Cancer Research

Tumor Suppressor HLJ1 Binds and Functionally Alters Nucleophosmin via Activating Enhancer Binding Protein 2α Complex Formation

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Abstract

HLJ1, a member of the heat shock protein 40 chaperone family, is a newly identified tumor suppressor that has been implicated in tumorigenesis and metastasis in non–small cell lung cancer. However, the mechanism of HLJ1 action is presently obscure. In this study, we report that HLJ1 specifically interacts with the nuclear protein nucleophosmin (NPM1), forming a multiprotein complex that alters the nucleolar distribution and oligomerization state of NPM1. Enforced accumulation of NPM1 oligomers by overexpression in weakly invasive but high HLJ1-expressing cells induced the activity of signal transducer and activator of transcription 3 (STAT3) and increased cellular migration, invasiveness, and colony formation. Furthermore, silencing HLJ1 accelerated NPM1 oligomerization, inhibited the activity of transcription corepressor activating enhancer binding protein 2α (AP-2α), and increased the activities of matrix metalloproteinase-2 (MMP-2) and STAT3. Our findings suggest that HLJ1 switches the role of NPM1, which can act as tumor suppressor or oncogene, by modulating the oligomerization of NPM1 via HLJ1-NPM1 heterodimer formation and recruiting AP-2α to the MMP-2 promoter. *Cancer Res; 70(4); 1656–67. ©2010 AACR.*

Introduction

With its high relapse and low cure rates, lung cancer is the most common cause of cancer deaths worldwide (1). Molecular-targeted therapy (MTT), which aims at specific molecular derangements in cancer cells or their microenvironment, has emerged recently as one of the most important modalities of cancer treatment (2, 3). Characterization of the putative genes involved and understanding the molecular pathogenesis of tumor development are needed urgently for identifying new molecular-targeted genes in lung cancer. In a previous study, we used microarrays to screen a series of lung cancer model cell lines with varying invasive capabilities and identified a panel of metastasis-associated genes, including human liver DnaJ-like protein (HLJ1, also known as DNAJB4; ref. 4).

We subsequently showed that HLJ1 is a newly identified tumor suppressor in non–small cell lung cancer (NSCLC) that can inhibit tumorigenesis and metastasis of lung cancer cells *in vitro* and *in vivo* and whose expression correlates with survival of patients (5). We also discovered a new mechanism whereby HLJ1 is transcriptionally upregulated via enhancer activator protein-1 binding to promoter Yin Yang-1 (YY1) and the coactivator p300 (6, 7). However, the mechanisms underlying the role of HLJ1 in tumor progression are unknown.

Nucleophosmin (NPM1) is a nucleolar phosphoprotein that localizes in granular regions of the nucleolus and is highly expressed in malignant and actively dividing cells. NPM1 shuttles continuously between the nucleus and cytoplasm (8) and acts as a multifunctional protein that plays an important role in the increased nucleolar activity needed for cell proliferation (9). Previous reports have revealed that NPM1 has DNA-binding activity (10), can bind nuclear and nucleolar localization signals (11), regulates the stability and transcriptional activity of p53 (12), and interacts with several transcription factors including transcription factor activating enhancer binding protein 2α (AP-2α), IFN regulatory factor-1, alternative reading frame, NF-κB, and YY1 (13–16). The NPM1 protein has been proposed to have both oncogenic and tumor-suppressing activities depending on its level of expression and cellular localization (17, 18).

The objective of this study was to identify the molecular mechanism responsible for the inhibition by HLJ1 of invasion and metastasis of lung cancer. Our results show that HLJ1

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downregulates the activities of signal transducer and activator of transcription 3 (STAT3) and matrix metalloproteinase-2 (MMP-2) by blocking NPM1 oligomerization and regulating the NPM1–AP-2α complex. These findings offer a new insight into tumor progression and an opportunity to discover potential genes that might act as targets in MTT.

Materials and Methods

Cell culture. Human lung adenocarcinoma cell lines CL1-0 and CL1-5, numbered in ascending order of invasive competence (4, 19), and their derivative transfectants were cultured in RPMI 1640 (Invitrogen) with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen).

Coimmunoprecipitation and Western blot analysis. The preparations of whole-cell lysates, cytoplasmic and nuclear extracts, and Western blot analysis (20) and the details of coimmunoprecipitation have been described previously (21). The primary antibodies used for Western blot analysis were monoclonal mouse anti-HLJ1 antibody (prepared in-house), monoclonal mouse anti-NPM1 antibody (Zymed Laboratories), polyclonal rabbit anti-AP-2α antibody (Santa Cruz Biotechnology), polyclonal rabbit anti-STAT3 antibody (Upstate Biotechnology, Inc.), monoclonal mouse anti-phospho-STAT3 (Tyr^{705}) antibody (Upstate Biotechnology), polyclonal rabbit anti-MMP-2 (Santa Cruz Biotechnology), polyclonal rabbit anti-TATA-box binding protein (TBP) antibody (Santa Cruz Biotechnology), polyclonal rabbit anti–enhanced green fluorescent protein (EGFP) antibody (Clontech), monoclonal mouse anti-β-tubulin (Upstate Biotechnology), and monoclonal mouse anti-α-tubulin (Upstate Biotechnology).

Construction of expression vectors. The full-length cDNA of *NPM1* was constructed in-frame into pVP16 vector (Clontech), pcDNA3 expression vector (Invitrogen), or pEGFP-C3 vector (Clontech). To identify the protein-protein interacting regions of HLJ1 with NPM1, one set of constructs was generated for a pull-down assay. All primers used in this study are listed in Supplementary Table S1. To overexpress HLJ1 in cells, the coding region of *HLJ1* cDNA was cloned into the constitutive mammalian expression vector pEF6/V5-His-TOPO (Invitrogen). To silence HLJ1 expression, the available small interfering RNA (siRNA) and scrambled siRNA sequences published previously (5) were constructed into pSilencer 3.1-H1 Puro vector (Invitrogen).

Pull-down and oligomerization assay. Recombinant HLJ1 and its deletion mutants with a glutathione *S*-transferase (GST) tag in the pGST4T1 vector (Amersham Biosciences) and full-length NPM1 with a His tag in the pQE31 vector (Qiagen) were expressed in *Escherichia coli*, and GST fusion proteins were purified using glutathione Sepharose (Amersham Biosciences) according to standard protocols. The purified HLJ1 recombinant proteins bound to beads were incubated with the indicated His-NPM1 bacterial lysates in binding buffer (0.1% Nonidet P-40, 150 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride) with rotation overnight at 4°C. After an extensive washing with binding buffer, the beads were boiled and resuspended in SDS loading buffer and then resolved by SDS-PAGE, followed by Western blot. All experiments were performed in triplicate. For oligomerization assay, we separately incubated recombinant His-NPM1 alone and His-NPM1 plus His-HLJ1 in binding buffer overnight at 4°C. After slowly mixing with native sample dye, the protein complexes were incubated for 5 min at room temperature and then resolved by native-PAGE, followed by Western blot.

Real-time PCR. The primer sets used in SYBR-Green real-time PCR were listed as follows: *HLJ1* forward primer, 5′-CCAGCAGACATTGTTTTTATCATT-3′ and reverse primer, 5′-CCATCCAGTGTTGGTACATTAATT-3′; *NPM1* forward primer, 5′-AGGTGGTTCTCTTCCCAAAGT-3′ and reverse primer, 5′-CACTGCCAGATCTTGAATAGC-3′. The *TBP* was used as an internal control (20). All experiments were performed in triplicate. The relative expression level of *HLJ1* and *NPM1* against that of *TBP* was calculated by the equation $-\Delta CT = -[CT_{HLJ1 \text{ or } NPM1} - CT_{TBP}]$. The RNA relative expression was calculated as $2^{-\Delta\Delta CT} \times K$, wherein K = constant.

Mammalian two-hybrid assay. The mammalian two-hybrid assay was performed using the BD Matchmaker Mammalian Assay kit (Clontech) according to the manufacturer's instructions. The recombinant pM-HLJ1 construct was cotransfected into CL1-0 cells with the pVP16-NPM1 construct along with the reporter plasmids (pG5SEAP) and the β-galactosidase reporter at a defined molar ratio (5:5:1:1). All experiments were performed in triplicate. The secreted alkaline phosphatase (SEAP) activity was measured by using BD Great EscAPe SEAP chemiluminescence detection kit (Clontech). The intensity of SEAP was normalized to β-galactosidase activity to calculate the transfection efficiency. Chemiluminescent β-galactosidase activity was detected by a Galacto-Light plus system (Applied Biosystems).

Immunofluorescence staining. To identify the cellular localization of NPM1 cells were seeded onto eight-well chamber slides. Cells were fixed and immunostained by anti-NPM1 antibody and rhodamine-labeled antimouse secondary antibody. Nuclei were demarcated with 4′,6-diamidino-2-phenylindole (DAPI) staining, and the cells were mounted onto slides and visualized by confocal microscopy (C1si; Nikon Instech).

Invasion and migration assay. The invasiveness of CL1-0 cells transfected with the NPM1 construct was performed using Transwell chambers (8-μm pore size; Costar,) and Transwell filters coated with Matrigel (Becton Dickinson), as described previously (5). The number of cells attached to the lower surface of the polycarbonate filter was counted at 400× magnification under a light microscope. The migratory capability of the transfectant was assessed using the wound healing approach or the modified Boyden chamber assay described previously (22). The number of cells migrating into the cell-free zone was counted under a light microscope. All experiments were performed in triplicate.

Anchorage-dependent and -independent growth assays. For the anchorage-dependent growth assay, 200 cells were resuspended in RPMI and seeded in six-well plates. For the

independent-growth assay, 1×10^4 cells were seeded and treated according to the procedures described previously (5). Triplicate samples were used in the experiment.

Gelatin zymography. Gelatinolytic activity was measured by gelatin zymography according to the methods described previously (23). The intensities of MMP-2 bands were quantified using ImageJ software (NIH). All experiments were performed in triplicate.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) was performed according to the manufacturer's instructions (Upstate Biotechnology). The ChIP primers of *MMP-2* and control glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) are also listed in Supplementary Table S1. For the sequential ChIP experiments, following an initial round of ChIP, the immunoprecipitated complexes were eluted and heated to 65°C for 15 min. Samples were diluted in dilution buffer, and the secondary ChIP was performed as described in the standard ChIP protocol. All experiments were performed in triplicate.

MMP-2 promoter assay. The promoter fragment of *MMP-2* (−1 to −902) relative to first nucleotide of the *MMP-2* was constructed into the luciferase reporter vector (pGL3-basic, Promega). *MMP-2* promoter primers (forward primer, 5′-CCGGGGTACCTCCCAAGGTGTATCTAG-CATCTCGCACT-3′; reverse primer, 5′-GGACTAGTCG-TAGCGCTCCCTGGCCCCGCGCGTC-3′) were used for amplification of *MMP-2* promoter. All transfections were carried out in triplicate in six-well plates. CL1-0 cells (1×10^5) were seeded 24 h before transfection. The luciferase reporter construct along with the control plasmid (pRL-TK Vector; Promega) were cotransfected into cells at the DNA ratio of 10:1 in the presence/absence of NPM1 and/or HLJ1 expressing vectors (0.5 μg for each vector and adding pcDNA 3.1 plasmid to make-up to 1 μg in total) as indicated by Lipofectamine 2000 (Invitrogen). After 48 h of incubation, the Dual-Glo luciferase substrate (Promega) was added to each well and the luminescent signals were measured by Victor3 multilabel counter (PerkinElmer) according to the manufacturer's instructions. The activity of *Renilla* luciferase was used as an internal control to normalize transfection efficiency.

Results

HLJ1 interacts with NPM1 through its COOH terminal domain. Several proteins that were differentially coimmunoprecipitated from highly HLJ1-expressed cells (CL1-0) compared with low ones (CL1-5) were subjected to mass spectrometry analysis. Preimmune serum was used as a negative control to exclude the nonspecific binding proteins. One dominant protein, NPM1, was coimmunoprecipitated with HLJ1 and was chosen for further study (Fig. 1A; Table 1). Reciprocal immunoprecipitation–Western blot analysis performed using CL1-0 cell lysates and the designated antibodies showed that HLJ1 interacted with NPM1 to form a protein complex (Fig. 1B). The mammalian two-hybrid assays revealed further that the HLJ1-NPM1 interaction occurred *in vivo* (Fig. 1C). The pull-down assay showed that HLJ1 could bind directly to NPM1 *in vitro* (see Supplementary Fig. S1). To identify the key domains of HLJ1 responsible for its interaction with NPM1, a set of HLJ1 deletion constructs was generated. The pull-down results revealed that all COOH terminus–truncated HLJ1 mutants (HLJ1ΔC1, HLJ1ΔC2, and HLJ1ΔC3) completely lost the NPM1 binding ability and that the COOH terminal domain of HLJ1 (HLJ1ΔN3) was sufficient to interact with NPM1 (Fig. 1D).

HLJ1 alters the nuclear distribution of NPM1. As we know, both expression and localization of NPM1 modulate its biological functions (17, 18). The expression of NPM1 was similar between both cell lines at the RNA and protein levels. However, CL1-0 had a higher HLJ1 expression than CL1-5 at both the RNA and protein levels (Fig. 2A). Furthermore, the cytoplasmic and nuclear protein fractions of CL1-0 were immunoprecipitated with anti-HLJ1 or anti-NPM1 antibody followed by Western blotting to identify in which compartment the HLJ1-NPM1 interaction occurs primarily. We found that both proteins interacted with each other principally in the nucleus (Fig. 2B).

Two HLJ1-silenced stable clones (siHLJ1-1 and siHLJ1-2) derived from the HLJ1-specific siRNAs and two scrambled controls (S-1 and S-2) were generated, and the effect of HLJ1 on NPM1 expression was examined by Western blot. The expression of HLJ1 was reduced to nearly undetectable levels in both HLJ1-silenced clones compared with the scrambled controls, but that of NPM1 was not affected in HLJ1-silenced clones (Fig. 2C). Next, to determine whether HLJ1 can modulate the subcellular localization of NPM1, the effect of HLJ1 on NPM1 nuclear distribution was examined by immunofluorescent staining. The scrambled controls, in which NPM1 localized in the nucleus, in particular, in the peripheral part of the nucleolus (i.e., the granular component), showed little donut-like distribution, and the nucleoli were round. In contrast, the HLJ1-silenced cells, in which NPM1 exhibited a diffuse pattern throughout the whole nucleus, possessed distinctly irregular or elongated nucleoli (Fig. 2D).

HLJ1 inhibits NPM1 oligomerization. To investigate whether HLJ1 inhibits the NPM1 oligomerization, which would disturb the NPM1 localization within nuclear compartments, on the *in vitro* study, the recombinant His-NPM1 protein alone or His-NPM1 plus His-HLJ1 protein mixture was separated by native gel followed by Western blotting. Therefore, according to the intensity of NPM1 oligomer pattern, we can understand whether HLJ1 disturbs NPM1 oligomerization or not. The data showed that HLJ1-NPM1 heterodimer turns up at ~78 kDa and HLJ1 conspicuously interfered with the formation of NPM1 oligomer so that the intensity of NPM1 oligomer decreased and that of NPM1 monomer increased in the reaction of His-NPM1 plus His-HLJ1 (Fig. 3A). In addition, on the *in vivo* study, the HLJ1-silenced cells and scrambled control cells were transfected with pEGFP-NPM1 or pEGFP plasmids, respectively, and the NPM1 oligomerization was detected by coimmunoprecipitation. The subcellular localization of the endogenous NPM1 and EGFP-NPM1 was similar and occurred mostly in

the nucleus, especially in the nucleolus. In contrast, transfection with EGFP alone produced a diffuse pattern (Fig. 3B). The protein extracts of HLJ1-silenced and scrambled cells transfected with either pEGFP or pEGFP-NPM1 were immunoprecipitated with anti-EGFP antibody. Interestingly, EGFP-NPM1, but not EGFP, captured more endogenous NPM1 in HLJ1-silenced cells than in scrambled controls (Fig. 3C). To further determine where the NPM1 oligomerization is taking place, the cytoplasmic and nuclear fractions of HLJ1-silenced cells transfected with pEGFP-NPM1 were immunoprecipitated with anti-EGFP antibody. The nuclear fraction/cytoplasmic fraction ratio of EGFP-NPM1 captured endogenous NPM1 is greater than that of the input (Fig. 3D). This result indicated that NPM1 oligomerization mainly occurs in nucleus. These findings imply that NPM1 forms oligomers in the nucleus and that HLJ1 would hinder NPM1 from forming oligomers and may further affect the NPM1 function.

NPM1 increases the malignancy of lung cancer cells. CL1-0 cells were transiently transfected with pcDNA3-NPM1 to mimic the accumulation of NPM1 oligomer, and the enforced expression of NPM1 was confirmed by Western blot (Fig. 4A). NPM1 significantly increased the cell-invading capability in a dose-dependent manner. The cell-invading capability of cells transfected with 10 μg of NPM1 expression vector was 2.5 times the value in the mock-transfected controls ($P = 0.013$; Fig. 4B). The migration capabilities at 6 and 12 hours after transfection were also significantly greater in NPM1 transfectants compared with the mock-transfected cells ($P = 0.031$ and 0.0017, respectively; Fig. 4C). In addition, NPM1 induced 2-fold or greater increases in colony formation compared with the mock in both anchorage-independent and -dependent growth assays ($P = 0.029$ and $P < 0.001$, respectively; Fig. 4D). However, HLJ1 expression did not only eliminate the oncogenic effects of NPM1 on colony formation ($P < 0.001$; see Supplementary Fig. S2) but also

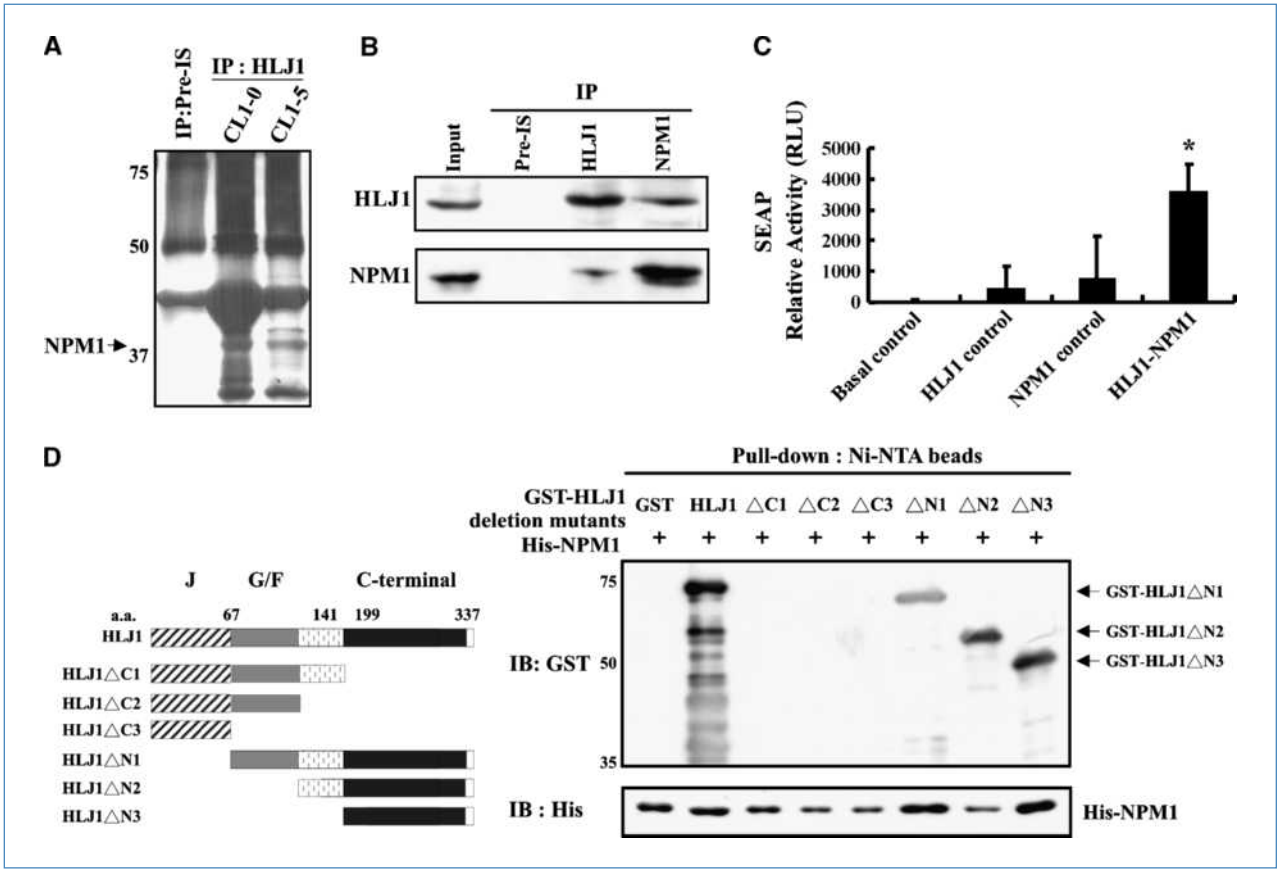

Figure 1. NPM1 acts as an HLJ1-interacting protein. A, the identification of HLJ1-interacting proteins by coimmunoprecipitation and mass spectrometry. The different proteins precipitated from CL1-0 and CL1-5 cells were subjected to mass spectrometry analysis. The position of NPM1 on the silver-stained SDS polyacrylamide gel is denoted by the arrow. B, determination of the intracellular interaction between HLJ1 and NPM1 by coimmunoprecipitation assays. CL1-0 cell lysates combined with preimmune serum (Pre-IS), anti-HLJ1, or anti-NPM1 antibody were subjected to immunoprecipitation, followed by immunoblotting with the antibodies indicated. Five percent of input was the positive control. C, determination of the interaction between HLJ1 and NPM1 *in vivo* using a mammalian two-hybrid assay. The SEAP activities of CL1-5 cells transfected with various combinations of plasmids, as indicated at the bottom, were determined. *, $P = 0.01$, compared with the HLJ1 control. D, identification of the domain of HLJ1 that interacted with NPM1. The HLJ1 mutant constructions with a series of NH_2 or COOH terminal truncations are illustrated (left). The full-length His-NPM1 was used as the bait to determine the interacting domain of HLJ1.

Table 1. The identification of HLJ1-interacting proteins by mass spectrometry

gi	Protein identification	MW	Score	Queries matched	Peptide
825671	B23 Nucleophosmin	30,919	434	44	K.VDNDENEHQLSLR.T K.DELHIVEAEAMNYEGSPIK.V
6631085	DnaJ (Hsp40) homologue, subfamily, member 4	37,783	32	2	R.DGSNIIYTAKISLR.E

neutralized the NPM1 effect on migration and invasion in both lower and higher invasive cells (see Supplementary Figs. S3 and S4).

HLJ1 regulates AP-2α activity by binding NPM1. A previous report showed that NPM1 acts as a novel AP-2α–binding transcriptional corepressor during retinoic acid–induced cell differentiation (16). Coimmunoprecipitation showed that both HLJ1 and NPM1 interact with AP-2α in CL1-0 cells (Fig. 5A). We hypothesized that HLJ1 interferes with NPM1 oligomerization and then releases NPM1 monomer, which increases the opportunity for interaction with AP-2α as a transcriptional corepressor. The reduction in HLJ1 by siRNA increased NPM1 oligomerization and then decreased the interaction between NPM1 and AP-2α (Fig. 5B).

Using the transcription element search software[6] (24), we predicted a putative AP-2α–binding consensus sequence in the promoter of *MMP-2* (Fig. 5C, top). To investigate whether HLJ1 regulates the transcription of *MMP-2* through the formation of an AP-2α complex, we performed a ChIP using anti-AP-2α antibody combined with PCR (Fig. 5C, middle). The *MMP-2* promoter-specific DNA fragments were eliminated in HLJ1-silenced cells compared with scrambled controls and were enriched in HLJ1 ectopically expressed cells compared with mock cells. A sequential ChIP experiment showed the cooccupancy of HLJ1, AP-2α, and NPM1 on the *MMP-2* promoter (Fig. 5C, bottom). To show the effect of HLJ1 on the transcriptionally suppressive activity of NPM1 directly, the *MMP-2* promoter assay was performed. Overexpression of NPM1 can enhance the transcription of *MMP-2* up to 3.6-fold compared with the mock control, but overexpression of HLJ1 can eliminate the transcriptional activity of NPM1 on *MMP-2* (see Supplementary Fig. S5).

HLJ1 downregulates MMP-2 expression by regulating NPM1 and AP-2α interaction. Because there is growing evidence that the STAT3 activity is associated with MMP-2 expression and metastasis (25, 26), we were interested in whether MMP-2 and STAT3 activities are dependent on HLJ1 expression. The low-invasive CL1-0 cells with abundant HLJ1 in the nucleus expressed less p-STAT3 (Tyr^{705}) than did the highly invasive CL1-5 cells with low HLJ1 expression (see Supplementary Fig. S6).

Silencing of HLJ1 markedly increased p-STAT3 phosphorylation (Tyr^{705}) and MMP-2 expression (Fig. 6A, left). In contrast, enforced expression of HLJ1 decreased the expression levels of p-STAT3 and MMP-2 appreciably (Fig. 6A, right). Furthermore, enforced expression of NPM1 in CL1-0 also induced MMP-2 expression and STAT3 phosphorylation compared with the mock control (Fig. 6B). Gelatin zymography provided functional evidence of an increase in MMP-2 gelatinase activity in HLJ1-silenced cells and a decrease in HLJ1 transfectants (Fig. 6C).

Discussion

Although HLJ1 has been characterized as a tumor suppressor and is related to relapse and survival of NSCLC patients (5), the molecular mechanisms responsible for the signaling pathways of HLJ1 and its involvement in cancer progression are still unclear. To understand the regulatory mechanisms underlying HLJ1 in tumorigenesis and metastasis, we identified the HLJ1-associated proteins and elucidated the biological significance of the interaction between HLJ1 and its partners, which might be important for anticancer therapy. Our results showed that HLJ1 can interact directly with NPM1 both *in vitro* and *in vivo*. We also found that HLJ1 binds competitively to NPM1 and then prevents NPM1 from forming oligomers in nucleus. The released HLJ1-NPM1 complex might bind further to tumor suppressor AP-2α and act as its corepressor, which would subsequently downregulate the expression of AP-2α-regulating genes, such as *MMP-2*. As a result, the capabilities of cell migration, invasion, and colonogenesis decrease, as HLJ1 is expressed ectopically.

HLJ1 was first identified from the human liver cDNA library and was classified as one member of the heat shock protein 40 family (Hsp40/DnaJ; ref. 27). HLJ1 belongs to the type II homologues of the Hsp40 family and comprises four conserved functional domains: a highly conserved J domain, glycine/phenylalanine-rich region, cysteine-rich region, and COOH terminal domain (28). Our study indicated that HLJ1 associates specifically with intact NPM1 through the COOH terminal region. The exact role of the COOH terminal domain of HLJ1 is still unknown and needs further exploration to clarify its biological significance.

In this work, we found that HLJ1-NPM1 complexes are located primarily in the nucleus and that reduction of HLJ1 alters the NPM1 distribution in the nucleus, causing an irregular, elongated shape and distortion of nuclear architecture. NPM1 is one structural component of chromosome scaffold, and the characteristics of nuclear architecture regulate gene expression (29). We speculated that the HLJ1-NPM1 interaction would disturb the nuclear distribution of NPM1 and

[6] http://www.cbil.upenn.edu/cgi-bin/tess/tess

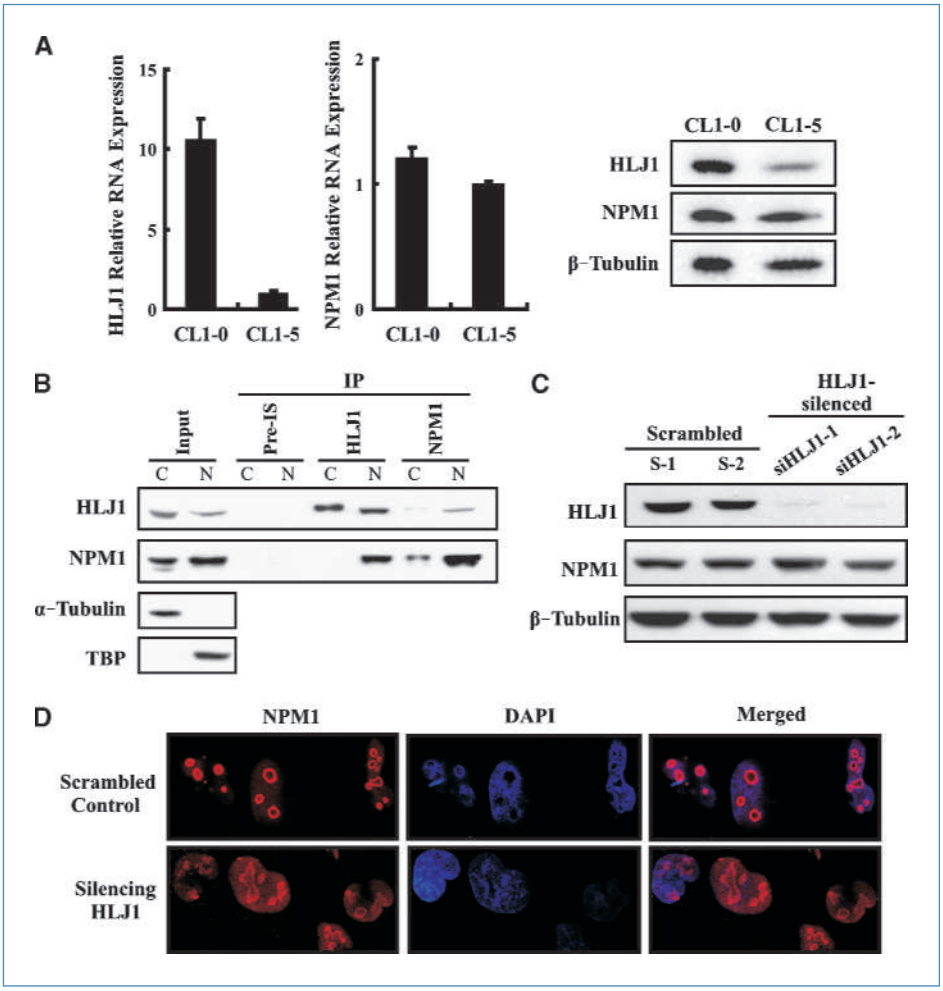

Figure 2. Effect of HLJ1 on the cellular distribution of NPM1. A, quantification of *HLJ1* and *NPM1* in cancer cell lines. The RNA and protein levels of HLJ1 or NPM1 in cancer cell lines with different degrees of invasiveness, CL1-0 and CL1-5, were measured by real-time SYBR-Green PCR and Western blot. TBP and β-tubulin were the controls for RNA and protein loading, respectively. B, cellular localization of the interaction between HLJ1 and NPM1. Cell extracts were separated into cytoplasmic (C) and nuclear (N) protein fractions and then immunoprecipitated with anti-HLJ1 or anti-NPM1 antibody, followed by Western blot. The input was used as a positive control to quantify the HLJ1 and NPM1 in the cytoplasmic and nuclear fractions. Pre-IS, preimmune serum. α-Tubulin and TBP served as the loading controls. C, expression of HLJ1 and NPM1 in HLJ1-silenced cells. The expression of HLJ1 and NPM1 was detected in two HLJ1-silenced stable cell lines (siHLJ1-1 and siHLJ1-2) and two scrambled stable controls (S-1 and S-2) by immunoblotting. β-Tubulin was used as the loading control. D, effect of HLJ1 on the cellular distribution of NPM1. HLJ1-silenced and scrambled cells were fixed and immunostained sequentially with anti-NPM1 and rhodamine-labeled antimouse secondary antibodies. The nuclei are demarcated with DAPI staining. Original magnification, 600×.

that the molar ratio of HLJ1 to NPM1 might be an important factor modulating cellular functions. This may explain why silencing HLJ1 or overexpressing NPM1 increases the invasiveness, migration ability, and colonogenesis of cancer cells. We also found that a low molar ratio of HLJ1 to NPM1 makes cells more malignant, because the amount of HLJ1 is insufficient to inhibit NPM1 oligomerization, which might strengthen the oncogenic activity of NPM1 (Supplementary Fig. S6, HLJ1/NPM1 ratio in nucleus). Two recent studies showed that blockade of endogenous NPM1 oligomerization by small molecular inhibitors or NPM1 peptides inhibits cell proliferation, induces apoptosis, and represses expression of the NPM1-controlling genes (30, 31). Hence, HLJ1 might act as a natural modulator to manipulate and fine-tune the biological activities of NPM1.

NPM1 tends to oligomerize *in vivo* via its NH_2 terminal domain (32) and exists as a hexamer (33). Oligomerization might modulate the various biological activities of NPM1 in different conditions. By perturbing the effect of HLJ1 on NPM1 oligomerization, we inferred that HLJ1 binding disrupts the oligomeric form, which changes into the monomeric form. Previous reports showed that NPM1 protein acts as both an oncogene and tumor suppressor, depending on its expression level and cellular localization (17, 18). Our findings that NPM1 overexpression by itself promotes oligomerization and that HLJ1 expression level alters NPM1 nuclear

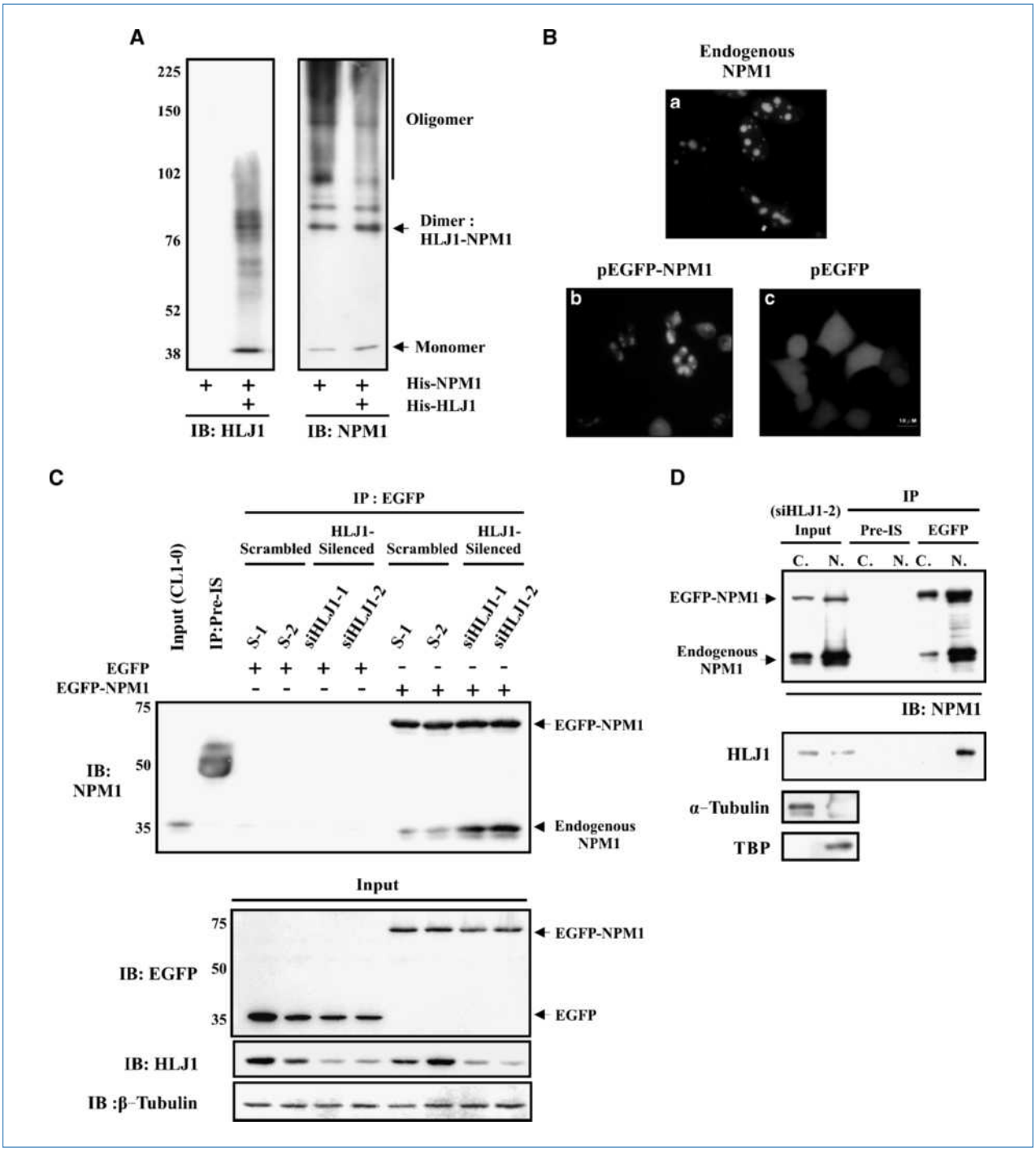

Figure 3. Effect of HLJ1 on NPM1 oligomerization. A, HLJ1 interferes with NPM1 oligomerization *in vitro*. Recombinant HLJ1 and NPM1 with a His-tag in the pQE31 vector were expressed in *E. coli*, and then the designated protein or mixtures were then separated by 6% native gel followed by Western blotting. B, cellular localization of endogenous NPM1 and exogenous EGFP-NPM1 fusion proteins. Endogenous NPM1 (a) is stained with anti-NPM1 antibody. Mock (pEGFP-C3) or the plasmids harboring full-length *NPM1* (pEGFP-NPM1) were transfected into CL1-0 cells. The localization of EGFP-NPM1 fusion proteins (b) and EGFP alone (c) was analyzed directly with fluorescence microscopy. C, effect of HLJ1 reduction on NPM1 oligomerization. Protein extracts prepared from scrambled controls (S-1 and S-2) and HLJ1-silenced cells (siHLJ1-1 and siHLJ1-2), which were transiently transfected with pEGFP-C3 or pEGFP-NPM1, were immunoprecipitated with anti-EGFP antibody and then reacted with anti-NPM1 antibody. In the input panel (bottom), EGFP and EGFP-NPM1 served as control of transfection efficiency, and β-tubulin served as the loading controls. Pre-IS, preimmune serum. D, cellular localization of HLJ1-NPM1 interaction and NPM1 oligomerization. Cell extracts were separated into cytoplasmic (C) and nuclear (N) protein fractions from HLJ1-silenced cells (siHLJ1-2) and then immunoprecipitated with anti-EGFP antibody, followed by Western blot. Pre-IS, preimmune serum. α-Tubulin and TBP served as the loading controls.

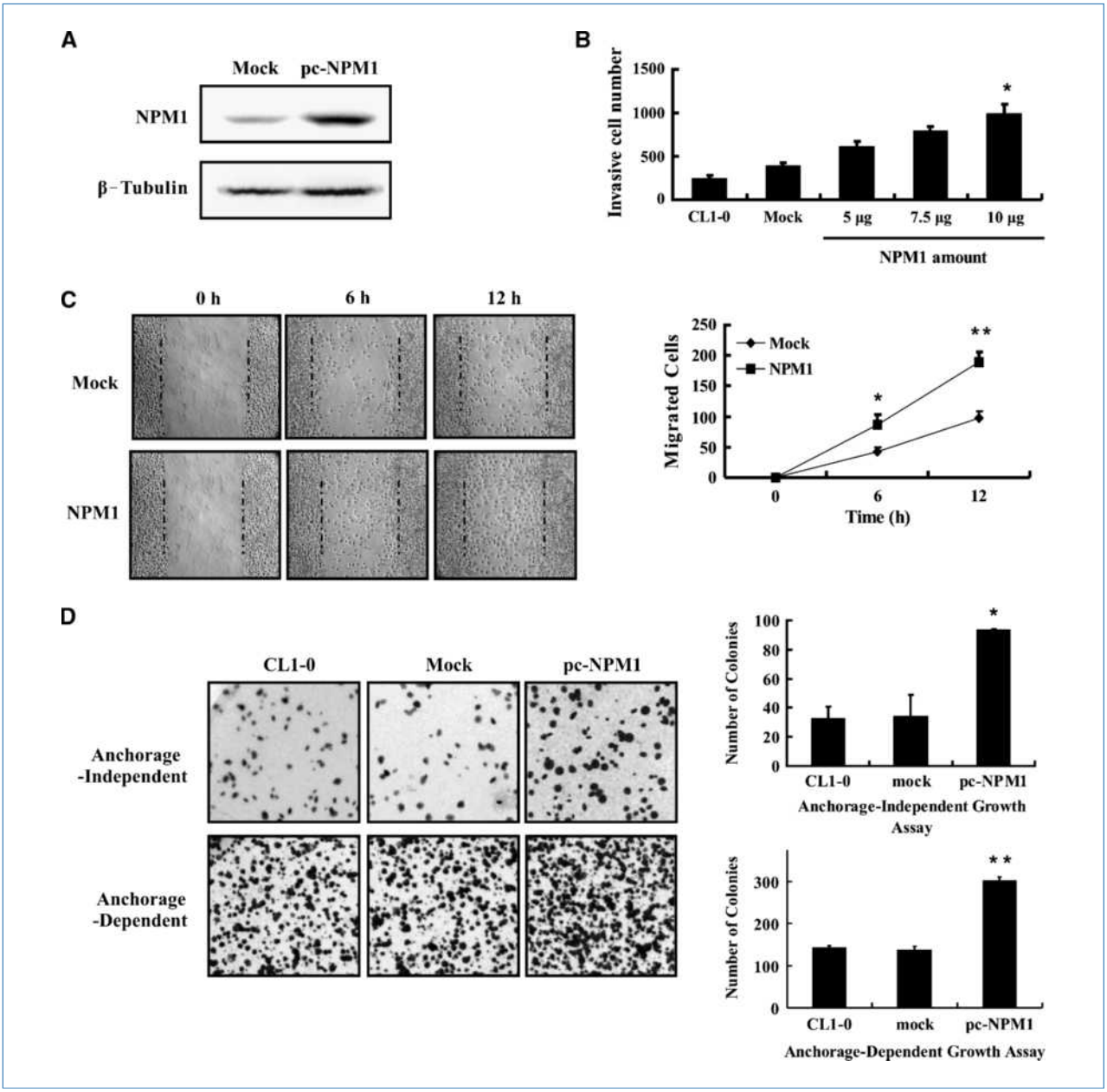

Figure 4. Effect of NPM1 on malignancy of lung cancer cells. A, enforced expression of NPM1. CL1-0 cells were transiently transfected with pcDNA-NPM1 (pc-NPM1) and pcDNA3 (mock) plasmids, and the expression of NPM1 and cellular functions were analyzed 24 h later. β-Tubulin was the loading control. B, effect of NPM1 on invasiveness. The invasiveness of CL1-0 cells transfected with pcDNA-NPM1 or mock plasmids was evaluated in a modified Boyden chamber assay. *, $P = 0.013$, compared with mock. C, effect of NPM1 on migration. The migration of NPM1 and mock transfectants was evaluated using the wound-healing assay. After wounding, the track was photographed immediately, 6 h, and 12 h later. The number of cells migrating into the cell-free zone was evaluated at each time point indicated. *, $P = 0.031$ and **, $P = 0.0017$, compared with corresponding mock. D, effect of NPM1 on colonogenesis. The colony formation of the NPM1 and mock transfectants was analyzed by anchorage-independent and anchorage-dependent growth assays. After transfection, the cells were harvested and seeded into six-well plates and grown for 2 wk at 37°C. Colonies larger than 1 mm were counted. *, $P = 0.029$ and **, $P < 0.001$, compared with mock.

distribution and oligomerization are consistent with these previous studies. Our results also suggest that the oncogenic or tumor-suppressive role of NPM1 might be switched according to the level of NPM1 oligomerization, which is modulated by HLJ1.

AP-2 proteins are involved in tumorigenesis and act as tumor suppressors in mammary carcinomas (34). The AP-2 family includes several highly homologous proteins, such as AP-2α, AP-2β, AP-2γ, AP-2ε, and AP-2δ, which would be able to form homodimers and heterodimers to recognize GC-rich

DNA sequences (5′-GCCN$_3$GGC-3′) of the target genes (34, 35). For instance, AP-2α plays a suppressive role in cancer development through its effects on invasion, migration, carcinomatosis, and changed cell morphology from the spindle to epithelioid type, which would increase E-cadherin and reduce MMP-2 levels (36, 37). A previous study also indicated that NPM1 can regulate cell growth and differentiation by acting as an AP-2α corepressor in a newly described mechanism (16). We found that HLJ1 induces the release of NPM1 monomer or HLJ1-NPM1 heterodimer, which acts as an AP-2α corepressor (Figs. 3 and 5). AP-2α would also bind to the *MMP-2* promoter, and HLJ1 would regulate the AP-2α binding to the *MMP-2* promoter.

The MMP family is associated with invasion and metastasis of cancer cells (38). MMP-2, which degrades type IV collagen, plays an important role in the malignant progression of cancer (39). A previous study revealed that AP-2 proteins regulate MMP-2, E-cadherin and p21WAF-1, and BCL-2 (40). In addition, AP-2α inactivation increases MMP-2 expression and activity and cell invasion in melanoma cells (41). Although the molecular basis of MMP-2 transcriptional regulation in tumor cells remains unclear, our results suggest that HLJ1 regulates MMP-2 expression and activity through NPM1 as an AP-2α corepressor (Fig. 6B and C). On the other hand, MMP-2 expression is also regulated by STAT3 activity in various cancers, such as melanoma, ovarian cancer, and colorectal carcinoma (25, 26). STAT3 is constitutively activated in many tumor cell lines and human cancers, including lung cancers (42), and its activation requires tyrosine or serine phosphorylation (43). The elevated levels of STAT3 phosphorylation are significantly associated with antiapoptosis, cell cycle regulation, metastasis, and angiogenesis (44). We found that HLJ1 can modulate STAT3 phosphorylation appreciably through the HLJ1-NPM1 interaction, by which HLJ1 or NPM1 might regulate both invasiveness and colonogenesis. Our results show that HLJ1 can repress MMP-2 expression through two distinct mechanisms: enhancement of AP-2α DNA binding and reduction of STAT3 activity.

In conclusion, we discovered a new mechanism of HLJ1 suppression of tumor progression, in which HLJ1 modulates

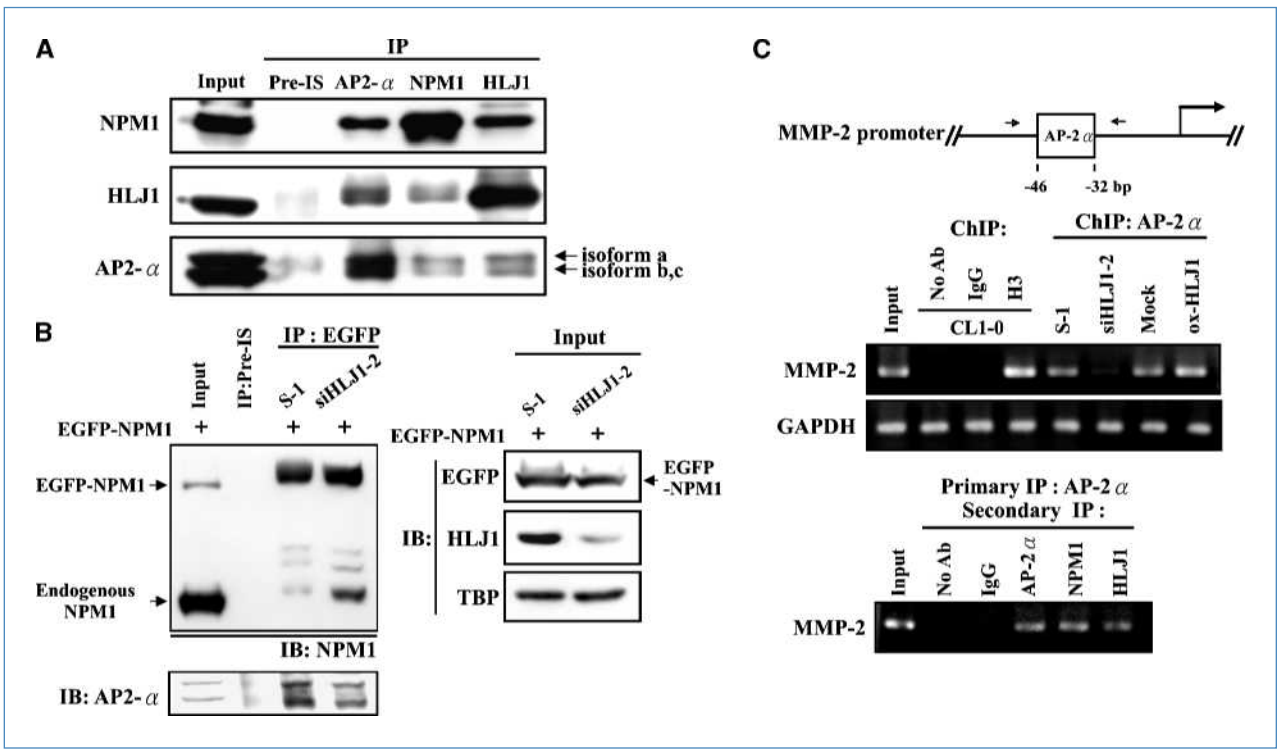

Figure 5. Involvement of HLJ1 in the interaction between NPM1 and AP-2α. A, determination of the intracellular interaction between NPM1, AP-2α, and HLJ1 by coimmunoprecipitation. CL1-0 cell nuclear lysates were reacted with preimmune serum (Pre-IS), NPM1, AP-2α, or HLJ1 antibody, and then subjected to immunoprecipitation, followed by immunoblotting with the antibodies indicated. Five percent of the input served as a positive control. B, retardation of NPM1 oligomerization and increase in the interaction between NPM1 and AP-2α by HLJ1. HLJ1-silenced (siHLJ1-2) and scrambled control (S-1) cells were transiently transfected with pEGFP-NPM1, subjected to isolate nuclear protein extracts, and then immunoprecipitated with anti-EGFP antibody, followed by Western blot with anti-NPM1 and anti-AP-2α antibodies. Input control derived from S-1. EGFP-NPM1 and TBP served as the loading controls (right). C, ChIP analysis of the *in vivo* binding of AP-2α in the *MMP-2* promoter. Top, scheme of the AP-2α regulatory element predicted in the *MMP-2* promoter region. Sheared chromatin fragments were immunoprecipitated with the antibodies indicated and were subjected to PCR amplification (middle). Mock, mock-transfected CL1-5 cells; ox-HLJ1, HLJ1-transfected CL1-5 cells; input, chromosomal DNA; No Ab, no antibody; IgG, rabbit preimmune IgG; H3, anti-acetyl histone H3 antibody, positive control; *GAPDH,* PCR product of glyceraldehyde-3-phosphate dehydrogenase, loading control. Bottom, the sequential ChIP analysis for the co-occupancy of AP-2α, NPM1, and HLJ1 in the *MMP-2* promoter. Fixed chromatin extracts from CL1-0 cells were immunoprecipitated with the AP-2α antibody and then subjected to a second round of immunoprecipitation with the antibodies indicated.

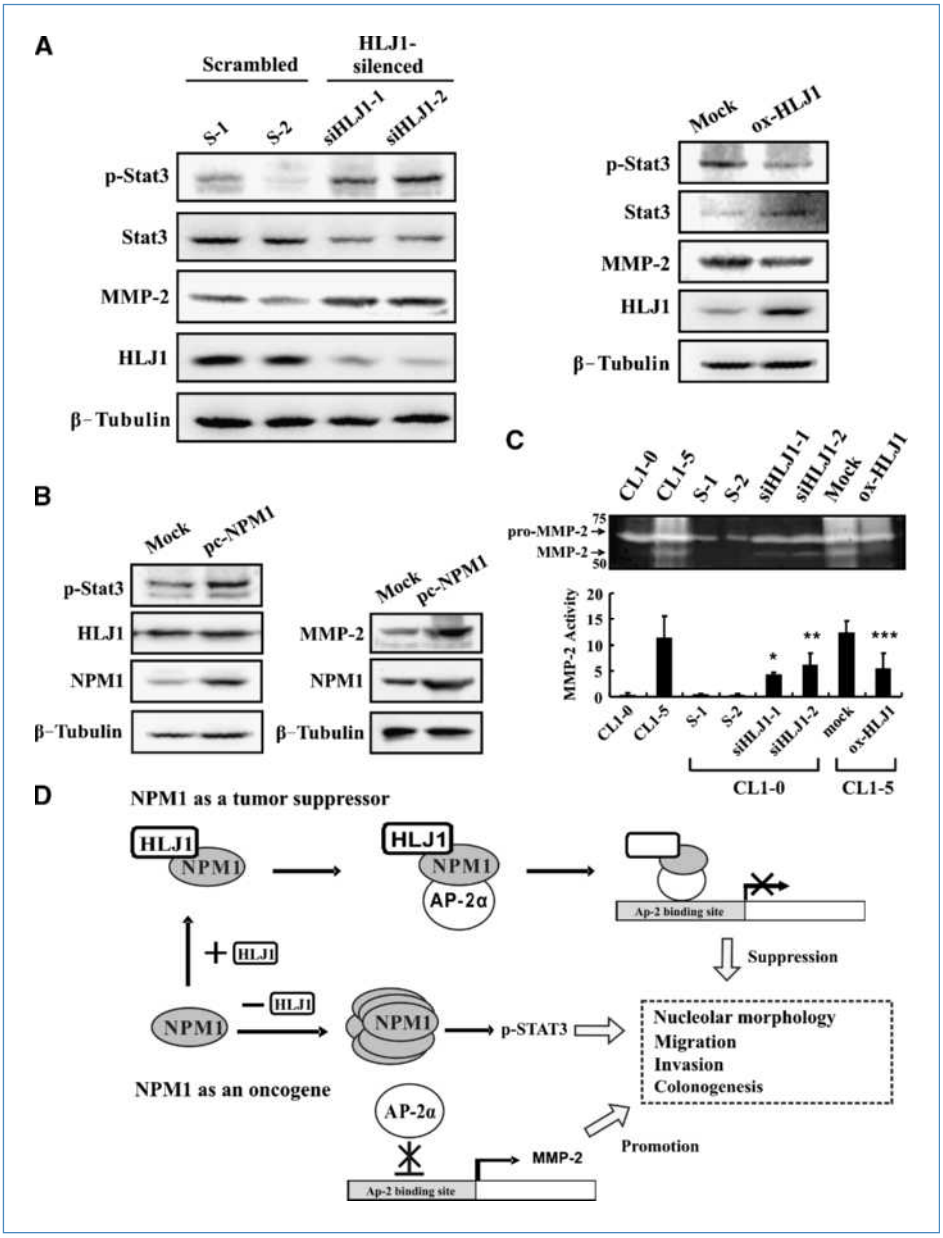

Figure 6. Effect of HLJ1 and NPM1 expression on the regulation of MMP-2 and STAT3. A, HLJ1 attenuates STAT3 activity and MMP-2 expression. HLJ1-silenced, scrambled control, HLJ1-overexpressed (ox-HLJ1), and mock control cell extracts were subjected to immunoblotting analysis with the designated antibodies. B, NPM1 activates STAT3 and increases MMP-2 expression. CL1-0 cells were transfected with pcDNA-NPM1 (pc-NPM1) or pcDNA3 (mock), harvested after 24 h, and subjected to immunoblotting analysis. C, HLJ1 negatively regulates gelatinolytic activity. The designated cell lines were cultured with serum-free medium, the media were subjected to zymographic analysis (top), and the density of each lytic band was quantified (bottom). *, $P < 0.001$; **, $P = 0.045$, compared with S-2. ***, $P = 0.032$, compared with mock. S-1 and S-2, CL1-0 cells transfected with scrambled siRNAs; siHLJ1-1 and siHLJ1-2, CL1-0 cells transfected with HLJ1 siRNAs; mock, mock-transfected CL1-5 cells; ox-HLJ1, HLJ1-transfected CL1-5 cells. D, a hypothetical model wherein HLJ1 switches the dual role of NPM1. HLJ1 may bind NPM1 though its COOH terminal domain and prevent NPM1 from oligomerization, which could change the role of NPM1 from proto-oncogene to tumor suppressor. The monomeric (NPM1 alone) or heterodimeric (HLJ1-NPM1) form of NPM1 would interact with AP-2α and act as an AP-2α corepressor and subsequently downregulate target genes, such as *MMP-2*. Therefore, silencing HLJ1 might facilitate NPM1 oligomerization and reduce the release of the NPM1 monomer, which would promote cancer cell invasion, migration, and colonogenesis.

NPM1 oligomerization and NPM1-AP-2α complex formation, which alter AP-2α transcriptional activity. These changes then suppress the downstream gene expression and activity of enzymes, such as MMP-2, and decrease cancer cell invasiveness, migration ability, and colonogenesis (Fig. 6D). HLJ1 might also affect the phosphorylation of STAT3 by altering NPM1 oligomerization, but the molecular mechanism remains unclear. The molar ratio of HLJ1 to NPM1 in

the nucleus might be important for the dual role of NPM1. It is reasonable to speculate that any disturbance in the balance between HLJ1 and NPM1 might dramatically change the role of *NPM1,* either as a tumor suppressor or an oncogene.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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