Title
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Permalink
https://escholarship.org/uc/item/2050m2zd

Journal
Developmental dynamics : an official publication of the American Association of Anatomists, 242(9)

ISSN
1058-8388

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Publication Date
2013-09-01

DOI
10.1002/dvdy.23990

Peer reviewed
Nuclear Phosphatase PPM1G in Cellular Survival and Neural Development

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Background: PPM1G is a nuclear localized serine/threonine phosphatase implicated to be a regulator of chromatin remodeling, mRNA splicing, and DNA damage. However, its in vivo function is unknown.

Results: Here we show that ppm1g expression is highly enriched in the central nervous system during mouse and zebrafish development. ppm1g−/− mice were embryonic lethal with incomplete penetrance after E12.5. Rostral defects, including neural tube and craniofacial defects were observed in ppm1g−/− embryos associated with increased cell death in the neural epithelium. In zebrafish, loss of ppm1g also led to neural defects with aberrant neural marker gene expression. Primary fibroblasts from ppm1g−/− embryos failed to grow without immortalization while immortalized ppm1g−/− fibroblasts had increased cell death upon oxidative and genotoxic stress when compared to wild type fibroblasts. Conclusions: Our in vivo and in vitro studies revealed a critical role for PPM1G in normal development and cell survival. Developmental Dynamics 242:1101-1109, 2013. © 2013 Wiley Periodicals, Inc.

Key words: PPM1G; neural tube; serine threonine phosphatase

Key Findings:
- PPM1G is essential for mouse and zebrafish embryonic development
- Loss of PPM1G leads to neuronal cell death and development defects in zebrafish and mice
- PPM1G regulates cell proliferation in vitro
- PPM1G regulates stress-induced cell death in vitro

Accepted 14 May 2013

INTRODUCTION

Type 2C phosphatase family (PP2C or PPM) (Guthridge et al., 1997; Travis and Welsh, 1997; Murray et al., 1999) have been implicated in cellular stress responses (Lu and Wang, 2008). All PPM phosphatases share a conserved pp2c domain and many display specific sub-cellular localization (Stern et al., 2007). The most extensively studied PPMs, PPM1A and PPM1B, are located in the cytosol (Lu and Wang, 2008), while three other members: PPM1D, PPM1G (pp2c7), and PPM1M are all located in nuclei (Komaki et al., 2003). The PPM1D coding gene ppm1d is an established oncogene amplified in breast cancer (Li et al., 2002) and PPM1M function is unknown.

PPM1G is a PP2C family member with a unique acidic domain inserted in the middle of the conserved PP2C phosphatase domain, which may direct substrate specificity (Travis and Welsh, 1997; Murray et al., 1999). In vitro work has implicated PPM1G in diverse nuclear functions including mRNA splicing (Murray et al., 1999; Allemand et al., 2007), snRNP assembly (Petri et al., 2007), Histone exchange (Kimura et al., 2006), and DNA damage and repair
onic lethality was observed in onic development. Significant embryonic defects and elevated stress-induced cell death when compared to wild type MEFs. All these results suggest that PPM1G is critical for cellular growth and survival in response to stress and has a conserved and essential role in vertebrate neural development.

**RESULTS**

**ppm1g Gene is Highly Expressed Across Neural Structures in Developing Embryos**

The ppm1g+/− mice were obtained from Deltagen in which one allele carrying a lacZ neomycin fusion gene replacing portions of ppm1g exons 4 and 5 by homologous recombination (Fig. 1A for schematic illustration and genomic PCR) were backcrossed into C57BL6 background. The ppm1g−/− had a complete loss of ppm1g expression as demonstrated at mRNA level by qRT-PCR on samples from whole embryos (Fig. 1B) and at protein level by immunoblot on samples from derived mouse embryonic fibroblasts (Fig. 1C). Since the lacZ/neomycin cassette was fused in-frame into the ppm1g coding sequence, the expression pattern of the ppm1g gene could be revealed via X-gal staining in the ppm1g+/− embryos. At E8.5, positive

LacZ staining was detected mostly along the neural plate and the neural folds (Fig. 2A, E). This expression persisted throughout the length of the neural tube in E9.5, E10.5, and E12.5 embryos (Fig. 2). LacZ staining in the E9.5 embryos indicated that ppm1g is broadly expressed at high levels in neural regions including the hindbrain, midbrain, otic vesicle, and somites (Fig. 2B, F). In E10.5 embryos, there was strong expression in the first two branchial arches, the frontal neural process and the telencephalon. Additional LacZ signal was also present in the midbrain and hindbrain areas (Fig. 2C, G). The limb buds at this stage were also strongly stained, with additional expression present in somites and weak expression in the heart. The E12.5 embryos revealed that ppm1g is expressed in most rostral structures including the maxilla, mandible, and neck regions (Fig. 2I). Sections of E10.5 embryos revealed that ppm1g expression in the developing embryo is enriched in neural structures. LacZ activities from adult ppm1g+/− mice were also measured utilizing the CPRG assay, which detected a high level of expression in the testes, small intestine, brain, and spleen (see Supp. Fig. S1A, which is available online).

**ppm1g−/− Mice Are Embryonic Lethal**

Only one ppm1g−/− mouse was obtained at weaning from all the crosses between heterozygous ppm1g+/− mice (0.72%, 1/138). Between E8.5–E12.5, the number of surviving ppm1g−/− embryos became progressively lower than the expected Mendelian ratios (21.4, 20.7, 16.4, and 15.5%, respectively) (Table 1). From embryonic stages after E13.5 until P0, the majority of ppm1g−/− embryos did not survive (7/304). Clearly, ppm1g deficiency leads to embryonic lethality between E12.5–E13.5.

**ppm1g−/− Embryos Have Rostral Defects**

Rostral neural tube defects were observed in 27.8% (5/18) of the...
ppm1g<sup>−/−</sup> embryos between E12 and E14.5. Shown in Figure 2 are several defects observed in the ppm1g<sup>−/−</sup> embryos including open neural tubes and exencephaly. At E14.5, we found that one surviving ppm1g<sup>−/−</sup> embryo had a severe rostral defect with failed neural tube closure (Fig. 2O). Therefore, consistent with its enriched expression pattern, significant defects were observed in the developing neural tube of the ppm1g<sup>−/−</sup> mouse embryos.

**The Surviving ppm1g<sup>−/−</sup> Adult Has a Craniofacial Defect**

We observed only one ppm1g<sup>−/−</sup> mouse from all the heterozygous crossings survived beyond birth and weaning. This ppm1g<sup>−/−</sup> mouse was severely runted and failed to grow at the same rate as the littermates (Supp. Fig. S2A). This mouse also exhibited circling behavior, suggestive of a neural defect. At 2 months of age, this mouse was euthanized due to lack of growth. Upon necropsy, it was observed that the ppm1g<sup>−/−</sup> mouse had craniofacial defects such as a deviated maxilla (Supp. Fig. S2B).

**Loss of ppm1g Leads to Increased Apoptosis of E9.5 and 10.5 Midbrain and Hindbrain Neural Epithelium**

To determine whether a change in proliferation or cell death occurred in the ppm1g<sup>−/−</sup> embryos, we performed immunohistochemistry for phospho-histone H3 serine 10 (H3P) and TUNEL staining. The distribution of proliferating cells in E10.5 brain was mostly observed at ventricle side as expected in both wild type and ppm1g<sup>−/−</sup> mice with no significant differences (Fig. 3). In contrast, a significant increase in TUNEL-positive apoptotic cells was observed in the midbrain neural epithelium of E9.5 and E10.5 ppm1g<sup>−/−</sup> embryos (Figs. 4 and 5). The level of apoptosis was highest in the rostral portion of the hindbrain (Fig. 5E), while the neural tube at the level of rhombomeres 4 and 5 showed similar levels of apoptosis when compared to wild-type controls (Fig. 4). This indicates that apoptotic events in ppm1g<sup>−/−</sup> embryos are concentrated around the rostral neural tube, particularly around the midbrain and hindbrain. In addition to apoptosis, we cannot exclude other forms of cell death, such as necrosis, are also implicated in the abnormalities observed in ppm1g<sup>−/−</sup> embryos.

**ppm1g<sup>−/−</sup> Embryos Have Increased Stress Signaling**

As ppm1g<sup>−/−</sup> embryos had increased cell death in the neural epithelium, we examined whether there was also a change in pro-apoptotic stress signaling pathways. Immunoblot analysis of E10.5 wild type, ppm1g<sup>+</sup>/+ and ppm1g<sup>−/−</sup> embryos revealed an

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**TABLE 1. Embryonic Lethality in ppm1g<sup>−/−</sup> Mice**

<table>
<thead>
<tr>
<th>Embryonic Stage</th>
<th>Genotype and Number of Surviving Embryos</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>+/+</td>
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<tr>
<td>8.5</td>
<td>3</td>
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<tr>
<td>9.5</td>
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<td>14.5</td>
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<td>15.5</td>
<td>3</td>
</tr>
<tr>
<td>19.5</td>
<td>3</td>
</tr>
<tr>
<td>P0</td>
<td>20</td>
</tr>
<tr>
<td>weaned</td>
<td>61</td>
</tr>
<tr>
<td>total</td>
<td>251</td>
</tr>
</tbody>
</table>

*ppm1g<sup>−/−</sup> mice were crossed and the resulting embryos of mice of different genotypes are listed along with the percentage of ppm1g<sup>−/−</sup> embryos.
increase in the stress-activated protein kinase activity in ppm1g
two
embryos as demonstrated by the higher levels of phospho-p38 MAPK when compared to wild type or heterozygous embryos (Fig. 5F).

**ppm1g’s Function in Zebrafish Neural Development**

The ppm1g gene is highly conserved in vertebrates based on peptide sequence alignment among human, mouse, chicken, and zebrafish (Supp. Fig. S3). By in-situ hybridization in zebrafish egg and embryos, ppm1g mRNA was detected ubiquitously at the onset of gastrulation (Fig. 6A) but became more restricted to the central nervous system and posterior somites, spinal cord (motorneurons), and floor plate as somitogenesis proceeds (Fig. 6B,C). By 24 hr post fertilization (hpf), ppm1g mRNA was strongly detected in the brain, spinal cord, eyes, and branchial arches (Fig. 6D, lateral; E, dorsal). At 48 hpf, ppm1g expression remained strong in the brain, eyes, and branchial arches (Fig. 6F). Overall, these observations were largely consistent with what were reported by Thisse et al. (2004). Therefore, the enriched expression of ppm1g in the central nervous system during embryonic development appears to be conserved in vertebrates.

To investigate the functional role of ppm1g in zebrafish, we established ppm1g knockdown fish using specific morpholino targeting the zebrafish homolog of ppm1g. Compared to control zebrafish or zebrafish injected with a random morpholino (Supp. Fig. S4), the ppm1g morphants showed central nervous system defects, with smaller heads and underinflated midbrain ventricles at 36 hr post fertilization (hpf) (Fig. 7A, B). Also, there was elevated cell death in the forebrain ventricular zone, and the rest of the central nervous system, including the midbrain, hindbrain, and spinal cord (Fig. 7C, D, Supp. Fig. S4C). Additionally, the ppm1g morphants exhibited abnormal expression of neural markers. In ppm1g morphants, the pax2 expression was extended anteriorly beyond the midbrain/hindbrain boundary to include the midbrain tectum (Fig. 7G). Increased pax2 expression was also detected in the choroid fissure and optic nerve of ppm1g morphants (Fig. 7G). Similarly, the expression level of notch1B was increased in the brains of the 48-hpf-old ppm1g morphants compared to the controls (Fig. 7H, I). Overall, ppm1g morphants exhibited abnormal neural marker expression patterns and neural defects associated with elevated cell death. Therefore, PPM1G has a conserved function in neural development and cell survival in vertebrates.

**PPM1G Regulates Cellular Survival and Stress Response In Vitro**

To explore the cellular effects of ppm1g function in vitro, MEF cells were derived from E10.5 embryos from wild type, ppm1g+/−, and ppm1g−− embryos. The genotyping and deficiency in Ppm1g protein expression were confirmed by genomic PCR and immunoblot (Fig. 1). Primary MEFs from the heterozygous and wild type embryos were readily established and expanded in cell culture. However, primary MEFs from ppm1g−− embryos failed to propagate beyond the second passage (from 24 well plates to a single 6 well plate) (Supp. Table S1). Therefore, only immortalized wild type and ppm1g−− MEFs were used for the following studies.

As PPM1G is reported to be a key regulator in ATM-mediated regulation of DNA damage response (Khorenkenova et al., 2012) and apoptosis, and our ppm1g−− MEFs exhibited elevated apoptosis and stress signaling, we tested the viability of MEFs in response to DNA damaging and oxidative stress. Genotoxic stress was
tested with the topoisomerase inhibitor doxorubicin (DOX at 1 mM) and oxidative stress was tested by hydrogen peroxide (H2O2, at 50 μM). There was significantly increased cell death in ppm1g−/− MEFs compared to wild type controls following H2O2 treatment (Fig. 8C–F). Conversely, Hela cells overexpressing PPM1G were less sensitive to H2O2-induced cell death (Supp. Figs. 5,6). As shown in Figure 8E, in response to treatment with Dox or H2O2, the cell viability of the ppm1g−/− MEFs was significantly lower than the wild type control (P < 0.05). This response was replicated in independently derived ppm1g−/− MEF and wildtype MEF lines (Supp. Fig. S6). These data suggest that PPM1G regulates stress-induced cell death.

We further determined whether there was an increase in stress signaling in the ppm1g−/− MEFs. Serum starved (2% FBS, 1% P/S DMEM) wild type and ppm1g−/− MEFs were subjected to Dox for 0–12 hr and examined for phosphorylation levels of the stress-activated MAP kinases, p38 (Fig. 8G). The ppm1g−/− MEFs have a more pronounced activation of p38 activity over the course of 12 hr when compared to wildtype MEFs.

**DISCUSSION**

PPM1G was reported to regulate DNA damage response (Kimura et al., 2006; Beli et al., 2012; Khoronenkova et al., 2012) and histone exchange (Kimura et al., 2006) based on in vitro studies. However, the in vivo role of PPM1G remained completely unknown. Here we show for the first time that PPM1G expression is highly enriched in embryonic neural tissues and is important for the survival of the developing embryo. In the ppm1g−/− deficient embryos, neural tube defects were observed alongside elevated neural apoptosis and increased stress signaling. The ppm1g−/− MEFs also showed an increased susceptibility to stress-induced cell death and stress signaling response. PPM1G’s role in cranial and neural development is best illustrated by the conservation of PPM1G’s function in zebrafish. In ppm1g morphants, there were rostral defects including ventricle under inflation, increased cell death, and dysregulation of the hindbrain markers pax2 and notch1B. Overall, our data has revealed the in vivo expression pattern and the functional importance of ppm1g during neural development.
development and demonstrated an important function for PPM1G in cell death regulation during development and genotoxic stress.

It is not entirely clear what constitutes the underlying mechanisms for the neural tube defects observed in the \( ppm1g^{2/2} \) embryos. PPM1G was found to interact with and regulate an alternative splicing factor YB1 (Allemand et al., 2007). Interestingly, the \( yb1^{2/2} \) mice also have neural tube defects as well as deficiency in cell growth (Uchiumi et al., 2006). Therefore, it would be worthwhile to investigate whether the loss of YB1 dephosphorylation and subsequent loss of proper YB1-mediated mRNA splicing contribute to the embryonic lethality and neural tube defects found in the \( ppm1g^{2/2} \) mice.

Another reported target of PPM1G is SMN, which modulates the nuclear/cytoplasmic transport for snRNP assembly and localization (Petri et al., 2007). Human SMN mutations cause a loss of motor neuron function and muscle weakness ranging from mortality to mild weakness (Humphrey et al., 2012), while deletion or catalytic defective mutants of SMN1 in mice result in defects in axonal growth (Rossoll et al., 2003; Gabanella et al., 2005). However, these are not as severe or early as the embryonic lethality observed in the \( ppm1g^{2/2} \) mice. Therefore, SMN phosphorylation defects may not represent the full spectrum of the downstream effects due to \( ppm1g \) inactivation.

Kimura et al. (2006) found that PPM1G directly bound histones H2A/H2B, and that PPM1G could dephosphorylate H2B ser14, H2A ser1, and gH2AX ser139 residues in response to DNA damage (Kimura et al., 2006). Other recent work also indicated that PPM1G may have a role in the DNA damage response downstream of ATM (Kimura et al., 2006; Beli et al., 2012; Khoronenkova et al., 2012). Beli et al. found that PPM1G was phosphorylated in response to DNA damage and recruited to the gH2AX foci of DNA damage in response to topoisomerase inhibition. Previous studies indicated that PPM1G overexpression impaired cell cycle progression (Suh et al., 2009), while knockdown of PPM1G with siRNA also lead to a proliferative defect (Allemand et al., 2007; Khoronenkova et al., 2012). Indeed, \( ppm1g^{2/2} \) MEFs showed an inability to divide without immortalization, which is consistent with the senescence phenotype observed in the MEFs with deficient DNA damage response, such as Ku70/−/−, Ku80/−/−, and ATM/−/− MEFs (Elson et al., 1996; Nussenzweig et al., 1996; Gu et al., 1997). However, our in vivo analysis...
did not detect any significant differences in cell proliferation between the wildtype and the ppm1g-deficient embryos. Therefore, it is unlikely that the developmental phenotype observed in the ppm1g−/−embryos is a direct result of defects in cell proliferation.

Embryos deficient for ppm1g had increased cell death as well as activation of stress kinase p38. To assess whether the increased cell death and stress activation was a cell autonomous effect in the ppm1g-deficient cells, we analyzed stress-induced cell death in the immortalized ppm1g−/−MEFs. Compared to wild type MEFs, the ppm1g−/−MEF cell lines had significantly reduced viability treated with doxorubicin or H2O2. The increased cell death in the stressed ppm1g−/−MEFs illustrates the essential and cell-autonomous role of ppm1g for cell survival under stress stimulations.

Combining both in vitro and in vivo analyses as presented in this report, it is clear that PPM1G-mediated signaling is a conserved and functionally important pathway for CNS development. These observations provide the physiological context for the reported function of PPM1G in chromatin remodeling, RNA processing, and DNA damage regulation.

**EXPERIMENTAL PROCEDURES**

**Animal Welfare**

All mice and zebrafish were housed and cared for by the staff of UCLA Division of Laboratory Animal Medicine according to current guidelines and policies set forth in Public Health Service Policy on Humane Care and Use of Laboratory Animals (2002). Euthanizing mice or zebrafish for tissue or cells was performed according to current guidelines and policies set forth in Public Health Service Policy on Humane Care and Use of Laboratory Animals (2002). Euthanizing mice or zebrafish for tissue or cells was performed following specific protocols approved by UCLA Institutional Animal Care and Use Committee (UCLA Animal Research Committee protocol 2003-105 for mice and 2000-051-33B for zebrafish).

**βGalactosidase Staining**

For LacZ staining of embryos, dissected embryos were fixed in 2% gluteraldehyde in PBS for 10 min followed by incubation in staining solution containing 100 mM Na phosphate pH 7.4, 0.01% deoxycholate, 0.02% NP40, 5 mM potassium ferrocyanate, 5 mM potassium ferricyanate, 2 mM MgCl2, and 3 μg/ml Indigal at 37°C.

**Zebrafish ppm1g and Morpholino Injection**

Zebrafish colonies (AB strain) were cared for and bred under standard conditions. The developmental stages were determined using morphological features of fish raised at 28.5°C (Westerfield, 2000). The zebrafish ppm1g (ACCESS Number: BC 052132) was cloned from a cDNA prepared from 2-pfd embryos using the following primers:

ppm1g-F: GGGGGCTTACTTGTCT
ppm1g-R: TTACTCAGTTTGGA

The cDNA fragment was then cloned into pCS2-myc vector for expression and riboprobe preparation.

The cassette contains a stop codon, a polyA and strong splicing acceptor to stop transcript 3′ to the insert. ppm1g+/− mice were backcrossed into C57BL background. Mice were housed under standard conditions with a 12-hr light dark cycle. Heterozygous mice were crossed and the embryos were harvested at different embryonic developmental time points as indicated. Genotyping was performed on genomic DNA based on PCR as illustrated in Figure 1A. PCR product ab represents wildtype allele with 297 bp in length and PCR product cb represents targeted allele with 585 bp in length.

Primer a: 5′-CATGACTATTGAAGAGCTGCTGAG-3′
Primer b: 5′-TTAGCAACACTCAGGCAGCTGTGAGCAG-3′
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A morpholino antisense oligonucleotide (Gene-Tools, Philomath, OR) complementary to the translation start site of ppm1g and its flanking sequence (pm1gMO, 5'-GAGACAAGTAAGCCCCCATGAGTGT-3') was synthesized. The lyophilized morpholino was reconstituted in 5 mM HEPES, pH 7.6, at a concentration of 8 ng/nl. Wildtype AB embryos were injected with 6 or 8 ng of the ppm1gMO at the 1-cell stage.

In Situ Hybridization of Zebrafish Embryos

Embryos for in situ hybridization were raised in embryo medium supplemented with 0.2 mM 1-phenyl-2-thiourea to maintain optical transparency. Whole-mount in situ hybridization was performed as previously described (Langenbacher et al., 2011). Antisense in situ hybridization probes were generated from ppm1g (purchased from Open Biosystems), or from notch1b and pax2 plasmids as described (Langenbacher et al., 2011).

Acridine Orange Staining

Live control and ppm1gMO-injected embryos were soaked in the vital dye acridine orange (5 µg/ml in embryo medium) for 15 min. The embryos were then rinsed with embryo medium and viewed with an epifluorescence equipped Stemi SV 11 (Zeiss, Thornwood, NY) using a GFP filter set. Images were captured with a Zeiss AxioCam and AxioVision software.

MEF Cells

Mouse embryonic fibroblasts (MEF) were derived from E10.5 embryos using an established protocol (Hogan, 1994). All cells were grown in DMEM medium with 1% fetal bovine serum and 1% penicillin/streptomycin under 5% CO₂ at 37°C. All MEFs used in this study were immortalized with SV40T antigen as described (Lu et al., 2009).

qRT-PCR

Quantitative RT-PCR was performed on embryos to measure ppm1g mRNA expression. RNA was isolated from the embryos using the Trizol (Invitrogen, Carlsbad, CA) reagent according to standard protocols. One microgram of total RNA was reverse transcribed with iscript RT (Bio-Rad, Hercules, CA). qPCR was performed on a MyiQ™ Real-Time PCR Detection System (Bio-Rad) using ssofast evagreen polymerase (Bio-Rad). All results are reported as fold ct (cycle difference) change normalized to GAPDH from PCR reactions that were validated by both melting curve analysis and agarose gel electrophoresis. The following primers sets were used for qPCR:

- ppm1g-F: GGACTAGCAGTCAACC
  ppm1g-R: ACACAACAGAGCACAG
  gapdh-F: TCCTGACCACCAACT
  gapdh-R: GATGACCTTGGCCACA

Immunohistochemistry and TUNEL Staining

Paraffin-embedded sections of embryos were utilized for immunohistochemistry and TUNEL staining. All samples were from stage-matched ppm1g−/− and control embryos. Immunohistochemistry was performed with antibodies against PH3 (Zymed) and counterstained with DAPI. TUNEL staining (Chemicon, Temecula, CA) was performed according to the manufacturer’s instructions and counterstained with DAPI.

Immunoblot

Immunoblots were performed by separating proteins in Lysis Buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, Triton X-100 1%, 1 mM EDTA, 1 mM EGTA, 2.5 mM Na pyrophosphate, 1 mM β-glycophosphate, 20 mM NaF, 1 mM Na orthovanadate, 1 mM PMSF, and protease inhibitor tablet (Roche, Indianapolis, IN)) on 12% SDS PAGE gels that were transferred onto nitrocellulose membranes. The following antibodies were then used to probe the membranes: PPM1G (BD Biosciences, San Jose, CA), actin (Santa Cruz Biotechnology, Dallas, TX), P-p38, p38a (Cell Signaling, Danvers, MA).

MTT Assay for Cell Viability

MEF cells were cultured in 2% FBS DMEM for 48 hr prior to stimulation with 50 µM H₂O₂ or 1 µM Dox for 24 hr. Fresh 10% FBS DMEM containing 0.5 mg/ml MTT was added to cells for 15 min prior to washing and dye extraction with methyl sulfioxide. Absorbance was then measured at 650 nm. Two independent ppm1g−/− and ppm1g+/+ MEF lines were used in this assay.

Statistical Analysis

For comparisons between ppm1g−/− and wild type, Student’s t-test was employed. All error bars are standard deviation.

ACKNOWLEDGMENTS

The authors acknowledge outstanding technical assistance from Haiying Pu, technical help from Dr. Shahab Danesh and Dr. Jean-Louis Plouhinec. This work was in part supported by NIH Grants HL070079, HL103205, HL098954 and HL108186 to Y.W. and UCLA Chancellor’s Fellowship to W.H.F. No additional external funding received for this study.

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