

Published in final edited form as:

Curr Biol. 2012 November 6; 22(21): 2017–2026. doi:10.1016/j.cub.2012.08.050.

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

Michele A. Wozniak¹, Catherine Q. Cheng¹, Colette J. Shen¹, Lin Gao¹, Anthony O. Olarerin-George², Kyoung-Jae Won³, John B. Hogenesch⁴, and Christopher S. Chen¹

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

²Genomics and Computational Biology Graduate Group, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA

³Department of Genetics, The Institute for Diabetes, Obesity, and Metabolism, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

⁴Department of Pharmacology and the Institute for Translational Medicine and Therapeutics, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA

Abstract

Background—The ternary complex factors (TCFs; Elk1, Net, and Sap-1) are growth factor-responsive transcription co-factors 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 G₀-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. ChIP studies showed increased occupancy of *egr1* and *fos* promoters by Sap-1 with high adhesion, while 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 co-regulated by Sap-1 and Net.

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

© 2012 Elsevier Inc. All rights reserved.

Corresponding author: Christopher S. Chen, Department of Bioengineering, University of Pennsylvania, 510 Skirkanich Hall, 210 South 33rd Street, Philadelphia, PA 19104, Phone: (215) 746-1754, Fax: (215) 746-1752, chrischen@seas.upenn.edu.

The authors declare no competing financial interests.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

adhesion; transcription; ternary complex factor; map kinase; proliferation; Sap-1/Elk4; Net/Elk3

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]. While 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 may 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. Amongst 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, 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 while Elk1 and Net can also be repressive [15, 16]. Phosphorylation of TCFs by the ERK, c-Jun N-terminal kinase (JNK), or p38 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 (non-lethal) 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, while 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 may 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.

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 cells could fully spread, or on 1225 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 cyclinD1 (*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 may regulate the proliferative response to changes in adhesion. To identify these candidate TFs, two microarray data sets were analyzed, from human umbilical vein endothelial cells (HUVECs [28]) and human mesenchymal stem cells (hMSCs) in which micropatterning was used to control adhesion. Each microarray dataset 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 (Table S1).

Given the central role of SRF in controlling proliferation, we determined if 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 performed. Reducing cell adhesion significantly decreased SRE-dependent luciferase activity compared to highly adherent, well-spread cells (Figure 1C and Figure 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 density was reduced from 20 $\mu\text{g}/\text{ml}$ to 1 $\mu\text{g}/\text{ml}$ 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 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,C,D). 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 *vc1*(vinculin). The immediate early genes *egr1* and *fos* contain the full SRE and are dependent on TCFs for their transcription, while *srf* and *vc1* lack the Ets site and are thought to act independent of TCFs [31, 32].

Unless otherwise noted, the remaining studies use 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 *vc1* showed no difference in expression (Figure 1D). The mRNA expression changes are likely due to changes in transcription and not changes in transcript stability since 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 co-factors 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). While 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 shRNAs against Elk1, Sap-1, or Net (ElkKD, SapKD, or NetKD). The SapKD and Net KD 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 if the TCFs modulate adhesion-dependent proliferation. TCF KD cells were synchronized by increased cell confluency and serum starving, and 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 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, while when adhesion is limited, Net blocks proliferation.

Adhesive context dictates Sap-1 and Net promoter binding and transcriptional activity

Because Net or Sap-1 regulated adhesion-dependent proliferation, we next wanted to determine if 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, then stimulated cells cultured under high or low adhesive contexts. SapKD cells lost the increase in serum-induced luciferase activity (Figure 3A) and *egr1* and *fos* expression (Figures 3B, 3C) in high vs. low adhesion conditions that was observed in control cells (Figures 3A, 3B, 3C). Interestingly, when adhesion was limited in the NetKD cells, *egr1* and *fos* expression were rescued (Figure 3A, 3B, 3C), suggesting that Net represses or weakly activates immediate early gene expression under low adhesive contexts. Although the NetKD cells plated on low adhesion showed a slight super-activation in the luciferase assays, endogenous SRE 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. As we were unable to further decrease Elk1 protein levels by siRNA 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, while 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, 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 ~1000bp upstream of the SREs, nor three different genomic regions that do not contain SREs showed enrichment in either ChIP assay (Figures 3D, 3E). These data show that differences in cell adhesion can trigger a switch in TCF promoter occupancy that correlate 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, if this MAPK activity mediated the gene expression changes resulting from limited adhesion. To determine MAPK activity, cell lysates were obtained from cells plated on micropatterned surfaces, serum starved overnight, then stimulated with serum for 45 minutes. 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 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) or the MEK1/2 inhibitor UO126 (data not shown) failed to abrogate luciferase activity in highly adhesive cells.

Therefore, we explored to the possibility that the dominant control point may lie with JNK or p38. In response to serum stimulation, phospho-JNK levels increased under conditions of high adhesion (Figure 4B), while 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 down to levels similar to low-adhesive conditions (Figure 4E, Figure S4B). Conversely, under limited adhesion, selective inhibition of p38 with SB-20358050 rescued luciferase activity (Figure 4F, Figure S4B). These data suggest a balance in signaling between JNK and p38 such that when adhesion is high JNK positively regulates SRE activity, while 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 if 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 adhesive 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 eluted and analyzed by western blotting for phospho-serine. Sap-1 phosphorylation was significantly increased under conditions of high adhesion, and this was blocked by inhibition of JNK but not 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 if these changes in TCF phosphorylation correlated with changes in TCF promoter binding, ChIP assays were used to test if Sap-1 promoter binding in highly adhesive cells is dependent on JNK activity and if 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 low adhesive 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 adhesive contexts (Figure 5E).

Microarray analysis confirms TCF regulation of proliferation and predicts additional TCF-regulated genes and functions

Giovane et al. has 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 vs. Net affected global gene expression using microarray analysis. NIH 3T3 cells were plated in a highly adherent context and serum starved overnight, then stimulated with serum for one hour the following day before RNA isolation and microarray analysis. Genes that changed by twofold 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 may be direct targets of SRF/TCFs using two different analyses. First, the mouse genome was searched 5000nt upstream of each transcription start site (TSS) 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 TCFs/SRF. From this list, 77 of the 81 genes were included in the microarray (Table S3). We then determined if these genes changed expression in the microarray, using a 0.1% FDR. 4.9% of predicted direct binders were differentially regulated in the NetKD cells, as compared to 1.9% of all genes. 10.4% of predicted direct binders were regulated in the

SapKD cells, as compared to 13.9% of all genes. These data confirm that TCF knockdown regulated 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). Out of the predicted direct TCF binders that showed a reciprocal regulation (up in SapKD/down in NetKD or vice versa), six out of nine 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 to only Sap-1, or genes downregulated in the NetKD cells only bound 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-Seq dataset [36] to our microarray analysis of SapKD cells. Thresholding at $1e-4$ Poisson p-value and 0.1% FDR, the replicate genes identified from the published data were combined (Table S4, Sheet1). 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, Sheet2). 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 non-direct binding targets, as expected.

In order to further examine Sap-1 and Net reciprocity, genes identified in the microarray up and downregulated in response to Sap-1 and Net knockdown (2 fold change, 0.1% FDR) were compared and the regulatory overlaps determined (Fig 6A). To determine the likelihood that these overlaps occurred by chance, one million random iterations were simulated using the R statistical program and the number of genes expected to show chance regulatory overlap were compared to the observed overlaps. For each case the overlap we observed had a probability of $p < 1 \times 10^{-6}$ of occurring by chance. These data demonstrate that Sap-1 and Net co-regulate many genes in addition to *egr1* and *fos*, some regulated antagonistically and others regulated 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 analysis predicted several physiological roles previously undescribed 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 adhesive-mediated proliferative regulation. First, in the microarray, *egr1* expression decreased in SapKD cells by -1.06 fold (\log_2 transformed), which corresponded to qPCR results in these cells plated in a highly adherent context. Second, the IPA analysis identified cellular growth and proliferation as the top function affected genes downregulated in the SapKD cells (Figure 6B) and genes upregulated in the NetKD cells (Figure 6C), verifying an antagonistic role for Sap-1 and Net TCFs in proliferative regulation. Last, 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.

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], while SRF 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 suggests that while 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 find that the actin polymerization-sensitive MRTF-regulated CArG-box only genes are not regulated by changes in adhesion. While 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 factors 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.

While 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 since 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, while under other circumstances they are not [37]. Here we knock down each TCF individually to compare their effect on adhesion-mediated gene expression and function and find 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 vs. 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 inhibits 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; while loss of adhesion regulates ERK, limiting adhesion regulates a switch in JNK and p38 MAPK activity. To our knowledge, this 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. While 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 κ B 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 future directions. 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 may play a role in adhesion-mediated SRE activity remains unclear. It will be important to determine if the three TCFs regulate other genes and functions using a similar switching mechanism we propose for immediate early genes. In conclusion, these 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 supplemental information for complete experimental procedures.

Cell culture

NIH 3T3 cell lines were maintained in growth media (GM: 10% bovine serum in DMEM).

Immunoprecipitation and Western blotting

Cells were plated for 2h, serum starved overnight, 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), anti-Net (Santa Cruz, clone A-20X) antibody overnight at 4 °C. Antibodies: phospho-serine (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 (abcam).

Chromatin immunoprecipitation (ChIP) analysis

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

Microarray Analysis

Log₂RMA expression data were deposited in NCBI's Gene Expression Omnibus (GEO Series accession number GSE26640).

Statistical analysis

Means \pm S.E.M. and P values were calculated using GraphPad Prism software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Ana Cristancho, Mitchell Lazar, and Michelle Lynch (University of Pennsylvania) for assistance with ChIP experiments, Guillermo García-Cardeña (Harvard University) for HUVECs, Donald Baldwin and John Tobias 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 deLanerolle (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 grants from NIH (EB00262, HL73305, and GM74048). M.A.W. acknowledges support from the Ruth L. Kirschstein National Research Service Award (F32 AR054219-01) and A.O.O. acknowledges support from the National Human Genome Research Institute training grant (T32HG000046).

References

1. Stoker M. Contact and short-range interactions affecting growth of animal cells in culture. *Curr Top Dev Biol.* 1967; 2:107–128. [PubMed: 4331870]
2. Chen CS, Mrksich M, Huang S, Whitesides GM, Ingber DE. Geometric control of cell life and death. *Science.* 1997; 276:1425–1428. [PubMed: 9162012]
3. Dike LE, Ingber DE. Integrin-dependent induction of early growth response genes in capillary endothelial cells. *J Cell Sci.* 1996; 109 (Pt 12):2855–2863. [PubMed: 9013333]
4. Folkman J, Moscona A. Role of cell shape in growth control. *Nature.* 1978; 273:345–349. [PubMed: 661946]
5. Sun Q, Chen G, Streb JW, Long X, Yang Y, Stoeckert CJ Jr, Miano JM. Defining the mammalian CArGome. *Genome Res.* 2006; 16:197–207. [PubMed: 16365378]
6. Connelly JT, Gautrot JE, Trappmann B, Tan DW, Donati G, Huck WT, Watt FM. Actin and serum response factor transduce physical cues from the microenvironment to regulate epidermal stem cell fate decisions. *Nat Cell Biol.* 2010; 12:711–718. [PubMed: 20581838]
7. Farra R, Dapas B, Pozzato G, Giansante C, Heidenreich O, Uxa L, Zennaro C, Guarnieri G, Grassi G. Serum response factor depletion affects the proliferation of the hepatocellular carcinoma cells HepG2 and JHH6. *Biochimie.* 2010; 92:455–463. [PubMed: 20144681]
8. Gauthier-Rouviere C, Fernandez A, Lamb NJ. ras-induced c-fos expression and proliferation in living rat fibroblasts involves C-kinase activation and the serum response element pathway. *Embo J.* 1990; 9:171–180. [PubMed: 2104799]

9. Liu N, Bezprozvannaya S, Williams AH, Qi X, Richardson JA, Bassel-Duby R, Olson EN. microRNA-133a regulates cardiomyocyte proliferation and suppresses smooth muscle gene expression in the heart. *Genes Dev.* 2008; 22:3242–3254. [PubMed: 19015276]
10. Wang DZ, Li S, Hockemeyer D, Sutherland L, Wang Z, Schratt G, Richardson JA, Nordheim A, Olson EN. Potentiation of serum response factor activity by a family of myocardin-related transcription factors. *Proc Natl Acad Sci U S A.* 2002; 99:14855–14860. [PubMed: 12397177]
11. Miralles F, Posern G, Zaromytidou AI, Treisman R. Actin dynamics control SRF activity by regulation of its coactivator MAL. *Cell.* 2003; 113:329–342. [PubMed: 12732141]
12. McBeath R, Pirone DM, Nelson CM, Bhadriraju K, Chen CS. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Dev Cell.* 2004; 6:483–495. [PubMed: 15068789]
13. Shaw PE, Schroter H, Nordheim A. The ability of a ternary complex to form over the serum response element correlates with serum inducibility of the human c-fos promoter. *Cell.* 1989; 56:563–572. [PubMed: 2492906]
14. Treisman R. Identification and purification of a polypeptide that binds to the c-fos serum response element. *Embo J.* 1987; 6:2711–2717. [PubMed: 3119326]
15. Maira SM, Wurtz JM, Wasylyk B. Net (ERP/SAP2) one of the Ras-inducible TCFs, has a novel inhibitory domain with resemblance to the helix-loop-helix motif. *Embo J.* 1996; 15:5849–5865. [PubMed: 8918463]
16. Yang SH, Vickers E, Brehm A, Kouzarides T, Sharrocks AD. Temporal recruitment of the mSin3A-histone deacetylase corepressor complex to the ETS domain transcription factor Elk-1. *Mol Cell Biol.* 2001; 21:2802–2814. [PubMed: 11283259]
17. Gille H, Sharrocks AD, Shaw PE. Phosphorylation of transcription factor p62TCF by MAP kinase stimulates ternary complex formation at c-fos promoter. *Nature.* 1992; 358:414–417. [PubMed: 1322499]
18. Janknecht R, Ernst WH, Pingoud V, Nordheim A. Activation of ternary complex factor Elk-1 by MAP kinases. *Embo J.* 1993; 12:5097–5104. [PubMed: 8262053]
19. Price MA, Rogers AE, Treisman R. Comparative analysis of the ternary complex factors Elk-1, SAP-1a and SAP-2 (ERP/NET). *Embo J.* 1995; 14:2589–2601. [PubMed: 7540136]
20. Whitmarsh AJ, Shore P, Sharrocks AD, Davis RJ. Integration of MAP kinase signal transduction pathways at the serum response element. *Science.* 1995; 269:403–407. [PubMed: 7618106]
21. Dalton S, Treisman R. Characterization of SAP-1, a protein recruited by serum response factor to the c-fos serum response element. *Cell.* 1992; 68:597–612. [PubMed: 1339307]
22. Giovane A, Pintzas A, Maira SM, Sobieszczuk P, Wasylyk B. Net, a new ets transcription factor that is activated by Ras. *Genes Dev.* 1994; 8:1502–1513. [PubMed: 7958835]
23. Hipskind RA, Rao VN, Mueller CG, Reddy ES, Nordheim A. Ets-related protein Elk-1 is homologous to the c-fos regulatory factor p62TCF. *Nature.* 1991; 354:531–534. [PubMed: 1722028]
24. Ayadi A, Zheng H, Sobieszczuk P, Buchwalter G, Moerman P, Alitalo K, Wasylyk B. Net-targeted mutant mice develop a vascular phenotype and up-regulate egr-1. *Embo J.* 2001; 20:5139–5152. [PubMed: 11566878]
25. Cesari F, Brecht S, Vintersten K, Vuong LG, Hofmann M, Klingel K, Schnorr JJ, Arsenian S, Schild H, Herdegen T, Wiebel FF, Nordheim A. Mice deficient for the ets transcription factor elk-1 show normal immune responses and mildly impaired neuronal gene activation. *Mol Cell Biol.* 2004; 24:294–305. [PubMed: 14673163]
26. Costello PS, Nicolas RH, Watanabe Y, Rosewell I, Treisman R. Ternary complex factor SAP-1 is required for Erk-mediated thymocyte positive selection. *Nat Immunol.* 2004; 5:289–298. [PubMed: 14770179]
27. Vickers ER, Kasza A, Kurnaz IA, Seifert A, Zeef LA, O'Donnell A, Hayes A, Sharrocks AD. Ternary complex factor-serum response factor complex-regulated gene activity is required for cellular proliferation and inhibition of apoptotic cell death. *Mol Cell Biol.* 2004; 24:10340–10351. [PubMed: 15542842]
28. Shen CJ, Raghavan S, Xu Z, Baranski JD, Yu X, Wozniak MA, Miller JS, Gupta M, Buckbinder L, Chen CS. Decreased cell adhesion promotes angiogenesis in a Pyk2-dependent manner. *Exp Cell Res.* 2011; 317:1860–1871. [PubMed: 21640103]

29. Haverty PM, Frith MC, Weng Z. CARRIE web service: automated transcriptional regulatory network inference and interactive analysis. *Nucleic Acids Res.* 2004; 32:W213–216. [PubMed: 15215383]
30. Paszek MJ, Zahir N, Johnson KR, Lakins JN, Rozenberg GI, Gefen A, Reinhart-King CA, Margulies SS, Dembo M, Boettiger D, Hammer DA, Weaver VM. Tensional homeostasis and the malignant phenotype. *Cancer Cell.* 2005; 8:241–254. [PubMed: 16169468]
31. Cen B, Selvaraj A, Burgess RC, Hitzler JK, Ma Z, Morris SW, Prywes R. Megakaryoblastic leukemia 1, a potent transcriptional coactivator for serum response factor (SRF), is required for serum induction of SRF target genes. *Mol Cell Biol.* 2003; 23:6597–6608. [PubMed: 12944485]
32. Gineitis D, Treisman R. Differential usage of signal transduction pathways defines two types of serum response factor target gene. *J Biol Chem.* 2001; 276:24531–24539. [PubMed: 11342553]
33. Aplin AE, Stewart SA, Assoian RK, Juliano RL. Integrin-mediated adhesion regulates ERK nuclear translocation and phosphorylation of Elk-1. *J Cell Biol.* 2001; 153:273–282. [PubMed: 11309409]
34. Oktay M, Wary KK, Dans M, Birge RB, Giancotti FG. 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.* 1999; 145:1461–1469. [PubMed: 10385525]
35. O'Donnell A, Yang SH, Sharrocks AD. MAP kinase-mediated c-fos regulation relies on a histone acetylation relay switch. *Mol Cell.* 2008; 29:780–785. [PubMed: 18374651]
36. O'Geen H, Lin YH, Xu X, Echipare L, Komashko VM, He D, Frieze S, Tanabe O, Shi L, Sartor MA, Engel JD, Farnham PJ. Genome-wide binding of the orphan nuclear receptor TR4 suggests its general role in fundamental biological processes. *BMC Genomics.* 2010; 11:689. [PubMed: 21126370]
37. Hollenhorst PC, McIntosh LP, Graves BJ. Genomic and biochemical insights into the specificity of ETS transcription factors. *Annu Rev Biochem.* 2011; 80:437–471. [PubMed: 21548782]
38. Schrott G, Weinhold B, Lundberg AS, Schuck S, Berger J, Schwarz H, Weinberg RA, Ruther U, Nordheim A. Serum response factor is required for immediate-early gene activation yet is dispensable for proliferation of embryonic stem cells. *Mol Cell Biol.* 2001; 21:2933–2943. [PubMed: 11283270]
39. Iyer D, Chang D, Marx J, Wei L, Olson EN, Parmacek MS, Balasubramanyam A, Schwartz RJ. Serum response factor MADS box serine-162 phosphorylation switches proliferation and myogenic gene programs. *Proc Natl Acad Sci U S A.* 2006; 103:4516–4521. [PubMed: 16537394]
40. Somogyi K, Rorth P. Evidence for tension-based regulation of *Drosophila* MAL and SRF during invasive cell migration. *Dev Cell.* 2004; 7:85–93. [PubMed: 15239956]
41. Dupont S, Morsut L, Aragona M, Enzo E, Giulitti S, Cordenonsi M, Zanconato F, Le Digabel J, Forcato M, Bicciato S, Elvassore N, Piccolo S. Role of YAP/TAZ in mechanotransduction. *Nature.* 474:179–183. [PubMed: 21654799]
42. Huddleson JP, Ahmad N, Srinivasan S, Lingrel JB. 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.* 2005; 280:23371–23379. [PubMed: 15834135]
43. Mammoto A, Connor KM, Mammoto T, Yung CW, Huh D, Aderman CM, Mostoslavsky G, Smith LE, Ingber DE. A mechanosensitive transcriptional mechanism that controls angiogenesis. *Nature.* 2009; 457:1103–1108. [PubMed: 19242469]
44. Huang S, Ingber DE. A discrete cell cycle checkpoint in late G(1) that is cytoskeleton-dependent and MAP kinase (Erk)-independent. *Exp Cell Res.* 2002; 275:255–264. [PubMed: 11969294]
45. Klein EA, Yin L, Kothapalli D, Castagnino P, Byfield FJ, Xu T, Levental I, Hawthorne E, Janmey PA, Assoian RK. Cell-cycle control by physiological matrix elasticity and in vivo tissue stiffening. *Curr Biol.* 2009; 19:1511–1518. [PubMed: 19765988]
46. Wang Z, Wang DZ, Hockemeyer D, McAnally J, Nordheim A, Olson EN. Myocardin and ternary complex factors compete for SRF to control smooth muscle gene expression. *Nature.* 2004; 428:185–189. [PubMed: 15014501]
47. de la Fuente V, Freudenthal R, Romano A. Reconsolidation or extinction: transcription factor switch in the determination of memory course after retrieval. *J Neurosci.* 31:5562–5573. [PubMed: 21490196]

48. Fletcher JC, D'Avino PP, Thummel CS. A steroid-triggered switch in E74 transcription factor isoforms regulates the timing of secondary-response gene expression. *Proc Natl Acad Sci U S A*. 1997; 94:4582–4586. [PubMed: 9114033]
49. Ducret C, Maira SM, Dierich A, Wasylyk B. The net repressor is regulated by nuclear export in response to anisomycin, UV, and heat shock. *Mol Cell Biol*. 1999; 19:7076–7087. [PubMed: 10490644]

Highlights

- The SRF cofactors, the TCFs, are controlled by changes in adhesion
- Adhesion activates JNK/Sap-1-mediated gene expression and proliferation
- Restricting adhesion triggers p38/Net to repress transcription and proliferation

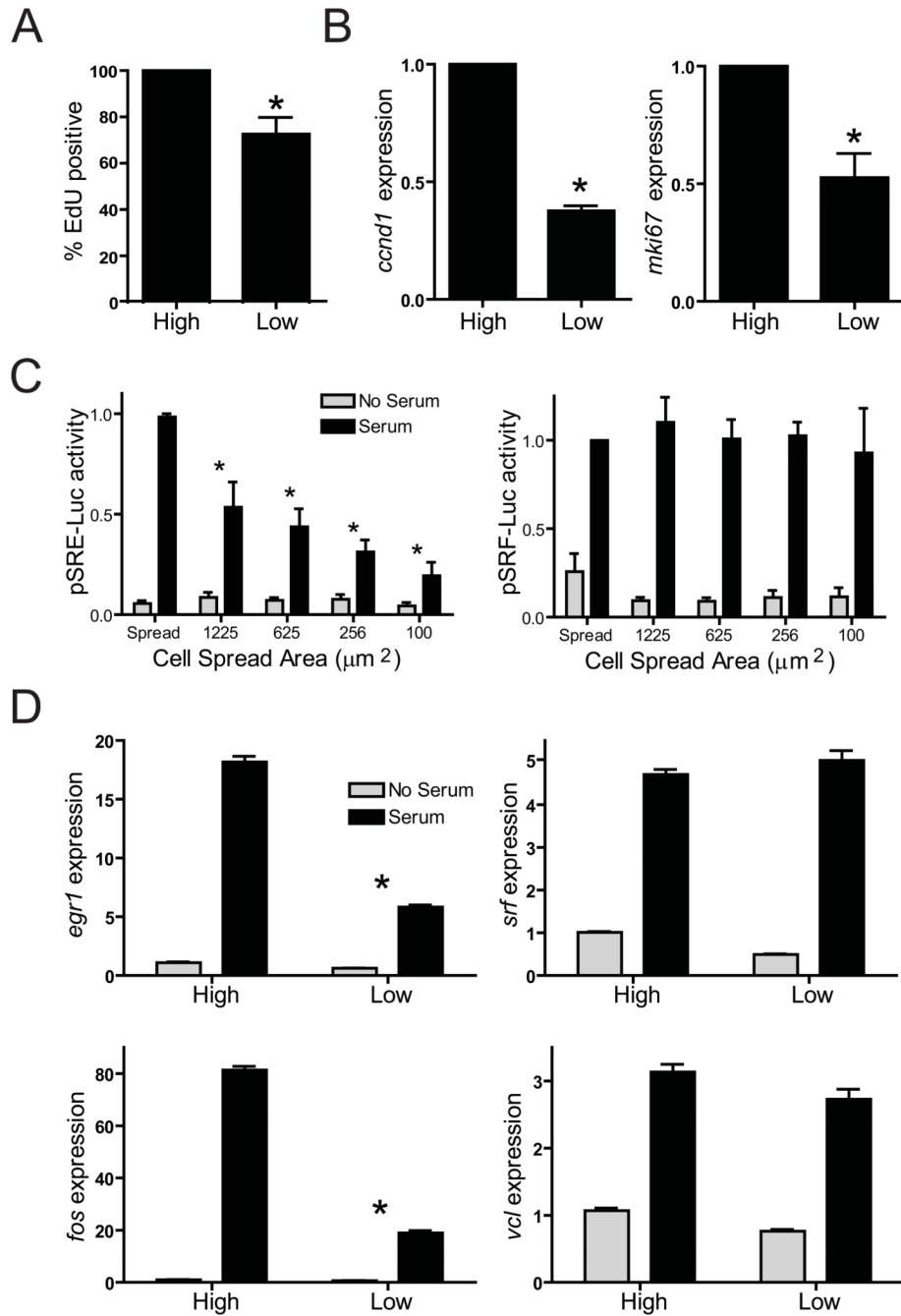


Figure 1. Cell adhesion regulates proliferation and SRE-dependent transcription

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 \pm S.E.M. 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, plated on micropatterned islands, and luciferase assays performed. Means \pm S.E.M., $n=4$. * $p < 0.05$, two-way ANOVA. (D) qPCR for *egr1*, *fossrf*, and *vcl*. Data are presented as means \pm S.E.M., $n=4$. * $p < 0.05$, two-way ANOVA. See also Figure S1.

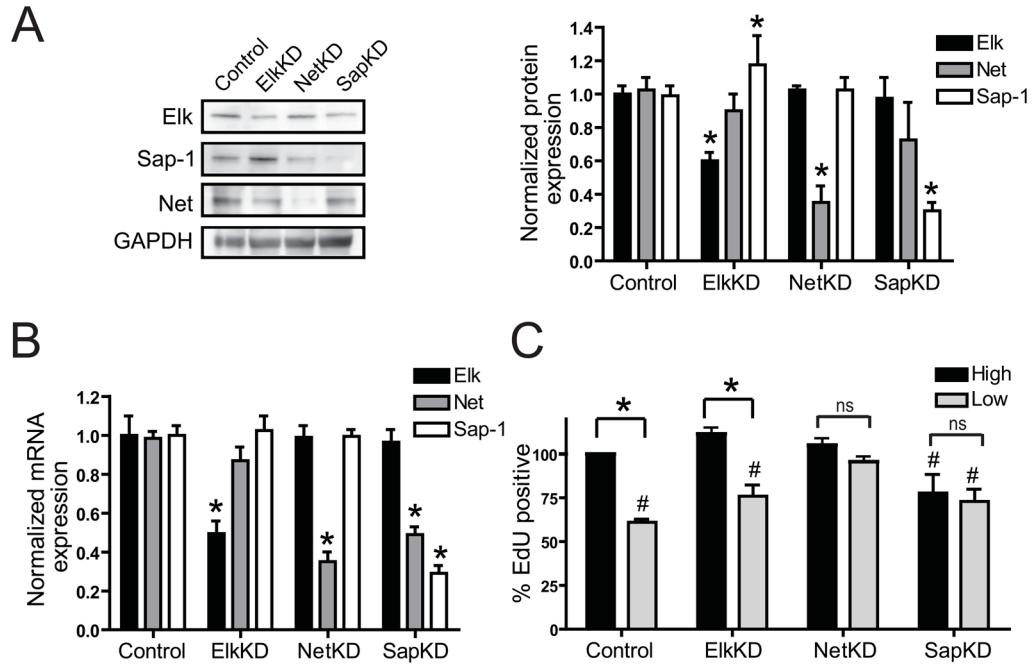


Figure 2. Adhesion regulates proliferation through the Sap-1 and Net TCFs
 Stable cell lines expressing shRNAs against Elk1, Net, and Sap-1 were generated to knockdown protein (A) and mRNA (B) levels. Protein levels were normalized to GAPDH and means \pm S.E.M are shown for n=5. mRNA levels were normalized to 18S and means \pm S.E.M. are shown for n=3. *p<0.05 vs. controls, paired t-test. (C) Cell lines were plated under high or low adhesive micropatterned conditions and EdU incorporation assays performed. All data are represented as means \pm S.E.M., n=4. *p<0.05 high vs. low for each cell line, #p<0.05 vs. high control, both by paired t-tests.

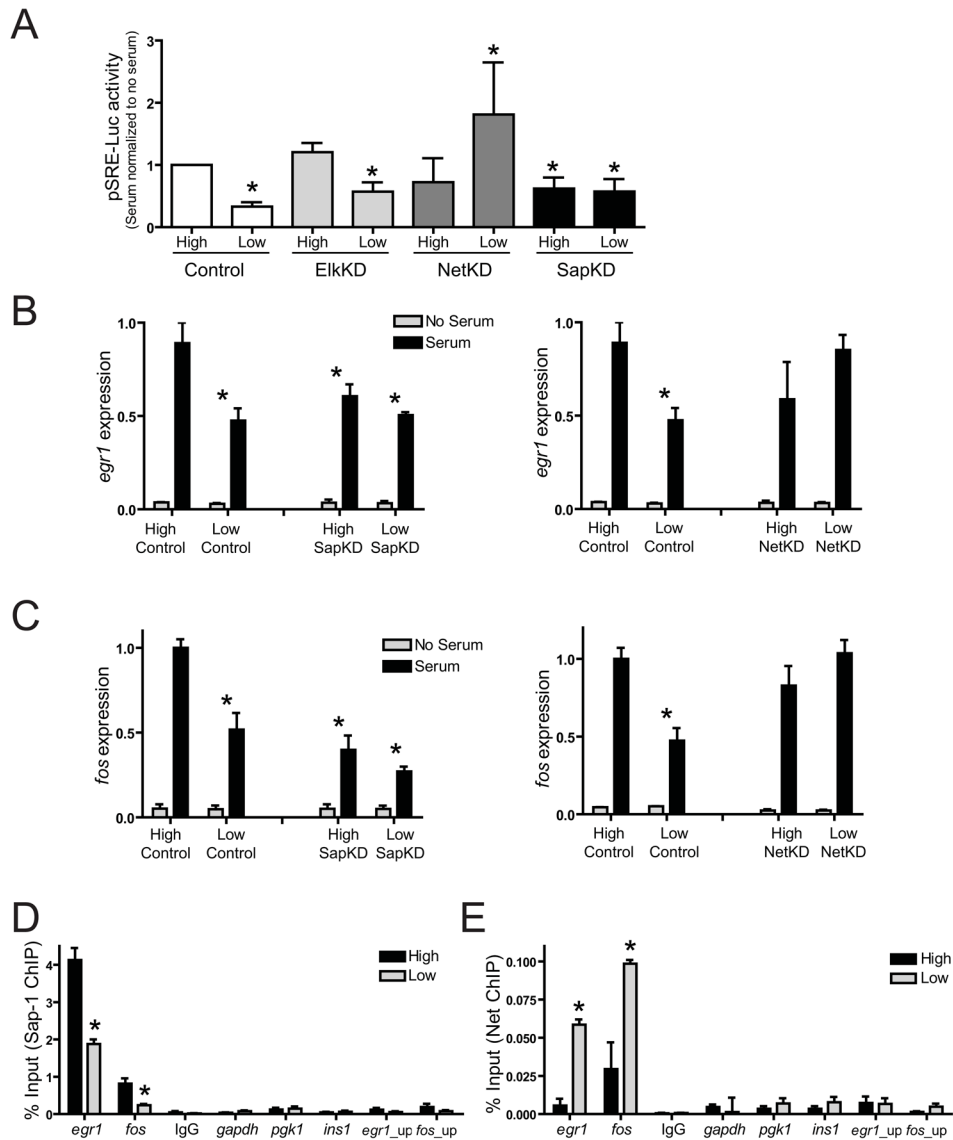


Figure 3. Adhesive context dictates Sap-1 and Net promoter binding and transcriptional activity (A) pSRE luciferase assays were performed in cells plated under high vs. low adhesion micropatterned conditions. Data are represented as serum values normalized to no serum values and means \pm S.E.M. are shown for $n=5$. * $p<0.05$ vs. control high, paired t-test. qPCR was performed for *egr1* (B) and *fos* (C) expression in SapKD (left panel) and NetKD (right panel) cells compared to control cells. Means \pm S.E.M. are shown for $n=4$. * $p<0.05$ vs. control high, two-way ANOVA. 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 are IgG binding to *egr1* containing two SREs and Sap or Net binding to *gapdh*, *pgk1*, *ins1*, and ~ 1000 bp upstream of *egr1* and *fos* TSSs, none of which contain SREs. Data shown are means \pm S.E.M. for $n=4$, * $p<0.05$ vs. high by paired t-test. See also Figure S2.

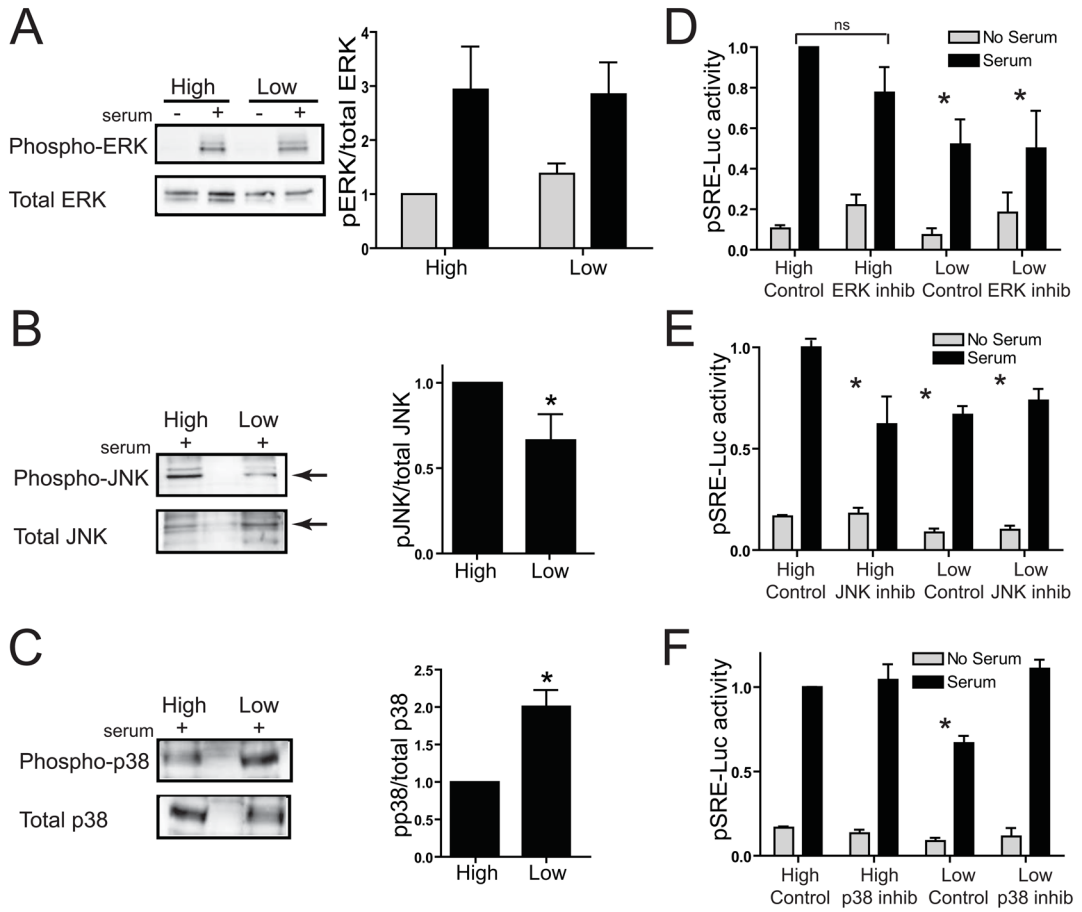


Figure 4. JNK and p38 play opposing roles in adhesion-regulated TCF-targeted transcription
 Western blotting and quantitation for phospho- and total ERK (A), JNK (see arrows) (B) and p38 (C) levels in cells micropatterned under conditions of high or low adhesion and stimulated by serum. pSRE luciferase assays were performed in cells treated with (D) 50 μ M PD98059 (ERK inhibitor), (E) 20 μ M SP 600125 (JNK inhibitor), (F) 10 μ M SB-203580 (p38 inhibitor), or vehicle control. Means \pm S.E.M. are shown for all graphs. 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, * $p<0.05$ vs. high control, two-way ANOVA. See also Figure S3.

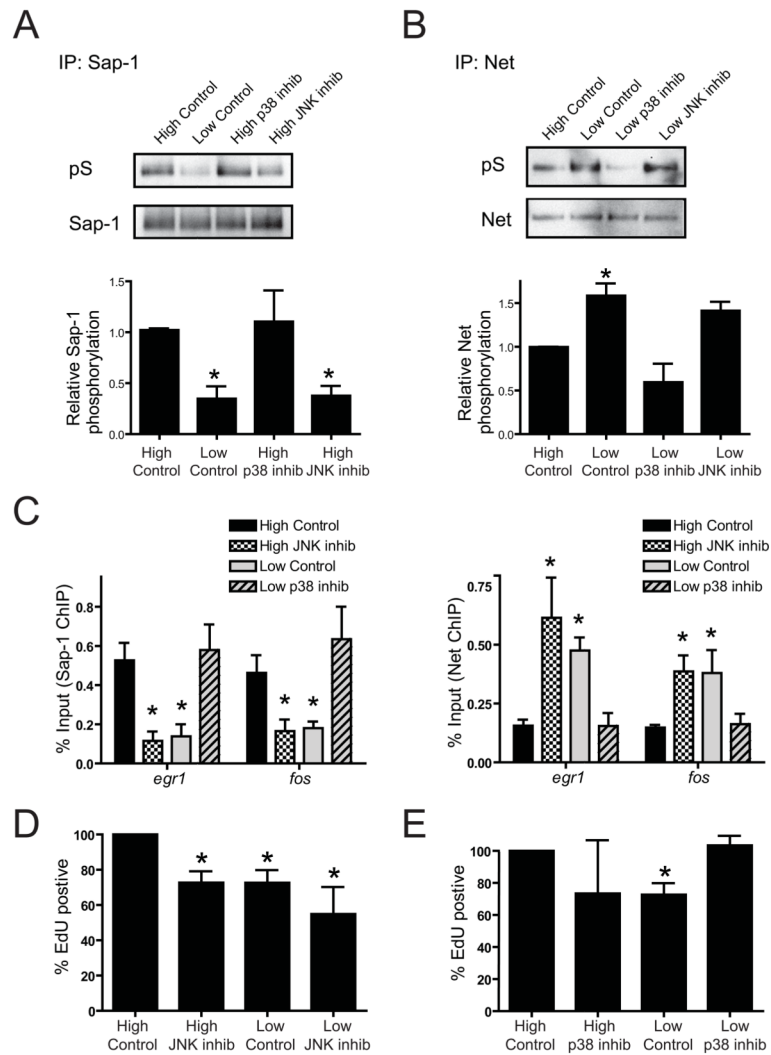


Figure 5. Changes in adhesion switch TCF phosphorylation, promoter occupancy, and proliferation in a MAPK-dependent manner

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 \pm S.E.M. are shown for $n=6$, $*p<0.05$ vs. High Control, paired t-test. (C) ChIPs were performed in high or low adhesive 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. Cells were plated on high or low adhesive micropatterned substrates with either DMSO control or SP 600125 (D) or SB-203580 (E) and EdU incorporation assays performed. All data are represented as means \pm S.E.M. and were repeated at least three times, $*p<0.05$ vs. High Control, paired t-test.

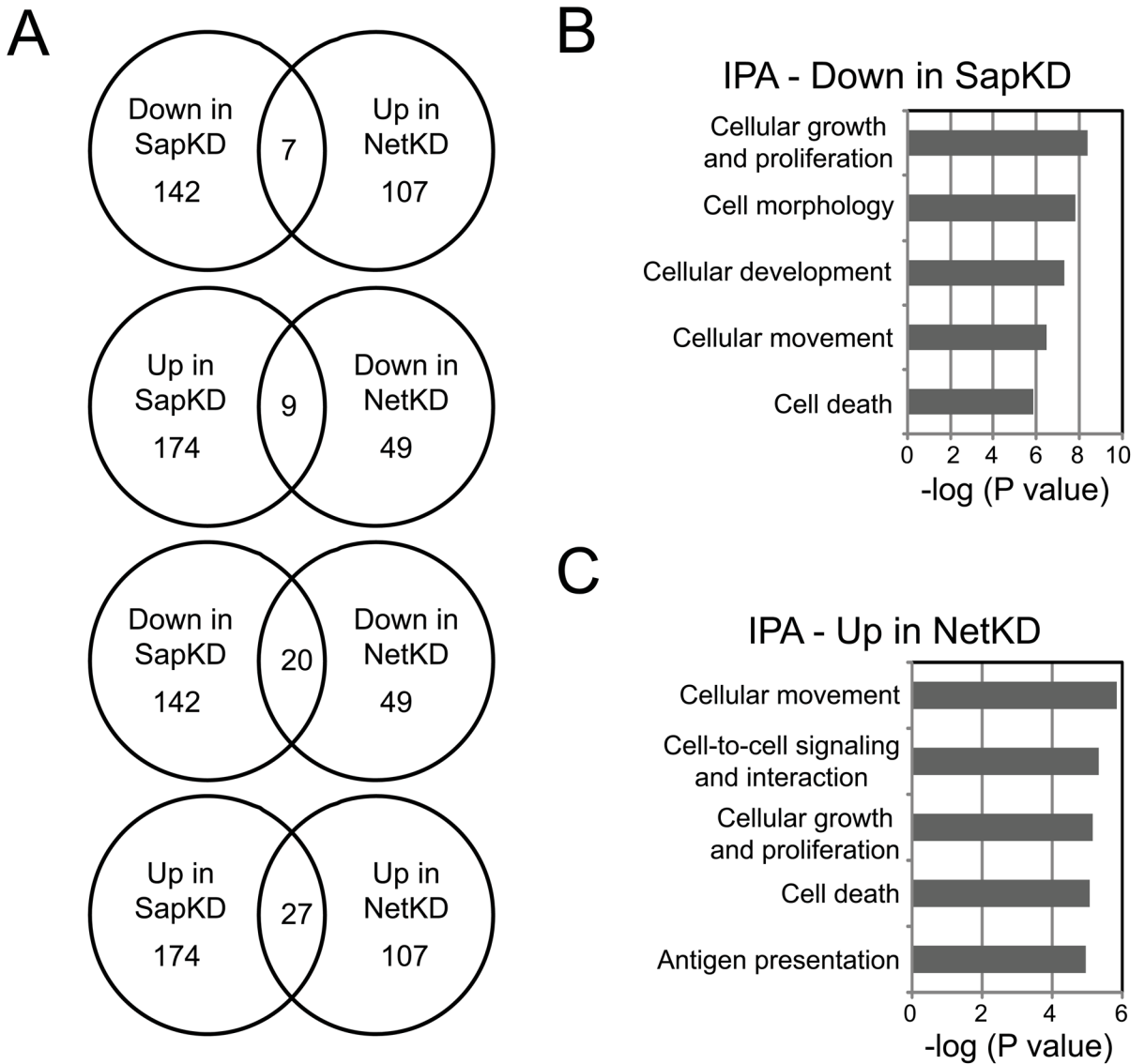


Figure 6. Microarray analysis confirms TCF regulation of proliferation and predicts new functions regulated by TCFs

(A) Venn diagrams showing the overlaps observed in NetKD and SapKD cells. Unique genes downregulated in the SapKD (B) or upregulated in the NetKD cells (C) were analyzed using IPA analysis. Functions are plotted against the log (P value). See also Figure S4, Tables S2, S3, and S4.

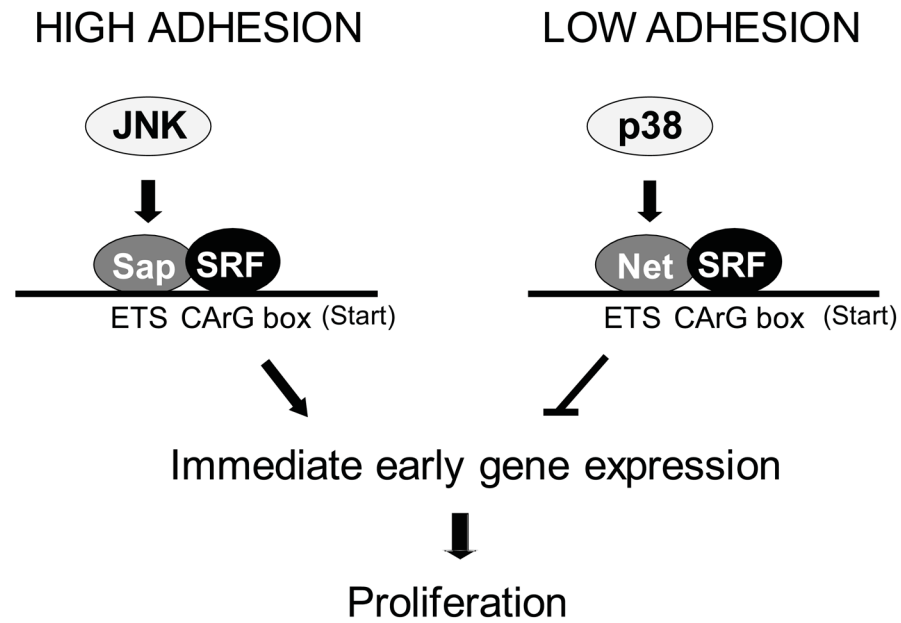


Figure 7. Proposed model of the adhesive regulation of the MAPK/TCF switch to control immediate early gene expression and proliferation

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.