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TGF- β regulates expression of KLF6 and its splice variants, and promotes cooperative transactivation of common target genes through a Smad3/Sp1/KLF6 interaction

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Running Title: TGF- β effect on KLF6 and target genes

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Abstract

KLF6 is a transcription factor and tumor suppressor with a growing range of biological activities and transcriptional targets. Among these, *KLF6* suppresses growth through transactivation of transforming growth factor- β 1. *KLF6* can be alternatively spliced, generating lower molecular weight isoforms that antagonize the full-length, wild type protein and promote growth. A key target gene of full length *KLF6* is *endoglin*, which is induced in vascular injury. Endoglin, a homodimeric cell membrane glycoprotein and TGF- β auxiliary receptor, has a pro-angiogenic role in endothelial cells and is also involved in malignant progression. The aim of the present work was to explore the effect of TGF- β on *KLF6* expression and splicing, and to define the contribution of TGF- β on promoters regulated by cooperation between *KLF6* and Sp1. Using co-transfection, co-immunoprecipitation and FRET, our data demonstrate that *KLF6* cooperates with Sp1 in transcriptionally regulating *KLF6*-responsive genes, and that this cooperation is further enhanced by TGF- β 1 through at least two mechanisms. First, in specific cell types TGF- β 1 may decrease *KLF6* alternative splicing, resulting in a net increase in full-length, growth-suppressive *KLF6* activity. Second, *KLF6*/Sp1 cooperation is further enhanced by the TGF- β /Smad pathway via the likely formation of a tripartite complex of *KLF6*, Sp1, and Smad3 in which *KLF6* interacts indirectly with Smad3 through Sp1, which may serve as a bridging molecule to coordinate this interaction. These findings unveil a finely tuned network of interactions between *KLF6*, Sp1, and TGF- β to regulate target genes.

Keywords: transforming growth factor- β , endoglin, transactivation, Sp1, alternative splicing, growth regulation

Introduction

KLF6 is a ubiquitous transcription factor with a growing range of activities and transcriptional targets. It is a Krüppel-like factor initially cloned from liver, placenta and leukocytes [1-3]. Induction of *KLF6* in activated hepatic stellate cells, the key fibrogenic cell, after liver injury, suggested it plays a major role in tissue remodelling [1-2]. This conclusion has been further supported by evidence that changes in the expression of specific genes regulating endothelial and hepatic injury are transactivated by *KLF6* through GC box motifs in their promoters. These motifs are the cognate recognition sequences for *KLF6* and Sp1, another well studied member of the Krüppel-like family.

Regulation of target gene expression often occurs through the cooperativity of *KLF6* and Sp1 through a direct physical interaction [4]. Genes induced by this cooperativity include the cytokine *transforming growth factor- β* (*TGF- β*), its receptors, *type I and II*, *endoglin*, a *TGF- β* auxiliary receptor [5] and the extracellular matrix component *collagen type I*, [2,6] as well as the urokinase plasminogen activator (uPA), which promotes the activation of latent *TGF- β* [7]. Through this cooperation *KLF6* may switch the function of Sp1 from regulating the basal transcriptional machinery to participating in the regulation of inducible gene expression involved in tissue repair.

On the other hand, *KLF6* has also been characterized as tumor suppressor gene mediating growth suppression through induction of p21 [8] and sequestration of cyclin D1 [9]. In this context *KLF6* is inactivated by allelic loss and somatic mutation in prostate cancer [10-11] and other human cancers including colorectal cancer [12], glioblastoma [13], gastric and nasopharyngeal carcinoma [14]. In addition, *KLF6* functional inactivation in other cancers including esophageal, primary lung cancer, and prostate cell lines, occurs through decreased *KLF6* expression via promoter methylation [15-17].

One unique regulatory mechanism for *KLF6* function is its inactivation through the generation of alternatively spliced products, which functionally antagonize the full length *KLF6* function through an unknown mechanism [8]. Expression of these splice variants (Sv) is increased in some cancers, is associated with a poor prognosis [18-19], and may contribute to functional inactivation of *KLF6* even in tumors in which there is no loss of heterozygosity or inactivating mutation. However, the role of the *KLF6* Svs in regulating full length *KLF6* activity in tissue remodelling is unclear.

TGF- β is induced through a KLF6/Sp1 interaction during vascular injury and inflammation [4], whereas it increases endoglin transcription through a Smad3/Sp1 interaction [20]. Smads are signal transducing and transcription factors mediating TGF- β signals [21]. Once TGF- β 1 binds to the serine-threonine kinase receptor type II, it forms a tetra-complex with another serine-threonine kinase receptor type I and phosphorylates it, which in turn phosphorylates Smads 2 and/or 3, depending on the cell type [21]. Phosphorylated Smads 2 and 3 bind to Smad 4, which together translocate into the nucleus, where they bind to their cognate binding motifs on the promoters of target genes, and activate them by cooperating with the general transcription factor machinery [21]. Therefore, the cooperation of KLF6/Sp1 in responsive genes might be further enhanced by TGF- β 1 through Smad3/Sp1 interaction, but this has not yet been established. Moreover, it is uncertain whether TGF- β directly affects KLF6 expression or splicing. If so, this would represent a potentially appealing way to enhance growth suppression through increased KLF6 activity.

Here, we explored the effect of TGF- β on KLF6 biology at three different levels: 1) KLF6 expression; 2) KLF6 alternative splicing; 3) promoter transactivation regulated by cooperation between KLF6 and Sp1.

Materials and Methods

Materials

Recombinant human TGF- β 1 was purchased from R&D Systems (Minneapolis, MN)

Cells

Human umbilical vein endothelial cells (HUVECs) were grown in medium 199 containing 20% fetal calf serum (FCS) and 50 μ g/mL bovine brain extracts on 0.5% gelatin-coated dishes. COS-7 monkey kidney cells, HeLa human epithelial cells from a cervical carcinoma, HepG2 human hepatic cells, and HEK 293T human epithelial kidney cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS. U-937 and THP-1 human monocytic cell lines were grown in RPMI-1640 medium supplemented with 10% FCS. *Drosophila* Schneider SL-2 cells were grown in Shield and Sang *Drosophila*-enriched Schneider (DES) insect medium (Sigma-Aldrich, St Louis, MO) supplemented with 10% FCS.

Where indicated, cells were treated with TGF- β (10 ng/ml) (R&D systems) for progressive incubation times. Wound healing treatments were made by disruption of confluent monolayers of HUVECs with micropipette tips so that ~75% of the surface was denuded.

Flow cytometry

For semi-quantitative analysis of the amount of KLF6 expression, U-937, THP-1, HepG2 and HUVECs were fixed in 3.5% formaldehyde and were permeabilized with 100 μ g/mL lysophosphatidyl choline before incubation with the primary antibody to KLF6 (Santa Cruz Biotechnology, CA) for 1h at 4°C. Cells were then incubated with fluorescein isothiocyanate (FITC)-labelled rabbit anti-mouse IgG (DAKO, Glostrup, Denmark) for 30 min at 4°C and washed with cold PBS. Fluorescence intensity of each cell type was estimated with an EPICS-XL (Coulter, Hialeah, FL) using logarithmic amplifiers. The amount of KLF6 expression is expressed as *Expression Index*, obtained by multiplying the percentage of KLF6 positive cells by the mean fluorescence intensity

of the total population of cells. A minimum of 10,000 cells was counted for each experimental timepoint.

RNA analysis by real-time polymerase chain reaction (PCR)

Total RNA was isolated from HUVECs, THP-1 and HepG2 cells using the RNeasy kit (Qiagen, Hilden, Germany) and was reverse transcribed by avian myeloblastosis virus reverse transcriptase (RT). The resultant cDNA was used as a template for PCR performed with a combination of specific oligonucleotide primers for wild-type human *KLF6*, forward, 5'-CGGACGCACACAGGAGAAAA-3' and reverse, 5'-CGGTGTGCTTTCGGAAGTG-3'; total human *KLF6*, forward, 5'-CTGCCGTCTCTGGAGGAGT-3' and reverse, 5'-TCCACAGATCTTCCTGGCTGTC-3'; Human *GAPDH*, forward, 5'-CAATGACCCCTTCATTGACC-3' and reverse, 5'-GATCTCGCTCCTGGAAGATG-3'.

Collagen type 1 forward 5'CTGCTGTCCTAAGGGTGAG3' reverse
5'TAGCACCATCATTTCACGA3'

Endoglin, forward 5'GCCCCGAGAGGTGCTTCT3' reverse 5'
TGCAGGAAGACACTGCTGTTTAC3'

All experiments were performed in triplicate and the amounts of both wild-type and total *KLF6* were normalized to *GAPDH* expression. To calculate the fold change in *KLF6* alternative splicing, the fold change in total *KLF6* mRNA levels (wild-type *KLF6* + alternatively spliced *KLF6* transcripts) was divided by the fold change in wild-type *KLF6* alone.

For Sp1 knock-down expression in endothelial cells (HUVECs), confluent cells were infected with supernatants of 293T cells transfected with the lentiviral system pSIH1 H1-Puro shRNA of Sp1 (targeting site for Sp1 knockdown: TGTACCAGTGGCCCTGAA) or a random sequence (non-target siRNA) as negative control, following the protocol recommended for the manufacturer (Cloning and Expression Lentivectors, User Manual, SBI System Biosciences)

Endoglin and collagen promoter constructs and transactivation assay.

Luciferase reporters for the *endoglin* promoter, *pCD105 (-350/+350)-pXP2*, and the *collagen type 1(I)* promoter, *pGL-Col1*, have been described elsewhere [4]. Transient transfection was performed using the Superfect transfection reagent (Qiagen) in serum-free medium containing 1 µg each of either *pCD105 (-350/+350)-pXP2* or *pGL-Col1*,

with or without indicated amounts of expression vectors for Sp1, wild-type KLF6, KLF6 splice variants KLF6-Sv1 and Sv2, Smad3, and TGF- β 's type I receptor. Sv1 and Sv2 alternatively spliced products, arise from the use of native cryptic splice sites within exon 2 (8). KLF6SV1 contains a novel 21 amino acid carboxyl domain, resulting from out-of-frame splicing of its terminal exon. KLF6SV2 has lost a Zn finger binding domain and part of the C-terminal transactivation domain. While KLF6 WT contains 283 aa, with 3 zinc fingers as its binding domain, SV1 has 195 aa and no zinc fingers, and SV2 has 241 aa and 2 zinc fingers. The expression vectors for Sp1 and KLF6 were in plasmids pAC for transfections into *Drosophila* cells or in pCneo for expression in mammalian cells. All transfections contained the same amount of total DNA (2 μ g), with the balance composed of the corresponding empty expression vectors. The KLF6-GAL4 and GAL4-LUC reporter were used as previously described [2]. Luciferase activity was determined in each cell lysate using a TD20/20 luminometer (Promega, Madison WI, USA). Correction for transfection efficiency was made by cotransfection with pCMV- β -galactosidase (BD Biosciences, San Jose, CA, USA) and galactolight (Tropix, Bedford MA, USA) as a substrate. Transactivation assay results were expressed as arbitrary units of luciferase activity or as a fold induction with respect to the corresponding untreated control samples.

GST-KLF6 pull down and co-immunoprecipitation experiments

Forty hours after transfection, cells (COS-7, Schneider SL-2, HUVECs or HEK-293T) were lysed as described previously [4], and total extracts were incubated with the specified antibody against the tag "Flag", anti-Flag (Sigma Co., Aldrich, Madrid, Spain). Immunocomplexes were precipitated with protein-G Sepharose and were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Proteins were transferred to Hybond-C extra nitrocellulose membranes (Amersham Biosciences [GE Healthcare, Denmark) and probed with antibodies indicated. Signals were developed using the Super Signal reagent (Pierce, Rockford, IL) for enhanced chemiluminescence. Experiments were repeated at least 3 independent times with similar results, and representative results are shown in the corresponding figures. The expression vector pCMV5-T β RI (ALK-5) which encodes for the constitutively active form of this receptor and the glutathione S-transferase (GST) fusion protein GST-KLF6 has been described before [7].

For sequential immunoprecipitation experiments, 75% confluent 100 mm plates of HEK 293T cells were either double transfected with 3 micrograms each of KLF6 and Smad3 expression vectors (pcDNA3.KLF6 and pcDNA3.Smad3.Flag, respectively), or triple transfected with these two vectors and additionally, 3 micrograms of pcDNA3.Sp1. First immunoprecipitation was performed with anti-KLF6 (Santa Cruz Biotechnology, CA). After washing, the precipitates were subjected to the 2nd immunoprecipitation with anti-Flag (Sigma Co., Aldrich, Madrid, Spain). Total protein extracts and first and second immunoprecipitates were subjected to 10% PAGE.SDS electrophoresis, and Western blotting. Western blots were incubated with Sp1, KLF6, and Smad3 antibodies, respectively (Santa Cruz Biotechnology, CA).

Plasmid constructions for FRET

KLF6-wt-ECFP vector was constructed by recloning a *Bam*HI/*Eco*RI fragment from the KLF6 expression vector into the pECFP vector (Clontech Laboratories Inc., Mountain View, CA) as described [22]. Sp1 Cter (amino acid 556-763)-EYFP and Smad3-EYFP or -ECFP vectors were made by recloning both a *Not*I/*Xba*I fragment from pEYFP or pECFP vector (Clontech Laboratories Inc., Mountain View, CA) plus an *Eco*RI/*Eco*RI fragment from the Sp1 and Smad3 expression vectors, respectively, into the pcDNA3 vector (Invitrogen Co., Carlsbad, CA).

Analysis of fluorescent resonance energy transfer (FRET)

HEK 293T cells were transfected with a combination of KLF6-ECFP and each of Sp1 Cter-EYFP, Smad3-EYFP, and control EYFP, or a combination of Smad3-ECFP and either Sp1 Cter-EYFP or control EYFP, using LipofectAMINE Plus reagent (Invitrogen Co., Carlsbad, CA). Cells were incubated in DMEM containing 10 % calf serum for 48 h, and then treated with 2.5 ng/ml TGF- β in the same serum containing medium for 6 h, or scratched by pipette tip as described [22] and the incubation was continued in the same serum-containing medium for 6 h. Cells were fixed with 10 % formaldehyde, and FRET was determined by a Carl Zeiss confocal microscopy, model LSM 510 (Carl Zeiss Inc., Thornwood, NY) at a combination of excitation wavelength of 458 nm and emission wavelength of 523 nm. For the photobleach method, pre-photobleach CFP (donor) images were first acquired at a combination of excitation wavelength of 458 nm and emission wavelength of 480 nm. Cells were then exposed to 514 nm laser for 20 seconds with maxim power to eliminate yellow color for YFP

(acceptor photobleaching). Thereafter, cells were re-exposed to 458 nm laser again. The intensity of the emission signal at 480 nm, recovered by acceptor bleaching, was detected. Fluorescence intensities at 480 nm obtained before and after the bleaching were compared in each sample.

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Results

Cell type-specific regulation of KLF6 expression by TGF- β 1

KLF6 up-regulates several genes involved in the TGF- β signalling pathway, including *TGF- β 1*, its types I and II receptors (*T β RI and II*), and the urokinase plasminogen activating enzyme, *uPA* [4,7]. However, it is unknown whether there is a reciprocal induction of KLF6 by TGF- β 1. To address this question we measured the effect of TGF- β 1 on the expression of KLF6 in several cell lines using flow cytometry. As seen in Figure 1, top *panel*, KLF6 expression in both monocytic U-937 cells and promonocytic THP-1 cells was upregulated in a time-dependent manner up to 8-fold after 24 h ($p < 0.001$). A similar effect was also observed in primary HUVEC cultures, although the increase was only sustained from 0 to 12 h of TGF- β 1 treatment accompanying an increment of only 50% at 12 h ($p < 0.01$), followed by a drop between 12 and 24 h to the basal level (Figure 1 middle *panel*). In contrast, the expression of KLF6 in HepG2 and HeLa cells was down-regulated after TGF- β 1 treatment and decreased to 30% and 80% of basal levels, respectively, after 24 h ($p < 0.001$) (Figure 1, bottom *panel*), suggesting that KLF6 responsiveness to TGF- β 1 was cell type-specific.

KLF6 alternative splice products functionally antagonize full length KLF6 in regulating wound repair genes

Because it was unclear if alternative splice variants (Svs) antagonize KLF6's transactivation of genes involved in tissue repair as they do in cancer, we explored the impact of KLF6 Sv1 and Sv2 on two target genes, *endoglin* and *collagen type 1*, whose promoters are transactivated by full length KLF6, (Figure 2A and 2B, respectively). While over-expression of wild-type (wt = full-length) KLF6 transactivated both *endoglin* and *collagen* promoters (*closed column 2*), over-expression of Sv1 and Sv2 downregulated them even below basal levels (*columns 3 and 4*, respectively). Compared to Sv1, Sv2 had a more prominent suppressive effect. Treatment with TGF- β (10 ng/ml) increased the transactivation of *endoglin* in the presence of KLF6 wt, but not when variants Sv1 and Sv2 of KLF6 were transfected (*columns 1 to 4*, hatched histograms), indicating a dominant negative effect of these variants. Furthermore, the combination of wt KLF6 with Sv1 and Sv2 attenuated the effect of exogenously over-expressed wt KLF6 (*columns 5 and 6*, respectively) and suppressed transactivation

activity down to the levels obtained with KLF6 siRNA (*open column 2*), suggesting KLF6 Svs could functionally antagonize full length KLF6 in tissue repair.

We next examined whether TGF- β 1 might affect KLF6 alternative splicing (Figure 2C). Using real time PCR with a primer set capable of specifically detecting only wt KLF6 and a primer set that detects both wt and all Svs, we quantified full length and total (full length + alternative splice forms) KLF6 mRNAs, and calculated the amount of Sv mRNA. To calculate the fold change in KLF6 alternative splicing, the fold change in the mRNA levels of total KLF6 (wild-type KLF6 + alternatively spliced KLF6 transcripts) was divided by the fold change in wild-type KLF6 alone. This method for determining KLF6 splice variant expression has been validated extensively [8, 31]. TGF- β 1 transiently increased KLF6 alternative splicing in THP-1 cells after 4 and 6 h-treatment, followed by more sustained and highly significant decreases after 8 h and longer treatment (*panel C*). In HUVECs, after a transient increase at 1h, there was a decrease in KLF6 splicing starting after 2 h-treatment (*panel D*). In contrast, in HepG2 TGF- β 1 had no significant or sustained effect on alternative splicing (*panel E*).

TGF- β 1 enhances Sp1/KLF6 cooperation through Smads

We previously reported that KLF6 and Sp1 may functionally and physically interact to regulate target gene expression [4]. Based upon these previous findings, we next explored if TGF- β 1 might regulate KLF6 target gene expression by modulating KLF6/Sp1 interactions through Smads.

To address this question, we utilized reporter constructs containing the proximal upstream regions of the *endoglin* and *collagen type I* promoters, both of which are TGF- β responsive through Smad3/Sp1 interactions [4,23]. The results of these experiments are shown in Figure 3. In HeLa cells, which express high levels of endogenous Sp1, transfection of either KLF6 (*sample 2*) or Smad3 (*sample 3*) alone led to 5- and 2.5-fold stimulation in endoglin (*panel A*) and collagen type I (*panel B*) promoters, respectively, whereas co-transfection of both KLF6 and Smad3 (*sample 4*) led to synergistic increases up to 18- and 10-fold for *endoglin* (*panel A*) and *collagen type I* (*panel 3B*) transactivation, respectively, suggesting that TGF β 1/Smad3 pathway further enhances Sp1/KLF6 transcriptional cooperation in mammalian cells. The over-expression of Smad3 in these experiments by co-transfection the effect of exogenous TGF- β

treatment, since Smad 3 is the effector of the TGF- β pathway. However, the strongest transactivation was obtained when using both TGF- β (10ng/ml) and transfecting Smad3 (*panel A, samples from 1-4*).

Because these findings did not directly establish a functional requirement for Sp1 in mediating KLF6/Smad3 synergy and involvement of Sp1 as an adaptor, we examined whether KLF6 and Smad3 could still functionally cooperate in the transcriptional regulation of *endoglin* when Sp1 expression was knocked down by transient transfection with siRNA (*panel C*). In 293T cells, which express very low levels of KLF6, simultaneous over-expression of Smad3 and KLF6 by transient co-transfection transactivated the *endoglin* promoter 5-fold (closed *column* in *sample 4*). However this apparent cooperation of KLF6 and Smad3 (compare closed *columns* in *samples 2 and 3* with *sample 4*), was abrogated by transfecting siRNA to Sp1, which decreased the transcriptional activity to the levels resulting from KLF6 transfection alone (2-fold) (open *column* in *sample 4*). Control siRNA to random sequences had no effect (slashed *column* in *sample 4*).

The preceding result was further supported by transfection of *endoglin* or *collagen type I* promoters in *Drosophila Schneider* cells (Figure 3D and 3E), which lack endogenous Sp1 and KLF6 [2]. In these cells lacking Sp1, transfected KLF6 and Smad3 showed no functional cooperation, but rather a slight competition (compare *samples 2 and 3* with *sample 4*-combination), whereas Sp1 and Smad3 showed an additive effect (*sample 5*). Similarly, Sp1 and KLF6 showed a cooperative effect (*sample 6*) in the transactivation of *endoglin* (Figure 3D) and *collagen I* (Figure 3E) promoters, as reported previously [4,20,23]. Smad3 additionally enhanced KLF6/Sp1 synergy from 28- and 22- fold, to 32- and 26- fold, respectively (*sample 8*) in transactivating the *endoglin* and *collagen I* promoters. These results suggested that KLF6 and Smad3 cooperate functionally in an indirect manner via their common partner Sp1 as an adaptor. These findings also suggested a novel mechanism in KLF6 target gene regulation involving the formation of KLF6-Sp1-Smad complexes on target promoters.

Next, the endogenous functional outcome of this cooperation between KLF6-Sp1-TGF- β /Smad, was assessed by treating HUVECs with TGF- β (10ng/ml), and by *in vitro* disruption of HUVEC monolayer (wound healing (WH)) at different times to induce KLF6. As shown in Figure 3 (*part II, panel A*), WH treatment significantly induced the expression of KLF6 mRNA between 2-5 fold, based on quantitative real time PCR.

Having validated that WH induced endogenous KLF6, levels of endoglin and collagen mRNA were measured either in untreated cells, after TGF- β treatment, or after KLF6 induction (by WH), or a combination of both stimuli. As seen in Figure 3 (*part II, panels B and C*), there was a synergistic effect of KLF6 and TGF- β , which significantly increased *endoglin* mRNA from 3 to 25 hours, with a peak of 22.5-fold induction. In the case of *type I collagen* (panel C), the cooperation between TGF- β and KLF6 (WH) was significant after 3 hours of combined treatment, while the effect of WH was predominant after 12 and 25 hours of combined treatment.

Together, these data indicate a synergistic effect between KLF6 and TGF- β in endothelial cells, measured by an increase in *endoglin* and *collagen* type I mRNAs.

KLF6 and Smad3 form a multimeric complex with Sp1 as a bridging molecule

The potential complexing of KLF6/Sp1/Smad3 was examined by GST pull-down experiments using total extracts from COS-7 cells with or without stimulation of the TGF- β 1/Smad3 signaling pathway (Figure 4A). When GST-KLF6 was precipitated with glutathione-Sepharose beads, both endogenous Sp1 and transfected Smad3 were pulled-down together (Fig 4A, *lanes 1 and 2*). The over-expression of Smads (Smad3/4 here) is sufficient to trigger TGF- β signalling (Smad3 phosphorylation) even in the absence of ligand stimulation (TGF- β) [24]; we additionally examined whether there was further stimulation following co-transfection of constitutively active T β RI receptor [24]. The activation of Smad3/4 by co-transfection of constitutively active TGF- β receptor I (T β RI) yielded only a small increase in the amount of Sp1 recovered (*lane 1*). When the transfection lacked Smad3, only Sp1 could be recruited by KLF6 (*lane 3*). The control pull down with GST-Sepharose beads alone did not give any bands (data not shown).

The co-immunoprecipitation of Sp1, Smad3/4 and KLF6 was further demonstrated in an endogenous system using HUVECs grown under control conditions (C) or 24 h after either wound healing and TGF- β treatment (T) as described previously. In both conditions, Sp1 immunoprecipitates also pulled down KLF6 and Smad 3/4 (Fig 4 B). When human IgG was used as a control antibody, no band of immunoprecipitation was detected. Further, the amount of KLF6 recovered was higher after treatment by either TGF- β or wound healing (*lane T*, Fig 4).

To reinforce this conclusion, *Drosophila Schneider* cells, which lack endogenous KLF6 and Sp1, were co-transfected with a combination of expression vectors for either

Sp1 or KLF6 and/or Flag-Smad3. Forty-eight hours after transfection, total extracts from these cells were subjected to immunoprecipitation using an anti-Flag antibody to identify Flag-Smad3 (Figure 4C, *part II*). Using this approach, only Sp1 could be co-immunoprecipitated with Smad3 while KLF6 was not recovered in the immunoprecipitates with Smad3 when Sp1 was not present. These data suggest that Sp1 is necessary for the formation of the complex, since there is no direct physical interaction KLF6/Smad3.

Altogether, the formation of two-component endogenous complexes (Sp1/KLF6 and Sp1/Smad3) has been demonstrated (Fig 4B). Figure 4C shows in addition that Sp1 is needed for Smad3/KLF6 complex formation in S2 cells. The functional data support cooperation between KLF6, Smad3 and Sp1. However, at this point there was still an over-statement to argue that a ternary complex is really formed. To claim such a complex, we performed sequential double co-IP/precipitation in HEK 293T cells, either double or triple transfected with expression vectors for KLF6 and Smad 3, or KLF6, Smad3 and Sp1. The performance of a triple transfection experiment in S2 and double sequential co-IP was not viable due to the lower transfection efficiency in these cells. The results of the experiment in HEK293T cells are shown in Fig 4D. First, we immunoprecipitated using an antibody for the 1st component, KLF6. Second, we eluted and made a subsequent IP using an antibody for the second Smad 3, using the Flag tag, and in this way we have been able to detect Sp1, the 3rd component, of the ternary complex. There is a substantial enrichment of the ternary complex after the second immunoprecipitation, being relevant the enrichment detected in Smad3, which is dependent on the transfection of Sp1 (Fig 4D: middle *panel*, right side). The negative control used double and triple transfections using IgG as irrelevant antibody, no immunoprecipitates were detected (data not shown). The detected increase in co-immunoprecipitated levels of Smad 3 when overexpressing Sp1 (triple transfection) argues in favour of a ternary complex.

We further characterized interactions between KLF6, Sp1 and Smad3 using a Gal4 one-hybrid system by expressing the combination of KLF6-Gal4DBD- and a Gal 4-responsive luciferase reporter (Supplementary Fig 1). These results indicated that KLF6 functionally interacts only with Sp1, but not with Smad3 (compare *samples 5 with 6*) and further indicated that in the KLF6/Sp1/Smad3 transcriptional complex, Sp1 is the

key partner, interacting both with KLF6 and with Smad3 simultaneously. This association is in agreement with the transcriptional synergy depicted in Figure 3.

In vivo interaction between KLF6, Sp1, and Smad3 assessed by FRET

To further confirm our findings of KLF6, Sp1 and Smad3 interaction, we used FRET to examine whether these interactions occurred in living cells. Cells expressing a combination of KLF6-ECFP/Sp1Cter-EYFP, Smad3-EYFP and control EYFP, or Smad3-ECFP and either Sp1Cter-EYFP or control EYFP were analyzed for FRET by a confocal microscopy. Supplementary Fig 2 shows FRET and corresponding bright field images. As shown in Supplementary Fig 2, weak FRET signals were observed in the cells co-transfected with combinations including KLF6-ECFP and Sp1Cter-EYFP (*panel a*), as well as Smad3-ECFP and Sp1Cter-EYFP (*panel c*), and almost no FRET signals for a combination of KLF6-ECFP and Smad3-EYFP (*panel e*). Strong signals were not detected in cells co-transfected with combinations of KLF6-ECFP and EYFP (*panel g*) or Smad3-ECFP and EYFP (*panel i*), indicating a functional interaction of KLF6 with Sp1 as well as Sp1 with Smad3 in these cells. This interaction was further amplified by employing the photobleach method (Supplementary figure 2B). This method is based on the principle that energy transfer is eliminated when the acceptor is bleached, thereby yielding an increase in donor fluorescence. Supplementary Fig 2B shows the fluorescence intensity of emission at 480 nm before and after bleaching. Recovery of emission at 480 nm was seen in the cells co-transfected with combinations of KLF6-ECFP and Sp1Cter-EYFP (*panel b*) and Smad3-ECFP and Sp1Cter-EYFP (*panel d*), but not in the cells co-transfected with combinations of KLF6-ECFP and Smad3-YFP (*panel f*), KLF6-ECFP and EYFP (*panel h*) or Smad3-ECFP and EYFP (*panel j*). The average numbers in the recovery rate in each sample are presented under each of corresponding photographs. In conclusion, FRET confirmed a direct interaction KLF6/Sp1-C-ter with Smad3/Sp1-Cter in living cells, but not between KLF6 and Smad3.

Sp1 interacts with the transactivation domain of KLF6

We previously demonstrated that Sp1 interacts with KLF6 through its C-terminal DNA binding domain [4]. However, the domain of KLF6 interacting with Sp1 has not been identified. We addressed this question by expressing a Flag-tagged KLF6 DNA binding domain (DBD KLF6) or activation domain (ZAD KLF6) [19] following transient transfection of COS-7 cells, and then immunoprecipitation with anti-Flag antibodies from

cell extracts. Co-immunoprecipitates were Western-blotted with an Sp1-specific antibody. As seen in Figure 5A, Sp1 was co-immunoprecipitated only when cells were transfected with the KLF6 transactivation domain (*lane 3* in the IP Flag). Combined with our previous finding [4], this result suggests that the Sp1/KLF6 interaction requires the DNA binding domain of Sp1 and the transactivation domain of KLF6. The specificity of the Flag immunoprecipitation was documented as shown in Figure 5B, where expression of both, KLF6 DBD and ZAD domains can be observed in the total extracts (*lanes 1* and *3*), but not when the Flag cDNA construct was omitted from the transfection (*lane 2*).

We also examined whether KLF6 Sv1 and Sv2 interacted with Sp1 by separately transfecting each Flag-tagged KLF6 Sv, using the Flag-tagged wild type KLF6 as positive control and the empty expression vector as negative control. Flag-immunoprecipitates were then subjected to Sp1 Western blot. As shown in Figure 5C, both Sv1 and Sv2 interacted with Sp1, similar to wt KLF6, although Sv2 appeared to do so more efficiently. These data are consistent with binding studies using wt KLF6, since both Sv1 and Sv2 retain all or part of the transactivation domain. Moreover, it localizes the binding site between KLF6 and Sp1 to amino acids 1 to 201, since this is the region shared by both the KLF6 splice variants and wild-type KLF6.

Discussion

Combined, these findings provide insight into the regulation of *endoglin*, a key target gene of KLF6 following vascular injury, and define a precise molecular arrangement that involves Sp1 and Smad3. The results also contribute to our understanding of tumorigenesis since there is an emerging role of *endoglin* in neoplasia. *Endoglin* (CD105) is a homodimeric cell membrane glycoprotein functioning as a TGF- β auxiliary receptor [5]. It plays an important role in vascular development, remodeling, and neo-angiogenesis. Endoglin is highly expressed in endothelial cells of the tumor vasculature and at much lower levels in tumor cells [25-26]. There is also evidence supporting its involvement in malignant progression by its direct effect on the tumor cells themselves. Thus, loss of endoglin expression in cultured human prostate cancer cells enhances cell migration and invasiveness [27], and endoglin-heterozygous (*eng*^{+/-}) mice

exhibit accelerated malignant progression during chemical skin carcinogenesis *in vivo* [28]. The latter results suggested that endoglin could act as a suppressor of malignancy in carcinogenesis.

The current and previous data [4] suggest that KLF6 cooperates with Sp1 in transcriptionally regulating KLF6-responsive genes, and that this cooperation is further enhanced by TGF- β 1 by at least two mechanisms (Figure 6A). First, in specific cell types TGF- β 1 may decrease KLF6 alternative splicing such that there is a net increase in full length, growth-suppressive KLF6 activity. TGF- β 1 may indirectly increase the relative expression of KLF6-regulated genes by favoring the expression of full-length relative to alternative splice forms of KLF6, the net effect of which might be to inhibit growth. Second, KLF6/Sp1 cooperation is further enhanced by the TGF- β /Smad pathway via the likely formation of a tripartite complex of KLF6, Sp1, and Smad3 in which KLF6 interacts indirectly with Smad3 through Sp1, as uncovered using sequential immunoprecipitation, albeit following triple transfection rather than assessing endogenous protein interactions. Sp1 appears to serve as a bridging molecule in this complex. The physical interaction between Sp1 and KLF6 requires the N-terminal transactivating domain of KLF6 and the C-terminal DNA binding domain of Sp1 [4]. On the other hand, Sp1 and Smad3 reportedly make physical contact between the two glutamine-rich transactivating domains of Sp1 and MH1 binding domain of Smad3 [29]

Based on accumulated data, a model of basal and injury-associated (or inflammatory stimulus mediated) *endoglin* gene regulation is proposed, which defines the sequence of events involving KLF6, Sp1 and Smad3 (Figure 6B). Figure 6B depicts the basal transcription of *endoglin* [20]. It must be pointed out that in the absence of Sp1 expression, when knocking down Sp1 by infection of endothelial cells with a siSp1 lentiviral vector, *endoglin* transcription is abolished (see supplementary Fig. 3).

Upon TGF- β 1 stimulation associated with injury, this cytokine signals through its cognate receptors to Smad3/4 (Figure 6C), which then translocates to the nucleus where they physically interact with Sp1. Smad3/4 also interact with the GTF (general transcription factor machinery), which is further reinforced through interaction with Sp1, thereby synergizing transcription [20]. Endothelial injury or inflammatory events stimulate *de novo* synthesis of KLF6 and its translocation to the nucleus (as an early event). In the nucleus, KLF6 interacts with Sp1, enhancing *endoglin* transcription (Figure 6D) and other KLF6-responsive promoters involved in the TGF- β 1 system [4],

thereby increasing expression and activation of TGF- β 1. This eventually may culminate in the formation of Smad3/Sp1/KLF6 multimeric complex (Figure 6E) (late event). As TGF- β 1 increases, alternative splicing of KLF6 is inhibited (Figure 6A), leading to more net unopposed KLF6-mediated growth suppression through induction of p21 [8] and sequestration of cyclin D1 [9]. Binding of Sv1 and Sv2 to Sp1 suggests a potential mechanism whereby these splice forms may antagonize endoglin gene expression through cytoplasmic sequestration of this bridging factor, which is critical to maximal *endoglin* gene transactivation.

The present model is not only relevant to injury or inflammatory events, but also in carcinogenesis. While *endoglin* is a co-receptor of the TGF- β signaling system, and its role in carcinogenesis is the modulation of the TGF- β signal, KLF6 regulates the promoters of *endoglin* and of TGF- β receptors including TGF- β itself, and is a tumor suppressor by activating cell cycle regulators including p21, arresting cell division in a p53-independent manner. The modulation of KLF6 splicing by TGF- β might suggest an additional pathway whereby TGF- β is tumor-suppressive through down-regulation of growth-promoting KLF6 splice forms. On the other hand TGF- β 's synergistic action on the KLF6/Sp1 cooperation through Smads is also relevant to tumorigenesis, since *endoglin* and *KLF6* are tumor suppressor genes, and TGF- β is a known modulator of the first stages of carcinogenesis [30]. In aggregate, the findings further refine our understanding of the intersecting pathways underlying growth regulation and injury responses by TGF- β , KLF6 and Sp1.

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Figure Legends

Figure 1. Expression of KLF6 after TGF- β treatment in different cells

After different types of cells had been treated with 10 ng/ml TGF- β 1 for various times up to 24 h, cells were fixed and the KLF6 protein content of each cell type was assayed by flow cytometry and are expressed as “**Expression Index**” as described in Materials and Methods. Representative results from 3 different experiments with similar results are shown. * Statistical significance, at least $p < 0.05$.

Figure 2. Changes in KLF6 alternative splicing after TGF- β 1 treatment in different cells

A and B, HEK 293T cells were cotransfected with *pCD105 (-50/+350)-pXP2 (pEng, A)* or *pGL-Col1 (pCol1, B)*, and 1 μ g of either KLF6wt (*closed column 2*), KLF6 Sv1 (*closed column 3*), KLF6 Sv2 (*closed column 4*), or a mix of 0.5 μ g wt and 0.5 μ g of Sv1 (*closed column 5*)/Sv2 (*closed column 6*). In the first two histograms in *panel A*, endogenous KLF6 was knocked down with KLF6 siRNA (*open columns*). In addition, the effect of TGF- β treatment (10 ng/ml) was assessed in histograms 1 to 4 (*hatched columns*), in *panel A*. The results are given in arbitrary units of luciferase activity. Representative results obtained from 3 different experiments with replicable results are shown. * Statistical significance, at least $p < 0.05$, between control bars (*pEng, pCol*) and KLF6 cotransfected bars. In case of the *open column (siRNA)*, the asterisk (*) means that the value was statistically significant ($p < 0.05$) compared to the corresponding *closed column 2*.

C~E, After THP-1 (C), HUVEC (D), and HepG2 (E) cells had been treated with 10 ng/ml TGF- β 1 for various times indicated, cell lysates were prepared. Total RNA was extracted from each cell lysate, and levels of wt KLF6 and its Svs in each sample were measured using real time PCR. To calculate the fold change in mRNA levels of KLF6 alternative splicing, the fold change in mRNA levels of total KLF6 (wild-type KLF6 + alternatively spliced KLF6 transcripts) was divided by the fold change in wild-type KLF6 alone. (*) Statistical significance, at least $p < 0.05$.

Figure 3. Effect of Sp1, KLF6 and Smad3 on transactivation of *ENG* and *COL* promoters in mammalian and insect cells

A and B, the transactivation effect of different expression vectors for KLF6, Smad3, and a combination on both on the *ENG* (A) and *COL* (B) promoter constructs, respectively, in HeLa cells. In each transfection 1 µg of reporters, 0.5 µg of KLF6 and 250 ng of Smad3 expression vectors were used. 24 hours after transfection, cell lysates were prepared and luciferase activity in each lysate was determined as described in Materials and Methods. The results are expressed as fold induction comparing with values obtained with vacant vector (*sample 1*). *Sample 1*, Reporter (*ENG* or *COL-1*); *sample 2*, Reporter + KLF-6; *sample 3*, Reporter + Smad3; *sample 4*, Reporter + KLF6+ Smad3. Black histograms represent the untreated samples, while hatched histograms represent samples that were in addition treated with TGF-β (10ng/ml).

C. The same experiment was carried out in HEK 293 T cells. The transactivation effect of different expression vectors for KLF6, Smad3, and a combination on *ENG* promoter construct. In each transfection 1 µg of reporters, 0.5 µg of KLF6 and 250 ng of Smad3 expression vectors were used. 24 hours after transfection, cell lysates were prepared and luciferase activity in each lysate was determined as described in Materials and Methods. The results are expressed as fold induction comparing with values obtained with vacant vector (*sample 1*). *Sample 1*, Reporter (*ENG*); *sample 2*, Reporter + KLF6; *sample 3*, Reporter + Smad3; *sample 4*, Reporter + KLF6+ Smad3.

Moreover, the cooperative effect among KLF6 and Smad3 was also assessed silencing Sp1 by transfection of 0.5 µg siRNA (siSp1, empty box) and using as control a random si RNA sequence (si Random, dashed box).

D and E, the same type of experiment as in A-C was performed, but instead using Schneider *Drosophila* cells which do not express Sp1, nor KLF6. Reporter (*ENG* or *COL-1*). The transactivation effect of different expression vectors for Sp1, KLF6, Smad3, and a combination of them, on both on the *ENG* (D) and *COL* (E) promoter constructs, respectively, was assayed. In each transfection 1 µg of reporters, 0.5 µg of KLF6 and Sp1, and 250 ng of Smad3 expression vectors were used. 48 hours after transfection, cell lysates were prepared and luciferase activity in each lysate was determined as described in Materials and Methods. The results are expressed as fold induction comparing with values obtained with vacant vector (*sample 1*).

1. Reporter alone (*ENG* or *COL-1*)
2. Reporter + KLF-6
3. Reporter + Smad3
4. Reporter + KLF6+ Smad3
5. Reporter + Sp1
6. Reporter + Sp1 + KLF6
7. Reporter + Sp1 + Smad3
8. Reporter + Sp1 + KLF6 + Smad3

The experiments were repeated 5 times, and each experiment had 2 or 3 replica points. The experiments shown are representative of the results obtained.

Part II

A. RNA from KLF6 was quantitated after 0, 3, 12 and 25 hours of wound healing (WH) in HUVECs by real time PCR. The increase in RNA is expressed as fold induction.

B and C. RNA from endoglin and type I collagen was quantitated after 0, 3, 12 and 25 hours in HUVECs either, untreated or subjected to TGF- β treatment (10 ng/ml), wound healing (WH), or the combination of both treatments by real time PCR. The increase in RNA is expressed as fold induction.

Asterisks show that the differences are significant at least with a $p < 0.05$.

Experiments were repeated at least twice, using triplicates, and referred to GAPDH RNA as endogenous control.

Figure 4. Interactions of Sp1, KLF6 and Smad3 based on co-immunoprecipitation

A. Cos-7 cells were transfected with expression vectors for Smad3, 4 in the presence or absence of constitutively active TGF- β receptor I, T β RI ca (Cos cells have very low amounts of this receptor and of Smad proteins). GST-KLF6 fusion pulldown was generated from the protein extracts of transfected cells. As a control for specificity of the method, when Smad3 was not transfected (*lane 3*), only Sp1 was recovered by GST-KLF6 pulldown.

B. Co-immunoprecipitation experiments (Co-IP) in HUVECs, either untreated (C) or after treatment with TGF- β (10ng/ml) and recovery from wound healing for 24 h (T). IgG is a control lane following immunoprecipitation using human Ig G.

C. Co-immunoprecipitation experiments (Co-IP) were carried out in Schneider cells, which do not express Sp1, or KLF6. Schneider cells were cotransfected with pPACSp1

and Flag Smad3 expression vectors on the left side of *panel B*, or with pPACKLKF6 and Flag Smad3 on the right side. When the immunoprecipitation was made using the Flag tag to recover Smad3, Sp1 could be revealed by Western Blot (WB) in the immunoprecipitate (*lane 1* left side). However, when transfecting with pPACKLKF6 and Smad3, in the Flag Smad3 immunoprecipitate we could not detect KLF6. In the upper part of *panel B*, WBs show the expression of the transfected proteins in all the cases transfected proteins. *Lane 3 C* (293T) is a control of the migration for KLF6.

The co-IP experiments were repeated three times. Figure 4 illustrates a representative result.

D. Sequential co-immunoprecipitation experiments (Co-IP) in HEK 293T. HEK 293T cells were double or triple transfected with KLF6 and Smad3, or KLF6, Smad 3 and Sp1 expression vectors, respectively. *Lanes 1* show results obtained with double transfection of KLF6 and Smad 3, while *Lanes 2*, displays the triple transfection results. On the left side Western blots of total extracts are shown. On the right side of Figure 4D, the results for the 1st IP using KLF6 antibody, and 2nd IP with the anti-Flag tag are shown. There is a substantial enrichment of Smad3 in the ternary complex after the second immunoprecipitation, which is dependent on the transfection of Sp1.

Figure 5. Physical interaction between Sp1 and the different domains of KLF6

COS-7 cells were transiently transfected with expression vectors for the DNA of KLF6 Binding Domain (DBD), pcDNA3.DBDKLF6-Flag, or for the KLF6 transactivation domain (ZAD), pcDNA3.DBDKLF6-Flag, both tagged with the Flag domain.

A. In the left side of *panel A*, a WB shows that Sp1 is present in all the cell extracts. As shown in part **B** left side, the corresponding proteins to the transfected vectors could be detected in total extracts. When these extracts were subjected to immunoprecipitation using anti-Flag as antibody, we could detect Sp1 by WB, only in the immunoprecipitate of cells transfected with the expression vector of KLF6 transactivation domain (ZAD)

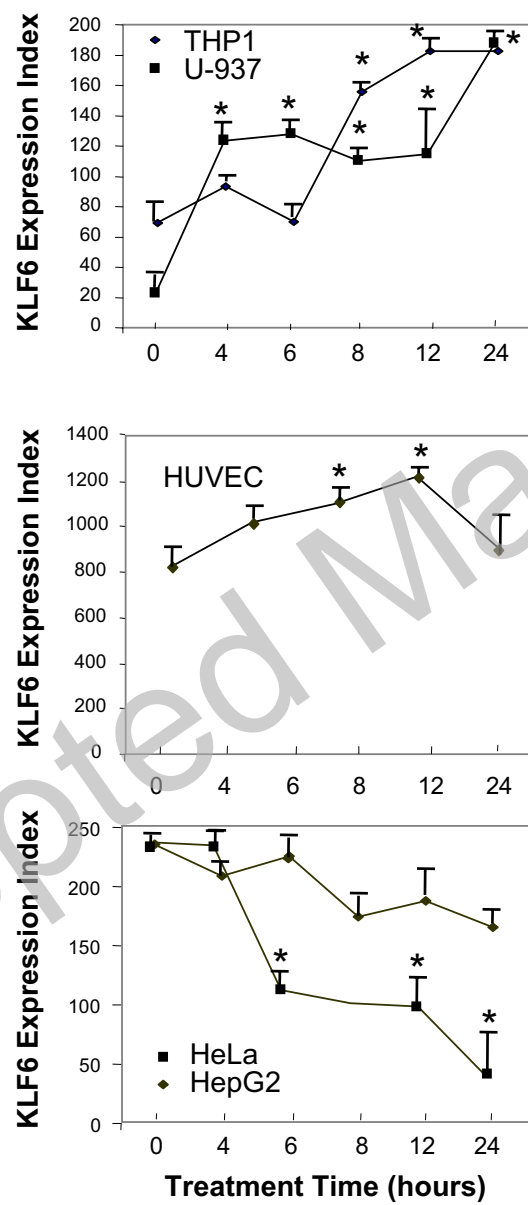
In **C**, COS-7 cells were transfected with the different pcDNA3 expression vectors of KLF6 including full length (wt), Splicing variants 1 and 2 (Sv1 and Sv2), and the DBD and ZAD KLF6 domains. As all the expression vectors were Flag-tagged, the immunoprecipitates after Flag incubation were separated and subjected to WB developed by Sp1 antibody. The experiments were repeated 3 times.

Figure 6. Model of basal and injury-associated Endoglin gene regulation.

This model defines the sequence of events involving KLF6, Sp1 and Smad3

A. As TGF- β increases, alternative splicing of KLF6 is inhibited leading to more net unopposed KLF6-mediated growth suppression. Binding of Sv1 and Sv2 to Sp1 suggests a potential mechanism whereby these splice forms may antagonize *Endoglin* gene expression through cytoplasmic sequestration of this bridging factor, which is critical to maximal endoglin gene transactivation.

B. Basal transcription of *Endoglin*. Upon TGF- β 1 stimulation associated with injury and/or inflammation, this cytokine signals through its cognate receptors to Smad3/4 (**C**), which translocate to the nucleus where they physically interact with Sp1. Smad3/4 also interact with the GTF (general transcription factor machinery) thereby synergizing transcription. Endothelial injury or inflammatory events stimulates de novo synthesis of KLF6 and its translocation to the nucleus (as an early event). In the nucleus, KLF6 interacts with Sp1, the binding domain (BD) or C-terminal part of Sp1 with the transactivating domain (AD) of KLF6 enhancing endoglin transcription (**D**) and other KLF6-responsive promoters involved in the TGF- β system thereby increasing expression and activation of TGF- β . This eventually may culminate in the formation of Smad3/Sp1/KLF6 multimeric complex (**E**) (as a late event), where Sp1 would be the bridge between the KLF6 AD and the MH1 domain of Smad3.

**Figure 1**

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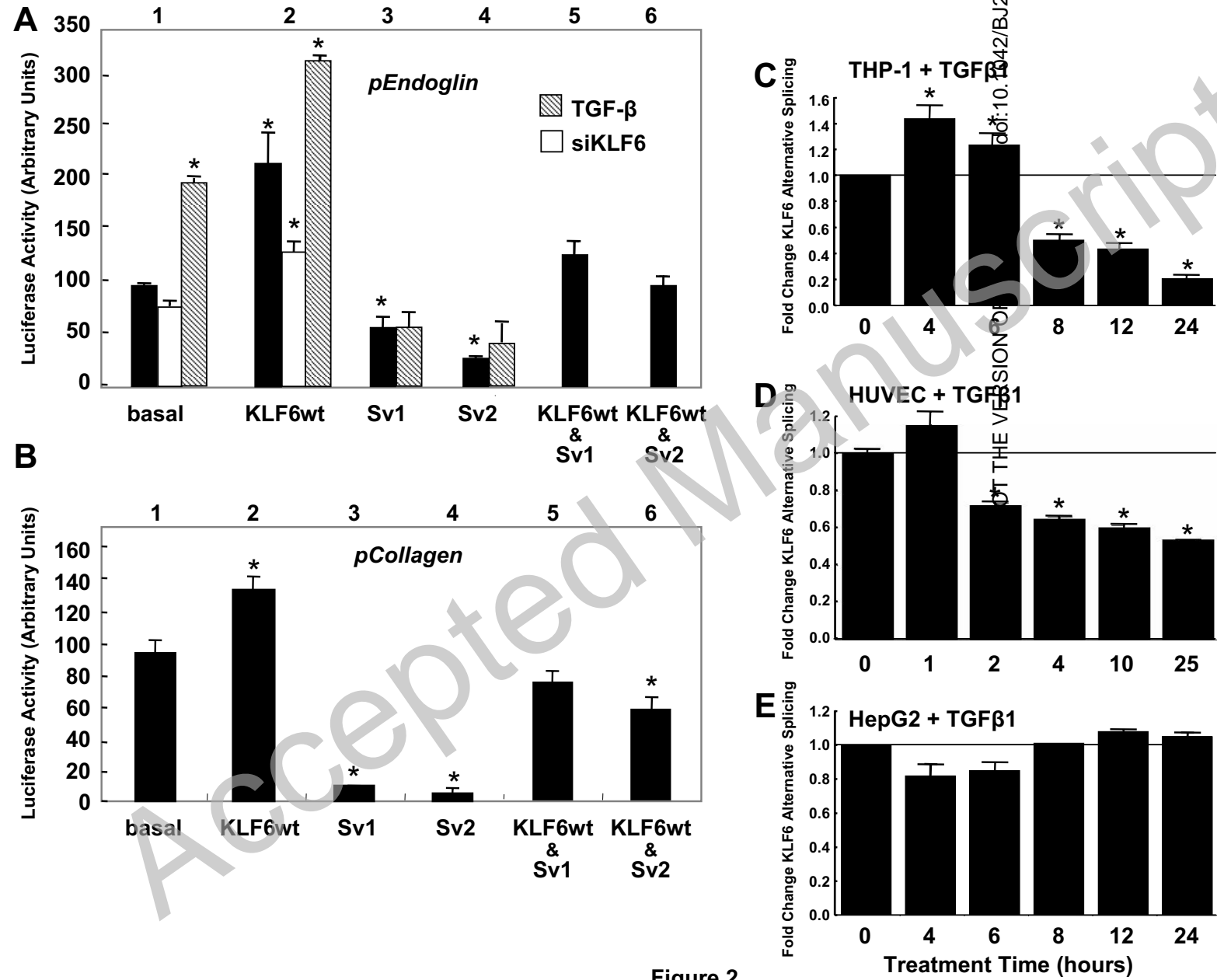


Figure 2

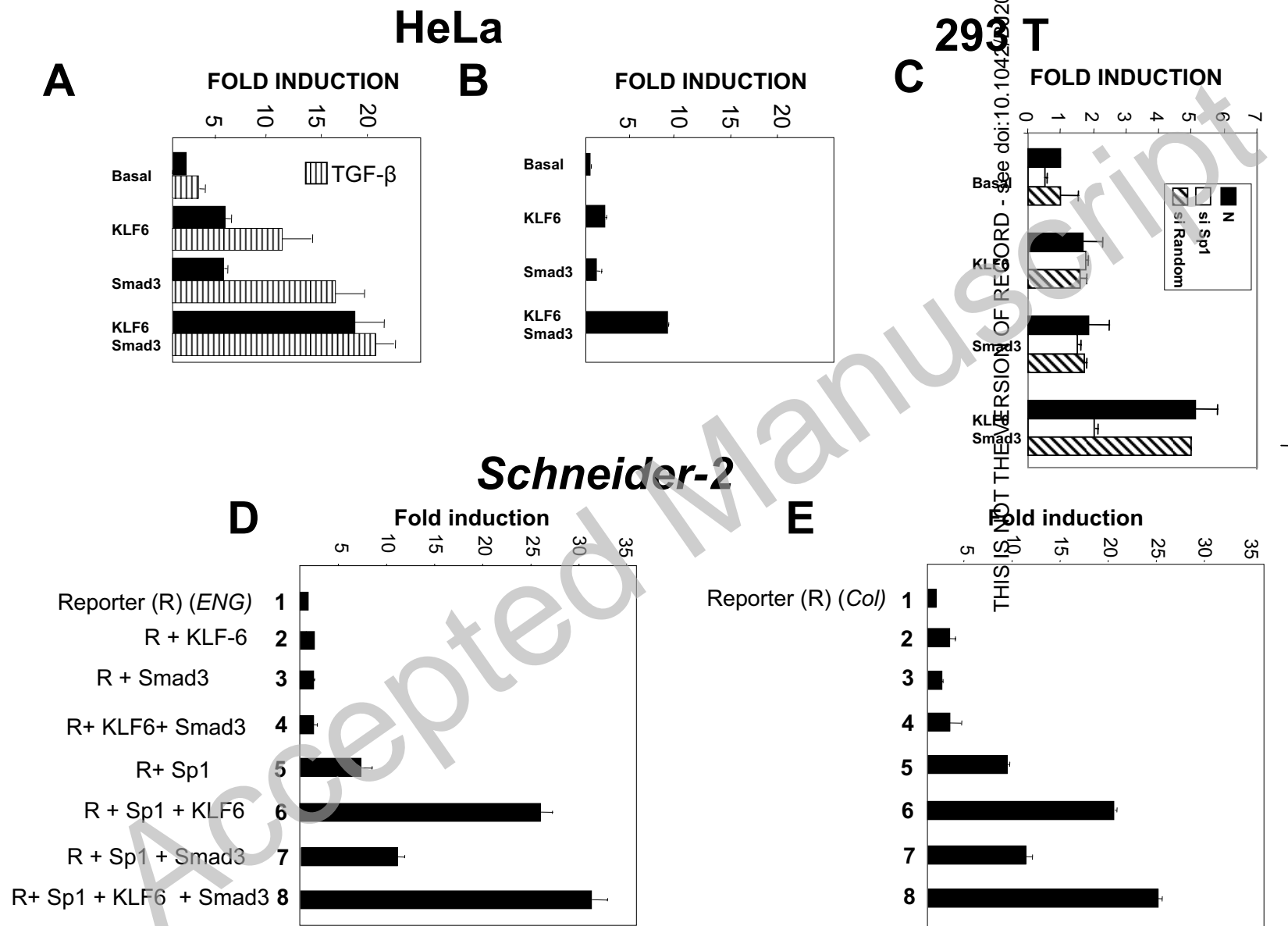
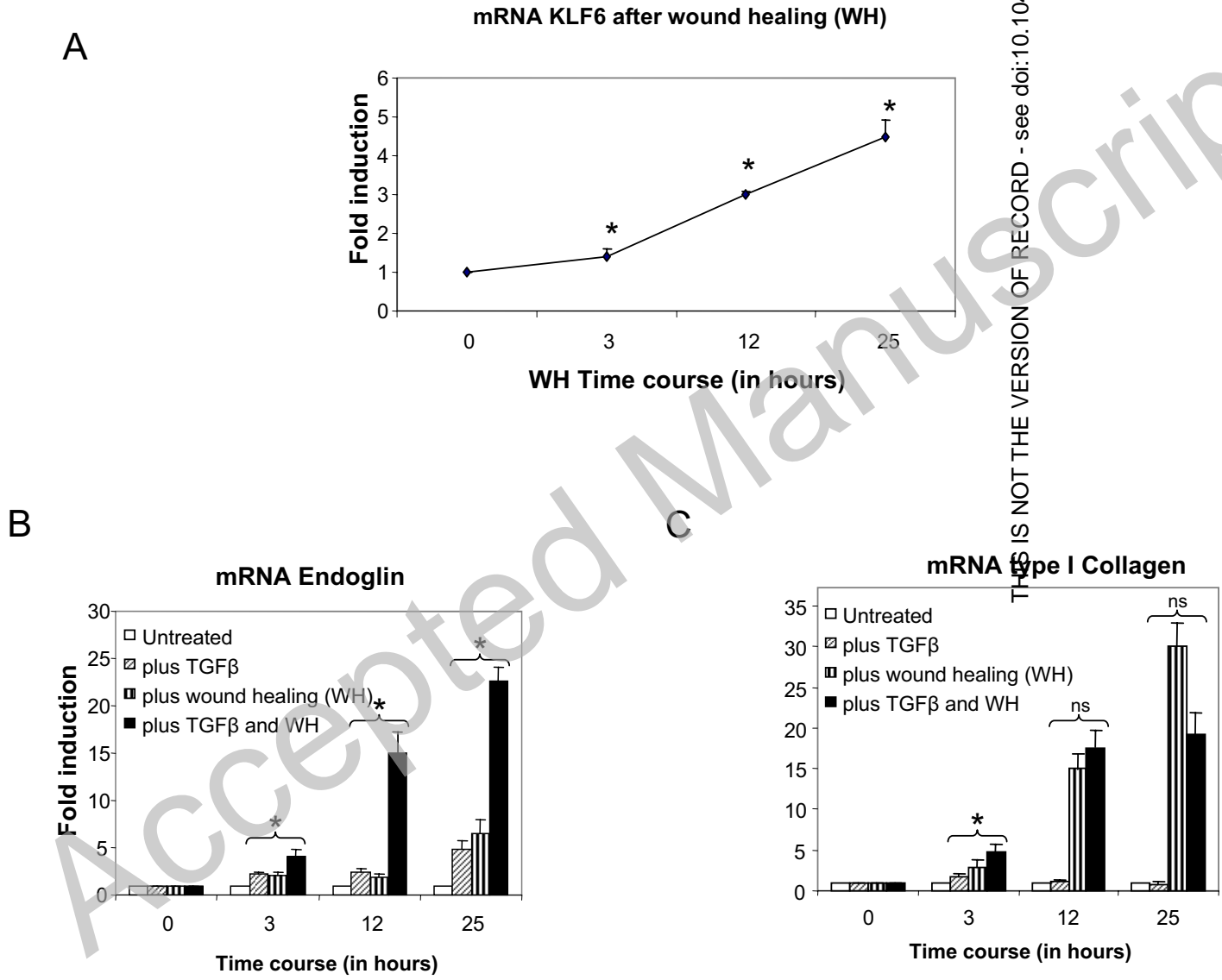
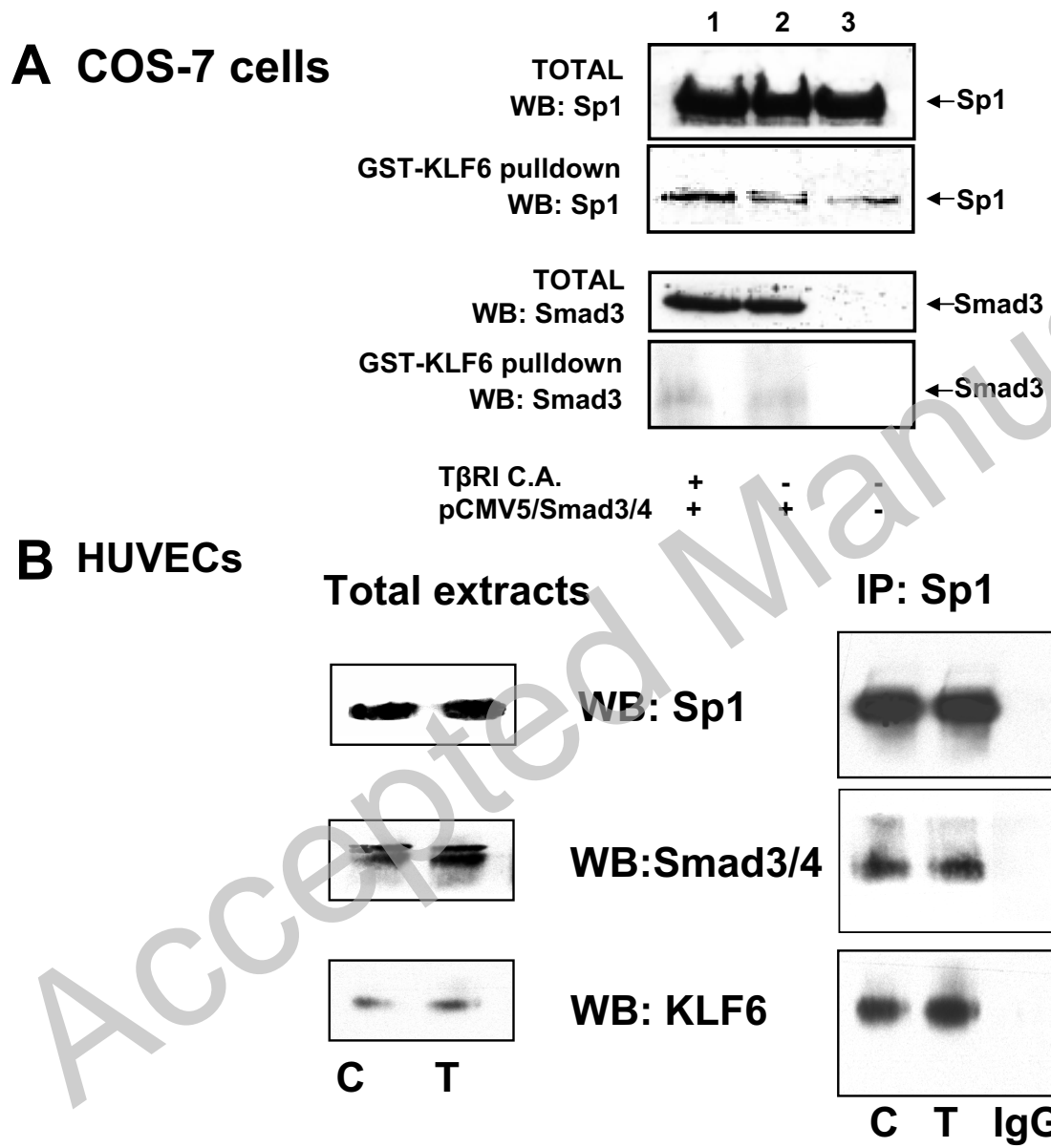


Figure 3

**Figure 3**



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Figure 4

C. Schneider-2 cells

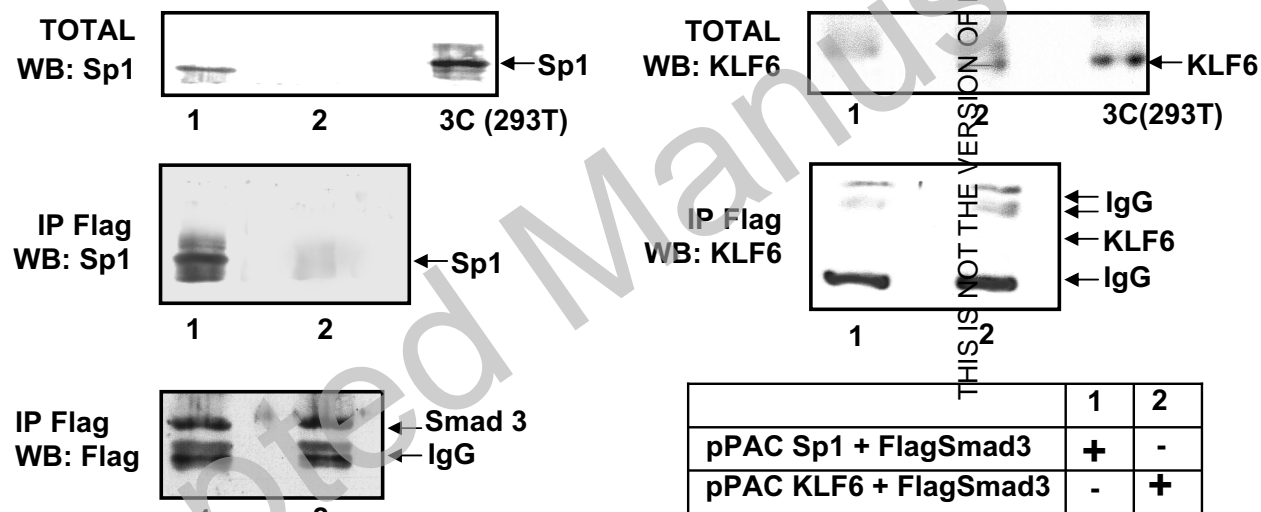


Figure 4

Figure 4

D

HEK 293T cells

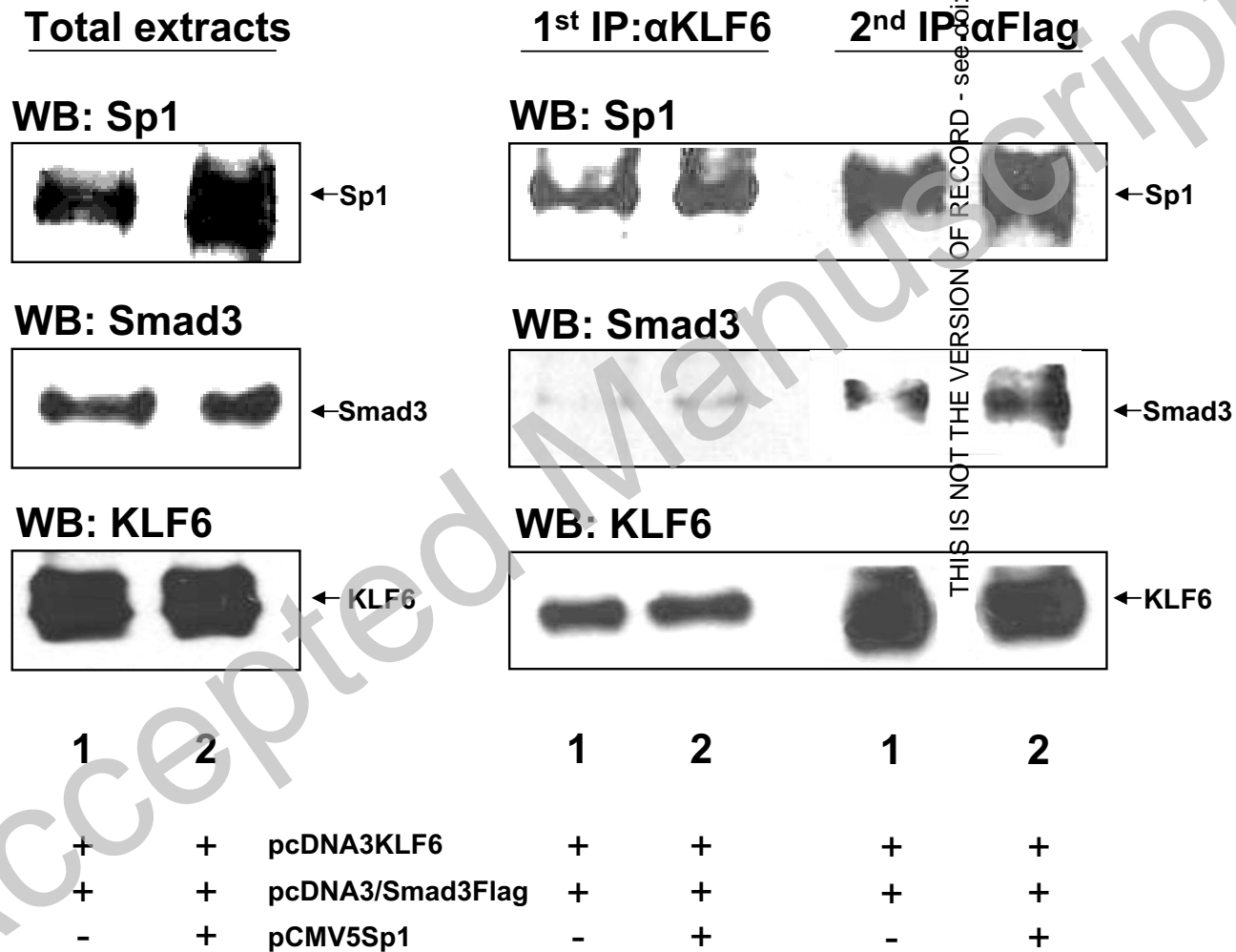
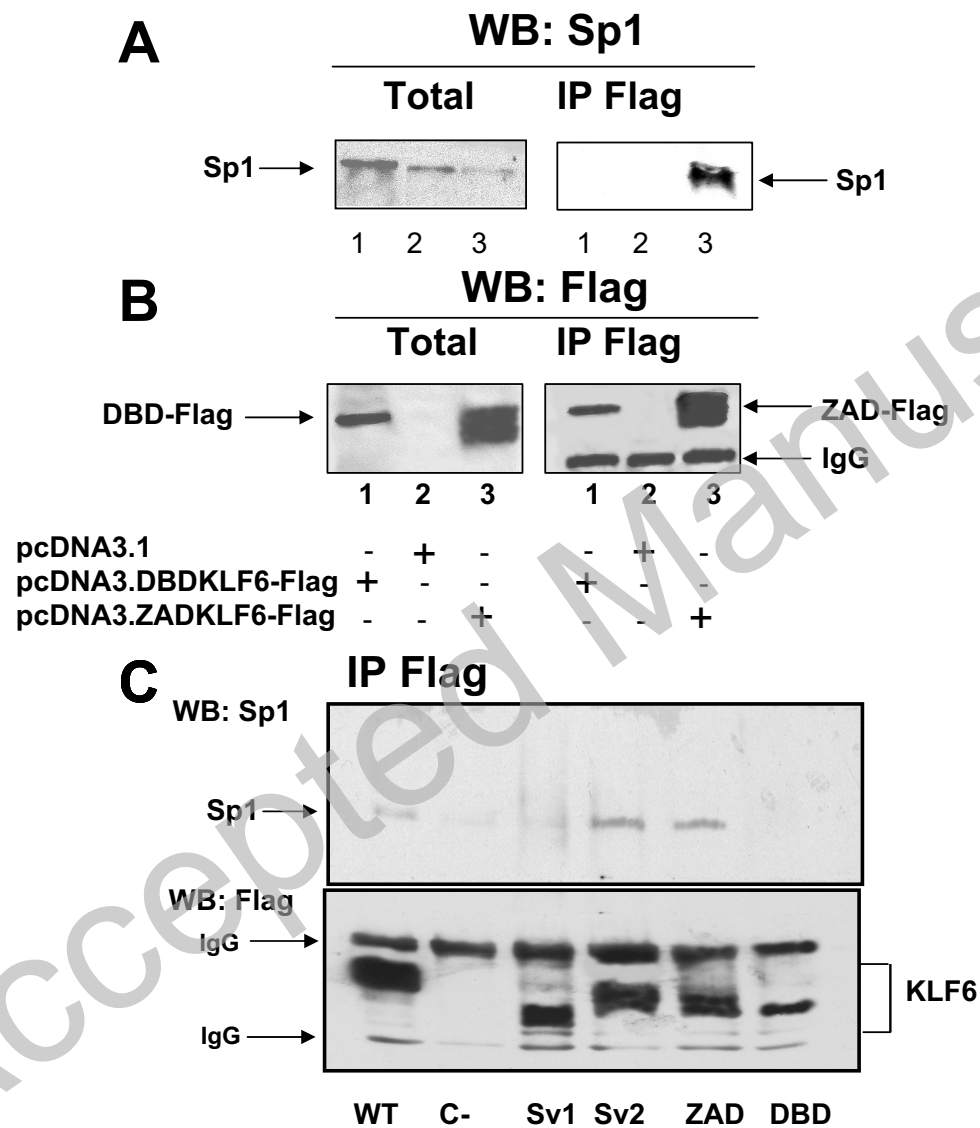


Figure 4



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Figure 5

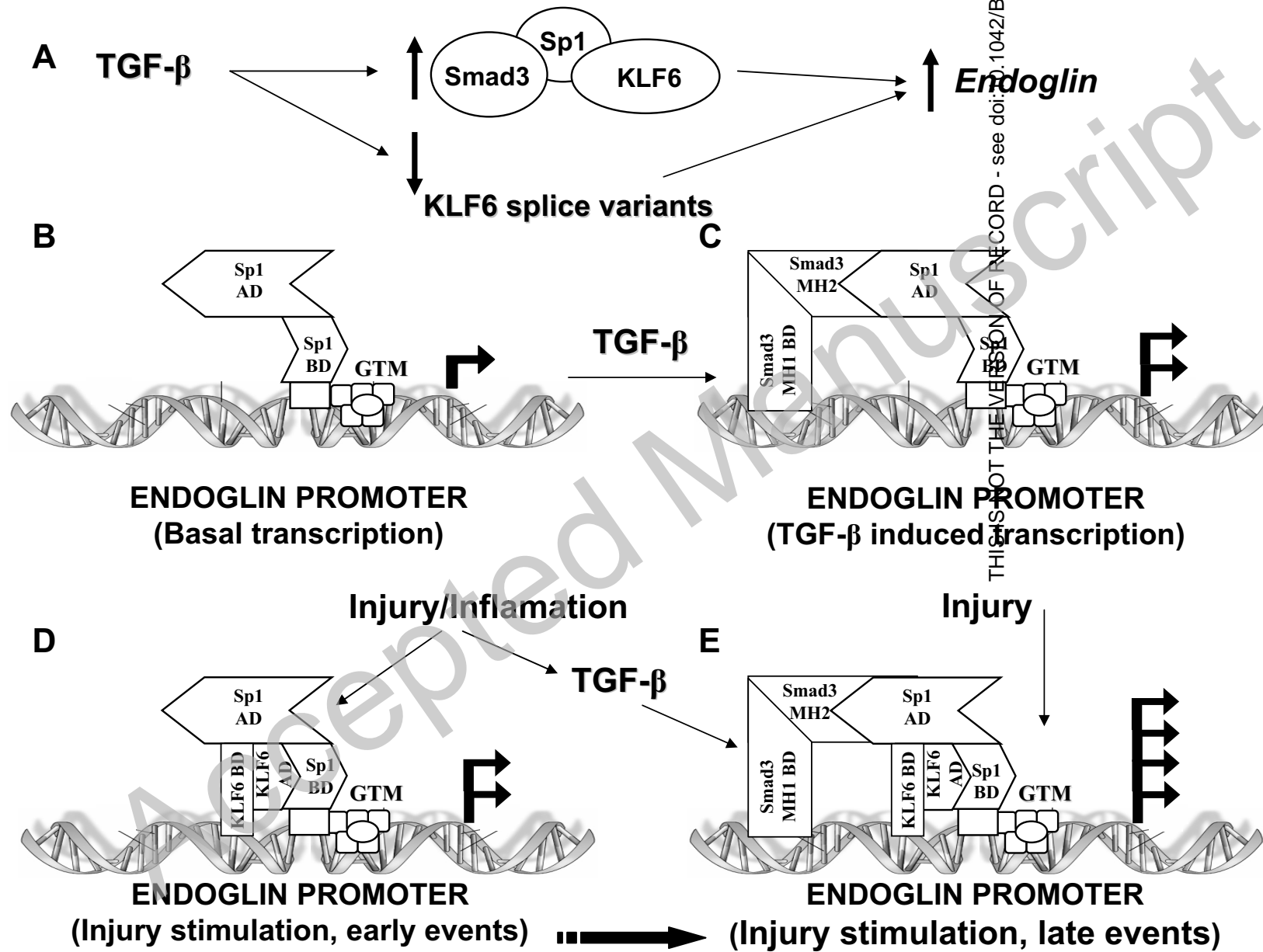


Figure 6

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20081434