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c-Myb negatively regulates Ras signaling through induction of dual phosphatase MKP-3 in NIH3T3 cells

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ABSTRACT

Mitogen-activated protein kinase phosphatase-3 (MKP-3) negatively regulates ERK1/2 MAPK in a feedback loop. However, little is known about the molecular mechanism by which Ras signaling induces *MKP-3* expression. In the present study, we demonstrate that exogenous expression of constitutively active H-Ras increases the level of *MKP-3* mRNA. A transfection study using a series of *MKP-3* promoter deletion constructs revealed that the c-Myb binding site is required for Ras-induced transcriptional activation of the *MKP-3* gene promoter. Furthermore, we show that c-Myb directly binds to the *MKP-3* promoter, as revealed by electrophoretic mobility shift assay and chromatin immunoprecipitation. Knock-down of c-Myb expression using siRNA abrogated Ras-induced *MKP-3* promoter activity. These findings propose a novel mechanism through which Ras signaling activates c-Myb-dependent transcriptional activation of the *MKP-3* gene.

1. Introduction

Extracellular signal-regulated kinase 1 and 2 (ERK1/2) are members of the mitogen-activated protein kinase (MAPK) family, which is involved in the regulation of cell growth, differentiation, and survival. ERK1/2 are phosphorylated in response to many different signals on both threonine and tyrosine residues by upstream kinases and MAPK/ERK kinase (MEK). These signals include stimulation of receptor tyrosine kinases, G-protein coupled receptors and integrins. Once activated, ERK1/2 translocate to the nucleus, where they regulate many downstream targets such as ELK-1 and c-JUN. It has been demonstrated that retained phosphorylation of ERK1/2 by nerve growth factor (NGF) leads to neuronal differentiation, whereas transient phosphorylation of ERK1/2 by epidermal growth factor (EGF) results in cell proliferation in cultured rat PC12 cells [1]. Therefore, it seems likely that the strength and duration of ERK1/2 activity determine the biological responses to any given mitogenic or stress stimuli for each cell type [2,3], which reflects a balance between kinases and phosphatases in the cell.

ERK1/2 activity is inhibited by dephosphorylation of either threonine or tyrosine residues. This can be achieved using the serine/threonine-specific phosphatase PP2A, tyrosine-specific phosphatases or by dual-specificity (threonine/tyrosine) phosphatases [4]. Mitogen-activated protein kinase phosphatase-3 (MKP-3; also known as dual-specificity phosphatase 6 (DUSP6), Pyst1, or rVH6) selectively dephosphorylates both threonine and tyrosine residues within a Thr-X-Tyr motif of ERK1/2 in the cytoplasm. The dephosphorylation prevents nuclear translocation of ERK1/2 [5], while showing little effect on c-Jun NH2-terminal kinase (JNK)/stress-activated protein kinase (SAPK) and p38 MAPK [6]. MKP-3 expression is upregulated by ERK1/2 via the Ets response element within the *MKP-3* promoter upon stimulation of both the fibroblast growth factor 2 (FGF2) in NIH3T3 cells [7] and the epidermal growth factor (EGF) in human lung cancer cells [8]. These findings suggest that growth factor-induced ERK1/2 activity is controlled by a negative feedback loop involving the upregulation of MKP-3 expression. Other studies have demonstrated that MKP-3 is overexpressed in breast epithelial H-Ras MCF10A cells that stably express activated H-Ras [9] as well as in human melanoma cell lines harboring activating mutations in B-RAF and N-Ras [10]. However, the molecular mechanism through which constitutively active Ras induces MKP-3 expression remains largely unknown. In this study, we examined the molecular mechanism by which exogenous expression of constitutively active H-Ras regulates *MKP-3* transcription in NIH3T3 cells and found that c-Myb plays an important role in H-Ras-induced *MKP-3* transcription.

Abbreviations: ERK1/2, extracellular signal-regulated kinase 1 and 2; MAPK, the mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MKP-3, mitogen-activated protein kinase phosphatase-3; ChIP, chromatin immunoprecipitation.

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2. Materials and methods

2.1. Cells and reagents

Tetracycline-inducible NIH3T3tet-on/H-RasG12R cells were generated as described elsewhere [11]. The expression plasmid for constitutively active Ras (pSG5/V12Ras) was donated by Julian Downward (Cancer Research UK London Research Institute, London, UK).

2.2. Western blot analysis

Cells were lysed in a buffer containing 20 mM HEPES (pH 7.2), 1% Triton X-100, 10% glycerol, 400 mM NaCl, 10 μg/mL leupeptin, and 1 mM PMSF. Western blot analysis was performed according to standard procedures using antibodies against H-Ras (1:500; Oncogene), phospho-ERK1/2 (Thr202/Tyr204; 1:1000; Cell Signaling), c-Myb (1:2000; Santa Cruz Biotechnology), MKP-3 (1:5000; Cell Signaling), and GAPDH (1:2000; Santa Cruz Biotechnology).

2.3. Northern blot analysis

Total RNA samples (10 μg) were separated by electrophoresis on a formaldehyde/agarose gel and transferred to a Hybond N^+ nylon membrane (Amersham Pharmacia Biotech). Northern blotting was performed with [γ-^{32}P]dCTP-labeled MKP-3 cDNA probes using a High Prime DNA Labeling Kit (Roche), followed by hybridization with a *glyceraldehydes-3-phosphate dehydrogenase* (*Gapdh*) cDNA probe.

2.4. Electrophoretic mobility shift assay (EMSA)

Synthetic deoxyoligonucleotides (4 pmol) corresponding to the Myb binding sequence (5′-acggcaacagccccttc-3′) within the *MKP-3* promoter were end-labeled with [γ-^{32}P]dATP (Amersham Biosciences) through incubation with 10 units of T4 polynucleotide kinase (Promega) and 5 μL of T4 polynucleotide kinase buffer for 30 min at 37 °C, followed by inactivation at 65 °C for 10 min. For EMSA, 10 μg of nuclear extract was mixed with the binding buffer (50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 2.5 mM dithiothreitol, 2.5 mM EDTA, 250 mM NaCl, 20% glycerol), and 1 μg of poly(dI-dC) (Amersham Biosciences) added as a non-specific competitor, as described previously [12].

2.5. Chromatin immunoprecipitation (ChIP) assay

NIH3T3tet-on/H-RasG12R cells cultured in the absence or presence of doxycycline (2 μg/mL) for 48 h were treated with 1% formaldehyde to cross-link the DNA. The cells were lysed and chromatin immunoprecipitated using a rabbit anti-c-Myb antibody or normal rabbit IgG. The cross-linking of protein to DNA and chromatin immunoprecipitation were performed as described previously [13]. The following promoter-specific primers were used to amplify the *MKP-3* gene promoter sequences by polymerase chain reaction (PCR): 5′-tgcactggggcttatccg-3′ (target region forward primer, −176/−158), 5′-gatacattctctcggtcagc-3′, (target region reverse primer, −42/−23), 5′-acaatagaaccgagcgcg-3′ (off-target region forward primer, −1475/−1458), 5′-agagacctggagcggaaaa-3′ (off-target region reverse primer, −1298/−1280).

2.6. Construction and mutagenesis of the MKP-3 promoter-reporter construct

A fragment of the mouse *MKP-3* gene spanning nucleotides −1597 to −10 (transcription start site numbered as +1) was amplified from mouse genomic DNA (Promega) by PCR using the primers 5′-agctccttttccctgggacc-3′ (forward; −1597/−10) and 5′-agagaatgtatccattgagacgc-3′ (reverse; −34/−10). The amplified PCR products were ligated into a T&A vector (RBC Bioscience), digested with *Hind*III, and then subcloned into the luciferase reporter plasmid pGL3-basic (Promega), yielding pMkp3-Luc(−1597/−10). A series of deletion constructs was generated using pMkp3-Luc(−1597/−10) as a template. Forward primer sequences were 5′-ctaacttaagattgtaagcgtcg-3′ (−386/−10), 5′-gcagcttgtttg-cactggggc -3′ (−186/−10), 5′-tgaatgacaaactcattaacaa-3′ (−133/−10), and 5′-cagcgcgctcattggctgacc-3′ (−56/−10). One reverse primer (−34/−10) was used for all deletion constructs. The amplified PCR products were ligated into the *Kpn*I/*Bgl*II sites of the pGL3-basic vector, yielding pMkp3-Luc(−386/−10), pMkp3-Luc(−186/−10), pMkp3-Luc(−133/−10), and pMkp3-Luc(−56/−10). The pMkp3-Luc(−1129/−10) and pMkp3-Luc(−712/−10) were generated by digestion of pMkp3-Luc(−1597/−10) with *Nhe*I/*Hind*II (−1129/−10) or *Sma*I/*Hind*III (−712/−10). Site-specific mutation of the Myb binding site was performed with a QuickChange site-directed mutagenesis system (Stratagene) using pMkp3-Luc(−186/−10) as a template. All mutations were verified by DNA sequencing.

2.7. Transient transfection and luciferase reporter assay

For the promoter reporter assay, cells were seeded into 12-well plates and transfected with 0.2 μg of the *MKP-3* promoter constructs using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. For Myb-dependent transcriptional activity, NIH3T3 cells cultured in 12-well plates were transfected with 0.1 μg of the pMyb-Luc plasmid (RIKEN BioResource Center, Ibaraki, Japan), along with 50 ng of the pRL-null plasmid encoding *Renilla* luciferase. To assess transfection efficiency, 50 ng of pRL-null plasmid encoding Renilla luciferase was included in all samples. At 24 h post-transfection, the levels of firefly and Renilla luciferase activities were measured using a Dual-Glo™ Luciferase assay system (Promega). Luminescence was also measured using a dual luminometer (Centro LB960; Berthold Tech). The firefly luciferase activity was normalized to the Renilla activity, and the relative amount of luciferase activity in the control cells was set to "1".

2.8. siRNA-mediated c-Myb silencing

A mixture of double-stranded RNA nucleotides targeting four different sequences of mouse Myb mRNA (ON-TARGETplus SMARTpool L-044112-00-0005) and a non-targeting negative control siRNA were obtained from Dharmacon (Thermo Fisher Scientific., Lafayette, CO). NIH3T3 cells were transfected with siRNA oligonucleotide pools using DharmaFECT reagent according to the manufacturer's protocol (Dharmacon).

2.9. Statistical analysis

Each experiment was performed at least three times. The results are plotted as means with SD. Statistical comparisons were made by a one-way ANOVA followed by the Turkey–Kramer Multiple Comparisons Test using GraphPad InStat v3.0 software. A p value of <0.05 was considered statistically significant.

3. Results

3.1. MKP-3 is induced by activation of Ras signaling pathway

We previously described a NIH3T3 cell line (NIH3T3tet-on/H-RasG12R), in which the expression of constitutively active Ras (H-RasG12R) can be induced by addition of doxycycline [11]. In

this system, the H-RasG12R protein was detectable within 12 h after the addition of 2 μg/mL doxycycline (Fig. 1A). To investigate whether MKP-3 expression is regulated by the Ras signaling pathway, serum-starved NIH3T3tet-on/H-RasG12R cells were exposed to doxycycline and Northern blot analysis was used to examine the *MKP-3* mRNA levels. A time kinetic study showed that *MKP-3* mRNA was induced within 24 h of doxycycline addition (Fig. 1B).

To determine whether Ras activates the *MKP-3* gene promoter, we isolated the 5′-regulatory region of the mouse *MKP-3* gene (nucleotides from −1597 to −10 relative to the transcription start site) and constructed a series of its deletion constructs (Fig. 1C). 5′-deletion of the promoter from −1597 to −1130 enhanced reporter activity by doxycycline more than the full-length construct (−1597/−10), suggesting the presence of negative regulatory sequences between −1597 and −1130 in response to Ras activation. Further deletion up to −132 steadily decreased reporter activity, suggesting that multiple regulatory elements might be involved in Ras-induced *MKP-3* transcription. Of note, deletion of the sequence between −133 and −57 resulted in a near complete loss of doxycycline-induced promoter reporter activity. These data suggest that the Ras-response element is located between −133 and −57.

3.2. Myb-binding plays an important role in Ras-induced MKP-3 promoter activation

Computational sequence analysis using the web-based program MatInspector (Genomatix) revealed the presence of Sox-5 and Myb sites at −119 and −73, respectively (Fig. 2A). Given that c-Myb is strongly induced by oncogenic RasV12 in NIH3T3 cells [14] and that its role in the regulation of *MKP-3* transcription has not been studied, we were particularly interested in whether c-Myb plays a role in *MKP-3* promoter activation. To determine whether the Ras pathway activates the Myb-binding element at −73, we introduced site-directed mutations (aacag → GGGag) within the putative Myb-binding motif in the pMkp3-Luc(−186/−10) construct, yielding pMkp3-Luc(−186/−10)mtMyb. After transient transfection into NIH3T3tet-on/H-RasG12R cells, the pMkp3-Luc(−186/−10)mtMyb reporter displayed low luciferase activity compared to the wild-type construct in response to doxycycline treatment (Fig. 2B). In contrast, point mutation of the conserved Sox-5 site (attca → GGCca) had little effect on doxycycline-induced reporter activity. To determine whether c-Myb transactivates the Myb-binding element within the MKP-3 promoter, we transiently co-transfected c-Myb expression plasmid with either wild-type −186/−10 construct or c-Myb site mutant −186/−10 construct (mtMyb) into NIH3T3 cells. Transfection of c-Myb elevated reporter activity in a c-Myb plasmid concentration-dependent fashion, while the c-Myb site mutant reporter had no effect (Fig. 2C). These data suggest that the Myb-binding element at −73 plays an important role in Ras-induced *MKP-3* promoter activation.

3.3. c-Myb directly interacts with the MKP3 promoter in response to activation of the Ras signaling

To further investigate whether c-Myb binds to the Myb-binding motif within the *MKP-3* promoter, EMSA was performed with an oligonucleotide probe containing Myb-binding core sequences. Nuclear extracts from doxycycline-treated NIH3T3tet-on/H-RasG12R cells were prepared and incubated with radiolabeled oligonucleotides containing a Myb-binding sequence corresponding to −73 to −59 within the *MKP-3* promoter. As shown in Fig. 3A, Myb-binding probes formed a protein–DNA complex, which was prevented by the addition of unlabeled oligonucleotide probe. The specificity of Myb binding was further confirmed by the failure of a radiolabeled probe carrying a mutation in the Myb binding core motif (aacag → GGGag) in forming a protein–DNA complex. To verify Myb protein binding to the *MKP-3* promoter at the chromatin level, we cross-linked cellular DNA and bound proteins in doxycycline-treated NIH3T3tet-on/H-RasG12R cells using formaldehyde. Cross-linked DNA–protein complexes were subjected to ChIP using a rabbit anti-Myb antibody or normal rabbit IgG. The resulting immunoprecipitated DNA was amplified by PCR using primers targeted to the promoter elements (−176 to −23) of the *MKP-3* gene. As shown in Fig. 3B, a noticeable increase in the intensity of the DNA band was observed

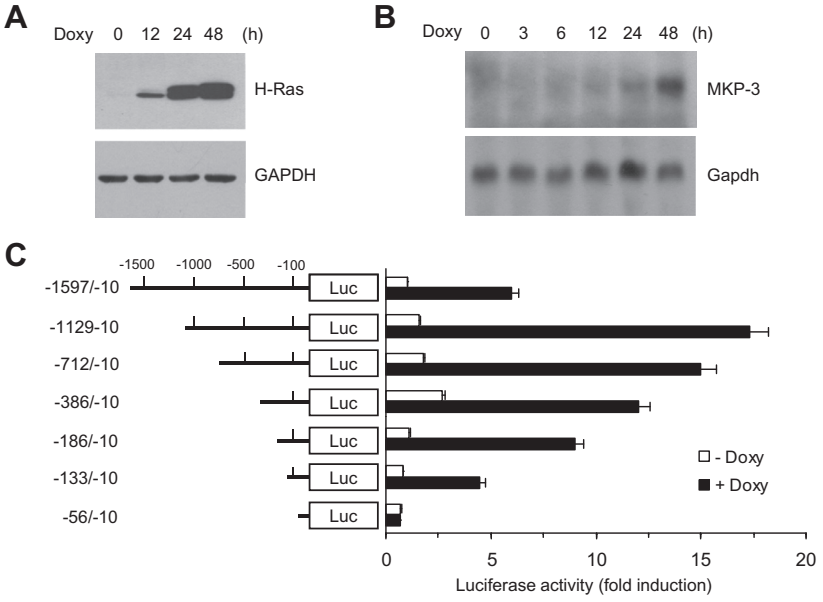

Fig. 1. Effect of constitutively active Ras expression on MKP-3 expression. (A) NIH3T3tet-on/H-RasG12R cells were cultured in the presence of doxycycline (Doxy; 2 μg/mL) for the indicated periods of time, and Western blot analysis was performed using antibodies against H-Ras and GAPDH (internal control). (B) Serum-starved NIH3T3tet-on/H-RasG12R cells cultured in the presence of doxycycline for the indicated times, and total RNA was isolated. Northern blot analysis was performed using MKP-3 and Gapdh cDNA probes. (C) Identification of Ras responsive region within the MKP-3 promoter. NIH3T3 cells were transiently transfected with 0.2 μg of a series of 5′-deletion constructs of mouse MKP-3 promoter reporter plasmids, along with 50 ng of the Renilla luciferase expression plasmid pRL-null to normalize transfection efficiency. After 24 h, the cells were treated with doxycycline (2 μg/mL) for an additional 24 h. The cells were collected and assayed for luciferase activity.

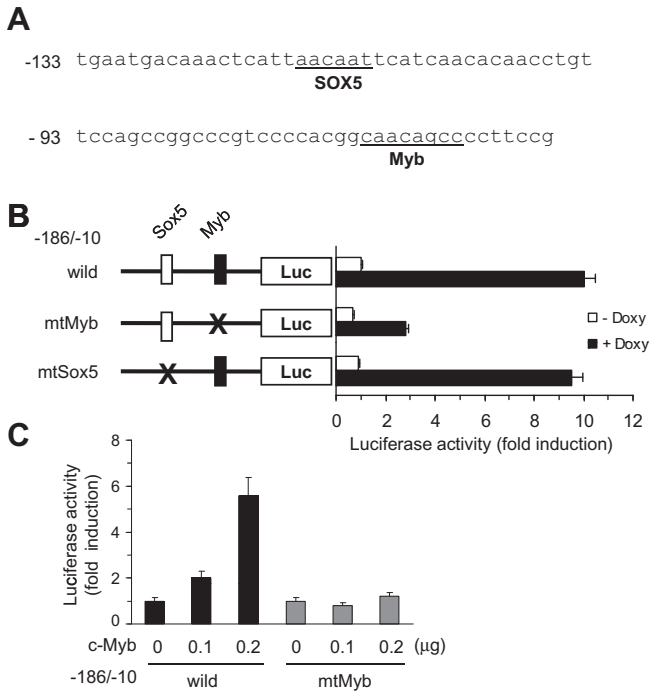

Fig. 2. Requirement of the Myb response element for oncogenic Ras-induced MKP-3 promoter activity. (A) Putative SOX5 and Myb-binding elements within the promoter region of the mouse MKP3 gene. (B) NIH3T3 cells were transfected with 0.2 μg of wild-type pMkp3-Luc(−186/−10), Myb site-specific mutant pMkp3-Luc(−186/−10)mtMyb, or Sox5 site-specific mutant pMkp3-Luc(−186/−10)mtSox5 construct. After 24 h, the cells were treated with doxycycline (2 μg/mL) for an additional 24 h. The cells were collected and assayed for luciferase activity afterwards. Schematics of the reporter constructs used in the transfection assay show the targeted mutations of the conserved Myb or Sox5 binding motif. Data represent means ± SD of three independent experiments performed in triplicate. (C) NIH3T3 cells were co-transfected with expression plasmid for c-Myb (0, 0.1, or 0.2 μg) and wild-type pMkp3-Luc(−186/−10) or Myb site-specific mutant pMkp3-Luc(−186/−10)mtMyb reporter, along with 50 ng pRL-null/Luc to normalize transfection efficiency. After 48 h, the cells were collected and assayed for luciferase activity.

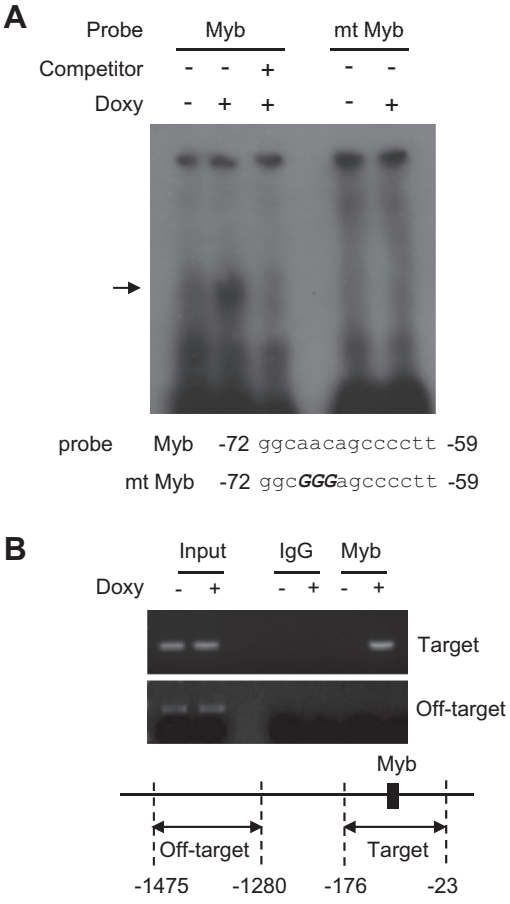

Fig. 3. Association of c-Myb with the MKP-3 promoter. (A) NIH3T3tet-on/H-RasG12R cells were cultured in the absence or presence of doxycycline (2 μg/mL) for 48 h. Nuclear extracts were prepared and incubated with a ^{32}P-labeled oligonucleotide probe containing the Myb-binding sequence (Myb; 5′-acggcaacagccccttc-3′) or mutated Myb-binding sequence (mt Myb; 5′-acggcGGGagccccttc-3′). For the competition assay, unlabeled oligonucleotide (*Competitor*) was incubated for 15 min after adding labeled probe at 20-fold excess. The resulting DNA–protein complexes were separated in 0.5 × TBE polyacrylamide gels. *Arrow*, DNA–protein complex. (B) NIH3T3tet-on/H-RasG12R cells were cultured in the absence or presence of doxycycline (2 μg/mL) for 48 h, cross-linked, lysed, and immunoprecipitated with a rabbit anti-c-Myb antibody or with normal rabbit IgG (negative control). The precipitated DNA was subjected to PCR with primers specific to the MKP3 promoter. An aliquot of input DNA was used as a positive control. The schematic representation shows the locations of the Myb-binding site and off-target region in the MKP-3 promoter.

following doxycycline treatment for the anti-Myb antibody compared to the untreated control. Doxycycline treatment had no effect on the normal rabbit IgG. The off-target region (from −1475 to −1280) was not amplified, although positive results were obtained from the input genomic DNA. These data indicate that c-Myb protein physically interacts with the *MKP-3* promoter in response to Ras activation.

3.4. c-Myb is upregulated by activation of Ras signaling

To investigate whether active Ras stimulates Myb-dependent transcription, we utilized a *cis*-acting reporter assay system. A pMyb-Luc plasmid containing four Myb-binding core sequences was transfected into NIH3T3tet-on/H-RasG12R cells and treated with doxycycline. Luciferase reporter activity was enhanced in a time-dependent manner after doxycycline treatment (Fig. 4A). We next evaluated whether active Ras expression up-regulates c-Myb expression. NIH3T3tet-on/H-RasG12R cells were cultured in the presence of doxycycline and the c-Myb protein level assessed by Western blotting. c-Myb gradually increased after 12 h of doxycycline treatment, which paralleled the elevation of MKP-3 levels (Fig. 4B). We also found that c-Myb expression alone increased the level of MKP3 protein in the absence of doxycycline treatment (Fig. 4C).

To further corroborate the role of c-Myb in Ras-induced MKP-3 expression, RNA interference was employed. Transient expression of c-Myb siRNA clearly attenuated doxycycline-induced accumulation of c-Myb and MKP-3 proteins (Fig. 4D). Doxycycline-induced *MKP-3* promoter activity was also strongly reduced by expression of c-Myb siRNA, but not by scrambled control siRNA (Fig. 4E). Furthermore, transient transfection of RasV12 also led to a strong increase in the promoter reporter activity of the pMkp3-Luc(−186/−10) construct, which was significantly abrogated by expression of c-Myb siRNA (Fig. 4F). These results demonstrate that Ras induction of c-Myb contributes to *MKP-3* transcriptional activity.

4. Discussion

Accumulating evidence demonstrates that MKP-3 expression is rapidly induced as a negative feedback regulatory mechanism by the MEK1/ERK1/2 pathway in response to growth factor stimulation [7,8,15,16]. The present study revealed an additional mechanism by which active Ras can modulate ERK1/2 activity through c-Myb-mediated induction of MKP-3 in NIH3T3 cells.

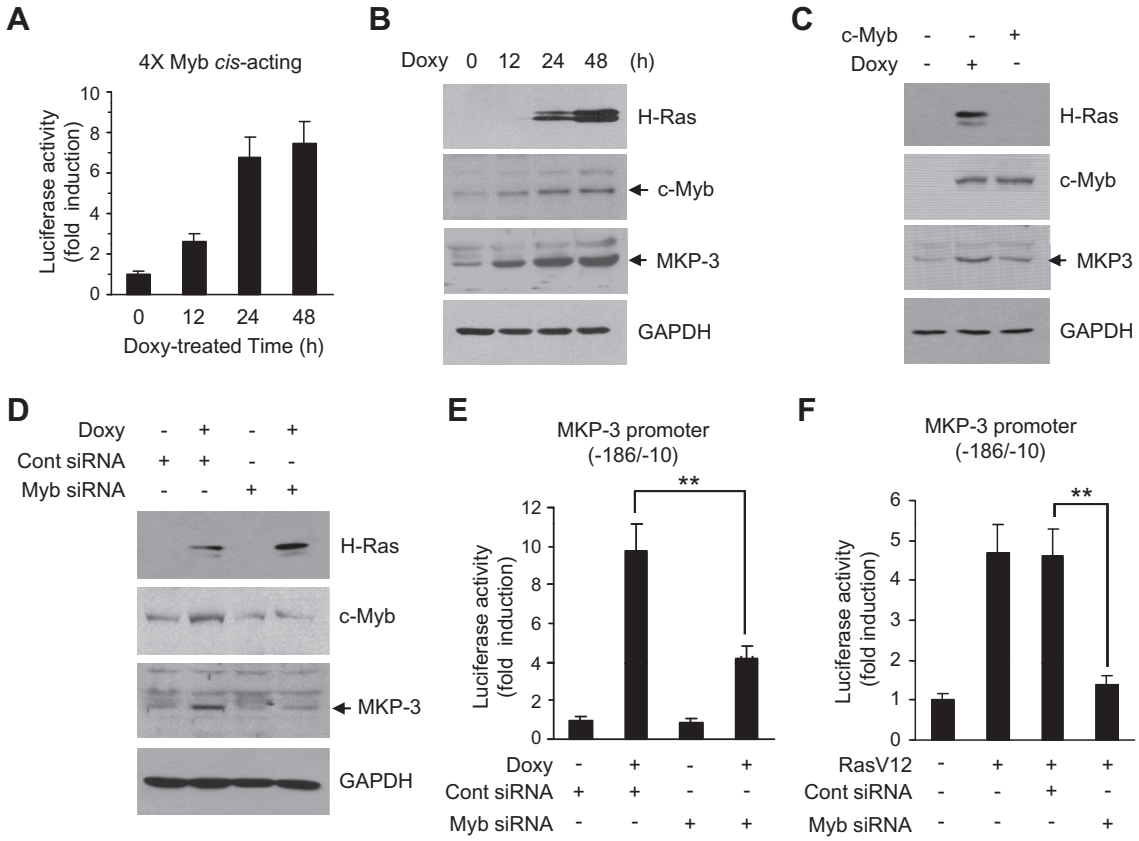

Fig. 4. Role of c-Myb in Ras-induced MKP-3 expression. (A) NIH3T3 cells were transfected with 50 ng pMyb-Luc, along with 50 ng pRL-null/Luc to normalize transfection efficiency. After 24 h, doxycycline (2 μg/mL) was added for the indicated times. The cells were collected and assayed for luciferase activity. (B) NIH3T3tet-on/H-RasG12R cells were treated with doxycycline (2 μg/mL) for the indicated periods of time, and Western blot analysis was performed using antibodies against H-Ras, c-Myb, MKP-3, and GAPDH (internal control). (C) NIH3T3tet-on/H-RasG12R cells were cultured in presence of doxycycline (2 μg/mL) or transfected with 0.5 μg expression plasmid for c-Myb as indicated. After 48 h, the cells were collected and Western blot analysis was performed using antibodies against H-Ras, c-Myb, MKP3, and GAPDH (internal control). (D) NIH3T3tet-on/H-RasG12R cells were cultured in the presence of doxycycline (2 μg/mL) for 24 h, followed by transfection with scrambled or c-Myb SMARTpool siRNA. After 24 h, cells were collected and Western blot analysis was performed using antibodies against H-Ras, c-Myb, MKP-3, and GAPDH (internal control). (E) NIH3T3tet-on/H-RasG12R cells were cultured in the presence of doxycycline (2 μg/mL) for 24 h, followed by transfection with pMkp3-Luc(−186/−10) along with scrambled siRNA or c-Myb SMARTpool siRNA. After 24 h, cells were collected and luciferase activity was measured. Firefly luciferase activity was normalized to the Renilla luciferase activity. The data shown represent the means ± SD of three independent experiments performed in triplicate (**$P < 0.01$). (F) NIH3T3 cells were transfected with active H-RasV12 and pMkp3-Luc(−186/−10), along with scrambled or c-Myb SMARTpool siRNA. After 24 h, cells were collected and luciferase activity was measured. Firefly luciferase activity was normalized to the Renilla luciferase activity. The data shown represent the means ± SD of three independent experiments performed in triplicate (**$P < 0.01$).

To determine the mechanism responsible for activating *MKP-3* transcription through the Ras signaling pathway, we used 5-deletion analysis of the *MKP-3* promoter to identify the participating *cis*-acting response elements. We observed that the promoter region spanning positions −133 to −56 was indispensable for Ras-triggered *MKP-3* promoter activity. Amongst the various transcription factor sites in this region, we were particularly interested in the c-Myb binding site because the role of c-Myb in the regulation of *MKP-3* transcription has yet to be studied. c-Myb has been identified in diverse cell types, including hematopoietic tissues, fibroblasts and smooth muscle cells [17,18], as well as several human tumors including colon carcinoma and small cell lung carcinoma [19,20]. Our promoter analyses demonstrated that mutation of the Myb consensus motif strongly abrogated activation of the *MKP-3* promoter by the expression of constitutively active H-Ras. EMSA and ChIP experiments showed that c-Myb bound to the *MKP-3* promoter in response to the expression of active Ras. Furthermore, the introduction of c-Myb-specific siRNA into NIH3T3 cells strongly attenuated Ras-induced *MKP-3* promoter activity and MKP-3 protein accumulation. Although we did not directly examine the roles of other regulatory factors, our results point to a prominent role for the conserved Myb binding site.

The Ras and PI3K pathways can be mutually regulated by several cross-talk mechanisms in different cellular contexts. For example, Ras interacts directly with the p110 catalytic subunit of PI3K and promotes the activation of PI3K signaling [21]. In addition, prolonged Ras activation down-regulates the expression of PTEN, a lipid phosphatase that negatively regulates PI3K signaling [22]. On the contrary, PI3K-Akt pathway inhibits the Ras pathway through the phosphorylation of Raf1 on Ser-259 [23]. During limb development, the PI3K pathway inhibits ERK1/2 activity through upregulation of MKP-3 expression in response to FGF8 stimulation [24]. However, other studies have demonstrated that FGF-induced MKP-3 expression is mediated by an ERK1/2-dependent Ets transcription factor, not by the PI3K pathway [7]. In our previous study, we reported that PI3K upregulates MKP-3 expression in NIH3T3 cells [25]. We also found that c-Myb and MKP-3 expression was attenuated when the PI3K inhibitor LY294002 was added during active H-Ras stimulation (data not shown). Furthermore, transfection of an Akt expression plasmid alone significantly stimulated *MKP-3* promoter activity of the −186/−10 construct. However, treatment with the Akt specific inhibitor API-2 strongly blocked the effect of H-RasV12C40 on promoter activity of the −186/−10 construct (data not shown). These data suggest that the PI3K/Akt

pathway might be involved in MKP-3 expression via the expression of c-Myb in response to prolonged expression of constitutively active Ras in NIH3T3 cells. Taking these observations into consideration, it is possible that growth factor induces MKP-3 expression through ERK1/2-dependent Ets, while long-term activation of oncogenic Ras induces MKP-3 expression through PI3K-mediated c-Myb expression.

In conclusion, we have demonstrated that the transcription factor c-Myb directly binds to the *MKP-3* promoter and stimulates the *MKP-3* gene transcription to negatively regulate Ras-Raf-MEK-ERK signaling pathway in NIH3T3 cells expressing constitutively active H-Ras.

Acknowledgments

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