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# Adhesion-dependent Skp2 transcription requires selenocysteine tRNA gene transcription-activating factor (STAF)

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## Abstract

Cell adhesion is essential for cell cycle progression in most normal cells. Loss of adhesion dependence is a hallmark of cellular transformation. The F-box protein Skp2 (S-phase kinase-associated protein 2) controls $G_1$–S-phase progression and is subject to adhesion-dependent transcriptional regulation, although the mechanisms are poorly understood. We identify two cross-species conserved binding elements for the STAF (selenocysteine tRNA gene transcription-activating factor) in the *Skp2* promoter that are essential for *Skp2* promoter activity. Endogenous STAF specifically binds these elements in EMSA (electrophoretic mobility-shift assay) and ChIP (chromatin immunoprecipitation) analysis. STAF is sufficient and necessary for *Skp2* promoter activity since exogenous STAF activates promoter activity and expression and STAF siRNA (small interfering RNA) inhibits *Skp2* promoter activity, mRNA and protein expression and cell proliferation. Furthermore, ectopic Skp2 expression completely reverses the inhibitory effects of STAF silencing on proliferation. Importantly, STAF expression and binding to the *Skp2* promoter is adhesion-dependent and associated with adhesion-dependent Skp2 expression in non-transformed cells. Ectopic STAF rescues Skp2 expression in suspension cells. Taken together, these results demonstrate that STAF is essential and sufficient for *Skp2* promoter activity and plays a role in the adhesion-dependent expression of Skp2 and ultimately cell proliferation.

### Keywords

adhesion; cell cycle; proliferation; S-phase kinase-associated protein 2 (Skp2); selenocysteine tRNA gene transcription-activating factor (STAF); ZNF76; ZNF143

## INTRODUCTION

Cell cycle progression requires timely degradation of cyclin-dependent kinase inhibitors and de-regulation of these degradative pathways causes hyper-proliferative diseases [1,2]. For



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example, proteolysis of $p27^{Kip1}$ is essential for $G_1$–S transition; it is accomplished by the UPS (ubiquitin-proteasome system) after ATP-dependent ubiquitination [3]. Ubiquitination of many $G_1$ regulatory proteins, including $p27^{Kip1}$, is mediated by SCF [Skp1 (S-phase kinase-associated protein 1)/cullin/F-box] E3 ubiquitin ligases that consist of a modular E3 core containing three constant proteins (CUL1, RBX1 and SKP1) and a variable FBP (F-box protein) that determines the substrate specificity. The FBP Skp2 is necessary for the ubiquitination and subsequent degradation of $p27^{Kip1}$ and plays a major role in regulating $G_1$–S-phase progression [4–8]. Inhibition of Skp2 function or expression elevates levels of $p27^{Kip1}$ and leads to $G_1$ arrest in many cell types [4,8–10]. Mice deficient in Skp2 exhibit retarded growth associated with nuclear enlargement, centrosome duplication, polyploidy and impaired cell proliferation [7]. This phenotype is largely reversed in mice doubly deficient in Skp2 and $p27^{Kip1}$ [11,12], although other substrates, including $p21^{Cip1}$, cyclin E, E2F-1 and c-Myc have also been described [13–17]. The above observations demonstrate that Skp2 is a major regulator of normal cell-cycle progression. Consistent with this, Skp2 has been implicated in regulating cell proliferation during numerous physiological processes and in the pathogenesis of many hyper-proliferative diseases. For example, we recently demonstrated a role for increased Skp2 expression in the pathological neointima development following vascular injury [9,10]. Numerous studies have also reported aberrant expression of Skp2 in tumours that is often inversely associated with $p27^{Kip1}$ expression and directly with poor clinical outcome [18,19].

Skp2 levels are regulated by both transcriptional and post-transcriptional mechanisms, although neither is well understood. In many cell types, mitogen stimulation increases Skp2 levels via stabilization of Skp2 protein with little effect on Skp2 transcription [8,9,17,20]. On the other hand, Skp2 transcription is adhesion-dependent in several cell types [5,8]. Non-adherent cells fail to transcribe Skp2 and do not proliferate. The fact that ectopic Skp2 expression can rescue cell proliferation in suspension cells suggests that adhesion-dependent regulation of Skp2 transcription is a major mechanism enforcing adhesion-dependence on $G_1$–S progression [5]; a regulatory mechanism that is often lost upon oncogenic transformation. To explore the molecular mechanisms regulating Skp2 adhesion-dependent transcription, we performed a detailed analysis of the *Skp2* promoter, identifying two positively acting *cis*-elements recognised by the transcription factor $STAF_{ZNF143}$ (selenocysteine tRNA gene transcription-activating factor; where ZNF143 is the human paralogue of the *Xenopus laevis* STAF) and the rat paralogue ZNF76 that play an essential role regulating adhesion-dependent Skp2 transcription and cell proliferation.

## MATERIALS AND METHODS

### Reporter constructs and expression vectors

*Skp2* promoter fragments were PCR amplified from the plasmid p3CAT [21] and directionally cloned into pGL2-Basic luciferase reporter vector (Promega). Site-directed mutagenesis was achieved using PCR primers incorporating the desired mutation, as described previously [22]. Plasmids pAc5.1 and pAc5.1-*lacZ* were obtained from Invitrogen. Plasmid pAC5.1-ZNF143 was provided by Dr Dieter Saur (Technische Universität München, Munich, Germany). The vector GAL4:STAF 13–152, expressing a chimaeric protein consisting of the GAL4 DBD (DNA-binding domain) (a.a. 1–94) fused to the N-terminal STAF transcriptional activation domain (amino acids 13–152) was provided by Professor Gary Kunkel. pGL-Skp2-STAFMUT contains the proximal *Skp2* promoter (−165/+136) in which both STAF elements are mutated to GAL4 elements (SBS1 5′-TCCCAGCAGGCCTTGGG-3′ to GAL4 5′-CGGAAGACTCTCCTCCG-3′ and SBS2 5′-CGCGGGGGGTTGTGGGT-3′ to GAL 4 5′-CGGAGGAGAGTCTTCCG-3′). Control adenovirus and wild-type Skp2-expressing adenovirus have been described previously [8]. Adenovirus expressing human wild-type $STAF_{ZNF143}$ was constructed by cloning human

$STAF_{ZNF143}$ cDNA with a C-terminal HA (haemagglutinin) tag between the EcoR1–BamH1 sites of pDC515 (Microbix) followed by homologous recombination with pBHGfrt viral genomic vector in HEK (human embryonic kidney)-293 cells.

### Purification of recombinant $STAF_{ZNF143}$ DBD

The DBD (a.a. 236–444) of human $STAF_{ZNF143}$ was cloned into the pGEX6P-1 prokaryotic expression vector (GE Healthcare). Recombinant GST (glutathione transferase)-tagged STAF DBD was purified using a glutathione–Sepharose column. The GST tag was cleaved using PreScission protease (GE Healthcare) and the liberated DBD was used for EMSAs (electrophoretic mobility-shift assays).

### EMSAs

Nuclear extracts were prepared by washing cells with ice-cold buffer A (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10mM KCl and 0.5 mM PMSF). Cells were homogenized and nuclei pelleted from cell homogenate at 1000 ***g*** for 10 min at 4 °C. Nuclear proteins were extracted in ice-cold buffer C (20 mM Hepes, pH 7.9, 25% glycerol, 420 mM KCl, 1.5 mM $MgCl_2$, 0.2mM EDTA, 0.5 mM dithiothreitol and 0.5 mM PMSF). For EMSA, 10 μg of nuclear extract or the indicated amount of purified recombinant STAF DBD was incubated in 20 μl of binding buffer [10 mM Hepes, pH 7.5, 5 mM $MgCl_2$, 10 mMKCl, 20 μM$ZnCl_2$, 5% glycerol, 0.1% Nonidet P40 and 1 μg poly(dI-dC)] with 20 fmol of the indicated biotinylated probe (generated by PCR using 5′ biotinylated primers) and in some of the experiments with 100-fold molar excess of unlabelled competitor oligonucleotides. Reactions were incubated for 20 min at room temperature (20 °C) and loaded on to a non-denaturing 4% polyacrylamide gel in 0.5× TBE [Tris/borate/EDTA (1×TBE 45 mM Tris/borate and 1 mM EDTA)] buffer. Electrophoresis was performed at 200 V in 0.5× TBE buffer at 4 °C and shifted complexes were detected using the Lightshift EMSA Chemiluminescent Nucleic Acid Detection Module (Pierce #89880) according to the manufacturer's instructions.

### Cell culture and transfection

Aortic VSMCs (vascular smooth muscle cells) were isolated from rat aorta as described previously [8]. All animal procedures were conducted in accordance with the U.K. Animal Scientific Procedures Act 1986. Male Wistar rats (300–400 g) were anesthetized with pentabarbitone followed by retrograde perfusion with phosphate-buffered saline via the abdominal aorta. The thoracic aorta was excised, cut into 4-mm sections and cultured in DMEM (100 units/ml streptomycin and 100 mg/ml penicillin) containing 10% FBS. HeLa cells and VSMCs were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 μg/ml penicillin and streptomycin. For transfection, cells were plated at $2 \times 10^5$ cells/well in 12-well plates. VSMCs were transfected the next day with 1 μg of *Skp2* promoter construct plus 0.25 μg of pTK-*Renilla* for normalization using 5 μl of TransIT-LT1 transfection reagent (Mirus). HeLa cells were transfected with the same amounts of plasmid using calcium phosphate-mediated transfection. Cell lysates prepared 48 h post-transfection were analysed for luciferase activity using the Dual Luciferase reporter assay kit (Promega). *Drosophila* S2 cells were cultured in Schneider's S2 media (Invitrogen) supplemented with 10% fetal bovine serum and 100 μg/ml penicillin and streptomycin. For transfection, $2 \times 10^6$ S2 cells were seeded into 6-well plates and transfected the next day with 8 μg of PEI (polyethyleneimine) and 1 μg of pGL2-Basic containing the *Skp2* promoter (−165 to +136), 500 ng of pAC5.1-LacZ for normalization and indicated amounts of pAC5.1-ZNF143 or pAC5.1-ZNF76 as indicated. The total DNA transfected was maintained at 2 μg with appropriate amounts of empty pAc5.1 vector.

### siRNA (small interfering RNA) mediated silencing and quantitative RT (reverse transcription)-PCR

Cells were transfected with either a single siRNA targeting human STAFZNF143 (Invitrogen, 10620312), rat STAFZNF143 (Invitrogen, 10620318), a pool of three rat ZNF76 siRNAs (Invitrogen, RSS324258;RSS324259;RSS324260) or an equal amount of negative control siRNA using either Dharmafect1 transfection reagent (Dharmacon) or Amaxa nucleofection (Lonza). For Dharmafect 1 transfection, cells were mixed with lipid:siRNA complexes in suspension, allowed to attach to the culture dish and incubated for a further 24 h. Where indicated, cells were infected with $2\times10^7$ pfu (plaque-forming units)/cell of either control or wild-type Skp2 adenovirus (previously described [8]) 24 h after siRNA transfection. Total RNA or protein was extracted 48 h post-transfection. cDNA was synthesised using QuantiTect first strand synthesis kit (Qiagen). Quantitative RT–PCR was performed using QuantiTect SYBR-green (Qiagen) using primers for Skp2, pre-spliced Skp2, $p27^{Kip1}$ and 18S (see Supplementary Table S1 at http://www.BiochemJ.org/bj/436/bj4360133add.htm for primer details). Fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method.

### Chromatin immunoprecipitation assays

Monoclonal anti-$STAF_{ZNF143}$ (Sigma, WH0007702M1) and rabbit anti-STAF [23] were used for ChIP (chromatin immunoprecipitation) assays using the EZ-ChIP kit (Millipore) according to the manufacturer's instructions. Purified DNA was analysed by quantitative real-time PCR using a Roche Lightcycler 1.5 with the primer pair (forward: 5'-GAGGGTTCGTCCAAAATAAGAGTG-3'; and reverse: 5'-ACTAGCAACGTTCCATCACCAAC-3') flanking the *Skp2* promoter SBS1 element. Additional primer pairs (see Supplementary Table S1) complementary to various sites along the *Skp2* promoter were also used to localise the $STAF_{ZNF143-}$ binding site. Control primers (forward: 5'-GCCTCAGTGGACAGGATGTGGAGAAT-3' and reverse 5'-GCCTCAGTGGACAGGATGTGGAGAAT-3') were complementary to a region approximately 10 kbp 3' to the Skp2 proximal promoter. Amplification of correctly sized amplicons was initially confirmed by agarose gel electrophoresis and subsequently by real-time melt curve analysis.

### Statistical analysis

Results are presented as means ± S.E.M. unless otherwise stated in the Figure legends. Data were analysed by two-tailed paired *t* test or ANOVA with Student-Newman-Keuls post test as indicated. Significant differences were taken when $P < 0.05$.

## RESULTS

### Identification of two positive *cis*-acting $STAF_{ZNF143}$elements in the proximal *Skp2* promoter

HeLa and primary VSMCs were transfected with progressive 5'truncations of the *Skp2* promoter driving expression of a luciferase reporter to identify functionally important elements (Figure 1). The full-length *Skp2* promoter fragment (−3800 to +136bp resulted in a large increase in luciferase activity compared with the empty reporter plasmid, indicating that this promoter fragment is functional in both cell types (Figures 1A and 1B). Truncation to −1518 resulted in a modest reduction of promoter activity that reached statistical significance in VSMCs (to 59.0%±10.2%, $n = 8$,$P = 0.0092$), consistent with previously recognised functional NF-κB (nuclear factor κB) elements in this region [24,25]. Further serial truncation to −325 had no significant effect on promoter activity in either cell type. However, truncation to + 32 resulted in a dramatic and significant loss of promoter activity

in both cell types (from 54.2±7.6% to 3.8 ±1.7% $n$ = 40, $p$=0.0075 in HeLa and from 67.1−14.4% to 4.6 ±0.8%, $Pn$ = 6, $P$ = 0.0066 in VSMCs), indicating presence of positive regulatory elements between −325 and +32 (Figures 1A and 1B).

In order to identify potential transcription factor binding elements within this region, we performed computational sequence analysis of orthologous regions from human, chimpanzee, rhesus monkey, cow, rat and mouse using the Genomatix software (http://www.genomatix.de) (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/436/bj4360133add.htm). This identified cross-species conserved binding elements for Sp-1 (specificity protein 1), GABP (GA-binding protein) and two binding elements for the transcription factor STAF (referred to as SBS1 and SBS2 in the present paper). STAF elements are located at −121 to −139 (SBS2, reverse orientation) and −24 to −42 (SBS1, forward orientation) and show 83% and 89% conservation across all species analysed respectively. No TATA element was identified, consistent with previous observations [21]. A second series of detailed 5' promoter truncations was analysed to define which of these elements are functionally important (Figures 1C and 1D). Truncation from −325 to −165, removing the Sp-1 and GABP elements, resulted in a small non-significant reduction in promoter activity in both cell types (from 67.7±16.6% to 50.2±11.8%, $P$ = 0.44, $n$ = 3 in HeLa from 62.1±10.0%, $P$ = 0.29, $n$ = 6 in VSMCs). Further truncation to −132 bp, bisecting SBS2, resulted in small non-significant reduction in promoter activity 50.3±11.8% to 25.4±2.9%, $n$ = 3, $P$=0.118 in HeLa and from 53.7±10.0% to 33.3±3.5%, $n$ = 9 $P$=0.052 in VSMC). Truncation to −95 bp resulted in a small increase in promoter activity despite no conserved elements being identified in this region. Further truncation from −95 to +32, completely removing SBS1, dramatically reduced promoter activity in both cell types (from 36.8±11.29% to 0.9±0.1%, $n$ = 3, $P$ = 0.0335 in HeLa and from 58.8±6.2% to 4.6±0.8%, $n$ = 12, $P$ < 0.001 in VSMC). We introduced mutations (illustrated in Figure 1G) into each STAF element alone and in combination to further test the function of these elements (Figures 1E, 1F and 2C). SBS1 mutation significantly reduced promoter activity in both cell types to background levels (to 3.0±0.43%, $n$ = 3, $P$<0.0001 in HeLa and to 15.0±3.2% of fragment −65/+136, $n$ = 4, $P$ = 0.0001 SBS2 mutation had no effect in HeLa but significantly reduced promoter activity in VSMCs) (to 55.5±13.6%, $n$ = 4, $P$ = 0.0468). Mutation of both SBS1 and SBS2 elements resulted in complete loss of promoter activity. These results indicate that the proximal STAF element (SBS1) is essential for basal *Skp2* promoter activity in both cell types and SBS2 has a facultative role in VSMCs.

## STAF binds to the *Skp2* promoter *in vitro* and in live cells

ZNF143 and ZNF76 represent the human and rat paralogues of the transcription factor STAF that was originally characterized in *X. laevis* [26]. Both factors have almost identical DBDs and bind STAF elements with identical binding affinities [26]. We therefore used the recombinant $STAF_{ZNF143}$ DBD to test STAF binding to the proximal *Skp2* promoter STAF elements using EMSAs. Incubation of recombinant STAF with a probe (−165 to +136) encompassing both STAF elements resulted in the formation of two retarded complexes (complexes C1 and C2) (Figure 2A). Complexes were competed by excess unlabelled probe but not by unrelated probe (Figure 2B, lanes 3 and 4). Complex C1 and C2 presumably represent occupancy of either one or both SBS elements respectively. Consistent with this, complex C2 was lost after mutation of either SBS1 or SBS2, whereas both complexes were lost after double mutation of SBS1 and SBS2 (Figure 2C).

We next tested whether endogenously expressed STAF in nuclear extracts of exponentially proliferating VSMCs is capable of binding a shorter probe (−54 to −25) containing only SBS1. Only a single retarded complex (C1) was formed, since the probe used in these EMSAs contained only SBS1 (Figure 2D). This complex was specifically competed by

excess unlabelled specific probe (lane 2) but not unrelated probe (lane 3) or probe containing a mutated SBS1 element (lane 4). Co-incubation of binding complexes with a mouse anti-$STAF_{ZNF143}$ antibody (Figure 2E;lane 2) but not non-immune IgG (lane 1) resulted in the formation of a super-shifted complex. Co-incubation with a rabbit anti-$STAF_{ZNF143}$ antibody (Figure 2E; lane 4) but not non-immune rabbit IgG (lane 5) reduced complex (C1) formation, indicating the presence of $STAF_{ZNF143}$ in the complex.

We next used ChIP assays to demonstrate binding of $STAF_{ZNF143}$ to the endogenous *Skp2* promoter in live cells. Amplification of correctly sized amplicons was initially confirmed by agarose gel electrophoresis (see Supplementary Figure S2A at http://www.BiochemJ.org/bj/436/bj4360133add.htm) and subsequently by real-time melt curve analysis. Quantitative PCR analysis demonstrated specific immunoprecipitation of the proximal *Skp2* promoter with mouse-anti-$STAF_{ZNF143}$ but not non-immune IgG (Figure 3A). Importantly, a DNA region 10 kbp downstream of the *Skp2* promoter that is not associated with a STAF element was not immunoprecipitated. Quantitative PCR analysis of multiple amplicons evenly spaced along the *Skp2* promoter demonstrated specific immuno-precipitation of only the *Skp2* promoter region corresponding to the proximal STAF elements (Supplementary Figure S2B), providing direct evidence of STAF binding to these sites in living cells. Given that standard ChIP does not have the resolution to discriminate binding of STAF between the closely spaced SBS1 and SBS2, we performed STAF immunoprecipitation on cells transiently transfected with *Skp2* promoter reporter plasmids carrying mutations in either SBS1 or SBS2, followed by quantitative PCR analysis of the immunoprecipitated plasmids (Figure 3B). Mutation of SBS1, and to a lesser extent SBS2, reduced recovery of reporter DNA, indicating that STAF does indeed bind both elements. The greater effect of SBS1 mutation is consistent with the greater functional role of this element observed in our reporter assays (Figures 1E and 1F).

## Effects of overexpression and knockdown of STAF on Skp2 expression and cell proliferation

We used three separate approaches to test if STAF activates *Skp2* promoter activity and expression. We initially used *Drosophila* S2 cells, which normally lack vertebrate transcription factors, including STAF (ZNF143 or ZNF76) [27]. *Drosophila* S2 cells were transfected with vectors expressing human $STAF_{ZNF143}$ or zNF76 and a −165/+136 *Skp2* promoter/luciferase reporter. Ectopic $STAF_{ZNF143}$ or ZNF76 expression significantly increased *Skp2* promoter activity, indicating that both STAF paralogues positively regulate the *Skp2* promoter activity (Figure 4A). Secondly, we co-transfected HeLa with a *Skp2* promoter/luciferase reporter plasmid in which both STAF elements were converted into GAL4-binding elements (pGLSkp2-STAFMUT), together with an expression plasmid encoding a chimaeric GAL4 DBD fused to the activation domains of $STAF_{ZNF143}$ [GAL4-STAF (13–152)] [28]. This allowed us to negate the effects of endogenous STAF and test if recruitment of the STAF transcriptional activation domain to the *Skp2* promoter increases its activity. Expression of GAL4-STAF (13–152) stimulated a significant 2.5-fold increase in promoter activity relative to cells expressing the GAL4 DBD alone (Figure 4B). Lastly, we used Ad:WT-$STAF_{ZNF143}$ (wild-type $STAF_{ZNF143}$− expressing adenovirus vector) in VSMCs. This demonstrated that infection with Ad:WT-$STAF_{ZNF143}$ resulted in a significant increase in endogenous Skp2 mRNA expression compared with Ad:Control (control adenovirus vector) infected cells (Figure 4C).

We next used siRNA-mediated silencing to test the role of endogenous STAF in the regulation of the *Skp2* promoter, mRNA, protein and cell proliferation. $STAF_{ZNF143}$ siRNA significantly reduced $STAF_{ZNF143}$ mRNA levels (to 25.5±0.78% of control, $n = 3$, $P<0.05$; Figure 5A), $STAF_{ZNF143}$ protein expression (Figures 5C and 5D) and activity of a synthetic promoter consisting of three STAF elements immediately upstream of a thymidine kinase

basic promoter (to 35.5±8.4% of controls, $n = 5$, $P<0.05$; Figure 5B) compared with control siRNA in HeLa cells. $STAF_{ZNF143}$ silencing also resulted in a significant reduction in Skp2 mRNA expression (Figure 5A) and activity of a *Skp2* promoter reporter (−165/+136) (Figure 5B). This translated into a significant reduction in Skp2 protein (Figures 5C and 5E) expression that was associated with an increase in the levels of the Skp2 substrate $p27_{Kip1}$ (Figure 5C). Importantly, levels of β-actin, β-tubulin or GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were not affected. Taken together, this indicates a major role for $STAF_{ZNF143}$ in Skp2 regulation in these cells. In VSMCs, silencing of $STAF_{ZNF143}$ or ZNF76 alone did not significantly affect Skp2 expression (results not shown). We hypothesised that this was due to functional redundancy between these two STAF paralogues. Consistent with this, silencing of both ZNF143 and ZNF76 together (referred to as $siSTAF_{ZNF143/76}$ in the present manuscript) resulted in a significant inhibition of Skp2 mRNA expression without affecting levels of 18S RNA, GAPDH mRNA or FBXW7 (another FBP related to Skp2) mRNA (Figure 5F), indicating a redundant role for these factors in Skp2 regulation in VSMCs. Use of $siSTAF_{ZNF143/76}$ also resulted in a significant reduction in the level of Skp2 protein (Figures 5G and 5I) that was again associated with an increase in $p27^{Kip1}$ levels (Figure 5G). Expression of β-actin, β-tubulin or GAPDH protein was not affected.

Given that Skp2 is a major regulator of $G_1$–S-phase progression, we analysed whether STAF silencing also resulted in an inhibition of proliferation. Consistent with this, $siSTAF_{ZNF143}$ in HeLa or $siSTAF_{ZNF143/76}$ in VSMCs resulted in a significant inhibition of cell growth measured by cell number in both cell types compared with control siRNA transfected cells (Figures 5J and 5K). Importantly, no change was observed in cell viability measured by Trypan Blue exclusion assay [results not shown; 4.35±1.75% compared with 2.54±0.55% dead cells for siNEG (negative control siRNA) and $siSTAF_{ZNF143/76}$ respectively in VSMCs]. S-phase entry measured by BrdU (bromodeoxyuridine) incorporation was also significantly inhibited in both cell types (Figures 5L and 5M). We next performed a Skp2 rescue experiment to determine the functional significance of Skp2 in mediating the effects of STAF on cell proliferation. VSMCs were transfected with siNEG or $siSTAF_{ZNF143/76}$ followed by infection with either Ad:Skp2 (Skp2-expressing adenovirus vector) or Ad:Control (Figure 5N). Ad:Skp2 infection in siNEG cells resulted in a small increase in BrdU incorporation, consistent with previous observations [9,29]. Importantly, Ad:Skp2 infection completely reversed the inhibition of proliferation induced by $siSTAF_{ZNF143/76}$, strongly suggesting that Skp2 is a major effector mediating the effects of $STAF_{ZNF143/76}$ on S-phase entry.

## STAF expression and binding to the Skp2 promoter is adhesion-dependent

Skp2 mRNA expression is adhesion-dependent in several cell types [5,8], including primary VSMCs [8]. We therefore determined the requirement of cell adhesion for the expression and binding of STAF to the Skp2 proximal promoter (Figure 6). First we confirmed that Skp2 mRNA and pre-spliced Skp2 hnRNA (heterogeneous nuclear RNA) were significantly reduced in suspension culture (to 24.0±3.7% and 24.7± 4.1% respectively, $P<0.05$) compared with adherent cultures (Figure 6A). Suspension culture did not significantly alter $STAF_{ZNF143}$ or ZNF76 mRNA levels ($P = 0.389$ and $P = 0.3$ respectively) or other genes, such as $p27^{Kip1}$ or 18S RNA, indicating that changes in Skp2 mRNA are not due to a generalised reduction in cell viability (results not shown), or transcription (Figure 6A). Wild-type *Skp2* promoter activity was also significantly reduced in suspension cells (Figure 6B). Most importantly, we show that suspension culture led a significant reduction in protein levels of both STAF paralogues (ZNF143 and ZNF76), together with significant reductions in Skp2 and increased $p27^{Kip1}$ protein levels (Figures 6C and 6D). GAPDH protein levels were unchanged. The reduction in $STAF_{ZNF143}$ protein but not mRNA in suspension cells

indicates a post-transcriptional adhesion-dependent mechanism controlling STAF. $STAF_{ZNF143}$ protein levels were reduced as early as 4 h in suspension, preceding the loss of *Skp2* mRNA, which was first detectable at 8 h (Figure 6E). ChIP analysis demonstrated significant reduction in $STAF_{ZNF143}$ binding to the *Skp2* promoter (from 5.5±7.7% to 1.10.±0.8% of input, $n = 3$, $P$ 0.0002) in suspension compared with adherent culture, demonstrating adhesion-dependent binding of $STAF_{ZNF143}$ to the proximal *Skp2* promoter (Figure 6F). Moreover, transient transfection with wild-type $STAF_{ZNF143}$ plasmid resulted in a significant ($P<0.05$) but partial rescue (to 42.7±15.6% of that in adherent cells) of endogenous *Skp2* mRNA expression in suspension cells, despite a modest 40% transfection efficiency achieved in these cells (Figure 6G). Taken together, this data indicates that STAF plays a role in the adhesion-dependent regulation of Skp2 expression.

## DISCUSSION

This study demonstrates a novel role for both paralogues of the STAF transcription factor in the adhesion-dependent expression of Skp2 and in cell proliferation. Silencing of STAF inhibits Skp2 expression and proliferation, which demonstrates the potential of this pathway as a target for therapy.

First we identified a stimulatory region (−325 to +136 bp) in the *Skp2* promoter. Sequence comparison with other species identified four conserved transcription factor-binding elements. These included the previously identified Sp-1 and GABP elements [30]. We identified two previously unrecognised elements for STAF. STAF was originally identified as a DNA-binding factor required for transcription of the RNA polymerase III-transcribed STAF in *Xenopus laevis* [27] as well as many snRNA (small nuclear RNA) and snRNA-like genes [31]. Two conserved paralogues of STAF exist in mammals that have previously been reported to regulate expression of a small number of RNA polymerase II-transcribed protein-coding genes in mammalian cells [32–40]. Genome-wide sequence analysis identified 938 promoters containing STAF-binding elements, suggesting a widespread role for this factor in regulating transcription responses [41]. The *Skp2* promoter contains several characteristic features in common with the promoters of these other putative STAF-regulated genes, including the absence of a recognisable TATA element, the presence of multiple copies of the STAF elements in close proximity to the transcriptional start site and the presence of an associated ACTACAA motif flanking the STAF element [41]. We present strong evidence that binding of STAF to the two conserved elements (SBS1 and SBS2) is essential and sufficient for *Skp2* promoter activity and ultimately cell proliferation. Using truncation and mutational promoter analysis we show an essential role for SBS1 in both HeLa and VSMCs. SBS2 appears to be utilized in a cell-type-specific manner, being functional in VSMCs but not in HeLa cells. Furthermore, the recombinant STAF DBD, which is almost identical in ZNF143 and ZNF76, binds both SBS1 and SBS2 in EMSAs. Nuclear extracts form specific binding complexes with the *Skp2* promoter SBS1 element in EMSA and ChIP analysis, demonstrating binding of $STAF_{ZNF143}$ to the endogenous *Skp2* promoter in living cells. Forced expression of either STAF paralogue (ZNF143 or ZNF76) stimulates *Skp2* promoter reporter activity in *Drosophila* S2 cells, and a similar stimulation of *Skp2* promoter activity occurs in mammalian cells upon forced expression of ZNF143. Taken together, these results demonstrate that the proximal *Skp2* promoter STAF elements are functional, that they bind STAF and that both STAF paralogues are capable of stimulating promoter activity. Moreover, siRNA-mediated silencing of $STAF_{ZNF143}$ in HeLa cells inhibits *Skp2* promoter activity, mRNA and protein expression and cell proliferation, indicating an important role for endogenous $STAF_{ZNF143}$ in regulating Skp2 expression and $G_1$–S progression. Interestingly, our results indicate that ZNF143 and ZNF76 are functionally redundant with respect to Skp2 regulation in VSMCs. Consistent with this, dual silencing of both paralogues in these cells inhibits Skp2 expression and S-phase entry.

This redundancy is rational given our data showing that both STAF paralogues are able to activate the *Skp2* promoter activity and are both expressed at similar levels in these cells (similar mRNA copy number, results not shown, and protein for both present). Other promoters that contain STAF elements have also been shown to be positively regulated by both ZNF143 and ZNF76, suggesting that this redundancy is not a unique feature of Skp2 regulation [39]. It is intriguing why a similar redundancy does not operate in HeLa cells. Our results indicate that similar mRNA levels for ZNF143 and ZNF76 are also expressed in these cells, indicating that this difference is not simply due to a cell-type specific expression of each paralogue. This cell-type specific redundancy presumably reflects differential expression of co-factors or post-translational modifications of ZNF76. For example, acetylation and SUMOylation of ZNF76 is known to play an important role in controlling whether ZNF76 acts as a transcriptional activator or repressor [42,43].

Although the present study clearly establishes an important role for STAF paralogues in controlling Skp2 expression, it is likely that STAF acts together with other factors, such as NF-$_{\kappa}$B and E2F factors, previously reported to regulate Skp2 transcription [21,24,25]. In our experiments above, deletion of distal NF-$_{\kappa}$B elements (between −3800 and −1518bp) resulted in a small reduction in promoter activity, consistent with other studies [24,25]. From previous work in HeLa, *Skp2* promoter constructs lacking the E2F element are also inactive even though the STAF elements are present [6]. We show here that promoter constructs containing E2F but lacking proximal STAF elements are also non-functional. Clearly co-operation between STAF and E2F must control *Skp2* promoter activity.

*In silico* identification of STAF element-containing promoters [41] and microarray analysis of STAF-regulated genes [44] indicates that STAF controls the expression of several genes involved in cell growth and proliferation. Consistent with this, our results from the present study and that of another recent report show that STAF silencing results in inhibition of cell growth [44]. Our results demonstrate that *Skp2* is also a STAF-regulated gene involved in mediating these effects of STAF on cell proliferation. The importance of Skp2 as a mediator of the proliferative function of these STAF paralogues is clearly demonstrated by the complete rescue of S-phase entry in $STAF_{ZNF143/76}$-silenced cells by ectopic expression of Skp2. This indicates that Skp2 is the major target for STAF in controlling $G_1$–S-phase progression but does not preclude the possibility that other STAF-regulated genes also control other parts of the cell cycle.

Skp2 has been implicated in both the physiological regulation of cell proliferation and its pathological deregulation in diseases such as cancer. Apart from its possible pharmacological value, discovery of STAF-dependent Skp2 regulation sheds light on the mechanisms responsible for the adhesion dependence of Skp2 expression [5]. Adhesion-dependent regulation of Skp2 is a major mechanism controlling Skp2 in response to tissue injury and enforcing adhesion-dependent proliferation in most non-transformed cells. Our results from the present study indicate that STAF is an important player in mediating this, since level of both STAF paralogues are adhesion-dependent in non-transformed cells and forced expression of $STAF_{ZNF143}$ in these cells rescues Skp2 expression. Acquisition of adhesion-independence is a fundamental property of transformed cancer cells, associated with increased invasiveness and metastasis. Deregulation of Skp2 is likely to be involved since ectopic Skp2 expression allows proliferation of non-adherent fibroblasts [5]. It is tempting to speculate, given a recent report demonstrating elevated STAF in numerous solid tumours [44], that deregulation of STAF may contribute to acquisition of adhesion-independence in transformed cancer cells.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Abbreviations used

| | |
|---|---|
| **Ad:Control** | control adenovirus vector |
| **Ad:Skp2** | Skp2-expressing adenovirus vector |
| **BrdU** | bromodeoxyuridine |
| **ChIP** | chromatin immunoprecipitation |
| **DBD** | DNA-binding domain |
| **EMSA** | electrophoretic mobility-shift assay |
| **FBP** | F-box protein |
| **GABP** | GA-binding protein |
| **GAPDH** | glyceraldehyde-3-phosphate dehydrogenase |
| **GST** | glutathione transferase |
| **NF-$_{\kappa}$B** | nuclear factor $_{\kappa}$B |
| **pfu** | plaque-forming units |
| **RT** | reverse transcription |
| **siRNA** | small interfering RNA |
| **siNEG** | negative control siRNA |
| **Skp** | S-phase kinase-associated protein |
| **snRNA** | small nuclear RNA |
| **STAF** | selenocysteine tRNA gene transcription-activating factor |
| **Ad:WT-STAF $_{ZNF143}$** | wild-type STAF$_{ZNF143}$-expressing adenovirus vector |
| **siSTAF** | siRNA specific to STAF |
| **VSMC** | vascular smooth muscle cell |

## REFERENCES

1. Chen C, Seth A, Aplin A. Genetic and expression aberrations of E3 ubiquitin ligases in human breast cancer. Mol. Cancer Res. 2006; 4:695–707. [PubMed: 17050664]
2. Versari D, Herrmann J, Gossl M, Mannheim D, Sattler K, Meyer F, Lerman L, Lerman A. Dysregulation of the ubiquitin-proteasome system in human carotid atherosclerosis. Arterioscler. Thromb. Vasc. Biol. 2006; 26:2132–2139.

3. Herrmann J, Ciechanover A, Lerman LO, Lerman A. The ubiquitin-proteasome system in cardiovascular diseases - a hypothesis extended. Cardiovasc. Res. 2004; 61:11–21. [PubMed: 14732197]
4. Carrano A, Eytan E, Hershko A, Pagano M. SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. Nat. Cell Biol. 1999; 1:193–199. [PubMed: 10559916]
5. Carrano AC, Pagano M. Role of the F-Box protein Skp2 in adhesion-dependent cell cycle progression. J. Cell. Biol. 2001; 153:1381–1389. [PubMed: 11425869]
6. Zhang H, LKobayashi R, Galaktionov K, Beach D. p19Skp-1 and p45Skp-2 are essential elements of the cyclin A-Cdk2 S phase kinase. Cell. 1995; 82:915–925. [PubMed: 7553852]
7. Nakayama K, Nagahama H, Minanishima Y, Matsumoto M, Nakamichi I, Kitagawa K, Shirane M, Tsunematsu R, Tsukiyama T, Ishida N, et al. Targeted disruption of Skp2 results in accumulation of cyclinE and p27kip1 and centrosome overduplication. EMBO J. 2000; 19:2069–2081. [PubMed: 10790373]
8. Bond M, Sala-Newby G, Newby A. Focal adhesion kinase (FAK)-dependent regulation of S-phase kinase-associated protein-2 (Skp-2) stability. J. Biol. Chem. 2004; 279:37304–37310. [PubMed: 15208331]
9. Wu Y, Bond M, Sala-Newby G, Newby A. Altered S-phase kinase-associated protein-2 levels are a major mediator of cyclic nucleotide-induced inhibition of vascular smooth muscle cell proliferation. Circ. Res. 2006; 98:1141–1150. [PubMed: 16574903]
10. Wu YJ, Sala-Newby GB, Shu KT, Yeh HI, Nakayama KI, Nakayama K, Newby AC, Bond M. S-phase kinase-associated protein-2 (Skp2) promotes vascular smooth muscle cell proliferation and neointima formation in vivo. J. Vasc. Surgery. 2009; 50:1135–1142.
11. Zhong L, Georgia S, Tschen S, Nakayama K, Nakayama K, Bhushan A. Essential role of Skp2-mediated p27 degradation on growth and adaptive expansion of pancreatic B cells. J. Clin. Investig. 2007; 117:2869–2876. [PubMed: 17823659]
12. Kossatz U, Dietrich N, Zender L, Buer J, Manns M, Nisar P. Skp2-dependent degradation of p27Kip1 is essential for cell cycle progression. Genes Dev. 2004; 18:2602–2607. [PubMed: 15520280]
13. Bornstein G, Bloom J, Sitry-Shevah D, Nakayama K, Pagano M, Hershko A. Role of the SCFskp2 ubiquitin ligase in the degradation of p21cip1 in S phase. J. Biol. Chem. 2003; 278:25752–25757. [PubMed: 12730199]
14. Nakayama K, Nakayama K. Regulation of the cell cycle by SCF-type ubiquitin ligases. Seminars Cell Dev. Biol. 2005; 16:323–333.
15. Marti A, Wirbelauer C, Scheffner M, Krek W. Interaction between ubiquitin-protein ligase SCFSKP2 and E2F-1 underlies the regulation of E2F-1 degradation. Nat. Cell Biol. 1999; 1:14–19. [PubMed: 10559858]
16. von der Lehr N, Johansson S, Wu SQ, Bahram F, Castell A, Cetinkaya C, Hydbring P, Weidung I, Nakayama K, Nakayama KI, et al. The F-box protein Skp2 participates in c-Myc proteosomal degradation and acts as a cofactor for c-Myc-regulated transcription. Mol. Cell. 2003; 11:1189–1200. [PubMed: 12769844]
17. Bond M, Wu Y, Sala-Newby G, Newby A. Biphasic effect of p21Cip1 on vascular smooth muscle cell proliferation: role of PI3-kinase signalling and Skp2-mediated degradation. Cardiovasc. Res. 2005; 69:198–206. [PubMed: 16212951]
18. Seki R, Okamura T, Koga H, Yakushiji K, Hashiguchi M, Yoshimoto K, Ogata H, Imamura R, Nakashima Y, Kage M, et al. Prognostic significance of the F-box protein Skp2 expression in diffuse large B-cell lymphoma. Am. J. Hematol. 2003; 73:230–235. [PubMed: 12879424]
19. Min YH, Cheong JW, Lee MH, Kim JY, Lee ST, Hahn JS, Ko YW. Elevated S-phase kinase-associated protein 2 protein expression in acute myelogenous leukemia: its association with constitutive phosphorylation of phosphatase and tensin homologue protein and poor prognosis. Clin. Cancer Res. 2004; 10:5123–5130. [PubMed: 15297415]
20. Wirbelauer C, Sutterluty H, Blonel M, Gstaiger M, Peter M, Reymond F, Krek W. The F-box protein Skp2 is a ubiquitinylation target of Cul1-based core ubiquitin ligase complex: evidence for a role of Cul1 in the suppression of Skp2 expression in quiescent fibroblasts. EMBO J. 2000; 19:5361–5375.

21. Zhang L, Wang C. F-box protein Skp2: a novel transcriptional target of E2F. Oncogene. 2006; 25:2615–2627. [PubMed: 16331253]
22. Sala-Newby GB, Campbell AK. Engineering a bioluminescent indicator for cyclic AMP-dependent protein kinase. Biochem. J. 1991; 279:727–732. [PubMed: 1953665]
23. Rincon J, Engler S, Hargrove B, Kunkel G. Molecular cloning of a cDNA encoding human SPH-binding factor, a conserverd protein that binds to the engancer-like regions of the U6 small nuclear RNA gene promoter. Nucleic Acids Res. 1998; 26:4846–4852. [PubMed: 9776743]
24. Barre B, Perkins ND. A cell cycle regulatory network controlling NF-$_{\kappa}$B subunit activity and function. EMBO J. 2007; 26:4841–4855. [PubMed: 17962807]
25. Barre B, Perkins ND. The Skp2 promoter integrates signaling through the NF-$_{\kappa}$B, p53, and Akt/GSK3β pathways to regulate autophagy and apoptosis. Mol. Cell. 2010; 38:524–538. [PubMed: 20513428]
26. Myslinski E, Krol A, Carbon P. ZNF143 and ZNF76 are two human homologs of the transcriptional activator Staf. J. Biol. Chem. 1998; 273:21998–22008. [PubMed: 9705341]
27. Schuster C, Myslinski E, Krol A, Carbon P. STAF, a novel zinc-finger protein that activates the RNA-polymerase-III promoter of the selenocysteine transfer-RNA gene. EMBO J. 1995; 14:3777–3787. [PubMed: 7641696]
28. Schuster C, Krol A, Carbon P. Two distinct domains in STAF to selectively activate small nuclear RNA-type and mRNA promoters. Mol. Cell. Biol. 1998; 18:2650–2658. [PubMed: 9566884]
29. Sutterluty H, Chatelain E, Marti A, Wirbelauer C, Senften M, Muller U, Krek W. p45skp2 promotes p27kip1 degradation and induces S phase in quiescent cells. Nat. Cell Biol. 1999; 1:207–214. [PubMed: 10559918]
30. Imaki H, Nakavama K, Deiehouzee S, Handa H, Kitagawa K, Kamagawa T, Nakayama K. Cell cycle-dependent regulation of the Skp2 promoter by GA-binding protein. Cancer Res. 2003; 63:4607–4613. [PubMed: 12907639]
31. Schaub M, Myslinski E, Schuster C, Krol A, Carbon P. STAF, a promiscuous activator for enhanced transcription by RNA polymerases II and III. EMBO J. 1997; 16:173–181. [PubMed: 9009278]
32. Kubota H, Yokota S, Yanagi H, Yura T. Transcriptional regulation of the mouse cytosolic chaperonin subunit gene Ccta/t-complex polypeptide 1 by selenocysteine tRNA gene transcription activating factor family zinc finger proteins. J. Biol. Chem. 2000; 275:28641–28648. [PubMed: 10893243]
33. Mach CM, Hargrove BW, Kunkel GR. The small RNA gene activator protein, SphI postoctamer homology-binding factor/selenocysteine tRNA gene transcription activating factor, stimulates transcription of the human interferon regulatory factor-3 gene. J. Biol. Chem. 2002; 277:4853–4858. [PubMed: 11724783]
34. Saur D, Seidler B, Paehge H, Schusdziarra V, Allescher HD. Complex regulation of human neuronal nitric-oxide synthase Exon 1c gene transcription - essential role of Sp and ZNF family members of transcription factors. J. Biol. Chem. 2002; 277:25798–25814. [PubMed: 11960979]
35. Barski OA, Papusha VZ, Kunkel GR, Gabbay KH. Regulation of aldehyde reductase expression by STAF and CHOP. Genomics. 2004; 83:119–129. [PubMed: 14667815]
36. Di Leva F, Ferrante MI, Demarchi F, Caravelli A, Matarazzo MR, Giacca M, D'Urso M, D'Esposito M, Franze A. Human synaptobrevin-like 1 gene basal transcription is regulated through the interaction of selenocysteine tRNA gene transcription activating factor-zinc finger 143 factors with evolutionary conserved cis-elements. J. Biol. Chem. 2004; 279:7734–7739. [PubMed: 14672948]
37. Grossman CE, Qian YM, Banki K, Perl A. ZNF143 mediates basal and tissue-specific expression of human transaldolase. J. Biol. Chem. 2004; 279:12190–12205. [PubMed: 14702349]
38. Ishiguchi H, Izumi H, Torigoe T, Yoshida Y, Kubota H, Tsuji S, Kohno K. ZNF143 activates gene expression in response to DNA damage and binds to cisplatin-modified DNA. Int. J. Cancer. 2004; 111:900–909. [PubMed: 15300802]
39. Myslinski E, Gerard MA, Krol A, Carbon P. Transcription of the human cell cycle regulated BUB1B gene requires hStaf/ZNF143. Nucleic Acids Res. 2007; 35:3453–3464. [PubMed: 17478512]

40. Gerard MA, Krol A, Carbon P. Transcription factor hStaf/ZNF 143 is required for expression of the human TFAM gene. Gene. 2007; 401:145–153. [PubMed: 17707600]

41. Myslinski E, Gerard MA, Krol A, Carbon P. A genome scale location analysis of human Staf/ZNF143-binding sites suggests a widespread role for human Staf/ZNF143 in mammalian promoters. J. Biol. Chem. 2006; 281:39953–39962. [PubMed: 17092945]

42. Zheng G, Yang Y. Acetylation and alternative splicing regulate ZNF76-mediated transcription. Biochem. Biophys. Res. Commun. 2006; 339:1069–1075. [PubMed: 16337145]

43. Zheng G, Yang Y. ZNF76, a novel transcriptional repressor targeting TATA-binding protein, is modulated by sumoylation. J. Biol. Chem. 2004; 279:42410–42421. [PubMed: 15280358]

44. Izumi H, Wakasugi T, Shimajiri S, Tanimoto A, Sasaguri Y, Kashiwagi E, Yasuniwa Y, Akiyama M, Han B, Wu Y, et al. Role of ZNF143 in tumor growth through transcriptional regulation of DNA replication and cell-cycle-associated genes. Cancer Sci. 2010; 101:2538–2545. [PubMed: 20860770]

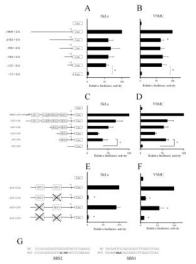

**Figure 1. Characterisation of the *Skp2* promoter in HeLa cells and primary VSMCs**

Indicated *Skp2* promoter/luciferase constructs were co-transfected with equal amounts of pTK-RL into either HeLa cells or VSMCs, as indicated. Reporter activity was quantified 48 h post-transfection and presented as means ± S.E.M. of firefly luciferase activity/*Renilla* luciferase activity relative to the −3800/+136 construct. (**A** and **B**) Broad range truncation analysis of the *Skp2* promoter. (**C** and **D**) Detailed truncation analysis of the proximal *Skp2* promoter. Results are from at least six independent experiments. **P* < 0.05 compared with full-length promoter. (**E** and **F**) Mutational analysis of STAF (SBS) elements in the proximal (−165/+136 bp) *Skp2* promoter. Results are presented relative to the wild-type −165/+136 promoter fragment and are from at least three independent experiments. **P* < 0.05 compared with wild-type fragment −165/+136. (**G**) Comparison of wild-type and mutated SBS1 and SBS2 elements.

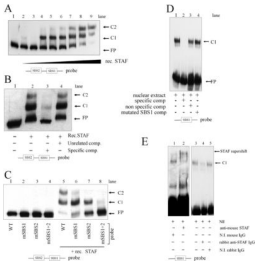

**Figure 2. EMSA analysis of recombinant $STAF_{ZNF143}$ binding to wild-type and mutated *Skp2* promoter SBS elements**

(**A**) EMSA were performed using a 5′ biotinylated probe corresponding to the −165/+136 fragment of the *Skp2* promoter containing both SBS2 and SBS1 elements. The probe was incubated with increasing amounts of the recombinant human $STAF_{ZNF143}$ DBD (rec. STAF) (lanes 2–9; 10 ng–6000 ng). (**B**) The probe was incubated alone (lane 1), in the presence of recombinant $STAF_{ZNF143}$ DBD (lanes 2–4), and with 100-fold molar excess of specific competitor (Specific comp., unlabelled −86/+217 promoter fragment, lane 3) or an unrelated competitor (Unrelated comp., unlabelled probe containing EBNA-binding element, lane 4) as indicated. (**C**) Biotinylated probes representing fragment −165/+136 with indicated mutations (m) in either SBS element or both together (mutations as indicated in Figure 1G) were incubated either alone (lanes 1–4) or together with the recombinant human $STAF_{ZNF143}$ DBD (lanes 5–8). (**D**) VSMC nuclear extract was incubated with biotinylated probe encompassing SBS1 together with a 100-fold molar excess of the indicated unlabelled probes. (**E**) Supershift assays were performed as in (**D**) but with the addition of non-immune (N.I.) mouse IgG (lane 1), mouse anti-STAF antibody (lane 2), rabbit anti-STAF polyclonal antibody (lane 4) or non-immune rabbit IgG (lane 5). FP, free unbound probe.

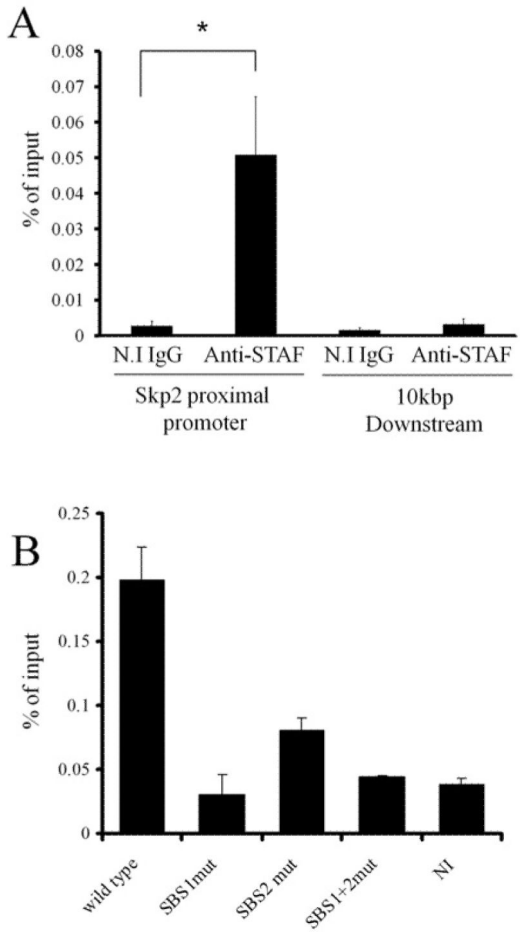


**Figure 3. ChIP analysis of endogenous STAF$_{ZNF143}$ binding to the *Skp2* promoter**

ChIP analysis was performed using either a 2 μg mouse anti-STAF$_{ZNF143}$ antibody or isotype matched pre-immune mouse IgG with sheared chromatin from 1 × 10$^6$ VSMCs. (**A**) Real-time quantitative PCR analysis of Skp2 proximal promoter or a control region approximately 10 kbp 3′ to the promoter in anti-STAF$_{ZNF143}$ or non-immune IgG (N.I IgG) immunoprecipitated samples and input chromatin, as indicated. Results are expressed as means ± S.E.M. of immunoprecipitated DNA as a percentage of total input DNA. (**B**) Cells were transiently transfected with equal amounts of *Skp2* promoter reporter plasmids containing either the wild-type −165/+136 promoter region or an equivalent region harbouring mutations (mut) in either SBS element as indicated, using Amaxa nucleofection. Chromatin prepared from 4 × 10$^5$ cells was immunoprecipitated with either 2 μg of mouse anti-STAF antibody or non-immune IgG (NI) and immunoprecipitated plasmids were quantified by real-time quantitative PCR using primers specific for the reporter plasmid. Results are expressed as means ± S.E.M. of immunoprecipitated DNA as a percentage of total input DNA.

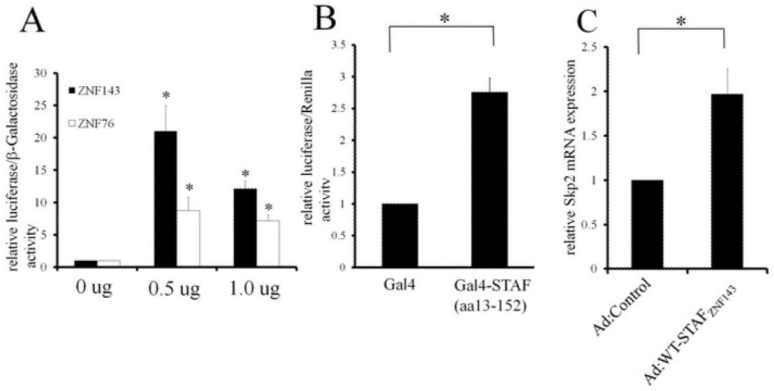


**Figure 4. $STAF_{ZNF143}$ activates the *Skp2* promoter activity and expression**

(**A**) *Drosophila* S2 cells were transiently transfected with either 1 μg of the human *Skp2* promoter fragment −325/+136 luciferase reporter construct together with the indicated amounts of vector expressing human $STAF_{ZNF143}$ (pAC5.1-$STAF_{ZNF143}$) or ZNF76 (pAC5.1-ZNF76) and 0.5 μg of pAC5.1-lacZ for normalization. Luciferase and β-galactosidase activity was quantified 48 h post-transfection. (**B**) HeLa cells were transfected with a 500 ng of Skp2 reporter in which both STAF elements were mutated to GAL4-binding elements (pGL-Skp2-STAFMUT) together with either 500 ng of vector expressing a GAL4 DBD (GAL4) or vector expressing a GAL4 DBD fused to the transcription activation domain of STAF [GAL4–STAF (aa13–152)]. pTK-*Renilla* reporter was used for normalization. Luciferase and *Renilla* activity were quantified 48 h post transfection. (**C**) VSMCs were infected with $1 \times 10^7$ pfu/ml of either Ad:Control or Ad:WT-$STAF_{ZNF143}$. Skp2 mRNA was quantified 48 h post infection by quantitative PCR. Results are expressed as means ± S.E.M. of at least three independent experiments. **P* <0.05, significantly different from control.

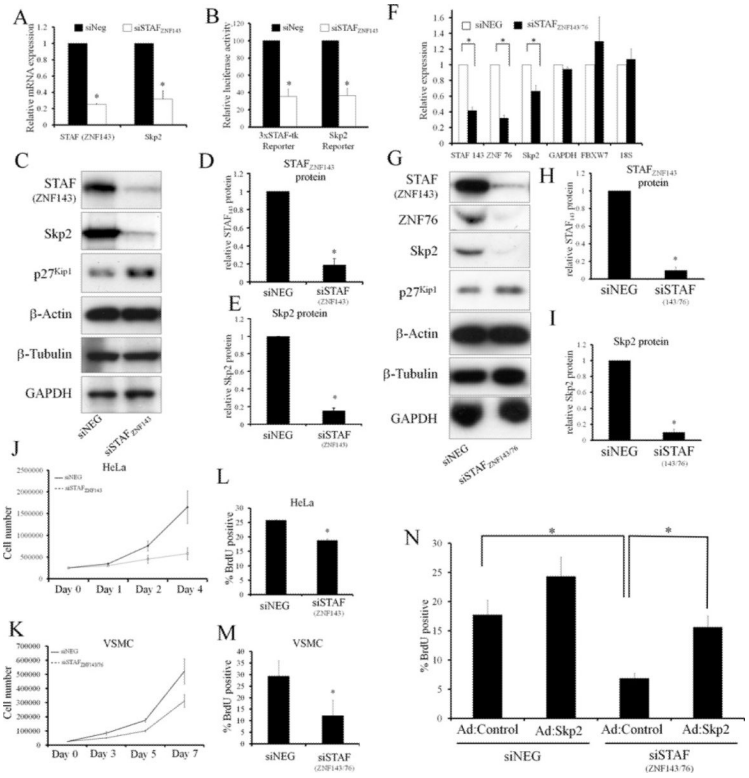


**Figure 5. STAF regulate Skp2 expression and proliferation**

(**A**) HeLa cells were transfected with 100 nM $STAF_{ZNF143}$ siRNA or negative control siRNA (siNeg) as indicated and analysed for $STAF_{ZNF143}$ and Skp2 mRNA expression by quantitative RT–PCR. (**B**) Activity of synthetic STAF promoter luciferase reporter and *Skp2* promoter luciferase reporter 48 h post-transfection with either negative control or $STAF_{ZNF143}$ siRNA. (**C**) Western blot analysis of $STAF_{ZNF143}$, Skp2, p27Kip1, β-actin, β-tubulin and GAPDH protein levels in HeLa cell lysates 48 h post-transfection with either negative control or $STAF_{ZNF143}$ siRNA. (**D** and **E**) Densitometric analysis of $STAF_{ZNF143}$ and Skp2 protein expression. (**F**) VSMCs (100 μl) were transfected with 1000 nM siNEG or a combination of siRNA for ZNF143 and ZNF76 ($siSTAF_{ZNF143}/76$) using nucleofection. mRNA levels for $STAF_{ZNF143}$, ZNF76, Skp2, GAPDH, FBXW7 and 18S were quantified 48 h post-transfection. (**G**) Western blot analysis of $STAF_{ZNF143}$, ZNF76, Skp2, $p27^{Kip1}$, β-actin, β-tubulin and GAPDH protein levels in VSMC lysates 48 h post transfection with either negative control or $STAF_{ZNF143/76}$ siRNA. (**H** and **I**) Densitometric analysis of $STAF_{ZNF143}$ and Skp2 protein expression.(**J** and **K**) Time course of increase in cell number in HeLa cells and VSMCs, as indicated, after transfection with $siSTAF_{ZNF143}$ or $siSTAF_{ZNF143/76}$ respectively. (**L** and **M**) BrdU incorporation in HeLa cells and VSMCs, as indicated, 48 h post transfection with $siSTAF_{ZNF143}$ or $siSTAF_{ZNF143/76}$ respectively. (**N**) Effect of ectopic wild-type Skp2 on BrdU incorporation in siNEG or $siSTAF_{ZNF143/76}$ transfected cells. $^{*}P < 0.05$.

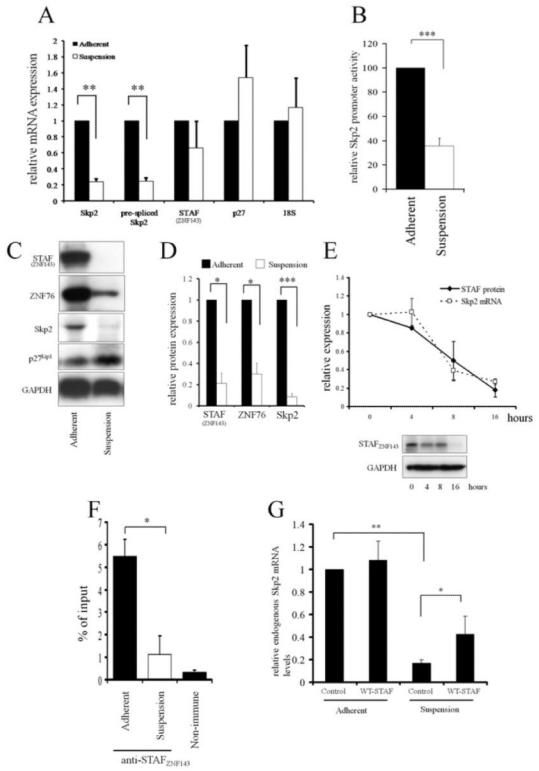


**Figure 6. Adhesion-dependent binding of STAF to the endogenous *Skp2* promoter**

(**A**) VSMCs were cultured in suspension or adhered to plastic for 16 h. Expression of Skp2 mRNA, Skp2 pre-spliced mRNA, $STAF_{ZNF143}$ mRNA, $p27^{Kip1}$ mRNA and 18S RNA was quantified by quantitative RT–PCR. (**B**) Cells were transiently transfected with indicated *Skp2* promoter luciferase reporter plasmid. At 24 h post-transfection, cells were trypsinized and re-plated into suspension or adherent culture for a further 16 h. (**C**) $STAF_{ZNF143}$, ZNF76, Skp2, $p27^{Kip1}$ and GAPH protein levels in adherent or suspension cultures were quantified by Western blotting and (**D**) densitometric analysis of $STAF_{ZNF143}$, ZNF76 and Skp2 protein in adherent or suspension cultures. (**E**) Time course of $STAF_{ZNF143}$ protein and Skp2 mRNA levels in adherent and suspension cells. Representative immunoblots showing $STAF_{ZNF143}$ protein and GAPDH protein are shown in the lower panel. (**F**) $STAF_{ZNF143}$ ChIP analysis of VSMCs cultured in suspension or adherence. Results are presented as means ± S.E.M. of % input chromatin of three independent experiments. (**G**) VSMCs were transiently transfected with either empty vector or wild-type $STAF_{ZNF143}$ expression vector as indicated. At 24 h post transfection, cells were trypsinized and either re-plated into adherent or suspension culture for a further 16 h. Endogenous Skp2 mRNA expression was quantified using quantitative RT–PCR. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ using ANOVA with Student-Newman-Keuls post test on log transformed data.