the level of gene expression. Prdx1 mRNA is upregulated by hypoxia, $H_20_2$, and tert-BOOH (Prosperi et al., 1998; Li et al., 2002; Rhee et al., 2005; Kim et al., 2007), as well as serum (Prosperi et al., 1998). Likewise, Prdx6 expression is induced by hyperoxia, $H_20_2$, and nitric oxide (Kim et al., 2002; Kim et al., 2003; Sparling and Phelan, 2003; Simeone and Phelan, 2005; Chowdhury et al., 2007; Diet et al., 2007; Gallagher and Phelan, 2007; Chowdhury et al., 2009), and also by growth stimuli including keratinocyte growth factor (KGF) (Frank and Werner, 1997; Munz et al., 1997; Sparling and Phelan, 2003; Gallagher and Phelan, 2007), lens epithelium-derived growth factor (LEDGF) (Fatma et al., 2001), and TNF-$\alpha$ (Kubo et al., 2006; Gallagher and Phelan, 2007). One of the most significant differences we observed between Prdx1 and Prdx6 in the present study is their expression in growing vs. quiescent cells. The observation that Prdx6 is three times higher in growing H2.35 cells vs. serum deprived cells suggests an important role for Prdx6 in this growth response. We previously reported the upregulation of Prdx6 in H2.35 cells by serum (Sparling and Phelan, 2003; Simeone and Phelan, 2005; Gallagher and Phelan, 2007), and others have demonstrated increased Prdx6 in hyperproliferative epithelium of wounded and psoriatic skin (Kumin et al., 2006). In addition, Prdx6 levels seem to be inversely correlated with TGF-$\beta$ levels in the lens (Kubo et al., 2006). Together, these data implicate Prdx6 in the maintenance of cellular homeostasis in proliferating cells.

*4.3 Regulation of Peroxiredoxins by TPA*

TPA is both a known tumor promoter and inducer of apoptosis in various cell types. Although the mechanism by which it elicits these two effects is unclear, the requirement of ROS in TPA-induced toxicity has been demonstrated (Lin et al., 2006). Induction of Prdx1 by TPA in H2.35 cells is not surprising given a prior study reporting Prdx1 induction by TPA in rat liver tissue macrophages and monocytic cells through a PKC/Ras/p38MAPK pathway (Hess et al., 2003). While this study reported nearly eight-fold induction by six hours, our results suggest a more modest effect on Prdx1 in H2.35 cells. The more dramatic induction of Prdx6 by TPA may represent the relative importance of these proteins in these cells, or

may simply suggest a more critical role for Prdx6 in the TPA response, as prior studies have not examined induction of Prdx6 by TPA in any cell line.

*4.4 Potential Role of Sp1 in Prdx6 Regulation by Serum*

Previous studies from our lab identified the proximal -184 bp of the mouse Prdx6 promoter as sufficient to drive reporter expression in growing H2.35 cells. Our present observations that mithramycin A inhibits this induction to a level comparable to that observed in serum-derived cells implicates Sp1 as a possible regulator of this activity. Sp1 is a redox-regulated transcription factor that has been implicated in the control of cell-cycle genes in normal cell proliferation and cancer (Safe and Abdelrahim, 2005). A prior study demonstrated that the middle Sp1 site bound to Sp1 in gel shift assays (Lee et al., 1999), although the other sites were not examined and corresponding transcriptional activity was never reported. Given its known role in proliferation, it is possible that Sp1 activity is upregulated in growing H2.35 cells and induces Prdx6 as a mechanism of balancing the increased cellular ROS. Since mithramycin A binds to many G-C rich DNA sequences, it is also possible that one or more other DNA binding proteins are responsible for, or contribute to, the serum-stimulated Prdx6 promoter activity seen in H2.35 cells. In addition to the Sp1 sites, the proximal promoter includes two consensus E-box sequences for Upstream Stimulatory Factor (USF) and Myc (shown in Figure 3). Interestingly, Prdx1 binds to the myc protein and suppresses its function (Mu et al., 2002), and a recent study found a myc-dependent regulation of Prdx5 by Prdx1 (Graves et al., 2009), demonstrating myc as a peroxiredoxin regulator. Mithramycin A is also known to suppress myc DNA binding activity, so the inhibitory effect of mithramycin A on Prdx6 induction by serum and TPA may be attributed to suppression of myc, rather than Sp1. This will require further investigation.

*4.5 Other Possible Mechanisms of Prdx6 Regulation*

The inhibitory effect of Mithramycin A on Prdx6 stimulation by TPA suggests that Sp1 may be involved in mediating part of this response. TPA has been shown to increase transcription of another antioxidant gene, manganese superoxide dismutase, through activation of Sp1 (Porntadavity et al., 2001). Despite a possible role for Sp1 in this activation, the inability of the proximal promoter to respond to TPA suggests that Prdx6 upregulation by TPA likely requires additional upstream regulatory elements and other transcription factors. A recent study revealed that the human Prdx6 gene is transcriptionally regulated through an antioxidant response element (Chowdhury et al., 2009), although a similar element is not found in the mouse promoter. In addition to the previously mentioned consensus sequences, two putative AP1 sites are found between 340 and 470 nucleotides upstream of the transcription start site. It is worth noting that the TPA-dependent induction of Prdx1 reported by Hess et al. was mediated by two proximal AP-1 sites targeted by c-Jun (Hess et al., 2003). The investigation of these potential regulatory elements and the corresponding DNA binding proteins will provide greater insight into the transcriptional regulation of Prdx6. Together with additional functional analyses these studies will help to reveal the precise role for this protein in liver cell physiology and stress responses.

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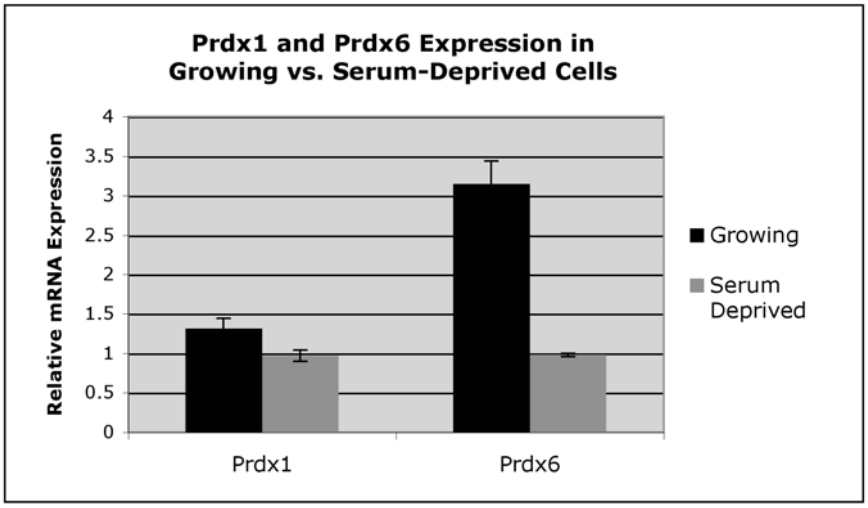
**A**




**B**

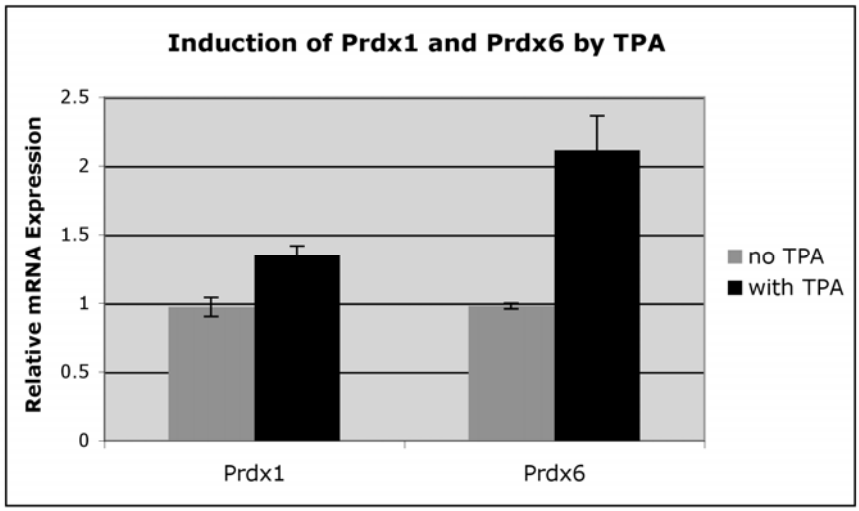
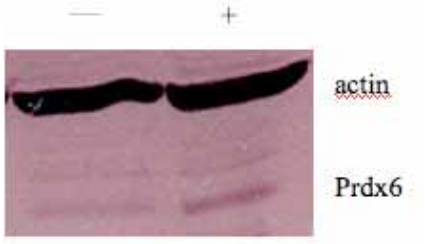



Figure 1. Induction of Prdx1 and Prdx6 by Serum and TPA

H2.35 cells were cultured in complete growth media or serum-free/dexamethasone-free media for 24 hours (A) and serum-free/dexamethasone-free treated cells were subsequently treated with or without 0.5 μM TPA for an additional eight hours (B). All cells were lysed, RT-reactions were performed, and *Prdx1*, *Prdx6*, and *beta-actin* mRNA levels were quantified by Real-Time PCR as described in Materials & Methods. Relative expression was calculated using the $C_T$ method, normalizing to actin and calibrating against the serum-deprived expression level for each gene. Averages of three independent replicates are shown (+/- SDM).

**A**

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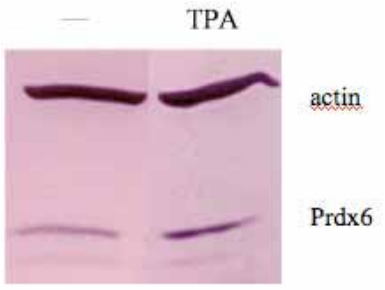



Figure 2. Prdx6 Protein Induction by Serum and TPA

(A) Western blot of Prdx6 and beta-actin in H2.35 cells under serum deprived and growing conditions. (B) Western blot of Prdx6 and beta-actin in serum deprived H2.35 cells in the absence or presence of 0.5 μM TPA for eight hours.

TACAAGTCCCCGCAATTCTCGGTCTTGCGCTTCCTTCCTATTGCACGTGGCCTTCTTTAGCTCCAGAATC
USF / MYC

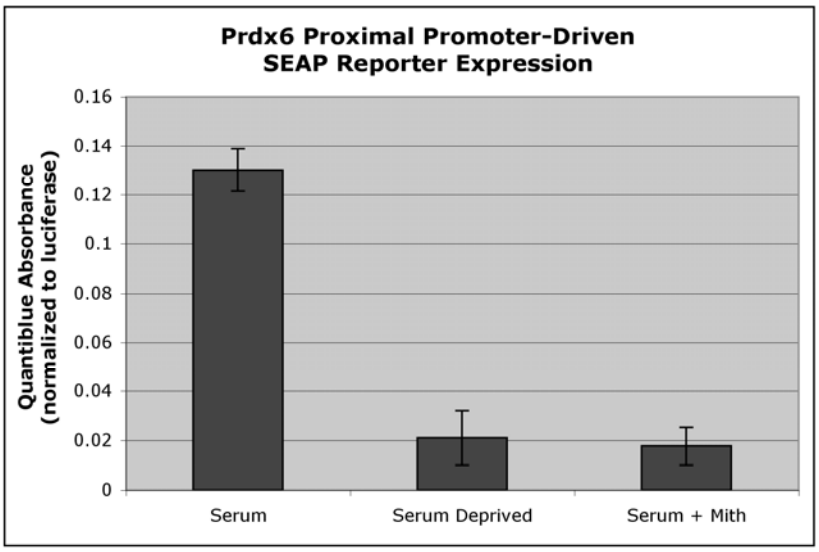
TCCCTTGCCTGCAAGCTCCACGTGAAGAACCGCAGCCGCCAGACTCGCGGTCGCCCCACCCCGCCCCA
USF / MYC Sp1

GCCCCGCCCCAGCCCCGCCCACTCGGCCAGCACTGATCTAGGTCTC
Sp1 Sp1

Figure 3. Prdx6 Proximal Promoter Sequence

Nucleotide sequence of the proximal 184 nucleotides of the mouse Prdx6 promoter. Nucleotide number is relative to the transcription start site for the gene. This sequence corresponds to the region used in the SEAP reporter analysis.

**A**

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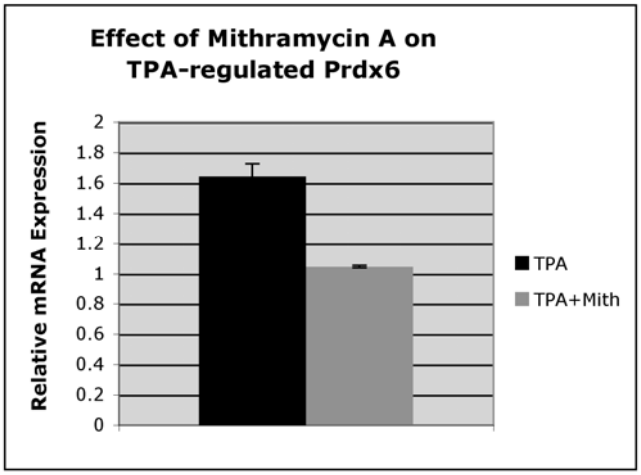


Figure 4. Effect of Mithramycin A on Prdx6 Regulation.

(A) H2.35 cells were transfected with pGluc control vector and SEAP2-184 reporter construct in the indicated media. Two hours after transfection, cells were treated with or without 100 nM mithramycin A, and subsequently cultured under the indicated conditions for five days. SEAP and luciferase activity were measured as described in Materials and Methods. Averages of normalized SEAP reporter expression for three independent replicates are shown (+/- SDM). (B) H2.35 cells were cultured in serum-free/dexamethasone-free media and treated with 0.5 μM TPA for eight hours in the presence or absence of 100 nM mithramycin A. All cells were lysed, RT-reactions were performed, and *Prdx1*, *Prdx6*, and *18S* mRNA levels were quantified by Real-Time PCR as described in Materials & Methods. Relative expression was calculated using the $C_T$ method, normalizing to 18s. Averages of three independent replicates are shown (+/- SDM).