Activation of ERK ½ mitogen-activated protein kinase cascade by dentin matrix protein1 promotes osteoblast differentiation

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Running Title: DMP1 activates ERK1/2 MAP kinase signaling

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Abstract:

DMP1 has been shown to play many roles in osteogenesis. We have recently demonstrated calcium-mediated stress kinase activation by DMP1 leads to osteoblast differentiation. In this study we demonstrate that DMP1 can also activate the extracellular signal-regulated kinase (ERK)-MAPK pathway. This activation was mediated through the RGD integrin binding domain in DMP1. Further, we demonstrate that Runx2 an essential transcription factor was stimulated by the ERK-MAPK pathway.

Introduction:

MAP kinases function in many cell types and are regulated by a diverse group of extracellular stimuli and mediate a variety of cellular responses (1). Published reports suggest that the duration and intensity of MAPK activation can profoundly influence the biological response observed (1, 2). There is evidence for cross talk between the proliferation/differentiation pathways activated by MAP kinases. The extracellular signal-regulated kinase (ERK)-MAPK pathway has important functions in the differentiation of post-mitotic cells. In addition, this pathway can regulate the activity of several lineage-specific transcription factors. In bone, the ERK-MAPK pathway is a major conduit for conveying information about the extracellular environment to the nucleus and has been implicated in the response of bone to a variety of signals, including hormone/growth factor stimulation, extracellular matrix-integrin binding and mechanical loading (3).

In a recent study we have demonstrated that DMP1 stimulation supports a stimulatory role in osteoblast differentiation (4). Quantitative PCR results showed that RUNX2 and osteocalcin mRNA expression levels were elevated by DMP1 treatment in MC3T3-E1 cell cultures. Four hour exposures with a p38 kinase inhibitor (SB203580) reduced the DMP1 elevated Runx2 & osteocalcin mRNA

expression levels suggesting the involvement of p38 kinase in the signal transduction pathway. Western blot analyses using an antibody that recognizes the phosphorylated (i.e., activated) form of p38 kinase show rapid elevated levels upon treatment with DMP1. Experiments with an IP3R antagonist (2APB) or a PLC inhibitor (U73122) showed that these elevated intracellular calcium transients are the result of calcium release from endoplasmic reticulum stores. Treatment with BAPTA-AM resulted in attenuation of p38 phosphorylation confirming the role of Ca2+ in downstream signaling. The addition of the calphostin C control clearly indicates that a PLC conversion of IP4 into IP3 & DAG (which would activate PKC) is involved in this DMP1 mediated calcium release (4).

In this study we sought to gain insight into additional signaling by other members of the MAP kinase family. We provide evidence for activation of the extracellular signal-regulated kinase- MAPK cascade in preosteoblasts in response to DMP1 stimulation. Thus, DMP1 stimulation can regulate osteoblast differentiation by activating both the ERK and p38 MAP kinase signaling pathways in preosteoblasts.

MATERIALS AND METHOD:

Expression and Purification of DMP-1

The recombinant DMP1 protein was expressed in E-coli as published earlier (5).

Cell Culture

Mouse calvarial preosteoblast MC3T3-E1 cells (a kind gift of Dr. R.T Franceschi, University of Michigan) were cultured in α-MEM medium supplemented with 10% FBS and 1% Penicillin-Streptomycin. They were seeded in six well plates. The cells were allowed to proliferate until it attained 70% confluence. Media was changed every 2 days. 12-16 hrs before the start of the experiment the cells were cultured in α-MEM medium supplemented with 1% FBS (Basal medium).

These cells were then stimulated with 250 ng rDMP1. The treated cells were then trypsinized and RNA was extracted for RT-qPCR. Total proteins were extracted at 30 min, 1h, and 2h respectively.

Quantitative Real Time PCR

RNA was extracted according to the manufacturer's recommended protocol by using Trizol (Invitrogen). RT-qPCR was performed with DNase I (Promega) treated RNA as published earlier (4). Expression of osteocalcin, Runx2 and GAPDH transcripts were analyzed by qPCR during its linear phase.

Detection of Protein Phosphorylation by Western Blot Analysis

Total proteins were extracted from rDMP1 stimulated MC3T3-E1 cells using M-per reagent (Pierce). 35 μg of the total proteins were resolved on a 10% SDS-polyacrylamide gel under reducing conditions. After electrophoresis, the proteins were electro-transferred onto nitrocellulose membrane (Bio-Rad Laboratories), blocked with 5% non-fat milk, probed with either anti-ERK1/2 (1:500) (Santa Cruz), anti-phospho-ERK1/2 (1:500 (Santa Cruz) anti- MEK1/2 (1:500) (Cell Signaling), anti-phospho-MEK1/2(1:500) (Cell Signaling), anti-Runx2(1:500) (Abcam) for 16h at 4ºC. Blots were then incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody (Chemicon International) or HRP-conjugated goat anti-mouse IgG secondary antibody (Sigma). They were washed three times with PBS containing 0.05% Tween 20 and once with PBS. The bands were visualized by the ECL-Western blot reagent (Perkin-Elmer Life Sciences). Each membrane was then carefully washed, treated for 10 min with a stripping buffer (Pierce), washed with PBS and western blot analysis performed with anti-tubulin antibody (Sigma) and HRP conjugated goat anti-mouse IgG secondary antibody.

Treatment of cells with PD98059 and blocking with RGD peptide

Cells were cultured as described above and were treated with 15μM of PD98059 (Biomol) - an inhibitor specific for ERK1/2 MAP kinase. PD98059 was added to the DMP1 containing basal media and was preincubated for 30 min at 37 °C prior to the start of the experiment. Total RNA and cell lysates were harvested at various time points and were analyzed by qPCR and western blotting performed as described above.

Immunofluorescence

Activation of ERK1/2 signaling by DMP1 was analyzed by immunofluorescence. MC3T3-E1 cells were seeded on glass slides and treated with 250ng/ml rDMP1 for 30min, 1h and 2h. The cells were then washed with 1X PBS, fixed with 4% paraformaldehyde for 30min. Fixed cells were then rinsed thrice with 1X PBS and then permeabilized with 0.05% Triton X-100 in PBS for 20min. The cells were then washed with PBS, and blocked with 5% BSA in PBS for 1 hr. After blocking, the cells were then incubated overnight with anti-phospho-ERK1/2 (1:100) (Santa-Cruz) followed by 1h incubation with a fluorescein-conjugated goat anti-rabbit IgG (Sigma). After washing with PBS the cover glass was mounted using mounting media (Vector shield) and labeling was detected with an Axio Observer D1 fluorescence microscope (Zeiss) equipped with Axiovision imaging software (Zeiss).

RESULTS

DMP1 stimulation induces the expression of osteocalcin and Runx2:

MC3T3-E1 cells stimulated with DMP1 can activate gene expression of Runx2 and osteocalcin. Results in Fig 1A showed a 4 fold and 8 fold increase of osteocalcin gene expression at 4 and 24 hrs respectively and a 3 and 6 fold increase in Runx2 gene expression respectively with DMP1 stimulation. In the presence of ERK1/2 inhibitor PD98059, these effects were abrogated. These results demonstrate that ERK1/2 activation is required for osteoblast differentiation. Western blot

analysis performed on total cell extracts after DMP1 stimulation confirmed the upregulation of Runx2 protein levels (Fig 1B).

DMP1 stimulation induces ERK1/2 activation:

We next measured the phosphorylation state of Erk1/2 mitogen-activated kinase in preosteoblasts stimulated by DMP1. Western blot analysis performed on DMP1 stimulated cells at 30min, 1 and 2 hr showed sustained activation from 30min to 2hr (Fig 2A). An increase in phosphorylation at Thr 202/204 demonstrates the activation of Erk1/2 in preosteoblasts. Presence of Erk1/2 inhibitor PD98059 suppressed this activation (Fig 2C).

DMP1 mediated Erk1/2 activation occurs through MEK1/2:

MEK has shown to be an upstream regulator of Erk1/2. Western blot analysis in Fig 2B shows the activation of MEK1/2 in response to DMP1 stimulation. MEK1/2 activation is characterized by activation by phosphorylation at two activation loop residues Ser 217 and 221. MEK activation was more in DMP1 stimulated cells when compared with the control.

DMP1 mediated ERK1/2 activation in preosteoblasts is suppressed by an integrin recognition site RGD peptide:

To investigate the role of integrin mediated intracellular signaling, we measured the effect of RGD peptide on the activation of ERK1/2. Western blot analysis presented in Fig 2D showed that blocking the integrin binding domain by an RGD peptide did suppress ERK1/2 activation. The ability of DMP1 to suppress ERK1/2 activation in the presence of the RGD peptide suggests a role for integrins in ERK1/2 activation.

Translocation of phospho ERK1/2 to the nucleus of preosteoblasts upon DMP1 stimulation:

Confocal analysis of DMP1 stimulated MC3T3-E1 cells stained for phospho-Erk1/2 show nuclear localization within 30min and an intense staining by 2 hr (Fig 3) while unstimulated cells showed a diffuse cytoplasmic staining. This is consistent with the immunoblotting analysis shown in Fig 2A which demonstrates an increase in the phosphorylation of Erk1/2 in MC3T3-E1 cells.

Discussion

The ability of DMP1 to induce phosphorylation and activation of Erk1/2 represents an important signaling mechanism in osteoblast maturation process. In this manuscript we have identified that stimulation of preosteoblasts by DMP1 leads to the activation of Erk1/2. In our previous study, we demonstrated that DMP1 stimulation induces the activation of p38 MAPK and interestingly p38 activation is required for all phases of osteoblast differentiation (4).

The Erk pathway is one of the best studied MAPK pathways in mammals(6, 7). Erk1/2 activation regulates proliferation, differentiation, survival, migration, angiogenesis and even chromatin remodeling through the phosphorylation of both cytoplasmic and nuclear targets including phosphatases, transcriptional factors and cytoskeletal proteins. In the canonical Erk1/2 pathway, receptor tyrosine kinases are activated by specific ligands and trigger guansine triphosphate (GTP) loading of the Ras protein which can then recruit the Raf kinases (A-Raf, B-Raf, c-Raf) (8). These kinases consecutively phosphorylates and activates MEK (MEK1 and MEK2) ultimately leading to the activation of Erk1/2. In addition to the canonical pathway, Erk1/2 has been shown to be activated by a variety of pathways depending on the individual ligand, cell surface receptor and cell type. Although

MAP kinases participate in different signaling pathways, they may be functionally related, and may be coactivated in response to certain stimuli.

DMP1 is a noncollagenous protein present in the bone and dentin matrix (9-11). We had shown earlier that DMP1 is also specifically localized in the nucleus of differentiating osteoblasts and odontoblasts (12). Translocation of DMP1 from the extracellular matrix is facilitated by the plasma membrane localized GRP78. An interesting observation was that internalization of DMP1 results in store-Ca2+ release and activation of p38 MAP kinase (4). Knockout models of DMP1 show predominant skeletal defects confirming a role for DMP1 in osteoblast differentiation and mineralization (13).

In this study we demonstrate that DMP1 induces ERK activation leading to osteoblast differentiation as the expression of osteocalcin and Runx2 could be abrogated by PD98059 an inhibitor of ERK1/2 kinase. Several studies have shown that ERK activation stimulates Runx2 activation and osteoblast differentiation (3, 14, 15). As DMP1 contains an integrin binding domain, our observation suggests that the phosphorylation state of Erk1/2 is mediated through integrins. Raf and MEK are known to be upstream regulators of Erk1/2. Results from this study showed activation of MEK1/2 in osteoblast cells stimulated with DMP1 when compared with control cells indicating that ERK activation mediated by DMP1 is through the canonical pathway.

In conclusion our results suggest that DMP1 can activate the ERK signaling pathway during osteoblast differentiation. Overall, the data suggests the possibility of a cross-talk between the p38 MAPK and the ERK pathways facilitating osteoblast differentiation.

Figure Legends:

Fig 1A: Runx2 and osteocalcin gene expression analysis in the presence and absence of Erk1/2 MAP kinase inhibitor PD 98095 as determined by Q-PCR analysis.

Fig 1B: Total cell lysates obtained with and without DMP1 stimulation was immunobloted with anti-Runx2 antibody.

Fig 2 A: Cell lysates obtained from control and MC3T3 cells stimulated with DMP1 were immunoblotted with total and phospho specific Erk1/2 antibody. Tubulin was used as a loading control.

Fig 2B: Cell lysates obtained from control and MC3T3 cells stimulated with DMP1 were immunoblotted with total and phospho specific MEK antibody. Tubulin was used as a loading control.

Fig 2C: Cell lysates obtained from control and MC3T3 cells treated with the Erk MAP kinase inhibitor PD98059 and then stimulated with DMP1 for 30min,1h &2h were immunoblotted with phospho specific Erk1/2 antibody. Tubulin was used as a loading control.

Fig 2D: MC3T3 cells were incubated with or without RGD blocking peptide and then stimulated with DMP1 for 1h and immunoblotted with phosphor specific Erk1/2 antibody. Tubulin was used as a loading control.

Fig 3: Confocal image showing subcellular localization of phospho ERK1/2. In the control unstimulated cells phospho ERK is predominantly cytoplasmic while the intensity of the nuclear staining increased from 30min to 2h.

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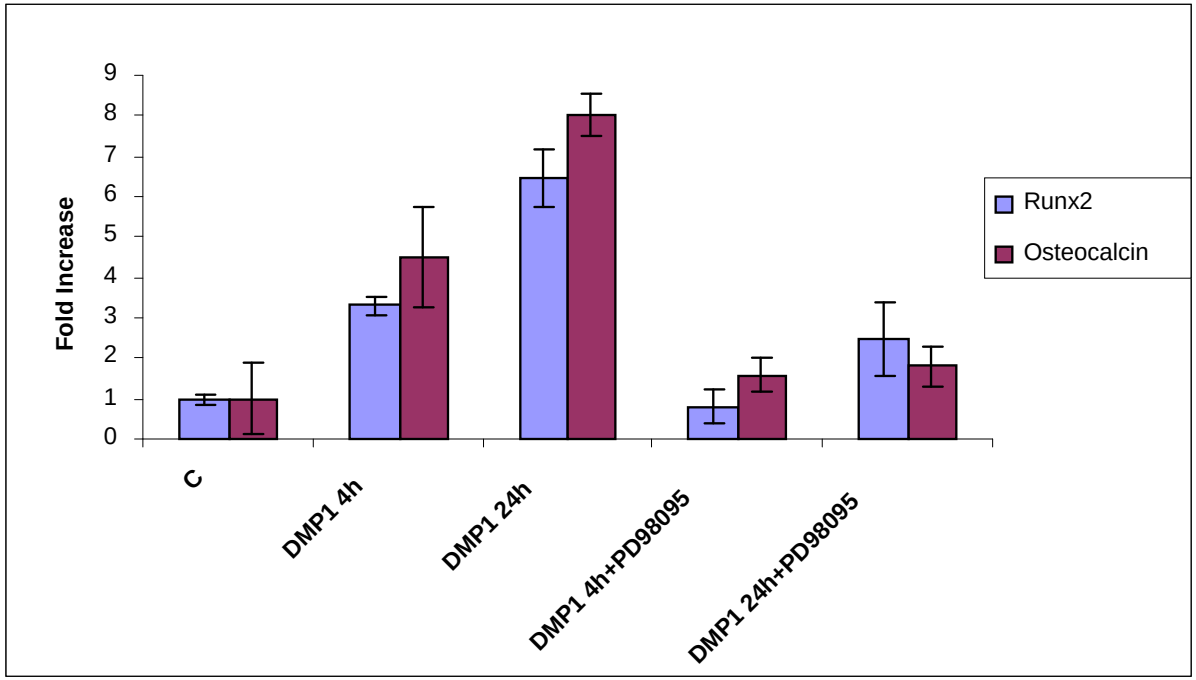
FIGURE 1

A


Fold Increase
9
8
7
6
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Runx2
Osteocalcin
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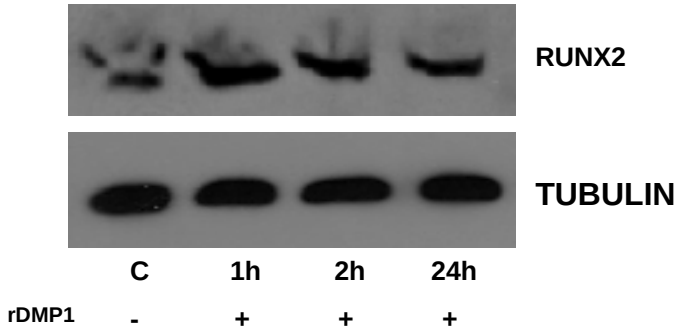
DMP1 4h+PD98095
DMP1 24h+PD98095

B


RUNX2
TUBULIN
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1h
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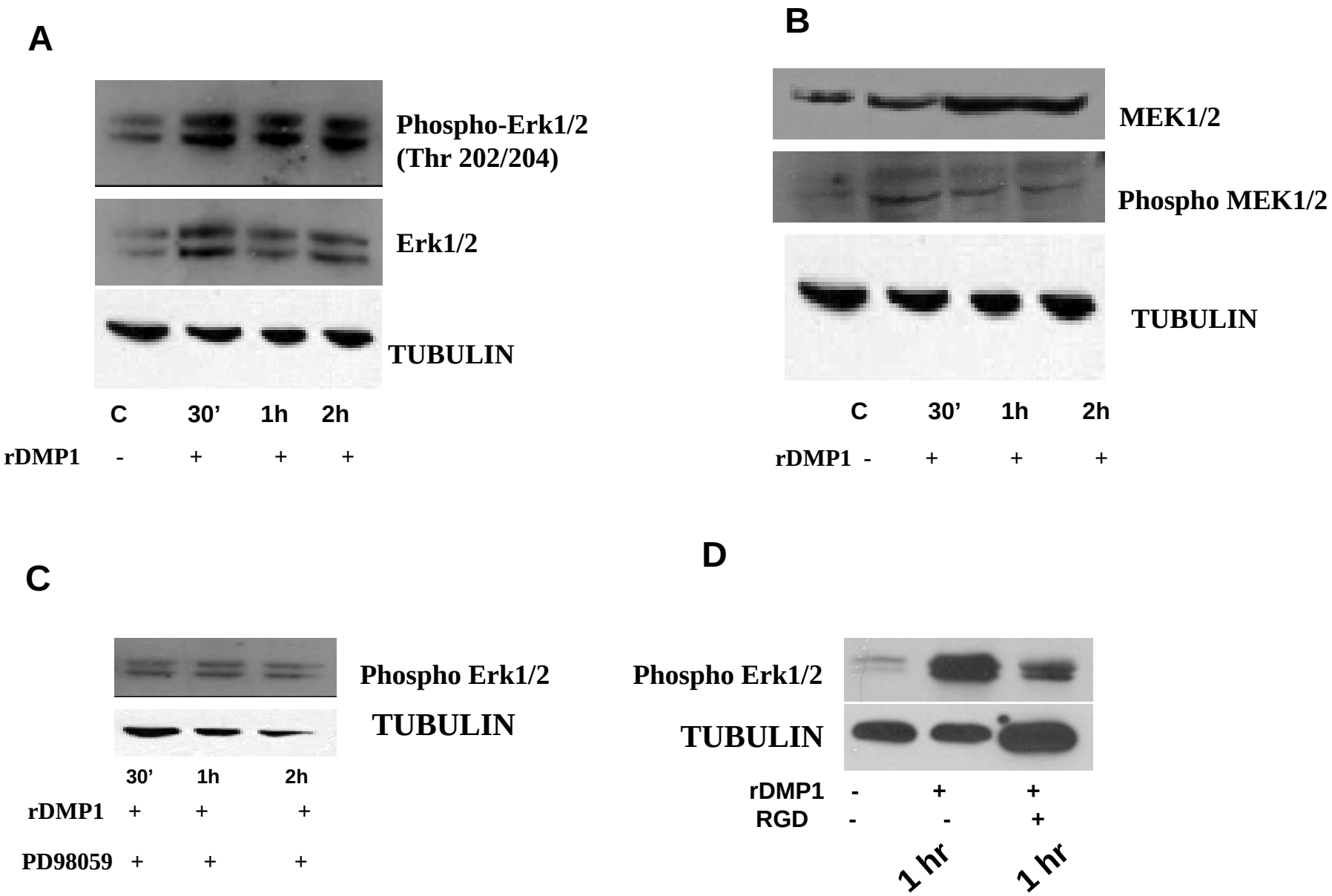
FIGURE 2


A
Phospho-Erk1/2
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Erk1/2
TUBULIN
C
30'
1h
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B
MEK1/2
Phospho MEK1/2
TUBULIN
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Phospho Erk1/2
TUBULIN
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rDMP1 + + +
PD98059 + + +
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Phospho Erk1/2
TUBULIN
rDMP1 - + +
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1 hr
1 hr

FIGURE 3