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Pdcd4 knockdown up-regulates MAP4K1 expression and activation of AP-1 dependent transcription through c-Myc

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Abstract

*P*rogramme*d c*ell *d*eath 4 (Pdcd4) is a novel tumor suppressor, whose expression is frequently down-regulated in several types of cancers. In the present study, we demonstrated that Pdcd4 knockdown up-regulates MAP kinase kinase kinase kinase 1 (MAP4K1) expression and increases phosphorylation of c-Jun. Over-expression of c-Myc in HEK293 cells increases the levels of MAP4K1, MAP4K1 promoter activity, and phospho-c-Jun. Mutation analysis showed that the c-Myc binding site at −536 bp (relative to the initiation ATG) of *map4k1* promoter responds to c-Myc regulation. In addition, chromatin immunoprecipitation demonstrated that c-Myc directly binds to *map4k1* promoter at this site. Down-regulation of c-Myc reverses MAP4K1 expression and AP-1 activation in Pdcd4 knockdown cells. Moreover, over-expression of dominant negative Tcf4 decreases expression of c-Myc and MAP4K1, JNK activation, and AP-1 dependent transcription. Thus, activation of β-catenin/Tcf dependent transcription in Pdcd4 knockdown cells up-regulates MAP4K1 expression and AP-1 activity via c-Myc. The study presented here further reveals in detail the mechanism of how Pdcd4 inhibits tumor cell invasion and provides a functional connection between β-catenin/Tcf and AP-1 dependent transcription.

Keywords

Pdcd4; MAP4K1; JNK signaling pathway; AP-1; c-Myc

1. Introduction

*P*rogramme*d c*ell *d*eath 4 (Pdcd4) is a novel tumor suppressor that is frequently down-regulated in several types of cancers. The *pdcd4* gene was first identified as a differentially expressed mRNA when cells were treated with apoptosis inducers [1]. Over-expression of Pdcd4 has been shown to induce apoptosis in breast MDA-MB-231 and hepatocellular carcinoma HCC cells [2,3]. In consistence with the induction of cell death, depletion of Pdcd4 promoted cell proliferation [4] and over-expression of Pdcd4 inhibited proliferation [5–7]. However, over-expression of Pdcd4 in human HEK293 and chicken DT40 cells had no effects on apoptosis or cell proliferation [8,9]. In addition, Eto et al. [10] showed that loss of Pdcd4 induced apoptosis in HeLa and C2C12 cells. Thus, the role of Pdcd4 in programmed cell death remains unclear.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2012.07.004.

Despite the action in programmed cell death, the inhibitory role of Pdcd4 in tumorigenesis has been clearly demonstrated in vitro and in vivo. Over-expression of *pdcd4* cDNA inhibits 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced transformation and tumor phenotype in mouse JB6 cells [11,12]. Pdcd4 transgenic mice that overexpress Pdcd4 in the epidermis show significant reductions in 7,12-dimethylbenz(a)anthracene (DMBA)/TPA induced skin papilloma formation and carcinoma incidence [13]. Conversely, knockout of Pdcd4 expression in mice increases DMBA/TPA induced skin papilloma formation and carcinoma occurrence [14]. In addition to inhibiting the tumor promotion stage, Pdcd4 has also been demonstrated to be involved in tumor progression stage. Overexpression of Pdcd4 inhibits colon, breast, and ovarian tumor cell invasion [5,15–18], while knockdown of Pdcd4 promotes tumor cell invasion [17,19,20]. Inhibition of tumor cell invasion by Pdcd4 is attributed, at least in part, by suppressing activator protein-1 (AP-1) dependent transcription that is through inhibiting the transactivation of c-Jun or c-Fos [8,12]. Pdcd4 blocks c-Jun activation by inhibiting the expression of mitogen-activated protein kinase kinase kinase kinase 1 (MAP4K1) (also known as hematopoietic progenitor kinase 1), a kinase upstream of Jun N-terminal kinase (JNK) [18]. MAP4K1 is a mammalian STE-20-like protein serine/threonine kinase which regulates the JNK signaling pathway [21]. MAP4K1 activates JNK through the signaling pathway MAP4K1 →TAKl →MKK4→JNK [22] and does not affect other MAPK signaling pathways, including the ERK and p38 signaling pathways [23]. MAP4K1 is involved in the stress response, proliferation, and apoptosis of hematopoietic cells; however, the expression regulation and functions of MAP4K1 outside of the hematopoietic cells is poorly understood. We previously reported that over-expression of Pdcd4 suppressed MAP4K1 expression, with consequent inhibition of c-Jun activation and AP-1-dependent transcription [18]. In addition, ectopic expression of *map4k1* cDNA enhanced c-Jun phosphorylation and activated AP-1 dependent transcription [18], suggesting that MAP4K1 plays a crucial role in c-Jun activation. However, how Pdcd4 regulates the expression of MAP4K1 remains unknown.

Recently, we demonstrated that knockdown of Pdcd4 expression in colon tumor GEO and HT29 cells led to a fibroblast-like morphological change and promoted invasion [19]. In addition, Pdcd4 knockdown resulted in down-regulation of E-cadherin expression, accumulation of β -catenin into the nuclei, and activation of β-catenin/Tcf and AP-1 dependent transcription [19]. Promoting tumor cell invasion by Pdcd4 knockdown was contributed at least in part by c-Myc elevation since knockdown of c-Myc inhibited invasion induced by Pdcd4 knockdown [20]. c-Myc, a proto-oncogene encoding transcription factor, frequently up-regulated protein in all types of human cancers, whose expression is highly correlated with high-grade premalignancy and invasive tumors [24]. c-Myc is an essential protein for embryogenesis and is involved in cell migration, invasion, and metastasis [24,25]. Being a transcription factor, c-Myc is able to function as transcription activator or repressor depending on the recruiting factors [26]. For example, c-Myc can stimulate cyclin D2 and cyclin-dependent kinase 4 expression as repressed $p27^{KIP1}$ expression for promoting cell cycle progression [24]. In addition, c-Myc also globally influences chromatin structure and affects genetic program [27],

In this study, we provide mechanistic insights of how c-Myc, a target of β-catenin/Tcf dependent transcription, regulates the expression of MAP4K1 and activation of AP-1 dependent transcription.

2. Materials and methods

2.1. Tissue culture

The colon GEO (a gift from Dr. Douglas Boyd, MD Anderson Cancer Center, Houston, TX, USA) and HT29 cells (American Type Culture Collection, ATCC, Manassas, VA) were

grown in McCoy's medium containing 10% FBS, 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin. HEK293 cells were purchased from ATCC and were grown in DMEM medium containing 10% FBS, 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air.

2.2. Western blot analysis

Aliquots containing 20 to 40 μg of protein were separated through SDS-PAGE, and transferred to nitrocellulose membranes as described previously [11]. Subsequently, the membrane was incubated with primary antibodies overnight followed by horseradish peroxidase-linked secondary antibody for 1 h. The target protein was visualized by chemiluminescence. The band intensity was quantified using VisionWork LS image acquisition and analysis software (UVP, Upland, CA). The following antibodies were used: MAP4K1 (1:200 dilution), Xpress (1:5000 dilution), c-Myc (1:1000 dilution), phospho-JNK (1:1000 dilution), phospho-c-Jun (Ser-73) (1:1000 dilution), phospho-ERK (1:1000 dilution),JNK (1:1000 dilution), ERK (1:1000 dilution), and c-Jun (1:1000 dilution). MAP4K1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Xpress antibody was from Life Technologies (Grand Island, NY), while the rest of the antibodies were purchased from Cell Signaling (Danvers, MA).

2.3. Site-specific mutagenesis

The −792 bp to −51 bp 5′-flanking region of human *map4k1* promoter was generated by PCR and ligated into pGL3-basic vector (Promega, Madison, WI) as described previously [18]. The consensus sequence of the c-Myc binding site at −536 bp on the *map4k1* promoter was mutated from CACGTG to TATATA (mutated nucleotides are underlined) using wild-type pMAP4K1(792)-LUC as the template and the produced mutant was named as 536m. The site-specific mutagenesis was performed using QuickChange II XL Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA). Dominant negative Tcf4 construct (pcDNA4-dnTcf4) was generated using wild-type Tcf4 cDNA as the template. The first 92 bp were deleted using QuickChange II XL Site-Directed Mutagenesis kit (Agilent Technologies) and sub-cloned into pcDNA4/HisMax vector (Life Technologies). The wild-type Tcf4 cDNA was purchased from Origene (Rockville, MD). All constructs were verified by DNA sequencing.

2.4. Cell transfection and luciferase activity assays

For *map4k1* promoter activity assays, 3×10^4 cells were transiently transfected with 0.2 μg of pMAP4Kl(792)-LUC (or 536m) along with 10 ng of pRL-SV40 using jetPRIME transfection reagent (Polyplus-Transfection Inc., New York, NY). For the specificity assay, 0.1 μg of pcDNA, pCMV-Myc, or pCMV-β-gal and 0.2 μg of pMAP4K1(792)-LUC were transfected along with 10 ng of pRL-SV40 using jetPRIME transfection reagent as above. For AP-1 dependent transcription assays, 3×10^4 cells were transfected with various amounts of dnTcf4 expression plasmid (pcDNA-dnTcf4) and 0.2 μg of 4× AP-1-Luc plasmid [11] and 10 ng of pRL-SV40 plasmid. After 48 h, the cells were lysed in 1 × lysis buffer (Promega) and the luciferase activity was determined as previously described [20].

For over-expression of c-Myc, 2 μg or otherwise indicated of c-Myc expression plasmid (Origene) was transiently transfected into HEK293 cells (2×10^5 cells/60 mm dish) using Fugene HD reagent (Promega). After 72 h, cells were collected for RNA extraction or cell lysates.

2.5. Real-time PCR (qPCR)

The total RNA isolation and real-time PCR were performed as described previously [19]. Briefly, after synthesis of the first strand cDNA using the Superscript First-Strand Kit (Invitrogen), mRNA levels of *map4k1, c-myc*, or *GAPDH* were quantified by real-time PCR in a LightCycler 480 (Roche Applied Science, Indianapolis, IN). The PCR cycling was performed at 95 °C for 6 min followed by 40 cycles of denaturation (95 °C for 15 s), annealing (61 °C for 30 s), and extension (72 °C for 20 s). To determine the specificity of the PCR, the amplified products were subjected to melt-curve analysis using the standard machine method. The target mRNA level was normalized to the internal control, GAPDH, using the formula $\Delta C_T = C_T$ (target) - C_T (GAPDH) (C_T: threshold cycle). The level of target gene expression in control cells was designated as 100%. The relative expression levels were calculated using the equation $100\times2^{-[\text{average }\Delta\text{CT (test)}-\text{average }\Delta\text{CT (control)}]}$ [28]. The primers used for amplifying *map4k1, c-myc*, and *GAPDH* were purchased from SA Biosciences (Frederick, MD).

2.6. Chromatin immunoprecipitation (ChIP) assay

GEO-shLacZ and GEO-shPdcd4 cells as well as HT29-shLacZ and HT29-shPdcd4 cells were grown to 70–80% confluence and fixed with formaldehyde. ChIP assay was performed using ChIP-IT™ Express kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. The c-Myc antibody was used and the preimmune mouse serum was used as the negative control. The immunoprecipitated DNA was quantitated by qPCR. The following primers were used to amplify human *map4ld* promoter: forward, 5′-CATCTGCCTGGATACCTGTG; reverse, 5′-TCTCCATTAGGTTCCGGTCT. The input DNA not subjected to immunoprecipitation was used as control in PCR reaction. PCR products were analyzed onto 2% agarose/Tris-borate EDTA gels.

2.7. Knock-down of c-Myc and expression of dnTcf4

GE0-shPdcd4 cells (5×10^5 cells on a 60 mm plate) were transfected with 4.4 μl of 10 μM siRNA (Santa Cruz Biotechnology) (or 0.8 μg of pcDNA-dnTcf4) along with 0.4 μg of pMACS K^k.II plasmid (Miltenyi Biotec, Auburn, CA) using jetPRlME transfection reagent (Polyplus-Transfection Inc.) according to the manufacturer's protocol. Twenty-four hours post-transfection, the transfected cells were enriched by H-$2K^k$ antibody conjugated magnetic beads according to the manufacturer's protocol (Miltenyi Biotec). The eluted cells were then cultured for additional 48 h for qPCR, Western blot, or transfection assays.

2.8. Statistical analysis

Statistical analyses were performed using one-way ANOVA (http://faculty.vassar.edu/lowry/anova1u.html). Data are shown as the mean±standard deviation (SD) with at least three replicates ($n\geq3$). Differences were considered statistically significant at the $P\leq0.05$ level. Each experiment was repeated at least twice to confirm the results.

3. Results

3.1. Pdcd4 knockdown stimulates MAP4K1 expression and activates the JNK signaling pathway

Previously, we have demonstrated that over-expression of Pdcd4 resulted in inhibition of MAP4K1 expression [18]. To test whether knockdown of Pdcd4 up-regulates MAP4K1 expression and activates JNK and c-Jun, the stable Pdcd4 knockdown (GEO-shPdcd4) and vector control (GEO-shLacZ) GEO cells were used [20]. The total mRNAs from these cells were isolated and the *map4k1* mRNA levels were compared between GEO-shLacZ and GEO-shPdcd4 cells by qPCR using MAP4K1 specific primers. As shown in Fig. 1A, the

map4k1 mRNA expression was increased by approximately 50% by Pdcd4 knockdown. In addition, the protein level of MAP4K1 also increased in GEO-shPdcd4 cells comparing to the GEO-shLacZ cells (Fig. 1B). MAP4K1 is a kinase upstream of JNK that regulates the activation of JNK [22]. In order to determine whether Pdcd4 knockdown activates the JNK signaling pathway, the Western blot analysis was performed. The phospho-JNK and phospho-c-Jun (ser-73) were expressed at a much higher level in GEO-shPdcd4 cells than in GEO-shLacZ cells in which they were barely detectable, whereas the levels of total JNK and c-Jun proteins were similar in GEO-shLacZ and GEO-shPdcd4 cells (Fig. 1B). These results suggest that Pdcd4 knockdown elevates MAP4K1 expression and activates the JNK signaling pathway. It is noteworthy that the level of phospho-c-Jun at Ser-63 is similar in both control and Pdcd4 knockdown cells [19]. Thus, Ser-73 is probably the primary phosphorylation site in the activated c-Jun in Pdcd4 knockdown cells. The differential phosphorylation at Ser-63 and Ser-73 of c-Jun has been reported previously [29].

3.2. Over-expression of c-Myc stimulates MAP4K1 expression and activates c-Jun

Recently, we reported that knockdown of Pdcd4 up-regulated c-Myc expression [20]. Since c-Myc regulates the expression of numerous genes, we thus hypothesize that up-regulation of c-Myc contributes to the stimulation of MAP4K1 expression. To test this, the c-Myc expression plasmid (pCMV6-Myc) was transiently transfected into HEK293 cells and the MAP4K1 expression and c-Jun activation were examined. After 72 h, the cells were harvested, and total RNA and cell lysate were prepared. The level of *map4k1* mRNA was assayed by qPCR. As shown in Fig. 2A, the mRNA level of *map4k1* is approximately 50% higher in the cells transfected with pCMV6-Myc (c-Myc) than in the cells transfected with empty vector (control). To test whether c-Myc also activates *map4k1* promoter activity, the *map4k1* promoter luciferase construct [pMAP4Kl(792)-LUC] was transfected along with pCMV6-Myc into HEK293 cells. After 48 h, cells were lysed and the luciferase activity was assayed. The −792 nt to −51 nt (relative to the initiation ATG) in the 5′ flanking region of human *map4k1* promoter was amplified by PCR and ligated into pGL3-basic vector and named as pMAP4Kl(792)-LUC. Transient co-transfection of pCMV6-Myc and pMAP4Kl(792)-LUC plas-mids stimulated *map4k1* promoter activity in a concentration dependent manner (Fig. 2B). The *map4k1* promoter was activated by approximately 2-folds when 100 ng of pCMV6-Myc plasmid was transfected. Activation of *map4k1* promoter by c-Myc is specific as transient expression of β-galactosidase did not stimulate *map4k1* promoter activity (Fig. 2C). To test whether c-Myc regulates c-Jun activation, the level of phospho-c-Jun was analyzed by Western blot analysis using phospho-c-Jun (ser-73) antibody. The level of phospho-c-Jun was approximately 3-folds higher in c-Myc expressing cells than that in control cells (Fig. 2D), revealing that over-expression of c-Myc not only stimulates MAP4K1 expression but also activates c-Jun.

3.3. The c-Myc binding site at −536 of the map4k1 promoter responds to c-Myc regulation

To investigate how MAP4K1 expression is regulated in the Pdcd4 knockdown cells, the pMAP4Kl(792)-LUC was transfected into GEO-shLacZ and GEO-shPdcd4 cells. The luciferase activity in GEO-shPdcd4 cells was approximately 4.5-folds of that seen in GEO-shLacZ cells (Fig. 3A, WT), indicating that *map4k1* promoter activity (−792 bp to −51 bp) is enhanced by Pdcd4 knockdown. This region *of map4k1* promoter contains a potential c-Myc binding site located at −536 bp (Fig. 3A). To test whether this potential c-Myc binding site mediates the stimulation of *map4k1* promoter activity in Pdcd4 knockdown cells, we mutated the c-Myc binding site and transfected the mutated construct (536m) into GEO-shLacZ and GEO-shPdcd4 cells. The 536m exhibited an approximately 6-fold reduction of *map4k1* promoter activity in the GEO-shPdcd4 cells (Fig. 3A, filled bars). A similar reduction was also observed when the 536m was transfected into GEO-shLacZ cells (Fig. 3A, open bars), suggesting that this c-Myc binding site is essential for MAP4K1 expression.

Transfection of 536m into HT29-shPdcd4 cells (HT29 cells with Pdcd4 knockdown) also showed a dramatic reduction of *map4k1* promoter activity (Supplementary Fig. 1). Since the c-Myc protein level in GEO-shPdcd4 cells is approximately 2.5-folds higher than that in GEO-shLacZ cells [20], it is expected that more c-Myc molecules bind to the *map4k1* promoter in GEO-shPdcd4 than in GEO-shLacZ cells. To test this, ChIP assays were performed to examine the binding of c-Myc to the *map4k1* promoter using *map4k1* primers which amplified the c-Myc binding site at position −536 bp. The input chromatin without immunoprecipitation was used as the control in PCR reaction. A high level of PCR products (185 bp) was observed using c-Myc antibody precipitated chromatin from lysates of GEO-shPdcd4 cells (Fig. 3B, lane 6), wherein the band intensity of the PCR product was approximately 20-folds higher than that of the pre-immune serum precipitated chromatin (Fig. 3C). However, using c-Myc antibody precipitated chromatin from GEO-shLacZ cell lysates, the same primers only generated a low level of PCR products (Fig. 3B, lane 3), whose band intensity is similar to that of the pre-immune serum precipitated chromatin (Fig. 3C). Similar results were also observed when c-Myc antibody was used to precipitate chromatin from HT29-shLacZ and HT29-shPdcd4 cell lysates (Supplementary Fig. 2). These results directly show that c-Myc binds to the *map4k1* promoter to stimulate MAP4K1 expression in Pdcd4 knockdown cells.

3.4. Down-regulation of c-Myc reverses MAP4K1 expression in Pdcd4 knockdown cells

If c-Myc contributes to MAP4K1 expression in Pdcd4 knockdown cells, knockdown of c-Myc should inhibit MAP4K1 expression. In order to enhance the population of c-Myc knockdown cells, pMACS K^k.II plasmid was co-transfected with *c-myc* siRNA into GEO-shPdcd4 cells. The pMACS K^k.II plasmid produces a mouse MHC class I H-$2K^k$ protein on the cell membrane with a truncated cytoplasmic domain. Twenty-four hours post-transfection, the cells with successful transfection were enriched by H-$2K^k$ antibody conjugated magnetic beads. As shown in Fig. 4A, transient transfection of *c-myc* siRNA resulted in reduction of approximately 50% of *c-myc* mRNA The level of *map4k1* mRNA is about 20% lower in the cells transfected with *c-myc* siRNA (si-Myc) than in the cells transfected with scramble siRNA (control), suggesting that down-regulation of c-Myc reverses MAP4K1 expression in Pdcd4 knockdown cells. Although knockdown of c-Myc inhibits about 20% of *map4k1* mRNA expression, this inhibition is significantly enough to affect the activation of downstream targets. The level of phospho-JNK and phospho-c-Jun in the si-Myc cells was approximately 30% and 50% of that observed in the control cells, respectively (Fig. 4B). In contrast, the total JNK protein level was similar between control and si-Myc cells (Fig. 4B). In addition, the levels of phospho-ERK were similar between si-Myc and control cells, suggesting that knockdown of c-Myc did not affect the ERK signaling pathway. Interestingly, the total c-Jun protein level was slightly decreased in the si-Myc cells, which might be due to the feedback inhibition of c-Jun expression by inactivating c-Jun [30]. Moreover, knockdown of c-Myc also inhibited approximately 50% of AP-1 dependent transcription (Fig. 4C). These results suggest that c-Myc enhances MAP4K1 expression which contributes to the activation of JNK, c-Jun, and AP-1 dependent transcription in Pdcd4 knockdown cells.

3.5. β-catenin/Tcf dependent transcription regulates MAP4K1 expression, JNK activation, and AP-1 dependent transcription

To study the functional significance of β-catenin/Tcf dependent transcription in regulating MAP4K1 expression, we tested whether dominant negative Tcf4 (dnTcf4) inhibits the expression of MAP4K1 and activation of the JNK signaling pathway. The dnTcf4 expression plasmid (pcDNA4-dnTcf4) and pMACS K^k.ll plasmids were transfected into GEO-shPdcd4 cells and the transfected cells were enriched by H-$2K^k$ antibody conjugated beads. The mRNA levels of *c-myc* and *map4k1* in control and dnTcf4 expressing (dnTcf4)

cells were determined by qPCR The dnTcf4 is lacking the β-catenin interaction domain and expression of dnTcf4 has been known to inhibit the β -catenin dependent transcription [31]. As shown in Fig. 5A, over-expression of dnTcf4 cDNA decreased by approximately 20% and 40% in *c-myc* and *map4k1* mRNA levels, respectively, indicating that dnTcf4 suppressed c-Myc and MAP4K1 expression. To further confirm that dnTcf4 regulates MAP4K1 expression, the pcDNA4-dnTcf4 was transfected along pMAP4Kl(792)-LUC into GEO-shPdcd4 cells. Expression of dnTcf4 inhibited *map4k1* promoter activity in a dose dependent manner (Fig. 5B). The *map4k1* promoter activity was reduced to approximately 35% when 0.6 μg of pcDNA4-dnTcf4 plasmid was transfected. To further investigate the effects of over-expressing dnTcf4 on the JNK signaling pathway, a series of Western blotting analyses was performed. The c-Myc protein expression was suppressed by about 3-folds by dnTcf4 (Fig. 5C). The levels of phospho-JNK and phospho-c-Jun in dnTcf4 cells were approximately 15% and 35% of that seen in control cells, respectively (Fig. 5C). The total JNK protein levels were similar between control and dnTcf4 cells while the total c-Jun levels were suppressed in the dnTcf4 cells. Moreover, over-expression of *dnTcf4* cDNA resulted in the inhibition of AP-1 dependent transcription (Fig. 5D). Transfection of 0.2 or 0.4 μg of pcDNA4-dnTcf4 plasmid displayed approximately 50% inhibition. It has been known that c-Myc is a target of the β-catenin-dependent transcription in the Pdcd4 knockdown cells [20]. Thus, these results suggest that the β-catenin/Tcf4 complex regulates the c-Myc expression to mediate the MAP4K1 expression in Pdcd4 knockdown cells resulting in the change of JNK activity and AP-1 dependent transcription.

4. Discussion

In this study, we demonstrated that MAP4K1 expression is upregulated by elevating c-Myc expression. Knockdown of c-Myc expression results in inhibition of MAP4K1 expression, c-Jun activation, and AP-1 dependent transcription. In addition, over-expression of dnTcf4 suppresses c-Myc and MAP4K1 expression as well as the activation of c-Jun and AP-1 dependent transcription. Our results suggest that activation of β-catenin/Tcf dependent transcription by Pdcd4 knockdown up-regulates MAP4K1 expression and activates the JNK signaling pathway through c-Myc (Fig. 6).

Elevated or deregulated expression of c-Myc has been detected in a wide range of human cancers, and is often associated with aggressive, poorly differentiated tumors [24]. In human colon cancer, c-Myc expression is frequently elevated at both early and late stages of colon carcinogenesis [32]. Although it has been well understood that c-Myc regulates cell cycle progression and cell proliferation, several studies also implicated that c-Myc might be involved in tumor cell invasion and metastasis. For example, *c-myc* mRNA levels were higher in metastatic lesions than in primary lesions [33,34]. Over-expression of c-Myc can reverse the inhibitory effect of F box only protein 8 on tumor invasion [35]. In addition, we recently demonstrated that c-Myc contributes to colon tumor cell invasion induced by Pdcd4 knockdown because down-regulation of c-Myc results in inhibition of invasion in the Pdcd4 knockdown cells [20]. These findings suggest that c-Myc is an important regulator of tumor cell invasion.

How does c-Myc regulate colon tumor cell invasion? The present study extends this mechanistic understanding to now implicate that MAP4K1 expression is regulated by c-Myc. Knockdown of Pdcd4 up-regulates MAP4K1 expression (Fig. 1) and stimulates MAP4K1 promoter activity (Fig. 3). Over-expression of c-Myc increases MAP4K1 mRNA level (Fig. 2A), while c-Myc knockdown decreases MAP4K1 mRNA level (Fig. 4A). Mutation of the c-Myc binding site on the MAP4K1 promoter decreases the promoter activity dramatically (Fig. 3A and Supplementary Fig. 1). In addition, the regulation of MAP4K1 expression by c-Myc is further supported by the direct binding of c-Myc to the

promoter region of MAP4K1 (Fig. 3B,C, and Supplementary Fig. 2). These findings collectively indicate that MAP4K1 expression is regulated by c-Myc. MAP4K1, a kinase three steps upstream of JNK, regulates JNK activation. In turn, JNK regulates the activation of c-Jun by phosphorylating it at Ser-63 and Ser-73 and subsequently activates AP-1 dependent transcription. Our data also suggest that regulation of the JNK signaling pathway by c-Myc is specific since knockdown of c-Myc did not affect ERK phosphorylation (Fig. 4B). Ectopic expression of dominant negative MAP4K1 in which methionine substituted the place of lysine 46 inhibits c-Jun phosphorylation, AP-1 dependent transcription, and invasion [18]. Conversely, overexpression of MAP4K1 cDNA increases phosphorylation of c-Jun [18]. AP-1 is a transcription factor complex composed of Jun-Jun homodimers or Jun-Fos heterodimers. The Jun protein family includes c-Jun, JunB, and JunD. The Fos protein family contains c-Fos, Fra-1, Fra-2, and FosB. Immunohistochemical studies of human colon cancer tissues revealed that c-Jun and Fra-1 expression is frequently elevated in adenoma, adenocarcinoma, and neuroendocrine carcinoma [36]. Activation of AP-1 activity by over-expression of Jun or Fos proteins enhances invasion and metastasis [30]. In addition, inhibition of AP-1 activity by the dominant negative c-Jun, TAM67, suppresses the invasive ability of a keratinocyte [37], fibroblast [38], and squamous carcinoma [39]. Thus, activation of AP-1 dependent transcription by Pdcd4 knockdown is likely to contribute, at least in part, to the promotion of colon tumor cell invasion through elevation of MAP4K1 expression, which is regulated by c-Myc.

The Tcf transcription factor family consists of four members, Tcf1, Tcf3, Tcf4, and LEF1. In the presence of Wnt signaling, β-catenin translocates into the nucleus and binds with a member of the Tcf family to form a β-catenin/Tcf complex resulting in the activation of transcription of the β-catenin/Tcf target genes, including c-Myc [40]. The finding that expression of dnTcf4 attenuates JNK phosphorylation, c-Jun phosphorylation, and AP-1 transactivation (Fig. 5) suggests that β-catenin/Tcf dependent transcription affects the JNK signaling pathway. On the other hand, it has been suggested that JNK/c-Jun regulates β-catenin/Tcf dependent transcription since the expression of Tcf4 is elevated in JNK1 transgenic mice while depletion of c-Jun expression significantly reduces Tcf4 expression [41]. These findings reveal a feedback mechanism of regulation between the Wnt signaling pathway and the JNK/c-Jun signaling pathway. It is noteworthy that the *map4k1* mRNA level decreases more in dnTcf4 expressing cells (Fig. 5A) than in c-Myc knockdown cells (Fig. 4A). This finding suggests that other target(s) of β-catenin/Tcf dependent transcription besides c-Myc may regulate MAP4K1 expression, which needs to be further investigated.

In conclusion, our results show that elevation of c-Myc expression by Pdcd4 knockdown stimulates MAP4K1 expression resulting in the activation of JNK, and c-Jun, and transactivation of AP-1. These findings provide a molecular explanation of how Pdcd4 knockdown activates AP-1 dependent transcription and connects β-catenin/Tcf dependent transcription and AP-1 dependent transcription.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Pdcd4	programmed cell death 4
MAP4K1	mitogen-activated protein kinase kinase kinase kinase 1
AP-1	activator protein-1
TPA	12-O-tetradecanoylphorbol-13-acetate
DMBA	7,12-dimethylbenz(a)anthracene
TAK1	transforming growth factor β-activated kinase 1
MKK4	mitogen-activated kinase kinase 4
JNK	Jun N-terminal kinase
ERK	extracellular signal-regulated kinases
ChIP	chromatin immunoprecipitation
siRNA	small interfering RNA

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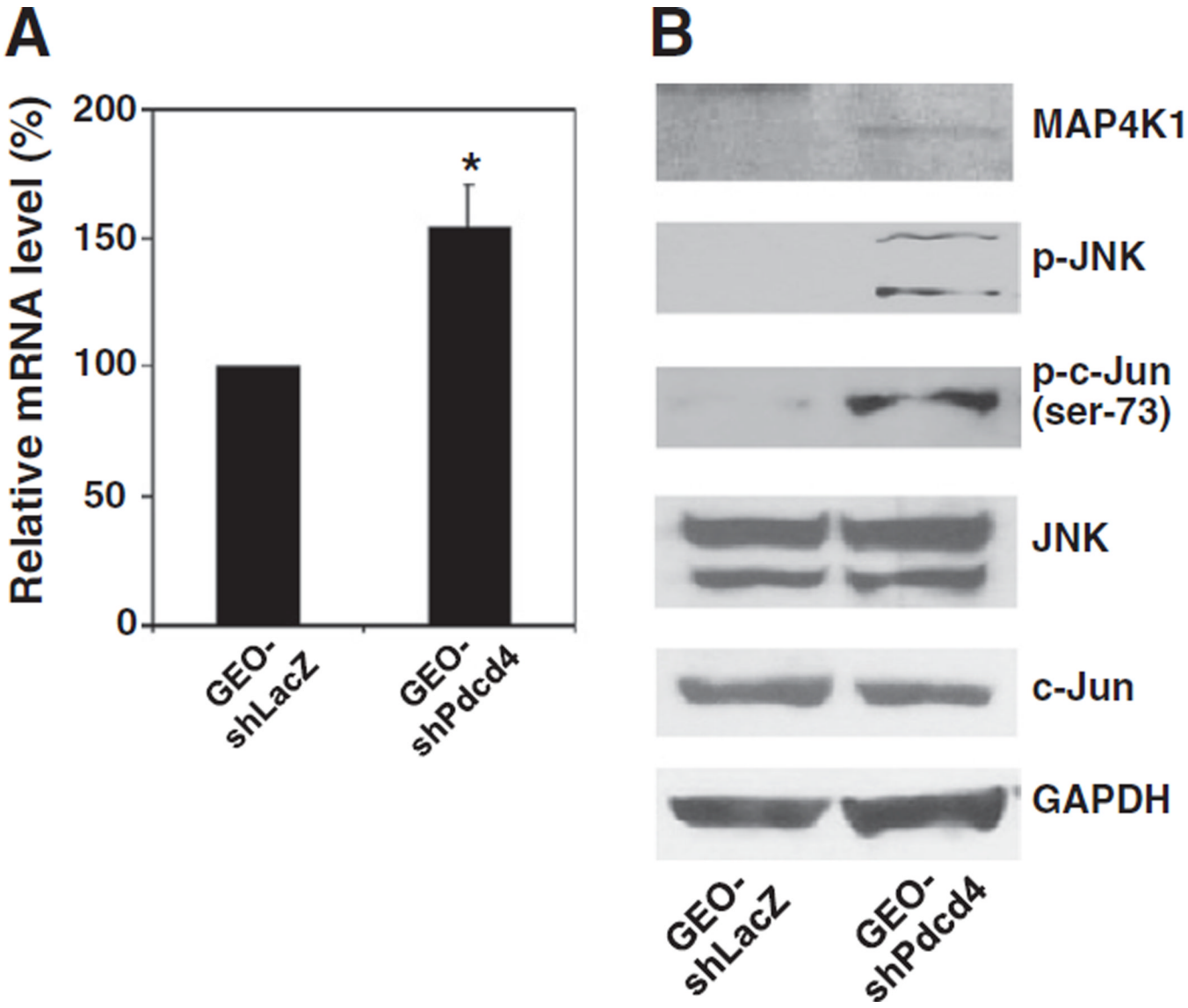

Fig. 1.

Pdcd4 knockdown stimulates MAP4K1 expression and activates the JNK signaling pathway. (A) The mRNA level of *map4k1* is up-regulated in Pdcd4 knockdown cells. The mRNA levels of *map4k1* and *GAPDH* were determined by qPCR using total RNA isolated from GEO-shLacZ and GEO-shPdcd4 cells. The ratio of *map4k1/GAPDH* in GEO-shLacZ cells is designated as 100%. Three independent experiments were performed with triplicates for each sample. The data are shown and expressed as mean ± standard deviation (SD). The asterisk indicates a significant difference as determined by one-way ANOVA ($P<0.01$). (B) The protein level of MAP4K1 is increased and its downstream targets are activated in Pdcd4 knockdown cells. Western blot analysis was performed using antibodies against MAP4K1, phospho-JNK, phospho-c-Jun (ser73), JNK, c-Jun, and GAPDH.

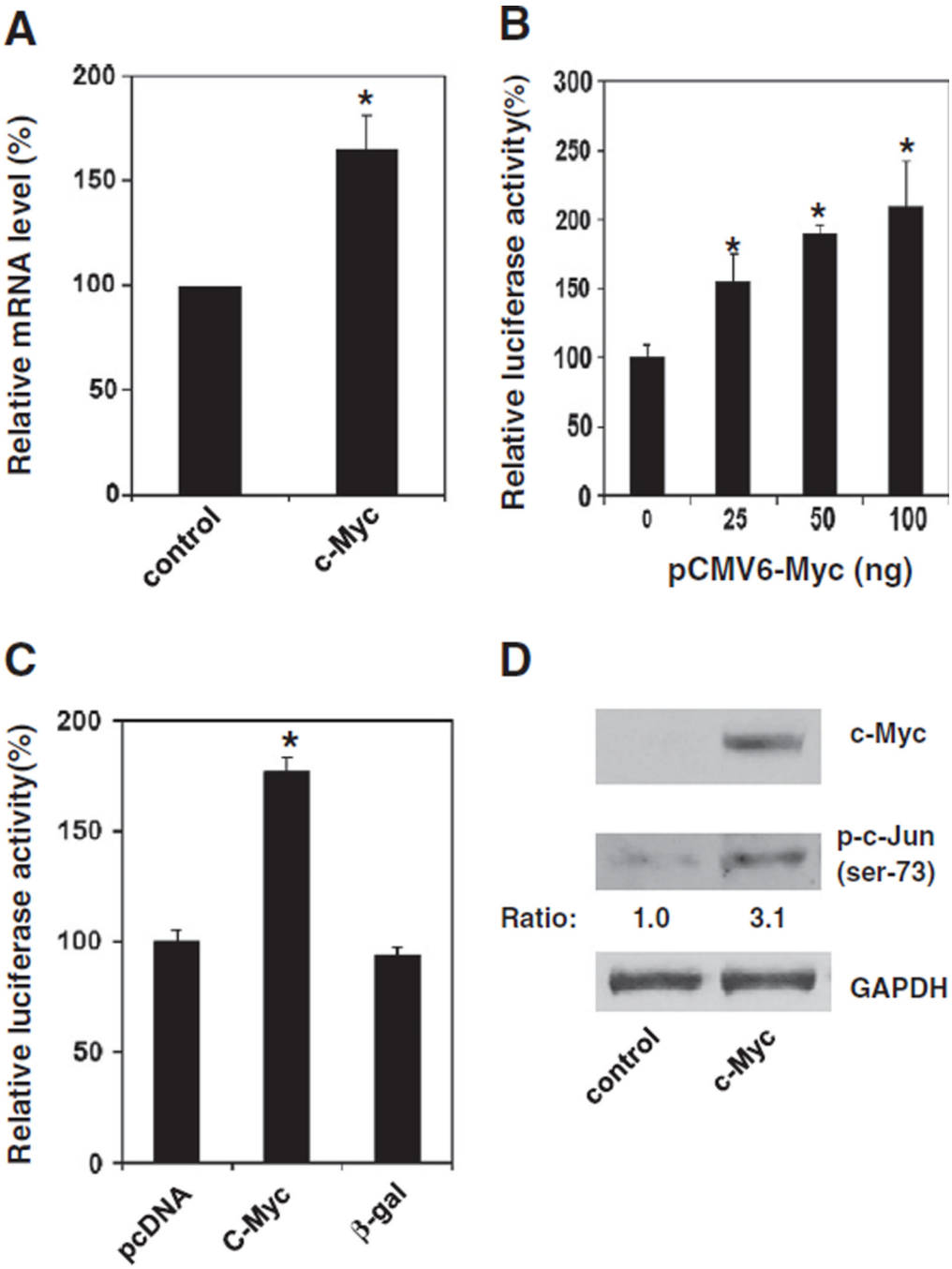

Fig. 2.

Over-expression of c-Myc stimulates MAP4K1 expression and activates c-Jun. (A) Over-expression of c-Myc increases *map4k1* mRNA The mRNA levels of *map4k1* and *GAPDH* were determined by qPCR using total RNA isolated from HEK293 cells transfected with either control or c-Myc expression plasmid for 72 h. The ratio of *map4kl/GAPDH* in cells transfected with the control vector is designated as 100%. Two independent experiments were performed with 3 replicates for each sample. The data are shown and expressed as mean±SD. The asterisk indicates a significant difference as determined by one-way ANOVA ($P<0.01$). (B) c-Myc enhances the promoter activity of MAP4K1. Increasing amounts (0–100 ng) of pCMV6-Myc plasmid and pMAP4Kl-LUC (0.2 μg) along with 10 ng

of pRL-SV40 were transfected into HEK293 cells. The total DNA was maintained at 0.3 μg by adding the empty vector pcDNA 3.1 DNA The activity of cells transfected with 0 ng of pCMV6-Myc is designated as 100%. Three independent experiments were performed with 5 replicates for each sample. The represented data are shown and expressed as mean±SD (n=5). The asterisk denotes a significant difference compared to transfection with 0 μg of pCMV6-Myc as determined by one-way ANOVA ($P<0.005$). (C) Stimulation of *map4k1* promoter by c-Myc is specific. The pcDNA pCMV-Myc, or pCMV-β-gal (0.1 μg) and pMAP4Kl-LUC (0.2 μg) along with 10 ng of pRL-SV40 were transfected into HEK293 cells as described in (B). The activity of cells transfected with pcDNA and pMAP4Kl-LUC is designated as 100%. The asterisk denotes a significant difference as determined by one-way ANOVA ($P<0.005$). (D) Over-expression of c-Myc elevates c-Jun phosphorylation. Cell lysate from HEK293 cells transfected with either control or c-Myc expression plasmid for 72 h was used. Western blot analysis was performed using antibodies against c-Myc, phospho-c-Jun (ser-73), and GAPDH. The ratio of phospho-c-Jun (ser-73)/GAPDH in control cells is designated as 1.0.

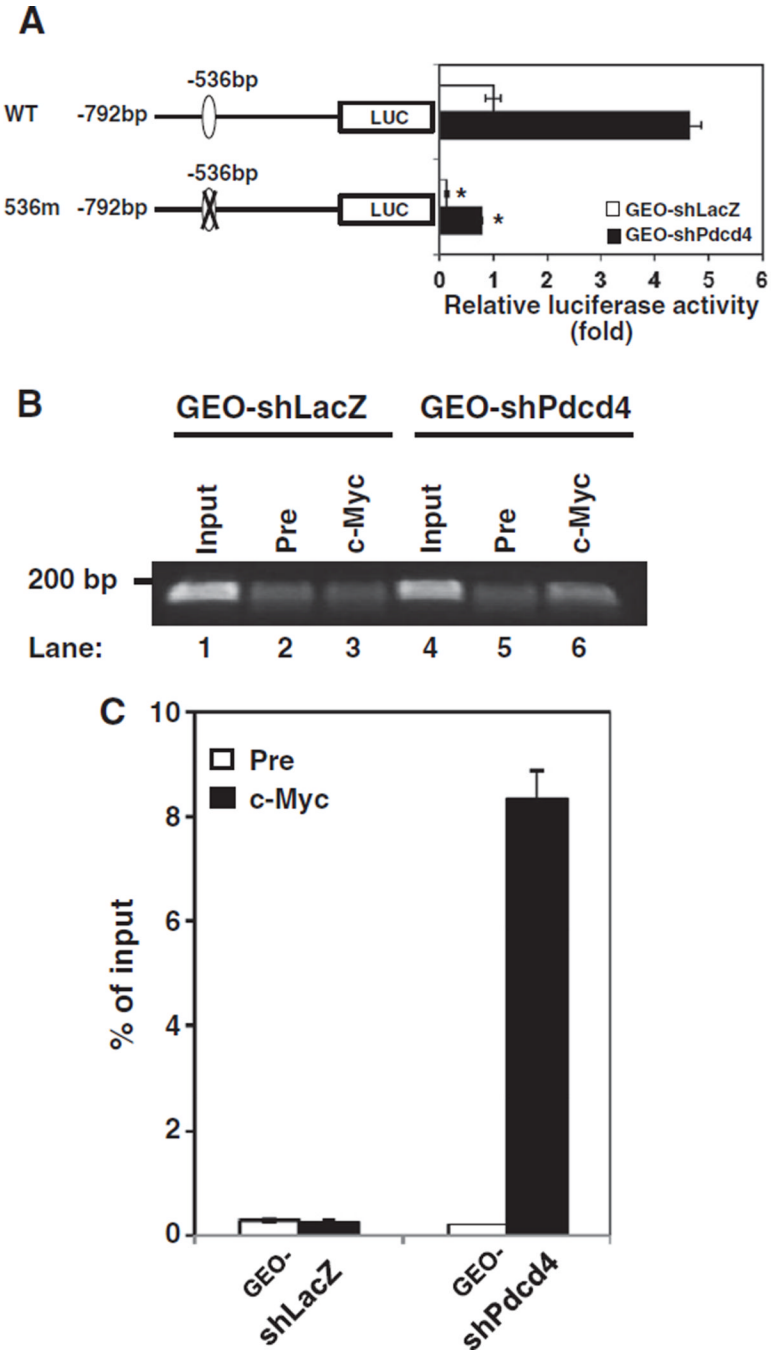

Fig. 3.

The c-Myc binding site at−536 of the *map4k1* promoter responds to c-Myc regulation. (A) Pdcd4 knockdown stimulates *map4k1* promoter activity. The pMAP4Kl(792)-LUC (WT) or 536m promoter construct (0.2 μg) was transfected into GEO-shLacZ and GEO-shPdcd4 cells along with 10 ng of pRL-SV40. The activity of GEO-shLacZ cells transfected with WT is designated as 1. Three independent experiments were performed with 5 replicates for each sample. The represented data are shown and expressed as mean±SD (n=5). The asterisk denotes a significant difference compared with cells transfected with WT as determined by one-way ANOVA (P<0.0001). (B and C) c-Myc directly binds to the *map4k1* promoter in Pdcd4 knock-down cells. ChIP assays were performed with cell lysates from either GEO-

shLacZ or GEO-shPdcd4 cells using control preimmune IgG (Pre) or anti-c-Myc antibody (c-Myc). The DNAs from cell lysate (input) and ChIP enriched were quantified by qPCR using primers for amplifying the c-Myc binding site at −536 bp on the promoter of MAP4K1. The representative PCR products were resolved onto 2% agarose gels (B). The level of the target gene in immunoprecipitated DNA of each sample is compared to that in input chromatin, which is equivalent to 100% (C).

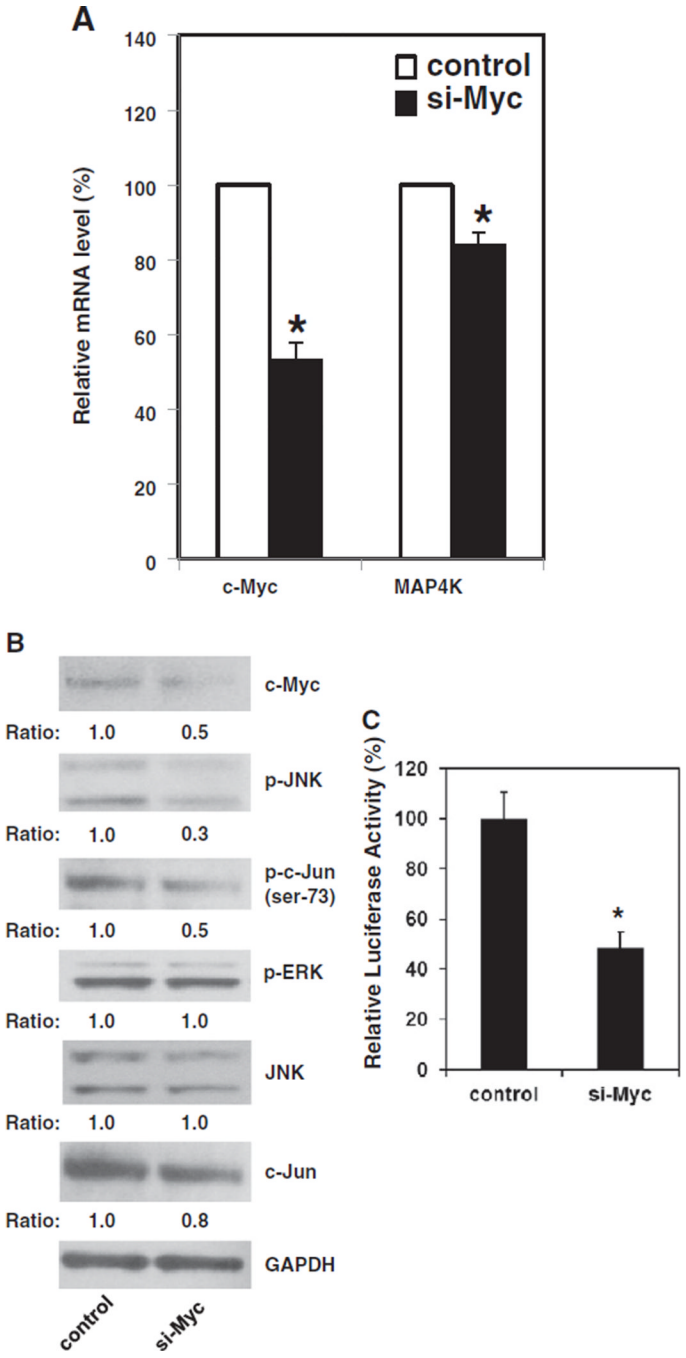

Fig. 4.

Down-regulation of c-Myc reverses MAP4K1 expression in Pdcd4 knock-down cells. The cells transfected with *c-myc* siRNA (si-Myc) or scramble siRNA (control) along with pMACS K^+.II plasmid were collected for extracting RNA or making cell ly-sates. The c-Myc knockdown cells were enriched by H-$2K^k$ antibody conjugated beads as described in Materials and methods. (A) The mRNA level of *map4k1* decreased in c-Myc knock-down cells. The total RNAs from control and si-Myc cells were reverse transcribed and subjected to qPCR. The ratio of *c-myc/GAPDH* and *map4k1/GAPDH* in control cells is designated as 100%. Two independent experiments were performed with 3 replicates for each sample. The data are shown and expressed as mean ± SD. The asterisk indicates a significant difference

compared with control cells as determined by one-way ANOVA ($P<0.05$). (B) c-Myc knockdown decreases the phosphorylation of JNK and c-Jun (ser-73). The cell lysates from control and si-Myc cells were subjected to Western blot analysis using various antibodies as indicated. The ratio of target protein/GAPDH in control cells is designated as 1.0. (C) Knockdown of c-Myc inhibits AP-1 dependent transcription. The relative luciferase activity in control cells is designated as 100%. Three independent experiments were performed with 5 replicates for each sample. The represented data are shown and expressed as mean ± SD ($n = 5$). The asterisk indicates a significant difference as determined by one-way ANOVA ($p<0.005$).

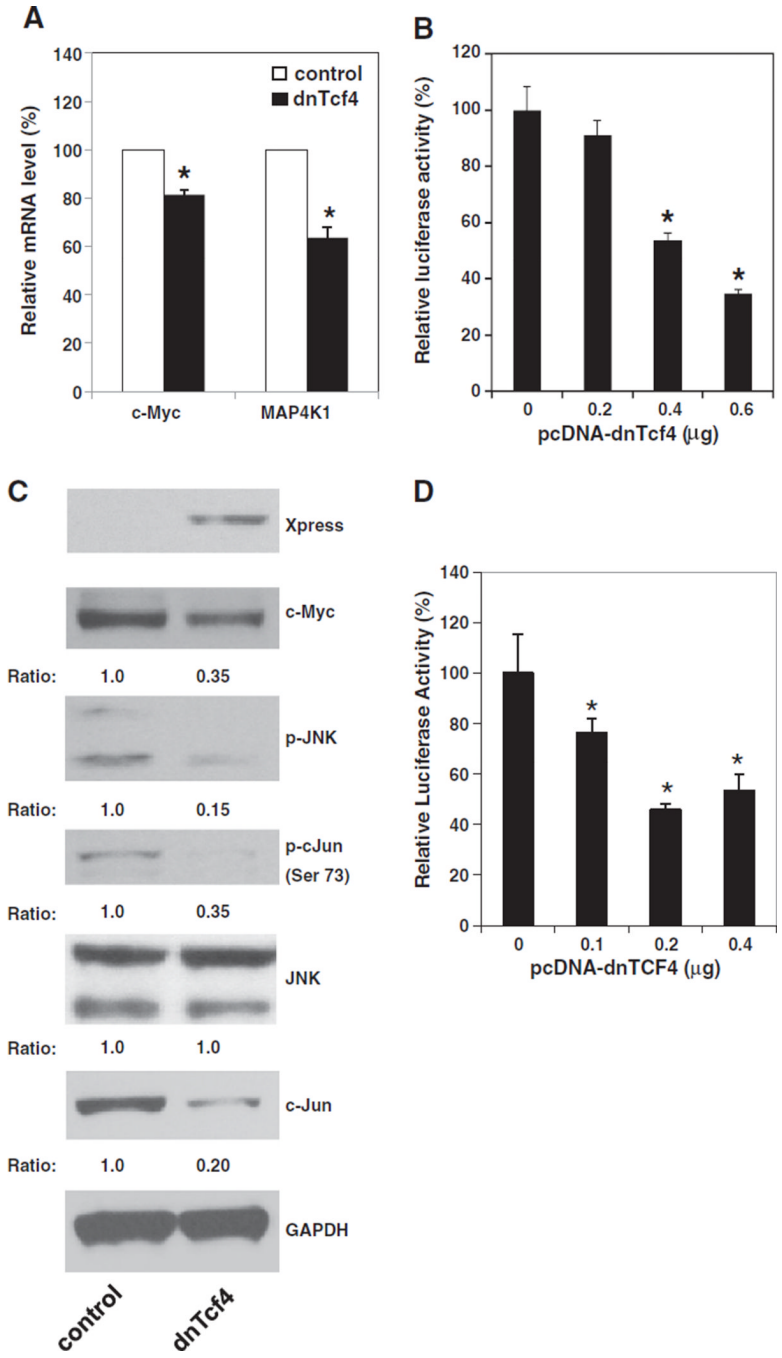

Fig. 5.

β-catenin/Tcf dependent transcription regulates MAP4K1 expression, JNK activation, and AP-1 dependent transcription. (A) Over-expression of dnTcf4 inhibits the expression of MAP4K1. GEO-shPdcd4 cells transfected with pcDNA4/Max (control) or pcDNA4-dnTcf4 (dnTcf4) plasmid along with pMACS K^+.II plasmid were collected for extracting RNA or making cell lysates. The dnTcf expressing cells were enriched by H-$2K^k$ antibody conjugated beads as described in Materials and methods. Total RNA was isolated and used in qPCR to examine the mRNA level of *c-myc* and *map4k1*. The ratio of *c-myc/GAPDH* and *map4k1/GAPDH* in cells transfected with pcDNA4/Max is designated as 100%. Two independent experiments were performed with 3 replicates for each sample. The data are

shown and expressed as mean ± SD. The asterisk indicates a significant difference compared with control cells as determined by one-way ANOVA ($P<0.05$). (B) Over-expression of dnTcf4 inhibits *map4k1* promoter activity. Indicated amount of pcDNA4-dnTcf4 plasmid was transfected into GEO-shPdcd4 cells while the total DNA for each transfection was maintained at 0.6 μg by adding pcDNA4/Max vector DNA. The activity of GEO-shPdcd4 cells transfected with 0 μg of dnTcf4 expression plasmid is designated as 100%. Three independent experiments were performed with 5 replicates for each sample. The represented data are shown and expressed as mean ± SD (n = 5). The asterisk denotes a significant difference compared with cells transfected with 0 μg of dnTcf4 expression plasmid as determined by one-way ANOVA ($P<0.001$). (C) Over-expression of dnTcf4 inhibits the activation of the JNK pathway. Western blot analysis was performed using the cell lysates from (A) with the indicated antibodies. The ratio of target protein/GAPDH in control cells is designated as 1.0. (D) Over-expression of dnTcf4 suppresses AP-1 dependent transcription. Indicated amount of pcDNA4-dnTcf4 plasmid was transfected into GEO-shPdcd4 cells while the total DNA for each transfection was maintained at 0.4 μg by adding pcDNA4/Max vector DNA The activity of GEO-shPdcd4 cells transfected with 0 μg of dnTcf4 expression plasmid is designated as 100%. Three independent experiments were performed with 5 replicates for each sample. The represented data are shown and expressed as mean ± SD (n = 5). The asterisk denotes a significant difference compared with cells transfected with 0 μg of dnTcf4 expression plasmid as determined by one-way ANOVA ($P<0.01$).

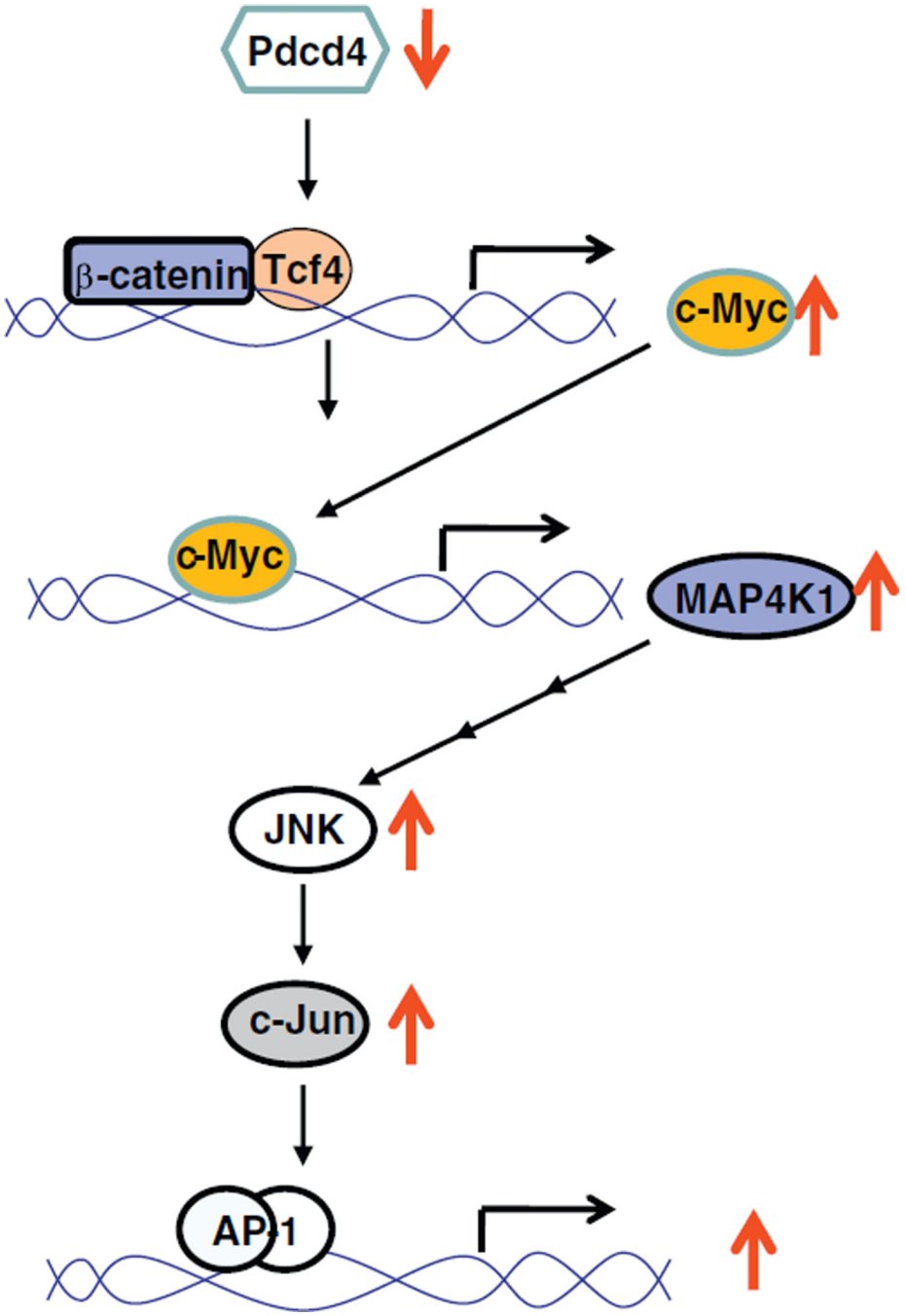

Fig. 6.
A model depicting the correlation between β-catenin and AP-1 dependent transcription in Pdcd4 knock-down cells.