

Involvement of RNA Helicases p68 and p72 in Colon Cancer

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Abstract

The homologous proteins p68 and p72 are members of the DEAD box family of RNA helicases. Here, we show that expression of both of these helicases strongly increases during the polyp→adenoma→adenocarcinoma transition in the colon. Furthermore, p68 and p72 form complexes with β -catenin and promote the ability of β -catenin to activate gene transcription. Conversely, simultaneous knockdown of p68 and p72 leads to reduced expression of the β -catenin-regulated genes, *c-Myc*, *cyclin D1*, *c-jun*, and *fra-1*, all of which are proto-oncogenes. Moreover, transcription of the cell cycle inhibitor p21^{WAF1/CIP1}, whose expression is suppressed by c-Myc, is enhanced on p68/p72 knockdown. Thus, p68/p72 may contribute to colon cancer formation by directly up-regulating proto-oncogenes and indirectly by down-regulating the growth suppressor p21^{WAF1/CIP1}. Accordingly, knockdown of p68 and p72 in colon cancer cells inhibits their proliferation and diminishes their ability to form tumors *in vivo*. Altogether, these results suggest that p68/p72 overexpression is not only a potential marker of colon cancer but is also causally linked to this disease. Therefore, p68 and p72 may be novel targets in the combat against colon cancer. [Cancer Res 2007;67(16):7572–8]

Introduction

Colorectal tumors constitute the third leading cause of cancer death in both men and women in the United States. In most cases, inappropriate activation of the proto-oncoprotein β -catenin is thought to trigger the onset of tumor formation. In normal cells, activation of β -catenin is mainly elicited through the Wnt signaling pathway that entails the inactivation of glycogen synthase kinase 3 β (GSK-3 β) in the adenomatous polyposis coli (APC)/axin/GSK-3 β complex. This leads to a cessation of β -catenin phosphorylation by GSK-3 β , which normally targets β -catenin for destruction by the proteasome. Consequently, β -catenin levels increase on Wnt stimulation, allowing β -catenin to enter the cell nucleus and affect gene transcription (1, 2).

Mutations in the tumor suppressor APC and axin that prevent β -catenin degradation or mutations in β -catenin itself, which protect it from being phosphorylated, are found in many colorectal tumors (1, 3). All of these mutations cause nuclear accumulation of β -catenin, its binding to T-cell factor transcription factors, and the up-regulation of genes such as the proto-oncogenes *c-Myc* and *cyclin D1* (4–6). Accordingly, the transcription factor c-Myc is overexpressed in the majority of colorectal tumors (7, 8), and

overexpression of the cell cycle protein cyclin D1 is also commonly observed in colon cancer (9, 10).

RNA helicases have various essential functions in basically all aspects of RNA metabolism, including RNA splicing, translation, and ribosome biogenesis. They can not only unwind RNA but also disturb the interaction of RNA with proteins (11, 12). p68 and p72 are two homologous proteins that belong to a subclass of RNA helicases characterized by the sequence Asp-Glu-Ala-Asp (D-E-A-D) in the Walker B motif required for ATP hydrolysis. Both p68 and p72 RNA helicases are involved in RNA splicing, splice site selection, and the rearrangement of secondary RNA structures (13–16). However, these two RNA helicases may also act in the regulation of gene transcription. They bind to estrogen receptor α and thereby stimulate its transactivation function (17, 18). Further, p68 and p72 coactivate MyoD, bind to p53 and RNA polymerase II, and collaborate with the coactivators CREB binding protein and p300 (19–22).

A previous report indicated that p68 RNA helicase is overexpressed in colorectal tumors (23). This prompted us to systematically study the expression and role of both p68 and p72 RNA helicases in colon cancer and uncover their importance for β -catenin-dependent transcription.

Materials and Methods

Antibodies and immunohistochemistry. Rabbit polyclonal antibodies were directed against amino acids 555 to 576 (21) or 501 to 524 of human p68 and amino acids 632 to 650 of human p72 and affinity purified. Monoclonal 6F9 antibodies directed against β -catenin were from GeneTex (ab6300). Colon cells were fixed with formaldehyde and then stained as described (24, 25). Paraffin-embedded colon tissues were stained according to standard procedures (26).

Coimmunoprecipitation assays. HCT-116 cells were lysed at 4°C in 5 mmol/L Tris, 15 mmol/L Na₂P₂O₇, 25 mmol/L NaCl, 25 mmol/L NaF, 0.5 mmol/L Na₂VO₄, 0.2 mmol/L DTT, 0.5% Triton X-100, 10 μ g/mL leupeptin, 2 μ g/mL aprotinin, 1 μ g/mL pepstatin A, 1 mmol/L phenylmethylsulfonyl fluoride (pH 7.1). After removal of debris and preclearing, 0.5 μ L of monoclonal antibodies was added and, 2 h later, 15 μ L of protein A-agarose beads were also added (27). After another 1 h of tumbling, beads were washed four times with lysis buffer and bound proteins resolved by SDS-PAGE (28). In case of ectopically expressed proteins, 293T cells grown in 6-cm dishes were transfected with 1 μ g of Flag- β -catenin and 1 μ g of Myc-p68 or p72 expression vectors and immunoprecipitations done as described (29).

Luciferase assays. HCT-116 cells grown in 12 wells were transiently transfected using the calcium phosphate coprecipitation method (30). Two hundred nanograms of reporter plasmids [Gal4₂-tk80-luc (31), TOPFlash or FOPFlash (32), or tk80-luc (33)], 400 ng of GAL4 (34) or GAL4- β -catenin expression vector, 150 ng of Myc- β -catenin (or 10 ng Myc- β -catenin-S33Y) or empty vector pCS³⁺-6Myc, and 400 ng of HA-p68, HA-p72, or empty vector pEV3S were used as indicated. Thirty-six hours after transfection, cells were lysed and luciferase activity was determined in a Berthold Lumat (35, 36).

Short hairpin RNA. Short hairpin RNAs (shRNA) targeting the sequence GAACTGCTCGCAGTACCAA in p68 and the sequence GAGACGCTGTGATGATCTG in p72 were cloned into pSIREN-RetroQ (Clontech). Cells were infected with retrovirus produced from these constructs according to

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standard procedures. Proliferation was assayed with WST-8 formazan (Cell counting kit-8, Alexis Biochemicals).

Reverse transcription-PCR. RNA was prepared with Trizol reagent (Invitrogen) and reverse transcribed and amplified with the Access Quick reverse transcription-PCR (RT-PCR) system (Promega; ref. 37). The PCR program used was 48°C for 45 min; 96°C for 2 min; 18 repeats (for cyclin D1) or 12 repeats [for glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] of 94°C for 30 s, 58°C for 45 s, and 68°C for 80 s; followed by a final extension at 68°C for 6 min. Generation of a 484-bp human cyclin D1 or a 226-bp human GAPDH DNA fragment was revealed on ethidium bromide-stained agarose gels (38). The following primers were used: cyclin D1-forward, 5'-CTGGCCATGAACTACCTGGAC-3'; cyclin D1-reverse, 5'-GGTCACACTTGATCACTCTGG-3'; GAPDH-forward, 5'-GAGCCACATCGCTCAGACACC-3'; and GAPDH-reverse, 5'-TGACAAGCTTCCCGTTCTCAGC-3'.

Chromatin immunoprecipitation assays. Chromatin immunoprecipitation assays were done in RKO cells essentially as described (39). The following primers were used to amplify a 361-bp fragment of the human cyclin D1 promoter: 5'-GTAACGTCACACGGACTACAGG-3' and 5'-GCACA-CATTTGAAGTAGGACACC-3'.

In vivo tumor formation. RKO cells were infected with retrovirus expressing control shRNA or shRNA for p68 and p72. These cells (3.5×10^6), resuspended in 0.3-mL PBS, were injected into the left flank of 6-week-old athymic *NCr-nu/nu* nude mice. Tumors were dissected 32 days thereafter and their mass determined. These experiments had been approved by the Mayo Clinic Institutional Animal Care and Use Committee.

Results

p68 and p72 overexpression in colorectal cancer. First, we studied the expression of p68 and p72 RNA helicases in colon cell lines by Western blotting. As shown in Fig. 1A, p68 was overexpressed in all four tumor cell lines studied compared with the three nontumor cell lines. Similarly, p72 and its isoform p82, both of which are generated from the same transcript but use different translation start codons (40), were overexpressed in tumor cells. Interestingly, the degree of p68/p72 overexpression was at least as high as the known overexpression of β -catenin in colon cancer cells (Fig. 1A).

Originally, p68 and p72 RNA helicases were reported to be nuclear resident proteins (41, 42), although later on some cytoplasmic staining of p68 was also detected in ovarian and breast cancer cell lines (21). Analysis of HCT-116 and SW620 colon cancer cells revealed that p68 is present in the cytoplasm and cell nucleus, whereas normal CCD-841-CoN colon cells displayed nearly exclusively nuclear staining (Fig. 1B, top). In contrast, p72 RNA helicase was predominantly localized to the nucleus in both normal and cancerous colon cells (Fig. 1B, bottom). Biochemical fractionation confirmed these differences in intracellular localization between p68 and p72 (Fig. 1C). Thus, a significant portion of p68 protein may translocate from the nucleus to the cytoplasm during the neoplastic transformation of colon cells, whereas p72 RNA helicase remains localized in the cell nucleus.

Next, we stained human colon specimens from different stages of cancer development with two anti-p68 antibodies (Fig. 2, top). Benign hyperplastic polyps and normal tissue showed no or minimal, exclusively nuclear p68 staining of epithelial cells; any strong p68 staining visible in hyperplastic polyps was confined to stromal cells that were mainly infiltrating leukocytes. In contrast, tubular and villous adenomas displayed strong cytoplasmic and focal nuclear p68 staining. In addition, generalized cytoplasmic and nuclear p68 RNA helicase overexpression was observable in adenocarcinomas. Similar to p68, we observed little to no p72 staining in hyperplastic polyps and normal tissue, whereas

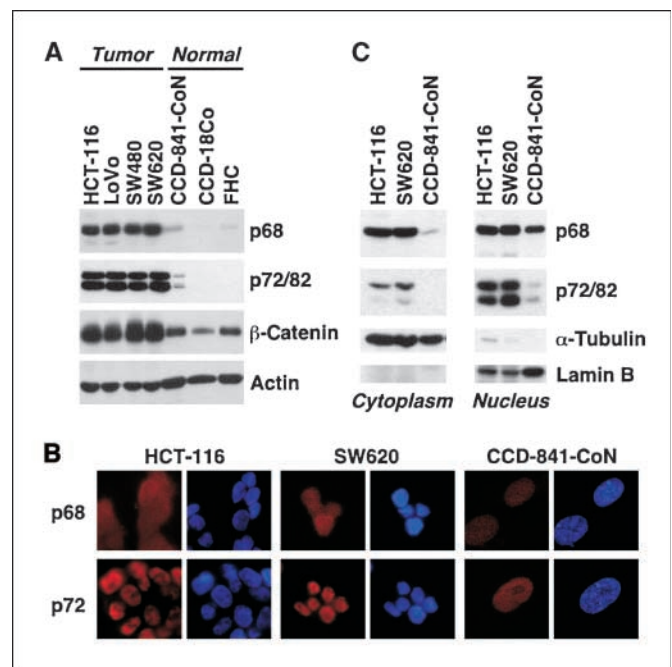


Figure 1. Analysis of tumor and normal colon cell lines. A, Western blots were probed with indicated antibodies. Actin served as a loading control. B, intracellular localization of p68 and p72 RNA helicases. HCT-116 and SW620 colon cancer cells and CCD-841-CoN normal colon cells were stained with anti-p68 or anti-p72 antibodies (red color). Simultaneously, DNA was stained with 4',6-diamidino-2-phenylindole (blue color). C, Western blots of indicated colon cells fractionated into cytoplasm and nucleus. Lamin B is a nuclear marker, and α -tubulin a cytoplasmic marker.

adenomas and adenocarcinomas showed high levels of p72 RNA helicase expression (Fig. 2, bottom); in contrast to p68, p72 immunostaining was always predominantly restricted to the cell nuclei.

Overall, we initially analyzed 9 polyps, 19 adenomas, and 5 adenocarcinomas. Only 0% to 10% of epithelial cells in polyps showed weak staining for p68 or p72 RNA helicase, whereas strong p68 and p72 staining was observed in 30% to 90% and 90% to 100% of the epithelial cells of the adenomas and adenocarcinomas, respectively. Subsequently, we analyzed another six adenocarcinomas and matching normal colon tissue. Again, whereas barely any p68 and p72 staining was observable in the control normal tissue, we observed strong staining in >90% of the epithelial cells of these six adenocarcinomas. Altogether, these data show that the degree of p68 and p72 RNA helicases expression increases within the sequence polyp \rightarrow adenoma \rightarrow adenocarcinoma and may therefore be indicative of disease progression.

Interaction of p68/p72 with β -catenin. When we stained consecutive cuts of the same colon cancer tissue specimen with antibodies against either p68 or β -catenin, these two proteins seemingly colocalized both in the cytoplasm and nucleus (Supplementary Fig. S1A). Moreover, we observed the same when analyzing established colon cancer cell lines (Supplementary Fig. S1B). This prompted us to analyze whether p68 and β -catenin might interact with each other. Thus, we carried out coimmunoprecipitation experiments and found that endogenous p68 RNA helicase coimmunoprecipitated with endogenous β -catenin (Fig. 3A). Similarly, p72 RNA helicase coimmunoprecipitated with β -catenin (Fig. 3A); note that the p82 isoform seems to less efficiently interact with β -catenin than with the p72 isoform.

Both p68 and p72 consist of a central helicase domain flanked by NH₂ and COOH termini that may regulate their function (see Fig. 3B for a sketch of p68 and p72). To map domains that mediate the interaction of p68/p72 with β -catenin, we produced Myc-tagged truncations of p68 and p72 and analyzed whether they interact with β -catenin. As expected, β -catenin coprecipitated with full-length p68₂₋₆₁₄ and p72₂₋₆₅₀ but not when control empty vector was cotransfected (Fig. 3B, left). In addition, the NH₂ termini of p68 and p72 were not required for the interaction with β -catenin (see p68₁₇₆₋₆₁₄ and p72₁₂₆₋₆₅₀). However, the COOH terminus of p68 (see p68₃₈₇₋₆₁₄) was incapable of interacting with β -catenin, whereas the COOH terminus of p72 still bound to β -catenin (see p72₄₃₈₋₆₅₀). Further, p68₂₋₃₈₈, but not p72₂₋₄₃₇, was able to form complexes with β -catenin. Altogether, this suggests that p68 interacts with β -catenin through its helicase domain, whereas p72 does so through its COOH terminus.

While this article was in preparation, it was reported that p68 interacts with β -catenin only when being phosphorylated on Tyr⁵⁹³ (43). However, we did not observe a difference in the ability of wild-type and the phosphorylation-deficient Y593F mutant of p68 to form complexes with β -catenin *in vivo* (Fig. 3B, right), which is consistent with the fact that the COOH terminus of p68 harboring Tyr⁵⁹³ does not contribute to the binding to β -catenin. Possibly, the utilization of different cell lines or culturing conditions may explain the different results obtained by us and Yang et al. (43).

Conversely, we attempted to map the interaction domain(s) for p68/p72 in β -catenin. To this end, we expressed several fragments of β -catenin together with p68 or p72 and carried out coimmunoprecipitation experiments (Fig. 3C). We found that both the NH₂

terminus (amino acids 1–223) and the COOH terminus (amino acids 583–781) of β -catenin did not interact with either p68 or p72, whereas β -catenin₁₋₅₈₂ did so, indicating that amino acids 224 to 582 mediate binding to p68/p72. We further subdivided amino acids 224 to 582 into amino acids 224 to 390 and 391 to 582. Unfortunately, β -catenin₂₂₄₋₃₉₀ was very poorly expressed, thus prohibiting us to study its interaction with p68/p72 *in vivo*, but we could show that β -catenin₃₉₁₋₅₈₂ did interact with p68 and p72 (Fig. 3C). However, because β -catenin₁₋₃₉₀ interacted with p68/p72 *in vitro* (see Fig. 3D), we conclude that both β -catenin amino acids 224 to 390, which encompass the armadillo repeats 3 to 6, and amino acids 391 to 582, which encompass the armadillo repeats 7 to 10, bind to p68 and p72.

Finally, we inquired whether β -catenin binds directly to p68 and p72. To this end, we used full-length p68 and p72 fused to glutathione *S*-transferase (GST) that were produced in *E. coli* and affinity purified (21, 22) and His-tagged β -catenin amino acids 1 to 390 and 391 to 781 that were also produced in *E. coli* and affinity purified on Ni²⁺-NTA agarose. As shown in Fig. 3D, both the NH₂-terminal half and the COOH-terminal half of β -catenin interacted with p68 and p72, indicating a direct interaction between these proteins.

Activation of β -catenin by p68/p72. Next, we investigated whether p68 and p72 RNA helicases may affect the transcriptional activity of β -catenin in HCT-116 colon cancer cells. Because β -catenin on its own does not bind to DNA, we fused β -catenin to the DNA binding domain of the yeast protein GAL4 and assessed the activity of the resulting fusion protein with a GAL4 binding site-driven luciferase reporter. Coexpression of p68 or p72 RNA helicase strongly enhanced GAL4- β -catenin-dependent transcription (Fig. 4A); no effect of p68

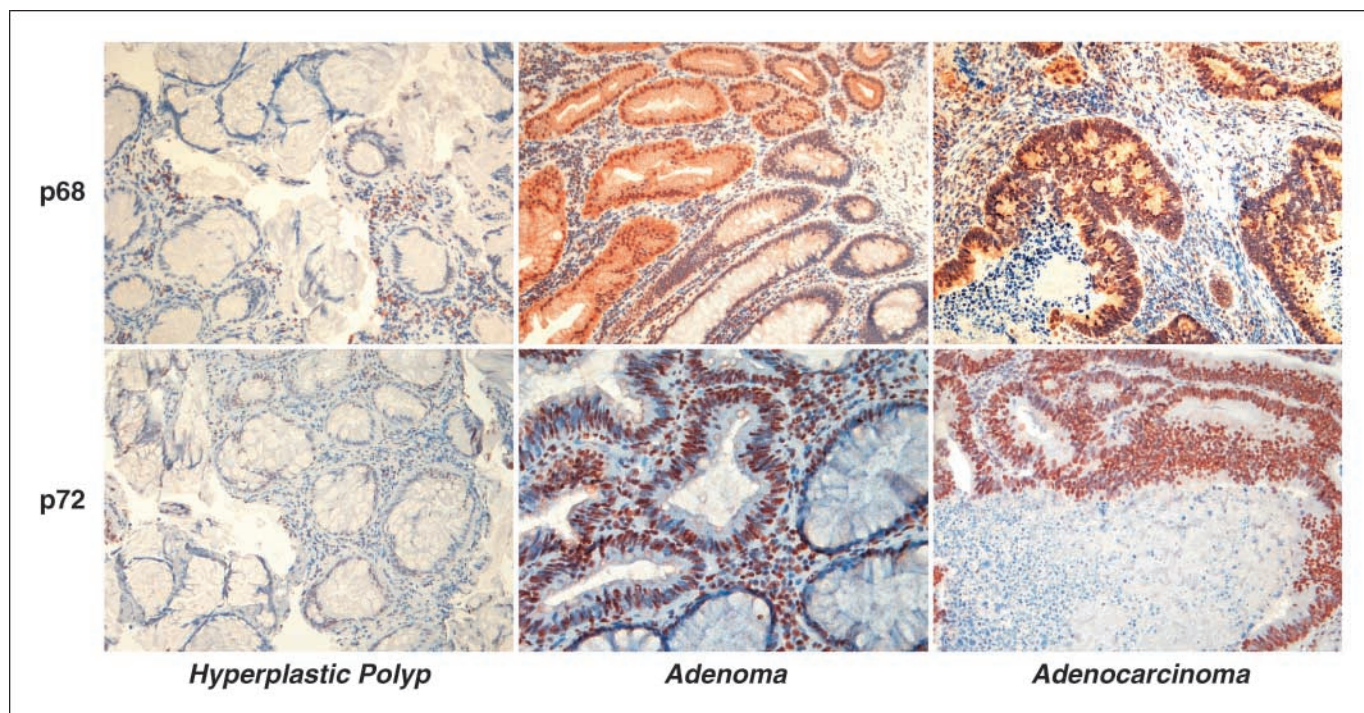


Figure 2. p68 and p72 RNA helicase overexpression in colon tumors. Human colon specimens were immunostained with anti-p68 (top) or anti-p72 (bottom) antibodies (brown color). Cell nuclei were counterstained with light hematoxylin (blue color). No significant staining was observable in the crypts of the depicted polyps. Top middle, a tubulovillous adenoma (top left area) and hyperplastic regions (bottom right area) that are devoid of significant staining. The villous adenoma (bottom middle) was photographed at twice the magnification ($\times 200$) compared with all other pictures. The adenocarcinoma is invading the muscular wall of the colon (top) or is on top of a necrotic core (bottom).

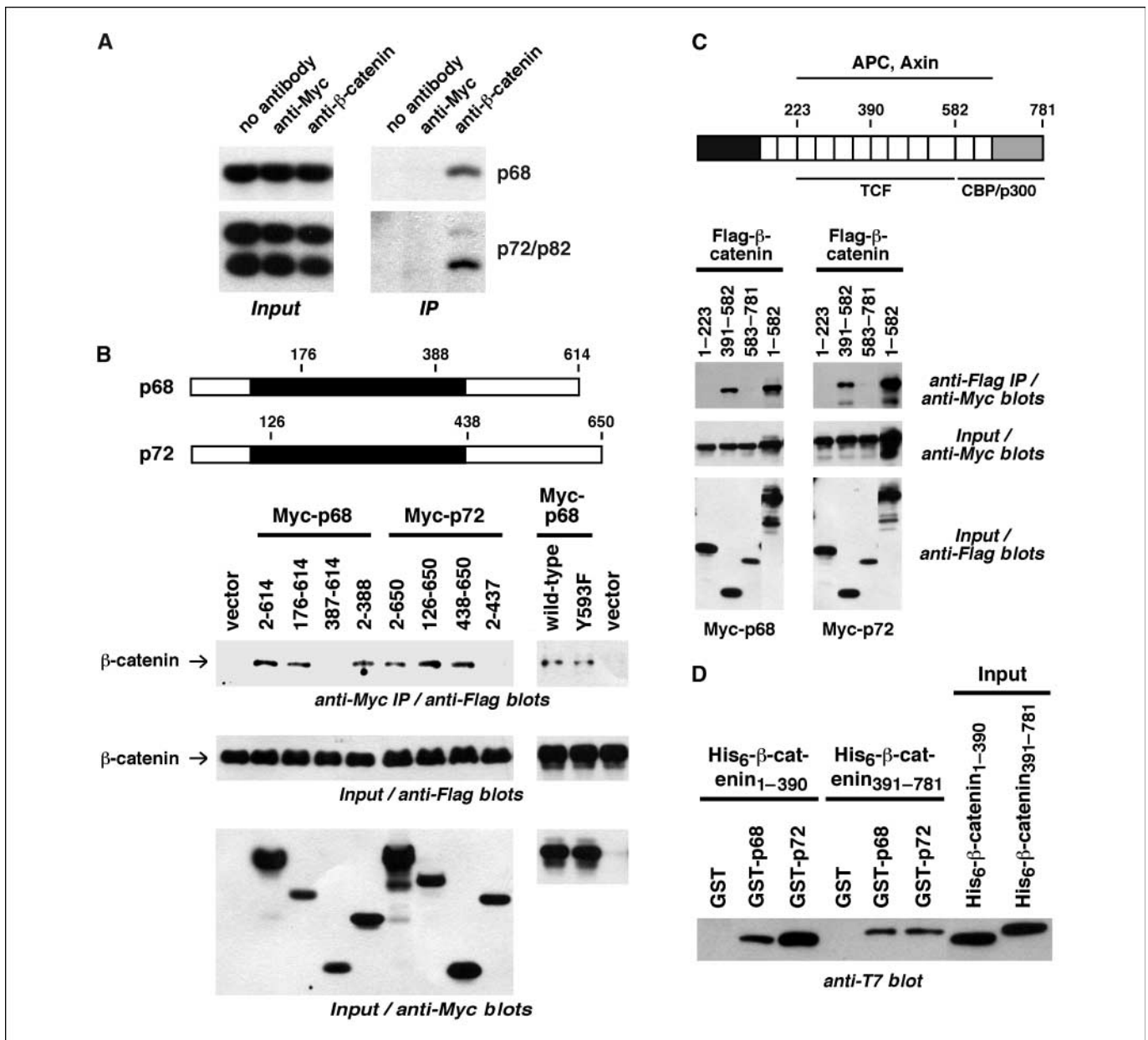


Figure 3. Complex formation between p68/p72 and β -catenin. **A**, coimmunoprecipitation of p68 and p72 with β -catenin. HCT-116 colon cancer cell lysates were immunoprecipitated with no antibody or with anti-Myc or anti- β -catenin antibodies. Coprecipitated p68 and p72/p82 were revealed by Western blotting. **B**, flag-tagged β -catenin was cotransfected with empty vector pCS³⁺-6Myc or indicated Myc-tagged amino acids of p68 or p72 into 293T cells. After anti-Myc immunoprecipitation, any coprecipitated β -catenin was revealed by anti-Flag Western blotting. *Bottom two*, input levels of β -catenin or Myc-tagged proteins. **C**, coimmunoprecipitation of indicated Flag-tagged β -catenin amino acids with Myc-tagged p68 or p72. *Top*, a sketch of β -catenin with the central 12 armadillo repeats indicated by white boxes. Binding domains of APC, axin, T-cell factor (TCF), and CREB binding protein (CBP)/p300 are pointed out. **D**, *in vitro* pull-down assays probing the binding of His₆-tagged β -catenin₁₋₃₉₀ and His₆-tagged β -catenin₃₉₁₋₇₈₁ to GST-p68 or GST-p72. A T7 tag present between the hexahistidine stretch and β -catenin amino acids allowed the detection of bound β -catenin₁₋₃₉₀ and β -catenin₃₉₁₋₇₈₁ by anti-T7 Western blotting.

and p72 RNA helicases was observed with the GAL4 DNA binding domain itself.

To further explore the effect of p68 and p72 on β -catenin function, we used the TOPFlash luciferase reporter (32) in which a luciferase gene is driven by T-cell factor binding sites and, accordingly, also by the T-cell factor cofactor β -catenin. p68 (or p72) alone stimulated the TOPFlash reporter by 2-fold (Fig. 4B, left), probably through endogenous β -catenin and T-cell factor; importantly, coexpression of β -catenin with p68 or p72 RNA helicase led to synergistic activation of transcription. This activation was

specific because mutation of the T-cell factor binding sites (FOPFlash luciferase reporter; Fig. 4B, middle) abolished any activation by β -catenin, p68, and p72; similarly, a basal thymidine kinase promoter was unaffected by these proteins (Fig. 4B, right). Moreover, we found that a mutant of β -catenin (S33Y) that escapes destruction dependent on GSK-3 β phosphorylation (1) was also activated by p68 and p72 (Fig. 4C), indicating that p68 and p72 act at the level of transcription and not by stabilizing β -catenin. In addition, we found that p68-Y593F does not differ from wild-type p68 in its ability to stimulate β -catenin-dependent transcription

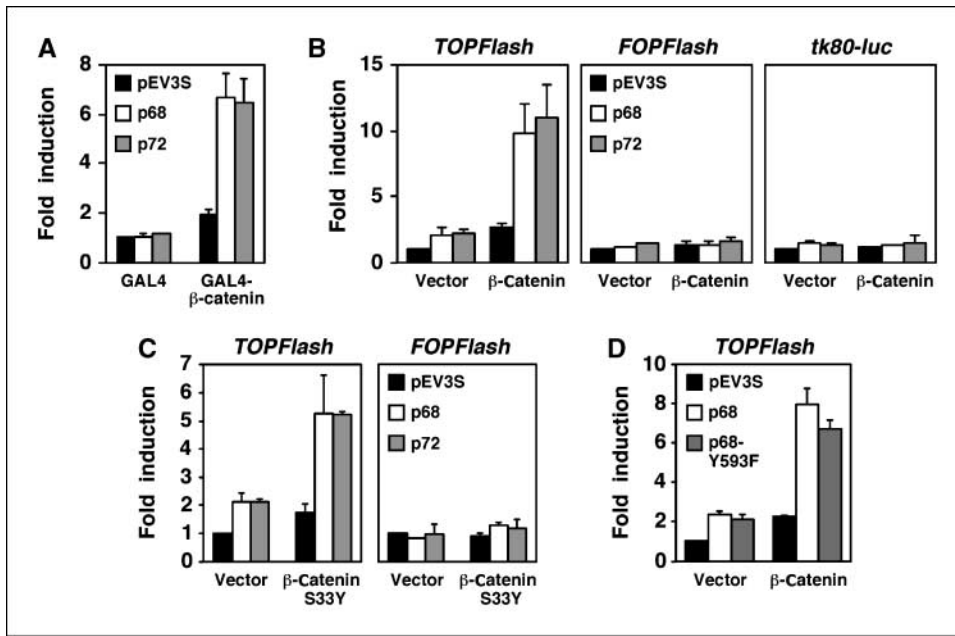


Figure 4. Transcriptional coactivation by β -catenin and p68/p72. *A*, HCT-116 cells were transfected with an expression vector for the GAL4 DNA binding domain or the GAL4- β -catenin fusion protein. Additionally, empty vector pEV3S or p68 or p72 expression vector was transfected. Activation of the cotransfected Gal4₂-tk80-luc reporter gene was measured. *B*, TOPFlash, FOPFlash, or tk80-luc luciferase reporter plasmids were transfected into HCT-116 cells. As indicated, β -catenin, p68, p72, or pEV3S vector was cotransfected. *C*, analogous with β -catenin S33Y. Note that we transfected 15 times less β -catenin S33Y expression vector than wild-type β -catenin expression vector in (*B*). *D*, comparable activation of the TOPFlash luciferase reporter gene by p68 and its Y593F mutant in HCT-116 cells.

(Fig. 4D). Altogether, these results indicate that p68 and p72 collaborate with β -catenin in activating transcription.

To prove an effect of p68 and p72 on endogenous gene transcription, we developed shRNAs specifically targeting these RNA helicases. When delivering p68 and p72 shRNA, either singly or in combination, to RKO colon cancer cells, they efficiently reduced p68 and/or p72 RNA helicase expression (Fig. 5A). Note that we observed a slight (~50%) up-regulation of p68 when p72

was knocked down, and vice versa, suggesting that cells try to compensate for the loss of p68 or p72, which may imply an important role of these RNA helicases in cell physiology. We also analyzed how the β -catenin/T-cell factor target gene *c-Myc* (4) was affected by p68/p72 down-regulation. No significant change of *c-Myc* expression was observed when p68 or p72 was knocked down alone; however, when both p68 and p72 were knocked down, *c-Myc* expression was reduced (Fig. 5A). Thus, probably due to the

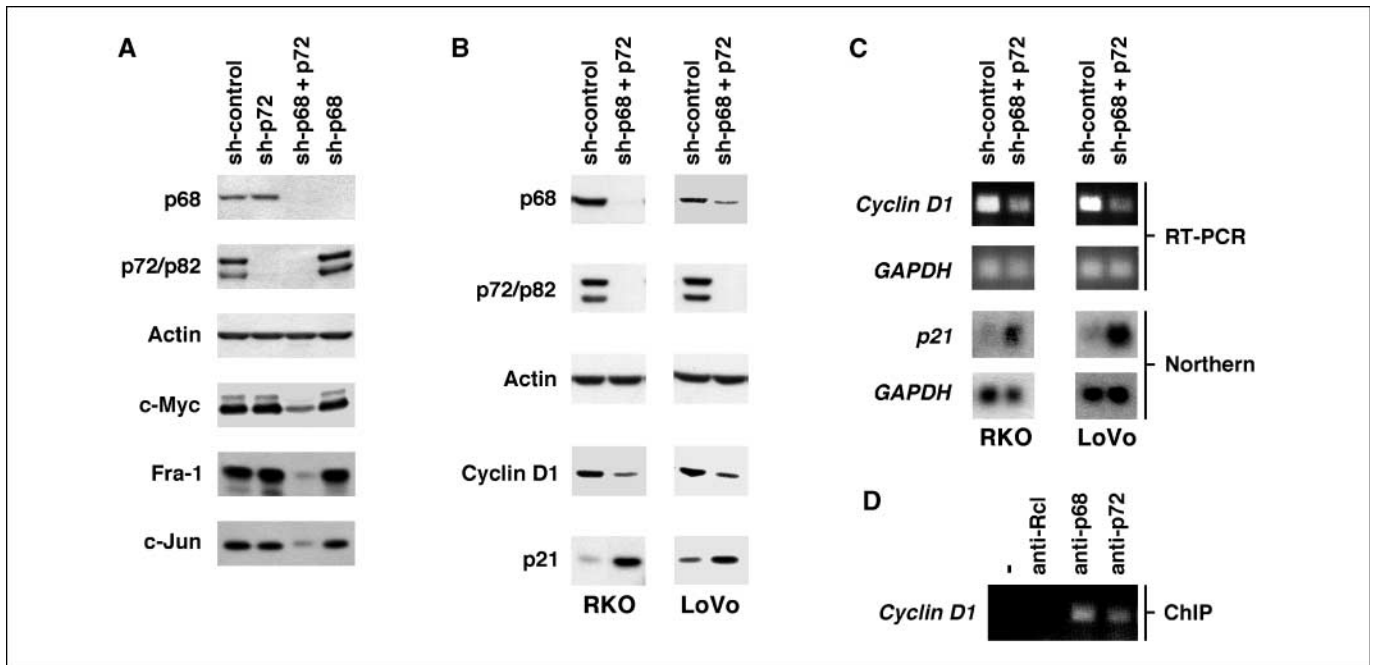


Figure 5. Down-regulation of p68 and p72 in colon cancer cells. *A*, RKO cells were infected with retrovirus directing the production of control, p68, or/and p72 shRNA as indicated. Western blots probed for p68, p72/p82, actin, c-Myc, Fra-1, or c-Jun. *B*, similarly, knockdown of p68 and p72 in RKO and LoVo colon cancer cells. *C*, regulation of cyclin D1 and p21^{WAF1/CIP1} gene transcription. RT-PCR results and Northern blots. *D*, chromatin immunoprecipitation (ChIP) assays. RKO cell extract was immunoprecipitated with no antibody or with anti-Rcl (control), anti-p68, or anti-p72 antibodies. Coprecipitated DNA was amplified with cyclin D1 primers.

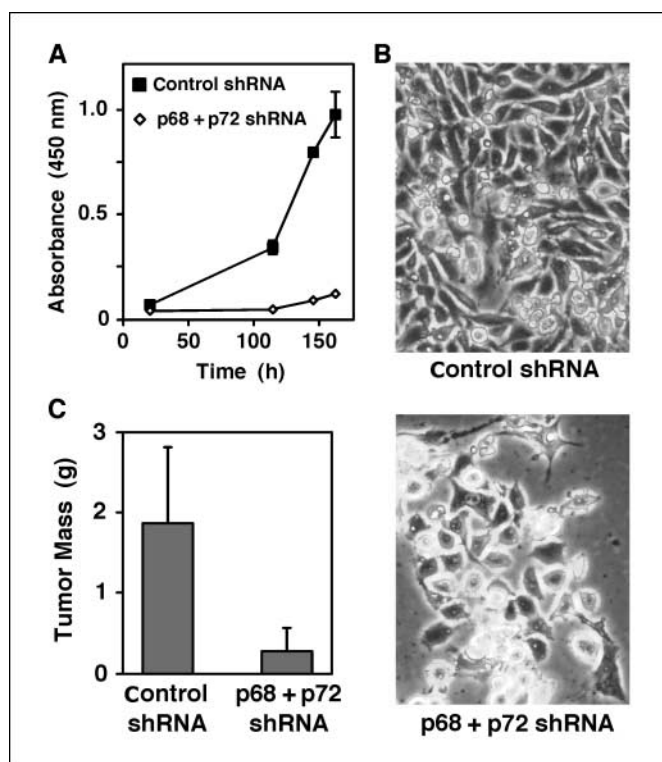


Figure 6. p68 and p72 shRNAs inhibit cell proliferation and tumor formation. RKO cells were treated with retrovirus expressing control shRNA or p68 and p72 shRNA. *A*, proliferation assay. Equal numbers of cells were plated at time 0 h and proliferation was assayed with WST-8 formazan. *B*, equal numbers of cells were plated at 25% confluency and photographed 2 d later. *C*, tumor mass in nude mice injected with control or p68/p72 shRNA-bearing cells. Columns, average of tumor mass from eight mice in each group; bars, SD.

functional equivalency of p68 and p72 and due to the compensatory up-regulation of the other helicase, knocking down of only one of these RNA helicases was without effect. Similar to *c-Myc*, two further β -catenin/T-cell factor target genes, *fra-1* and *c-jun* (44), which are overexpressed in colorectal tumors (45–48), were down-regulated on joint p68 and p72 knockdown (Fig. 5A).

We further analyzed the effect of joint p68 and p72 knockdown in two colon cancer cell lines (Fig. 5B). First, we observed that another β -catenin/T-cell factor target gene, *cyclin D1* (5, 6), was down-regulated on p68/p72 knockdown, again indicating that p68 and p72 are indeed cofactors for β -catenin-mediated transcription. Second, we also analyzed the expression of the cell cycle inhibitor p21^{WAF1/CIP1}, which is normally repressed by c-Myc in colonic crypts (49), and found it to be overexpressed on p68/p72 knockdown (Fig. 5B). Both the decrease in cyclin D1 and the increase in p21^{WAF1/CIP1} protein levels were reflected by a respective decrease in *cyclin D1* and an increase in p21^{WAF1/CIP1} mRNA levels (Fig. 5C), indicating that p68 and p72 act at the level of transcription and not posttranslationally. Finally, we did chromatin immunoprecipitation assays and found that p68 and p72 bound to the *cyclin D1* promoter in close proximity to its T-cell factor binding site (Fig. 5D). Altogether, these results indicate that p68 and p72 augment β -catenin/T-cell factor to induce transcription.

Physiologic role of p68/p72. The fact that p68 and p72 are involved in the regulation of oncogenes (*c-Myc*, *cyclin D1*, *fra-1*, and *c-jun*) suggested that they could affect cell proliferation. And

indeed, down-regulating p68/p72 in RKO colon cancer cells severely compromised their ability to proliferate (Fig. 6A). Moreover, we found changes in cell morphology with the appearance of round bodies that may represent cell carcasses indicative of apoptosis (Fig. 6B). These data strongly suggest that p68 and p72 are required for efficient proliferation of colon cancer cells. Notably, normal colon cell lines such as CCD-18Co and FHC are basically devoid of p68 and p72 (see Fig. 1A) and proliferate well. This implicates that the proliferation block induced by the p68/p72 knockdown in RKO cells is a cancer cell-specific effect, further supporting the notion that p68/p72 overexpression is causally linked to colon cancer formation.

Finally, we studied the relevance of p68 and p72 for tumor formation. To this end, we injected RKO cells bearing control shRNA or p68 + p72 shRNA into nude mice and measured the mass of tumors being formed. A striking ~7-fold reduction in tumor mass was observed in the presence of p68 + p72 shRNA (Fig. 6C). These data indicate that p68 and p72 are needed for efficient tumor growth *in vivo*.

Discussion

In this report, we have shown that both p68 and p72 RNA helicases are overexpressed in colon cancer and that the degree of their overexpression correlates with the progression of the disease from polyp to adenoma to adenocarcinoma. Thus, p68 and p72 expression may serve as diagnostic or even prognostic markers for colon cancer. Notably, overexpression of p68 and p72 is not a general tumor marker as none was detected in tumors of the prostate or endometrium (data not shown). The overexpression of p68/p72 in colon tumors can be a consequence or a contributing cause of cancer. We argue that the latter is the case because p68/p72 down-regulation reduces proliferation of colon cancer cells and compromises their ability to form tumors *in vivo*. Therefore, our data point at p68 and p72 RNA helicases to be novel important effectors of colon carcinogenesis.

How do p68 and p72 become overexpressed in colon tumors? One possibility is that both of their promoters are up-regulated at the same time. This is unlikely because p68 mRNA levels do not seem to be enhanced in colorectal tumors (23). Rather, p68 and p72 may be posttranslationally modified at homologous amino acid residues, which could boost their stability. Enhanced ubiquitylation (not branched polyubiquitylation that leads to proteasomal destruction) or tyrosine phosphorylation might be responsible because these posttranslational modifications have been reported to be increased in p68 in cancer cells (23, 50). Posttranslational modifications might also be the underlying cause for p68 being prominently present in the cytoplasm of cancerous but not normal colon cells. However, it remains to be determined why only p68 and not p72 intracellular localization is different between normal and cancer cells.

A possible mechanism by which p68 and p72 contribute to colon carcinogenesis is through coactivation of β -catenin-mediated transcription. Indeed, our reporter gene studies clearly show that p68 and p72 augment the transcriptional activity of β -catenin, and down-regulation of p68/p72 diminished the expression of four prominent β -catenin/T-cell factor target genes (*c-Myc*, *cyclin D1*, *fra-1*, and *c-jun*) and, accordingly, indirectly increased the expression of p21^{WAF1/CIP1}, whose promoter is normally repressed by c-Myc in colonic crypts (49). Mechanistically, both endogenous p68 and p72 can form complexes with

endogenous β -catenin *in vivo* and bind to the *cyclin D1* promoter, suggesting that p68 and p72 are bona fide transcriptional coactivators. It was reported that ectopically expressed, Y593 phosphorylated p68 binds to β -catenin and can induce its nuclear translocation through disruption of its interaction with axin (43). Thus, p68 may affect β -catenin in two ways in cancer cells: in the cytoplasm by protecting it from degradation through dissociation of β -catenin from the cytoplasmic APC/axin/GSK-3 β complex, and in the nucleus by augmenting β -catenin transcriptional activity. In contrast, p68 in normal colon cells and p72 in both normal and cancerous colon cells are unlikely to protect

β -catenin from degradation because we observed no significant cytoplasmic localization of p68/p72 in these cases.

In conclusion, our study has identified both p68 and p72 as potential markers and compelling causative agents of colon cancer, which may guide in the development of novel anticancer therapies.

Acknowledgments

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