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Research Paper

The RUNX2 Transcription Factor Cooperates with the YES-Associated Protein, YAP65, to Promote Cell Transformation

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KEY WORDS

transformation, cell cycle, inhibitor, transcription, coactivator, RUNX2, YAP65

ABBREVIATIONS

cdk	cyclin-dependent kinase
RUNX2	runt box transcription factor-2
RBS	Runx-binding sequence
YAP65	yes-associated protein

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ABSTRACT

The Runt box domain DNA-binding transcription factors (RUNX) play key roles in hematopoietic, bone, and gastric development. These factors regulate angiogenesis and tumorigenic events, functioning as either activators or repressors of target genes. Although RUNX2 is an essential bone maturation factor, it has also been found to promote transformation in vivo and cell proliferation in vitro, perhaps by associating with specific coactivators or corepressors. Adenoviral-mediated overexpression of dominant negative RUNX2 or specific reduction of RUNX2 with RNA-interference inhibits cell proliferation. To determine whether RUNX2 also plays a role in cell transformation, RUNX2 interactions with the coactivator Yes-associated protein (YAP65) were examined. RUNX2 associated with YAP65 via a proline-rich segment in the C-terminal domain (PPPY) and coexpression of RUNX2 and YAP65 significantly increased foci formation and anchorage-independent growth relative to each factor alone. However, in contrast to wild-type RUNX2, a mutant RUNX2(P409A), which does not bind YAP65, did not cooperate with YAP65 to promote anchorage-independent growth. RUNX2 is a strong repressor of the cyclin-dependent kinase inhibitor p21^{CIP1}, which is known to mediate cell transformation. Overexpression of YAP65 prevented RUNX2-dependent downregulation of p21^{CIP1} protein expression while promoting cell transformation. The RUNX2(P409A) mutant retained the ability to bind DNA and repress the p21^{CIP1} promoter as shown by DNA precipitation and luciferase-reporter assays, respectively, but was not able to relieve repression of the p21^{CIP1} promoter. Therefore, these results reveal a novel function of the RUNX2 and YAP65 interaction in oncogenic transformation that may be mediated by modulation of p21^{CIP1} protein expression.

INTRODUCTION

The RUNX family of transcription factors is composed of three homologous genes (RUNX1, RUNX2, and RUNX3) each containing a conserved Runt DNA binding domain. The RUNX proteins are critical for regulation of mammalian developmental events related to hematopoiesis (RUNX1),¹ osteogenesis (RUNX2)² or epithelial cell maturation (RUNX3).³ Although RUNX1 has been implicated in certain human leukemias due to translocation events and mutations, and silencing of the RUNX3 promoter is a causal event in gastric carcinomas, the role of RUNX2 in tumorigenesis is less characterized. The *Runx2* gene is a common target for retroviral insertion and overexpression resulting in T-cell lymphomas.⁴ Overexpression of the RUNX2 gene has been linked to T-cell oncogenesis since RUNX2 transgenic mice developed lymphomas in combination with c-myc or Pim1, or in the absence of p53.⁴⁻⁶ Recently, Runx2 was shown to collaborate with MYC in lymphoma development by suppressing apoptotic pathways.⁷ Normal human mammary epithelial cells express low levels of endogenous RUNX2. However, elevated RUNX2 in malignant breast cancers was found to activate expression of bone sialoprotein⁸ and to mediate the formation of osteolytic lesions in bone metastases.⁹ Since bone sialoprotein has been associated clinically with skeletal metastasis and breast cancer cells preferentially metastasize to the bone,¹⁰ RUNX2 may play an important role in breast cancer tumorigenesis. RUNX2 mRNA and protein were also elevated in high-grade human melanomas,¹¹ prostate cancer specimens,¹² and in the bone metastatic PC-3 prostate cell line.¹³ Induction of RUNX2 and RUNX2-regulated gene expression in metastatic prostate tumor cells suggests that activation of osteomimetic properties may confer a survival advantage in the bone microenvironment.¹⁴

RUNX2 is a strong transcriptional activator or repressor of target genes depending on the recruitment of associating proteins. Corepressors such as mSin3a, histone

deacetylases, and the TLE1/Groucho factor, do not bind DNA, but are RUNX2-associating proteins that inhibit transactivation.¹⁵⁻¹⁷ Coactivator proteins such as p300/CBP and CBF β also do not bind to DNA, but associate with RUNX2 to mediate transactivation.¹⁸⁻²² One such coactivator, the Yes-associated protein, YAP65, is a phosphoprotein that interacts with the proto-oncogene *c-yes*, a nonreceptor tyrosine kinase of the Src family. YAP65 contains two SH3 domains, a PDZ domain, and a WW domain,²³ all of which mediate specific protein interactions. The WW domain of YAP65 consists of three anti-parallel β strands forming a hydrophobic pocket that binds proline containing motifs such as the peptide ligand motif PPxY.^{24,25} It has been determined that the WW domain of YAP65 binds the sequence PPPY in RUNX1²⁶ and RUNX2,²⁷ and may interact with RUNX3 because of identical PPPY motifs in these RUNX family members. Upon phosphorylation, YAP65 is sequestered in the cytoplasm by 14-3-3. However, when YAP65 is unphosphorylated, it is localized to the nucleus where it can interact with transcription factors. YAP65 is known to be a strong coactivator of the TEAD/TEF family of transcription factors²⁸ and p73.^{29,30} However, the functional significance of the RUNX-YAP65 interaction has not been elucidated.

We showed previously that ectopic expression of RUNX2 prevents TGF β -mediated inhibition of cell growth.³¹ RUNX2 DNA-binding activity, cell cycle progression, pRb phosphorylation, and DNA synthesis correlated with cell proliferation. RUNX2 also repressed p21^{CIP1} promoter activity and reduced p21^{CIP1} protein levels. RUNX2 modulation of p21^{CIP1} expression and promotion of EC proliferation suggested that RUNX2 might promote cellular transformation. Recently, we reported that shRNA-mediated downregulation of RUNX2 inhibited cell proliferation and that RUNX2 overexpression increased 3T3 fibroblast growth in soft agar, a transforming activity mediated, in part, through cdk1-dependent phosphorylation of RUNX2.³² Further, adenoviral-mediated overexpression of a dominant negative factor, which inhibits endogenous RUNX2, inhibited EC proliferation. We now identify the transcriptional coactivator, YAP65, as a synergistic factor that cooperates with RUNX2 to increase cell transformation. RUNX2 promoted transcriptional repression of the cdk inhibitor p21^{CIP1}, while YAP65 alleviated this repression at the promoter level through a direct interaction with RUNX2. Further, we show that, although RUNX2 reduced p21^{CIP1} protein levels, the addition of YAP65 prevented this downregulation. Taken together these data suggest that RUNX2 and YAP65 increase oncogenic transformation by direct protein:protein interaction and reveal, for the first time, that p21^{CIP1} regulation is one possible mechanism for this transforming activity.

MATERIALS AND METHODS

Cell culture and reagents. Human bone marrow endothelial cells (HBME), HEK293, 293T, and NIH3T3 fibroblasts were cultured in DMEM (Biofluids) and 10% FBS (Biofluids) and used until passage 20. Stable NIH3T3 cell lines were selected in 1 mg/ml G418 (Invitrogen) and only used for three more passages after selection. HCT116, p21^{-/-} and parental cells were cultured in McCoy's medium containing 10% FBS. Monoclonal anti-flag M2 antibody (Sigma) was used to detect or immunoprecipitate the flag-tagged RUNX2 or RUNX2(P409A) mutant proteins. Monoclonal HA.11 antibody (Covance) was used to detect or immunoprecipitate the HA-tagged YAP65. Anti-p21^{CIP1}, anti-cdk4, anti-PCNA and anti-actin antibodies were obtained from Santa Cruz, Inc. (Santa Cruz, CA). Anti-RUNX2 antibody (AML3 Ab) was from Oncogene Research Products (Cambridge, MA).

Plasmids. pCMV-tag2a (NEO) was purchased from Stratagene. The full length RUNX2 cDNA was inserted into the BamH1/Xho1 sites of the pCMV-tag2a as previously described.³¹ Flag-tag RUNX2 or flag-tag RUNX2DN (dominant negative; missing exon8) was subcloned into the pShuttle vector (Clontech) before restriction enzyme cloning into the Adeno-X adenoviral vector (BD Biosciences, Palo Alto, CA). The dominant negative activity of RUNX2DN was previously quantified using p21^{CIP1} promoter-luciferase assays.³¹ Adenoviruses were prepared in HEK293 packaging cells by sequential transfection/infection and viral titers of HEK293 supernatants were determined by cell lysis as recommended by the manufacturer (BD Biosciences). The YAP65 expression vector and the empty vector control (XJ540-HA) were gifts from Dr. Iain Farrance (University of Maryland, Baltimore, MD). The p21^{CIP1} promoter luciferase plasmid (WWP-LUC) was a gift from Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). Using a site-directed mutagenesis kit (Invitrogen), a point mutation was introduced into the RUNX2 cDNA, which changed the proline at position 409 to an alanine, to create the RUNX2(P409A) mutant. The mutation was verified by sequencing.

Immunoprecipitation and western blot analysis. Nuclear proteins were isolated using NucBuster (Novagen). Protein concentration was determined with the Bio-Rad Protein Assay. One mg of protein was diluted into 500 μ l of immunoprecipitation (IP) buffer [20 mM Tris, pH 7.5, 2 mM CaCl₂, 1% Triton X-100 and 1X protease inhibitor cocktail (Roche)] and was precleared with 30 μ l of protein G sepharose. For immunoprecipitations, 1 μ g of antibody (M2 or HA) pre-bound to 30 μ l of Protein G sepharose was combined with Protein G precleared nuclear extracts and incubated on an orbital shaker for at least 12 hr at 4°C. The mixture was centrifuged, and the pellet was washed three times with the IP buffer. All excess fluid was removed and 2.5 μ l reducing agent (Invitrogen) and 22.5 μ l of 4x Laemmli buffer were added to the pellet. Samples were boiled for 10 min and centrifuged. The supernatant was loaded on a 4–12% Nu-PAGE gel (Invitrogen), and electrotransferred to PVDF membranes (Invitrogen). The blots were incubated with either anti-M2 antibody (1:1000) or anti-HA antibody (1:5000) followed by horseradish peroxidase-conjugated goat anti-mouse IgG (KPL, Gaithersburg, MD). All other antibodies were used at concentrations recommended by the manufacturer (Santa Cruz, Inc., Santa Cruz, CA). Specific proteins were detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Buckinghamshire, England).

DNA precipitation assays. Two single stranded, biotin labeled oligonucleotides corresponding to RUNX binding site A in the distal p21^{CIP1} promoter were hybridized to generate a double-stranded probe. For the wild-type probe (Runx2 binding site in bold), the specific oligonucleotides used were 5'GCTCAGTAC CACAAAAATTC-biotin 3' (sense) and 5'GAATTTTTGTGGTA CTGAGC-biotin 3' (antisense). For the mutant probe, the specific oligonucleotides used were 5'GCTCAGTCGAACAAAAATTC-biotin 3' (sense) and 5'GAATTTTTGTTCGACTGAGC-biotin 3' (antisense). Equal concentrations of sense oligo and antisense oligo were added in annealing buffer for a final concentration of 3.33 μ M of double-stranded oligo in 0.1 M Tris, pH 7.6, 0.01 M MgCl₂, 0.0034 M DTT. The mixture was heated to 95°C for 10 min, allowed to cool slowly to 65°C, then allowed to cool to room temperature. One mg of nuclear protein was diluted into 500 μ l of DNAP buffer (10 mM Hepes, pH 7.0, 100 mM KCl, 5 mM MgCl₂, 10% glycerol, 1 mM DTT, 0.5% NP40, and 1X protease inhibitor cocktail), and samples were precleared with 30 μ l streptavidin-agarose

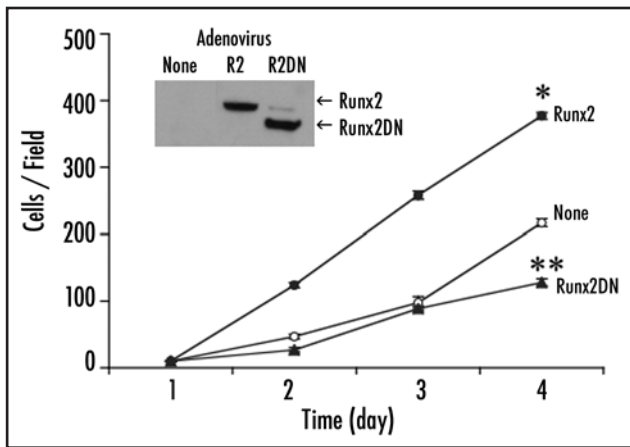


Figure 1. RUNX2 regulates cell proliferation. Human bone marrow EC (HBME) were infected with adenoviral vectors expressing RUNX2 or dominant negative RUNX2 (RUNX2DN). After infection (days 1–4), cells were photographed and cell number (cells/field) was quantified. Nuclear proteins from HBME cells were prepared after viral infection and the expression of RUNX2 or RUNX2DN on day two was confirmed by Western blot using anti-Flag RUNX2 antibody (inset). Results are mean \pm SEM of triplicate samples and are representative of three separate experiments. *At four days, $p \leq 0.006$ for RUNX2 relative to control; ** $p \leq 0.003$ for RUNX2DN versus control.

beads (Pierce). The biotinylated, double stranded DNA probe (10 μ l of a 3.33 μ M stock) and 10 μ g of poly dI/dC were added to the supernatant and incubated at 4°C overnight. To the mixture, 30 μ l of the streptavidin beads were added and the incubation continued at 4°C for at least 1 hr. The supernatants were then removed and the beads were washed three times with 0.5 ml of the DNA precipitation buffer. Laemmli buffer plus reducing agent were added to the beads and the mixture was boiled for 10 min. After centrifugation at 14,000 rpm for 2 min, the supernatant was loaded on a 4–12% NuPage gel to resolve proteins bound to the DNA.

Luciferase assays. Nontransformed, early passage NIH3T3 cells were plated in 6-well plates at a density of 10^5 cells per well. Cells were allowed to recover for 24 hr and then transfected with the indicated combination of plasmids. For all luciferase assays, the WWP-LUC plasmid was used at a concentration of 1 μ g per well and the pTK-renilla was used at a concentration of 50 ng per well. Cells were incubated at 37°C in a 5% CO₂ incubator for 48 hr. The cells were lysed with 1X passive lysis buffer (Promega). Lysates were analyzed using the Dual Luciferase Kit (Promega) and a Turner Design TD 20/20 dual-wavelength luminometer.

Cell proliferation, soft agar assays and foci formation. HBME or 3T3 fibroblasts were transfected with the Mirus LT1 transfection reagent (Mirus Corporation) as described previously.³¹ HBME cells were infected with dilutions of HEK293 viral supernatants corresponding to equivalent viral titers for 24 hours and harvested and replated in 24-well plates for proliferation assays. Cells were photographed after 1, 2, 3 and 4 days and cell numbers in each representative field were counted. For growth in soft agar, DMEM, 10% FBS, and agar (0.5%) mixture (2 ml) were added and allowed to cool and solidify at 25°C for at least 30 min. Cells (20,000) in 0.5 ml DMEM, 10% FBS, and agar (0.33%) were carefully overlaid on the solidified agar base in each well. This mixture was allowed to solidify at 25°C for 30 min. The plates were then incubated for ten days in a 37°C, 5% CO₂. Colony formation was compared and photos of representative regions from each well were taken using

a Zeiss microscope, and video camera, and images were processed with Oncor Image software. Each photo contains multiple computer images fitted together to give a larger representative view of the colonies in each well. For measurement of foci formation, NIH3T3 cells and transfectants were cultured in 100 mm dishes and allowed to reach confluence. Cells growing above the fibroblast monolayer were photographed 25 days after culturing.

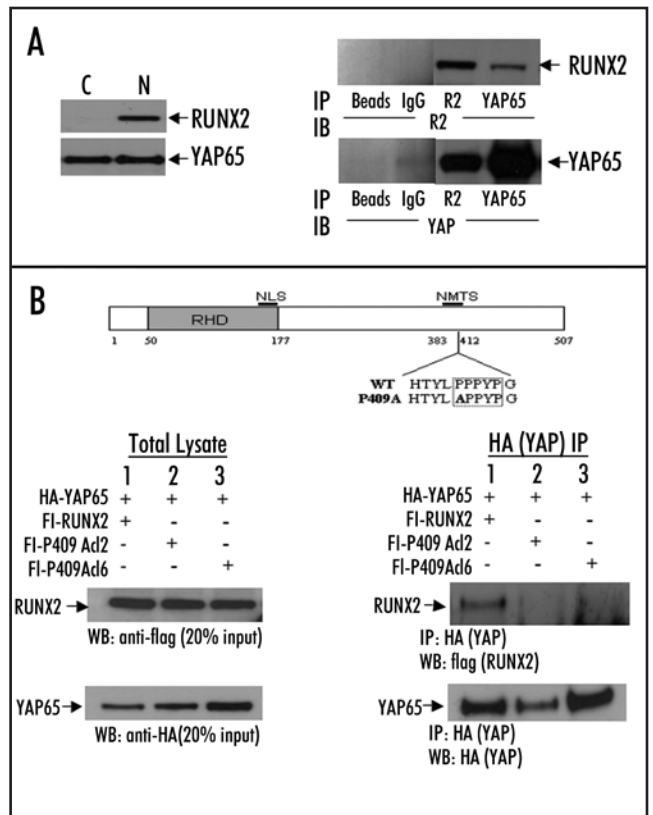
Statistical analysis. Results were analyzed using Microsoft Excel spreadsheets and are expressed as mean values \pm SD from at least three determinations. Significance was calculated using paired analysis (Excel Office Suite) and expressed as p values.

RESULTS

RUNX2 promotes cell proliferation. We showed previously that RUNX2 protein expression and DNA-binding activity correlated with EC proliferation.^{31,33} Human bone marrow EC (HBME) expressed RUNX2 when subconfluent (proliferating), but not at confluence (growth arrested). We further showed that overexpression of RUNX2 in bovine aortic EC (BAEC), stimulated cell proliferation, DNA synthesis and pRb phosphorylation,³¹ while a dominant negative (DN) RUNX2 variant (with a deletion of exon8) inhibited cell proliferation. To further define the role of RUNX2 in EC proliferation, HBME cells were infected with adenoviral vectors encoding DN or wild-type RUNX2 for 1–4 days. RUNX2 expression was confirmed on day 2 after infection (Fig. 1). Uninfected cells were used as controls during the 4-day period. RUNX2 increased HBME proliferation relative to controls by 2.8-fold (day 1), 2.5-fold (day 2) and 1.8-fold (day 4), while proliferation in cells expressing DN RUNX2 was inhibited by 50% on day 4 (Fig. 1). In similar experiments, HBME cells were also infected with adenoviral vector encoding a control green fluorescent protein (GFP). The growth rates of control (GFP) infected cells were essentially the same as the growth rates of uninfected cells (data not shown). Since HBME express RUNX2, inhibition of proliferation by DN RUNX2 suggests that endogenous RUNX2 promotes EC proliferation. These data are consistent with the observation that targeted knockdown of RUNX2 by specific RNA interference inhibited EC proliferation and cell cycle progression.³²

Specific association of RUNX2 and YAP65. RUNX2 oncogenic activity depends on the interaction of cooperating oncogenes.⁵ It has been reported that RUNX2 interacts with YAP65, a c-yes associating coactivator and putative proto-oncogene. Yeast two-hybrid screening showed that the WW domain of YAP65 binds to the PPxY motif (PPYP) of RUNX1²⁶ and RUNX2.²⁷ To verify a direct interaction between RUNX2 and YAP65, Flag.RUNX2 and HA.YAP65 were expressed in 293T cells. Lysates were separated into cytoplasmic (C) and nuclear (N) fractions (Fig. 2A). Ectopic RUNX2 was localized to the nucleus, while YAP65 was observed in both cytoplasmic and nuclear fractions. Immunoprecipitation (IP) assays were performed with nuclear lysates using either a Flag.Tag or HA.Tag antibody and the proteins were detected by immunoblotting (Fig. 2A). The IP with Flag.Tag antibody or the reciprocal IP with HA.Tag antibody identified Flag.RUNX2 and YAP65 in the complexes. Negative controls (beads alone or nonspecific IgG coupled to beads) did not reveal either RUNX2 or YAP65 in the immunoprecipitated fractions. The immunodepleted lysates were analyzed for possible unbound Flag.RUNX2 or HA.YAP65 by Western blotting. Neither protein could be detected in the immunodepleted nuclear lysates (data not shown). If YAP65 was directly interacting with RUNX2 via the PPxY motif, a mutation of the first proline to an alanine in RUNX2 would

Figure 2. RUNX2 and mutant RUNX2(P409A) association with YAP65. (A) 293T cells were cotransfected with Flag-Tag RUNX2 and HA-Tag YAP65. Western blot analysis was performed on the cytoplasmic, C; and nuclear lysates, N. RUNX2 localizes to the nucleus, while YAP65 is detected in the cytoplasmic and nuclear extracts (left panel). Nuclear lysate from the cotransfected 293T cells was either immunoprecipitated with the Flag-specific antibody (RUNX2) or the HA-specific antibody (YAP65) coupled to protein A-Sepharose beads. Beads alone or beads coupled to isotype-matched IgG were used as negative controls. Western blot analysis was performed with the immunoprecipitated proteins (right panel). (B) Schematic of wild-type RUNX2 and mutant RUNX2(P409A) proteins (RHD, Runt Homology Domain; NLS, Nuclear Localization Sequence; NMTS, Nuclear Matrix Targeting Sequence). 293T cells were cotransfected with flag-tagged RUNX2 (lane 1), RUNX2(P409A), clone 2 (lane 2), or RUNX2(P409A), clone 6 (lane 3) and HA-tagged YAP65. Input proteins are shown on the left panel. Nuclear lysates were immunoprecipitated with the HA-tag antibody coupled to protein A-Sepharose beads (right panel). Western blot analysis was performed with either Flag.RUNX2 or HA.YAP65-specific antibodies.



reduce YAP65 binding. Therefore, the ability of the RUNX2(P409A) mutant to interact with YAP65 was assessed (Fig. 2B). Co-IP assays with the Flag.RUNX2 or Flag.RUNX2(P409A) and HA.YAP65 showed that YAP65 could associate with wild-type RUNX2 but not with either of two mutant RUNX2 clones (Fig. 2B).

RUNX2 and YAP65 synergistically increase oncogenic transformation. We showed recently that RUNX2 promotes cell growth in soft agar.³² To define the biological significance of the RUNX2/YAP65 interaction, NIH3T3 cells were transfected with RUNX2 or RUNX2 and YAP65 and the appropriate control vectors. Expression of RUNX2 or YAP65 was verified with antibodies specific for the Flag or HA-tag (Fig. 3A). Examination of post-confluent cultures of transfected NIH3T3 cells revealed that RUNX2 or RUNX2 + YAP65-transfected cells grew on top of the confluent cell monolayers forming cell foci, indicative of transformation (Fig. 3B). Freshly transfected cells were prepared and suspended in soft agar to measure anchorage-independent growth, another indicator of transformation (Fig. 3C).

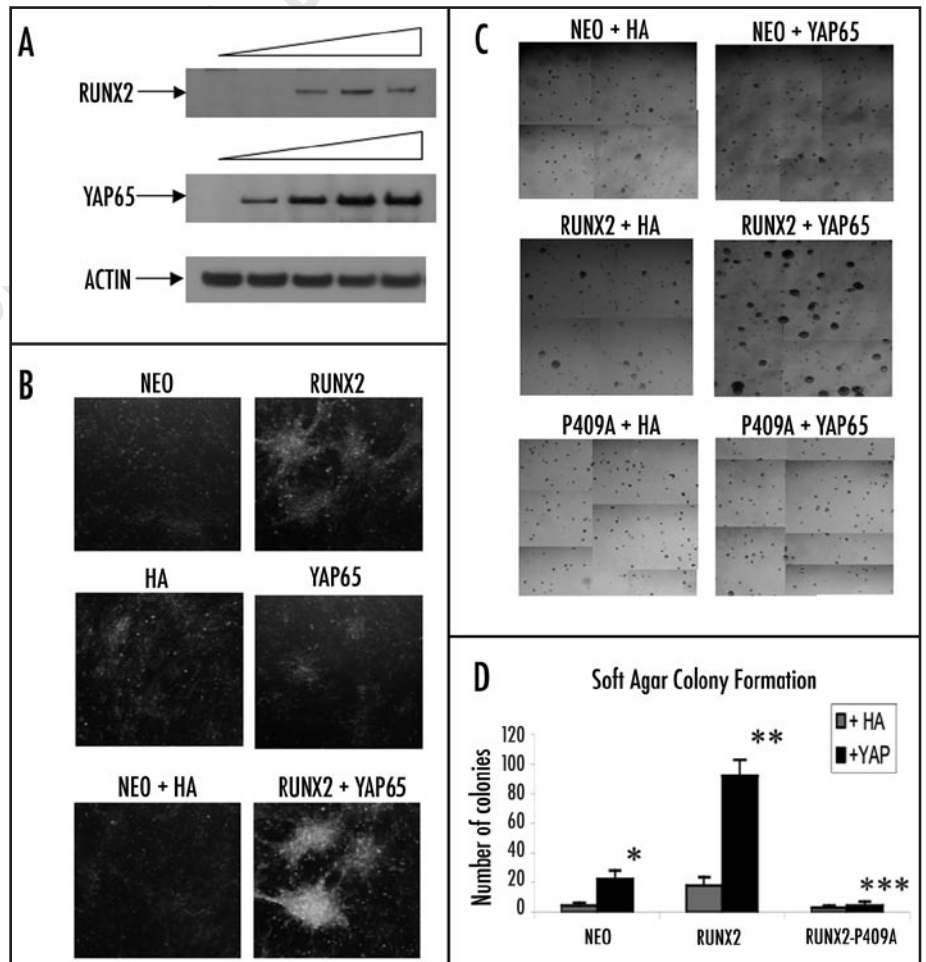


Figure 3. RUNX2 and YAP65 cooperate to promote NIH3T3 cell transformation. (A) Western blot of NIH3T3 cells transfected with an increasing amount of RUNX2 or YAP65 (0 μg, 1 μg, 2.5 μg, 5 μg, 10 μg). (B) NIH3T3 cells were transfected with control vectors (NEO and/or HA), vectors expressing RUNX2 or YAP65, or a combination of RUNX2 and YAP65 (2.5 μg each). Foci growing above the fibroblast monolayers were photographed after 25 days. RUNX2 or RUNX2 and YAP65 transfected cells continued to grow and form foci atop the monolayers. (C) Parental NIH3T3 cells transfected with control vectors (NEO + HA) or vectors expressing RUNX2, RUNX2 and YAP65, or the mutant RUNX2(P409A) and YAP65 were cultured in soft agar and photographed after 14 days. (D) The number of colonies from the NIH3T3 cells growing in soft agar was counted. Colony number for each treatment was the average of quadruplicate wells and included five fields per well (n = 20). *p ≤ 0.004 for YAP65 + NEO vs. HA control; **p ≤ 0.0007 for RUNX2 + YAP65 vs. HA control; ***p ≤ 0.002 for RUNX2(P409A) + YAP65 vs. NEO + YAP65.

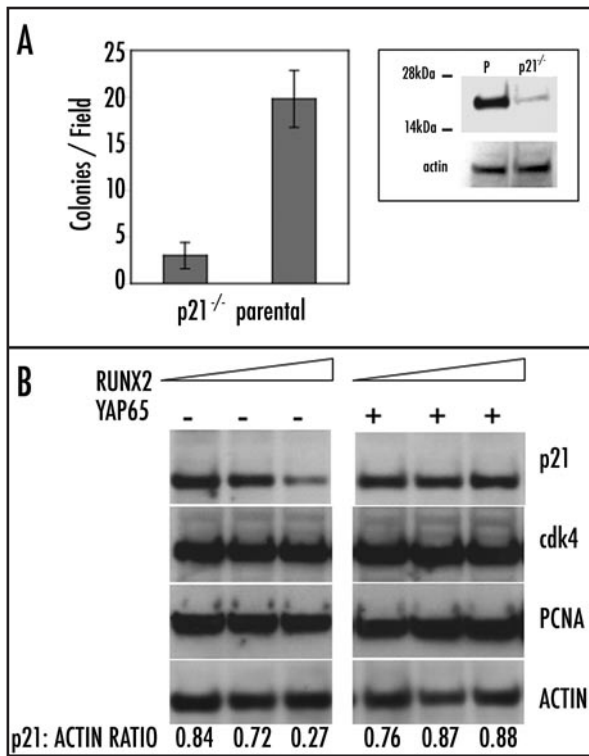


Figure 4. YAP65-mediated regulation of p21^{CIP1} cdk inhibitor expression. (A) HCT116 (p21^{-/-}) or parental cells were harvested and cultured in soft agar as described in Figure 3. Representative fields from triplicate wells were photographed and the number of colonies per well was calculated. Inset shows Western blot confirming reduction of p21^{CIP1} protein in HCT116 (p21^{-/-}) cells. (B) Expression of p21^{CIP1}, cdk4, PCNA and actin was measured by Western blot with specific antibodies in cells infected with increasing doses of RUNX2-expressing adenovirus as described in the Experimental Procedures. Cells had been previously transfected with control plasmid or YAP65 (1 μg/well). Western blots were scanned and density of bands was measured with Adobe Photoshop 7.0 to calculate the p21^{CIP1}: actin ratios.

After two weeks in soft agar, the RUNX2 transfected cells formed five times more colonies than the NEO (vector alone) controls (Fig. 3D). Moreover, the colonies formed by the combination of RUNX2 and YAP65 were two to five-fold larger than those of RUNX2 expressing cells. The number of colonies formed in the presence of RUNX2+YAP65 was greater than the number of colonies expected from expression of RUNX2 or YAP65 separately, indicative of a synergistic increase in oncogenic transformation. Previous mutational analysis of the YAP65 binding site of RUNX1 showed that the first two Pro and the Tyr residues in the YAP65 binding domain (PPYP) were necessary for transcriptional activation of a tk-promoter containing a GAL4 binding site.²⁶ Mutation of the first proline to an alanine completely abolished transcriptional activity. To determine whether the increased cellular transformation was mediated by the YAP65 binding site on RUNX2, cells transfected with a RUNX2(P409A) vector in which the YAP65 binding site had been mutated (Fig. 2B) were used in the soft agar assay (Fig. 3C). The RUNX2(P409A) mutant was incapable of increasing cellular transformation in the absence of YAP65 and even inhibited growth in soft agar below the levels of the NEO control in the presence of YAP65 (Fig. 3C and D). These results suggest that a direct interaction between RUNX2 and YAP65 is necessary to induce loss of contact inhibition and growth in soft agar, established indicators of cellular transformation.

Requirement for p21^{CIP1} protein expression in anchorage independent tumor growth. It was shown that p21^{CIP1} expression is permissive for cellular transformation.³⁴ We found previously that RUNX2 could inhibit expression of p21^{CIP1} protein in HBME cells in response to the DNA-damaging agent (and p53 activator) doxorubicin.³¹ To determine whether p21^{CIP1} expression was involved in the ability of RUNX2 and YAP65 to increase cell transformation, we first determined whether p21^{CIP1} expression could mediate anchorage-independent growth in an HCT116 tumor cell line from which the p21^{CIP1} gene had been deleted.³⁵ Parental and p21^{CIP1}-mutant HCT116 cells were cultured in suspension in soft agar. After two weeks, p21^{CIP1}-negative cells (p21^{-/-}) formed few colonies in soft agar (Fig. 4A). However, parental HCT116 cells, which express p21^{CIP1} protein, formed 6-fold more colonies in soft agar, indicating that p21^{CIP1} is essential for anchorage-independent growth. HCT116 p21^{CIP1}-mutant and parental cells exhibited identical growth rates in culture (data not shown), as reported previously.³⁵ Therefore, the difference in growth in soft agar may be due to the ability of p21^{CIP1} to promote anchorage-independent growth and/or cell survival, which are well-known properties of transformed cells.

To determine whether regulation of p21^{CIP1} protein expression by RUNX2 and YAP65 was also permissive for oncogenic transformation, control HBME cells or HBME cells overexpressing YAP65 were infected with increasing amounts of adenovirus encoding RUNX2. Confluent cells were harvested and nuclear fractions were analyzed for p21^{CIP1} protein by Western blotting (Fig. 4B). As expected, RUNX2 inhibited p21^{CIP1} protein expression in a dose-dependent manner. However, RUNX2 did not repress p21^{CIP1} protein in YAP65 overexpressing cells, consistent with the enhanced ability of YAP65 to promote cell transformation (Fig. 3). From quantitative densitometry, the p21^{CIP1}/Actin ratios declined with increasing RUNX2 expression, but were unchanged in the presence of YAP65. The Western blots were stripped and reprobed for cell cycle and proliferation-regulatory proteins (Fig. 4B). The levels of the cell cycle kinase cdk4 or proliferating cell nuclear antigen, PCNA, were not altered by RUNX2 or YAP65 overexpression.

Repression of the p21^{CIP1} promoter in response to RUNX2 and YAP65. To determine whether YAP65 prevents RUNX2 repression of p21^{CIP1} expression at the transcriptional level by inhibiting repression of the p21^{CIP1} promoter, we first determined whether the RUNX2(P409A) mutant that does not interact with YAP65 would retain its ability to bind DNA. A 20 nucleotide synthetic oligonucleotide was created containing the consensus RUNX binding sequence (RBS) and the flanking sequences from the distal site A in the p21^{CIP1} promoter (Fig. 5A, a).^{15,16,31} DNA precipitation assays showed that RUNX2 was able to bind the wild type p21^{CIP1} oligonucleotide but not the mutant oligonucleotide in which the RBS had been altered (Fig. 5A, b). We expected that the RUNX2(P409A) mutant would retain its ability to bind DNA since the P409A mutation was downstream of the Runt DNA binding domain (amino acids 50–177). To measure RUNX2(P409A) DNA binding, nuclear lysates from 293T cells transfected with either of two mutant Flag.RUNX2(P409A) clones were isolated. Both RUNX2(P409A) mutant clones retained DNA-binding activity (Fig. 5A, c).

Ectopic expression of RUNX2 is known to repress p21^{CIP1} promoter activity in nontransformed NIH3T3 cells.³¹ Consistent with published results,¹⁶ Flag.RUNX2 repressed a p21^{CIP1} promoter-luciferase construct in NIH3T3 cells (Fig. 5B). NIH3T3 cells were also transfected with two different clones of the mutant RUNX2(P409A) and luciferase activity was measured. Both mutant RUNX2 clones repressed p21^{CIP1} promoter activity to the

same extent as wild type RUNX2 (Fig. 5B). Since YAP65 associates with RUNX2, we wanted to assess whether YAP65 could regulate repression of the p21^{CIP1} promoter by RUNX2. At a constant input (0.25 μ g) of NEO control or Flag-RUNX2 vector, increasing amounts of HA.YAP65 were added to each sample (Fig. 5C). HA.YAP65 in the presence of NEO control had no significant effect on p21^{CIP1} promoter activity (Fig. 5C, gray bars). Increasing concentrations of HA.YAP65 alleviated the RUNX2 repression of p21^{CIP1} promoter activity in a dose dependent manner, resulting in complete relief of p21^{CIP1} promoter repression at 1.0 μ g DNA (Fig. 5C, dark bars), consistent with the observed expression of p21^{CIP1} protein (Fig. 4B). However, HA.YAP65 was unable to relieve p21^{CIP1} promoter repression by the mutant Flag-RUNX2(P409A) (Fig. 5C, white bars). A YAP65 control vector had no effect on RUNX2 repression and did not alleviate repression of the p21^{CIP1} promoter by RUNX2 or RUNX2(P409A) (data not shown). Since YAP65 relieved the RUNX2-mediated, but not RUNX2(P409A)-mediated repression of the p21^{CIP1} promoter, these data suggest that direct YAP65 binding to RUNX2 is necessary to relieve RUNX2 repression of the p21^{CIP1} promoter.

DISCUSSION

Tumor progression is characterized by dysregulation of normal cellular growth controls. Our studies were designed to determine the biological consequences of the interaction of RUNX2 with the transcriptional coactivator YAP65 in the context of anchorage-independent growth and cellular transformation. YAP65 interacts with RUNX2 through the PPPY domain of RUNX2. We show for the first time that one physiological consequence of the RUNX2-YAP65 interaction is to increase cell transformation since NIH3T3 cells overexpressing RUNX2 and YAP65 exhibited a synergistic increase in growth in soft agar. Although RUNX2 is a strong transcriptional repressor of the p21^{CIP1} promoter, the presence of YAP65 relieved this repression at both the promoter and protein levels. Further, the ability of YAP65 to relieve p21^{CIP1} repression depended on a direct RUNX2-YAP65 interaction since mutation of the PPPY YAP65-binding site on RUNX2 resulted in failure of YAP65 to relieve

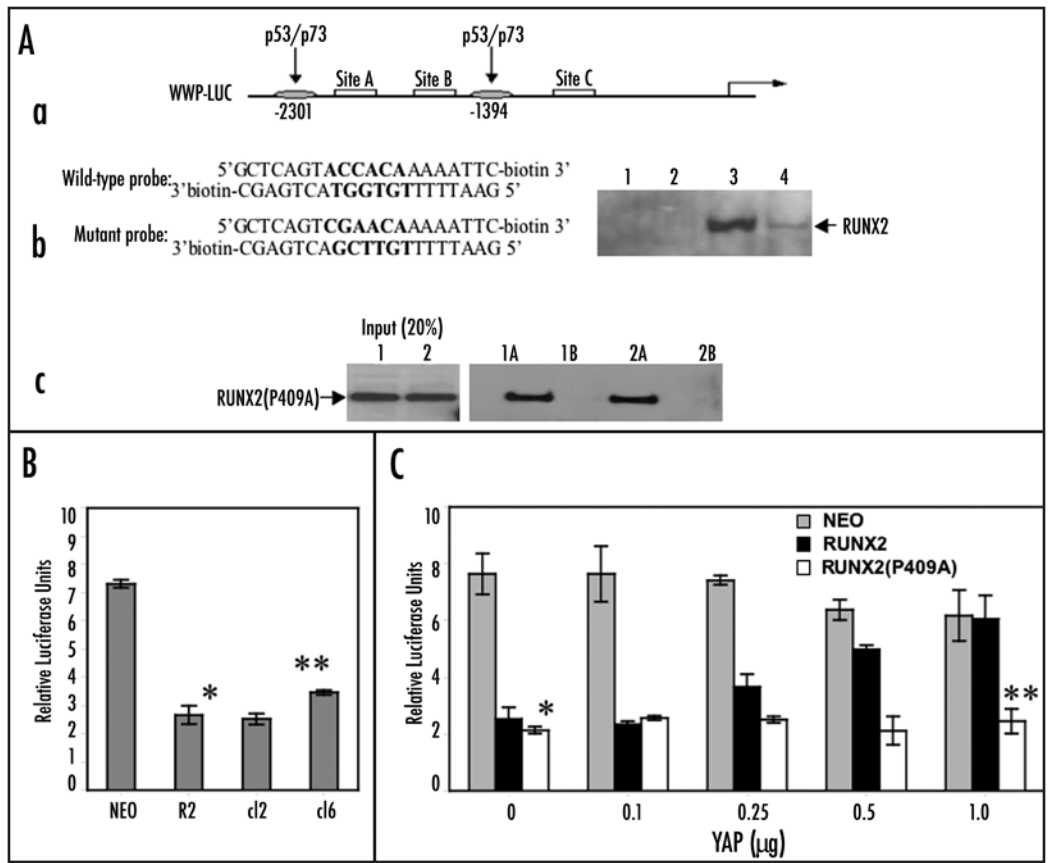


Figure 5. Modulation of RUNX2-mediated repression of the p21^{CIP1} promoter by YAP65. (A) Location of RUNX2 binding sites in the p21^{CIP1} promoter (sites A–C) in relation to the p53/p73 binding sites (a). The sequences of the wild type and mutant p21^{CIP1} double-stranded, biotinylated DNA probes are shown (b). DNA precipitation and Western blot analyses (b, lanes 1,2 no nuclear lysates) show the ability of RUNX2 to bind the double stranded oligonucleotide containing the RUNX consensus binding site (b, lane 3). However, RUNX2 is not able to bind the mutant binding sequence (b, lane 4). (c) 293T cells were transfected with Flag-Tag RUNX2(P409A), clone 2 (lane 1) or RUNX2(P409A), clone 6 (lane 2). Nuclear lysates from the transfectants were incubated with DNA oligos containing a RUNX2 binding site and streptavidin beads were used to precipitate the bound complexes. Western blot analysis was performed with the DNA-precipitated proteins. Both mutant RUNX2 clones maintained the ability to bind the p21^{CIP1} oligonucleotide (lanes 1A, 2A) with no detectable mutant RUNX2 remaining in the supernatant after precipitation (lanes 1B, 2B). (B) NIH3T3 cells were transfected with pTK-renilla (0.05 μ g), WWP-LUC (1.0 μ g), and 1.0 μ g of control NEO vector (NEO), RUNX2 (R2), or two different clones of RUNX2(P409A), clone 2 (cl2) or clone 6 (cl6). Both RUNX2(P409A) clones repressed p21^{CIP1}-promoter luciferase activity. *p < 0.002 for P409A_cl2 vs NEO or for RUNX2 vs NEO; ** p < 0.003 for P409A_cl6 vs NEO. (C) NIH3T3 cells were transfected with pTK-renilla, WWP-LUC, a constant amount of RUNX2, NEO control, or P409A clone 2 DNA (0.25 μ g), and increasing amounts of YAP65 (0 μ g, 0.1 μ g, 0.25 μ g, 0.5 μ g, 1.0 μ g). YAP65 alleviated the RUNX2 repression of the p21^{CIP1} promoter. Results represent a combination of data from three separate experiments performed in duplicate or triplicate (n = 8). *p < 0.004 for RUNX2 vs. NEO control at 0 μ g YAP65. YAP65 was unable to relieve the RUNX2(P409A) repression of the p21^{CIP1} promoter. **p < 0.004 for RUNX2(P409A) vs NEO control at 1.0 μ g YAP65. Results represent a combination of data from three separate experiments performed in triplicate (n = 9).

RUNX2 repression of the p21^{CIP1} promoter. These data suggest that the RUNX2 DNA-binding factor may regulate repression or activation of specific growth-modulating genes and promote oncogenesis in the presence of an appropriate transcriptional coactivator.

GST pull-down assays have confirmed that the WW domain of RUNX1 interacts directly with YAP65.²⁶ Since all members of the RUNX family contain a perfectly conserved 10 amino acids containing the PY motif, RUNX2 was believed to directly interact with YAP65. In addition, the TAZ coactivator, which is highly similar to YAP65, interacts with RUNX2.³⁶ Immunoprecipitation assays verified the interaction between RUNX2 and YAP65 (Fig. 2).

Published data have shown that Ala substitutions of the first, second, or fourth amino acid (PPPY) in the PY motif abolish binding to the WW domain of YAP65.^{26,37} Mutation of the first proline residue to alanine (P409A) resulted in the inability of YAP65 to associate with the mutated RUNX2 as shown by immunoprecipitation assays (Fig. 2) and failure to stimulate growth in soft agar (Fig. 3). As expected, RUNX2(P409A) retained the ability to bind DNA and, similar to wild-type RUNX2, RUNX2(P409A) also repressed the p21^{CIP1} promoter as shown using transactivation assays (Fig. 5). Therefore, the point mutation in RUNX2(P409A) did not affect RUNX2 DNA binding or transcriptional repression activity. However, YAP65 could not bind RUNX2(P409A), and YAP65 was not able to relieve the repression mediated by the RUNX2(P409A) mutant indicating that direct binding of YAP65 to RUNX2 is necessary to relieve repression of the p21^{CIP1} promoter.

RUNX2 contains multiple lysine residues that are the targets of protein acetylation and ubiquitination.³⁸ The E3 ligases, smad ubiquitination-related factors (Smurfs) interact with the PPxY motif of RUNX2 through their WW domains leading to RUNX2 ubiquitination and degradation by the proteasome pathway.^{39,40} Since YAP65 interacts with the same PPxY motif on RUNX2, YAP65 binding could block ubiquitination and stabilize RUNX2 levels, thus increasing cell transformation. Further studies to address whether any of these mechanisms are operative in RUNX2-mediated cell transformation are being pursued. Experiments in rat osteosarcoma (ROS 17/2.8) cells showed that YAP65 was able to suppress the ability of RUNX2 to activate the osteocalcin gene promoter, but had no effect on p21^{CIP1} promoter activity.³⁷ Since we have found that YAP65 inhibits the repression of the p21^{CIP1} promoter in NIH3T3 cells, it is possible that p21^{CIP1} promoter regulation by RUNX2 and YAP65 is cell-dependent. However, in both cases YAP65 inhibits RUNX2 transcriptional activity of either the osteocalcin or p21^{CIP1} promoter.

We, and others have shown that RUNX2 represses the p21^{CIP1} promoter^{16,31} and reduces expression of p21^{CIP1} protein.^{16,31} A segment of DNA containing the consensus sequence for the distal RUNX binding site plus the flanking sequence in the p21^{CIP1} promoter (site A) was used in DNA precipitation assays (Fig. 5). The role of the other two RUNX binding sites in the p21^{CIP1} promoter (sites B and C) is not known. However, deletion of site A completely abrogated the ability of RUNX1 to repress the p21^{CIP1} promoter.¹⁵ We have found that the expression of YAP65 relieved the RUNX2 repression of the p21^{CIP1} promoter in a dose dependent manner. Although HA.YAP65 completely relieved the RUNX2 repression of the p21^{CIP1} promoter, no increased activation, beyond initial basal levels, was observed with higher doses of HA.YAP65. The 2.4 kb segment of p21^{CIP1} promoter contains a p73 site (Fig. 5A). Therefore, we considered the possibility that YAP65 could interact with endogenous p73 to transactivate p21^{CIP1} since the WW domain of YAP65 can also bind the PY motif in p73 and enhance p73 transcriptional activity.²⁹ However, increasing concentrations of YAP65 in the absence of RUNX2 had no effect on p21^{CIP1} promoter activity (Fig. 5C). Therefore, it is unlikely that an interaction between YAP65 and p73 is responsible for increased transcriptional activity in the presence of YAP65.

It has been suggested that p21^{CIP1} acts as a cdk inhibitor to restrain cell cycle progression, but it may also function as an assembly factor for cdk/cyclin complexes to promote cell cycle progression.⁴¹⁻⁴³ Further, p21^{CIP1} has been shown in a variety of studies to promote cell transformation,⁴⁴⁻⁴⁶ including the transformation observed in mouse fibroblasts expressing the Src oncoprotein⁴⁷ or

the putative oncogene MCT-1.⁴¹ In addition, several reports have shown that p21^{CIP1} expression may contribute to tumor progression in prostate,⁴⁸ ovarian,⁴⁹ cervical,⁵⁰ breast⁵¹ and esophageal⁵² carcinomas, and brain tumors⁵³ perhaps by promoting the synthesis of genes involved in cell survival.⁴⁶ Consistent with these observations, YAP65 interaction with RUNX2 relieves p21^{CIP1} repression (Fig. 5), and maintains p21^{CIP1} protein expression (Fig. 4B), which could mediate cell survival in the context of anchorage independent growth. Recent reports have shown that p21^{CIP1} reduces the effectiveness of DNA damaging agents in HCT116 colorectal cancer cells by inhibiting DNA damage-induced apoptotic events.⁵⁴ These observations are consistent with our results showing that HCT116 cells, which express high levels of p21^{CIP1}, form colonies in soft agar while cells with low levels of p21^{CIP1} are deficient in colony formation (Fig. 4A). CDK inhibitors may, therefore, contribute to cell transformation by reducing cell sensitivity to proapoptotic signals.

In conclusion, we have demonstrated that the RUNX2 transcription factor is a repressor of the cdk inhibitor p21^{CIP1} and that YAP65 alleviates p21^{CIP1} promoter and protein repression by direct interaction with RUNX2. YAP65 and RUNX2 expression reduced cell: cell contact inhibition and synergistically enhanced anchorage-independent growth of NIH3T3 fibroblasts. Although the association of RUNX2 and YAP65 on the p21^{CIP1} promoter may contribute to cellular transformation, it is possible that RUNX2-YAP65 may control the expression of other genes that regulate cellular transformation. Therefore, future studies using this model of tumor progression will include a comparative analysis of gene expression patterns from parental cells and RUNX2-YAP65 expressing cells to further elucidate the mechanisms regulating cell transformation and to identify inhibitory therapeutic agents.

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