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ERK Oscillation-Dependent Gene Expression Patterns and Deregulation by Stress Response

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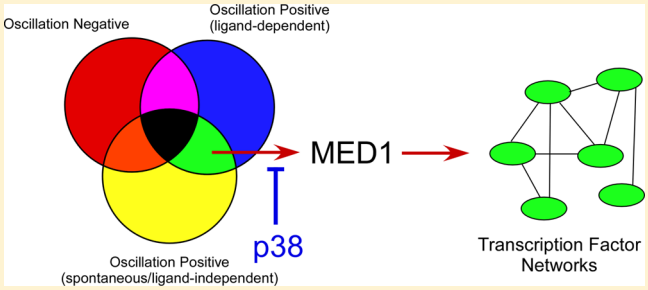
Supporting Information

ABSTRACT: Studies were undertaken to determine whether extracellular signal regulated kinase (ERK) oscillations regulate a unique subset of genes in human keratinocytes and subsequently whether the p38 stress response inhibits ERK oscillations. A DNA microarray identified many genes that were unique to ERK oscillations, and network reconstruction predicted an important role for the mediator complex subunit 1 (MED1) node in mediating ERK oscillation-dependent gene expression. Increased ERK-dependent phosphorylation of MED1 was observed in oscillating cells compared to nonoscillating counterparts as validation. Treatment of keratinocytes with a p38 inhibitor (SB203580) increased ERK oscillation amplitudes and MED1 and phospho-MED1 protein levels. Bromate is a probable human carcinogen that activates p38. Bromate inhibited ERK oscillations in human keratinocytes and JB6 cells and induced an increase in phospho-p38 and a decrease in phospho-MED1 protein levels. Treatment of normal rat kidney cells and primary salivary gland epithelial cells with bromate decreased phospho-MED1 levels in a reversible fashion upon treatment with p38 inhibitors (SB202190; SB203580). Our results indicate that oscillatory behavior in the ERK pathway alters homeostatic gene regulation patterns and that the cellular response to perturbation may manifest differently in oscillating vs nonoscillating cells.



INTRODUCTION

Signaling through the mitogen activated protein kinase (MAPK) network affects virtually every major aspect of cell physiology and is among the most intensely studied cell signaling pathways in biology. Multiple MAPK regulatory features related to signal strength, signal duration, compartmental localization, coordination through scaffolds/inhibitors, and dynamic behaviors have been characterized.[1−5] Four MAPK branches have been defined and are referenced according to their terminal kinase (p38, extracellular signal regulated kinase (ERK), JNK, and ERK5).[6] MAPK branches are frequently associated with opposing cellular programs, such as p38/JNK regulating differentiation, stress-responsive signaling/apoptosis, and ERK/ERK5 regulating mitogenic signaling.[7,8] Exceptions have been observed, such as a role for p38 in proliferation[9,10] or ERK in cell death,[11] making it difficult to rely on measurements of kinase activities for predicting biological response.

Our group has defined oscillatory behavior in the ERK pathway,[12] and the function of this dynamic behavior in cells and tissues is currently unknown. ERK oscillations are observed in primary cell culture and become deregulated with prolonged passage.[13] The mechanisms by which ERK oscillations are deregulated *in vitro* remain unclear; however, these observations suggest that the oscillating phenotype is underrepresented in cell culture models used for toxicological investigations. ERK oscillations regulate a tumor suppressor activity that is conserved *in vitro*/*in vivo*,[14] suggesting that oscillatory behavior can significantly impact biological outcomes. Therefore, it is prudent to consider this regulatory feature in toxicological investigations, and there is a need to bridge the molecular dynamics observed *in vitro* with complex biology in tissues to improve the interpretation of experimental models used for hazard assessment and risk prediction. ERK oscillations are measured at the single cell level *in vitro*, which is not experimentally tractable in animal models. In contrast, genes that are uniquely regulated by ERK oscillations could provide a bridge that can be examined *in vivo* by standard molecular/histological methods.

To date, the molecular features regulating canonical ERK signaling also regulate oscillatory behavior.[5,12] Stress-responsive signaling is known to antagonize the ERK pathway,[15] but this cross-talk has not yet been demonstrated to regulate oscillatory

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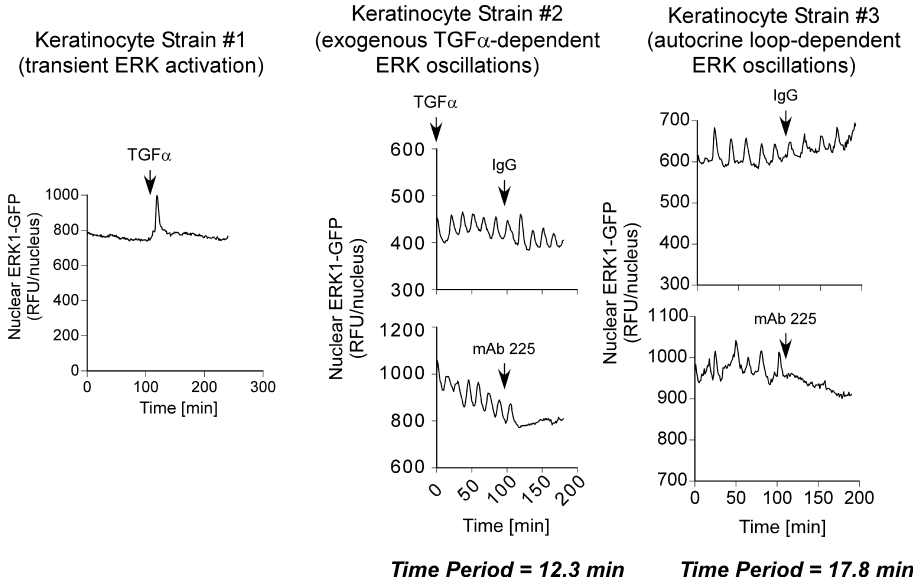

Figure 1. Human keratinocyte model system. Three independent cell strains were subcloned from an hTERT-immortalized normal human keratinocyte cell line that had been transduced with an ERK1-GFP chimera to monitor ERK nuclear-cytoplasmic shuttling. Strain #1 shows transient ERK activation and nuclear translocation following TGFα treatment with no detectable oscillations. Strain #2 displays persistent ERK oscillations that are dependent on exogenous TGFα and inhibited by mAb 225, indicating dependence on the EGFR. Strain #3 displays spontaneous ERK oscillations that are inhibited by mAb 225, indicating dependence on the EGFR. The time period is different for ERK oscillations regulated in strain #1 compared to strain #2.

behavior and may be of toxicological interest. As a first step toward advancing our understanding of ERK dynamics in complex tissues, we have defined gene expression patterns regulated by ERK oscillations and provide an initial assessment of regulatory nodes that may mediate oscillation-specific gene expression that are also targets of stress-responsive signaling.

MATERIALS AND METHODS

Cell Culture. hTERT-immortalized normal human keratinocytes (kindly provided by Dr. Jerry Shay, The University of Texas Southwestern Medical Center) with and without stable transfection with an ERK1-green fluorescent protein (ERK-GFP) chimera were maintained as previously described.[16] The parental human keratinocyte line was maintained in Keratinocyte Serum Free Medium (InVitrogen; Carlsbad, CA) supplemented with bovine pituitary extract and epidermal growth factor (EGF) according to the manufacturer's directions. A subclone of the parental human keratinocyte line that self-regulates persistent and sustained ERK oscillations via an EGF receptor (EGFR)-dependent mechanism without the need for the exogenous EGFR ligand was maintained in keratinocyte serum free medium supplemented with pituitary extract alone. It is unclear whether this self-maintaining subclone regulates ERK oscillations via an EGFR autocrine loop or mutant EGFR. Keratinocytes were subcultured using 0.025% trypsin and 3 volumes of trypsin neutralizing solution. From this parental keratinocyte cell line, we subcloned 3 distinct strains (#1−3) that differ in their ability to regulate ERK oscillations. The characteristics of these strains are outlined in Figure 1. Normal rat kidney cells (NRK CRL-6509, proximal tubule in origin) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic antimycotic solution (Sigma Chemical, St. Louis, MO). Cells were seeded in 12-well plates at 150,000 cells/mL and allowed to grow for 24 h (90% confluence). Cells were then exposed to 10 μM PD98059 or concentrations of bromate ($KBrO_3$; Sigma Chemical, St. Loius, MO) ranging from 0.06−0.3 mM for the indicated time points before being washed and harvested using a rubber policeman in immunoblot buffer (40 mM Tris [pH 7.5], 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA), 5% NP-40, 1 μM activated orthovanadate). In select experiments, cells were treated with pharmacological inhibitors of p38 (SB203580, SB202190), JNK (SP600125), or MEK (PD98059) (Enzo Life Sciences, Farmingdale, NY). We have previously demonstrated that bromate activates the p38 pathway at concentrations employed in our studies,[15,22] and this toxicant was used to further interrogate p38-dependent antagonism of ERK signaling.

Isolation and Culture of Primary Salivary Epithelial Cells. Primary salivary gland epithelial cells were isolated from adult male Sprague−Dawley rat submaxillary glands. Glands were removed aseptically; the outer connective tissue was excised followed by washing in Hank's balanced salt solution (HBSS) supplemented with 1× antibiotic/antimycotic (Life Technologies 100× antibiotic−antimycotic solution, Grand Island, NY). Glands were cut into cubes of approximately 1 mm^3 followed by washing 2× in HBSS using gravity settling. Cubes were then suspended in HBSS supplemented with collagenase (CLSPA, 150 units/mL) and hyaluronidase (150 units/mL) (Worthington Biochemical Corporation, Lakewood, NJ) and dissociated for 45 min at 37 °C with gentle rocking. The digests were passed through a 70 μm nylon mesh, and isolated cells were collected by centrifugation at 1300 rpm for 5 min. Cell pellets were resuspended in advanced DMEM:F12 supplemented with 2.5% FBS, 2 mM Glutamax, 100 units/mL penicillin, 100 units/mL streptomycin, 0.25 μg/mL fungizone, and 10 ng/mL epidermal growth factor (EGF). Cells were subcultured by trypsinization and used at passage 3.

ERK Oscillation Assay. An oscillation assay that monitors persistent ERK nuclear-cytoplasmic shuttling at the single cell level was used as described.[12,16] Persistent nuclear-cytoplasmic shuttling requires coordinated regulation by both positive and negative feedback processes[17] and is an appropriate index for long-term monitoring of ERK activity in live cells. Oscillation characteristics included amplitude, time period, rise time, and decay time. Two independent experiments were pooled for statistical analysis (approximately 100 cells). Results from p38 inhibitor studies in pooled experiments were validated separately at the single cell level as described in Results.

Microarray Analysis. Gene expression patterns using the Affymetrix system were defined as previously described.[14] Transcriptomics for each cell strain was defined for conditions of low vs high ERK activity. This was accomplished by maintaining strains 1 and 2 in the presence (high ERK activity) or absence (low ERK activity) of transforming growth factor alpha (TGFα). For strain #3 (self

regulated ERK oscillations), cells were maintained in the presence of nonspecific immunoglobulin G (IgG) (high ERK activity) or EGFR neutralizing antibody (mAb 225; low ERK activity; Figure 1). The microarray experimental design employed 5 independent biological replicates ($n = 5$) for each condition to ensure robust statistical power. All microarray data used in this study have been deposited into the Gene Expression Omnibus database under accession number GSE55823.

Western Blot. Western blot analysis was performed as previously described.[12] Titers for primary and secondary antibodies were 1:3000 and 1:5000, respectively. Antibodies used included mediator complex subunit 1/phospho-MED1 (MED1/P-MED1) T^{1457} (abcam, Cambridge, MA), P-p38 (Cell Signaling Technologies, Beverly, MA), actin (EMD Millipore, Billerica, MA), and ZO-1 (Invitrogen, Camarillo, CA).

Statistical Analysis. Individual comparisons were made using Student's t test or ANOVA with a posthoc Student's Newman−Keul test, as appropriate. The $p < 0.05$ level was accepted as significant.

RESULTS

Three independent cell strains have been subcloned from an hTERT immortalized normal human keratinocyte cell line. Strain #1 exhibits transient ERK activation induced by TGFα, without detectable oscillations (Figure 1). Strain #2 exhibits persistent ERK oscillations that are dependent on stimulation with exogenous TGFα. Strain #3 exhibits spontaneous ERK oscillations in the absence of exogenous TGFα. An EGFR neutralizing antibody (mAb 225)[18] fully inhibits ERK oscillations in strains #2 and #3 (Figure 1), indicating dependence on the EGFR in both cases. The time period for ERK oscillations is slightly prolonged in strain #3, as compared with strain #2, as might be expected for an autocrine process requiring the shedding of the ligand. However, we cannot distinguish whether strain #3 is regulating ERK oscillations via an EGFR autocrine loop or a mutant EGFR based on results using mAb225 alone. Collectively, all cell strains share the same genetic background and EGFR-dependent ERK activation, which is an ideal scenario for direct comparison of groups in a microarray experiment.

DNA microarray was used to define transcriptomics of the human keratinocyte cell lines described in Figure 1. Conditions for high and low ERK activity were defined for each cell strain as described in Materials and Methods, and gene expression patterns for the low ERK activity condition were subtracted from the high ERK activity condition to enrich for ERK-dependent gene expression patterns. We then defined the genes that were common or unique to each cell strain from this enriched data set. As illustrated in the Venn diagram (Figure 2A), we identified 45 genes that are common to the two oscillation proficient strains (#2 and #3) and are absent in the oscillation deficient strain (#1). The Metacore annotated database was used to predict the transcription factor networks encompassed within the 45 genes common to ERK oscillations (Figure 2B). Network reconstruction suggests that ERK oscillations are coupled to transcription factor networks through the MED1 coactivator and CD44.

Studies were conducted to determine whether an index of MED1 activation showed differential regulation under oscillating vs nonoscillating conditions as validation. ERK phosphorylates MED1 on T^{1457}, which increases MED1 stability and activity,[19] and we examined MED1 T^{1457} phosphorylation status (P-MED1) by Western blot analysis in cell strains #1−3. P-MED1 levels were below detection in strains #1 and #2 under serum-starved conditions, and treatment with EGF for

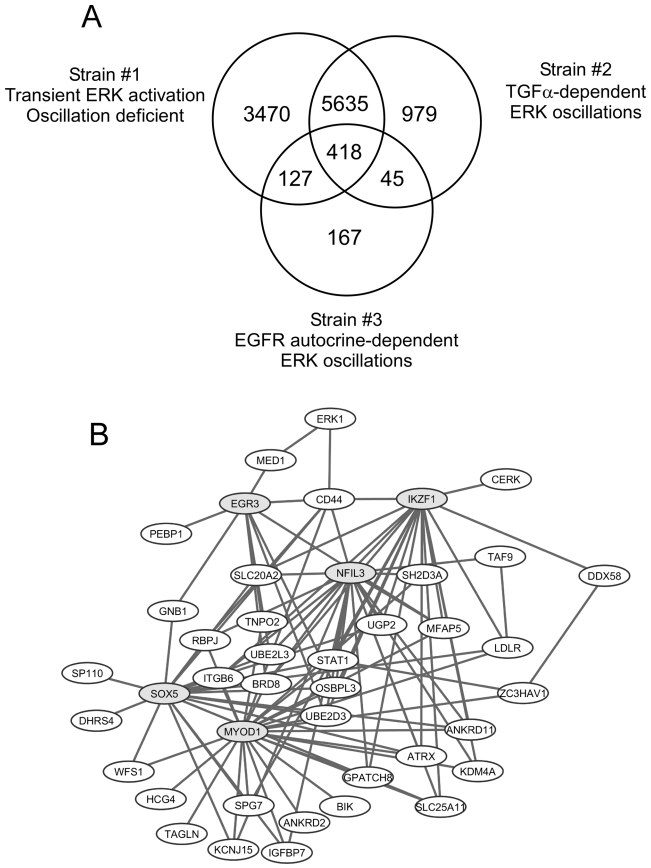

Figure 2. Microarray comparing oscillating vs nonoscillating cell strains. Panel A: Venn diagram showing subset of genes enriched for ERK-dependent regulation in oscillating vs nonoscillating cell strains and overlap between experimental groups. Panel B: Bioinformatics was used to predict the major transcription factor networks represented by the 45 genes common to two independent cell strains exhibiting ERK oscillations. MED1 and CD44 represent regulatory nodes predicted to couple ERK oscillations to inferred transcription factor networks.

10 min resulted in a dramatic increase in P-MED1 levels (Figure 3A). At 8 h post-EGF stimulation, cell strain #2

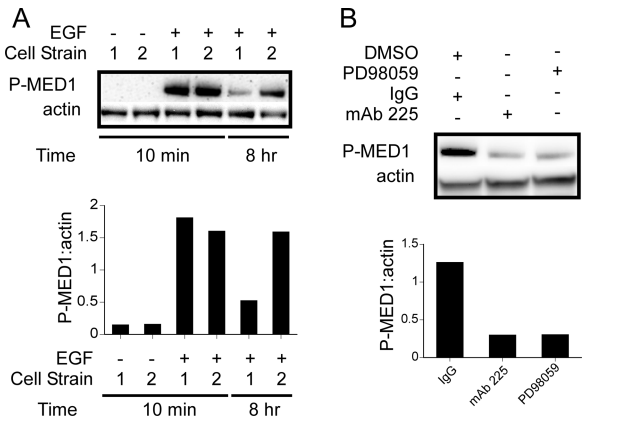

Figure 3. P-MED1 patterns in cell strains 1−3. Panel A: Cell strains #1 and #2 were serum-starved for 24 h, followed by stimulation with 10 ng/mL EGF for 10 min or 8 h. Panel B: Cell strain #3 (spontaneous ERK oscillations) was treated with IgG, mAb 225, or PD98059 for 60 min, and P-MED1 levels were determined by Western blot. Similar results were observed in 2 separate experiments.

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(ligand-induced oscillations) displayed higher P-MED1 levels compared with that of cell strain #1 (nonoscillating). Cell strain #3 (spontaneous oscillations) displayed constitutive P-MED1 levels that were reduced by treatment with mAb 225 or ERK inhibitor (PD98059) for 60 min (Figure 3B). Thus, oscillatory behavior in the ERK pathway is associated with increased P-MED1 status at an established ERK-responsive site.

We subsequently considered the interplay between growth- and stress-responsive signaling on the ERK-MED1 axis. Stress-responsive p38 signaling can antagonize the ERK pathway,[15] and high oxygen tension *in vitro* can induce tonic stress.[20] Thus, we asked whether p38-dependent antagonism of ERK oscillations was apparent under standard cell culture conditions. TGFα-stimulated keratinocytes were treated with DMSO or 1 μM SB203580 (p38 inhibitor), and ERK oscillation characteristics were quantified. Treatment with SB203580 resulted in a marked increase in TGFα-dependent ERK oscillation amplitudes but did not alter the time period, rise time, or decay time (Figure 4A, white bar; rise/decay time not shown). Additional

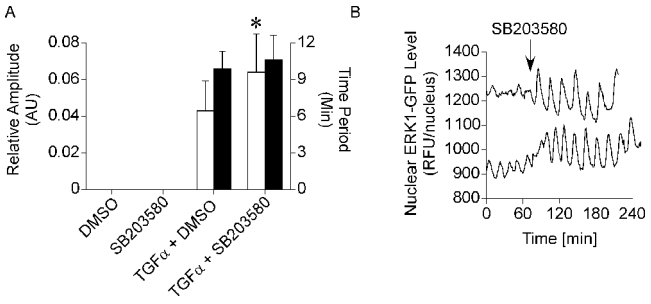

Figure 4. ERK oscillation amplitudes are increased following treatment with the p38 inhibitor. Panel A: Pooled results from two independent experiments showing that SB203580 treatment selectively increases ERK oscillation amplitudes (white bar) but does not alter other ERK oscillation characteristics (time period is shown by the black bar; other oscillation characteristics are not shown). Values represent the mean ± SE. *Significantly different from TGFα-treated cells, $p < 0.05$. Panel B: Single cell trace confirming increase in ERK oscillation amplitude following SB203580 treatment. Traces representing noisy (top) and clean (bottom) oscillators are shown to illustrate that oscillation amplitude is increased by SB203580 in both types.

evidence that was consistent with p38-dependent negative feedback included a small increase in the number of TGFα-stimulated cells displaying ERK oscillations (45% of population for TGFα-stimulated cells → 53% of population in cells treated with TGFα + SB203580). Treatment of keratinocytes with vehicle (DMSO) or a JNK inhibitor (100 nM SP600125)[21] did not alter ERK oscillation characteristics in the presence or absence of TGFα (data not shown). We then confirmed that SB203580 increased ERK oscillation amplitudes at the single cell level. ERK oscillations were monitored in TGFα-treated human keratinocytes for approximately 1 h prior to the addition of 1 μM SB203580. ERK oscillation amplitudes were clearly increased at the single cell level following SB203580 treatment, as compared with the pretreatment time period (Figure 4B), confirming the effects of the p38 inhibitor on oscillation amplitudes. In mathematical terms, ERK oscillations are classified into two categories, termed clean and noisy oscillations.[22] At present, it is unclear whether any biological difference exists between clean and noisy oscillators, and Figure 4B illustrates that both clean (bottom trace) and noisy (top trace) oscillators show a comparable response to the p38 inhibitor.

The increase in ERK oscillation amplitude induced by the p38 inhibitor suggests increased ERK activation under this condition since this index requires a quantitative increase in nuclear ERK-GFP protein levels. We next examined nuclear P-MED1 levels to determine whether the ERK-MED1 axis was regulated in a similar manner. Human keratinocytes were treated with SB203580 for 5−10 min, and nuclear P-ERK and P-MED1 levels were defined by epifluorescence microscopy following *in situ* staining with the appropriate antibodies. SB203580 treatment resulted in a significant increase in nuclear P-ERK levels (Figure 5A square), which preceded increased nuclear P-MED1 levels (Figure 5A, circle). P-MED1 and MED1 protein levels in nuclear extracts were increased following treatment of human keratinocytes with SB203580 for 20−60 min, while actin levels served as the loading control (Figure 5B). The increase in P-MED1 protein level induced by the p38 inhibitor is suppressed by an ERK inhibitor (PD98059) but not by a JNK inhibitor (SP600125) (Figure 5C). Thus, p38 inhibition enhances the ERK-dependent phosphorylation and stabilization of MED1.

Bromate is a probable human carcinogen produced as a disinfection byproduct from the ozonation of water containing bromide. The stress response to bromate has been carefully characterized and includes activation of p38.[23,24] ERK oscillations in human keratinocytes and JB6 cells treated with 1 mM bromate were inhibited following bromate treatment (Figure 6). Western blot analysis demonstrated that 1 mM bromate increased phospho-p38 (P-p38) and decreased P-

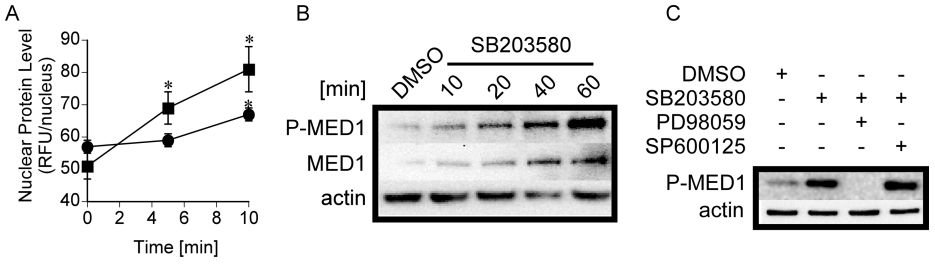

Figure 5. p38 inhibitor increases ERK activation that precedes MED1 phosphorylation. Panel A: Treatment of human keratinocytes with the p38 inhibitor (SB203580) for 5−10 min results in increased nuclear P-ERK levels (square) that precede detection of increased nuclear P-MED1 levels (circle). Values represent the mean ± SE. *Significantly different from the respective control, $p < 0.05$. Panel B: Treatment of human keratinocytes with SB203580 for 10−60 min results in a time-dependent increase in both P-MED1 and MED1 levels. Panel C: The increase in P-MED1 levels induced by the p38 inhibitor at 60 min can be blocked by cotreatment with the ERK inhibitor (PD98059) but not the JNK inhibitor (SP600125). Similar results were observed in two separate experiments.

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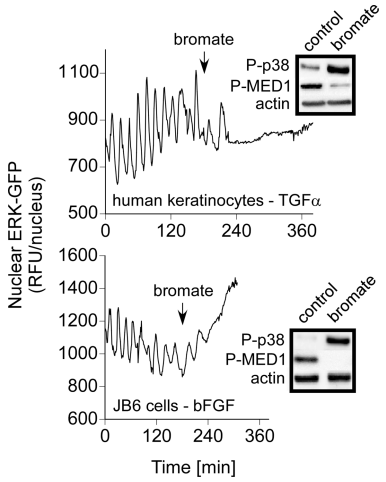

Figure 6. Bromate inhibits the ERK-MED1 axis in human and murine model systems displaying ERK oscillations. ERK oscillations induced by TGFα (human keratinocytes) or bFGF (JB6 cells) were monitored for several hours to demonstrate sustained regulation followed by treatment with 1 mM bromate. Following bromate treatment, oscillations were observed to cease in both model systems. Bromate treatment induced an increase in P-p38 and decrease in P-MED1 protein levels as detected by Western blot. Similar results were observed in two separate experiments.

MED1 protein levels at 60 min in both model systems. Basal P-p38 levels were clearly detectable in human keratinocytes, consistent with results illustrated in Figure 4 showing that p38 inhibitor alone increases ERK oscillation amplitudes in human keratinocytes. We have observed that JB6 cells are highly sensitive to hydrogen peroxide-mediated toxicity as compared with other *in vitro* models we have used (Weber et al., unpublished observation). Bromate treatment induced the accumulation of ERK-GFP in the nucleus immediately following the cessation of oscillations to a greater extent in JB6 cells as compared with human keratinocytes. Although the mechanism for nuclear accumulation is beyond our present scope, bromate induces toxicity via the generation of an oxidative stress.[23] Oxidative stress inhibits nuclear export regulated by the CRM1 export protein,[25] which is relevant because CRM1 regulates the export of ERK from the nucleus.[12] Thus, it is plausible that oxidative stress may modulate multiple mechanisms (e.g., p38 and CRM1) capable of inhibiting ERK oscillations, and the antioxidant status of the cell system may be an additional variable for consideration. Movies illustrating ERK oscillations in human keratinocytes and JB6 cells before and after the addition of bromate can be found in Supporting Information (S1 and S2).

We attempted to extend our investigation to normal rat kidney cells (NRK) and primary salivary gland epithelial cells but met technical limitations that constrained our interpretation of p38-dependent cross-talk. Specifically, we used our established retroviral transduction system[12] for ERK-GFP expression in both model systems. NRK cells showed efficient transduction with ERK-GFP; however, NRK cells rapidly acquired a malignant phenotype (within 2−3 passages) characterized by a stellate morphology and loose adherence to tissue culture plates (data not shown). Safety protocols dictate that cells cannot be removed from the virus laboratory until after 3 passages, and the NRK ERK-GFP cells we were able to examine under this condition did not display ERK oscillations in response to EGF treatment (data not shown). We have shown that JB6 cells transformed to a malignant phenotype by phorbol ester treatment lose the ability to regulate oscillatory behavior;[12] therefore, the lack of oscillations in NRK cells are difficult to interpret because the transformed phenotype may inhibit this dynamic behavior. Primary salivary gland epithelial cells were also examined for comparison with immortalized cell line responses; however, the primary cells were difficult to transduce, and we were unable to generate a stable model system for analysis.

Although the status of ERK oscillations remains unclear in NRK and salivary epithelial model systems, we believe that the antagonistic cross-talk between p38 and the ERK pathways is relevant to both canonical and dynamic signaling features and used these models to further investigate the ERK-MED1 axis. P-MED1 levels are detected in NRK cells and inhibited by 10 μM PD98059 (Figure 7A), indicating that ERK-dependent

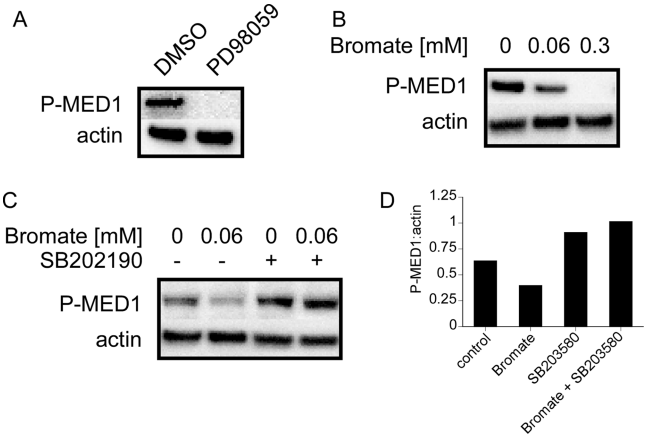

Figure 7. Bromate decreases P-MED1 levels in NRK cells. Panel A: Inhibition of MED1 phosphorylation by the ERK inhibitor (PD98059), confirming ERK-dependent regulation. Panel B: Treatment of NRK cells with 0.06−0.3 mM bromate for 1 h results in a dose-dependent decrease in P-MED1 levels. Decreased P-MED1 levels induced by bromate were observed in three separate experiments. Panels C and D: P-MED1 profile for NRK cells treated with bromate with or without pretreatment with SB202190 and quantified values. Results shown in panels C and D are for the 1 h time point, and similar results were observed at 3 and 24 h time points (data not shown).

regulation of MED1 was conserved in this model. Treatment of NRK cells with 0.06−0.3 mM bromate for 1 h reduced P-MED1 levels (Figure 7B), consistent with the activation of p38 by bromate in NRK cells as previously established.[14,22] P-MED1 levels remained depressed in bromate-treated cells at 24 h (data not shown). NRK cells were then treated with 0.06 mM bromate with or without pretreatment with a p38 inhibitor (10 μM SB202190). Bromate treatment reduced P-MED1 levels, and this effect was inhibited by SB202190 (Figure 7C and D).

Salivary gland epithelial cells afforded a comparison of the ERK-MED1 axis in a primary cell system. Salivary epithelial cells grow as epithelial islands and show localization of the tight junction protein ZO-1 at points of cell−cell contact (Figure 8A). Treatment of salivary epithelial cells with 1 mM bromate for 60 min reduced P-MED1 levels, and this effect was inhibited by the p38 inhibitor SB203580 (Figure 8B and C).

DISCUSSION

ERK oscillations represent a new regulatory mode in the ERK pathway[5,12] whose biological function remains unclear. The

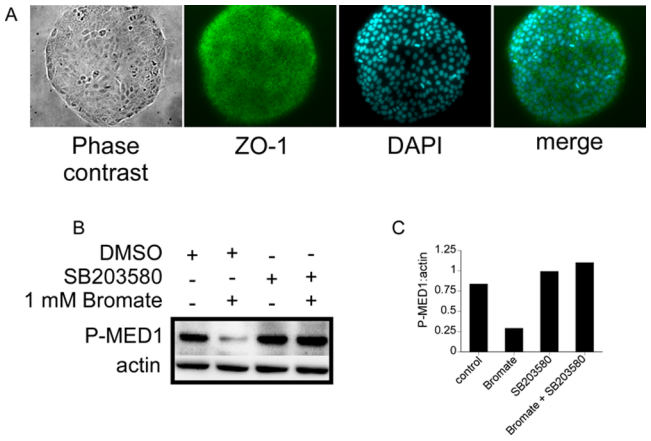

Figure 8. Bromate decreases P-MED1 levels in primary salivary epithelial cells. Panel A: Primary salivary epithelial cells grow as islands and express significant levels of the ZO-1 tight junction protein localized to points of cell−cell contact. Panels B and C: P-MED1 profile for salivary epithelial cells treated with 1 mM bromate with or without pretreatment with SB203580 and quantified values. Similar results were observed in 2 separate experiments.

results of the present study advance our understanding of ERK oscillations through the identification of unique gene expression patterns that can be used to infer possible biological function. The sensitivity of MED1 phosphorylation status to stress-responsive signaling raises the possibility that MED1 may represent a transcriptional node for signal integration that is relevant to both canonical and dynamic ERK signaling.

ERK phosphorylates MED1 on T^{1032}/T^{1457}, which imparts two distinct functions: (1) it stimulates MED1 transcriptional activity, and (2) it increases the stability and half-life of MED1.[19] Therefore, the activation of MED1 by ERK is established, and what remains to be determined is how oscillatory behavior uniquely impacts transcriptional regulation through the MED1 node. MED1 regulates the activity of a battery of transcription factors;[26−30] therefore, sustained MED1 activation by ERK oscillations could impact a broad transcriptional program. The stabilization of MED1 protein levels by ERK oscillations might result in the development of a MED1 gradient (Figure 3A), and future studies will determine the relationship between MED1 protein and ERK activity levels. Molecular gradients associated with pathways exhibiting oscillatory behavior have been documented,[31,32] and morphogens demonstrate the importance of gradients as a regulatory mode in biology.[33,34] Theoretically, an increase in MED1 stoichiometry is expected to permit interaction of MED1 with low abundance transcription factors following saturation of MED1 binding sites on high abundance transcription factors. Other mechanisms are possible, and additional studies focused on the MED1 node are warranted.

p38 antagonizes canonical ERK signaling,[15] and it was prudent to demonstrate that this antagonism extended to ERK oscillations and the ERK-MED1 axis (Figures 6−8). Prior work by Wiley's group[22] has demonstrated an important balance between positive and negative feedback loops in regulating oscillatory behavior. In fact, they demonstrated that treatment of cells with a phosphatase inhibitor alone in the absence of growth factor was sufficient to induce ERK oscillations. p38-dependent antagonism of ERK oscillations illustrates another example of negative feedback that is relevant to toxicological investigations in light of the broad association of p38 with stress-responsive signaling. Because ERK oscillations regulate a unique subset of genes, these observations raise the possibility that antagonistic signaling by p38 might manifest differently in cells displaying transient versus dynamic ERK signaling behaviors.

Bromate was chosen as a test agent because it is a probable human carcinogen that activates p38 via the generation of oxidative stress.[23,24] We have previously shown that reactive oxygen species and ionizing radiation inhibit ERK oscillations;[13] therefore, the inhibition of ERK oscillations by bromate is consistent with our previous observations. Prior studies have demonstrated that a bolus dose of hydrogen peroxide inhibits ERK oscillations and induces ERK accumulation in the nucleus, while a low dose of ionizing radiation inhibits ERK oscillations without nuclear ERK accumulation.[13] Bromate at a high dose (1 mM) inhibits ERK oscillations in association with both p38 activation and nuclear accumulation of ERK in JB6 cells (Figure 6). Thus, it appears that multiple mechanisms can contribute to the inhibition of ERK oscillations and that the p38 stress response may be activated at lower doses of free radicals, relative to other possible mechanisms, such as the inhibition of CRM1-dependent nuclear export. This may provide a useful gauge for interpreting the level of oxygen free radicals produced by toxicants *in vivo*, based on a comparison of p38 activation, the nuclear accumulation of ERK/CRM1, and target oscillation-specific gene expression patterns for oscillation positive cell types.

In the context of carcinogenesis, bFGF-dependent ERK oscillations regulate tumor suppressor activity in JB6 cells that is conserved in mouse skin carcinogenesis.[14] We have defined gene expression patterns that are unique to bFGF in JB6 cells and not associated with the fully transformed JB6 phenotype (RT101 cells).[14] We have analyzed this data set and identified tumor suppressors unique to bFGF-dependent ERK oscillations (lysyl oxidase,[35] ≈7-fold ↑; aspartoacylase 2,[36] ≈9-fold ↑; insulin-like growth factor binding protein 5,[37] ≈9-fold ↑; and scavenger receptor class A, member 5,[38] ≈7-fold ↑). In human keratinocytes (Figure 2B), ERK oscillations were uniquely associated with increased tumor suppressor mRNA expression, including PDCD6,[39] CD44,[40] WNT7A,[41] HOPX,[42] WNT5A,[43] and others. Thus, the regulation of multiple tumor suppressor genes was a common function of ERK oscillations in murine and human model systems. Antagonism of ERK oscillations might decrease tumor suppressor activity, which has obvious implications for increasing cancer risk.

In considering the ERK-MED1 axis, MED1 is known to form a complex with tumor suppressor genes such as BRCA1.[44] Alternatively, MED1 is a transcriptional coactivator for several nuclear hormone receptors, including members of the vitamin D and retinoic acid families.[45] Our experimental model systems are derived from skin, and both the vitamin D receptor[46] and retinoid receptors[47−49] are implicated as tumor suppressors in skin. Therefore, increasing MED1 transcriptional coactivation is expected to increase tumor suppressor activities mediated by vitamin D and retinoic acid pathways. In the murine model system where bFGF-dependent ERK oscillations directly inhibit the cell transformation response,[14] retinoid X receptor alpha mRNA expression is increased (2-fold), which could further sensitize retinoid-mediated tumor suppression. In human keratinocytes, retinoid receptor beta (RXRB) mRNA showed decreased expression with ERK oscillations, and a RXRB-causal network in human cancer has been hypothesized by independent investigators.[50] Therefore, retinoid signaling is a

common theme in microarray data sets defining oscillation-specific gene expression.

In summary, we have demonstrated that oscillatory behavior in the ERK pathway couples to the regulation of a unique subset of genes, including many tumor suppressor genes. Network reconstruction suggests that ERK oscillations are decoded into a transcriptional response, at least in part, through MED1 whose activation is antagonized by p38. Our results indicate that oscillatory behavior in the ERK pathway can alter homeostatic gene regulation patterns and that the cellular response to perturbation may manifest differently in oscillating vs nonoscillating cells.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Two movies illustrating normal human keratinocytes treated with 10 ng/mL TGFα with cells displaying observable oscillations (movie S1) and JB6 cells treated with 10 ng/mL bFGF with cells displaying observable oscillations (Movie S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

$KBrO_3$, bromate; EGFR, epidermal growth factor receptor; ERK, extracellular signal regulated kinase; ERK-GFP, ERK1-green fluorescent protein; HBSS, Hank's balanced salt solution; MED1, mediator complex subunit 1; MAPK, mitogen activated protein kinase; NRK, normal rat kidney cells; P-MED1, phospho-MED1; P-p38, phospho-p38; TGFα, transforming growth factor alpha

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