The Wnt–NLK Signaling Pathway Inhibits A-Myb Activity by Inhibiting the Association with Coactivator CBP and Methylating Histone H3

Toshihiro Kurahashi,^{*,†} Teruaki Nomura,^{*,†} Chie Kanei-Ishii,^{*} Yoichi Shinkai,[‡] and Shunsuke Ishii^{*,†}

^{*}Laboratory of Molecular Genetics, RIKEN Tsukuba Institute, Tsukuba, Ibaraki 305-0074, Japan; [†]Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8577, Japan; and [‡]Experimental Research Center for Infectious Diseases, Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan

Submitted May 31, 2005; Revised July 13, 2005; Accepted July 18, 2005
Monitoring Editor: William Tansey

The *c-myb* proto-oncogene product (c-Myb) regulates proliferation and differentiation of hematopoietic cells. Recently we have shown that c-Myb is degraded in response to Wnt-1 stimulation via a pathway involving TAK1 (TGF- β -activated kinase), HIPK2 (homeodomain-interacting protein kinase 2), and NLK (Nemo-like kinase). NLK and HIPK2 bind directly to c-Myb and phosphorylate c-Myb at multiple sites, inducing its ubiquitination and proteasome-dependent degradation. The mammalian *myb* gene family contains two members in addition to *c-myb*, *A-myb*, and *B-myb*. Here, we report that the Wnt–NLK pathway also inhibits A-Myb activity, but by a different mechanism. As in the case of c-Myb, both NLK and HIPK2 bound directly to A-Myb and inhibited its activity. NLK phosphorylated A-Myb, but did not induce A-Myb degradation. Overexpression of NLK inhibited the association between A-Myb and the coactivator CBP, thus, blocking A-Myb-induced *trans*-activation. The kinase activity of NLK is required for the efficient inhibition of the association between A-Myb and CBP, although the kinase-negative form of NLK also partly inhibits the interaction between A-Myb and CBP. Furthermore, NLK induced the methylation of histone H3 at lysine-9 at A-Myb-bound promoter regions. Thus, the Wnt–NLK pathway inhibits the activity of each Myb family member by different mechanisms.

INTRODUCTION

The *c-myb* proto-oncogene is the cellular progenitor of the *v-myb* oncogenes carried by the chicken AMV (avian myeloblastosis virus) and E26 retroviruses, which cause acute myeloblastic leukemia and erythroblastosis, respectively (Klempnauer *et al.*, 1982; Leprince *et al.*, 1983). A study of *c-myb*-deficient mice indicated that *c-myb* is essential for proliferation of immature hematopoietic cells (Mucenski *et al.*, 1991), and analysis of tissue-specific *c-myb* knockout mice revealed that *c-myb* is also required for T-cell development at several stages (Bender *et al.*, 2004). The vertebrate *myb* gene family contains two other members, *A-myb* and *B-myb* (Nomura *et al.*, 1988). *A-myb* is highly expressed in a few cell types, including male germ cells and female breast ductal epithelium (Trauth *et al.*, 1994). Consistent with this expression pattern, *A-myb* is required for spermatogenesis and development of breast tissue after pregnancy (Toscani *et al.*,

1997). *B-myb* expression is broader than that of *c-myb* or *A-myb* (Nomura *et al.*, 1989), and *B-myb* is essential for inner cell mass (ICM) formation in the early stages of development (Tanaka *et al.*, 1999).

All three members of the vertebrate *myb* family proteins have a conserved DNA-binding domain (DBD), which consists of three imperfect tandem repeats of 51–52 amino acids that recognize the specific DNA sequence 5'-AACNG-3' (Biedenapp *et al.*, 1988; Ogata *et al.*, 1994). The transcriptional activation domain containing the acidic amino acid-rich region is also localized adjacent to the DBD in all three *myb* family members (Sakura *et al.*, 1989; Nakagoshi *et al.*, 1993; Takahashi *et al.*, 1995). The transcriptional coactivator CBP binds to this activation domain (Dai *et al.*, 1996; Oelgeschlager *et al.*, 1996; Facchinetti *et al.*, 1997; Bessa *et al.*, 2001). The vertebrate *myb* family proteins regulate transcription of various target genes such as *c-myc*, which are involved in cell cycle control, suppression of apoptosis, or differentiation control (Ness *et al.*, 1989; Nakagoshi *et al.*, 1992; Frampton *et al.*, 1996; Taylor *et al.*, 1996; Kowenz-Leuz *et al.*, 1997). The carboxy (C)-terminal portions of c-Myb and A-Myb have a negative regulator domain (NRD), and its deletion increases *trans*-activation by c-Myb and A-Myb (Sakura *et al.*, 1989; Takahashi *et al.*, 1995). The NRD of c-Myb contains two $\Phi\text{XX}\Phi\Phi$ (Φ : hydrophobic amino acids) motifs (Kanei-Ishii *et al.*, 1992) and binds directly to two corepressors TIF1 β and BS69 (Ladendorff *et al.*, 2001; Nomura *et al.*, 2004). B-Myb activity is also suppressed by the corepressors N-CoR and SMRT (Li and McDonnell, 2002). The *trans*-activating capacity of both A-Myb and B-Myb is also positively regulated

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E05-05-0470>) on July 29, 2005.

Address correspondence to: Shunsuke Ishii (sishii@rtc.riken.jp).

Abbreviations used: A-Myb, *A-myb* gene product; ChIP, chromatin immunoprecipitation; c-Myb, *c-myb* proto-oncogene product; DBD, DNA-binding domain; H3K9, lysine-9 of histone H3; HIPK2, homeodomain-interacting protein kinase 2; HMTase, histone methyltransferase; NLK, Nemo-like kinase; NRD, negative regulatory domain; TAK1, TGF- β -activated kinase 1; TSA, trichostatin A.

through phosphorylation by cyclin A/Cdk2 (Ziebold and Klempnauer, 1997; Saville and Watson, 1998).

The functions of the nuclear oncogene products Jun/Fos and NF- κ B are regulated by signaling pathways, which include JNK/p38 and I κ B kinases, respectively (Ghosh and Karin, 2002; Weston and Davis, 2002). In contrast, the specific signaling pathways that regulate c-Myb activity have been unknown. Recently, we showed that c-Myb is phosphorylated and degraded via the Wnt-1 signaling pathway involving TAK1 (TGF- β -activated kinase), HIPK2 (homeodomain-interacting protein kinase 2), and NLK (Nemo-like kinase) (Kanei-Ishii *et al.*, 2004a). NLK and HIPK2 each bind directly to c-Myb, which results in phosphorylation of c-Myb at multiple sites, ubiquitination, and proteasome-dependent degradation. Because Wnt signaling controls differentiation or apoptosis in many cell types, including hematopoietic cells (Wodarz and Nusse, 1998; Peifer and Polakis, 2000), Wnt-induced c-Myb degradation may play some role in the proliferation and differentiation of hematopoietic cells. However, it is unknown whether the Wnt-NLK pathway also inhibits A-Myb and B-Myb activity. Because A-Myb and B-Myb function in different cell types than c-Myb, it is important to analyze the effect of the Wnt-1 signal on these members of the *myb* family proteins.

Here, we demonstrate that both A-Myb and B-Myb are inhibited by the Wnt-NLK pathway. However, NLK does not induce the degradation of A-Myb, but inhibits the interaction between A-Myb and the coactivator CBP and induces the methylation of histone H3 to block A-Myb activity.

MATERIALS AND METHODS

In Vitro Binding Assays

GST pulldown assays using GST-NLK and GST-HIPK2C were performed, as described previously (Kanei-Ishii *et al.*, 2004a). To increase the solubility of GST fusion proteins expressed in bacteria, the thioredoxin coexpression system (Yasukawa *et al.*, 1995) was used. The binding buffer used for most experiments consists of 20 mM HEPES, pH 7.5, 1 mM dithiothreitol (DTT), 0.1% NP-40, and 100 mM NaCl (for interactions between A-Myb and NLK or HIPK2) or 150 mM NaCl (for interactions between A-Myb and deletion mutants of CBP). GST pulldown assays using GST-CBP-KIX was performed, as described previously (Kanei-Ishii *et al.*, 2004b).

Coimmunoprecipitation

To investigate the *in vivo* interaction between A-Myb and NLK, CV-1 cells (5×10^5 cells per 100-mm dish) were transfected by LipofectAMINE Plus (Invitrogen, Carlsbad, CA) with the plasmids to express FLAG-A-Myb (2 μ g) or HA-NLK (1 μ g). Total plasmid amounts were adjusted to 8.0 μ g by adding empty plasmid. The FLAG-A-Myb expression vector contains four tandem repeats of the FLAG tag at the N-terminus of A-Myb and the chicken cytoplasmic β -actin promoter. Transfectants were incubated for 24–36 h and lysed in TNE buffer (10 mM Tris-HCl, pH 7.8, 1 mM EDTA, 1% NP-40, protease inhibitor cocktail, 50 mM NaF, 25 mM β -glycerophosphate) containing 500 mM NaCl. Anti-HA (12CA5, Roche Diagnostics, Indianapolis, IN) or normal mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) were used for immunoprecipitation. The immunocomplexes were washed three times with TNE buffer containing 1 M NaCl. To examine the effect of NLK on the A-Myb-CBP or B-Myb-CBP interaction, CV-1 cells were transfected with the plasmid encoding HA-tagged CBP (4 μ g), A-Myb or B-Myb (2 μ g), NLK (1 μ g) or the control plasmid, and the internal control pCMV-luc (0.01 μ g; total of 8.01 μ g DNA) using LipofectAMINE Plus. In the case of B-Myb, transfected cells were treated with MG132 (50 μ M) for 7 h before preparation of lysates. The immunoprecipitation was performed as described above. For immunoblotting, immunoprecipitates or whole cell lysates were resolved on SDS-PAGE and transferred to Hybond-P membranes (Amersham, Piscataway, NJ). The membranes were immunoblotted with various antibodies and the bound antibodies were visualized by horseradish peroxidase-conjugated antibodies against rabbit or mouse IgG using ECL (Amersham).

Western Blotting

To examine the effect of NLK on the A-Myb levels, CV-1 cells were transfected with a mixture of FLAG-A-Myb expression plasmid (4 μ g), NLK expression plasmid (2 μ g), and the internal control plasmid pact- β -gal (0.3 μ g). Total plasmid amounts were adjusted to 8.3 μ g by adding empty plasmid. To

investigate the effect of NLK on the B-Myb levels, CV-1 cells were transfected with a mixture of FLAG-B-Myb expression plasmid pact-4xFLAG-B-Myb (5 μ g), NLK expression plasmid (2 μ g), and the internal control plasmid pact- β -gal (0.3 μ g). In some cases, the transfected cells were treated with MG132 (50 μ M) for 7 h before preparation of lysates. To examine the effect of various components of the Wnt-NLK pathway on the A-Myb levels, CV-1 cells were transfected with a mixture of FLAG-A-Myb expression plasmid (2 μ g), the plasmid to express each component (1 μ g), and the internal control pCMV-luc (0.01 μ g; total 8.01 μ g). Cells were cultured for 40 h after transfection and then lysed in SDS sample buffer with mild sonication and subjected to SDS-PAGE, Western blotting with an anti-FLAG monoclonal antibody (Sigma, St. Louis, MO), and ECL detection. Aliquots of the cells were used to determine the transfection efficiency by measuring β -galactosidase or luciferase activity and the amounts of lysates used for Western blotting were normalized based on the β -galactosidase or luciferase activity.

Kinase Assays

For phosphorylation of A-Myb by NLK (Figure 2B), 293 cells were transfected with the FLAG-A-Myb expression plasmid (pact-FLAG-A-Myb) or the FLAG-NLK expression plasmid. The lysates were prepared from the transfected cells using NET buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% NP-40, protease inhibitor cocktail) containing 150 mM NaCl and immunoprecipitated with anti-FLAG antibody (M2, Sigma). The immunocomplexes were sequentially washed with washing buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 M NaCl, 2% NP-40, and 10% glycerol) and then NLK buffer (10 mM HEPES, pH 7.4, 1 mM DTT, and 5 mM MgCl₂). An aliquot of the A-Myb immunocomplexes was used for Western blotting. FLAG-NLK proteins were eluted from the immunocomplexes with excess FLAG peptide. To phosphorylate A-Myb proteins, the A-Myb immunocomplexes were mixed with the eluted FLAG-NLK proteins and [γ -³²P]ATP and incubated at 25°C for 2 h. The amount of A-Myb protein used in the kinase reaction was adjusted based on the Western blotting data. Phosphorylated proteins were analyzed by SDS-PAGE and autoradiography.

Luciferase Reporter Assays

Using LipofectAMINE PLUS, CV-1 cells (1×10^5 cells per 60-mm dish) were transfected with the 6MBS-I-SV40-luc reporter (0.5 μ g), the A-Myb or B-Myb expression plasmid (0.2 μ g), the NLK (0.03, 0.1 or 0.3 μ g), or HIPK2 (0.1, 0.3, or 1.0 μ g) expression plasmid, and the internal control plasmid pCMV-luc (0.1 μ g), followed by luciferase assays. The chicken β -actin promoter was used to express A-Myb and B-Myb. In experiments to determine the effect of Wnt-NLK pathway components on A-Myb activity (Figure 3), the pGL3-R2.2–6MBS-I-TK-luc reporter, which was made using the Rapid Response Reporter Vectors (Promega, Madison, WI) was used. CV-1 cells (2×10^5 cells per 60-mm dish) were transfected with the pGL3-R2.2–6MBS-I-TK-luc reporter (0.5 μ g), and plasmids to express A-Myb (0.2 μ g), R-Fz1 (0.1, 0.3, or 1.0 μ g), R-Fz2 (0.1, 0.3, or 1.0 μ g), TAK1/TAB1 (0.03, 0.1 or 0.3 μ g), or the dominant-negative form of HIPK2 (1.0 μ g), and the internal control plasmid pact- β -gal (0.05 μ g). Luciferase assays were performed 24 h after transfection. The total amount of plasmid DNA was adjusted to 2.05 μ g by adding empty plasmid.

In Vivo Acetylation Assay

To investigate the *in vivo* acetylation of A-Myb by CBP, CV-1 cells were transfected with the CBP expression plasmid pcDNA3-CBP-FLAG (4 μ g) together with pact-FLAG-A-Myb (1 μ g) and pCMV-FLAG-NLK or a control plasmid (1 μ g). Cells were culture for 2 d after transfection, lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 mM sodium butyrate) and centrifuged at 15,000 rpm. The supernatants were subjected to SDS-PAGE and Western blotting. The acetylated forms of A-Myb were detected with an anti-acetylated lysine polyclonal antibody (Cell Signaling, Beverly, MA), and a mixture of acetylated and nonacetylated forms of A-Myb were detected with the anti-FLAG M2 antibody.

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation (ChIP) assays were carried out essentially as described (Tanikawa *et al.*, 2004) and in the Upstate Biotechnology protocol (Lake Placid, NY), with minor modifications. In brief, 1.5×10^5 CV-1 cells were transfected with FLAG-A-Myb expression plasmid or control plasmid (0.5 μ g), CBP-HA expression plasmid (1.0 μ g), NLK expression plasmid or control plasmid (0.5 μ g), pGL3–6MBS-I-TK-luc reporter plasmid (0.1 μ g), and the internal control plasmid pCMV-luc (0.01 μ g). The cytomegalovirus promoter was used in all expression plasmids. Cells were cultured for 40 h after transfection and then cross-linked and processed according to the manufacturer's protocol. Immunoprecipitation was performed overnight at 4°C with 6 μ g of anti-FLAG antibody (M2, Sigma) or normal mouse IgG (Santa Cruz) as a negative control. The amounts of lysates used for immunoprecipitation was normalized based on the transfection efficiency (luciferase activity of pCMV-luc). In the ChIP assays to detect methylated histone H3, immunoprecipitation was performed using 2 μ g of K9-H3m3 antibody (Abcam, Cambridge, UK). The immunocomplexes were washed and incubated at 65°C in

Figure 1. NLK and HIPK2 bind to the DBD of A-Myb. (A) GST pulldown assay. (Top) The functional domains of A-Myb and the deletion mutants used are shown schematically. The results of binding assays shown below are indicated on the right. (Bottom) The input lanes show the ^{35}S -A-Myb variants, which were translated in vitro. The ^{35}S -A-Myb variant indicated above each lane was mixed with GST-NLK or GST-HIPK2C affinity resin, which have been described previously (Kanei-Ishii *et al.*, 2004a), and the bound proteins analyzed by SDS-PAGE and autoradiography. (B) Coimmunoprecipitation of A-Myb and NLK. Lysates were prepared from CV-1 cells transfected with a mixture of FLAG-A-Myb and HA-NLK expression plasmids. Lysates were precipitated by anti-HA antibody or control IgG and the immunocomplexes were analyzed by Western blotting using anti-FLAG or anti-HA antibodies. HA-NLK and FLAG-A-Myb in the whole cell extracts were detected by Western blotting (lower two panels). (C) Binding of B-Myb to NLK and HIPK2. In vitro-translated ^{35}S -B-Myb was mixed with the GST-NLK or GST-HIPK2C affinity resin, and the bound proteins were analyzed by SDS-PAGE and autoradiography.

100 μl of IP elution buffer (1% SDS, 0.1 M NaHCO_3 , 250 mM NaCl, 0.2 $\mu\text{g}/\mu\text{l}$ proteinase K, 10 mM DTT) to release the proteins. In immunocomplexes containing FLAG-A-Myb, the complexes were incubated in IP elution buffer in the presence of the FLAG peptide (300 $\mu\text{g}/\text{ml}$) to enhance the elution efficiency. The de-cross-linked chromatin DNA was further purified using QIAquick PCR Purification Kit (QIAGEN, Santa Clarita, CA) and eluted in 40 μl sterile water. The eluted DNA samples (5 μl) were used for real-time PCR (7500 Real Time PCR System, Applied Biosystems, Foster City, CA). The primers and TaqMan probe (QIAGEN) used for the amplification of the 6MBS-I tk promoter were as follows: forward (5'-AATTCGAACACGCA-GATGCA-3'), reverse (5'-GCCACACGCGTCACCTTAAT-3'), TaqMan probe (5'-CGCGGTCCCAGGTCCACTTCG-3').

RESULTS

Both NLK and HIPK2 Bind Directly to A-Myb and B-Myb

In the case of c-Myb, two kinases, NLK and HIPK2, bind directly to c-Myb (Kanei-Ishii *et al.*, 2004a). To examine whether A-Myb also directly interacts with these kinases, we performed GST pulldown assays. Wild-type A-Myb translated in vitro bound to GST-NLK and GST-HIPK2C resins (Figure 1A). The A-Myb mutant translated in vitro, which lacked repeats 2 and 3 in the DBD, failed to bind to GST-NLK and GST-HIPK2C. Further, an in vitro-translated R23

domain of A-Myb efficiently bound to GST-NLK and GST-HIPK2C. The in vivo association between A-Myb and NLK was investigated by coimmunoprecipitation, using lysates from CV-1 cells transfected with plasmids expressing FLAG-tagged A-Myb and HA-tagged NLK (Figure 1B). Anti-HA antibody coprecipitated FLAG-A-Myb, whereas control IgG did not. Thus, like c-Myb, A-Myb also binds to NLK and HIPK2 via its DBD. We also tested whether B-Myb, another member of the *myb* protein family, interacts with NLK and HIPK2. In vitro-translated B-Myb bound to GST-NLK and GST-HIPK2C (Figure 1C). These results indicate that direct interactions with NLK and HIPK2 are a common feature of all three members of the mammalian *myb* family proteins.

NLK Inhibits A-Myb Activity via Direct Phosphorylation without Protein Degradation

NLK phosphorylates c-Myb, leading to its proteasome-dependent degradation (Kanei-Ishii *et al.*, 2004a). In contrast, coexpression of NLK with A-Myb in CV-1 cells did not reduce A-Myb levels (Figure 2A, lanes 1 and 2), indicating that NLK does not induce degradation of A-Myb. The mobility of A-Myb coexpressed with NLK in SDS-PAGE was slower than that of A-Myb expressed alone or A-Myb coexpressed with kinase-negative NLK (Figure 2A). The change in mobility was blocked when the lysates were treated with λ phosphatase before electrophoresis. These results suggest that A-Myb is efficiently phosphorylated by NLK, resulting in its slower migration in the gel. The phosphatase treated A-Myb migrated faster than A-Myb expressed alone without NLK, indicating that uncharacterized kinase(s) other than NLK also phosphorylates A-Myb. This is not surprising because many kinases may phosphorylate transcription factor, such as A-Myb, although the physiological role of those kinases remains unknown. To confirm NLK phosphorylate A-Myb, in vitro kinase reactions were performed (Figure 2B). 293 cells were transfected with the FLAG-A-Myb or FLAG-NLK (wild-type or kinase-negative form), and A-Myb and NLK were purified by immunoprecipitation under high stringency (1 M NaCl, 2% NP-40). Under this high stringency, most of the associated proteins are likely to be dissociated, and in fact we confirmed that HIPK2 was not included in the immunopurified NLK sample (unpublished data). When the purified A-Myb was incubated with the purified NLK in the presence of [γ - ^{32}P]ATP, A-Myb became phosphorylated, whereas kinase-negative NLK did not phosphorylate A-Myb (Figure 2B).

Although NLK did not induce the degradation of A-Myb, we found that NLK inhibits A-Myb activity in luciferase reporter assays. NLK inhibited A-Myb-dependent luciferase expression from the 6MBS-I-luc reporter, which contains six tandem repeats of the Myb-binding site, in a dose-dependent manner (Figure 2C, left). NLK also suppressed A-Myb-induced luciferase expression from the *c-myc* promoter, which is an endogenous one target gene of Myb (Nakagoshi *et al.*, 1992; Figure 2C, right). These results indicate that NLK phosphorylates A-Myb and inhibits its activity without inducing its degradation. The kinase-negative NLK more weakly inhibited the luciferase expression from the 6MBS-I-luc and *c-myc*-luc reporters than wild-type NLK, when the same amounts of the expression vector was used (0.3 μg ; Figure 2C). However, kinase-negative NLK had still some inhibitory activity. These results suggest that NLK inhibits the A-Myb activity in the kinase-dependent and -independent manner.

When similar experiments were performed using B-Myb, coexpression of NLK with B-Myb partially decreased the B-Myb levels (Figure 2D), but did not lead to complete

Figure 2. NLK phosphorylates A-Myb and inhibits A-Myb-dependent *trans*-activation without A-Myb degradation. (A) In vivo phosphorylation of A-Myb. CV-1 cells were transfected with the FLAG-A-Myb expression vector, with or without the expression vector for FLAG-tagged wild-type (WT) or kinase-negative (KN) NLK. Anti-FLAG antibody was used to detect FLAG-A-Myb and FLAG-NLK in whole cell lysates. In lane 3, cell lysates were incubated with λ protein phosphatase (PPase). (B) NLK phosphorylates A-Myb in vitro. 293 cells were transfected with the expression plasmids for FLAG-A-Myb or FLAG-tagged wild-type or kinase-negative NLK, and cell lysates were immunoprecipitated with anti-FLAG antibody. The immunocomplexes were mixed with ³²P-ATP, as indicated above each lane, and analyzed by SDS-PAGE and autoradiography. (C) NLK negatively regulates the *trans*-activating capacity of A-Myb. CV-1 cells were transfected with a mixture of the luciferase reporter bearing six Myb-binding sites (6MBS-I-luc) or containing the *c-myc* promoter (*c-myc-luc*) and either the A-Myb expression plasmid or the control plasmid, with or without increasing amounts of plasmid to express wild-type or kinase-negative NLK. Luciferase activity was then measured. The relative *trans*-activation capacity of A-Myb (average of 3 experiments) is shown. The degree of activation by A-Myb using 6MBS-I-luc and *c-myc-luc* was 14.0 ± 0.9- and 25.4 ± 2.1-fold, respectively. (D) Induction of B-Myb degradation by NLK. CV-1 cells were transfected with the FLAG-B-Myb expression vector, with or without the FLAG-NLK expression plasmid. Anti-FLAG antibody was used to detect FLAG-B-Myb and FLAG-NLK in whole cell lysates. In lane 2, transfected cells were treated with the proteasome inhibitor MG132 (50 μM) for 7 h before lysate preparation. (E) Negative regulation of B-Myb-dependent *trans*-activation by NLK. The effect of NLK on B-Myb-dependent transcription from the 6MBS-I-luc reporter was examined as described in C.

degradation of the protein, as seen with c-Myb (Kanei-Ishii *et al.*, 2004a). In luciferase reporter assays, NLK also inhibited B-Myb-dependent luciferase expression from the 6MBS-I reporter (Figure 2E). Thus, NLK appears to negatively regulate B-Myb activity both by induction of B-Myb degradation and by a mechanism similar to that of A-Myb.

Figure 3. Wnt signaling negatively regulates A-Myb-dependent *trans*-activation. (A–D) Effect of R-Fz1 (A), R-Fz2 (B), TAK1+TAB1 (C), or HIPK2 (D) on the *trans*-activating capacity of A-Myb. CV-1 cells were transfected with a mixture of 6MBS-I-luc and either the A-Myb expression plasmid or control plasmid, with or without increasing amounts of the R-Fz1, R-Fz2, HIPK2, or TAK1/TAB1 expression plasmids. Luciferase activity was then measured. The relative *trans*-activation capacity of A-Myb is indicated. The degree of activation by A-Myb was 8.0 ± 0.6-fold when no component of Wnt-TAK1 signaling was coexpressed. Averages of three experiments ± SD are shown. (E) Wnt1-TAK1 signaling suppresses A-Myb activity via HIPK2. CV-1 cells were transfected with a mixture of 6MBS-I-luc reporter, A-Myb expression plasmid or control plasmid, with or without the R-Fz1 or TAK1+TAB1 expression plasmids. In some cases, the kinase-negative form of HIPK2 was added. Luciferase activity was then measured, and the relative *trans*-activation capacity of A-Myb is indicated as described above. (F) Coexpression of Wnt-NLK pathway components do not affect the A-Myb levels. CV-1 cells were transfected with the FLAG-A-Myb expression plasmid and the internal control plasmid pact-β-gal together with a plasmid expressing the protein indicated above each lane. The total cell lysates were prepared and analyzed by Western blotting using anti-FLAG antibody.

The Wnt-NLK Pathway Inhibits A-Myb Activity

Because the mechanism by which NLK suppresses A-Myb activity is completely different from that of c-Myb, we asked whether the Wnt-TAK1-HIPK2-NLK pathway inhibits A-Myb activity, as it does c-Myb. In reporter assays using the 6MBS-I-luc reporter expressed in CV-1 cells, A-Myb activity was inhibited in a dose-dependent manner by coexpression of rat Frizzled-1 and Frizzled-2 (R-Fz1 and R-Fz2), which act

as receptors for Wnt-1 and Wnt5a in the canonical Wnt and noncanonical Wnt/ Ca^{2+} pathways, respectively (Figure 3, A and B). Coexpression of TAK1/TAB1 and HIPK2 also suppressed A-Myb activity (Figure 3, C and D). To examine whether R-Fz1, R-Fz2, and TAK1/TAB1 function in the upstream region of HIPK2 in the same signaling pathway, we analyzed the effect of the kinase-negative form of HIPK2 on the suppression of A-Myb activity by R-Fz1, R-Fz2, and TAK1/TAB1. The inhibitory effect of R-Fz1, R-Fz2, and TAK1/TAB1 was blocked by coexpressing the kinase-negative HIPK2 mutant (Figure 3E). Because kinase-negative NLK partially blocks the A-Myb activity, this cannot be used in the similar experiments to examine whether NLK acts in the same signaling pathway as R-Fz1, R-Fz2, TAK1/TAB1, and HIPK2. However, our recent report clearly demonstrated that NLK plays a role in the downstream of the Wnt-TAK1/TAB1-HIPK2 signaling pathway (Kanei-Ishii *et al.*, 2004a). Thus, A-Myb-dependent transcriptional activation is inhibited by the Wnt-TAK1-HIPK2-NLK pathway.

To further confirm that the degradation of A-Myb is not induced by the Wnt-TAK1/TAB1-HIPK2 signaling pathway, the effect of coexpression of each component of this pathway on the A-Myb levels was examined. Coexpression of HIPK2, TAK1/TAB1, R-Fz1, or R-Fz2 with A-Myb did not affect the A-Myb levels (Figure 3F).

NLK Inhibits the Interaction between A-Myb and CBP

The transcriptional coactivator CBP binds directly to the activation domain of all three members of the Myb family and mediates Myb-dependent transcriptional activation (Dai *et al.*, 1996; Oelgeschlager *et al.*, 1996; Facchinetti *et al.*, 1997; Bessa *et al.*, 2001). These data suggest that NLK may block the interaction between A-Myb and CBP. To test this, we coimmunoprecipitated lysates from CV-1 cells expressing FLAG-A-Myb and CBP, in the presence or absence of NLK expression vector (Figure 4A). In the absence of NLK, the anti-CBP antibody efficiently coprecipitated A-Myb, demonstrating complex formation between A-Myb and CBP. In contrast, coexpression of NLK almost completely blocked the A-Myb-CBP complex formation. Interestingly, coexpression of kinase-negative NLK partially inhibited the association between A-Myb and CBP. These results suggest that NLK inhibits the interaction between A-Myb and CBP by both kinase-dependent and -independent mechanisms. This is consistent with the observation that NLK inhibits the A-Myb-induced luciferase expression from the *c-myc* promoter in the kinase-dependent and kinase-independent manner (Figure 2C).

Previously, we found that kinase-negative NLK inhibits the interaction between c-Myb and CBP (Kanei-Ishii *et al.*, 2004b). To test whether kinase-negative NLK also competes with CBP for binding to A-Myb we performed GST pull-down assay (Figure 4B). In vitro-translated A-Myb efficiently bound to the GST-CBP resin and addition of an in vitro-translated kinase-negative NLK inhibited the binding of A-Myb to GST-CBP in a dose-dependent manner. Thus, NLK blocks the interaction between A-Myb and CBP by directly binding to A-Myb.

We also examined whether NLK blocks the interaction between B-Myb and CBP by similar coimmunoprecipitation experiments using lysates from CV-1 cells expressing FLAG-B-Myb and CBP, in the presence or absence of NLK expression vector (Figure 4C). In the absence of NLK, the anti-CBP antibody efficiently coprecipitated B-Myb, whereas coexpression of NLK dramatically diminished the B-Myb-CBP complex formation. Thus, NLK also inhibits the interaction between B-Myb and CBP.

Figure 4. NLK inhibits the association of A-Myb with CBP. (A) Coimmunoprecipitation of A-Myb and CBP. CV-1 cells were transfected with a mixture of FLAG-A-Myb and CBP expression plasmids together with or without the plasmid to express FLAG-tagged wild-type or kinase-negative (K155M) NLK. Lysates were precipitated by anti-CBP antibody (CT, Upstate) or control IgG and the immunocomplexes were analyzed by Western blotting with anti-FLAG or anti-CBP antibody (C1, Santa Cruz). FLAG-A-Myb, FLAG-NLK, and CBP in whole cell extracts were detected by Western blotting (lower three panels). (B) NLK blocks the binding of A-Myb to GST-CBP. Left, the binding of in vitro-translated A-Myb to GST-CBP-KIX in the presence of increasing amounts of either in vitro-translated NLK-K155M or control lysates was examined. Right, the relative amount of A-Myb bound to GST-CBP is indicated. (C) Inhibition of B-Myb-CBP interaction by NLK. CV-1 cells were transfected with a mixture of FLAG-B-Myb and CBP expression plasmids together with or without the NLK expression plasmid. Coimmunoprecipitation and Western blotting were performed as described in A. FLAG-B-Myb and CBP in whole cell extracts were detected by Western blotting (left panels).

To further confirm that NLK blocks the interaction between CBP and A-Myb, we investigated the effect of NLK on the acetylation of A-Myb by CBP. Previous work has shown that CBP binds to c-Myb via its KIX and C/H2 domains, and acetylates c-Myb at multiple sites, leading to enhanced c-Myb-dependent transcriptional activation (Dai *et al.*, 1996; Sano and Ishii, 2001). In vitro-translated A-Myb also bound to GST-CBP resins containing either the KIX or C/H2 domains (Figure 5A). Coexpression of increasing amounts of CBP induced acetylation of A-Myb in a dose-dependent manner (Figure 5B). However, when A-Myb and CBP were

Figure 5. NLK inhibits the CBP-induced acetylation of A-Myb. (A) A-Myb binds to two regions of CBP. Top, schematic representation of the GST-CBP fusion proteins used. Top, the domain structure of CBP is shown schematically: C/H1, cysteine- and histidine-rich domain; KIX, CREB-binding domain; Bromo, bromo domain. Structures of the three GST-CBP fusion proteins used are shown below. The results of binding assays shown below are indicated on the right. Bottom, GST pull-down assays. In the right panel, the GST-CBP fusion proteins bound by glutathione-Sepharose resin were analyzed by 10% SDS-PAGE followed by Coomassie Brilliant Blue staining. In the left panel, 35 S-labeled A-Myb was synthesized *in vitro* and mixed with various GST-CBP fusion proteins, and bound proteins were analyzed by SDS-PAGE and autoradiography. (B) A-Myb is acetylated by CBP *in vivo*. Left, CV-1 cells were transfected with the FLAG-A-Myb expression plasmid, with or without increasing amounts of the CBP-FLAG expression plasmid. Cell lysates were prepared and analyzed by SDS-PAGE and Western blotting using anti-acetylated lysine (top panel) or anti-FLAG antibody (middle and bottom panels). Right, comparison of the sequence surrounding the CBP-induced acetylation sites between mouse c-Myb and human A-Myb. Lys-438 and Lys-441 of mouse c-Myb, which are acetylated by CBP and indicated by bold letters, are conserved in human A-Myb. (C) Acetylation of A-Myb is inhibited by NLK. CV-1 cells were transfected with the FLAG-A-Myb and CBP-FLAG expression plasmids, with or without different amounts of the plasmid to express FLAG-tagged wild-type or kinase-negative NLK. Cell lysates were prepared and analyzed by a 4–12% gradient SDS-PAGE and Western blotting using anti-acetylated lysine (top panel) or anti-FLAG antibody (middle and bottom panels).

coexpressed with NLK, acetylation of A-Myb was inhibited (Figure 5C). Thus, NLK inhibits the CBP-induced acetylation of A-Myb by blocking the interaction between A-Myb and

CBP. Interestingly, kinase-dead NLK had only weak inhibitory activity on the CBP-induced acetylation of A-Myb (Figure 5C), although this NLK mutant significantly inhibits the A-Myb-CBP interaction (Figure 4A). These results may suggest that the relatively strong interaction between A-Myb and CBP may be necessary for the CBP-induced acetylation of A-Myb. One possibility is that kinase-negative NLK may specifically affects interaction of one of the domains (KIX domain) of CBP with A-Myb.

NLK Also Induces Methylation of Histone H3 at Lysine-9

One major activity of CBP is acetylation of histones, and the degree of histone acetylation can be controlled by a balance between histone acetyltransferase (HAT) and histone deacetylase (HDAC) activity. Therefore, if the major mechanism of NLK action is to block CBP recruitment, the NLK action would not be attenuated by the HDAC inhibitor, because there would be no acetylation to be removed by HDACs. To investigate this, we analyzed the effect of trichostatin A (TSA), an inhibitor of histone deacetylase (HDAC), on NLK activity. Because it was reported that transiently transfected cells can be used to examine the effect of TSA (Li and McDonnell, 2002), we also used the CV-1 cells transiently transfected with Myb sites containing luciferase reporter. NLK inhibited A-Myb-dependent luciferase expression from the 6MBS-I-luc reporter as shown above (Figure 2C). Treatment of transfected cells with TSA did not block the effect of NLK (Figure 6A). These results are consistent with the data indicating that NLK inhibits A-Myb-dependent *trans*-activation by inhibiting the recruitment of CBP.

In addition to the deacetylation of histones, methylation of histones plays an important role in repression of transcription. Specifically, methylation of the histone H3 tail at lysine-9 (H3-K9) is associated with gene silencing (Lachner *et al.*, 2001; Nakayama *et al.*, 2001; Schotta *et al.*, 2002). Lysines can accept three methyl groups and can therefore be monomethylated, dimethylated, or trimethylated (denoted as m, m2, and m3; Dutnall, 2003). In mammalian cells, H3-K9m3 is preferentially localized to pericentromeric heterochromatin, whereas H3-K9m and H3-K9m2 are localized to euchromatin (Peters *et al.*, 2003; Rice *et al.*, 2003). We examined whether NLK affects histone H3-K9 methylation using ChIP assays. Because it was reported that transiently transfected cells can be used for ChIP experiments (Lee *et al.*, 2002; Wells and Farnham, 2002), we also used the CV-1 cells transiently transfected with Myb sites containing luciferase reporter. CV-1 cells were transfected with the 6MBS-I-luc reporter and the FLAG-A-Myb expression plasmid or the control plasmid, with or without the NLK expression plasmid. The amounts of Myb site-containing promoter DNA fragment precipitated with anti-H3K9m3 antibody increased with coexpression of NLK (Figure 6B, left panel). It is thought that H3-K9 acetylation and methylation are mutually exclusive, and the high degree of methylation of H3K9m3 had been expected in the absence of A-Myb. However, the degree of methylation of H3K9m3 in the absence of A-Myb was not so high, and the degree of methylation was apparently enhanced by NLK. These results suggest that NLK inhibits A-Myb-dependent *trans*-activation by inducing methylation of histone H3 at lysine-9. In ChIP assays, the anti-FLAG antibody precipitated 45% less Myb site-containing promoter DNA when NLK was coexpressed (Figure 6B, right panel), indicating that the amounts of A-Myb bound to the promoter decreased in the presence of NLK. NLK blocks interaction between A-Myb and CBP, which may destabilize the A-Myb complex on Myb-binding sites.

Figure 6. NLK blocks the recruitment of CBP and induces histone H3 methylation. (A) TSA does not block the NLK-induced inhibition of A-Myb activity. CV-1 cells were transfected with a mixture of the 6MBS-I-luc reporter and the A-Myb expression plasmid, with or without different amounts of the NLK expression plasmid. Transfected cells were treated with TSA (30 nM) for 18 h before lysate preparation. Luciferase activity was then measured. The relative *trans*-activation capacity of A-Myb (average of 3 experiments) is shown. The degree of activation by A-Myb was 11.2 ± 2.9 -fold. (B) ChIP assays. CV-1 cells were transfected with the 6MBS-I-luc reporter and the FLAG-A-Myb expression plasmid or the control plasmid, with or without the NLK expression plasmid. Soluble chromatin was prepared from the transfected cells, and immunoprecipitated with anti-H3-K9m3 (left) or anti-FLAG (right) antibody. The final DNA extraction was amplified by real-time PCR using primers that cover the promoter region linked to the Myb-binding sites of 6MBS-I-luc. The relative densities of bands are indicated below.

DISCUSSION

The present study demonstrated that, like c-Myb, *trans*-activation by both A-Myb and B-Myb is negatively regulated by the Wnt-NLK pathway. However, unlike c-Myb, A-Myb is not degraded by the Wnt-NLK pathway. Overexpression of NLK inhibits the association between the coactivator CBP and A-Myb; this inhibition is partly dependent on the kinase activity of NLK, because the kinase-negative form of NLK only partially inhibited A-Myb activity. NLK bound to A-Myb also induced methylation of histone H3-K9 (Figure 7). B-Myb levels were slightly decreased by coex-

Figure 7. The Wnt-NLK pathway inhibits the activity of Myb family of proteins by two different mechanisms. The Wnt-NLK pathway inhibits A-Myb activity by blocking the interaction between A-Myb and CBP and inducing the methylation of histone H3-K9. In contrast, the Wnt-NLK pathway inhibits c-Myb activity by inducing proteasome-dependent c-Myb degradation.

pression of NLK, suggesting that NLK weakly induces B-Myb degradation. These results suggest that the Wnt-NLK pathway inhibits B-Myb activity by two mechanisms; degradation of B-Myb, similar to c-Myb, and inhibition of B-Myb-CBP interaction and histone H3-K9 methylation, similar to A-Myb.

NLK is a member of the MAP kinase super family that is known to phosphorylate the Ser or Thr residues next to Pro. A-Myb contains 16 putative NLK phosphorylation sites, and introducing the Ala mutation into six sites in the C-terminal region conserved among Myb family proteins did not completely abrogate the phosphorylation by NLK (unpublished data), suggesting the presence of more NLK phosphorylation sites. Thus, NLK phosphorylates both A-Myb and c-Myb at multiple sites in CV-1 cells, but NLK only induces the degradation of c-Myb, not A-Myb. We have recently identified the specific ubiquitin E3 ligase that induces the ubiquitination and proteasome-dependent degradation of the NLK-phosphorylated c-Myb (unpublished data). It is possible that we did not detect A-Myb degradation by NLK because the E3 ligase does not recognize NLK-phosphorylated A-Myb, or alternatively, that CV-1 cells lack an E3 ligase, which binds to NLK-phosphorylated A-Myb. Therefore, we cannot exclude the possibility that NLK induces A-Myb degradation in some cell types expressing an E3 ligase, which recognizes NLK-phosphorylated A-Myb. Similarly, it is possible that the Wnt-NLK pathway inhibits c-Myb-dependent *trans*-activation in some cell types by blocking CBP recruitment and inducing histone methylation.

Multiple histone methylases (HMTases), including Suv39h1, Suv39h2, and G9a, can methylate H3-K9 (Rea *et al.*, 2000; Tachibana *et al.*, 2001). A hallmark of this class of HMTases is the presence of a 130-amino acid SET domain

(Jenuwein, 2001), which is crucial for catalytic activity but requires an adjacent cysteine-rich domain (Rea *et al.*, 2000). The Suv39h enzymes display an exquisite site-selectivity toward H3-K9 methylation that is highest for an unmodified H3 amino terminus (Rea *et al.*, 2000). G9a is a “dual” HMTase methylating the K9 and K27 positions and seems broadly dispersed in interphase chromatin without enrichment at heterochromatic foci (Tachibana *et al.*, 2002). Because G9a is one of the major HMTase localized in euchromatin, we investigated whether G9a is involved in NLK-mediated histone H3K9 methylation. G9a efficiently bound to NLK in the coimmunoprecipitation assays, but the dominant-negative form of G9a did not block NLK-dependent inhibition of *trans*-activation by A-Myb (unpublished data). These results suggest that G9a could be partly involved in the NLK-mediated inhibition of A-Myb activity, but G9a is not the primary HMTase recruited by NLK. Interestingly, the ESET HMTase is highly expressed in testis (Yang *et al.*, 2002), a tissue that expresses A-*myb*.

A-*myb* is highly expressed in a limited range of cell types, including male germ cells and female breast ductal epithelium (Trauth *et al.*, 1994). A-*myb*-deficient males are infertile because of a block in spermatogenesis, and A-*myb* null females show underdevelopment of breast tissue after pregnancy (Toscani *et al.*, 1997). Wnt signaling was reported to have a critical role in the growth regulation of mammary epithelial cells and testis development (Miyoshi *et al.*, 2002; Civenni *et al.*, 2003; Jordan *et al.*, 2003). Therefore, the Wnt-dependent negative regulation of A-Myb activity demonstrated in this study may play important role in control of the proliferation and differentiation of mammary epithelial cells and germ cells by Wnt signals.

ACKNOWLEDGMENTS

We are grateful to K. Matsumoto for the TAK1, TAB1, and NLK cDNAs; R. T. Moon for the R-Fz1 and R-Fz2 cDNA; and Y. Kim for the HIPK2 cDNA. This work was supported in part by the Grants-in-Aid for Scientific Research of the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

REFERENCES

- Bender, T. P., Kremer, C. S., Kraus, M., Buch, T., and Rajewsky, K. (2004). Critical functions for c-Myb at three checkpoints during thymocyte development. *Nature Immunol.* 5, 721–729.
- Bessa, M., Saville, M. K., and Watson, R. J. (2001). Inhibition of cyclin A/Cdk2 phosphorylation impairs B-Myb transactivation function without affecting interactions with DNA or the CBP coactivator. *Oncogene* 20, 3376–3386.
- Biedenkapp, H., Borgmeyer, U., Sippel, A. E., and Klempnauer, K. H. (1988). Viral *myb* oncogene encodes a sequence-specific DNA-binding activity. *Nature* 335, 835–837.
- Civenni, G., Holbro, T., and Hynes, N. E. (2003). Wnt1 and Wnt5a induce cyclin D1 expression through ErbB1 transactivation in HC11 mammary epithelial cells. *EMBO Rep.* 4, 166–171.
- Dai, P., Akimaru, H., Tanaka, Y., Hou, D. X., Yasukawa, T., Kanei-Ishii, C., Takahashi, T., and Ishii, S. (1996). CBP as a transcriptional coactivator of c-Myb. *Genes Dev.* 10, 528–540.
- Dutnall, R. N. (2003). Cracking the histone code: one, two, three methyls, you're out! *Mol. Cell* 12, 3–4.
- Facchinetti, V., Loffarelli, L., Schreek, S., Oelgeschlager, M., Luscher, B., Introna, M., and Golay, J. (1997). Regulatory domains of the A-Myb transcription factor and its interaction with the CBP/p300 adaptor molecules. *Biochem. J.* 324, 729–736.
- Frampton, J., Ramqvist, T., and Graf, T. (1996). v-Myb of E26 leukemia virus up-regulates bcl-2 and suppresses apoptosis in myeloid cells. *Genes Dev.* 10, 2720–2731.
- Ghosh, S., and Karin, M. (2002). Missing pieces in the NF- κ B puzzle. *Cell* 109, Supplement 81–96.
- Jenuwein, T. (2001). Re-SET-ting heterochromatin by histone methyltransferases. *Trends Cell. Biol.* 11, 266–273.
- Jordan, B. K., Shen, J. H., Olaso, R., Ingraham, H. A., and Vilain, E. (2003). Wnt4 overexpression disrupts normal testicular vasculature and inhibits testosterone synthesis by repressing steroidogenic factor 1/ β -catenin synergy. *Proc. Natl. Acad. Sci. USA* 100, 10866–10871.
- Kanei-Ishii, C., MacMillan, E. M., Nomura, T., Sarai, A., Ramsay, R. G., Aimoto, S., Ishii, S., and Gonda, T. J. (1992). Transactivation and transformation by Myb are negatively regulated by a leucine-zipper structure. *Proc. Natl. Acad. Sci. USA* 89, 3088–3092.
- Kanei-Ishii, C. *et al.* (2004a). Wnt-1 signal induces phosphorylation and degradation of c-Myb protein via TAK1, HIPK2, and NLK. *Genes Dev.* 18, 816–829.
- Kanei-Ishii, C., Nomura, T., Tanikawa, J., Ichikawa-Iwata, E., and Ishii, S. (2004b). Differential sensitivity of v-Myb and c-Myb to Wnt-1-induced protein degradation. *J. Biol. Chem.* 279, 44582–44589.
- Klempnauer, K. H., Gonda, T. J., and Bishop, J. M. (1982). Nucleotide sequence of the retroviral leukemia gene v-*myb* and its cellular progenitor c-*myb*: the architecture of a transduced oncogene. *Cell* 31, 453–463.
- Kowenz-Leutz, E., Herr, P., Niss, K., and Leutz, A. (1997). The homeobox gene GBX2, a target of the myb oncogene, mediates autocrine growth and monocytic differentiation. *Cell* 91, 185–195.
- Lachner, M., O'Carroll, D., Rea, S., Mechtler, K., and Jenuwein, T. (2001). Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* 410, 116–120.
- Ladendorff, N. E., Wu, S., and Lipsick, J. S. (2001). BS69, an adenovirus E1A-associated protein, inhibits the transcriptional activity of c-Myb. *Oncogene* 20, 125–132.
- Lee, D., Kim, H. Z., Jeong, K. W., Shim, Y. S., Horikawa, I., Barrett, J. C., and Choe, J. (2002). Human papillomavirus E2 down-regulates the human telomerase reverse transcriptase promoter. *J. Biol. Chem.* 277, 27748–27756.
- Leprince, D., Gegonne, A., Coll, J., de Taisne, C., Schneeberger, A., Lagrou, C., and Stehelin, D. (1983). A putative second cell-derived oncogene of the avian leukaemia retrovirus E26. *Nature* 306, 395–397.
- Li, X., and McDonnell, D. P. (2002). The transcription factor B-Myb is maintained in an inhibited state in target cells through its interaction with the nuclear corepressors N-CoR and SMRT. *Mol. Cell. Biol.* 22, 3663–3673.
- Miyoshi, K. *et al.* (2002). Activation of different Wnt/ β -catenin signaling components in mammary epithelium induces transdifferentiation and the formation of pilar tumors. *Oncogene* 21, 5548–5556.
- Mucenski, M. L., McLain, K., Kier, A. B., Swerdlow, S. H., Schreiner, C. M., Miller, T. A., Pietryga, D. W., Scott, W. J., and Potter, S. S. (1991). A functional c-*myb* gene is required for normal murine fetal hepatic hematopoiesis. *Cell* 65, 677–689.
- Nakagoshi, H., Kanei-Ishii, C., Sawazaki, T., Mizuguchi, G., and Ishii, S. (1992). Transcriptional activation of the c-*myc* gene by the c-*myb* and B-*myb* gene products. *Oncogene* 7, 1233–1240.
- Nakagoshi, H., Takemoto, Y., and Ishii, S. (1993). Functional domains of the human B-*myb* gene product. *J. Biol. Chem.* 268, 14161–14167.
- Nakayama, J., Rice, J. C., Strahl, B. D., Allis, C. D., and Grewal, S. I. (2001). Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. *Science* 292, 110–113.
- Ness, S. A., Marknell, A., and Graf, T. (1989). The v-*myb* oncogene product binds to and activates the promyelocyte-specific *mim-1* gene. *Cell* 59, 1115–1125.
- Nomura, N., Takahashi, M., Matsui, M., Ishii, S., Date, T., Sasamoto, S., and Ishizaki, R. (1988). Isolation of human cDNA clones of myb-related genes, A-*myb* and B-*myb*. *Nucleic Acids Res.* 16, 11075–11089.
- Nomura, T., Tanikawa, J., Akimaru, H., Kanei-Ishii, C., Ichikawa-Iwata, E., Khan, M. M., Ito, H., and Ishii, S. (2004). Oncogenic activation of c-Myb correlates with a loss of negative regulation by TIF1 β and Ski. *J. Biol. Chem.* 279, 16715–16726.
- Oelgeschlager, M., Janknecht, R., Krieg, J., Schreek, S., and Lüscher, B. (1996). Interaction of the co-activator CBP with Myb proteins: effects on Myb-specific transactivation and on the cooperativity with NF-M. *EMBO J.* 15, 2771–2780.
- Ogata, K., Morikawa, S., Nakamura, H., Sekikawa, A., Inoue, T., Kanai, H., Sarai, A., Ishii, S., and Nishimura, Y. (1994). Solution structure of a specific DNA complex of the Myb DNA-binding domain with cooperative recognition helices. *Cell* 79, 639–648.
- Peifer, M., and Polakis, P. (2000). Wnt signaling in oncogenesis and embryogenesis—a look outside the nucleus. *Science* 287, 1606–1609.

- Peters, A. H. *et al.* (2003). Partitioning and plasticity of repressive histone methylation states in mammalian chromatin. *Mol. Cell* 12, 1577–1589.
- Rea, S. *et al.* (2000). Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature* 406, 593–599.
- Rice, J. C., Briggs, S. D., Ueberheide, B., Barber, C. M., Shabanowitz, J., Hunt, D. F., Shinkai, Y., and Allis, C. D. (2003). Histone methyltransferases direct different degrees of methylation to define distinct chromatin domains. *Mol. Cell* 12, 1591–1598.
- Sano, Y., and Ishii, S. (2001). Increased affinity of c-Myb for CREB-binding protein (CBP) after CBP-induced acetylation. *J. Biol. Chem.* 276, 3674–3682.
- Sakura, H., Kanei-Ishii, C., Nagase, T., Nakagoshi, H., Gonda, T. J., and Ishii, S. (1989). Delineation of three functional domains of the transcriptional activator encoded by the *c-myb* protooncogene. *Proc. Natl. Acad. Sci. USA* 86, 5758–5762.
- Saville, M. K., and Watson, R. J. (1998). The cell-cycle regulated transcription factor B-Myb is phosphorylated by cyclin A/Cdk2 at sites that enhance its transactivation properties. *Oncogene* 17, 2679–2689.
- Schotta, G., Ebert, A., Krauss, V., Fischer, A., Hoffmann, J., Rea, S., Jenuwein, T., Dorn, R., and Reuter, G. (2002). Central role of *Drosophila* SU(VAR)3–9 in histone H3–K9 methylation and heterochromatic gene silencing. *EMBO J.* 21, 1121–1131.
- Tachibana, M., Sugimoto, K., Fukushima, T., and Shinkai, Y. (2001). Set domain-containing protein, G9a, is a novel lysine-preferring mammalian histone methyltransferase with hyperactivity and specific selectivity to lysines 9 and 27 of histone H3. *J. Biol. Chem.* 276, 25309–25317.
- Tachibana, M. *et al.* (2002). G9a histone methyltransferase plays a dominant role in euchromatic histone H3 lysine 9 methylation and is essential for early embryogenesis. *Genes Dev.* 16, 1779–1791.
- Takahashi, T., Nakagoshi, H., Sarai, A., Nomura, N., Yamamoto, T., and Ishii, S. (1995). Human *A-myb* gene encodes a transcriptional activator containing the negative regulatory domains. *FEBS Lett.* 358, 89–96.
- Tanaka, Y., Patestos, N. P., Maekawa, T., and Ishii, S. (1999). *B-myb* is required for inner cell mass formation at an early stage of development. *J. Biol. Chem.* 274, 28067–28070.
- Tanikawa, J., Nomura, T., Macmillan, E. M., Shinagawa, T., Jin, W., Kokura, K., Baba, D., Shirakawa, M., Gonda, T. J., and Ishii, S. (2004). p53 suppresses c-Myb-induced trans-activation and transformation by recruiting the corepressor mSin3A. *J. Biol. Chem.* 279, 55393–55400.
- Taylor, D., Badiani, P., and Weston, K. (1996). A dominant interfering Myb mutant causes apoptosis in T cells. *Genes Dev.* 10, 2732–2744.
- Toscani, A., Mettus, R. V., Coupland, R., Simpkins, H., Letvin, J., Orth, J., Hatton, K. S., and Reddy, E. P. (1997). Arrest of spermatogenesis and defective breast development in mice lacking A-myb. *Nature* 386, 713–717.
- Trauth, K., Mutschler, B., Jenkins, N. A., Gilbert, D. J., Copeland, N. G., and Klempnauer, K.-H. (1994). Mouse *A-myb* encodes a *trans*-activator and is expressed in mitotically active cells of the developing central nervous system, adult testis and B lymphocytes. *EMBO J.* 13, 5994–6005.
- Wells, J., and Farnham, P. J. (2002). Characterizing transcription factor binding sites using formaldehyde crosslinking and immunoprecipitation. *Methods* 26, 48–56.
- Weston, C. R., and Davis, R. J. (2002). The JNK signal transduction pathway. *Curr. Opin. Genet. Dev.* 12, 14–21.
- Wodarz, A., and Nusse, R. (1998). Mechanisms of Wnt signaling in development. *Annu. Rev. Cell Dev. Biol.* 14, 59–88.
- Yasukawa, T., Kanei-Ishii, C., Maekawa, T., Fujimoto, J., Yamamoto, T., and Ishii, S. (1995). Increase of solubility of foreign proteins in *Escherichia coli* by coproduction of the bacterial thioredoxin. *J. Biol. Chem.* 270, 25328–25331.
- Yang, L., Xia, L., Wu, D. Y., Wang, H., Chansky, H. A., Schubach, W. H., Hickstein, D. D., and Zhang, Y. (2002). Molecular cloning of ESET, a novel histone H3-specific methyltransferase that interacts with ERG transcription factor. *Oncogene* 21, 148–152.
- Ziebold, U., and Klempnauer, K.-H. (1997). Linking Myb to the cell cycle: cyclin-dependent phosphorylation and regulation of A-Myb activity. *Oncogene* 15, 1011–1019.