Current Biology 22, 2017–2026, November 6, 2012 ©2012 Elsevier Ltd All rights reserved http://dx.doi.org/10.1016/j.cub.2012.08.050

Article

Adhesion Regulates MAP Kinase/Ternary Complex Factor Exchange to Control a Proliferative Transcriptional Switch

Michele A. Wozniak,[1] Catherine Q. Cheng,[1] Colette J. Shen,[1] Lin Gao,[1] Anthony O. Olarerin-George,[2] Kyoung-Jae Won,[3] John B. Hogenesch,[4] and Christopher S. Chen[1,*]

[1]Department of Bioengineering, University of Pennsylvania, Philadelphia, PA 19104, USA

[2]Genomics and Computational Biology Graduate Group

[3]Department of Genetics, The Institute for Diabetes, Obesity and Metabolism

[4]Department of Pharmacology and the Institute for Translational Medicine and Therapeutics

Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA

Summary

Background: The ternary complex factors (TCFs; Elk1, Net, and Sap-1) are growth factor-responsive transcription cofactors of serum response factor (SRF) and are activated by MAP kinase (MAPK) phosphorylation to regulate immediate early gene transcription. Although cell adhesion also can regulate immediate early genes and proliferation, the mechanism for this effect has remained unexplored.

Results: Restricting adhesion and spreading of G_0-synchronized cells on substrates with decreasing size of micropatterned islands of fibronectin suppressed serum-induced immediate early gene expression and S phase entry. Knockdown of Sap-1 decreased expression of the immediate early genes *egr1* and *fos* and subsequent proliferation normally present with high adhesion, whereas knockdown of Net rescued *egr1* and *fos* expression and proliferation normally suppressed by low adhesion. Chromatin immunoprecipitation studies showed increased occupancy of *egr1* and *fos* promoters by Sap-1 with high adhesion, whereas low adhesion increased Net occupancy. This switch in TCF promoter binding was regulated by an adhesion-mediated switch in MAPK activity. Increasing adhesion enhanced serum-induced JNK activity while suppressing p38 activity, leading to increased Sap-1 phosphorylation and Net dephosphorylation, and switching Net with Sap-1 at *egr1* and *fos* promoters to support proliferation. Microarray studies confirmed this switch in TCF regulation of proliferative genes and uncovered novel gene targets and functions coregulated by Sap-1 and Net.

Conclusions: These data demonstrate a key role for the TCFs in adhesion-induced transcription and proliferation and reveal a novel MAPK/TCF transcriptional switch that controls this process.

Introduction

Cell adhesion to the extracellular matrix (ECM) is a principal control point for proliferation. Not only do normal cells require adhesion to proliferate [1], but the extent of cell adhesion provides an additional regulatory point for proliferation. Reducing ECM ligand density or using micropatterned surfaces to limit the degree of cell spreading and adhesion results in decreased immediate early gene expression and proliferation [2–4]. Although proliferation is dependent on the regulated transcription of the immediate early genes and components of the cell-cycle machinery, it is unclear how these transcriptional changes are regulated by adhesion.

To begin to address how changes in adhesion might control proliferative gene expression, we used unbiased computational methods to predict what transcription factors (TFs) were most likely responsible for gene expression changes observed in microarrays obtained from cells under different adhesive conditions. Among the top TFs identified, serum response factor (SRF) has a number of features that suggested it might be an important target. It is involved in regulating the expression of numerous cytoskeletal genes important for cell adhesion [5], is important for differentiation programs that are known to be affected by changes in adhesion or cell shape [6], and is involved in proliferative regulation [7–9].

Two major mechanisms for regulation of SRF activity have been described. One involves the myocardin-related transcription factor (MRTF) family of cofactors, which stimulate SRF activity at the CC A/T-rich GG promoter sequence (CArG box) [10]. It has been shown that MAL, or MRTF-A, is activated by Rho-mediated shifts in actin polymerization [11], and cell adhesion and spreading are important regulators of Rho signaling [12]. In addition, SRF activity is also regulated by the ternary complex factor (TCF) family, a subclass of the ETS transcription factor family, members of which bind to Ets sites near the SRF-binding CArG box [13]; this promoter element that contains the CArG box and Ets site is called the serum response element (SRE) [14]. The TCF family includes the three TFs Elk1, Sap-1 (Elk4), and Net (Elk3); all activate transcription with SRF, although Elk1 and Net can also be repressive [15, 16]. Phosphorylation of TCFs by the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), or p38 mitogen-activated protein kinases (MAPKs) induces a conformational change in the TCF that is proposed to enhance DNA binding and transcriptional activity [17–20]. The similarity in protein structure, ability to drive immediate early gene expression in vitro [21–23], and limited (nonlethal) effects of individual TCF mouse knockouts [24–26] have led many to hypothesize that the three TCFs may be functionally redundant. The TCFs are implicated in growth control given that dominant-negative Elk, which blocks all three TCFs, decreases immediate early gene expression [27]. Thus, MRTF-A-dependent SRF signaling has been closely tied to adhesion signaling but not proliferative regulation, whereas TCF-dependent activity is associated with proliferative control but possesses no known link to adhesion. As such, although SRF signaling exhibits features that could link adhesion to proliferative regulation, a clear mechanism for such a link is absent.

Here we set out to determine how SRF signaling might be involved in adhesion-dependent proliferation and found differential roles for specific TCFs in this regulation. We show that limiting cell adhesion and spreading controls a previously undescribed switch in JNK/p38 and Sap-1/Net activities to regulate SRE promoter occupancy, immediate early gene transcription, and proliferation.

*Correspondence: chrischen@seas.upenn.edu

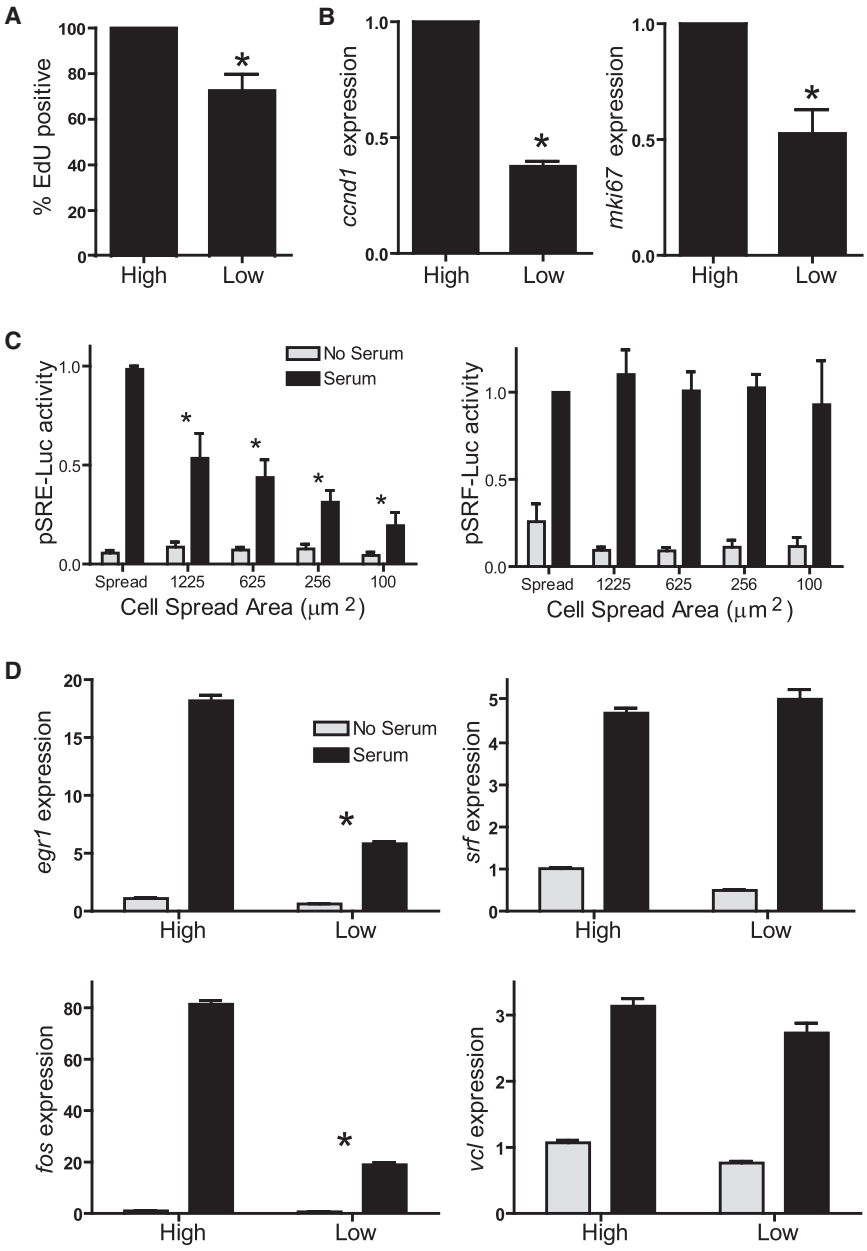

Figure 1. Cell Adhesion Regulates Proliferation and SRE-Dependent Transcription

(A and B) To assay cell proliferation under conditions of high and low adhesion, EdU incorporation assays (A) or qPCR for *ccnd1* and *mki67* (B) were performed. Means ± SEM from at least four independent experiments are shown. *p < 0.05, paired t test.

(C) Cells were transfected with pSRE-Luc (left panel), which contains the Ets site and CARG box, or pSRF-Luc (right panel), which lacks the Ets site, and plated on micropatterned islands, and luciferase assays were performed. Data are presented as means ± SEM, n = 4. *p < 0.05, two-way ANOVA.

(D) qPCR for *egr1*, *fos*, *srf*, and *vcl*. Data are presented as means ± SEM, n = 4. *p < 0.05, two-way ANOVA.

See also Figure S1.

Results

Cell Adhesion Regulates Proliferation and SRE-Dependent Transcription

We have previously shown that decreasing cell adhesion and spreading suppresses proliferation of endothelial cells [2]. In this study, we first confirmed that NIH 3T3 fibroblast proliferation is similarly sensitive to adhesion using microcontact printing. Cells were plated on large areas of fibronectin (FN) so that cells could fully spread, or on 1,225 μm^2 islands of FN to limit cell adhesion by directly restricting cell spreading. Proliferation was assessed by measuring incorporation of the thymidine analog 5-ethynyl-2′-deoxyuridine (EdU) or expression of cyclin D1 (*ccnd1*) and Ki67 (*mki67*) transcripts. As expected, limiting cell adhesion decreased EdU incorporation (Figure 1A) and *ccnd1* and *mki67* expression (Figure 1B).

We hypothesized that specific TFs might regulate the proliferative response to changes in adhesion. To identify these candidate TFs, we analyzed two microarray data sets, from human umbilical vein endothelial cells (HUVECs [28]) and human mesenchymal stem cells (hMSCs) in which micropatterning was used to control adhesion. Each microarray data set was individually processed using Computational Ascertainment of Regulatory Relationships Inferred from Expression (CARRIE), which identifies TFs with significant expression changes or promoter binding site overabundance [29]. The CARRIE-identified TFs in HUVECs and hMSCs were then compared to find the TFs common to both cell types. One of the top TFs identified was SRF (see Table S1 available online).

Given the central role of SRF in controlling proliferation, we determined whether changes in cell adhesion regulate SRF activity. NIH 3T3 cells were transfected with pSRE-Luc, in which the luciferase promoter included the full SRE (CArG box and Ets site), or pSRF-Luc, which contains the CARG box but lacks the Ets site. Cells were plated on micropatterned substrates, serum starved overnight, and then stimulated with serum, and luciferase assays were performed. Reducing cell adhesion significantly decreased SRE-dependent luciferase activity compared to highly adherent, well-spread cells (Figures 1C and S1A). Moreover, micropatterning smaller areas of FN to progressively restrict cell adhesion and spreading resulted in further decreases in luciferase activity (Figure 1C). Surprisingly, restricting cell spreading had no effect on activity of pSRF-Luc (Figure 1C). Although the differences in luciferase activity due to spreading were most pronounced after acute serum exposure following starvation, the differences were still present in cells continuously cultured with serum (data not shown).

We next examined whether other manipulations of cell adhesion and spreading could regulate SRF. First, FN coating

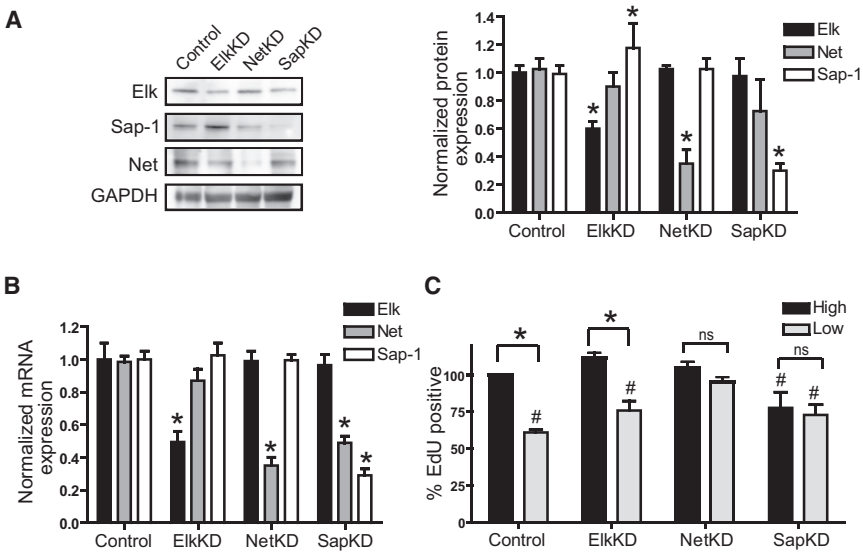

Figure 2. Adhesion Regulates Proliferation through the Sap-1 and Net TCFs

(A and B) Stable cell lines expressing short hairpin RNAs against Elk1, Net, and Sap-1 were generated to knock down protein (A) and mRNA (B) levels. Protein levels were normalized to GAPDH; means ± SEM are shown for n = 5. mRNA levels were normalized to 18S; means ± SEM are shown for n = 3. *p < 0.05 versus controls, paired t test.

(C) Cell lines were plated under high- or low-adhesion micropatterned conditions, and EdU incorporation assays were performed. Data are presented as means ± SEM, n = 4. *p < 0.05 high versus low for each cell line, #p < 0.05 versus high control, both by paired t tests.

density was reduced from 20 μg/ml to 1 μg/ml in order to modulate integrin binding and clustering and to reduce cell spreading (Figure S1B). Second, cell confluency was increased (Figure S1C), which indirectly reduces the extent of contact and spread area that cells have with the ECM. Finally, FN-crosslinked polyacrylamide gels were used to change substrate stiffness (Figure S1D), which can regulate integrin activation, cell adhesion, and spreading [30]. When cell adhesion was reduced by any of these manipulations, SRE luciferase activity decreased while SRF luciferase activity remained unchanged (Figures S1B–S1D). Together, these data indicate that limiting cell adhesion to the ECM regulates genes that contain the full SRE as compared with the CARG box alone.

Because transcriptional regulation of plasmid-borne genes may not reflect endogenous genomic regulation, real-time quantitative PCR (qPCR) was used to analyze endogenous expression of four SRF-target genes, *egr1*, *fos*, *srf*, and *vcl* (vinculin). The immediate early genes *egr1* and *fos* contain the full SRE and are dependent on TCFs for their transcription, whereas *srf* and *vcl* lack the Ets site and are thought to act independently of TCFs [31, 32]. Unless otherwise noted, the studies described below used small islands to generate "low" adhesion or large areas of FN to generate "high" adhesion. The expression of *egr1* and *fos* was reduced when cell adhesion was restricted (Figure 1D). However, *srf* and *vcl* showed no difference in expression (Figure 1D). The mRNA expression changes are likely due to changes in transcription and not changes in transcript stability, because qPCR for the unspliced *egr1* transcript versus a single *egr1* intron detected similar expression levels (Figure S1E). These data further supported a model whereby the Ets site in the SRE confers adhesion-dependent regulation of SRF and implicated the TCF, and not the MRTF, family of cofactors in this process. We also analyzed Rho signaling, which is known to regulate MRTF activity through its effects on actin polymerization [11]. Inhibition of Rho with C3 exoenzyme decreased CArG box-specific pSRF-Luc activity to the same degree in both high- and low-adhesion conditions (Figure S1F). Although this confirmed the requirement for Rho in regulating CArG box-dependent transcription, it also demonstrated that this role of Rho is not modulated by adhesion. Supporting this, we also found no change in Rho activity when adhesion was changed (data not shown).

Adhesion Regulates Proliferation through the Sap-1 and Net TCFs

Because the TCFs bind to the Ets site to regulate the transcription of SRE-containing genes, we next examined which TCFs were involved in adhesion-dependent proliferation. To test each TCF's involvement, NIH 3T3 cell lines were generated that stably expressed short hairpin RNAs (shRNAs) against Elk1, Sap-1, or Net (ElkKD, SapKD, or NetKD). The SapKD and NetKD cell lines showed a 60% specific reduction at the protein level (Figure 2A), and all three cell lines showed at least a 50% knockdown at the mRNA level (Figure 2B). EdU assays were used to test whether the TCFs modulate adhesion-dependent proliferation. TCF KD cells were synchronized by increased cell confluency and serum starving and were plated on micropatterned substrates in the presence of serum. In the control and ElkKD cell lines, restricting cell adhesion decreased proliferation (Figure 2C). However, both the SapKD and NetKD cell lines showed a loss of regulated proliferation by cell adhesion. Specifically, knockdown of Net rescued proliferation in adhesion-limited conditions to levels similar to those in highly adhesive cells (Figure 2C). Conversely, knockdown of Sap-1 abrogated the increase in proliferation normally present in highly adhesive cells (Figure 2C). These data suggest that when adhesion is high, Sap-1 positively regulates proliferation, whereas when adhesion is limited, Net blocks proliferation.

Adhesive Context Dictates Sap-1 and Net Promoter Binding and Transcriptional Activity

Because Net and Sap-1 regulated adhesion-dependent proliferation, we next wanted to determine whether they similarly regulated the adhesion-dependent transcription of their SRE-containing immediate early gene targets *egr1* and *fos*. Luciferase assays with the pSRE-Luc construct, as well as qPCR analysis for *egr1* and *fos*, were performed in serum-starved and then stimulated cells cultured under high- or low-adhesion contexts. SapKD cells lost the increase in serum-induced luciferase activity (Figure 3A) and *egr1* and *fos* expression (Figures 3B and 3C) in high- versus low-adhesion conditions that was observed in control cells (Figures 3A–3C). Interestingly, when adhesion was limited in the NetKD cells, *egr1* and *fos* expression were rescued (Figures 3A–3C), suggesting that Net represses or weakly activates immediate early gene expression under low-adhesion contexts. Although the NetKD cells plated on low adhesion showed a slight super-activation in the luciferase assays, the endogenous SRE

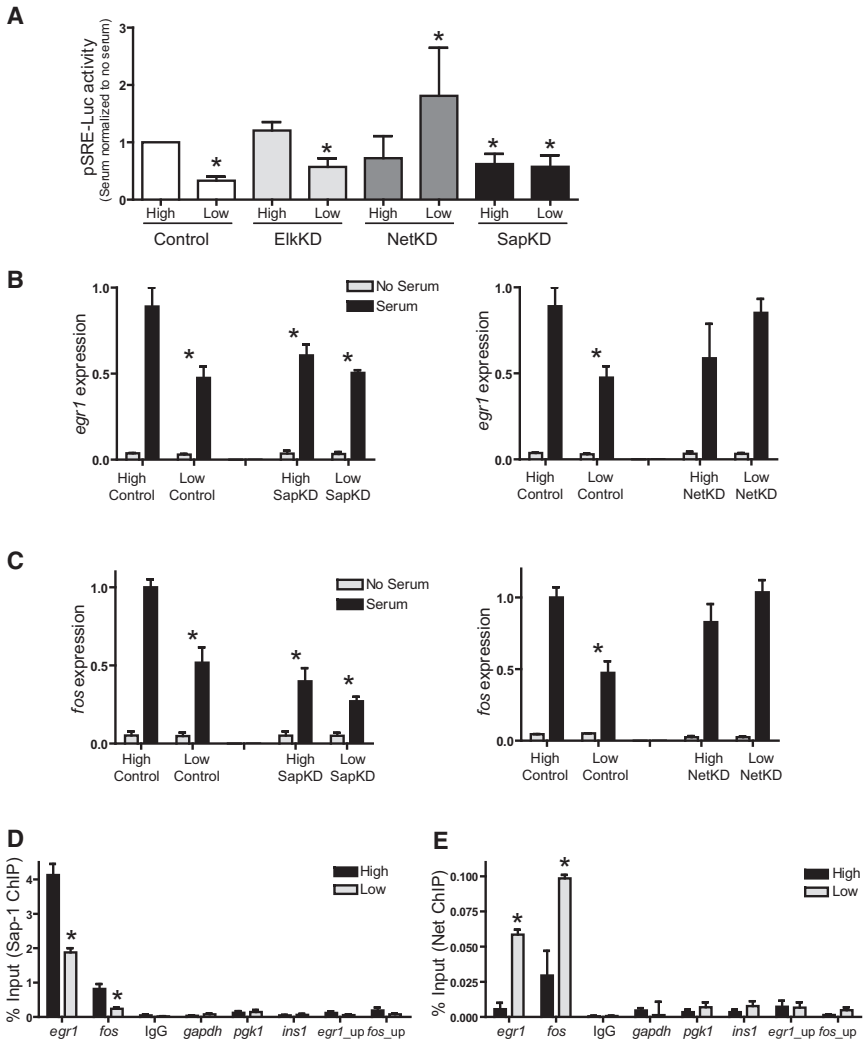

Figure 3. Adhesive Context Dictates Sap-1 and Net Promoter Binding and Transcriptional Activity

(A) pSRE luciferase assays were performed in cells plated under high- versus low-adhesion micropatterned conditions. Data are presented as serum values normalized to no serum values; means ± SEM are shown for n = 5. *p < 0.05 versus control high, paired t test.

(B and C) qPCR was performed for *egr1* (B) and *fos* (C) expression in SapKD (left panel) and NetKD (right panel) cells compared to control cells. Means ± SEM are shown for n = 4. *p < 0.05 versus control high, two-way ANOVA.

(D and E) Chromatin immunoprecipitations (ChIPs) detected Sap-1 (D) or Net (E) binding to an *egr1* promoter region containing two SREs ~100 nt upstream of the transcription start site (TSS) and a SRE-containing region of the *fos* promoter ~350 nt upstream of the TSS under conditions of high or low adhesion (generated by changing cell confluency) and stimulated with serum. Controls were IgG binding to *egr1* containing two SREs and Sap or Net binding to *gapdh*, *pgk1*, *ins1*, and ~1,000 bp upstream of *egr1* and *fos* TSSs, none of which contain SREs. Data shown are means ± SEM for n = 4. *p < 0.05 versus high by paired t test.

See also Figure S2.

targets *egr1* and *fos* showed a simple rescue. Thus, the effects of Sap-1 and Net knockdown on immediate early gene expression were similar to their effects on proliferation. However, the ElkKD cells exhibited no changes in *egr1* or *fos* expression (Figure S3) or adhesion-dependent luciferase activity (Figure 3A). Although this suggests that Elk1 may not be involved in adhesion-dependent immediate early gene expression or proliferation, it is also possible that the ~35%–40% reduction in Elk1 protein expression may be insufficient to affect Elk1 signaling. Because we were unable to further decrease Elk1 protein levels by small interfering RNA or shRNA, we cannot definitively conclude that Elk1 is not involved in these processes.

These data suggest that when adhesion is high and cells are spread, Sap-1 positively regulates immediate early gene expression, whereas when adhesion and spreading are limited, Net blocks immediate early gene expression. Because Sap-1 and Net should differentially bind to the promoter to activate or repress transcription, respectively, as a function of cell adhesion, chromatin immunoprecipitation (ChIP) experiments were performed to analyze TCF binding on SRE-containing regions of the *egr1* and *fos* promoters. For this study, changes in cell confluency were used to control cell adhesion and spreading (as in Figure S1C) because micropatterning could not feasibly provide sufficient amounts of chromatin for analysis. When adhesion was limited, Sap-1 showed decreased binding to the *egr1* and *fos* promoters as compared to highly adhesive cells (Figure 3D). Conversely, Net showed enhanced binding to the *egr1* and *fos* promoters under conditions of low adhesion (Figure 3E). As controls, neither IgG alone, regions of the *egr1* or *fos* promoters ~1,000 bp upstream of the SREs, nor three different genomic regions that do not contain SREs showed enrichment in either ChIP assay (Figures 3D and 3E). These data show that differences in cell adhesion can trigger a switch in TCF promoter occupancy that correlates with changes in *egr1* and *fos* expression.

JNK and p38 Play Opposing Roles to Regulate Adhesion-Mediated TCF Activity

It is not clear, mechanistically, how adhesion might regulate changes in TCF activity and promoter occupancy. However, the MAPKs are known to phosphorylate and activate the TCFs [17–20], and separate studies have shown that changes in adhesion induce changes in MAPK activity [33, 34]. We therefore wanted to determine, first, which MAPKs were regulated by changes in adhesion and, second, whether this MAPK activity mediated the gene expression changes resulting from limited adhesion. To determine MAPK activity, we obtained cell lysates from cells that were plated on micropatterned surfaces, serum starved overnight, then stimulated with serum for 45 min. Western blotting showed that serum-induced phospho-ERK levels were not significantly different when adhesion was restricted (Figure 4A). Additionally, ERK translocated to the nucleus equally efficiently after serum stimulation for

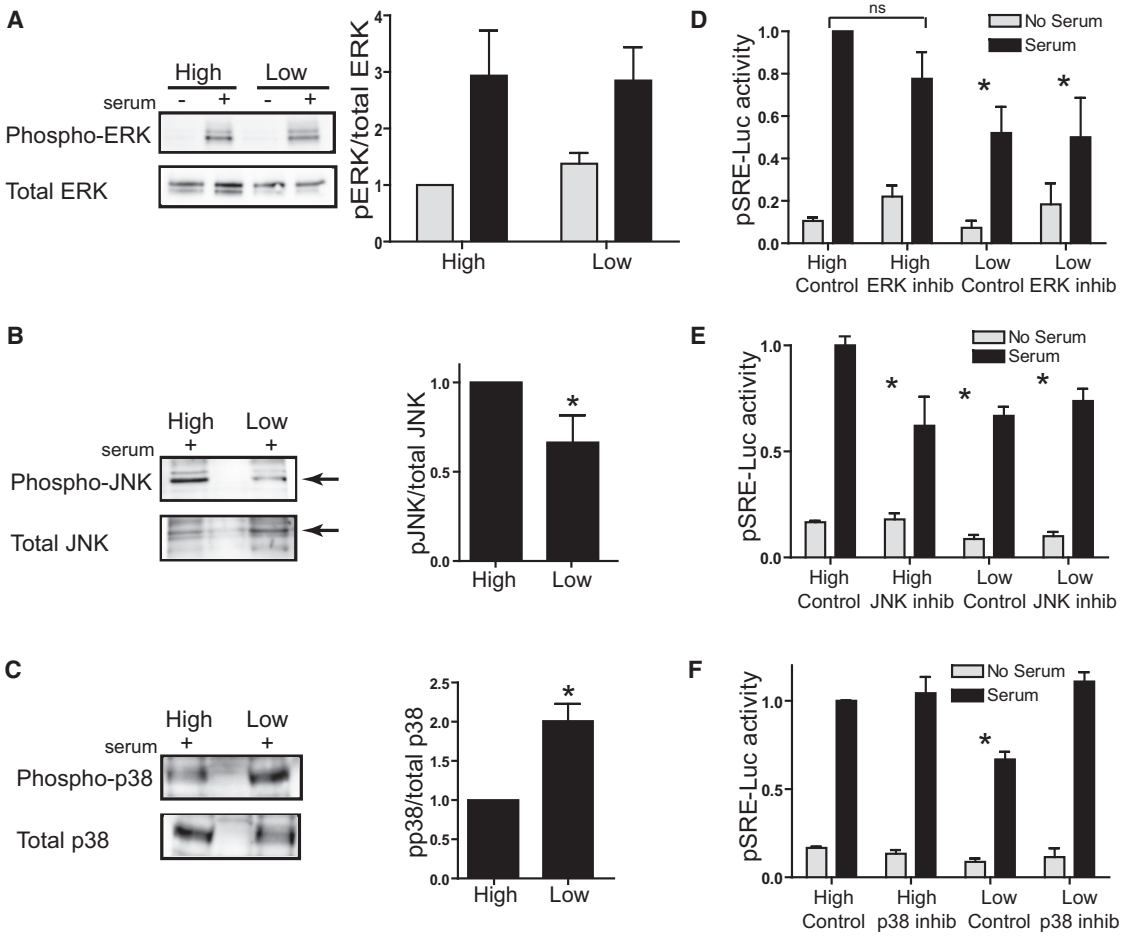

Figure 4. JNK and p38 Play Opposing Roles in Adhesion-Regulated TCF-Targeted Transcription

(A–C) Western blotting and quantitation for phospho-ERK and total ERK (A), JNK (B, see arrows), and p38 (C) levels in cells micropatterned under conditions of high or low adhesion and stimulated by serum.

(D–F) pSRE luciferase assays were performed in cells treated with 50 μM PD98059 (ERK inhibitor; D), 20 μM SP 600125 (JNK inhibitor; E), 10 μM SB 203580 (p38 inhibitor; F), or vehicle control.

Means ± SEM are shown in all panels. For western blots, n = 6; *p < 0.05, paired t test or two-way ANOVA (for ERK). Luciferase assays were repeated at least four times; *p < 0.05 versus high control, two-way ANOVA. See also Figure S3.

both high- and low-adhesion conditions (Figure S4A). These data raised the possibility that ERK did not mediate adhesion-dependent SRF/TCF activity. In support of this, the upstream MEK1 inhibitor PD98059 (Figure 4D) and the MEK1/2 inhibitor UO126 (data not shown) failed to abrogate luciferase activity in highly adhesive cells.

Therefore, we explored the possibility that the dominant control point might lie with JNK or p38. In response to serum stimulation, phospho-JNK levels increased under conditions of high adhesion (Figure 4B), whereas phospho-p38 levels increased under conditions of low adhesion (Figure 4C). Performing pSRE-Luc luciferase assays with pharmacological inhibitors revealed that selective inhibition of JNK with SP 600125 significantly decreased luciferase activity of highly adherent cells to levels similar to low-adhesion conditions (Figures 4E and S4B). Conversely, under limited adhesion, selective inhibition of p38 with SB 203580 rescued luciferase activity (Figures 4F and S4B). These data suggest a balance in signaling between JNK and p38 such that when adhesion is high, JNK positively regulates SRE activity, whereas under limited adhesion and spreading, active p38 represses SRE activity.

Because MAPK-mediated serine and threonine TCF phosphorylation is proposed to potentiate transcriptional activity by enhancing TCF binding to DNA [17–20], we next determined whether JNK and p38 regulate Sap-1 and Net phosphorylation and promoter binding in an adhesion-dependent manner. We first tested TCF phosphorylation in NIH 3T3 cells that were plated under high- or low-adhesion conditions, serum starved overnight, and stimulated with serum and DMSO control or MAPK inhibitors the following day. To measure phosphorylation states, we developed an assay in which Sap-1 and Net immunoprecipitations were performed and bound proteins were eluted and analyzed by western blotting for phosphoserine. Sap-1 phosphorylation was significantly increased under conditions of high adhesion, and this was blocked by inhibition of JNK, but not by inhibition of p38 (Figure 5A). Conversely, Net phosphorylation was increased when adhesion was limited, and inhibition of p38 but not JNK abrogated this increase (Figure 5B).

To determine whether these changes in TCF phosphorylation correlate with changes in TCF promoter binding, we used ChIP assays to test whether Sap-1 promoter binding in

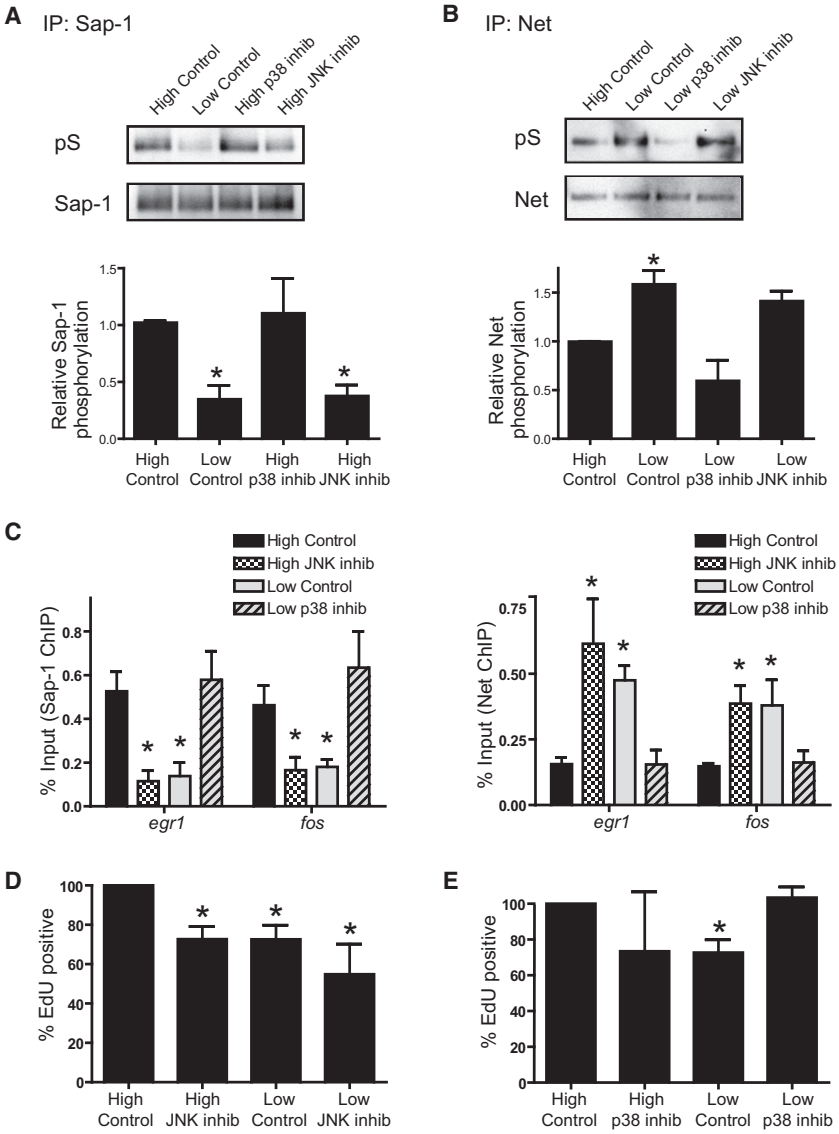

Figure 5. Changes in Adhesion Switch TCF Phosphorylation, Promoter Occupancy, and Proliferation in a MAPK-Dependent Manner

(A and B) Sap-1 (A) or Net (B) immunoprecipitations in serum-starved micropatterned cells treated with DMSO control, SP 600125, or SB 203580 and stimulated with serum. Means ± SEM are shown for n = 6. *p < 0.05 versus high control, paired t test.

(C) ChIPs were performed in high- or low-adhesion cells treated with the inhibitors and stimulated with serum. ChIPs tested Sap-1 (left panel) and Net (right panel) binding to the same SRE-containing *egr1* and *fos* promoter sites analyzed in Figure 3. ChIP data in (C)–(E) are presented as means ± SEM, and all experiments were repeated at least three times. *p < 0.05 versus high control, paired t test.

(D and E) Cells were plated on high- or low-adhesion micropatterned substrates with DMSO control, SP 600125 (D), or SB 203580 (E), and EdU incorporation assays were performed.

highly adhesive cells is dependent on JNK activity and whether Net promoter binding is dependent on p38 activity when adhesion is reduced. Under conditions of high adhesion, inhibition of JNK decreased Sap-1 binding to the *egr1* and *fos* promoters to levels similar to those in low-adhesion cells (Figure 5C). Interestingly, JNK inhibition also caused a concomitant increase in Net promoter binding (Figure 5C). Conversely, under conditions of low adhesion, inhibition of p38 decreased Net promoter occupancy to levels observed in highly adhesive cells (Figure 5C), while also increasing Sap-1 promoter binding (Figure 5C). These data indicate that changes in cell adhesion and spreading switch TCF phosphorylation and promoter occupancy in a JNK/p38-dependent manner.

Given that changes in adhesion regulate proliferation through a switch in TCF activity, we hypothesized that JNK and p38 would also regulate adhesion-dependent proliferation. JNK inhibition decreased EdU incorporation in highly adhesive cells but had no effect when adhesion was limited, suggesting that JNK promotes proliferation in well-adherent cells (Figure 5D). Conversely, inhibition of p38 had little effect when adhesion was high but rescued EdU incorporation when adhesion was limited, suggesting that p38 represses proliferation under low-adhesion contexts (Figure 5E).

Microarray Analysis Confirms TCF Regulation of Proliferation and Predicts Additional TCF-Regulated Genes and Functions

Giovane et al. have shown that the Net TCF can change from a transcriptional repressor to an activator upon Ras activation [22]. However, here we show a different type of TCF antagonism in which Net and Sap-1 act antagonistically to each other, on the same promoter, due to changes in adhesion. To identify other genes and functions that Sap-1 and Net regulate, antagonistically or similarly, in response to adhesion, we compared how knockdown of Sap-1 versus Net affects global gene expression via microarray analysis. NIH 3T3 cells were plated in a highly adherent context and serum starved overnight, then stimulated with serum for 1 hr the following day before RNA isolation and microarray analysis. Genes that changed by 2-fold or more with a false discovery rate (FDR) of 0.1% were identified, and qPCR was used to confirm the top ten genes downregulated in response to Sap-1 or Net knockdown, or upregulated in response to Net knockdown (Table S2).

We next determined what genes might be direct targets of SRF/TCFs via two different analyses. First, the mouse genome was searched 5,000 nt upstream of each transcription start site for the consensus SRE promoter binding site GGA(A/T)XXCC(A/T)6GG, with GGA being the invariant core of the TCF promoter binding site and the CArG box being the binding site for SRF. This analysis identified 82 promoter sites matching this sequence consensus, corresponding to 81 unique genes including *egr1* and *fos*, known targets of the SRF/TCFs. From this list, 77 of the 81 genes were included in the microarray (Table S3). We then determined whether these genes changed expression in the microarray using a 0.1% FDR. Of the predicted direct binders, 4.9% were

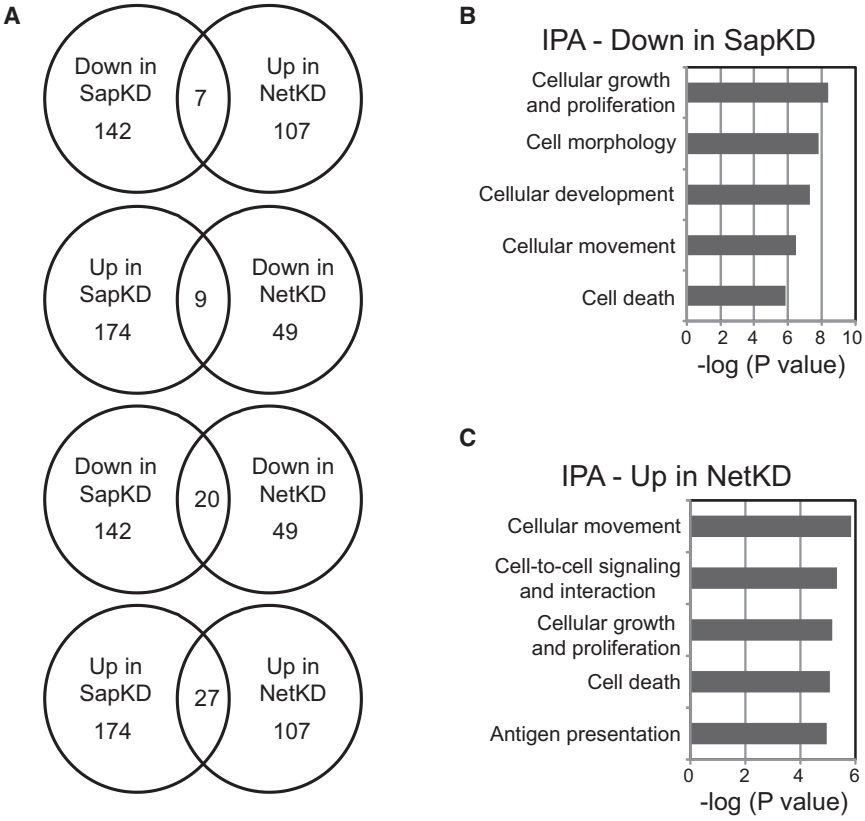

Figure 6. Microarray Analysis Confirms TCF Regulation of Proliferation and Predicts New Functions Regulated by TCFs

(A) Venn diagrams showing the overlaps observed in NetKD and SapKD cells.

(B and C) Unique genes downregulated in SapKD (B) or upregulated in NetKD (C) cells were analyzed using the Ingenuity Pathway Analysis program. Functions are plotted against the −log (p value). See also Figure S4 and Tables S2, S3, and S4.

differentially regulated in the NetKD cells, as compared to 1.9% of all genes. Of the predicted direct binders, 10.4% were regulated in the SapKD cells, as compared to 13.9% of all genes. These data confirm that TCF knockdown regulates direct TCF targets.

One-third of the 77 predicted candidates were tested for direct TCF binding using Net and Sap-1 ChIPs. This analysis uncovered five candidates that did not bind Net or Sap-1; the remaining 19 genes bound to at least one TCF (Table S3). Of the predicted direct TCF binders that showed a reciprocal regulation (up in SapKD/down in NetKD or vice versa), 6 out of 9 were able to bind to both Sap-1 and Net in ChIP assays, as expected. However, there were not simple correlations such that genes downregulated in the SapKD cells and unchanged in the NetKD cells bound only to Sap-1, or genes downregulated in the NetKD cells bound only to Net. This confirms that TCF promoter binding alone is not sufficient to induce transcriptional changes; rather, there are likely other signals such as phosphorylation [17–20] or acetylation [16, 35] that must occur.

We next compared Sap-1 targets identified by a published human Sap-1 ChIP sequencing data set [36] to our microarray analysis of SapKD cells. Thresholding at 1×10^{-4} Poisson p value and 0.1% FDR, we combined the replicate genes identified from the published data (Table S4, Sheet 1). 15,970 binding sites were detected, which corresponded to 7,746 unique genes. In comparison to our microarray, 1,034 were human genes that did not have a corresponding mouse gene and thus could not be compared. Of the remaining 5,383 genes, 807 were changed in the SapKD microarray (Table S4, Sheet 2). This list included genes that were both up- and downregulated in the microarray, some of which showed high fold changes (*casc4*, *rgs4*, *gpr39*, and *serpinb1a*). Taken together, these two analyses suggest that Sap-1 and Net knockdown alter gene expression of direct and nondirect binding targets, as expected.

In order to further examine Sap-1 and Net reciprocity, genes identified in the microarray as up- and downregulated in response to Sap-1 and Net knockdown (2-fold change, 0.1% FDR) were compared and the regulatory overlaps determined (Figure 6A). To determine the likelihood that these overlaps occurred by chance, we simulated one million random iterations using the R statistical program and compared the number of genes expected to show chance regulatory overlap to the observed overlaps. For each case, the overlap that we observed had a probability of $p < 1 \times 10^{-6}$ of occurring by chance. These data demonstrate that Sap-1 and Net coregulate many genes in addition to *egr1* and *fos*, some antagonistically and others similarly.

The biological consequences of TCF knockdown were predicted using the Ingenuity Pathway Analysis (IPA) program to provide a network analysis of relationships and functions and the Database for Annotation, Visualization, and Integrated Discovery (DAVID) program for gene ontology analysis. The DAVID and IPA analyses predicted known TCF functions, including locomotion, development, and inflammatory/immune response [37]. Additionally, the analyses predicted several physiological roles not previously described to be regulated by the TCFs, including cell-cell signaling and adhesion, ECM organization, immune signaling, chemotaxis, and transport (Figure S4).

Finally, results of the microarray analyses supported our data suggesting that Sap-1 and Net have opposing roles in adhesion-mediated proliferative regulation. First, in the microarray, *egr1* expression decreased in SapKD cells by 1.06-fold (log2 transformed), which corresponded to qPCR results in these cells plated in a highly adherent context. Second, the IPA analysis predicted cellular growth and proliferation to be downregulated in the SapKD cells (Figure 6B) and upregulated in the NetKD cells (Figure 6C), verifying an antagonistic role for Sap-1 and Net TCFs in proliferative regulation. Finally, cell adhesion was identified by DAVID as a top function affected in the common and unique genes regulated by Sap-1 and Net knockdown (Figure S4), confirming that the TCFs are significant mediators of adhesion-induced cell behavior.

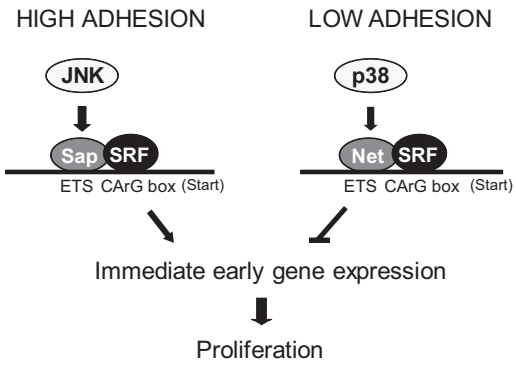

Figure 7. Proposed Model of Adhesive Regulation of the MAPK/TCF Switch to Control Immediate Early Gene Expression and Proliferation

The model most consistent with our results is as follows. Upon serum stimulation, conditions of high adhesion induce Sap-1 phosphorylation and promoter binding in a JNK-dependent manner, which stimulates SRF-targeted immediate early gene transcription and subsequent proliferation. When adhesion is restricted, MAPK/TCF activity is switched; increased p38 activity leads to Net phosphorylation and promoter occupancy of SRF target genes to inhibit their transcription, leading to decreased proliferation.

Discussion

Although the TCFs have classically been studied as growth factor-responsive transcription factors, here we show that this response is regulated by adhesion, and we present a model in which changes in adhesion regulate a Sap-1/Net transcriptional switch to control proliferation (Figure 7). This characterization of an adhesion-mediated switch in Sap-1/Net activity is a mechanism that has not been previously described for any of the known soluble triggers of the TCF pathway and therefore points to a new means by which TCFs can regulate SRF target genes.

Interestingly, there is no clear consensus on the requirement for SRF in cell proliferation. SRF appears to be necessary for proliferation in some cells, such as hepatocellular carcinoma cells [7], cardiomyocytes [9], and fibroblasts [8], whereas it is dispensable for proliferation in others, including embryonic stem cells [38]. These differences may be due to cell-type-specific requirements for SRF or SRF phosphorylation status [39]. Our data suggest that although SRF is required for fibroblast proliferation, the adhesive context is what ultimately provides the signaling permissive for SRF-mediated proliferation.

Although changes in adhesion are often associated with cytoskeletal reorganization, we surprisingly found that that the actin polymerization-sensitive MRTF-regulated CArG box-only genes are not regulated by changes in adhesion. Although this does not completely exclude MRTFs from adhesive regulation, this and the observation that the Rho pathway is insensitive to changes in adhesion suggest that, at least in fibroblasts, adhesive regulation of SRF signaling may not be regulated only through Rho-mediated tension. This TCF mechanism for transcriptional regulation by adhesion is different from other transcription factor pathways that are mechanically regulated, such as MRTF-A [6, 40], YAP/TAZ [41], KLF2 [42], and GATA2 and TFII-I [43]. Together, these findings suggest that changes in adhesion can regulate transcription through both tension-sensitive and -insensitive pathways. Interestingly, SRF may exploit both of these pathways, via the MRTF and TCF cofactors, to regulate cell behavior.

Although the TCF family regulates many genes, whether the TCFs fulfill redundant or independent roles is not well understood. The TCFs were originally proposed to be functionally redundant because much of the early research used exogenous *c-fos* as a template to understand TCF promoter binding and regulation. However, recent work has shown that in certain contexts (depending on cell type, relative levels of TCFs, etc.) TCFs can be redundant, whereas under other circumstances they are not [37]. Here we knocked down each TCF individually to compare their effect on adhesion-mediated gene expression and function and found that two of the TCFs, Net and Sap-1, play opposing roles in proliferative gene expression and growth control.

Mechanistically, we propose that limiting adhesion regulates a switch in JNK and p38 MAPK activity, which in turn controls TCF phosphorylation and promoter occupancy, immediate early gene expression, and proliferation. Unexpectedly, we did not find a role for ERK signaling in this process even though ERK has been implicated in serum-induced SRE activity [32] and ERK has classically been described as the MAPK whose activity is dependent on adhesion [33]. However, this model of adhesion-regulated proliferation is largely based on studies comparing suspended versus adherent cells, in which suspension abrogates ERK activation and proliferation [33]. Interestingly, others have reported that more subtle changes in the extent of adhesion (on micropatterned surfaces or polyacrylamide gels of decreasing stiffness) also inhibit proliferation, but without suppressing ERK activity [44, 45]. We extend these observations and propose that limiting adhesion is not the same as complete loss of adhesion due to suspension; whereas loss of adhesion regulates ERK, limiting adhesion regulates a switch in JNK and p38 MAPK activity. To our knowledge, our study is the first demonstration that this antagonism can be controlled through changes in the adhesive microenvironment. Thus, shifts in these three MAPKs may provide a paradigm for how cells are able to distinguish different types of changes in adhesive environment and respond appropriately.

We show Sap-1 and Net TCF switching at the promoters of immediate early genes to antagonistically control gene expression. Although it is known that SRF switches cofactors from the MRTF myocardin to the TCF Elk1 to repress smooth muscle gene expression [46], we unexpectedly show that switching between TCF family isoforms can also change gene expression. Although other transcription factors have demonstrated switching behavior, such as NFAT and NF-κB during fear memory reconsolidation [47] or GATA-2 and TFII-I competing on the VEGFR promoter [43], the TCF switch is different in that Sap-1 and Net are highly homologous isoforms competing for the same promoter binding site. This switch is also unique compared to the ETS domain-containing E74 transcription factor isoforms, E74A and E74B, which are turned on at different developmental stages to regulate the timing of early and late response genes [48]. Thus, we are not aware of another example of transcription factor switching similar to the TCF model described here.

This transcriptional switch model evokes several intriguing directions for future research. It is not understood why JNK targets Sap-1 in highly adhesive cells, yet p38 targets Net when adhesion is reduced. One possibility is that changes in adhesion alter the levels or localization of the TCFs. For instance, JNK activity can stimulate Net nuclear export [49];

perhaps the increased JNK activity induced by high adhesion increases Net export so that there is more nuclear Sap-1 (and less Net) to form ternary complexes at the SRE. Additionally, given experimental limitations, how Elk1 might play a role in adhesion-mediated SRE activity remains unclear. It will be important to determine whether the three TCFs regulate other genes and functions using a switching mechanism similar to the one we propose for immediate early genes. In conclusion, our findings highlight how changes in adhesion can regulate SRF signaling to control proliferation and describe a novel TCF transcriptional switch mechanism through which this occurs.

Experimental Procedures

Please refer to the Supplemental Experimental Procedures for complete experimental procedures.

Cell Culture

NIH 3T3 cell lines were maintained in growth medium (GM; 10% bovine serum in Dulbecco's modified Eagle's medium).

Immunoprecipitation and Western Blotting

Cells were plated for 2 hr, serum starved overnight, and then stimulated with GM. For TCF immunoprecipitations, cell lysates were incubated with Protein A/G beads and anti-Sap-1 (Santa Cruz, clone H-167X) or anti-Net (Santa Cruz, clone A-20X) antibodies overnight at 4°C. Antibodies used were phosphoserine (EMD Biosciences, 16B4), Elk1 (Santa Cruz, I-20), active MAPK (Promega), phospho-JNK (BioSource), phospho-p38 (Cell Signaling), ERK (Upstate), JNK (Santa Cruz), p38 (Cell Signaling), and GAPDH (Ambion).

ChIP Analysis

Cell pellets were resuspended in 50 mM HEPES (pH 7.5), 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate, 0.1% SDS, protease/phosphatase inhibitors (Thermo Scientific), and 1 mM phenylmethylsulfonyl fluoride and Dounce homogenized; nuclei were collected by centrifugation; and DNA was sheared by Bioruptor sonication (Diagenode). Sepharose Protein A/G beads and anti-Sap-1 antibody, anti-Net antibody, or IgG control (Sigma) were added to the chromatin suspension overnight at 4°C, collected, and eluted; crosslinks were reversed; and proteins were digested with Proteinase K (Active Motif). qPCR detected binding; see Table S5 for sequences.

Statistical Analysis

Means ± SEM and p values were calculated using GraphPad Prism software.

Accession Numbers

Log2 robust multiarray average expression data reported in this paper have been deposited in the NCBI Gene Expression Omnibus with the GEO series accession number GSE26640.

Supplemental Information

Supplemental Information includes four figures, five tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.08.050.

Acknowledgments

The authors thank Ana Cristancho, Mitchell Lazar, and Michelle Lynch (University of Pennsylvania) for assistance with ChIP experiments; Donald Baldwin and John Tobias (University of Pennsylvania) for assistance with the microarray experiment and analyses; Srivatsan Raghavan and Daniel Cohen (University of Pennsylvania) for the HUVEC and hMSC array data; and Primal de Lanerolle (University of Chicago), Richard Assoian, Brendon Baker, Brandon Blakely, Mitchell Lazar, Jennifer Leight, Xiang Yu, Colin Choi, and Jeroen Eyckmans (University of Pennsylvania) for helpful discussions and critical reading of the manuscript. This project was supported by NIH grants EB00262, HL73305, and GM74048 (to C.S.C.). M.A.W. acknowledges support from the Ruth L. Kirschstein National Research Service Award (grant F32 AR054219-01), and A.O.O. acknowledges support from the National Human Genome Research Institute (training grant T32HG000046).

Received: December 5, 2011
Revised: August 22, 2012
Accepted: August 28, 2012
Published online: October 11, 2012

References

1. Stoker, M. (1967). Contact and short-range interactions affecting growth of animal cells in culture. Curr. Top. Dev. Biol. *2*, 107–128.
2. Chen, C.S., Mrksich, M., Huang, S., Whitesides, G.M., and Ingber, D.E. (1997). Geometric control of cell life and death. Science *276*, 1425–1428.
3. Dike, L.E., and Ingber, D.E. (1996). Integrin-dependent induction of early growth response genes in capillary endothelial cells. J. Cell Sci. *109*, 2855–2863.
4. Folkman, J., and Moscona, A. (1978). Role of cell shape in growth control. Nature *273*, 345–349.
5. Sun, Q., Chen, G., Streb, J.W., Long, X., Yang, Y., Stoeckert, C.J., Jr., and Miano, J.M. (2006). Defining the mammalian CArGome. Genome Res. *16*, 197–207.
6. Connelly, J.T., Gautrot, J.E., Trappmann, B., Tan, D.W., Donati, G., Huck, W.T., and Watt, F.M. (2010). Actin and serum response factor transduce physical cues from the microenvironment to regulate epidermal stem cell fate decisions. Nat. Cell Biol. *12*, 711–718.
7. Farra, R., Dapas, B., Pozzato, G., Giansante, C., Heidenreich, O., Uxa, L., Zennaro, C., Guarnieri, G., and Grassi, G. (2010). Serum response factor depletion affects the proliferation of the hepatocellular carcinoma cells HepG2 and JHH6. Biochimie *92*, 455–463.
8. Gauthier-Rouvière, C., Fernandez, A., and Lamb, N.J. (1990). ras-induced c-fos expression and proliferation in living rat fibroblasts involves C-kinase activation and the serum response element pathway. EMBO J. *9*, 171–180.
9. Liu, N., Bezprozvannaya, S., Williams, A.H., Qi, X., Richardson, J.A., Bassel-Duby, R., and Olson, E.N. (2008). microRNA-133a regulates cardiomyocyte proliferation and suppresses smooth muscle gene expression in the heart. Genes Dev. *22*, 3242–3254.
10. Wang, D.Z., Li, S., Hockemeyer, D., Sutherland, L., Wang, Z., Schratt, G., Richardson, J.A., Nordheim, A., and Olson, E.N. (2002). Potentiation of serum response factor activity by a family of myocardin-related transcription factors. Proc. Natl. Acad. Sci. USA *99*, 14855–14860.
11. Miralles, F., Posern, G., Zaromytidou, A.I., and Treisman, R. (2003). Actin dynamics control SRF activity by regulation of its coactivator MAL. Cell *113*, 329–342.
12. McBeath, R., Pirone, D.M., Nelson, C.M., Bhadriraju, K., and Chen, C.S. (2004). Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. Dev. Cell *6*, 483–495.
13. Shaw, P.E., Schröter, H., and Nordheim, A. (1989). The ability of a ternary complex to form over the serum response element correlates with serum inducibility of the human c-fos promoter. Cell *56*, 563–572.
14. Treisman, R. (1987). Identification and purification of a polypeptide that binds to the c-fos serum response element. EMBO J. *6*, 2711–2717.
15. Maira, S.M., Wurtz, J.M., and Wasylyk, B. (1996). Net (ERP/SAP2) one of the Ras-inducible TCFs, has a novel inhibitory domain with resemblance to the helix-loop-helix motif. EMBO J. *15*, 5849–5865.
16. Yang, S.H., Vickers, E., Brehm, A., Kouzarides, T., and Sharrocks, A.D. (2001). Temporal recruitment of the mSin3A-histone deacetylase corepressor complex to the ETS domain transcription factor Elk-1. Mol. Cell. Biol. *21*, 2802–2814.
17. Gille, H., Sharrocks, A.D., and Shaw, P.E. (1992). Phosphorylation of transcription factor p62TCF by MAP kinase stimulates ternary complex formation at c-fos promoter. Nature *358*, 414–417.
18. Janknecht, R., Ernst, W.H., Pingoud, V., and Nordheim, A. (1993). Activation of ternary complex factor Elk-1 by MAP kinases. EMBO J. *12*, 5097–5104.
19. Price, M.A., Rogers, A.E., and Treisman, R. (1995). Comparative analysis of the ternary complex factors Elk-1, SAP-1a and SAP-2 (ERP/NET). EMBO J. *14*, 2589–2601.
20. Whitmarsh, A.J., Shore, P., Sharrocks, A.D., and Davis, R.J. (1995). Integration of MAP kinase signal transduction pathways at the serum response element. Science *269*, 403–407.

21. Dalton, S., and Treisman, R. (1992). Characterization of SAP-1, a protein recruited by serum response factor to the c-fos serum response element. Cell *68*, 597–612.
22. Giovane, A., Pintzas, A., Maira, S.M., Sobieszczuk, P., and Wasylyk, B. (1994). Net, a new ets transcription factor that is activated by Ras. Genes Dev. *8*, 1502–1513.
23. Hipskind, R.A., Rao, V.N., Mueller, C.G., Reddy, E.S., and Nordheim, A. (1991). Ets-related protein Elk-1 is homologous to the c-fos regulatory factor p62TCF. Nature *354*, 531–534.
24. Ayadi, A., Zheng, H., Sobieszczuk, P., Buchwalter, G., Moerman, P., Alitalo, K., and Wasylyk, B. (2001). Net-targeted mutant mice develop a vascular phenotype and up-regulate egr-1. EMBO J. *20*, 5139–5152.
25. Cesari, F., Brecht, S., Vintersten, K., Vuong, L.G., Hofmann, M., Klingel, K., Schnorr, J.J., Arsenian, S., Schild, H., Herdegen, T., et al. (2004). Mice deficient for the ets transcription factor elk-1 show normal immune responses and mildly impaired neuronal gene activation. Mol. Cell. Biol. *24*, 294–305.
26. Costello, P.S., Nicolas, R.H., Watanabe, Y., Rosewell, I., and Treisman, R. (2004). Ternary complex factor SAP-1 is required for Erk-mediated thymocyte positive selection. Nat. Immunol. *5*, 289–298.
27. Vickers, E.R., Kasza, A., Kurnaz, I.A., Seifert, A., Zeef, L.A., O'donnell, A., Hayes, A., and Sharrocks, A.D. (2004). Ternary complex factor-serum response factor complex-regulated gene activity is required for cellular proliferation and inhibition of apoptotic cell death. Mol. Cell. Biol. *24*, 10340–10351.
28. Shen, C.J., Raghavan, S., Xu, Z., Baranski, J.D., Yu, X., Wozniak, M.A., Miller, J.S., Gupta, M., Buckbinder, L., and Chen, C.S. (2011). Decreased cell adhesion promotes angiogenesis in a Pyk2-dependent manner. Exp. Cell Res. *317*, 1860–1871.
29. Haverty, P.M., Frith, M.C., and Weng, Z. (2004). CARRIE web service: automated transcriptional regulatory network inference and interactive analysis. Nucleic Acids Res. *32* (Web Server issue), W213-6.
30. Paszek, M.J., Zahir, N., Johnson, K.R., Lakins, J.N., Rozenberg, G.I., Gefen, A., Reinhart-King, C.A., Margulies, S.S., Dembo, M., Boettiger, D., et al. (2005). Tensional homeostasis and the malignant phenotype. Cancer Cell *8*, 241–254.
31. Cen, B., Selvaraj, A., Burgess, R.C., Hitzler, J.K., Ma, Z., Morris, S.W., and Prywes, R. (2003). Megakaryoblastic leukemia 1, a potent transcriptional coactivator for serum response factor (SRF), is required for serum induction of SRF target genes. Mol. Cell. Biol. *23*, 6597–6608.
32. Gineitis, D., and Treisman, R. (2001). Differential usage of signal transduction pathways defines two types of serum response factor target gene. J. Biol. Chem. *276*, 24531–24539.
33. Aplin, A.E., Stewart, S.A., Assoian, R.K., and Juliano, R.L. (2001). Integrin-mediated adhesion regulates ERK nuclear translocation and phosphorylation of Elk-1. J. Cell Biol. *153*, 273–282.
34. Oktay, M., Wary, K.K., Dans, M., Birge, R.B., and Giancotti, F.G. (1999). Integrin-mediated activation of focal adhesion kinase is required for signaling to Jun NH2-terminal kinase and progression through the G1 phase of the cell cycle. J. Cell Biol. *145*, 1461–1469.
35. O'Donnell, A., Yang, S.H., and Sharrocks, A.D. (2008). MAP kinase-mediated c-fos regulation relies on a histone acetylation relay switch. Mol. Cell *29*, 780–785.
36. O'Geen, H., Lin, Y.H., Xu, X., Echipare, L., Komashko, V.M., He, D., Frietze, S., Tanabe, O., Shi, L., Sartor, M.A., et al. (2010). Genome-wide binding of the orphan nuclear receptor TR4 suggests its general role in fundamental biological processes. BMC Genomics *11*, 689.
37. Hollenhorst, P.C., McIntosh, L.P., and Graves, B.J. (2011). Genomic and biochemical insights into the specificity of ETS transcription factors. Annu. Rev. Biochem. *80*, 437–471.
38. Schratt, G., Weinhold, B., Lundberg, A.S., Schuck, S., Berger, J., Schwarz, H., Weinberg, R.A., Rüther, U., and Nordheim, A. (2001). Serum response factor is required for immediate-early gene activation yet is dispensable for proliferation of embryonic stem cells. Mol. Cell. Biol. *21*, 2933–2943.
39. Iyer, D., Chang, D., Marx, J., Wei, L., Olson, E.N., Parmacek, M.S., Balasubramanyam, A., and Schwartz, R.J. (2006). Serum response factor MADS box serine-162 phosphorylation switches proliferation and myogenic gene programs. Proc. Natl. Acad. Sci. USA *103*, 4516–4521.
40. Somogyi, K., and Rørth, P. (2004). Evidence for tension-based regulation of Drosophila MAL and SRF during invasive cell migration. Dev. Cell *7*, 85–93.
41. Dupont, S., Morsut, L., Aragona, M., Enzo, E., Giulitti, S., Cordenonsi, M., Zanconato, F., Le Digabel, J., Forcato, M., Bicciato, S., et al. (2011). Role of YAP/TAZ in mechanotransduction. Nature *474*, 179–183.
42. Huddleson, J.P., Ahmad, N., Srinivasan, S., and Lingrel, J.B. (2005). Induction of KLF2 by fluid shear stress requires a novel promoter element activated by a phosphatidylinositol 3-kinase-dependent chromatin-remodeling pathway. J. Biol. Chem. *280*, 23371–23379.
43. Mammoto, A., Connor, K.M., Mammoto, T., Yung, C.W., Huh, D., Aderman, C.M., Mostoslavsky, G., Smith, L.E., and Ingber, D.E. (2009). A mechanosensitive transcriptional mechanism that controls angiogenesis. Nature *457*, 1103–1108.
44. Huang, S., and Ingber, D.E. (2002). A discrete cell cycle checkpoint in late G(1) that is cytoskeleton-dependent and MAP kinase (Erk)-independent. Exp. Cell Res. *275*, 255–264.
45. Klein, E.A., Yin, L., Kothapalli, D., Castagnino, P., Byfield, F.J., Xu, T., Levental, I., Hawthorne, E., Janmey, P.A., and Assoian, R.K. (2009). Cell-cycle control by physiological matrix elasticity and in vivo tissue stiffening. Curr. Biol. *19*, 1511–1518.
46. Wang, Z., Wang, D.Z., Hockemeyer, D., McAnally, J., Nordheim, A., and Olson, E.N. (2004). Myocardin and ternary complex factors compete for SRF to control smooth muscle gene expression. Nature *428*, 185–189.
47. de la Fuente, V., Freudenthal, R., and Romano, A. (2011). Reconsolidation or extinction: transcription factor switch in the determination of memory course after retrieval. J. Neurosci. *31*, 5562–5573.
48. Fletcher, J.C., D'Avino, P.P., and Thummel, C.S. (1997). A steroid-triggered switch in E74 transcription factor isoforms regulates the timing of secondary-response gene expression. Proc. Natl. Acad. Sci. USA *94*, 4582–4586.
49. Ducret, C., Maira, S.M., Dierich, A., and Wasylyk, B. (1999). The net repressor is regulated by nuclear export in response to anisomycin, UV, and heat shock. Mol. Cell. Biol. *19*, 7076–7087.