Zinc-finger protein YY1 suppresses tumor growth of human nasopharyngeal carcinoma by inactivating c-Myc–mediated microRNA-141 transcription

Mengna Li1,2, Yukun Liu1,2, Yanmei Wei1,2, Chunchun Wu1,2, Hanbing Meng1,2, Weihong Niu1,2, Yao Zhou1,2, Heran Wang1, Qiuyuan Wen3, Songqing Fan3, Zheng Li2,5, Xiayu Li6, Jianda Zhou6, Ke Cae6, Wei Xiong2, Zhaoyang Zeng2, Xiaoling Li2, Yuanyu Zheng1,2, Guiyuan Li1,2 and Ming Zhou*1,2

1Hunan Cancer Hospital and the Affiliated Tumor Hospital of Xiangya Medical School, Central South University, Changsha, Hunan 410013, P.R. China; 2The Key Laboratory of Carcinogenesis of the Chinese Ministry of Health, The Key Laboratory of Carcinogenesis and Cancer Invasion of the Chinese Ministry of Education, Cancer Research Institute, Central South University, Changsha, Hunan 410078, P.R. China; 3The Second XiangYa Hospital, Central South University, Changsha, Hunan 410011, P. R. China; 4Department of Otolaryngology Head and Neck Surgery, The Xiangya Hospital, Central South University, Changsha, Hunan 410008, P. R. China; 5High Resolution Mass Spectrometry Laboratory of Advanced Research Center, Central South University, Changsha, Hunan, 410013, P. R. China; 6The Third XiangYa Hospital, Central South University, Changsha, Hunan 410008, P. R. China

Running title: YY1 suppresses cell proliferation and tumor growth in NPC

*Corresponding author:
Ming Zhou, The Affiliated Tumor Hospital of Xiangya Medical School, Central South University, Changsha, Hunan 410013, P. R. China. Tel: +86-731-84805383; Fax: +86-731-84805383; E-mail: zhouming2001@163.com (MZ).

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ABSTRACT
YY1 (Yin Yang 1) is a zinc-finger protein that plays critical roles in various biological processes by interacting with DNA and numerous protein partners. YY1 has been reported to play dual biological functions as either an oncogene or tumor suppressor in the development and progression of multiple cancers, but its role in nasopharyngeal carcinoma (NPC) has not yet been revealed. In the present study, we found that YY1 overexpression significantly inhibits cell proliferation and cell cycle progression from G1 to S and promotes
apoptosis in NPC cells. Moreover, we identified YY1 as a component of the c-Myc complex and observed that ectopic expression of YY1 inhibits c-Myc transcriptional activity, as well as the promoter activity and expression of the c-Myc target gene microRNA-141 (miR-141). Furthermore, restoring miR-141 expression could at least partially reverse the inhibitory effect of YY1 on cell proliferation and tumor growth and on the expression of some critical c-Myc targets, such as PTEN/AKT pathway components both in vitro and in vivo. We also found that YY1 expression is reduced in NPC tissues, negatively correlates with miR-141 expression and clinical stages in NPC patients, and positively correlates with survival prognosis. Our results reveal a previously unappreciated mechanism in which the YY1/c-Myc/miR-141 axis plays a critical role in NPC progression and may provide some potential and valuable targets for the diagnosis and treatment of NPC.

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Nasopharyngeal carcinoma (NPC) is a head-and-neck epithelial malignancy that occurs frequently in Southern China, which is a most peculiar cancer with a distinctly skewed geographic and ethnic distribution (1). A multifactorial etiology has been suggested for NPC, including a dynamic interplay between genetic predisposition, Epstein-Barr virus (EBV) infection and environmental carcinogens (2). However, the molecular mechanisms that regulate NPC initiation and promote its malignant progression remain obscure. Despite marked improvements in NPC patient prognosis in recent decades (3), most patients are still diagnosed with advanced stage NPC (4). Therefore, identifying sensitive biomarkers and drug targets is essential to enable early diagnosis and improve treatment outcomes for early-stage NPC patients.

c-Myc is an important oncoprotein whose abnormal expression contributes to 30~50% of human malignancies, being overexpressed in more than 70% of human malignancies, including nasopharyngeal carcinoma, lymphomas, neuroblastomas, melanomas, breast, ovarian, prostate and liver cancers (5-7). In c-Myc dependent cancer models, c-Myc inactivation can not only regress tumor growth but also induce cell death, suggesting that c-Myc inactivation can impact a range of human cancers (8). As a critical transcription factor, c-Myc is widely involved in cell proliferation, apoptosis, differentiation, metabolism,
somatic cell reprogramming and other key processes under normal or pathological conditions via the regulation of numerous target genes (9). Therefore, strategies for downregulating the c-Myc transcriptional activity are thus widely sought after. We previously found that miR-141 functions as an oncogenic miRNA in NPC and plays critical roles in NPC development and progression (10). Meanwhile, c-Myc is known to specifically bind the miR-141 promoter region and thus regulate the transcription activation of miR-141, while c-Myc knockdown has been shown to activate PTEN/AKT signaling and suppress cell proliferation and tumor growth via transcriptional downregulation of miR-141 in NPC cells (11-13).

C-Myc always exerts its functions through the transcriptional regulation of its downstream target genes, which depends on the formation of the Myc/Max/Mad complex. c-Myc binds Max through its bHLHZip domain, and these heterodimers bind specifically to 5′-CACGTG-3′ E-box sequences to activate transcription (14,15). Alternatively, transcriptional repression by Mad is mediated through its interaction with mSin3, which results in the recruitment of histone deacetylases (HDACs) and corepressor molecules and thus leads to the transcriptional repression of target genes (16). Further exploration of the molecular mechanism of c-Myc in NPC using bioinformatics analyses revealed Yin Yang-1 (YY1) as a potential c-Myc-interacting protein that might be involved in the regulation of c-Myc target genes (17,18). YY1 is a ubiquitously expressed member of the GLI-Kruppel family of zinc-finger transcription factors that is abnormally expressed in most human tumors and exerts dual biological functions as a tumor suppressor or oncogene through the regulation of different target genes or signaling pathways (19,20). These dual functions in different cancers are probably because YY1 can both positively and negatively regulate gene expression, as well as interact with a multitude of proteins with diverse functions (21). Crystal structures of YY1 with different binding partners reveal that YY1 is a key scaffold protein that functionally interfaces with various partners to regulate gene transcription and participate in multiple signaling pathway. However, the precise biological function of YY1 in NPC remains unclear.

In the present study, we revealed that YY1 significantly inhibits cell
proliferation and cell cycle progression and induces apoptosis in NPC cells. Moreover, YY1 was identified as a component of the c-Myc complex, and ectopic expression of YY1 is able to inhibit c-Myc transcriptional activity, as well as the promoter activity and expression of the critical downstream target gene miR-141. Furthermore, restoring the expression of miR-141 at least partially reverses the inhibitory effects of YY1 on cell proliferation, cell cycle progression, apoptosis and tumor growth, as well as the expression of some critical c-Myc targets, such as the PTEN/AKT pathway, both in vitro and in vivo. In addition, YY1 is also down-regulated in NPC tissues, and YY1 protein level is negatively correlated with clinical stage in NPC patients and miR-141 expression, while positively correlated with survival prognosis. Taken together, our results demonstrate that the YY1/c-Myc/miR-141 axis plays a critical role in the development and progression of NPC, thereby providing potential targets for the diagnosis and treatment of NPC.

Results

YY1 inhibits cell proliferation and promotes apoptosis in NPC cells

As an important transcription factor, YY1 plays dual biological roles as an oncogene or tumor suppressor in different tumors. However, its role in nasopharyngeal carcinoma has not been defined. To confirm the role of YY1 in NPC progression, 5-8F and HNE2 cell lines stably overexpressing YY1 were constructed, and the expression of exogenous YY1 was confirmed by western blotting (Fig. 1A). Then, we investigated the effect of YY1 on cell proliferation. CCK-8 assays show that YY1 overexpression inhibits cell proliferation in both 5-8F and HNE2 cell lines (Fig. 1B), which was further confirmed by colony-forming assays. YY1-overexpressing 5-8F and HNE2 cells formed approximately 40 and 50% fewer colonies, respectively, compared with their negative controls (Fig. 1C). To define the underlying biological mechanisms, we further analyzed the effect of YY1 overexpression on cell-cycle progression (Fig. 1D) and found that ectopic expression of YY1 significantly increases the G0/G1 population, followed by a decrease in the S-phase, in both 5-8F and HNE2 cells compared with control cells. Furthermore, we examined the effect of YY1 on cell apoptosis by conducting Annexin V-PE/7-AAD double staining and flow-cytometry analysis after the cells were serum-starved for 24 h, and...
the results show that both of 5-8F and HNE2 cells overexpressing YY1 have significantly higher percentages of apoptotic cells compared with negative control (Fig. 1E). Furthermore, we investigated the effect of YY1 knockdown on cell biological functions by RNA interference technology with si-YY1 (Fig. S1, 2A), and the results show that knockdown of YY1 increases cell proliferation, colony formation and cell cycle G1/S transition (Fig. 2B-D), and inhibits cell apoptosis (Fig. 2E). Taken together, these data suggest that YY1 inhibits cell proliferation and the G1-S transition and promotes apoptosis in both 5-8F and HNE2 cells and thus functions as a potential tumor suppressor in NPC cells.

**YY1 negatively regulates c-Myc transcriptional activity via the c-Myc/Max/Mad network.**

Our previous work showed that knockdown of c-Myc significantly inhibits cell proliferation and promotes apoptosis in NPC cells. Further bioinformatics analysis revealed YY1 as a potential c-Myc-interacting protein. To confirm the interaction between c-Myc and YY1, we analyzed the subcellular localization of YY1 and c-Myc in 5-8F and HNE2 cells by immunofluorescence and the results show that YY1 is colocalized with c-Myc in the nucleus (Fig. 3A), and YY1 was also found to be interacted with c-Myc performed by co-immunoprecipitation experiments with the lysates from HEK293 cells co-transfected with HA-c-Myc and Flag-YY1 (Fig. 3B and C). Since both YY1 and c-Myc are critical transcription factors, we then explored the effect of YY1 on the expression of c-Myc as well as the effect of c-Myc on the expression of YY1. Western blotting and qRT-PCR indicated that overexpression or knockdown of YY1 has no effect on c-Myc protein or mRNA levels (Fig. S2 A, B and E). Meanwhile, knockdown of c-Myc similarly fails to alter YY1 protein or mRNA levels (Fig. S2C and D). Therefore, these results suggest that YY1 is a component of the c-Myc transcriptional complex and a negative regulator of c-Myc transcriptional activity.

c-Myc is a helix-loop-helix leucine zipper protein that dimerizes with an obligate partner, Max, to bind DNA sites, 5’-CACGTG-3’, termed E-boxes. Therefore, we first confirmed the promoter activity of a c-Myc target consensus sequence containing 3 E-boxes in NPC cells (Fig. 3D) and then investigated the effect of YY1 on...
c-Myc transcriptional activity using double luciferase reporter assays. The results show that c-Myc activity was significantly downregulated in YY1-overexpressing cells compared with control cells (Fig. 3E), suggesting that YY1 might directly play a negative role in c-Myc-mediated transcription. Furthermore, co-IP experiments were performed on total protein extracts to confirm that overexpression of YY1 reduces the formation of Max and c-Myc heterodimers approximately 71% compared with the negative control. Conversely, the Mad and c-Myc protein complex increases approximately 37% (Fig. 3F). These results indicate that YY1 inhibits c-Myc transcriptional activity towards target gene by decreasing the binding of the c-Myc/Max/Mad complex to E-box sequences.

**YY1 negatively regulates the transcription and expression of c-Myc oncogenic target miR-141.**

Since YY1 is a component of the c-Myc transcriptional complex and negatively regulates c-Myc transcriptional activity, and miR-141 was previously identified as a critical oncogenic miRNA which is directly transcriptionally regulated by c-Myc. We further verified the functions of miR-141 in HNE2 cells used in this study and found that miR-141 promotes cell proliferation and colony formation (Fig. S3A-B). Thus, we hypothesized that YY1 is a negative regulator of the c-Myc/miR-141 transcriptional axis and thus plays a critical role in the progression of NPC. Therefore, we first used dual luciferase reporter assays and real-time PCR to detect the effect of YY1 on c-Myc-dependent miR-141 transcription and expression. As a result, ectopic expression of c-Myc led to the upregulation of miR-141 promoter activity and expression, which is consistent with previous results (12), whereas YY1 overexpression decreases the effect of c-Myc on miR-141 promoter activity and expression in 5-8F and HNE2 cells (Fig. 4A and B). Next, we investigated the effect of YY1 on the expression of pri-, pre- and mature miR-141, as well as the promoter activity of miR-141 driven by endogenous c-Myc in NPC cells. The qRT-PCR results show that overexpressing YY1 downregulates the expression of pri-, pre- and mature miR-141, as well as miR-141 promoter activity (Fig. 4C and D), but present no effect on the expression levels of Drosha and Dicer, which are involved in miRNA processing (Fig. 4E). Inversely, knockdown of YY1 increases
the expression of miR-141 (Fig. S2F). In order to verify whether the regulation of YY1 on miR-141 depends on c-Myc, we examined the effect of YY1 overexpression on the promoter activity and expression of miR-141 in both 5-8F and HNE2 cells with c-Myc knockdown, and the results show that c-Myc knockdown causes the deregulation of YY1 on the promoter activity and expression of miR-141 (Fig. 4F-H). These findings demonstrate that YY1 transcriptionally downregulates miR-141 expression by negatively regulating c-Myc transcriptional activity.

**Restoring miR-141 levels reverses the effect of YY1 on cell proliferation and apoptosis in NPC cells.**

Given that YY1 functions as a tumor suppressor in NPC by inhibiting cell proliferation and inducing apoptosis and that YY1 downregulates oncogenic miR-141 expression, we wondered whether the effect of YY1 on cell proliferation and apoptosis is mediated by its negative regulation of miR-141. Therefore, rescue assays were performed by co-transfecting 5-8F/YY1 and HNE2/YY1 cells with miR-141 mimic. The expression of exogenous miR-141 was confirmed by qRT-PCR (Fig. S4). To determine the impact of miR-141 restoration on cell proliferation and colony formation by NPC cells, we performed CCK-8 and colony-forming assays. As expected, ectopic expression of YY1 obviously inhibits cell proliferation and colony formation in 5-8F and HNE2 cells (YY1), while restoring the expression of miR-141 in YY1-overexpressing cells (YY1+miR-141) markedly rescues cell proliferation and colony formation compared with YY1-overexpressing cells (YY1) (Fig. 5B and C). Furthermore, we analyzed the effect of miR-141 rescue on cell cycle progression, and the results show that overexpression of YY1 arrests cell cycle cells in G0/G1 phase, while restoring the expression of miR-141 causes significant decrease in the G0/G1 population, followed by an increase in S-phase cells compared with YY1 overexpression alone (Fig. 6A). Furthermore, overexpression of YY1 significantly increases the percentage of apoptotic cells in the NPC cells when compared with the negative controls, while restoring miR-141 expression dramatically rescues the effect of YY1 on apoptosis induction in NPC cells (Fig. 6B). Consistent with the results from flow cytometry assays, western blotting results show that overexpression of YY1 results in a
significant increase of c-PARP, a critical apoptosis marker (11), while restoring the expression of miR-141 dramatically recovers the expression of c-PARP (Fig. 5A). Taken together, these findings demonstrate that rescuing miR-141 expression at least partially reverses the tumor-suppressive effect of YY1 on cell proliferation and apoptosis in vitro, suggesting that YY1 plays critical roles in cell-cycle arrest and initiation of apoptosis by negatively regulating miR-141 transcription during NPC progression.

**miR-141 can rescue the tumor-suppressive effect of YY1 on tumor growth in vivo.**

Since our in vitro results show that YY1 regulates miR-141 to affect NPC cell proliferation and apoptosis, we next investigated whether YY1 inhibits tumor growth through downregulation of miR-141 in vivo. Therefore, we performed in vivo experiments using xenograft tumor models in nude mice. 5-8F/control, 5-8F/YY1 and 5-8F/YY1 with transfection of miR-141 mimic were injected subcutaneously into the flanks of 5-week-old female nude mice, respectively. All xenograft model mice were killed on day 38 to examine the final tumor volume, and the results show that tumor size in nude mice injected with 5-8F/YY1 cells obviously reduces compared with the negative control, while the size could be dramatically recovered when miR-141 expression was restored in 5-8F/YY1 cells (Fig. 7A). Meanwhile, xenograft tumor weight and growth rate with miR-141 restoration significantly increase compared with the 5-8 F/YY1 group (Fig. 7B and C). In addition, the expression of YY1 and miR-141 in xenograft tumor tissues was confirmed by western blotting and qRT-PCR (Fig. 7D and E). To further confirm the effect of miR-141 restoration on tumor growth and apoptosis in vivo, we analyzed the percentage of cells expressing the proliferation marker Ki-67, and the results show that ectopic expression of YY1 significantly reduces the number of Ki67-positive cells, whereas miR-141 restoration significantly recovers the number of Ki67-positive cells, which was also confirmed by H&E staining (Fig. 7F). These results indicate that YY1 inhibits tumor growth and presents anti-tumor effects in vivo, while restoring the expression of miR-141 significantly reverses these effects.

**YY1 inhibits tumor growth by repressing miR-141/PTEN/AKT signaling.**
We previously reported that PTEN functions as a tumor suppressor by negatively regulating the AKT signaling pathway. PTEN was identified to be a direct target of miR-141 and post-transcriptionally downregulated by miR-141 in NPC cells. Moreover, increasing evidence indicated that aberrant PTEN/AKT pathway activation is essential for the development and progression of NPC. Therefore, we performed western blotting and immunohistochemical (IHC) to detect the expression of some critical components of PTEN/AKT pathway in 5-8F cells and corresponding xenograft tumor tissues. As a result, PTEN protein increases and p-AKT decreases in YY1-overexpressed NPC cells and their xenograft tumor tissues compared with the negative controls, whereas restoring miR-141 expression causes a significant reversion. In addition, we detected the expression of some molecules related to cell cycle and apoptosis, and the results reveal that CCND1 is largely decreased, while p21 and c-PARP are obviously increased. Moreover, restoring miR-141 expression in YY1-overexpressing cells results in a significant reversion, while CDK4 levels remain unaltered (Fig. 8A and B). Consequently, these data show that YY1 inhibits cell proliferation and induces apoptosis by downregulating the miR-141/PTEN/AKT pathway in vitro and in vivo.

**YY1 expression was negatively correlated with miR-141 and survival in human NPC patients.**

To confirm the role of YY1 and miR-141 in NPC, we detected their expression in NPC tissues derived from 29 healthy donors and 126 patients varying in age, gender, histological type and clinical stage using IHC staining and in situ hybridization (ISH). As a result, most patients exhibit significant reduction of YY1 protein, and YY1 expression is decreasing with increasing clinical TNM stage in NPC patients (Table 1, Fig. 9A and B). We also analyzed the association between YY1 expression and the survival of NPC patients with follow-up data selected from the total of NPC samples. The survival time ranged from 3 to 120 months, and the OS rate of patients with high YY1 expression is significantly higher compared with low YY1 expression (Fig. 9C). These results indicate that YY1 plays an essential role in NPC development. However, average miR-141 levels in NPC tissues were higher than those in non-tumor NPC control tissues (Fig. S5A-B), and the OS rate of patients with low
miR-141 expression is significantly higher than that in patients with high miR-141 expression (Fig. S5C). and the expression level of miR-141 is negatively correlated with the protein level of YY1 in NPC patients (r=-0.207, P<0.001, Table 2) (Fig. 9D). We then assessed the relationship between the expression levels of YY1 and miR-141 and prognosis. The results show that low YY1 and high miR-141 expression in NPC patients are both associated with poorer survival rates. Conversely, high YY1 and low miR-141 expression correlated with favorable survival in NPC patients (Fig. 9E). These findings indicate that both YY1 and miR-141 might be involved in NPC progression and that YY1 might be a negative regulator of miR-141 in NPC. In conclusion, YY1 is a component of the c-Myc transcriptional complex and negatively regulates c-Myc transcriptional activity by disrupting the c-Myc/Max complex, therefore YY1 inhibits cell proliferation and tumor growth and initiates apoptosis though inactivation of c-Myc/miR-141/PTEN/AKT axis in NPC (Fig. 9F).

Discussion
The Yin Yang 1 (YY1) transcription factor plays pivotal roles in normal biological processes such as development, differentiation, replication and cell proliferation (25). The roles of YY1 in cancer have been explored recently. Interestingly, although YY1 function as an oncogene in most cancers (26), it also acts as a tumor suppressor, such as in pancreatic cancer (27) and breast cancer (28), while its function in NPC has not been reported. In the present study, we found that the expression of YY1 significantly decreased in NPC tissues compared with normal controls, and that low YY1 levels inversely correlated with OS in NPC patients. Furthermore, overexpressing YY1 effectively inhibited cell proliferation and promoted apoptosis in NPC cell lines, and xenograft data in nude mice also confirmed that ectopic expression of YY1 can inhibit tumor growth in vivo. These data support a role for YY1 as a tumor suppressor in NPC and provide us with a new perspective on the function of YY1. In recent years, more and more genes with dual functions as oncogenes or tumor suppressors have been found in different of tumor types, as well as different stages of tumor development and progression, such as KLF4 (29), ATF-2 (30), and TGF-β (31), indicating that the function of some genes is spatiotemporally dynamic and
cannot be simply considered as inherently oncogenic or tumor suppressive for functional mapping. To our knowledge, this is the first report to define the function of YY1 in NPC and provides more exact data to support the dual roles of YY1 in different types of tumors.

YY1 was first discovered as a multifunctional transcription factor that can selectively initiate, activate, or repress transcription depending upon the context in which it binds to the recruited cofactors (20). Previously, Wu S, Sankar N et al. reported that YY1 regulates the transcriptional activity of p53 by inhibiting its interaction with the co-activator p300 and by enhancing its interaction with the negative regulator Mdm2 (32,33). More recently, Schlisio S et al. reported that YY1 and E2F2/E2F3 constitutes a protein complex to regulate the Cdc6 promoter activity (34). In the present study, YY1 was identified to be a c-Myc co-factor and downregulate c-Myc transcriptional activity. As we all know, c-Myc is a critical growth regulatory that is commonly overexpressed in a wide range of cancers. Mechanistically, c-Myc is a helix-loop-helix leucine zipper protein that dimerizes with an obligate partner, Max, to bind DNA sites, 5'-CACGTG-3', termed E-box. Because binding to Max is necessary for all known c-Myc activities, the establishment of a link between YY1 and c-Myc in tumorigenesis is very interesting (35). In the current study, YY1 was identified as a potent inhibitor of c-Myc transcriptional activity through reducing the formation of c-Myc and Max heterodimers, and overexpression of YY1 inhibits the activity of the promoters containing E-boxes. Therefore, YY1 acts as the most direct inhibitor for the interaction between c-Myc and Max, which suggests that YY1 reduces c-Myc-mediated transcription through disrupting c-Myc/Max complex. It is expected to provide a new target for clinical treatment.

Impressively, accumulating evidence indicates that YY1 might be involved in the regulation of c-Myc-mediated target gene expression. Previous studies have shown that knocking down c-Myc significantly inhibits cell proliferation and tumor growth and downregulates miR141 in NPC cells by directly binding to its promoter (11). miR141 is a member of the miR200 family that has been shown to be dysregulated in a wide variety of cancers and plays critical oncogenic roles in NPC (10). Meanwhile, c-Myc was identified as a negative regulator of
the Bromodomain containing protein 7 (BRD7) gene, which is a tumor suppressor in multiple types of cancers, as well as a cofactor of c-Myc (36-38); therefore, BRD7 expression and c-Myc activation form a double-negative feedback loop that controls cell proliferation and tumor growth in NPC by targeting the downstream gene miR-141 (12). In the current study, we found that YY1 overexpression does not affect the mRNA and protein levels of c-Myc, but significantly reduces miR-141 expression, including pri-, pre- and mature-miR-141, as well as miR-141 promoter activity, suggesting that YY1 inhibits the activation of the c-Myc/miR-141 transcriptional axis. Additionally, the restoration of miR-141 significantly rescues the tumor-suppressive effect of YY1 overexpression in NPC cells. These results demonstrate that YY1 inhibits cell proliferation and tumor growth in NPC at least partially by inactivating the c-Myc/miR-141 axis. PTEN was reported as a direct target of miR-141 and it is post-transcriptionally downregulated by miR-141 in NPC cells. Here, we confirmed that ectopic expression of YY1 in NPC cells results in increased PTEN protein level and decreased AKT phosphorylation, while restoring miR-141 rescues the alterations in PTEN expression and AKT phosphorylation, which demonstrates that the PTEN/AKT pathway might play critical roles in YY1-mediated tumor suppression.

Taken together, our results reveal a precise mechanism by which YY1 functions as a critical regulatory factor for c-Myc-mediated miR-141 transcription in NPC development and progression. As c-Myc is known to be deregulated in 30–50% of human malignancies with poor prognosis, finding more c-Myc targeting genes that are coregulated by YY1 and further investigating their function in NPC development and progression remain critical goals in the future.

**Experimental procedures**

**Tissue samples.** NPC samples (n=126) and noncancerous nasopharyngeal tissues (n=29) were all collected at the Second Xiangya Hospital of Central South University (Changsha, China); noncancerous nasopharyngeal tissues were collected from independent patients with chronic inflammation of the nasopharyngeal mucosa or polyps. The clinicopathological characteristics of the NPC patients are shown in Table 1. The NPC patients range in age from 23 to 74 years old, and 85 of the 126 patients have valid follow-up data. The
longest survival time is 104 months. Overall survival (OS) is defined as the time from diagnosis to the date of death or the date that the patient was last known to be alive. All tissue samples were immediately snap-frozen in liquid nitrogen and stored in at -80°C. Clinicopathological data is reviewed, and TNM staging classifications are based on the criteria of the American Joint Committee on Cancer (AJCC, 6th edition).

Cell lines. The human NPC cell lines 5-8F and HNE2 were preserved in our laboratory and were cultured in RPMI-1640 (Gibco-BRL, Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL, Invitrogen, Paisley, UK) in a humidified incubator with 5% CO₂ at 37 °C. The embryonic kidney cell lines HEK293 and HEK293T were also preserved in our laboratory and maintained at 37°C with a 5% CO₂ atmosphere in Dulbecco’s-modified Eagle’s medium (DMEM) (Gibco-BRL, Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum (Gibco-BRL, Invitrogen, Paisley, UK).

Cell transfections. Exponentially growing cells were trypsinized, counted and seeded a 6-well plates to ensure 60~80% cell confluence on the following day for transfection. All of the constructs and oligonucleotides were transfected into the indicated cells using Lipofectamine 3000 (Invitrogen) according to the manufacturer’s instructions. The hsa-miR-141 mimic (miR-141) and negative control (miR-NC) were purchased from RiBoBio (Guangdong, China), which was used to restore the expression of miR-141 in YY1 overexpressing cells according to the manufacturer’s instructions. The specific small interfering RNAs (siRNAs) for YY1 were purchased from RiBoBio, and the siRNA sequences are as following (5’ → 3’): siRNA#1: CGACGACTACATT GAACAA, siRNA#2: GCACAAAGATGTTCAGGGA. The two recombinant plasmids of c-Myc knockdown (pRNAU6.1/sh-c-Myc) and c-Myc overexpression (PIRES2-EGFP/2Flag-c-Myc) were constructed by our laboratory as described previously (11).

Stable cell lines. As for recombined lentiviral expression vector (pCDH-EF1-MCS-T2A-copGFP/YY1), the full-length open reading frame of human YY1 with 3Flag tags was linked to lentivirus expression vector pCDH-EF1-MCS-T2A-copGFP. For the construction of NPC stable cell lines
with YY1 overexpression (HNE2/YY1 and 5-8F/YY1), the packaging vectors pMD2.G, psPAX2 and the recombined lentiviral expression vector were co-transfected into HEK293T packaging cells using Lipofectamine3000 Reagent (Invitrogen). After transfection for 6 h, the medium was replaced with fresh medium with 10% FBS. After 30 h, virus particles in the medium were collected, filtered and transduced into target cells. Then, flow cytometry assay was further used to screen positive cells. Finally, the NPC stable cell lines with YY1 overexpression were obtained.

**RNA extraction and quantitative real-time PCR.** Total RNA was isolated from tissue samples and cell lines using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. cDNA was synthesized with 2ug of total RNA using PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). Quantitative real-time PCR was performed using the miDETECT A Track miRNA qRT-PCR Kit (RiBoBio) and the iCycler Real-time PCR Detection System (Bio-Rad) following the manufacturer’s protocol. The U6 small nuclear RNA was used for normalization. The relative expression ratio of mature miR-141, pre-miR-141 and pri-miR-141 was calculated using the $2^{-\Delta\Delta Ct}$ method. The primers for miR-141, pre-miR141, U6, pri-miR141, c-Myc, and GAPDH were described previously (11,12). The relative amount of mRNA or gene to internal control was calculated using the equation $2^{-\Delta\Delta Ct}$ method. And each sample was conducted in triplicate.

**Western blot analysis.** Cells and tissue samples were lysed in RIPA buffer in the presence of Protease Inhibitor Cocktail and PhoSTOP (Roche, Basel, Switzerland). Protein was quantified using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). Protein (30~80 μg) was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (PVDF) (Millipore, Billerica, MA, USA). The membranes were then blocked with 5% non-fat milk in Tris-buffered saline and then incubated with primary antibodies anti-YY1(dilution 1:500, Santa Cruz, SC-734), anti-c-Myc(dilution 1:1000, CST, D84C12), anti-Flag(dilution 1:2000, Sigma,F-10084), anti-MAX (dilution 1:500, Santa Cruz, SC-8011), anti-Mad (dilution 1:500, Santa Cruz, SC-8012), anti-PARP (dilution 1:1000, CST,46011), anti-c-PRAP (dilution
1:1000, CST, ASP214), anti-CCND1 (dilution 1:500, Santa Cruz, SC-753), anti-CDK4 (dilution 1:500, Santa Cruz, SC-260), anti-PTEN (dilution 1:500, Bio world Technology, BS1305), anti-pS473AKT (dilution 1:500, Bioworld Technology, BS4007), anti-AKT (dilution 1:500, Bioworld Technology, BS1810), anti-p21(dilution 1:1000, CST,12D1), anti-Drosha (dilution 1:1000 , proteintech, 55001-1-AP), anti-Dicer ( dilution 1:500 ,Santa Cruz,SC-136979 ) or Anti-GAPDH ( dilution 1:1000, proteintech, 10494-1-Ig) at 4° C overnight. The membranes incubated with primary antibodies were then washed with PBST solution for 10 min in triplicate, and then incubated with secondary antibodies and washed. Signals were detected by an enhanced chemiluminescence detection system as the manufacturer’s protocol (Bio-Rad, Hercules, CA, USA).

**Immunofluorescence assay (IF).** Subcellular localization of endogenous c-Myc and exogenous YY1 was analyzed by immunofluorescence assay (IF). 5-8F and HNE2 cells were seeded on glass coverslips, and then fixed with 4% paraformaldehyde for 15 min at room temperature and washed with PBS. Cells were then permeabilized with 0.3% Triton X100 for 5 min at room temperature. Cells were then washed 3 times with PBS and incubated for 15 min in 5% goat serum and then incubated at 4 °C overnight with the primary antibody anti-c-Myc (dilution 1:800; CST) and anti-YY1 (dilution 1:100; Santa Cruz). After three washes for 5min with PBS, cells were incubated for 1 h at 37 °C in the dark with the secondary antibody DyLight 488 AffiniPure Goat Anti-Rabbit IgG ( H+L ) (dilution 1:1000; abbkine,A23220) and DyLight 594 AffiniPure Goat Anti-Mouse IgG ( H+L ) (dilution 1:1000; abbkine,A23410). Coverslips were washed with PBS and visualized using an epifluorescence microscope.

**Co-immunoprecipitation assay (Co-IP).** After the transfection for approximately 36 hours, the cells were lysed with lysis buffer for co-immunoprecipitation assay (Co-IP) using the Co-IP kit (Beyotime, Beijing, China). The cell lysates were then premixed with protease inhibitor cocktail (Roche) on ice for 20 min and then centrifuged at 12,000 g for 15 min. Equal amounts of protein (1 mg) with 30 μl of protein A/G magnetic beads (Selleck, Houston, TX, USA) precoated with anti-Flag antibodies (Sigma) or anti-mouse IgG
(Santa Cruz) were immunoprecipitated for 16~18 hours at 4°C. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane, and then followed by Western blot assay probed with corresponding antibody.

**Luciferase reporter assays.** The luciferase vector with E-box was a gift from Prof. Guoliang Qing (Wuhan University of Medical Research Institute, China) (22). The recombinant reporter vectors fused with the miR141 promoter (pGL3 enhancer /miR-141P) were constructed by our laboratory as described previously (11). And the proper insertions were further confirmed by sequencing. The cells were seeded in 24-well plates. After 12 hours, pIRES2-EGFP/2Flag-cMyc and the luciferase vector with E-box or pGL3 enhancer/miR-141P were transiently co-transfected into 5-8Fand HNE2 stable NPC cells with YY1 overexpression in triplicates using Lipofectamine 3000, respectively, and pRL-TK vector (Promega) containing Renilla luciferase was used as an internal control. Cells were harvested 36h post transfection. Firefly and Renilla luciferase activities were measured using a Dual-luciferase reporter kit (Promega) according to the manufacturer’s protocol. Firefly luciferase activity was normalized to Renilla luciferase activity.

**CCK-8 and colony-forming assays.** Cell proliferation assays were performed using the Cell Counting Kit-8 (Selleck, Houston, Texas, USA) according to the manufacturer’s instructions. Briefly, cells were seeded into 96-well plates (1000 cells per well). After the indicated time points (0 h, 24 h, 48 h, 72 h, 96 h), 10 μl of CCK-8 reagent was added to each well containing 100 μl of growth medium. After incubation at 37 °C for 3 h, the number of viable cells was determined by measuring the optical density at 450 nm on a Beckman microplate reader (Beckman, Brea, CA, USA).

For colony-forming assays, approximately 800 cells per well were added to a 6-well plate, with three wells per sample. After incubation for 14 days, the cells were washed twice with PBS and stained with Crystal Violet Staining Solution (Beyotime, Beijing, China). Colonies containing more than 50 cells were counted as 1 positive colony. The plate clone formation efficiency was calculated as (number of colonies/number of cells inoculated) × 100%. All experiments were performed in triplicate.
**Immunohistochemical (IHC) staining and in situ hybridization (ISH).** For IHC, paraffin-embedded specimens were cut into 4-μm sections and baked for 1 h at 65 °C. After deparaffinization and rehydration, endogenous peroxidase was blocked using 0.3% hydrogen-peroxide methanol for 20 min, and then heat-induced antigen retrieval was performed for 15 min in a microwave oven. Next, the sections were blocked with 5% goat serum for 30 min and then incubated overnight with anti-YY1 (dilution 1:200, Abcam, EPR4652), anti-c-PRAP (dilution 1:100, CST, ASP214), anti-CCND1 (dilution 1:100, Santa Cruz, SC-753), anti-PTEN (dilution 1:100, Bioworld Technology, BS1305), anti-pS473AKT (dilution 1:100, Bioworld Technology, BS4007), and anti-p21 (dilution 1:100, CST,12D1) primary antibodies using predetermined optimal dilutions and incubation times. Immune complexes were visualized using the MaxVison HRP-polymer IHC Kit Detection System, Peroxidase/DAB, Rabbit/Mouse (MaxVison, Fuzhou, China), according to the manufacturer’s protocol. Nuclei were counterstained with hematoxylin (Beyotime). Positive-control slides were included in every experiment in addition to the internal positive control. Antibody specificity was determined using a matched IgG isotype antibody as a negative control.

For in situ hybridization (ISH) staining, the mature *Hsa-miR-141-specific* probe and negative control (Scramble) were purchased from Sangon (Sangon Biotech, Shanghai, China). An in-situ hybridization kit (Boster, Wuhan, China) was used to perform probe hybridization according to the manufacturer’s instructions.

The results of IHC and ISH were analyzed by examination and photography under microscopy with ×400 magnification. All the slices were evaluated by two pathologists without knowledge of the clinical outcome. The percentage of immune-positive cells and the staining intensities were evaluated in each sample. The percentage of immune-positive cells was graded on a scale of 0 to 4, where no staining was scored as 0; 1–10% of cells stained was scored as 1; 11–50% was scored as 2; 51–80% was scored as 3; and 81–100% was scored as 4. The staining intensities were graded from 0 to 3, where 0 was defined as negative; 1 as weak; 2 as moderate; and 3 as strong. The score of the molecule of interest in samples was calculated as the product of intensity and percentage scores.
ranging from 0 to 12. The expression level was divided as low or high by the median total score (23,24).

5-8F tumor xenograft model. All animal studies were approved by the Institutional Animal Care and Use Committee of Central South University (Changsha, China). For xenograft experiments, a total volume of 150 µl (8 x 10⁶ cells in 0.9% saline solution with Matrigel) of 5-8F cells stably expressing YY1 or YY1 plus miR-141 mimic or negative control were injected subcutaneously into 5-week-old male BALB/C nude mice (n=5 for each group). Mice were checked every 4 days, and tumor nodules were measured with a caliper. Tumor volume was evaluated using the following formula: volume = (length x width²)/2. Tumor growth curves were calculated. The three experimental groups were killed after 38 days. All tumor grafts were excised, weighed, harvested, fixed and embedded. Anti-Ki-67 (dilution 1:100, Bioworld), anti-c-PARP, anti-PTEN, anti-p-AKT, anti-P21 and anti-CCND1 antibodies were used to detect the expression of the corresponding molecules related to cell proliferation, apoptosis or the PTEN/AKT pathway using immunohistochemistry. Samples were observed on an Olympus microscope (Olympus, Tokyo, Japan), and the results were calculated as the mean percentage of cells positive for Ki-67 or other molecules in 10 different 200x fields.

Statistical analysis. GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS 18.0 (SPSS, Chicago, IL, USA) were used to perform statistical analyses. The relationships between miR-141 and YY1 expression levels and clinical pathological characteristics in NPC were assessed using the chi-square test. Spearman’s rank correlation coefficient was used to assess the significance of the correlation between YY1 and miR-141 expression in NPC. Kaplan-Meier analysis was performed for OS curves, and statistical significance was assessed using the log-rank test. Differences between groups were analyzed using Student’s t-test for pairs of groups or using one-way ANOVA for more than two groups. A two-tailed value of P<0.05 was considered statistically significant.
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Author contributions: ML mainly performed the experiments, analyzed the data, and wrote the paper. YL, YW, WN and CW constructed the vectors. CW, HW and HM helped with cells culture and transfection. YZ, QW, YQ, ZL, XL, JZ, KC and SF helped with the experiments and prepared the samples. XL, WX, ZZ and MZ helped to approve the final version and correct the spelling mistakes. MZ and GL carried out the experiment design and manuscript drafting. All authors read and approved the final manuscript.
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**FOOTNOTES**
The abbreviations used are: NPC, Nasopharyngeal carcinoma; BRD7, Bromodomain containing protein 7; FBS, fetal bovine serum; PVDF, polyvinylidene difluoride; IP, immunoprecipitation; IHC, immunohistochemical staining; ISH, in situ hybridization
## TABLE 1: Association between the expression of YY1, miR-141 and NPC clinical pathological features (n = 126)

| Characteristics(N)       | YY1            | miR-141       | YY1/miR-141 |
|--------------------------|----------------|---------------|-------------|
|                          | H (%) | L (%) | P-value          | H (%) | L (%) | P-value          | H-L (%) | L-H (%) | P-value          |
| Age (year)               |       |       |                  |       |       |                  |         |         |                  |
| ≤40 (n= 52)              | 29    | 71    | 0.7156           | 88    | 12    | 0.8325           | 6        | 67      | 0.6587           |
| >40 (n= 74)              | 23    | 77    | 0.5013           | 84    | 16    | 0.9316           | 8        | 68      | 0.8474           |
| Gender                   |       |       |                  |       |       |                  |         |         |                  |
| Female ( n= 25 )         | 23    | 77    | 0.3481           | 82    | 18    | 0.664            | 9        | 61      | 0.5624           |
| Male ( n= 101 )          | 32    | 68    | 0.0247*          | 85    | 15    | 0.001***         | 6        | 56      | 0.0158*          |
| Histological type        |       |       |                  |       |       |                  |         |         |                  |
| DNC (n= 33)              | 18    | 82    | <                | 97    | 3     | 0.001***         | 6        | 74      | 0.0158*          |
| UDNC (n= 93)             | 28    | 72    | 0.0247*          | 77    | 23    | 0.001***         | 6        | 56      | 0.0158*          |
| Clinical stages          |       |       |                  |       |       |                  |         |         |                  |
| Stage I (n= 15)          | 60    | 40    | 0.0247*          | 67    | 33    | <                | 6        | 56      | 0.0158*          |
| Stage II (n= 52)         | 25    | 75    | 0.0247*          | 77    | 23    | <                | 6        | 56      | 0.0158*          |
| Stage III (n= 34)        | 24    | 76    | 0.0247*          | 97    | 3     | 0.001***         | 6        | 74      | 0.0158*          |
| Stage IV (n= 25)         | 8     | 92    | 0.0247*          | 92    | 8     | 0.001***         | 6        | 74      | 0.0158*          |

Abbreviations: DNC, differentiated non-keratinized nasopharyngeal carcinoma; UDNC, undifferentiated non-keratinized nasopharyngeal carcinoma; H, high expression; L, low expression
TABLE 2: The expression level of *miR-141* was negatively correlated with YY1 in NPC patients (n= 126)

| miR-141 expression | YY1 expression |  |  |  |
|-------------------|----------------|---|---|---|
|                   | High (n= 28)   | Low (n= 98)               |
| High (n= 108)     | 22             | 86            | $r = -0.2078$ |
| Low (n= 18)       | 6              | 12            | ***p < 0.001 |
Figure 1. YY1 functions as a tumor suppressor in nasopharyngeal carcinoma. (A) Western blot using antibodies against YY1 and Flag tag to confirm exogenous YY1 protein levels. GAPDH served as an internal control. (B) CCK-8 assays of 5-8F and HNE2 stably overexpressing YY1 or negative control cells. (C) Colony-forming assays (upper panel) and quantification of colony 800/well (lower panel). (D) Cell-cycle analysis by flow cytometry. (E) Flow cytometry analysis of cell apoptosis via Annexin V-PE and 7-AAD double staining. Error bars represent the mean ± SD. *P<0.05, **P<0.01, ***P<0.001; NS, no significance. All experiments were performed in triplicate.
Figure 2. Depletion of YY1 by siRNA promotes cell proliferation and inhibits apoptosis in NPC cell lines. (A) 5-8F or HNE2 cells were transfected with scramble siRNA (negative control) and YY1 siRNA pool, respectively, and western blotting was used to analyze the silence efficiency of YY1, and GAPDH served as an internal control. (B) CCK-8 assays of 5-8F and HNE2 with transfection of YY1 siRNAs or negative control. (C) Colony-forming assays and quantification of colony 800/well (lower panel). (D) Cell-cycle analysis by flow cytometry. (E) Flow cytometry analysis of cell apoptosis via Annexin V-PE and 7-AAD double staining. Error bars represent the mean ± SD. *P<0.05, **P<0.01, ***P<0.001; NS, no significance. All experiments were performed in triplicate.
Figure 3. YY1 regulates c-Myc transcriptional activity via the c-Myc/Max/Mad network. (A) Subcellular colocalization of endogenous YY1 and c-Myc detected by immunofluorescence in 5-8F and HNE2 cell lines. Images were obtained via confocal microscopy. (B) and (C) Co-IP assays and western blotting confirmed the interaction between YY1 and c-Myc in HEK293 cells. (D) and (E) Dual-luciferase reporter assays confirmed the transcriptional activity of c-Myc towards the consensus E-box sequence (D), and the effect of YY1 overexpression on c-Myc activity (E) in HNE2 and 5-8F cells. Data are normalized to Renilla activity (pRL-TK). (F) The effect of YY1 on c-Myc binding to Max or Mad was investigated by Co-IP and western blotting, and the amount of c-Myc binding to Max or Mad reflected the transcriptional activity intensity of c-Myc. Error bars represent the mean ± SD. *P<0.05, **P<0.01, ***P<0.001. All experiments were performed in triplicate.
Figure 4. YY1 negatively regulates c-Myc-mediated miR-141 transcription. (A) Dual-luciferase reporter assay shows the effect of YY1 overexpression on miR-141 promoter activity regulated by c-Myc in 5-8F and HNE2 cells. Data are normalized to Renilla activity (pRL-TK). (B) qRT-PCR analysis shows the effect of YY1 overexpression on miR-141 levels regulated by c-Myc in 5-8F and HNE2 cells. U6 served as an internal control. (C) and (D) Dual-luciferase reporter assay and q-PCR analysis shows miR-141 promoter activity and pri-miR-141, pre-miR-141 and mature...
miR-141 levels in YY1-overexpressing 5-8F (C) and HNE2 (D) cells. U6 served as an internal control. (E) Western blot of Dicer and Drosha protein levels in YY1-overexpressing 5-8F and HNE2 cells. GAPDH served as an internal control. (F) Western blot was performed to confirm the protein levels of exogenous YY1 and endogenous c-Myc by using antibodies against Flag tag and c-Myc in 5-8F and HNE2 cells. GAPDH served as an internal control. (G) Dual-luciferase reporter assay shows the effect of YY1 overexpression on miR-141 promoter activity in c-Myc-knockdown 5-8F and HNE2 cells. Data are normalized to Renilla activity (pRL-TK). (H) qRT-PCR analysis shows the effect of YY1 overexpression on miR-141 levels in c-Myc-knockdown 5-8F and HNE2 cells. U6 served as an internal control. Error bars represent the mean ± SD. *P<0.05, **P<0.01, ***P<0.001. All experiments were performed in triplicate.
Figure 5. Restoring miR-141 levels reverses the effect of YY1 on cell proliferation and colony formation in NPC cells. (A) Western blot using antibodies against Flag and the apoptosis marker c-PARP. GAPDH served as an internal control. (B) CCK-8 assays of 5-8F and HNE2 stably overexpressing YY1 or negative control. (C) Colony-forming assays and quantification of colony 800/well. Error bars represent the mean ± SD. *P<0.05, **P<0.01, ***P<0.001. All experiments were performed in triplicate.
Figure 6. Restoring miR-141 levels reverses the effect of YY1 on cell cycle and apoptosis in NPC cells. (A) Cell-cycle analysis by flow cytometry. (B) Flow cytometry analysis of cell apoptosis by Annexin V-PE and 7-AAD double staining. Error bars represent the mean ± SD. *P<0.05, **P<0.01, ***P<0.001. All experiments were performed in triplicate.
**Figure 7.** YY1 inhibits tumor growth by downregulating *miR-141* expression *in vivo*. (A) Representative images (top panel) and tumor images (bottom panel) of the 5-8F xenograft model in nude mice. (B) Tumor weight quantification (n=5). (C) Tumor growth curve (n=5). (D) Western blot using anti-Flag antibodies confirmed YY1 protein levels in xenograft tumor tissues. GAPDH served as an internal control. (E) qRT-PCR assay confirming the expression of mature *miR-141* in xenograft tumor tissues. U6 served as an internal control. (F) Representative IHC images of tumor samples that were stained with hematoxylin and eosin (H&E) and Ki67 (left). The percentages of Ki67-positive cells were measured (right). Three tumors were analyzed per group. Original magnification, 200 ×; scale bars represent 50 μm. Error bars represent the mean± SEM. *P<0.05, **P<0.01, ***P<0.001.
**Figure 8.** YY1 inhibits tumor growth by repressing miR-141/PTEN/AKT signaling. (A) Relative protein expression levels in miR-141 mimic transfected 5-8F and HNE2 stable cell lines were analyzed by western blotting. GAPDH served as an internal control. (B) IHC (DAB staining) for PTEN, p-AKT, p21, CCND1 and cleaved-PARP in the 5-8F xenograft model. Three tumors were analyzed per group. Original magnification, 200×; scale bars represent 50 μm. Error bars represent the mean± SD. Control: Vector+miR-NC, YY1: YY1+miR-NC. Error bars represent the mean± SD. *P<0.05, **P<0.01, ***P<0.001.
Figure 9. YY1 expression is negatively correlated with miR-141 and poor prognosis in NPC patients. (A) Representative images of YY1 expression in non-cancerous NP tissues and different clinical TNM stages of NPC detected by IHC. (B) Box diagram of YY1 expression in noncancerous control (n= 29) and NPC tissues (n= 126). (C) and (E) Kaplan-Meier overall survival analysis of NPC patients by expression of YY1 (C) or both of YY1 and miR-141 (E). Clinicopathological characteristics and statistical significance were assessed using the log-rank test. Kaplan-Meier curves show that worse survival rates in 64 NPC patients with low YY1 protein expression and high miR-141 expression compared with the related controls. (D) YY1 and miR-141 expression in NPC. (F) Working model showing the mechanism of YY1 in negatively regulating cell proliferation and tumor growth in NPC through the inactivation of the c-Myc/miR-141/PTEN/AKT pathway. Error bars represent the mean± SD. *P<0.05, **P<0.01, ***P<0.001.
Zinc-finger protein YY1 suppresses tumor growth of human nasopharyngeal carcinoma by inactivating c-Myc–mediated microRNA-141 transcription

Mengna Li, Yukun Liu, Yanmei Wei, Chunchun Wu, Hanbing Meng, Weihong Niu, Yao Zhou, Heran Wang, Qiuyuan Wen, Songqing Fan, Zheng Li, Xiayu Li, Jianda Zhou, Ke Cao, Wei Xiong, Zhaoyang Zeng, Xiaolong Li, Yuanzheng Qiu, Guiyuan Li and Ming Zhou

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