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YAP is a candidate oncogene for esophageal squamous cell carcinoma

Tomoki Muramatsu[1,2], Issei Imoto[1,3], Takeshi Matsui[4], Ken-ichi Kozaki[1,5], Shigeo Haruki[1,6], Marius Sudol[7], Yutaka Shimada[8], Hitoshi Tsuda[5,9], Tatsuyuki Kawano[6] and Johji Inazawa[1,2,5,*]

[1]Department of Molecular Cytogenetics, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan, [2]Global Center of Excellence Program for Frontier Research on Molecular Destruction and Reconstitution of Tooth and Bone, Tokyo Medical and Dental University, Tokyo 113-8510, Japan, [3]Department of Human Genetics and Public Health, Institute of Health Biosciences, The University of Tokushima, Graduate school, Tokushima 770-8503, Japan, [4]Medical Top Track Program, Medical Research Institute and School of Biomedical Science, Tokyo Medical and Dental University; Tokyo 113-8510, Japan, [5]Hard Tissue Genome Research Center, Tokyo Medical and Dental University; Tokyo 113-8510, Japan, [6]Department of Surgery, Graduate School, Tokyo Medical and Dental University; Tokyo 113-8510, Japan, [7]Laboratory of Signal Transduction and Proteomic Profiling, Weis Center for Research, Geisinger Clinic, Danville, PA 17822, USA, [8]Department of Surgery and Science, Graduate School of Medicine and Pharmaceutical Sciences for Research, University of Toyama, Toyama 930-0194, Japan and [9]Clinical Laboratory Division, National Cancer Center Hospital, Tokyo 104-0045, Japan

*To whom correspondence should be addressed. Tel: +81 3 5803 5820; Fax: +81 3 5803 0244;
Email: johinaz.cgen@mri.tmd.ac.jp

Yes-associated protein (YAP), the nuclear effector of the Hippo pathway, is a key regulator of organ size and a candidate human oncogene located at chromosome 11q22. Since we previously reported amplification of 11q22 region in esophageal squamous cell carcinoma (ESCC), in this study we focused on the clinical significance and biological functions of YAP in this tumor. Frequent overexpression of YAP protein was observed in ESCC cells including those with a robust amplicon at position 11q22. Overexpression of the YAP protein was frequently detected in primary tumors of ESCC as well. Patients with YAP-overexpressing tumors had a worse overall rate of survival than those with non-expressing tumors, and YAP positivity was independently associated with a worse outcome in the multivariate analysis. Further analyses in cells in which YAP was either overexpressed or depleted confirmed that cell proliferation was promoted in a YAP isoform-independent but YAP expression level-dependent manner. YAP depletion inhibited cell proliferation mainly in the G_0–G_1 phase and induced an increase in *CDKN1A/p21* transcription but a decrease in *BIRC5/survivin* transcription. Our results indicate that YAP is a putative oncogene in ESCC and it represents a potential diagnostic and therapeutic target.

Introduction

The Hippo signaling pathway regulates the balance between cell proliferation and apoptosis (1). Yes-associated protein (YAP) is the mammalian ortholog of *Drosophila* Yorkie (yki), which is a negatively regulated effector of the Hippo pathway and functions as a transcriptional coactivator or corepressor in the regulation of cell growth, proliferation and apoptosis (2). Given the essential role of YAP in cellular growth, knockout of the *YAP* gene in mice leads to early embryonic death (3). YAP was described to increase the ability of p73 to induce apoptosis as a consequence of DNA damage and be important for c-Jun-dependent apoptosis, suggesting that, under certain conditions of stress, YAP acts as a tumor suppressor (4–9). However, a majority of recent studies document YAP as a bona fide oncogene: YAP amplification and overexpression were observed in various human cancers and mouse models of cancer (10–16); overexpression of YAP in non-transformed mammary epithelial cells induced epithelial–mesenchymal transition, suppression of apoptosis, growth factor-independent proliferation and anchorage-independent growth (11); YAP cooperated with *myc* oncogene to stimulate tumor growth in nude mice (12) and transgenic mice with liver-targeted YAP overexpression showed a dramatic increase in liver size that eventually resulted in hepatic tumors (17,18). Considering those findings, YAP may have a dual role in signaling, which depends on context, stress and/or cell lineage because it is placed at the crossroad of many signaling pathways (19). In the current study, we focused on the role of *YAP* gene in a cancer that is prevalent in our country.

Esophageal carcinoma is the sixth most frequent cause of cancer-related death worldwide (20), and esophageal squamous cell carcinoma (ESCC) accounts for ∼90% of esophageal carcinomas diagnosed in Asian countries including Japan. Although accumulated evidence suggests that multiple genetic alterations occurring sequentially in a cell lineage underlie the carcinogenesis of ESCC, the repertoire of genetic alterations identified so far in ESCC cannot fully account for the pathogenesis. Among the genetic alterations detected in cancer, the amplification of chromosomal DNA is one of the mechanisms capable of activating genes whose overexpression contributes to the development and progression of human cancer, including ESCC (21). As such, the amplification event points to candidate tumor-promoting genes.

Before the YAP gene was mapped precisely in the human genome, in ESCC cell lines, we detected amplification at 11q22, which contains *YAP* gene and we also identified *cellular inhibitor of apoptosis 1* (*cIAP1*, *BIRC2*) within the amplicon as a possible tumor-promoting gene (22). Since *YAP* is located ∼100 kb centromeric to *cIAP1/BIRC2* according to the human genome database (http://www.ncbi.nlm.nih.gov) and seems to be amplified with *cIAP1* within the 11q amplicon in ESCC (22), *YAP* in concert with *cIAP1* could contribute to the neoplastic phenotype of ESCC. In the non-neoplastic esophageal mucosa, YAP immunoreactivity, especially in the nucleus, was shown to be positive in the proliferating basal layer of the epithelium but negative in the terminally differentiated mature squamous epithelium toward the surface (10). These are novel observations as, to our knowledge, the expression status and functional significance of YAP in the tumorigenesis of ESCC have not been previously characterized.

Here, we demonstrate that four isoforms of YAP, including one newly identified isoform, were frequently overexpressed in ESCC. We also showed that patients with YAP-overexpressing tumors had a worse overall rate of survival than those with non-expressing tumors. We also report proliferation-promoting activity of YAP, at least in part, through the inhibition of *CDKN1A/p21* transcription and induction of *BIRC5/survivin* transcription, either directly or indirectly. Interestingly, the YAP activity was isoform independent. These finding suggest YAP to be a potential target in the treatment of ESCC.

Abbreviations: cIAP1, cellular inhibitor of apoptosis 1; ESCC, esophageal squamous cell carcinoma; HCC, hepatocellular carcinoma; mRNA, messenger RNA; RT–PCR, reverse transcription–polymerase chain reaction; siRNA, small interfering RNA; siRNA-NC, negative control siRNA; YAP, Yes-associated protein; Yki, yorkie.

Materials and methods

Cell culture and primary tissue samples

A total of 43 ESCC cell lines were used, of which 31 belonged to the KYSE series established from surgically resected tumors and 12 were TE series lines provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (23).

All 120 primary tumor samples of ESCC had been obtained from ESCC patients treated at Tokyo Medical and Dental University (Tokyo, Japan) between 2000 and 2005 and embedded in paraffin after 24 h of formalin fixation. Relevant clinical and survival data were available for all patients (supplementary Tables S1 and S2 are available at *Carcinogenesis* Online). After approval by the local ethics committee, a formal written consent was always obtained

from patients. None of these patients underwent endoscopic mucosal resection, palliative resection, preoperative chemotherapy or radiotherapy, and none had synchronous or metachronous multiple cancers in other organs. Disease stage was defined in accordance with the International Union against Cancer and following the tumor-lymph node-metastases classification. In this series, all the M1 tumors had distant lymph node metastases and there was no organ metastasis. The median follow-up period for the surviving patients was 19 (ranging from 1 to 103) months.

Western blotting

The anti-YAP (H-125), anti-cIAP1 (H-83), anti-p21 (C-19), anti-survivin (D-8) and Lamin B (C-20) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-β-actin antibody from Sigma (St Louis, MO); anti-Ki67 antibody from DAKO (Carpinteria, CA) and anti-phospho-YAP (Ser127) and anti-β-tubulin antibodies from Cell Signaling Technology (Danvers, MA). Cells were lysed in Tris buffer (50 mM, pH 7.5) containing 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 0.5% NP-40, 10% glycerol, 100 mM NaF, 10 mM sodium pyrophosphate, 2 mM Na_2VO_3 and a protease inhibitor cocktail (Roche, Tokyo, Japan), and lysates were analyzed as described elsewhere (23). Nuclear and cytoplasmic proteins were extracted from cells separately using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL).

Immunofluorescence microscopy

Cells were fixed in 10% trichloroacetic acid, permeabilized with 0.2% Triton X-100 and treated with blocking solution (1% bovine serum albumin, in phosphate-buffered saline) and then incubated with the primary antibodies (YAP, 1:100 and/or Ki67, 1:200) for 1 h. The bound antibody was visualized using a fluorescein isothiocyanate-conjugated or Cy3-conjugated secondary antibody (1:1000). After being mounted with 4′,6′-diamidino-2-phenylindole to stain nuclei, the cells were observed under a fluorescence microscope (BZ-8100; Keyence, Osaka, Japan).

Immunohistochemistry

Tumor samples were fixed with 10% formaldehyde in phosphate-buffered saline, embedded in paraffin, sectioned into 4 μm thick slices and subjected to immunohistochemical staining of YAP protein with the avidin–biotin–peroxidase method (24). In brief, endogenous peroxidases were quenched by incubating the sections for 20 min in 3% H_2O_2. Antigen retrieval was performed by heating the samples in 10 mM citrate buffer (pH 6.0) at 120°C for 15 min using an autoclave. After treatment with Block Ace (Dainippon Sumitomo Pharmaceutical, Osaka, Japan) for 30 min at room temperature, the sections were incubated at 4°C overnight with an anti-YAP (1:100) antibody. The avidin–biotin–peroxidase complex system (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) was used for color development with diaminobenzidine tetrahydrochloride. The slides were counterstained with Mayer's hematoxylin. A formalin-fixed ESCC cell line overexpressing YAP (KYSE170) was used as a positive control, whereas a formalin-fixed ESCC cell line with low expression of YAP (KYSE1240) was included as a negative control. The percentage of the total cell population that expressed YAP was evaluated for each case at ×200 magnification. Expression of YAP protein was graded as either positive (≥30% of the nucleus or cytoplasm showing immunopositivity; cytoplasmic or nuclear positive, respectively) or negative (<30% of the nucleus or cytoplasm showing immunopositivity or no staining; cytoplasmic or nuclear negative, respectively).

Reverse transcription–polymerase chain reaction

Single-stranded complementary DNA was generated from total RNA. The messenger RNA (mRNA) expression pattern was analyzed by reverse transcription–polymerase chain reaction (RT–PCR) using gene-specific primer sets (supplementary Table S3 is available at *Carcinogenesis* Online). PCR products were electrophoresed in 3% agarose gels, and bands were quantified using LAS-3000 (Fujifilm, Tokyo, Japan) and Multi Gauge software (Fujifilm). Levels of mRNA expression were measured with a quantitative real-time fluorescence detection method (ABI PRISM 7500 sequence detection System; Applied Biosystems, Foster City, CA) using TaqMan Gene Expression Assays (Hs00193201_m1 and Hs00231069_m1 for *CDKN1A*/p*21* and *BIRC5*/*survivin*, respectively; Applied Biosystems) according to the manufacturer's instructions. Gene expression values are expressed as ratios between the genes of interest and an internal reference gene (Hs99999903_m1 for *beta-actin*, *ACTB*; Applied Biosystems) that provides a normalization factor for the amount of RNA isolated from a specimen and subsequently normalized with the value in the controls (relative expression level). Each assay was performed in duplicate for each sample.

Expression constructs and colony formation assay

Plasmids expressing each of the YAP isoforms (pcDNA3.1-YAP-α, -β, -γ and -δ) were obtained by cloning the full coding sequences for wild-type YAP-α, -β, -γ and -δ, respectively, into the vector pcDNA3.1(−) (Invitrogen, St Louis, MO). pcDNA3.1-YAP-α, -β, -γ or -δ or the empty vector (pcDNA3.1-mock), as a control, was introduced into ESCC cells as described previously (23). The expression of YAP protein in transfected cells was confirmed by western blotting. After 3 weeks of incubation with appropriate concentrations of G418, cells were fixed and stained with crystal violet.

Loss-of-function by small interfering RNA and cell growth analysis

Loss-of-function screening was done using small interfering RNAs (siRNAs) purchased from Invitrogen targeting the *YAP* gene (HSS115942, HSS115943 or HSS115944) or the *cIAP1* gene (HSS100559) and a universal negative control (46-2001). Each siRNA (total 20 nM) was transfected into ESCC cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The knockdown of a target gene was confirmed by quantitative real-time RT–PCR and western blotting. The numbers of viable cells at various time points after transfection were assessed by a colorimetric water-soluble tetrazolium salt assay as described elsewhere (23). The cell cycle was evaluated 72 h after transfection by fluorescence-activated cell sorting as described elsewhere (23).

Statistical analysis

The clinicopathological variables pertaining to the corresponding patients were analyzed for statistical significance by the χ^2 or Fisher's exact test. For the analysis of survival, Kaplan–Meier survival curves were constructed for groups based on univariate predictors and differences between the groups were tested with the log-rank test. Univariate and multivariate survival analyses were performed using the likelihood ratio test of the stratified Cox proportional hazards model. Differences between subgroups were tested with the Student's *t*-test. For multiple group comparisons, a one-way analysis of variance, followed by Scheffé's post-hoc test, was used. Differences were assessed with a two-sided test and considered significant at the $P < 0.05$ level.

Results

YAP protein is frequently overexpressed in human ESCC

To determine whether YAP may be involved in human ESCC, we first performed protein expression analyses in a panel of cell lines of ESCC using an antibody raised against 125 amino acid long, C-terminal fragment of YAP. We observed frequent high expression of the YAP protein in ESCC cell lines (Figure 1A) including those with high-copy amplification at 11q22 (KYSE170 and KYSE2270) (22). At least two bands (arrowheads in Figure 1A) at ∼75 kD (major) and 65 kD (minor) were detected. Since YAP protein were overexpressed in cells with remarkable amplification of this gene (22) as well as in cells without amplification (Figure 1A), YAP amplification seems to be only part of the mechanism to induce its overexpression/activation. The YAP protein was predominantly located in the cytoplasm of ESCC cells with the 11q22 amplification (Figure 1B and C).

Immunohistochemical analysis (Figure 1D–J) revealed that YAP protein is frequently overexpressed in primary tumors of ESCC compared with nontumorous esophageal epithelia in either the cytoplasm or nucleus. In 120 primary ESCC tumors, positive nuclear and cytoplasmic YAP immunoreactivity was detected in 69 (57.5%) and 74 (61.7%) cases, respectively (supplementary Tables S1 and S2 are available at *Carcinogenesis* Online). Notably, basal cell layer cells of nontumorous epithelia also clearly expressed YAP protein, predominantly in the nucleus (Figure 1F).

Nuclear YAP protein overexpression associated with short overall survival

We examined the clinicopathological significance of the nuclear or cytoplasmic overexpression of YAP in 120 primary ESCC tumors based on the immunohistochemical staining pattern of this protein (supplementary Tables S1 and S2 are available at *Carcinogenesis* Online). No significant association between any clinicopathological characteristics and nuclear or cytoplasmic YAP immunoreactivity was observed in our set of primary tumors. However, Kaplan–Meier survival estimates (Figure 1K) showed that nuclear immunoreactivity of YAP in tumor cells was significantly associated with a worse overall survival in all cases ($P = 0.0213$, log-rank test). In the Cox proportional hazard regression model (Table I), univariate analyses demonstrated that YAP protein expression, gender and pN category and stage

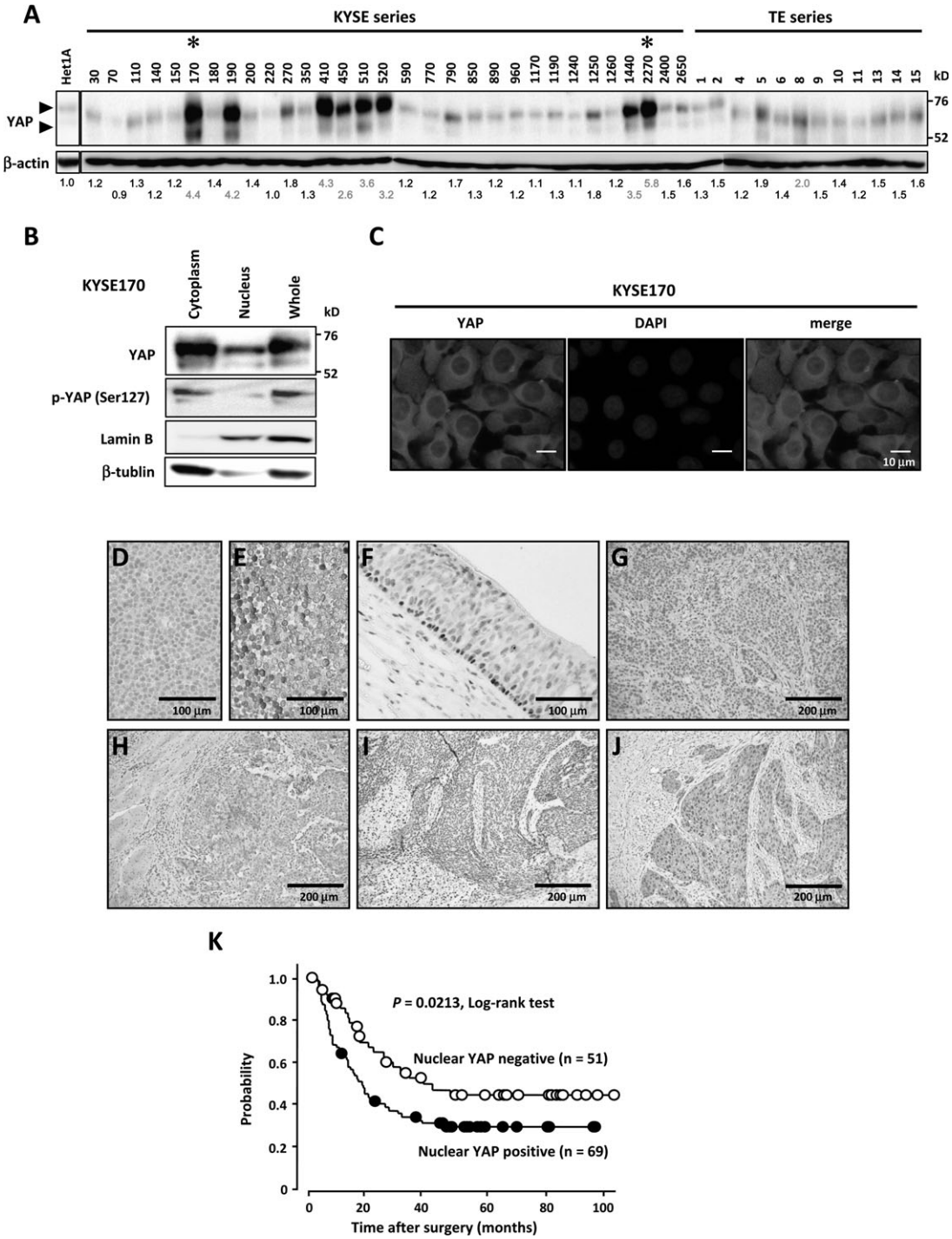

Fig. 1. Expression of YAP in cell lines and primary tumors of ESCC. (**A**) Expression of YAP protein detected by western blotting in a panel of ESCC cell lines with an immortalized esophageal epithelial cell line Het-1A [American Type Culture Collection (Manassas, VA)]. Asterisks indicate cell lines with remarkable 11q22 amplification (22). Note that at least two bands (arrowheads) at ∼75 kD (major) and 65 kD (minor) were detected. The bands were quantified by densitometry using an image analyzer (LAS3000; Fujifilm), and results of YAP protein expression level normalized with β-actin are shown with values relative to that for the control Het-1A cell line. Red, ≥2-fold increase in YAP protein expression compared with the Het-1A cell line. (**B**) Immunoblotting using separately extracted nuclear and cytoplasmic proteins and whole cell lysate of KYSE170, both YAP and phospho-YAP (Ser127). (**C**) Immunofluorescent cytochemical staining of endogenous YAP using anti-YAP antibody (Red). (**D–J**) Representative results of immunohistochemical staining of YAP in KYSE1240 cells [negative control, (D)], KYSE170 cells [positive control, (E)], normal esophageal mucosa (F), ESCC with negative staining (G), ESCC with positive staining in the cytoplasm (H), ESCC with positive staining in the nucleus (I) and ESCC with positive staining in both the nucleus and cytoplasm (J). Bars, 100 or 200 μm. (**K**) Kaplan–Meier curves for overall survival rates of patients with primary ESCC at all stages according to the nuclear expression of YAP. Nuclear YAP immunoreactivity of tumor cells was significantly associated with a worse overall survival at all stages ($P = 0.0213$, log-rank test).

in the tumor-lymph node-metastases classification were significantly associated with overall survival. In the multivariate analysis using a stepwise Cox regression procedure, nuclear YAP expression status, gender and tumor stage according to the tumor-lymph node-metastases classification were identified as independently selected predictive factors for overall survival in both forward and backward procedures

Table I. Coxproportional hazard regression analysis for overall survival

Factor	Univariate			Multivariate[a]
	Hazard ratio	(95% confidence interval)	*P*-value[*]	*P*-value[*]
Gender				
Male versus female	5.000	(1.225–20.408)	0.0249	0.0452
Age (years)				
≥60 versus <60	1.385	(0.819–2.342)	0.2238	—
Histopathological grading				
Poor versus well-moderate	1.381	(0.857–2.227)	0.1847	—
Venous invasion				
1–3 versus 0	2.309	(0.320–1.667)	0.4064	—
Lymphatic invasion				
1–3 versus 0	1.818	(0.833–3.984)	0.1334	—
TNM classification				
pT categories				
pT2–4 versus pT1	1.795	(0.564–5.741)	0.3217	—
pN categories				
pN1 versus pN0	2.564	(1.377–4.785)	0.0030	—
pM categories				
pM1 versus pM0	1.520	(0.934–2.469)	0.0921	—
pStage				
III + IV versus I + II	3.610	(1.845–7.042)	0.0002	0.002
Nuclear YAP expression[b]				
Positive versus negative	1.764	(1.081–2.882)	0.0232	0.0420

Note. Statistically significant values are in boldface type.
[a]Forward- and backward-stepwise analyses were used for mutivariate analysis.
[b]YAP expression was evaluated by immunohistochemical analysis as described in Materials and methods.
[*]*P*-values are from two-sided tests and were statistically significant at <0.05.

(P = 0.0420, 0.0452 and 0.002, respectively). In contrast to the nuclear YAP immunoreactivity association with survival, no significant association was observed between cytoplasmic immunoreactivity of YAP in tumor cells and overall survival (supplementary Figure S1 is available at *Carcinogenesis* Online).

These observations are consistent with the prevailing notion that proliferative or oncogenic signaling of the Hippo pathway requires nuclear localization of YAP (18).

Four major isoforms of YAP include two novel types

Since several forms of YAP protein were observed in ESCC cells (Figure 1A and data not shown) and some isoforms of human YAP were previously reported (24,25), we decided to clone the full-length coding sequence for each of the YAP isoforms using RT–PCR. Consequently, we identified four variants (Figure 2A and B), designated YAP-α, -β, -γ and -δ in order of increasing size. YAP-α and -δ have been reported as isoforms 1 and 2, respectively, in the human genome database (http://www.ncbi.nlm.nih.gov/), and YAP-δ is the same as YAP2L reported previously (24,25). YAP-β and YAP-γ (GenBank accession number, AB567720 and AB567721, respectively) are novel isoforms. YAP-γ and -δ contain two WW domains, whereas YAP-α and -β contain one. YAP-β and -δ contain a short insert sequence (16 amino acids) compared with YAP-α and -γ (Figure 2B). To determine the relative transcription levels of the four *YAP* isoforms, we designed primer sets (Figure 2B; supplementary Table S3 is available at *Carcinogenesis* Online) for RT–PCR. All four isoforms were detectable in normal esophagus and the ESCC cell line with the 11q22 amplification (KYSE170), although YAP-γ was predominantly expressed in the normal esophagus, whereas YAP-δ was predominantly expressed in KYSE170 cells (Figure 2C). The mRNA expression pattern of the four isoforms of YAP was similar among all ESCC cell lines examined regardless of the 11q22 amplification (Figure 2D). Since we did not detect smaller protein products of previously identified two putative, short isoforms of human YAP (GenBank accession number, BAG62143 and BAG65295), which miss in part, or completely, the WW domain region, in ESCC cell lines and tissues (data not shown), we did not follow the analysis of these two isoforms extensively.

Proliferation-promoting effect of YAP overexpression in ESCC cells

Increased expression was observed for all four major isoforms in ESCC cell lines, and the pattern of distribution was similar among cell lines examined, making it unclear whether all the YAP isoforms or some specific isoforms play a role in the tumorigenesis of ESCC through overexpression. To assess the effects of YAP on cell proliferation especially in ESCC cells, we conducted colony formation assays using expression construct encoding full-length sequence of each YAP isoform in an ESCC cell line with relatively low endogenous expression of this gene (KYSE1240; Figures 1A and 2D). Transient transfection of the expression construct for each isoform produced the corresponding protein, which localized predominantly in the cytoplasm, and faint band of endogenous YAP expression was also observed (Figure 3A and B). YAP-expressing constructs produced consistently more colonies than did the empty plasmid, regardless of the isoform (Figure 3C). In addition, no difference was observed in the occupancy of the stained area among the YAP isoforms (Figure 3D), suggesting that each isoform promotes cell proliferation in ESCC cells independently of the number of WW domains and the existence of short insert sequences.

Suppression of cell growth by downregulation of YAP expression in ESCC cells

We next performed a cell growth assay using YAP-specific siRNA to investigate whether the knockdown of YAP expression would suppress the proliferation of KYSE170 cells showing amplification/overexpression of YAP. Since each YAP isoform seems to show similar growth-promoting activity in ESCC cells, we used YAP-specific siRNA, which can knockdown all isoforms of YAP. In KYSE170 cells, the expression of all the isoforms was efficiently knocked down 24–72 h after the transient introduction of YAP-specific siRNAs (siRNA-*YAP#1*, *#2* and *#3*) compared with a negative control siRNA (siRNA-NC, Figure 4A). The proliferation of KYSE170 cells was decreased after the knockdown of endogenous YAP expression compared with the control (Figure 4B). On the other hand, knockdown of YAP had a minimal effect on proliferation in KYSE1240 cells expressing relatively low levels of this gene (Figure 4Aand B),

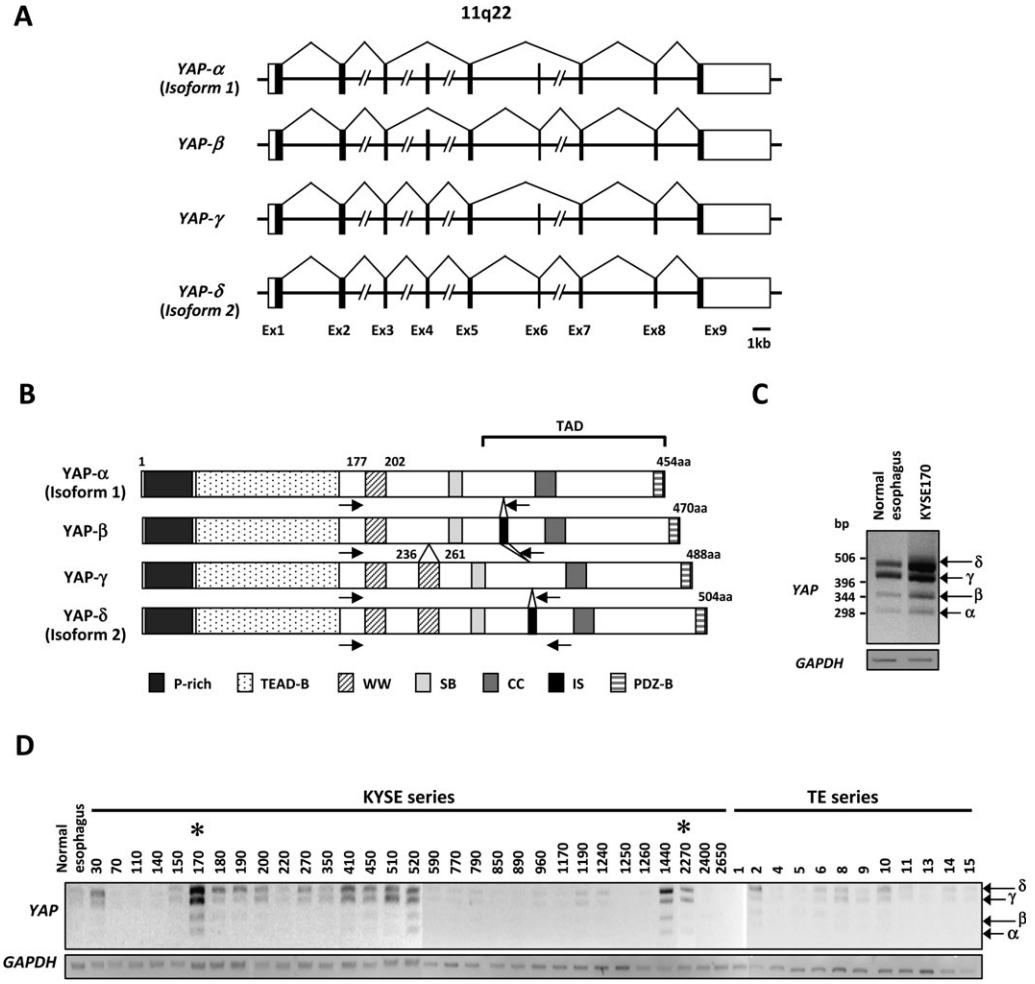

Fig. 2. YAP has four isoforms. (**A** and **B**) Schematic structure of the YAP-α and YAP-δ genes (**A**) and proteins (**B**), together with the novel isoforms YAP-β and YAP-γ. P-rich, proline-rich domain; TEAD-B, TEAD-binding region; WW, WW domain; SB, SH3-binding motif; IS, uncharacterized insertion sequence; CC, a coiled-coil domain; TAD, transcription activation domain and PDZ-B, PDZ-binding motif. (**C**) mRNA expression of *YAP* isoforms (arrows) detected by RT–PCR using the primer set producing four products, which can be recognized by size (see Figure 2B). (**D**) Expression pattern of *YAP* isoforms detected by RT–PCR (arrows) using the same primer set as in Figure 2D in a panel of ESCC cell lines. Asterisks indicate cell lines with remarkable 11q22 amplification (22).

suggesting that the growth-promoting effect of YAP in ESCC cells depends on its expression level. In order to unequivocally confirm the specificity of siRNA for YAP and assess the difference in the growth-promoting effect of each YAP isoform, we performed YAP-siRNA rescue experiments using cells with siRNA-induced YAP-depletion (supplementary Figure S2 is available at *Carcinogenesis* Online). We stably introduced individual expression constructs producing each of the four isoforms of YAP, which are refractory to siRNA-*YAP#2*, into KYSE170 cells and then depleted the endogenous YAP by transiently introducing siRNA-*YAP#2*. The expression of each YAP isoform was detected, and each isoform partially restored YAP depletion inhibited cell proliferation possibly due to lower expression level of each isoform in stable transfectants compared with their parental cells. However, no significant differences were observed among YAP isoforms. This result is consistent with that observed in colony formation assays using transiently introduced constructs producing each YAP isoform.

Since our previous studies and the current study demonstrated that YAP and cIAP1, two possible oncogenes located at 11q22, were co-overexpressed in some of the ESCC cell lines, including KYSE170 and KYSE2270 having the 11q22 amplification, we tested whether the two genes could cooperate to promote the proliferation of ESCC cells. We accomplished that test by using a double-knockdown with siRNA specific for each gene (supplementary Figure S3 is available at *Carcinogenesis* Online). In KYSE170 cells, the transient introduction of 10 nM of YAP- or cIAP1-specific siRNA alone with 10 nM of an siRNA-NC inhibited cell proliferation compared with that of 20 nM of siRNA-NC. In addition, the introduction of both YAP- and cIAP1-specific siRNAs (10 nM each) induced a larger decrease in cell proliferation compared with that of each siRNA (10 nM with 10 nM of a siRNA-NC), suggesting YAP and cIAP1 cooperatively promote tumorigenesis in ESCC.

Mechanisms of YAP-induced growth-promoting activity in ESCC cells

To gain further insight into the potential mechanism of YAP as an oncogene in esophageal carcinogenesis, we performed fluorescence-activated cell sorting, fluorescence immunocytochemical and expression analyses in ESCC cells treated with YAP-specific siRNA. Consistent with the results of the cell proliferation experiment, siRNA-*YAP* treatment resulted in an accumulation of cells in the G_0–G_1 phase compared with siRNA-NC-treated counterparts in the KYSE170 cell line in a fluorescence-activated cell sorting analysis, whereas a minimal effect was observed in siRNA-*YAP*-treated KYSE1240 cells (Figure 4C). In addition, we stained siRNA-*YAP*-treated KYSE170 cells with anti-YAP antibody and a cell proliferation marker, anti-Ki67 antibody, and found that Ki67 expression status was clearly correlated with YAP expression status: cells which retained the expression of YAP were positive for Ki67, whereas cells in which YAP was effectively knocked down tested negative (Figure 4D). Since *CDKN1A/p21*

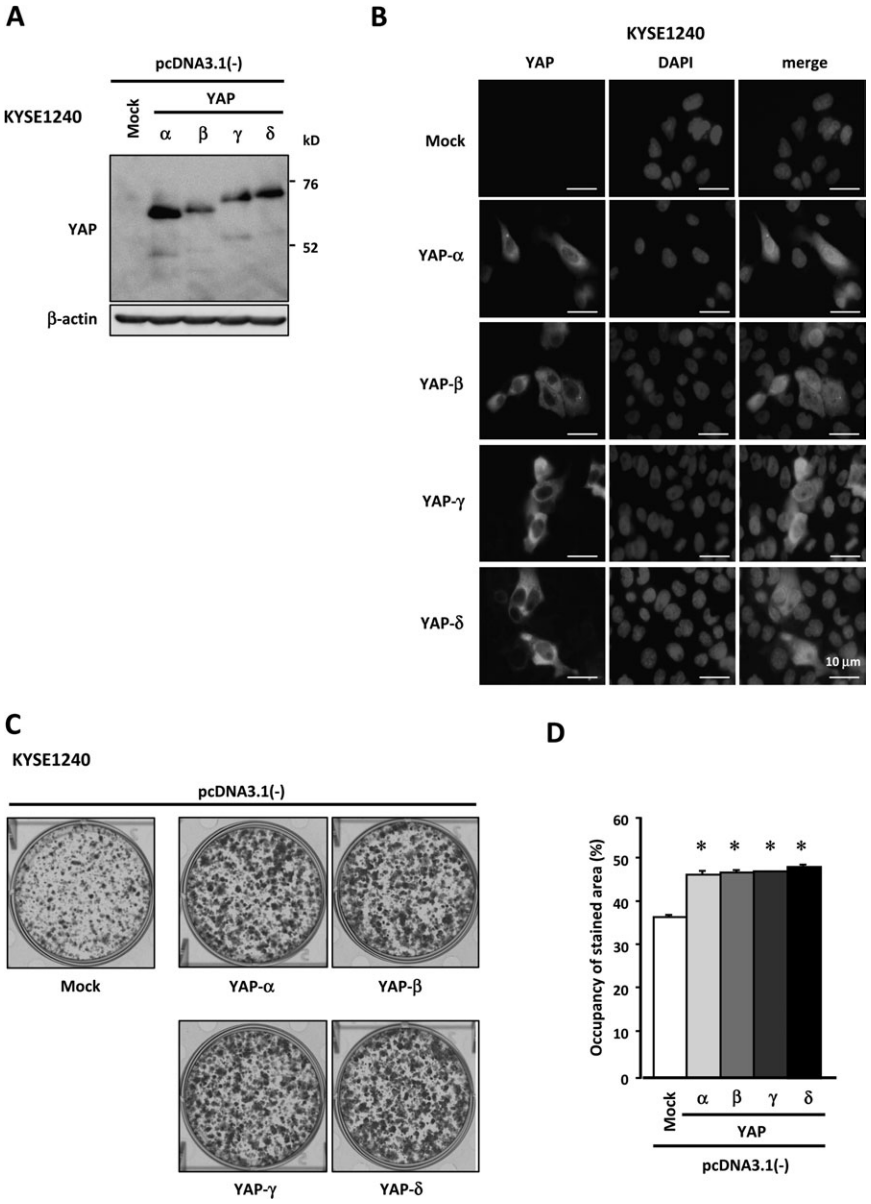

Fig. 3. Colony formation assays using the KYSE1240 cell line showing relatively low YAP expression. (**A**) KYSE1240 cells were transiently transfected with expression constructs containing empty vector [pcDNA3.1(−)-mock] or each isoform of YAP [pcDNA3.1(−)-YAP-α, -β, -γ or -δ]. YAP expression was detected by immunoblotting using 10 μg of protein extract and anti-YAP antibody. (**B**) Immunofluorescence staining of each exogenously expressed YAP isoform in KYSE1240 cells using anti-YAP antibody (Red). All isoforms were predominantly detected in the cytoplasm. (**C**) Representative image of a colony formation assay. Cells were transiently transfected with each construct and selected with appropriate concentrations of G418 for 3 weeks. The drug-resistant colonies formed by the YAP-transfected cells were more numerous than those formed by empty vector-transfected cells. (**D**) Quantitative analysis of occupancy of the stained area. The stained area was calculated by densitometry using an image analyzer (LAS3000; Fujifilm) and Black area calculation STD software (Gougasha, Kyoto, Japan). Columns, means of three separate experiments, each performed in triplicate; bars, standard deviation (histogram). Differences among multiple comparisons were analyzed by one-way analysis of variance with subsequent Scheffé's tests: asterisks represents $P < 0.05$ versus empty vector (Mock).

and *BIRC5/survivin* were reported as possible cell cycle-associated targets for transcriptional suppression and activation by YAP, respectively (13,26–28), we investigated CDKN1A/p21 and BIRC5/survivin expression after treatment with siRNA for YAP. After the downregulation of YAP expression, CDKN1A/p21 and BIRC5/survivin expression was remarkably induced and suppressed, respectively, at both the mRNA and protein levels in KYSE170 cells (Figure 5A and B) but only slightly affected in KYSE1240 cells (supplementary Figure S4 is available at *Carcinogenesis* Online). The results confirm YAP to induce and reduce transcription of *CDKN1A/p21* and *BIRC5/survivin* in a YAP expression level-dependent manner, resulting in G_0–G_1 arrest, although it remains unclear whether these are direct or indirect effects of YAP.

Discussion

The Hippo signaling pathway is gaining recognition as an important player in both organ size control and tumorigenesis, which are physiological and pathological processes that share common cellular signaling mechanisms (29). Since the disruption of any factor in this pathway can lead to tumorigenesis, it is not surprising that YAP functions as an oncogene and the major downstream effector of the Hippo pathway. As it is the case for ESCC, the *YAP* locus is also amplified in other human cancers, including intracranial ependymomas, oral squamous cell carcinomas and medulloblastomas (16,30–32). Two reports identified YAP as a driving oncogene of the 11q22 amplicon in human hepatocellular carcinoma (HCC) and breast cancer (11,12). Elevated

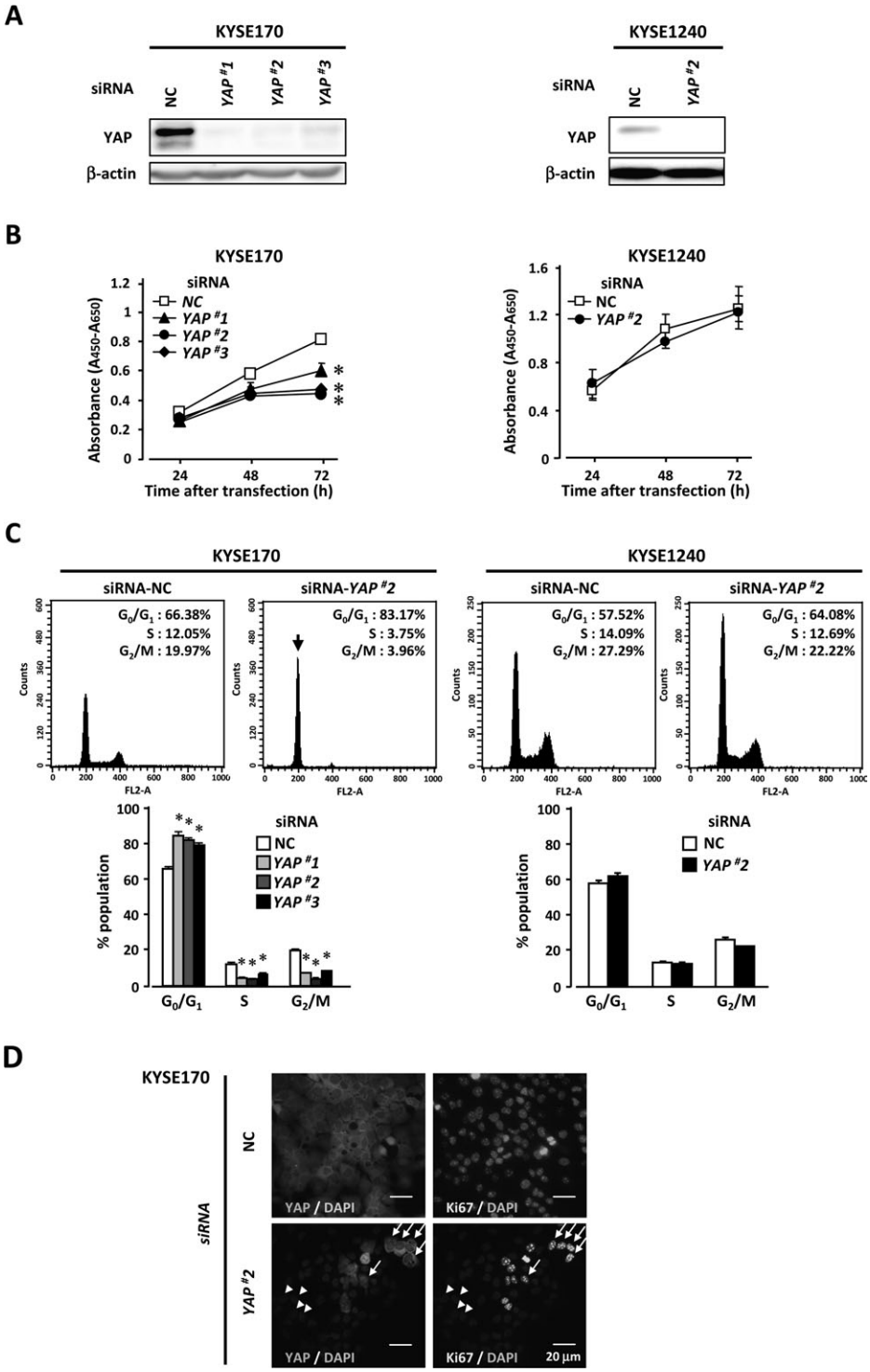

Fig. 4. YAP overexpression-dependent proliferation of KYSE170 cells. (**A**) knockdown of YAP protein expression by YAP-specific siRNA as confirmed by western blotting in KYSE170 (left) and KYSE1240 cells (right). The protein expression of all endogenous YAP isoforms was downregulated by either siRNA-*YAP#1*, *#2* or *#3* in KYSE170 cells. Since siRNA-*YAP#2* had the greatest effect, it was in further knockdown experiments. (**B**) The number of viable cells 24–72 h after transfection of YAP-specific siRNAs or an siRNA-NC was determined by the water-soluble tetrazolium salt assay at the indicated times. Results are shown with means ± standard deviations (bars) for quadruplicate experiments. Differences among multiple comparisons were analyzed by one-way analysis of variance with subsequent Scheffé's tests: asterisks represent $P < 0.05$ versus siRNA-NC transfectants. (**C**) Representative results of the population in each phase of the cell cycle in ESCC cell lines assessed by fluorescence-activated cell sorting 72 h after treatment with siRNA-NC or siRNA-*YAP#2* in KYSE170 (left) or KYSE1240 (right) cells. Note that the transfection of YAP-specific siRNA mainly induced the accumulation of G_0/G_1 phase cells and a decrease in S and G_2/M phase cells in the KYSE170 cell line. In KYSE1240 cells, a much smaller alteration was observed in the population in each phase compared with KYSE170 cells. Results of quantitative analysis are shown with means ± standard deviations (bars) for quadruplicate experiments. Differences among multiple comparisons were analyzed by one-way analysis of variance with subsequent Scheffé's tests (KYSE170 cells) or Student's *t*-test (KYSE1240 cells): asterisks represents $P < 0.05$ versus siRNA-NC transfectants. (**D**) Expression status of YAP and Ki67 assessed with immunofluorescence staining in KYSE170 cells treated with siRNA-NC or siRNA-*YAP#2* for 72 h. Ki67 immunopositivity was clearly correlated with YAP expression: cells which retained the expression of treatment with YAP even after YAP-specific siRNA showed positive Ki67 immunoreactivity (arrows), whereas cells that effectively lost the YAP protein showed negative Ki67 immunoreactivity (arrowheads).

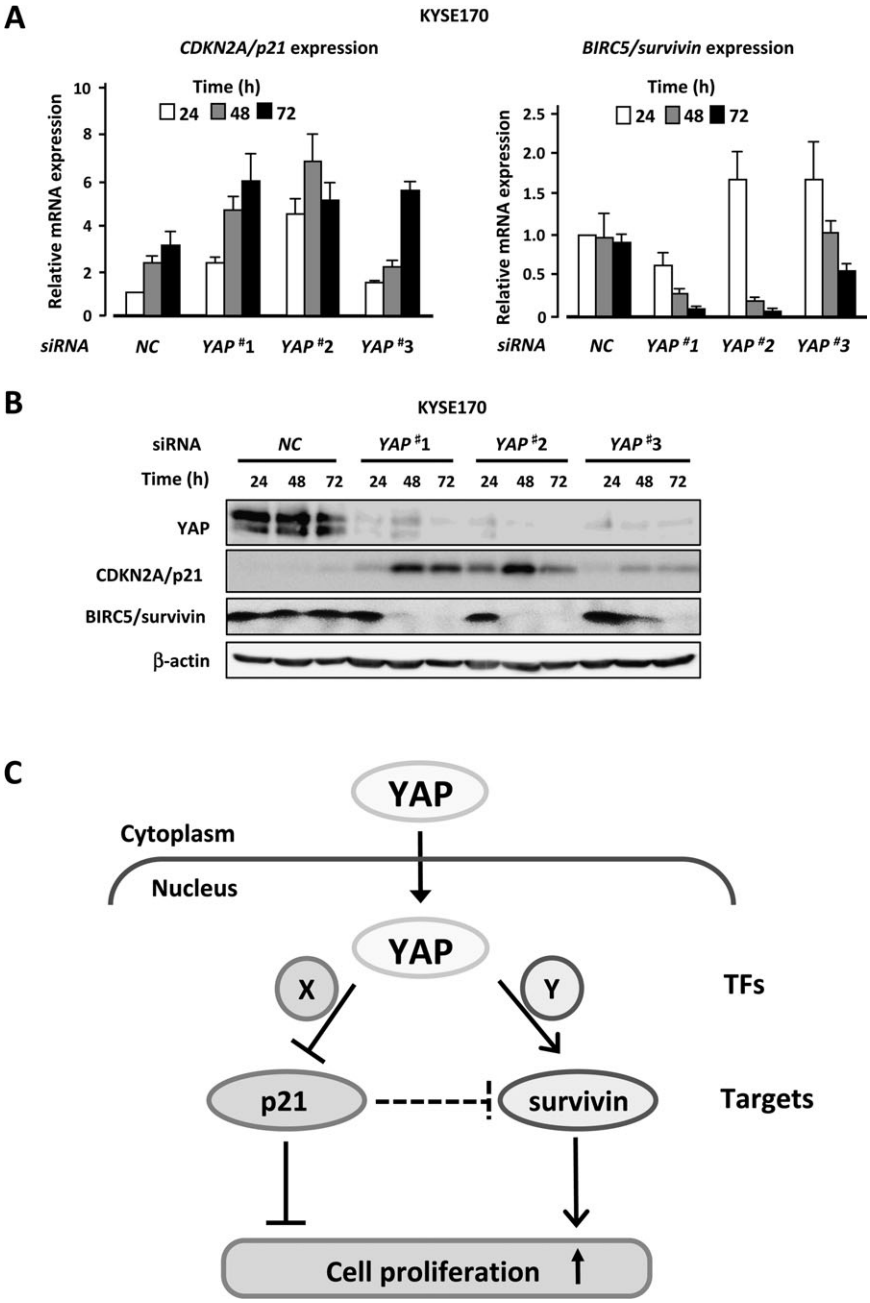

Fig. 5. YAP regulates transcription of *CDKN1A/p21* and *BIRC5/survivin*. (**A**) Relative mRNA levels of *CDKN1A/p21* or *BIRC5/survivin* 24–72 h after transfection of YAP-specific siRNA (siRNA-*YAP#1*, *#2* or *#3*) or an siRNA-NC in KYSE170 cells, determined by real-time RT–PCR. Results are shown with means ± standard deviations (bars) for triplicate experiments relative to the value for KYSE170 cells treated with siRNA-NC for 24 h. Although a different pattern was observed more or less in both genes among siRNAs specific for YAP, knockdown of YAP expression predominantly induced *CDKN1A/p21* mRNA expression and inhibited *BIRC5/survivin* mRNA expression in KYSE170 cells. (**B**) Representative results of CDKN1A/p21 and BIRC5/survivin protein expression in KYSE170 cells 24–72 h after treatment with YAP-specific siRNA (siRNA-*YAP#1*, *#2* and *#3*) or an siRNA-NC by western blotting. Efficient knockdown of YAP was observed in cells 24–72 h after the transfection of each YAP-specific siRNA compared with the siRNA-NC-transfected counterparts. Note that an increase in CDKN1A/p21 protein and a decrease in BIRC5/survivin protein were observed in cells transfected with YAP-specific siRNA (siRNA-*YAP#1*, *#2* and *#3*), whereas almost no change was observed in the expression of either protein in the siRNA-NC-transfected counterparts. (**C**) Hypothetical model of the overexpression/activation of YAP in ESCC cells. Overexpressed/activated YAP possibly binds to transcription factors (TFs, X or Y), cooperatively represses or enhances transcription of target genes, such as *CDKN1A/p21* or *BIRC5/survivin*, respectively, and promote cell proliferation although it remains unknown which transcription factors work cooperatively and whether those candidate targets are regulated directly or indirectly by YAP-mediated transcriptional regulation. The dotted line indicates the repression of BIRC5/survivin expression by CDKN1A/p21 because CDKN1A/21 was reported to mediate negative regulation of gene expression including BIRC5/survivin expression by p53 (28).

YAP expression and nuclear localization have been observed in multiple types of cancer, including HCC, colon cancer, ovarian cancer, lung cancer and prostate cancer (12,13,14,18,33,34). Only for HCC patients, however, YAP was determined to be an independent prognostic marker for overall survival and disease-free survival (33). In this study, we demonstrated that overexpression and nuclear localization of YAP in ESCC tissues was associated with a poor prognosis for patients with this disease. In addition, we revealed that YAP's overexpression in ESCC cells promoted cell proliferation, whereas its depletion impaired cell viability through the transcriptional regulation

of putative target genes. At present, the available data are still fragmentary and insufficient to delineate the tissue specificity and frequency of mutations/alterations affecting the components of the Hippo pathway, including YAP, in human cancers. A previous report (33) and the present study indicate that dysregulation of YAP plays a role in tumorigenesis and may be useful as an independent prognostic marker, at least in HCC and ESCC. Although it remains unclear whether this is due to a tissue-specific function of the Hippo pathway or simply due to a lack of studies of this pathway in other tissues.

In this study, we identified two novel isoforms of YAP, which have not been described in previous reports, although no functional differences was found among the YAP isoforms in cell growth-promoting activity assay. YAP has an N-terminal proline-rich domain, a TEAD-binding region, one (YAP-α and -β) or two (YAP-γ and -δ) WW domains, a Src homology domain 3- (SH3-) binding motif, an insertion sequence of unknown function (YAP-β and -δ), a coiled-coil domain, a transcription activation domain and a C-terminal PDZ-binding motif. Our results suggest that neither the number of WW domains nor the uncharacterized insertion sequence affects the growth-promoting activity of the YAP protein. Although it is still not clear how cells use the YAP isoforms differently (35), normal esophagus and ESCC cells predominantly express YAP-γ and -δ unrelated to the YAP level. Among these two isoforms, interestingly, the normal esophagus mainly expresses YAP-γ, whereas ESCC cells mostly express YAP-δ. Therefore, it is possible that unknown functions of YAP other than in cell proliferation, which differ among the isoforms, are differentially used in a tissue- and/or context-specific manner through transcriptional regulation.

Knockdown of the overexpressed YAP inhibits the proliferation of ESCC cells mainly by inducing G_0–G_1 arrest, not by inducing apoptosis, in an expression-dependent manner, suggesting that ESCC cells overexpressing YAP follow a highly activated YAP-mediated pathway of proliferation. Since *CDKN1A/p21* transcription was induced by YAP's knockdown and YAP is a transcription cofactor, which itself has no DNA-binding activity, YAP may directly inhibit *CDKN1A/p21* transcription as a transcription corepressor regulating specific transcription factors and resulting in the promotion of cell proliferation (Figure 5C). One candidate for such transcription factors is RUNX2, which was reported recently (27). In a preliminary experiment using a nuclear extract from KYSE170 cells, indeed, endogenous RUNX2 was co-immunoprecipitated with YAP (supplementary Figure S5 is available at *Carcinogenesis* Online). However, it is unclear whether RUNX2 is a critical transcription factor for YAP-induced CDKN1A/p21 suppression in the esophagus because the expression level of RUNX2 is relatively low in the esophagus and Vitolo *et al.* (27) showed that YAP overexpression promoted cell proliferation but prevented RUNX2-mediated repression of *CDKN1A/p21* transcription. TEAD family (TEAD1–4), four highly homologous proteins sharing a conserved DNA-binding TEA domain in humans, has been demonstrated to be important for the growth-promoting function of YAP (2,36). Knockdown of TEAD aborts expression of the majority of YAP-inducible genes and largely attenuates YAP-induced overgrowth, epithelial–mesenchymal transition and oncogenic transformation (2). Furthermore, the phenotype of TEAD1/TEAD2 double-knockout mice resembles that of YAP-knockout mice (37), suggesting the TEAD proteins to be a key downstream transcription factors mediating YAP's cellular function. However, in *Drosophila*, yk*i* mutant cells have more severe growth defects than scalloped, a *Drosophila* TEAD homolog, mutant cells (38) and overexpression of the scalloped-binding-defective Yki-S97A elicits a reduced but still obvious overgrowth in *Drosophila* eyes and wings (2). Consistently, the TEAD-binding-defective YAP-S94A mutant can still induce an expression of a fraction of YAP-regulated genes (2), indicating that besides TEAD, additional transcription factors may be used by YAP/yki to stimulate cell and tissue growth. Since all four functionally redundant members of the TEAD family are ubiquitously expressed in ESCC cells (data not shown), it is difficult to knockdown all TEAD proteins to assess whether the inactivation of TEAD can abort expression of the majority of YAP-inducible genes and largely attenuate YAP-induced growth promotion in ESCC cells. Further analysis will be needed to screen all possible transcription factors, which activate or repress transcription of target genes, working with YAP in ESCC cells.

Dong *et al.* (13) used a microarray to identify YAP-induced gene expression in murine livers and demonstrated that YAP induced the transcription of many genes, which are normally associated with hepatocyte proliferation, such as Ki67, c-myc, SOX4, H19 and AFP, and several negative regulators of apoptosis, such as the IAP family members BIRC5/survivin and BIRC2/cIAP1. Our expression analyses using real-time RT–PCR in ESCC cells with the amplification/overexpression of YAP (KYSE170) demonstrated that knockdown of YAP reduced survivin transcription but did not affect cIAP1 expression (supplementary Figure S4A is available at *Carcinogenesis* Online), suggesting that survivin but not cIAP1 is a possible direct target mediating YAP function at least in ESCC cells, possibly due to cellular context. Our double-knockdown experiments in ESCC cells (supplementary Figure S4 is available at *Carcinogenesis* Online) and a previous study with in mouse models (12) demonstrate that YAP and cIAP1 cooperatively contribute to tumorigenesis and progression possibly by mediating different pathways. Although diap1, a Drosophila homologue of cIAP1, is known to be a direct target for yki and contributes to tissue overgrowth, our results in ESCC cells indicate that cIAP1 is co-overexpressed with YAP through co-amplification within the same amplicon and not through direct targeting of YAP at least in esophageal carcinogenesis.

Supplementary material

Supplementary Figures S1–S5 and Tables S1–S3 can be found at http://carcin.oxfordjournals.org/

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References

1. Harvey,K. *et al.* (2007) The Salvador-Warts-Hippo pathway: an emerging tumor-suppressor network. *Nat. Rev. Cancer*, **7**, 182–191.
2. Zhao,B. *et al.* (2008) TEAD mediates YAP-dependent gene induction and growth control. *Genes Dev.*, **22**, 1962–1971.
3. Morin-Kensicki,E.M. *et al.* (2006) Defects in yolk sac vasculogenesis, chorioallantoic fusion, and embryonic axis elongation in mice with targeted disruption of Yap65. *Mol. Cell. Biol.*, **26**, 77–87.
4. Strano,S.*et al.* (2001) Physical interaction with Yes-associated protein enhances p73 transcriptional activity. *J. Biol. Chem.*, **276**, 15164–15173.
5. Matallanas,D. *et al.* (2007) RASSF1A elicits apoptosis through an MST2 pathway directing proapoptotic transcription by the p73 tumor suppressor protein. *Mol. Cell*, **27**, 962–975.
6. Danovi,S.A. *et al.* (2008) Yes-associated protein (YAP) is a critical mediator of c-Jun-dependent apoptosis. *Cell Death Differ.*, **15**, 217–219.
7. Lapi,E. *et al.* (2008) PML, YAP, and p73 are components of a proapoptotic autoregulatory feedback loop. *Mol. Cell*, **32**, 803–814.
8. Oka,T. *et al.* (2008) Mst2 and Lats kinases regulate apoptotic function of Yes kinase-associated protein (YAP). *J. Biol. Chem.*, **283**, 27534–27546.

9. Yuan,M. *et al*. (2008) Yes-associated protein (YAP) functions as a tumor suppressor in breast. *Cell Death Differ.*, **15**, 1752–1759.
10. Lam-Himlin,D. M. *et al*. (2006) The hippo pathway in human upper gastrointestinal dysplasia and carcinoma: a novel oncogenic pathway. *Int. J. Gastrointest. Cancer*, **37**, 103–109.
11. Overholtzer,M. *et al*. (2006) Transforming properties of YAP, a candidate oncogene on the chromosome 11q22 amplicon. *Proc. Natl Acad. Sci. USA*, **103**, 12405–124010.
12. Zender,L. *et al*. (2006) Identification and validation of oncogenes in liver cancer using an integrative oncogenomic approach. *Cell*, **125**, 1253–1267.
13. Dong,J. *et al*. (2007) Elucidation of a universal size-control mechanism in Drosophila and mammals. *Cell*, **130**, 1120–1133.
14. Steinhardt,A.A. *et al*. (2008) Expression of Yes-associated protein in common solid tumors. *Hum. Pathol.*, **39**, 1582–1589.
15. Da,C.L. *et al*. (2009) Significance and relationship between Yes-associated protein and survivin expression in gastric carcinoma and precancerous lesions. *World J. Gastroenterol.*, **15**, 4055–4061.
16. Fernandez-L,A. *et al*. (2009) YAP1 is amplified and up-regulated in hedgehog-associated medulloblastomas and mediates Sonic hedgehog-driven neural precursor proliferation. *Genes Dev.*, **23**, 2729–2741.
17. Camargo,F.D. *et al*. (2007) YAP1 increases organ size and expands undifferentiated progenitor cells. *Curr. Biol.*, **17**, 2054–2060.
18. Zhao,B. *et al*. (2007) Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. *Genes Dev.*, **21**, 2747–2761.
19. Bertini,E. *et al*. (2009) YAP at the crossroad between transformation and tumor suppression. *Cell Cycle*, **8**, 49–57.
20. Parkin,D.M. *et al*. (2005) Global cancer Statistics, 2002. *CA Cancer J. Clin.*, **55**, 74–108.
21. Lam,A.K. (2000) Molecular biology of esophageal squamous cell carcinoma. *Crit. Rev. Oncol. Hematol.*, **33**, 71–90.
22. Imoto,I. *et al*. (2001) Identification of cIAP1 as a candidate target gene within an amplicon at 11q22 in esophageal squamous cell carcinomas. *Cancer Res.*, **61**, 6629–6634.
23. Komatsu,S. *et al*. (2009) Overexpression of SMYD2 relates to tumor cell proliferation and malignant outcome of esophageal squamous cell carcinoma. *Carcinogenesis*, **30**, 1139–1146.
24. Naoi,Y. *et al*. (2008) Connexin26 expression is associated with aggressive phenotype in human papillary and follicular thyroid cancers. *Cancer Lett.*, **268**, 248–256.
25. Sudol,M. *et al*. (1995) Characterization of the mammalian YAP (Yes-associated Protein) gene and Its role in defining a novel protein module, the WW Domain. *J. Biol. Chem.*, **270**, 14733–14741.
26. Komuro,A. *et al*. (2003) WW domain-containing protein YAP associates with ErbB-4 and acts as a co-transcriptional activator for the carboxy-terminal fragment of ErbB4 that translocates to the nucleus. *J. Biol. Chem.*, **278**, 33334–33341.
27. Vitolo,M.I. *et al*. (2007) The RUNX2 transcription factor cooperates with the YES-associated protein, YAP65, to promote cell transformation. *Cancer Biol. Ther.*, **6**, 856–863.
28. Löhr,K. *et al*. (2003) p21/CDKN1A mediates negative regulation of transcription by p53. *J. Biol. Chem.*, **278**, 32507–32516.
29. Zhao,B. *et al*. (2010) The Hippo-YAP pathway in organ size control and tumorigenesis: an updated version. *Genes Dev.*, **24**, 862–874.
30. Baldwin,C. *et al*. (2005) Multiple microalterations detected at high frequency in oral cancer. *Cancer Res.*, **65**, 7561–7567.
31. Snijders,A.M. *et al*. (2005) Rare amplicons implicate frequent deregulation of cell fate specification pathways in oral squamous cell carcinoma. *Oncogene*, **24**, 4232–4242.
32. Modena,P. *et al*. (2006) Identification of tumor-specific molecular signatures in intracranial ependymoma and association with clinical characteristics. *J. Clin. Oncol.*, **24**, 5223–5233.
33. Xu,M.Z. *et al*. (2009) Yes-associated protein is an independent prognostic marker in hepatocellular carcinoma. *Cancer*, **115**, 4576–4585.
34. Wang,Y. *et al*. (2010) Overexpression of yes-associated protein contributes to progression and poor prognosis of non-small-cell lung cancer. *Cancer Sci.*, **101**, 1279–1285.
35. Sudol,M. *et al*. (2010) Modularity in the Hippo signaling pathway. *Trends Biochem. Sci.*, **35**, 627–633.
36. Ota,M. *et al*. (2008) Mammalian Tead proteins regulate cell proliferation and contact inhibition as transcriptional mediators of Hippo signaling. *Development*, **135**, 4059–4069.
37. Sawada,A. *et al*. (2008) Redundant roles of Tead1 and Tead2 in notochord development and the regulation of cell proliferation and survival. *Mol. Cell. Biol.*, **28**, 3177–3189.
38. Huang,J. *et al*. (2005) The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila homolog of YAP. *Cell*, **122**, 421–434.

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