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YAP1 is involved in mesothelioma development and negatively regulated by Merlin through phosphorylation

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We previously reported the results of bacterial artificial chromosome array comprehensive genomic hybridization of malignant pleural mesotheliomas (MPMs), including two cases with high-level amplification in the 11q22 locus. In this study, we found that the *YAP1* gene encoding a transcriptional coactivator was localized in this amplified region and overexpressed in both cases, suggesting it as a candidate oncogene in this region. We analyzed the involvement of YAP1 in MPM proliferation, as well as its functional and physical interaction with Merlin encoded by the *neurofibromatosis type 2* (*NF2*) tumor suppressor gene, which is frequently mutated in MPMs. YAP1-RNA interference suppressed growth of a mesothelioma cell line NCI-H290 with *NF2* homozygous deletion, probably through cell-cycle arrest and apoptosis induction, whereas YAP1 transfection promoted the growth of MeT-5A, an immortalized mesothelial cell line. We also found that the introduction of *NF2* into NCI-H290 induced phosphorylation at serine 127 of YAP1, which was accompanied by reduction of nuclear localization of YAP1, whereas nuclear localization of a YAP1 S 127A mutant was not affected. Furthermore, results of immunoprecipitation and *in vitro* pull-down assays indicated a physical interaction between Merlin and YAP1. These results suggest that YAP1 is involved in mesothelial cell growth and that the transcriptional coactivator activity of YAP1 is functionally inhibited by Merlin through the induction of phosphorylation and cytoplasmic retention of YAP1. This is the first report of negative regulatory signaling from Merlin to YAP1 in mammalian cells. Future studies of transcriptional targets of YAP1 in MPMs may shed light on the molecular mechanisms of MPM development and lead to new therapeutic strategies.

Introduction

A malignant pleural mesothelioma (MPM) is a highly lethal neoplasm that is thought to develop from pleural mesothelial cells, with exposure to asbestos playing a crucial role in tumor development (1–4). Patients with an MPM are usually diagnosed at an advanced stage and the tumors are refractory to conventional therapeutic modalities; thus, their prognosis is very poor, even though advancements in chemotherapeutical modalities that combine cisplatin and antifolate, such as pemetrexed or raltitrexed, have been made (5,6). The long latency period between asbestos exposure and tumor appearance implies that multiple genetic changes are required for malignant transformation of mesothelial cells (7,8). Accumulated genetic studies have identified that tumor suppressor genes are crucial for MPM development, including frequent inactivation of $p16^{INK4a}/p14^{ARF}$ at 9p21 (9–11) and neurofibromatosis type 2 (*NF2*) at 22q12 (12–14). The *NF2* gene is responsible for NF2 syndrome (15) and encodes Merlin (also known as schwannomin), an ezrin/radixin/moesin family protein that has been shown to be involved in cytoskeletal dynamics, growth factor receptor signaling and cell adhesion (16,17).

To further elucidate the alterations of oncogenes and tumor suppressor genes responsible for MPM development, we previously carried out bacterial artificial chromosome (BAC) array comprehensive genomic hybridization (CGH) analyses of MPM specimens from a total of 22 individuals and reported several distinct chromosomal alterations including high copy amplification of 11q22 (18). In the present study, we found that the *YAP1* gene, which was originally cloned as a partner of Yes kinase (19), resides within the 11q22 amplification region and that YAP1 is involved in mesothelial cell growth. Furthermore, we found that YAP1 activity may be negatively regulated via Merlin signaling in mesothelial cells. To our knowledge, this is the first known report of the existence of negative regulatory signaling from Merlin to YAP1 in mammalian cells, which may play a crucial role in growth regulation of mesothelial cells and development of malignant mesothelioma.

Abbreviations: BAC, bacterial artificial chromosome; CGH, comprehensive genomic hybridization; EGFP, enhanced green fluorescent protein; GST, glutathione S-transferase; MPM, malignant pleural mesothelioma; NF2, neurofibromatosis type 2; NHERF1, Na(+)/H(+) exchanger regulatory factor 1; PCR, polymerase chain reaction; RNAi, RNA interference; SDS, sodium dodecyl sulfate; sh, short hairpin.

Materials and methods

Array CGH analysis and quantitative polymerase chain reaction analyses of copy number and expression

Genome-wide array CGH analysis of 22 individual MPMs using microarrays with 2304 BAC and P-1 phage-derived artificial chromosome clones covering the whole human genome at a resolution of roughly 1.3 Mb was previously reported (18). To determine the precise copy numbers within the amplification, quantitative polymerase chain reaction (PCR) using custom TaqMan probes (Applied Biosystems, Foster City, CA) corresponding to the genomic sequences of seven genes (*PGR*, *TRPC6*, *ANGPTL5*, *YAP1*, *BIRC2*, *MMP13* and *AB08258*) dispersed within the 3 Mb region were designed and used together with TaqMan PCR master mix (Applied Biosystems) and an ABI7500 system (Applied Biosystems), according to the manufacturer's instructions. The copy number of the leucine-rich repeat containing the *4C* gene (also called *NGL1*) localized at 11p12 was used as a control. To examine the expression of genes within each amplification, TaqMan expression probes (Applied Biosystems) for *ANGPTL5*, *YAP1*, *BIRC2* and *BIRC3* were used, and quantification was performed as above.

Construction of RNA interference vectors and expression vectors

To construct RNA interference (RNAi) vectors, short hairpin (sh) oligonucleotides were inserted into a plasmid containing the U6 promoter and a puromycin-resistant gene (20). Two sh oligonucleotides were designed for two different sequences within the YAP1 open reading frame (YAP1-sh1, GGCCATGCTGTCCCAGATGAAT and YAP1-sh2, GGAGATGGAATGAACATAGAAT). In addition, control vectors, YAP1-scr1 and green fluorescent protein (GFP)-sh, were constructed using oligonucleotides with scrambled sequences for YAP1-sh1 (GGCTGCCATTCGCGACATGAAT) and GFP open reading frame (GFP-sh, GCAAGCTGACCCTGAAGTTCA). YAP1 cDNA was purchased from OriGene (Rockville, MD) and inserted into pcDNA (Invitrogen, Carlsbad, CA) and pEGFP-C1 (Clontech, Mountain View, CA) vectors. The phosphorylation-defective mutant YAP1 was constructed by *in vitro* mutagenesis at codon 127 from serine to alanine (S 127A), as the phosphorylation of serine 127 was reported to induce an interaction between 14-3-3 and cytoplasmic retention (21). *NF2* cDNA was amplified with reverse transcription–PCR and cloned into pcDNA (Invitrogen) and the lentivirus vector pLentiLox3.7. The *Na(+)/H(+) exchanger regulatory factor 1 (NHERF1)/*ezrin/radixin/moesin-*binding phosphoprotein 50 kD* gene expression constructs

were kindly provided by Dr Maria-Magdalena Georgescu (University of Texas M. D. Anderson Cancer Center) and Dr Martha C. Nowycky (University of Medicine and Dentistry of New Jersey).

Cell culture and colorimetric and flow cytometry analyses

A malignant mesothelioma cell line (NCI-H290), a gift from Dr Adi F. Gazdar (University of Texas Southwestern Medical Center), and a non-malignant mesothelial cell line (MeT-5A), purchased from American Type Culture Collection (Rockville, MD), were cultured as described previously (18). YAP1-RNAi vectors were transfected into NCI-H290 or MeT-5A cells using Lipofectamine 2000 (Invitrogen). For cell proliferation analysis, transfected cells were treated with puromycin at 1 μg/ml for 10 days, then stained using TetracolorOne (Seikagaku, Tokyo, Japan), after which absorbance was determined at 450 nm. For analysis of the cell cycle and sub-G_1 population, transfected cells were treated with puromycin at 1 μg/ml for 24 h, after which the culture medium and dead cells were removed. Residual and viable cells were further cultured without puromycin for 24 h, then harvested and stained with propidium iodide for flow cytometry analysis, as described previously (20).

Immunoblotting analysis

For preparation of nuclear and cytoplasmic fractions, cells were incubated in hypotonic buffer with 0.5% NP-40, then the nuclei were pelleted using a brief centrifugation, as described previously (22). For immunoblotting analysis, after harvesting the cells with lysis buffer, protein concentration was determined with a DC Protein assay kit (Bio-Rad, Hercules, CA). The same amounts of protein samples were applied to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis, then electrotransferred to Immobilon-P polyvinylidene difluoride membranes. Each membrane was incubated with anti-V5-tag (Invitrogen) for V5-tagged NF2, anti-YAP1 (Cell Signaling and Abnova, Taipei, Taiwan) and anti-S 127 phospho-YAP1 (Cell Signaling, Danvers, MA) antibodies, then visualized using an ECL detection kit (GE Healthcare, Amersham Place, UK).

Immunofluorescent microscopic analysis

NCI-H290 cells were transfected with expression vectors for the enhanced green fluorescent protein (EGFP)-fused YAP1 wild-type or S 127A mutant together with V5-tagged NF2 or an empty vector and cultured on cover glass slides. The transfected cells were then fixed, permeabilized and incubated with anti-V5 and Alexa Fluor568-conjugated anti-mouse antibodies and examined with a confocal microscope (LSM510, Carl Zeiss MicroImaging GmbH, Jena, Germany).

Immunoprecipitation and in vitro pull-down assays

For immunoprecipitation analyses, 293T cells were transfected with the EGFP-fused wild-type or S 127A mutant YAP1 constructs together with V5-tagged NF2 or an empty vector. Immunoprecipitates of lysates transfected with the anti-V5 antibody were subjected to SDS–polyacrylamide gel electrophoresis and immunoblotting with various antibodies.

For *in vitro* pull-down assays, human NF2 full-length cDNAs were inserted into a pGEX-KG vector (Amersham Pharmacia Biotech, Uppsala, Sweden) to express bacterial glutathione S-transferase (GST)–Merlin fusion protein. GST–Merlin or GST-alone proteins were purified from the transformed bacterial lysates by incubation with glutathione sepharose beads (GE Healthcare). The YAP1 protein expressed with an *in vitro* transcription/translation system (Promega, Madison, WI) or cell lysate of 293T transfectants were incubated with beads containing 3 μg of immobilized GST-alone or GST–Merlin fusion proteins for 3 h at 4°C, then washed four times. Proteins bound to GST proteins were eluted by boiling in SDS sample buffer, then separated by SDS–polyacrylamide gel electrophoresis and immunoblotted with various antibodies.

Results

Precise mapping of 11q22 amplification region in malignant mesotheliomas

We previously reported the results of genome-wide array CGH analyses of MPMs derived from 22 individuals, in which it was notable that two primary MPM cases (KD1033 and KD 1041) showed two discrete and significant high-level amplifications in the chromosome 1p32 and 11q22 regions (18). Since we demonstrated that the *JUN* proto-oncogene resided in the 1p32 amplification region, whose expression was shown to be induced by asbestos exposure in rat pleural mesothelial cells (23) and whose amplification was recently demonstrated in aggressive sarcomas (24), we speculated that the 11q22 amplification region may also harbor an important target gene whose overexpression is involved in MPM cell growth. To identify the target gene, we precisely determined the extent of the amplified regions from these two cases that were overlapped and bounded by RP11-40B14 and RP11-652L13 BAC probes, with only two BAC probes (RP11-203C2 and RP11-864G5) included between them (Figure 1A). With quantitative PCR analysis using TaqMan probes, the copy numbers of seven genes (*PGR*, *TRPC6*, *ANGPTL5*, *YAP1*, *BIRC2*, *MMP13* and *AB08258*) which dispersed within the 3-Mb-long region were investigated. As expected, both tumors were shown to carry high copy numbers of the *ANGPTL5*, *YAP1*, *BIRC2* and *MMP13* genes, while no gains were detected in *TRPC6* and *AB08258* genes, indicating that the extent of the common amplification region was ~1 Mb in length including 14 candidate genes (Figure 1B). In addition, comparing with each gene amplification level carefully, both *ANGPTL5* and *YAP1* showed about a 2-fold greater increase in copy numbers than *BIRC2* and *MMP13* in KD1033, while each amplification level of the four genes was similar in KD1041 (Figures 1B and 2C). This result suggested that the amplification of the centromeric half region including *ANGPTL5* and *YAP1* might be more important than that of telomeric region including the *BIRC2* and *MMP* cluster during the development of those MPMs, at least in KD1033.

Overexpression of YAP1 and BIRC2 in malignant mesotheliomas

To determine which gene residing in the 1 Mb amplification region was the most responsible for the development of these MPM cases, we next studied the expression levels of each gene using real-time reverse transcription–PCR analysis. Among the four genes in the centromeric half amplification region, *ANGPTL5*, *YAP1* and *BIRC2* were overexpressed in KD1033, whereas *YAP1*, *BIRC2* and *BIRC3* in KD1041, indicating that only *YAP1* and *BIRC2*, but not *ANGPTL5* or *BIRC3*, were commonly overexpressed in these tumors (Figure 2A). These results strongly suggest that the most probable target genes in this amplification region are *YAP1* and *BIRC2*. Meanwhile, we also examined the expression levels of several *MMP* cluster genes, but did not detect any overexpression, again suggesting that amplification of the telomeric half region was not significant (data not shown). The immunohistochemical staining results also clearly demonstrated the overexpression of YAP1 in KD1033 (Figure 2B, left panel), though normal pleural mesothelial cells did not show any YAP1 signals (arrowheads in Figure 2B, right panel).

Next, to determine whether other MPMs not shown to have clear amplification in BAC/P-1 phage derived artificial chromosome array CGH analysis may also have a more confined amplification or deregulation of the *YAP1* and *BIRC2* genes, we examined the copy numbers as well as expression levels of these genes using 12 additional primary MPM cases (Figure 2C and D) and 13 MPM cell lines (data not shown). However, amplification of these genes in these MPM specimens and cell lines was not detected, except for KD1033 and KD1041, nor was there significant upregulation of the others observed (Figure 2C and D). These results suggest that even though significant overexpression of the *YAP1* and *BIRC2* genes can occur, amplification of these genes is a relatively infrequent event in MPMs.

Involvement of YAP1 in mesothelial cell proliferation

To determine cancer-promoting roles of these genes in mesothelioma cells, we first focused on the *YAP1* gene since its positive role has also been suggested in other malignancies (25,26). We synthesized two YAP1-RNAi vectors, YAP1-sh1 and YAP1-sh2, to suppress endogenous YAP1 expression, a scramble control vector, YAP1-scr1, and a GFP-RNAi vector, GFP-sh. These vectors were transfected into MPM cell line NCI-H290 cells, and expression levels of endogenous YAP1 protein were examined. Both YAP1-RNAi vectors effectively reduced the expression levels of YAP1 to 30 and 10%, respectively, whereas the control vectors (GFP-sh and YAP1-scr1) did not demonstrate any suppression (Figure 3A). We then studied the effects of YAP1-RNAi on cell proliferation of mesothelioma. Both YAP1-RNAi vectors demonstrated significant suppression of cell proliferation, with YAP1-sh2 showing complete abolition of cell proliferation (Figure 3B). Flow cytometry analysis revealed that cells transfected by YAP1-sh2 showed G_1 arrest and induction of a sub-G_1 population

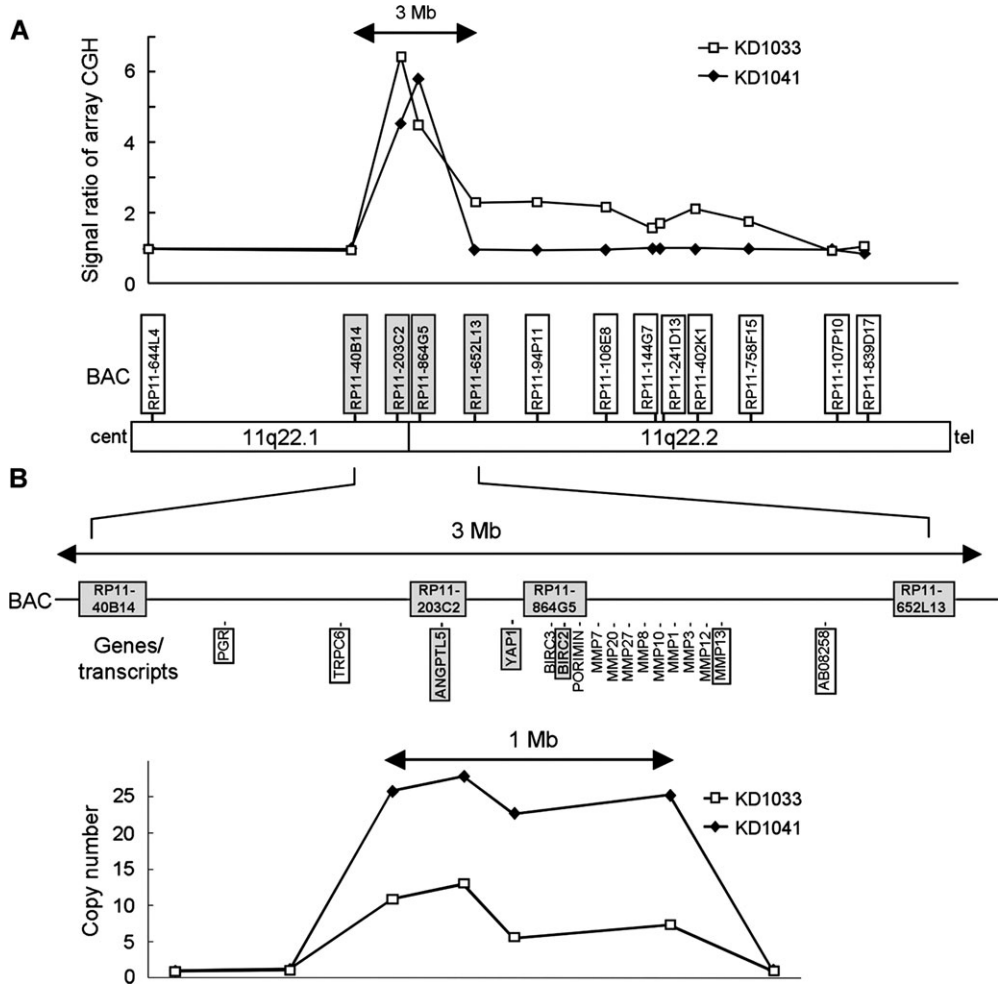

Fig. 1. Mapping of amplified region of 11q22 locus in two MPM cases. (**A**) Details of array-CGH results of 11q22 amplification in two MPM cases. The signal ratios from array CGH analyses of two primary MPM cases (KD1033 and KD 1041) were plotted for all BAC clones based on chromosome position, and the results indicated discrete and significant amplifications at the 11q22 region. Amplifications in both cases were similar within a 3-Mb-long region, which was bounded by RP11-40B14 and RP11-652L13, and included only two BAC probes (RP11-203C2 and RP11-864G5), represented by open squares and closed diamonds, respectively. (**B**) Copy number analyses using quantitative PCR with TaqMan probes. Genes and registered transcripts within the 3-Mb-long region are shown. To further determine the boundaries of the amplified regions, the copy numbers of seven genes (*PGR*, *TRPC6*, *ANGPTL5*, *YAP1*, *BIRC2*, *MMP13* and *AB08258*, indicated by boxes) were investigated using TaqMan probes. Four genes (*ANGPTL5*, *YAP1*, *BIRC2* and *MMP13*) showed high copy numbers in the two MPM cases, suggesting that both carried quite similar 1-Mb-long amplifications.

and those by YAP1-sh1 a moderate induction of the sub-G_1 population (Figure 3C and D). In contrast, the control vectors did not show any growth-suppressive effect.

Furthermore, we transfected a YAP1 expression vector into the immortalized mesothelial cell line MeT-5A to figure out whether YAP1 has growth-promoting activity in mesothelial cells. YAP1 overexpression moderately supported cell proliferation in a low-serum condition of 1% fetal calf serum, whereas it did not demonstrate clear promotion of cell proliferation in the usual condition (fetal calf serum 5%) (Figure 3E).

Functional interaction between YAP1 and Merlin

Since the Merlin-encoding *NF2* gene is frequently altered in MPMs, and the Merlin–Hippo–Warts pathway in *Drosophila* is known to negatively regulate Yorkie, the *Drosophila* ortholog of YAP1 through its phosphorylation (27), the results shown above strongly suggested that Merlin, as an upstream molecule, may functionally interact with and also suppress YAP1 in human mesothelial cells. In order to confirm this hypothesis, we cotransfected the *NF2* and *YAP1* expression vectors into NCI-H290 cells carrying a homozygous deletion of the *NF2* gene and studied whether exogenous Merlin has an effect on the phosphorylation status of YAP1 using the antibody against phosphorylated serine 127 (S 127) of YAP1, a critical phosphorylation site that has been indicated to induce inactivation of YAP1 as transcription coactivator through the induction of cytoplasmic retention (21). We found that cotransfection significantly induced the phosphorylation of YAP1 at S 127 (Figure 4A). To further demonstrate YAP1 S 127 phosphorylation by the activated form of Merlin, we also synthesized lentivirus vectors for full-length NF2 and truncated four-point-one/ezrin/radixin/moesin (FERM)-NF2 which translates 340 amino acids of the amino terminal and transfected into NCI-H290 cells. As expected, S 127 phosphorylation of YAP1 was induced by full-length NF2 in a dose-dependent manner, but not with truncated FERM-NF2 (Figure 4B).

Next, we determined whether phosphorylation of YAP1 protein induced its cytoplasmic localization, resulting in YAP1 inactivation as a transcriptional coactivator. We transfected NCI-H290 cells with EGFP-fused wild-type or S 127A mutant *YAP1*, together with *NF2* or an empty vector, and studied the subcellular localization using immunoblotting (Figure 4C) and immunofluorescence (Figure 4D). Immunoblotting of fractionated lysates, also depicted as a bar graph in the figure, clearly showed that wild-type phospho-YAP1 was scarcely detectable in the nuclear fractions, whereas the total YAP1 protein was localized in both the nucleus and cytoplasm (Figure 4C). In addition, the nuclear proportion of mutant YAP1 was higher than that of wild-type YAP1 (Figure 4C). These results suggested that

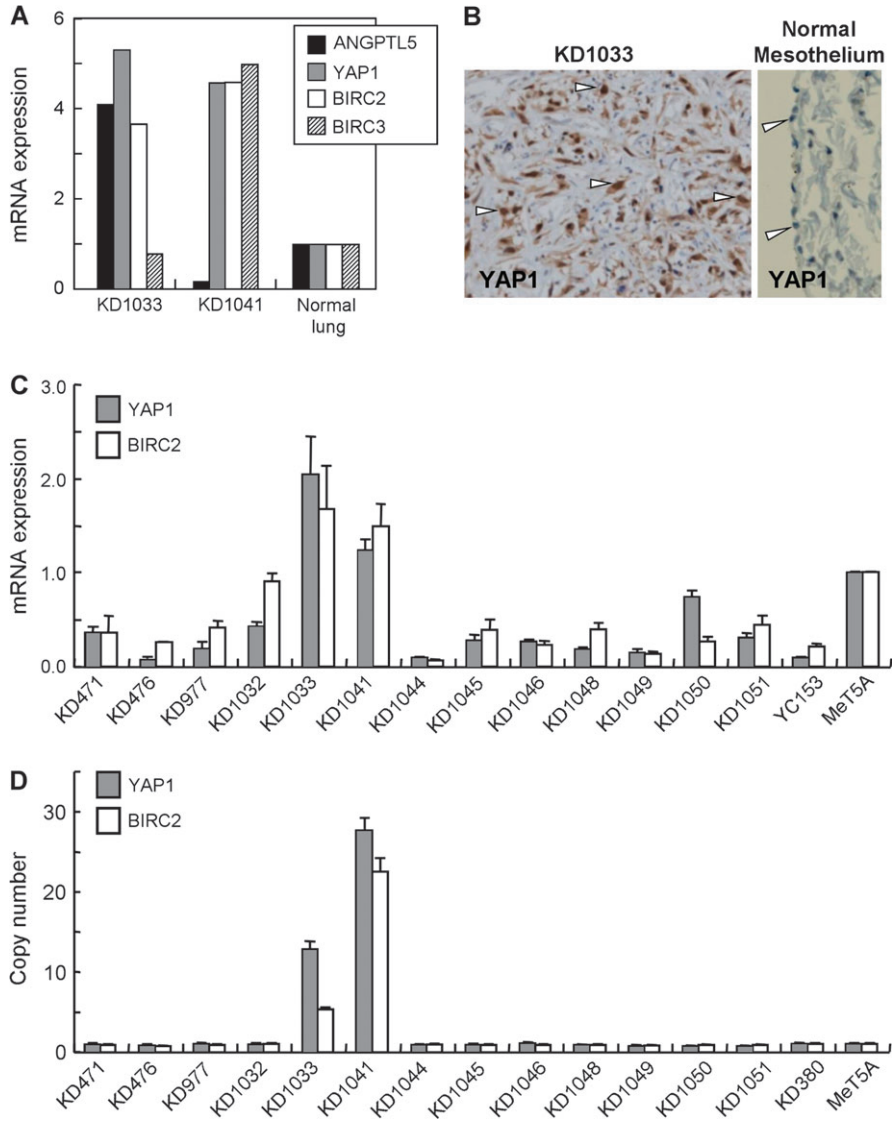

Fig. 2. Alterations of copy number and expression of *YAP1* and *BIRC2*. (**A**) Expression analyses of the *ANGPTL5*, *YAP1*, *BIRC2* and *BIRC3* genes indicated that *YAP1* and *BIRC2* were overexpressed in common in both MPM cases with amplification. (**B**) Immunohistochemical staining of YAP1. Immunohistochemical analysis clearly demonstrated overexpression and nuclear accumulation of YAP1 in KD1033 (left panel), whereas normal pleural mesothelial cells did not show any YAP1 signals (arrowheads, right panel). (**C**) Expression analysis in primary MPM cases. In an examination of 14 primary MPM cases and the normal mesothelial cell line MeT-5A, the two MPM cases showed the greatest amount of upregulated expressions of the *YAP1* and *BIRC2* genes with amplification. (**D**) Copy number analysis of primary MPM cases. In an examination of 14 MPM cases and the normal mesothelial cell line MeT-5A, only the two MPM cases demonstrated amplification of the *YAP1* and *BIRC2* genes.

phosphorylation negatively regulates nuclear localization and transcriptional activity of YAP1 protein. Although Merlin induced phosphorylation of YAP1, the nuclear proportion of wild-type total YAP1 was not significantly reduced, probably because of the existence of YAP1-alone transfectants.

Next, we performed immunofluorescence to further confirm that the subcellular localization of YAP1 protein is dependent on phosphorylation induced by Merlin. NCI-H290 cells were transfected with an EGFP-fused wild-type or S 127A mutant *YAP1* construct, together with *NF2* or an empty vector. Both the wild-type and mutant YAP1 proteins were found to be localized in both the nuclei and cytoplasm of the empty vector-cotransfected cells (Figure 4D). In contrast, cotransfection of the *NF2* vector clearly reduced nuclear localization of the wild-type YAP1 proteins, but not the mutant YAP1 protein. In addition, immunohistochemical staining of nuclear accumulation of YAP1 in the MPM case with YAP1 gene amplification (arrowheads in Figure 2B, left panel) also supported the idea that YAP1 localization in nuclei of the tested cell lines was not due to an artificial event.

Physical interaction between YAP1 and Merlin

Since these transfection experiments indicated a functional relationship between Merlin and YAP1, we next studied whether these molecules physically interact with each other. We immunoprecipitated Merlin from the lysates of 293T cells that were cotransfected with *NF2* and/or *YAP1* expression vectors and then investigated whether YAP1 could be coprecipitated with Merlin (Figure 5A). The results clearly demonstrated the coprecipitation of YAP1 with Merlin, indicating a physical interaction between them. Interestingly, the anti-phospho-YAP1 antibody did not show any signals, while the S 127A mutant YAP1 also interacted with Merlin as did wild-type YAP1 (Figure 5A), suggesting that Merlin may also interact with unphosphorylated YAP1.

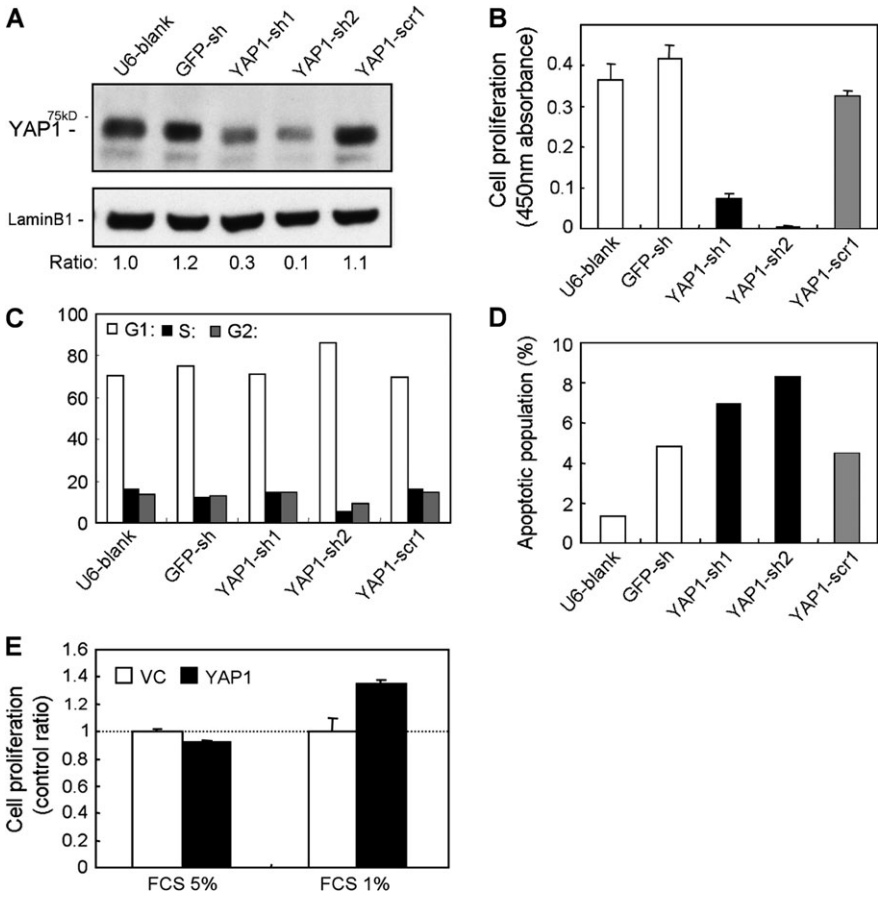

Fig. 3. Involvement of YAP1 in cell proliferation. (**A**) Knockdown of endogenous YAP1 in MPM cell line NCI-H290. Both YAP1-RNAi vectors, YAP1-sh1 and YAP1-sh2, showed effective suppression of the level of YAP1 protein, whereas the control vectors, GFP-sh and YAP1-scr1, showed no inhibition. (**B**) Inhibition of NCI-H290 proliferation by YAP1-RNAi. Colorimetric assay results demonstrated that both the YAP1-sh1 and YAP1-sh2 vectors induced significant suppression of cell proliferation. YAP1-sh2 inhibited proliferation to a greater degree as compared with YAP1-sh1, consistent with the RNAi effect. (**C**) Cell-cycle arrest by YAP1-RNAi. YAP1-sh2 clearly induced G_1 arrest of NCI-H290 cells. (**D**) Induction of sub-G_1 population by YAP1-RNAi. The sub-G_1 population of NCI-H290 cells was induced by both YAP1-RNAi vectors, though induction by YAP1-sh2 was greater. (**E**) Promotion of MeT-5A cell proliferation by YAP1 overexpression. Although YAP1 overexpression did not show a clear effect in the usual condition [fetal calf serum (FCS) 5%], YAP1 overexpression moderately promoted cell proliferation in the low-serum condition (FCS 1%).

To further confirm the physical interaction between YAP1 and Merlin, we prepared GST-alone or GST—Merlin-bound glutathione beads and then performed *in vitro* pull-down assays (Figure 5B). First, we conducted a pull-down assay using *in vitro*-translated YAP1 protein; however, no association between YAP1 and GST–Merlin was detected (Supplementary Figure 1), which suggested that YAP1 was not directly associated with Merlin. Next, we performed a pull-down assay using the lysate of 293T cells transfected with the YAP1 expression vector, as we considered that the cell lysate possibly contained endogenous molecules that could bridge YAP1 and Merlin. The pull-down assay using the 293T cell lysate clearly demonstrated that YAP1 was associated with GST–Merlin (Figure 5B, lane 2). In addition, we also studied the effects of NHERF1/ezrin/radixin/moesin-binding phosphoprotein 50 kD because it was reported to be associated with YAP1 (28) as well as with Merlin (29), and we considered that it might bridge the YAP1 and Merlin proteins. However, NHERF1 seemed unable to enhance the YAP1–Merlin association, though NHERF1 bound to GST–Merlin (Figure 5B, lane 4). These results indicate that YAP1 is indirectly associated with Merlin, probably through an endogenous bridging molecule other than NHERF1.

Discussion

In the present study, we demonstrated that the *YAP1* gene is localized in the high-level 11q22 amplification region, which we previously reported in a study of two cases with MPMs, and that *YAP1* together with *BIRC2* are overexpressed in these tumors. We also found that upregulation of YAP1 induced mesothelial cell proliferation, whereas its downregulation inhibited that proliferation. Furthermore, Merlin-dependent phosphorylation inhibits the nuclear localization of YAP1, which might result in inactivation of YAP1 transcriptional activity.

Amplifications at the 11q22 locus have been reported for several different types of human cancer (25,30–33). Amplifications at mouse chromosome 9qA1, the syntenic region of human chromosome 11q22, have also been shown in mouse mammary and liver cancers (25,26). Furthermore, during preparation of the present manuscript, MPM cell lines carrying chromosomal gain at the 11q22 locus were also reported (34). These findings, together with our previous array CGH analysis on malignant mesotheliomas, suggested a significant role for 11q22 amplification in carcinogenesis. In the present study, we demonstrated that, among the several candidate oncogenes at the 11q22 amplification region, both the *YAP1* and *BIRC2* genes were commonly overexpressed in the two MPM tissues, suggesting that *YAP1* and *BIRC2* were the most likely target genes. We focused on YAP1 primarily because previous reports have suggested its oncogenic activity of YAP1 (25,26). *In vitro* transfection assay that utilized knockdown or overexpression of YAP1 indicated that YAP1 promotes growth of mesothelial lineage cells, and cotransfection experiments strongly suggested that Merlin inhibits the transcriptional activator activity of YAP1 through induction of phosphorylation and inhibition

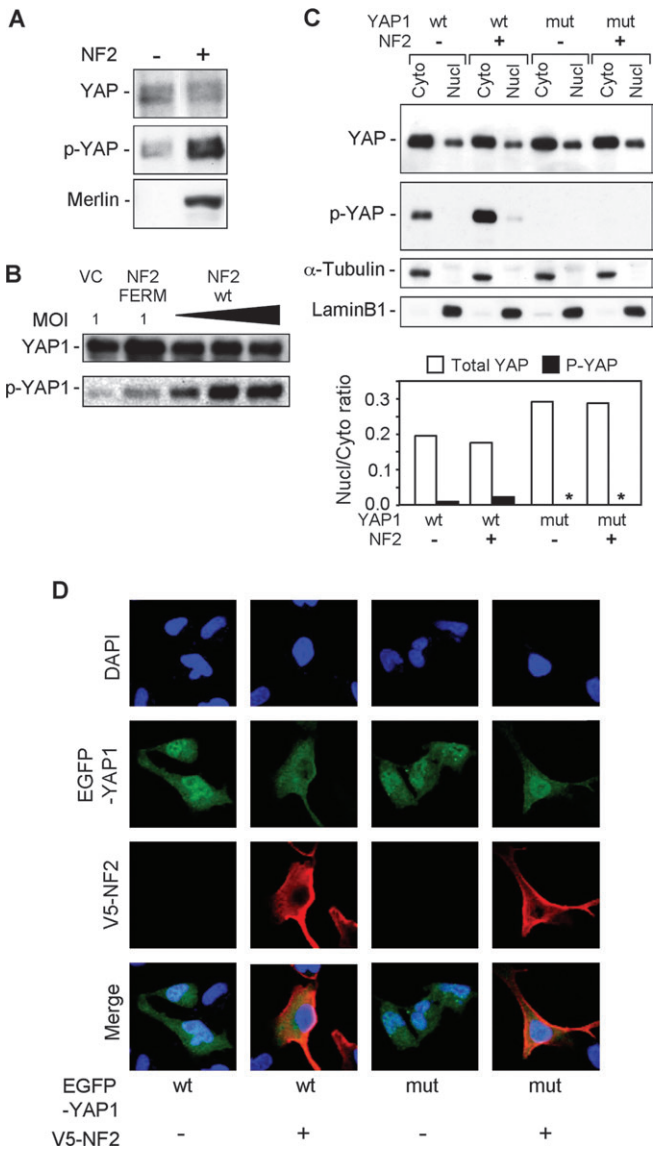

Fig. 4. Functional interaction between YAP1 and Merlin. (**A** and **B**) Induction of YAP1 phosphorylation by Merlin. (**A**) NF2-null NCI-H290 cells were transfected with YAP1 and NF2 expression vectors. Cotransfection of NF2 significantly induced phosphorylation of the YAP1 S 127 residue. (**B**) Either full-length or truncated NF2 was introduced into NCI-H290 cells with a lentivirus. Phosphorylation of YAP1 was induced by the full-length NF2 lentivirus in a dose-dependent manner, whereas the truncated NF2 FERM lentivirus did not have any clear effect. (**C**) Immunoblotting of cytoplasmic and nuclear fractions. NCI-H290 cells were transfected with wild-type (wt) or phosphorylation-defective S 127A mutant (mut) YAP1 and NF2 expression vectors. Immunoblots of nuclear and cytoplasmic fractions of transfectants clearly showed that phospho-YAP1 was mainly localized in the cytoplasm, as detected by the anti-S 127 phospho-YAP1 antibody, though total YAP1 was localized in both the nucleus and cytoplasm. In addition, the nuclear/cytoplasmic ratio of YAP1 and phospho-YAP1 proteins was measured with a densitometer and indicated with a bar graph, which clearly demonstrates the tight cytoplasmic retention of wild-type phospho-YAP1 protein. No signals were detected in mutant YAP1 transfectants with the anti-phospho-YAP1 antibody (asterisks). Results of immunoblotting with antibodies against α-tubulin and nuclear laminB1 indicated that proper fractionation occurred. (**D**) Reduction of YAP1 nuclear localization by NF2. NCI-H290 cells were transfected with expression vectors of the EGFP-fused wild-type (wt) or S 127A mutant (mut) YAP1, together with the vector control or V5-tagged NF2. Both the wild-type and mutant YAP1 proteins localize in both the nuclei and cytoplasm of H290 cells. Cotransfection of NF2 reduced nuclear localization of wild-type YAP1, whereas the localization of mutant YAP1 was not affected by NF2.

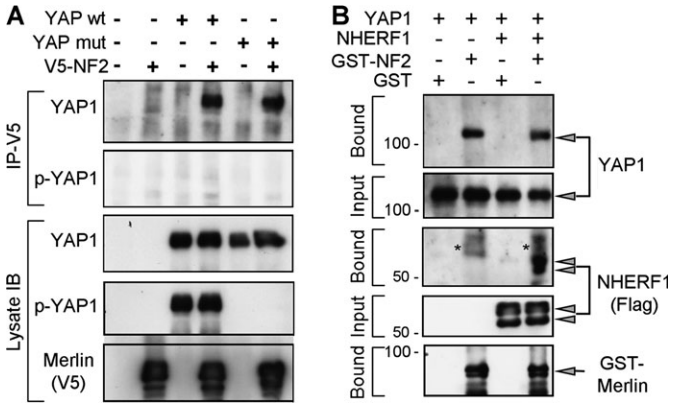

Fig. 5. Physical interaction between YAP1 and Merlin. (**A**) Immunoprecipitation: 293T cells were transfected with V5-tagged *NF2* and/or *YAP1* expression vectors, then Merlin was immunoprecipitated with the anti-V5 antibody and coprecipitation with YAP1 was studied. Both wild-type and S 127A mutant YAP1 were coprecipitated with Merlin. The anti-phospho-YAP1 antibody did not show any clear signals. (**B**) *In vitro* pull-down assay. Cell lysates of 293T cells were transfected with a *YAP1* or *NHERF1* vector, then incubated with GST-alone or GST—Merlin-bound beads. Immunoblots of bead-bound proteins (Bound) and the initial cell lysates (Input) are shown. Both YAP1 and NHERF1 proteins bound to GST–Merlin, though NHERF1 did not enhance the association of YAP1 with GST–Merlin. In the immunoblot of bound NHERF1, non-specific bands are indicated by asterisks.

of nuclear localization. To our knowledge, the present results are the first to show that YAP1 is regulated by Merlin through induction of phosphorylation, indicating that YAP1 is a downstream effector of Merlin tumor suppressor signaling in mammals. Our findings also suggest that YAP1 may play a crucial role in MPM development because Merlin tumor suppressor signaling is frequently altered in those tumors.

Recent genetic and biochemical analyses of *Drosophila* demonstrated that cell proliferation and organ size are negatively regulated by a kinase cascade of Hippo and Warts (also called Lats) and that two membrane-associated proteins, Merlin and Expanded, function upstream of this kinase cascade (35). Yorkie, the *Drosophila* ortholog of YAP1, is a critical target of the growth-inhibitory Hippo–Warts/Lats kinase cascade and a potential oncogene because its overexpression induces tissue overgrowth and apoptosis inhibition through the transactivation of cycE and diap1 expression (27,36). *Drosophila* rescue experiments also indicated evolutionary conservation of the signaling components, that is the mammalian *LATS1*, *MOB1*, *MST2* and *YAP* genes, which are orthologs of the *Drosophila Warts*, *Mats*, *Hippo* and *Yorki* genes, respectively. During the preparation of this manuscript, Zhao *et al.* (37) clearly demonstrated that the S 127 phosphorylation of YAP1 was catalyzed by LATS1 and that YAP1 inactivation plays significant roles in cell contact inhibition and tissue growth regulation. However, the signaling pathway from Merlin to YAP1 has not been clearly demonstrated in mammalian cells.

YAP1 has been reported to bind to and regulate the activities of various transcriptional regulators, including p73, RUNX2, ERBB4, and several TEA domain/transcription enhancer factor-type transcription factors, and also shown to function as an oncogene in mammals (25,26), possibly through an association with RUNX2 (38,39) and ERBB4 (19,40). YAP1 is phosphorylated at S 127, leading to its association with 14-3-3, which sequestrates YAP1 in the cytoplasm and inhibits its coactivator activity (21,41). The present results demonstrated that Merlin induces phosphorylation of YAP1 S 127 and inhibits its nuclear localization. Merlin–YAP1 signaling is conceivable, because, based on conserved *Drosophila* signaling, Merlin may activate the MST2–LATS1 kinase cascade, while it also appears to bind to unphosphorylated YAP1 protein.

YAP1 has also been reported to promote apoptosis through an association with p73 (21,42,43). In addition, a recent report demonstrated that LATS1 was activated by tumor suppressor RASSF1A to phosphorylate YAP1 and promote nuclear localization of the YAP1–p73 complex, resulting in apoptosis induction (44). Therefore, YAP1 may promote both cell growth and apoptosis, depending on the associated transcription factors. In this context, overexpression of *BIRC2* and *BIRC3* genes, which colocalize at 11q22 and encode apoptosis inhibitors, might be essential for exhibition of the oncogenic activity of YAP1 in cancers with 11q22 amplification, with our cases being consistent with this idea.

The *NF2* gene is frequently inactivated in MPMs, indicating that downregulation of Merlin signaling is essential for MPM development. The antiproliferative effect of Merlin in *NF2*-deficient mesothelioma cells has been suggested to be induced by repressing cyclin D1 expression (45), attenuating focal adhesion kinase phosphorylation (46) or interacting with NF2-associated guanosine triphosphate-binding protein (47). *Nf2*(+/−) mice exposed to asbestos exhibit accelerated formation of highly malignant mesothelial tumors (48,49). In addition, in a recent study, conditional knockout mouse models developed by inactivating *Nf2* together with $p16^{Ink4a}/p19^{arf}$, *p53* or both, developed malignant mesotheliomas at a high incidence, supporting the notion that *Nf2* inactivation is important for the pathogenesis of these tumors (50). Our results revealed that the *YAP1* gene can also be an activating target for a subset of MPMs, which was coincidentally found to be a downstream effector of the Merlin cascade. However, though YAP1 may be important in the subset of MPM with 11q22 amplification, its relevance in the vast majority of MPM cases is unknown at present. Therefore, precise analysis of tumor suppressor signaling in NF2–MST–WARTS/LATS–YAP1 is needed to shed light on the molecular mechanisms of MPM development in greater detail. Preliminary analysis of immunohistochemical staining of YAP1 revealed overexpression and nuclear localization of YAP1 in a subset of MPM cases, indicating the frequent involvement of YAP1 in MPM development. Nevertheless, the present results provide new insights into genetic alterations in MPMs and clues for development of a new molecular target therapy for patients with these tumors.

Supplementary material

Supplementary Figure 1 can be found at http://carcin.oxfordjournals.org/

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