HMGA1 Controls Transcription of Insulin Receptor to Regulate Cyclin D1 Translation in Pancreatic Cancer Cells

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Abstract

The HMGA1 proteins act as architectural transcription factors and are involved in the regulation of genes important in the process of carcinogenesis. Although HMGA1 proteins are overexpressed in most types of cancer, signaling circuits regulated by HMGA1 are not clarified in detail. In this study, we show that HMGA1 proteins promote proliferation of pancreatic cancer cells by accelerating G_1 phase progression. Transfection of HMGA1-specific small interfering RNA (siRNA) activates the RB-dependent G_1-phase checkpoint due to the impaired expression of cyclin D1. Down-regulation of cyclin D1 after the HMGA1 knockdown is due to translational control and involves the repressor of the eukaryotic translation initiation factor 4E (eIF4E) 4E-BP1. We show that 4E-BP1 and cyclin D1 act downstream of the insulin receptor (IR) in pancreatic cancer cells. At the molecular level transcription of the IR is controlled by a CAAT/enhancer binding protein β (C/EBPβ)/HMGA1 complex. Together, this work defines a novel pathway regulated by HMGA1, which contributes to the proliferation of pancreatic cancer cells. [Cancer Res 2007;67(10):4679–86]

Introduction

HMGA1 is involved in numerous biological processes, such as transcription, embryogenesis, differentiation, and neoplastic transformation (1, 2). HMGA1a (formerly HMG I) and HMGA1b (formerly HMG Y) are encoded by the same gene and are generated by alternative RNA splicing. HMGA1 proteins function as architectural transcription factors by binding to the minor groove of AT-rich elements via the positively charged basic AT-hook DNA-binding motif (3). Overexpression of HMGA1 proteins results in transformation or increased malignancy (4, 5). Furthermore, transgenic overexpression of HMGA1 results in tumor formation *in vivo* (6, 7). Analogous to experimental findings, HMGA1 is overexpressed in numerous tumors (2). Pancreatic ductal adenocarcinoma cells express high levels of HMGA1 mRNA and protein (8, 9). Furthermore, lowering HMGA1 expression in pancreatic ductal adenocarcinoma (PDAC) cells is a potential therapeutic strategy (10).

Disturbed cell cycle regulation is a hallmark of cancer. Pancreatic ductal adenocarcinoma cells are characterized by a functionally inactivated RB-dependent G_1-phase checkpoint (11). RB inactivation occurs by the concerted action of G_1-phase cyclins and cyclin-dependent kinases (CDK). The D-type cyclin, cyclin D1, is overexpressed in PDAC cells (12–14). Cyclin D1-CDK4/6 complexes phosphorylate RB and thereby contribute to the observed RB inactivation in PDAC cells. Although transcriptional regulation is an important mode of control for cyclin D1 expression, cyclin D1 is negatively regulated by proteasomal degradation, nucleocytoplasmic shuttling, and translation. Thr^{286} phosphorylation of cyclin D1 induces the interaction with the nuclear exportin CRM1 and the nuclear export of cyclin D1 (15). In the cytoplasm, cyclin D1 is polyubiquitinated and proteasomal degraded (16, 17). In addition, several signaling pathways, including the phosphoinositide-3-kinase (PI3K)/Akt pathway, regulate cyclin D1 translation (18, 19). Translational control of cyclin D1 occurs via the eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1). 4E-BP1 normally binds to the eIF4E, inhibiting cap-dependent translation. Hyperphosphorylation of 4E-BP1, a target of the mammalian target of rapamycin kinase, disrupts this binding, and activates cap-dependent translation (20, 21). Inactivation of 4E-BP1 and activation of S6K1 occur after turning on insulin receptor (IR) signaling (22).

The *IR* gene is supposed to be a housekeeping gene, which participates in cell growth, proliferation, and gene expression. Although the IR is universally expressed, *IR* gene transcription is governed by a broad range of factors under different environmental conditions. Recently, regulation of *IR* gene transcription has been shown to be controlled by a nucleoprotein complex containing HMGA1, SP1, AP2, and CAAT/enhancer binding protein β (C/EBPβ) in several cell lines (23, 24).

Because molecular pathways and target genes controlled by HMGA1 were not investigated in detail in PDAC cells, we used RNA interference to elucidate HMGA1 function. We show that HMGA1 regulates proliferation of PDAC cells by transcriptional control of the *IR* gene. Furthermore, we provide evidence that translational control of cyclin D1 abundance act downstream of IR signaling in pancreatic cancer cells.

Materials and Methods

Cell culture, reagents, transfection, and siRNAs. Pancreatic cancer cells were cultivated as described (25). MG132 (Sigma-Aldrich) was dissolved in DMSO and stored at −20°C. Double-stranded small interfering RNAs (siRNA) were transfected in a concentration 200 nmol using OligofectAMINE (Invitrogen) as recently described (25). For titration experiments, double-stranded siRNAs were transfected in a concentration of 1, 10, and 20 nmol. siRNA sequences used (target sequence, sense strand) scramble control siRNA 5′-CAGTCGCGTTTGCGACTGGdTdT-3′, HMGA1 siRNA 5′-GACCCGGAAAACCACCACAdTdT-3′, HMGA1 siRNA-2 5′-CTGGAGAAGGAGGAAGAdTdT-3′, IR siRNA 5′-GGACCCAGTATGCCATCTTdTdT-3′. siRNAs were purchased from Ambion and were stored in a 20 μmol stock at −80°C.

Cell proliferation assay. Proliferation was measured *in vitro* using a bromodeoxyuridine (BrdUrd) cell proliferation ELISA (Roche) as recently described (25).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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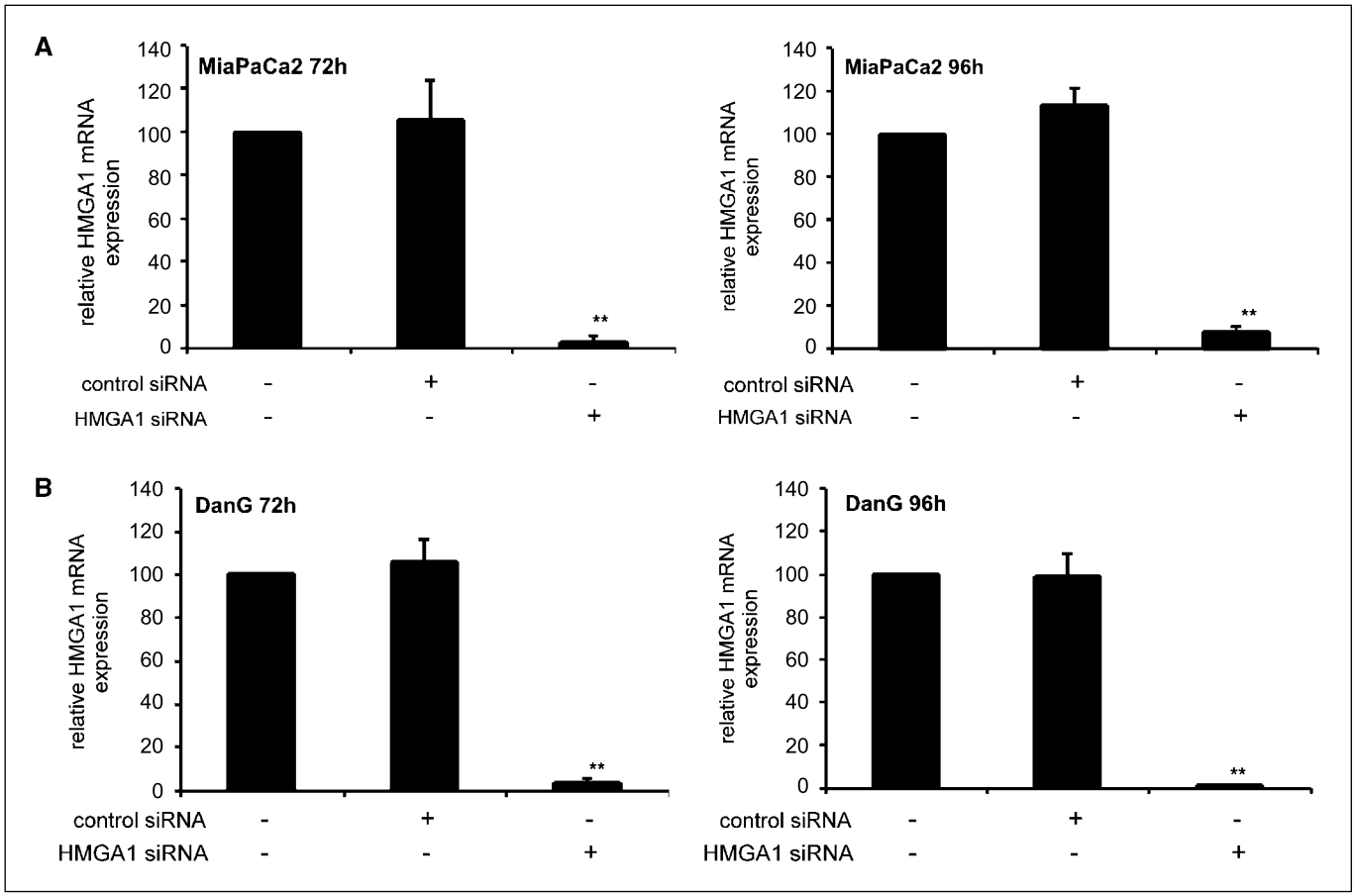

Figure 1. Knockdown of HMGA1 in PDAC cells. Total RNA was prepared 72 h (*left*) and 96 h (*right*) post-transfection of MiaPaCa2 (*A*) and DanG cells (*B*) with the indicated siRNAs. HMGA1 mRNA levels were quantified using real-time PCR analysis and normalized to cyclophilin expression levels. **, $P < 0.001$ versus controls by Student's *t* test.

Cell cycle analysis. For cell cycle analysis, the cells were washed twice in PBS and redissolved in propidium iodide staining buffer containing 0.1% sodium citrate, 0.1% Triton X-100, and 50 μg/mL propidium iodide. After 1 h of incubation at 4°C, flow cytometry was done using a BD Biosciences FACScan. The distribution of cells in different cell cycle stages (G_1-S/G_2 + M) was determined according to their DNA content. Thus, cells possessing 2n (diploid) DNA were assigned to be in G_1, and those having a DNA content between 2n and 4n (tetraploid) were defined as S-phase cells. Finally, cells having ~4n DNA content were assumed to be in G_2 + M. The sum of all cells in fluorescence-activated cell sorting (FACS) analysis was set to 100%, and the distribution into G_1, S, and G_2 + M phases was determined by counting the cells in each window according to the definitions above.

Preparation of total cell lysates. Whole-cell lysates were prepared by incubating cell pellets for 30 min at 4°C in immunoprecipitation buffer (50 mmol/L HEPES, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.5% NP40, 10% glycerol, 1 mmol/L DTT, 1 mmol/L phenylmethysulfonyl fluoride, and 5 mmol/L NaF). Insoluble material was removed by centrifugation, and lysates were aliquoted and stored at −80°C.

Western blot analysis. Extracts were normalized for protein and heated at 95°C for 5 min in Lämmli buffer. Proteins were resolved on 10% SDS-polyacrylamide gels and electrophoretically transferred to polyvinylidene diflouride (Millipore) membranes in a semidry blotting system. Membranes were blocked in PBS supplemented with 5% skim milk and 0.1% NP40 and incubated with antibodies against RB, cyclin A (BD PharMingen), cyclin D1 (Oncogene Research Products), 4E-BP1, IR (Cell Signaling Technology), and β-actin (Sigma-Aldrich). Proteins recognized by the antibodies were detected by the Odyssey IR Imaging System (Licor) using Alexa680-coupled (Molecular Probes) or IRDeye800-coupled (Rockland) secondary antibodies.

Quantitative reverse transcription-PCR. Total RNA was isolated from the pancreatic cancer cell lines using the RNeasy kit (Qiagen) following the manufacturer's instructions. Total RNA (100 ng) was used for cDNA synthesis using the TaqMan Reverse Transcription reagents (Applied Biosystems). Quantitative mRNA analysis was done using real-time PCR analysis (TaqMan, PE Applied Biosystems) as previously described (25). Primer sequences are as followed: HMGA1, forward 5′-CAGCGAAGTGCCAACACCTAAG-3′, HMGA1, reverse 5′-CCTTGGTTTCCTTCCTGGAGTT-3′; cyclin D1, forward 5′-GCCGAGAAGCTGTGCATCTAC-3′, cyclin D1, reverse 5′-GTTCAATGAAATCGTGCGGG-3′; IR, forward 5′-ACCTGCACCACAAATGCAAGA-3′, IR, reverse 5′-AGACGTCACCGAGTCGATGGT-3′.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assays were done as recently described (25). An equal amount of chromatin (50–100 μg) was used for each precipitation. The antibodies used were as follows: HMGA1 and C/EBPβ (Santa Cruz Biotechnology) and β-actin (Sigma-Aldrich). One-twentieth of the precipitated chromatin was used for each PCR reaction. To ensure linearity, 28 to 38 cycles were done, and one representative result out of at least three independent experiments is shown. Sequences of the promoter-specific primers are IR promoter E3 region, forward 5′-AGATCTGGCCATTGCACTCCA-3′; IR promoter E3 region, reverse 5′-ATACCTGCATGCCAGTTCTGGG-3′; cFlip, forward 5′-GGGCCACAGAGCGAGACTCTG-3′; cFlip, reverse 5′-CTCACTGTGTGAGGCCGAGTCC-3′.

Statistical methods. All data were obtained from at least three independent experiments done in duplicate, and the results are presented as mean and SE. To show statistical significance, Student's *t* test was used. *P* values are indicated in the figure legends.

Results

Knockdown of HMGA1 inhibits proliferation of PDAC cells. Increased HMGA1 protein levels have been reported in PDACs (8, 9). To elucidate the role of HMGA1 proteins in PDAC, we used RNA interference. Band-shift experiments using the high-affinity HMGA1 binding site of the interleukin 4 promoter revealed a knockdown of HMGA1 protein occurring 72 h after the transfection of the HMGA1 siRNA (data not shown). At this time point, HMGA1 mRNA expression is reduced to 2.8% in MiaPaCa2 and to 3.3% in DanG cells (Fig. 1) compared with untransfected controls. Ninety-six hours after the transfection HMGA1, mRNA was reduced to 7.9% in MiaPaCa2 and to 1.3% in DanG cells compared with untransfected controls (Fig. 1). HMGA1 proteins are known to play a critical role in cell proliferation (26). In this line, we found reduced BrdUrd incorporation 72 h after transfection of the HMGA1 siRNA to 57.9% compared with untransfected controls in MiaPaCa2 cells (Fig. 2*A*) and to 45.5% compared with untransfected controls in DanG cells (Fig. 2*B*). Ninety-six hours after the transfection of the HMGA1-specific siRNA, BrdUrd incorporation was reduced to 55.9% compared with untransfected controls in MiaPaCa2 cells (Fig. 2*A*) and to 59% compared with untransfected controls in DanG cells (Fig. 2*B*). In agreement with this, growth curves revealed an impaired proliferation rate after the knockdown of HMGA1 in MiaPaCa2 and DanG cells (Fig. 2*C*) compared with untransfected or control siRNA-transfected cells. Reduced proliferation rates are due to an accumulation of the cells in the G_1 phase of the cell cycle. The G_1-phase fraction was increased to 78.2% after the knockdown of HMGA1 in MiaPaCa2 cells compared with untransfected or control siRNA-transfected cells (Fig. 2*D*). Similarly, an increase of the G_1-phase fraction to 51.4% was observed after the transfection of the HMGA1 siRNA into DanG cells (Fig. 2*D*). In addition, an increase of the G_2-M–phase fraction was observed after the HMGA1 knockdown in DanG cells. These findings show that HMGA1 proteins control cell cycle progression

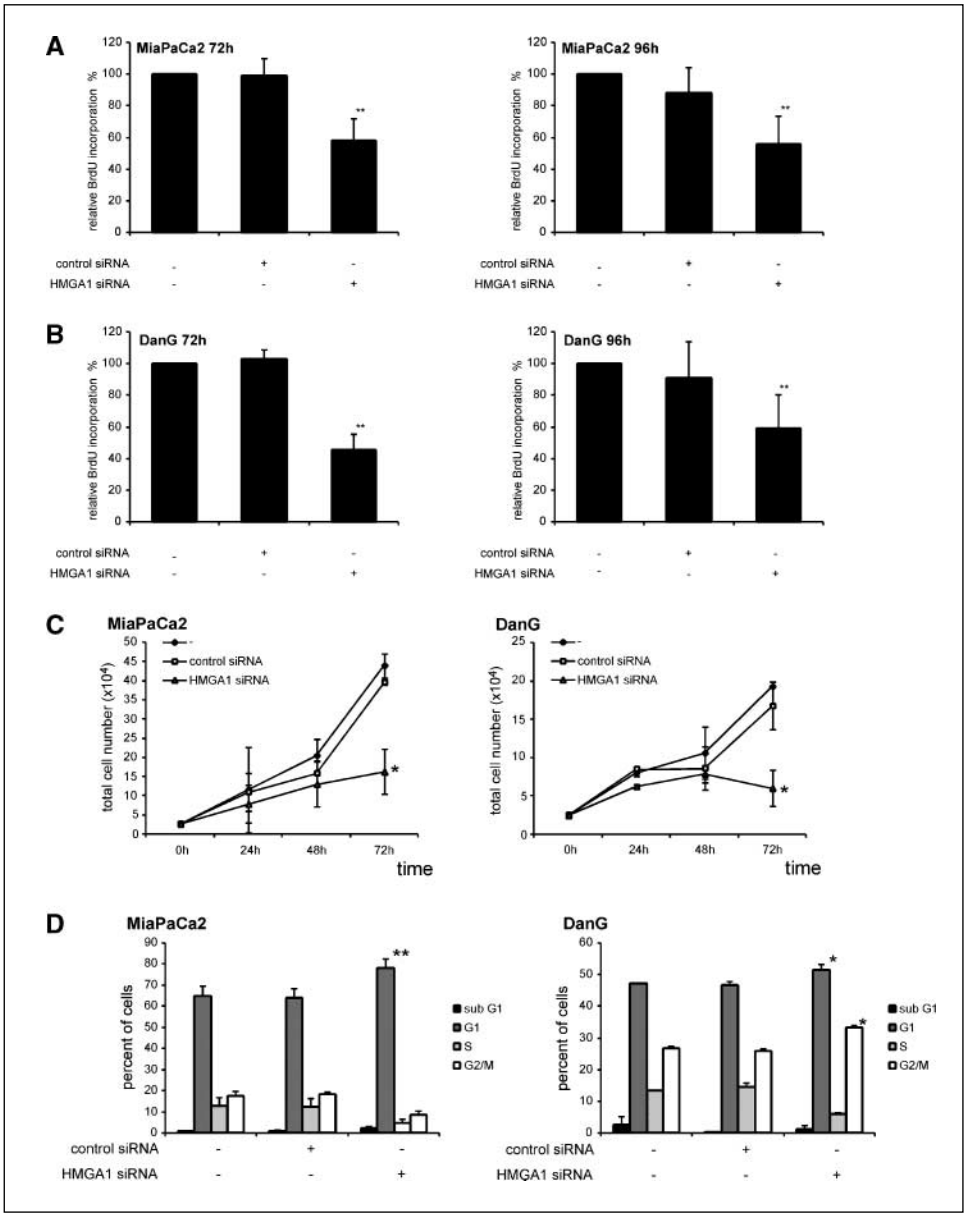

Figure 2. Knockdown of HMGA1 inhibits proliferation of PDAC cells. *A,* MiaPaCa2 cells were transfected with no siRNA, a control, and a HMGA1-specific siRNA. Relative BrdUrd incorporation was investigated 72 h (*left*) and 96 h (*right*) post-transfection. **, $P < 0.001$ versus controls by Student's *t* test. *B,* DanG cells were transfected with no siRNA, a control, and a HMGA1-specific siRNA. Relative BrdUrd incorporation was investigated 72 h (*left*) and 96 h (*right*) post-transfection **, $P < 0.001$ versus controls by Student's *t* test. *C,* MiaPaCa2 and DanG cells were transfected with no siRNA, a control, and a HMGA1-specific siRNA. Cell numbers were counted every 24 h until 72 h post-transfection. *, $P < 0.05$ versus controls by Student's *t* test. *D,* MiaPaCa2 and DanG cells were transfected with no siRNA, a control, and a HMGA1-specific siRNA and were stained with propidium iodide 72 h post-transfection. The cell cycle phase was measured by FACS analysis. *, $P < 0.05$; **, $P < 0.001$ versus controls by Student's *t* test.

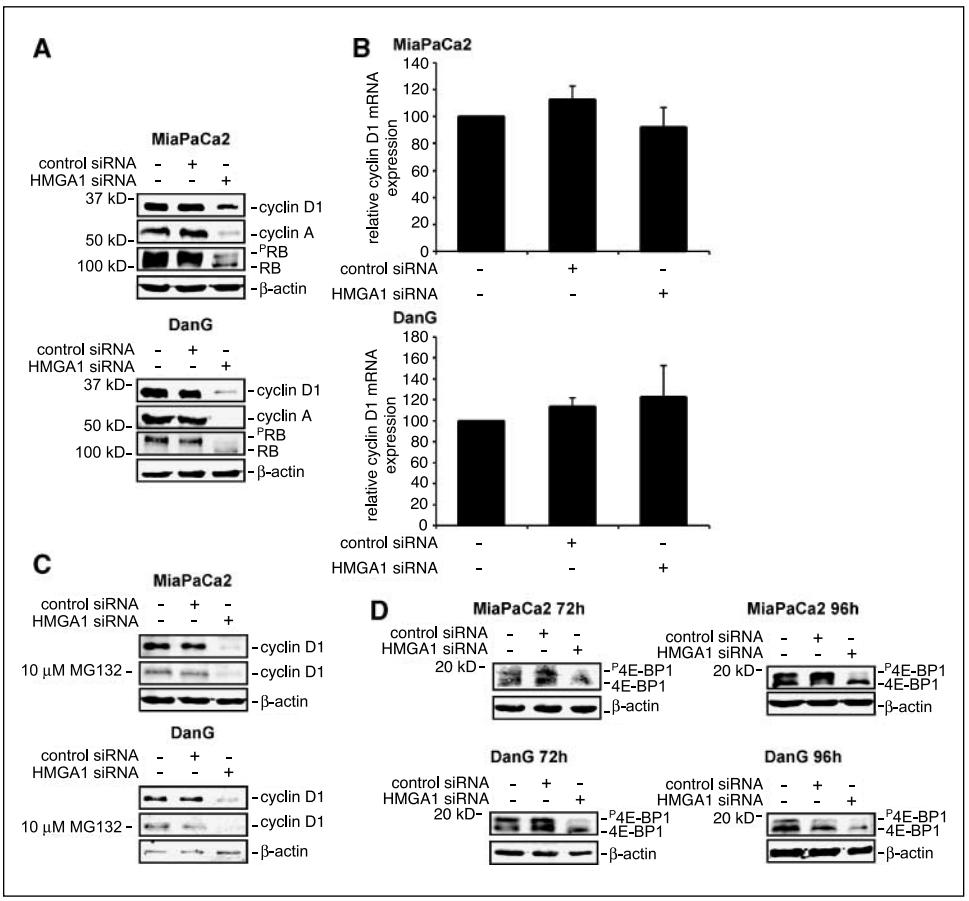

Figure 3. Cyclin D1 is down-regulated after the HMGA1 knockdown. *A,* MiaPaCa2 and DanG cells were transfected with no siRNA, a control, and a HMGA1-specific siRNA. Seventy-two hours after the transfection, Western blot analyses show the expression of cyclin D1, cyclin A, and the phosphorylation status of RB. β-Actin control equals protein loading. *B,* MiaPaCa2 and DanG cells were transfected as indicated. Cyclin D1 mRNA levels were quantified using real-time PCR analysis and normalized to cyclophilin expression levels. $P > 0.05$ versus controls by Student's t test. *C,* MiaPaCa2 and DanG cells were transfected as indicated. After 60 h, half of the cells were incubated with 10 μmol MG132. Seventy-two hours after the transfection, Western blot analysis displays the expression of cyclin D1. β-Actin control equals protein loading. *D,* MiaPaCa2 and DanG cells were transfected as indicated. About 72 and 96 h after the transfection, Western blot analyses show the phosphorylation status of 4E-BP1. β-Actin control equals protein loading.

of PDAC cells. Because the HMGA1 knockdown revealed a G_1-phase arrest in both cell lines investigated, we characterized the molecular mechanism in detail.

HMGA1 regulates translation of cyclin D1. To investigate the observed G_1-phase arrest after the knockdown of HMGA1, we did Western blot analysis of key G_1-phase regulators. Compatible to the accumulation of the cells in the G_1 phase, a decrease of cyclin A protein levels and hypophosphorylation of the tumor suppressor RB was observed 72 h after the transfection of the HMGA1-specific siRNA in MiaPaCa2 and DanG cells (Fig. 3*A*). RB is phosphorylated and inactivated by the complex interaction of G_1 cyclins with CDK4 and CDK6. In this line, a distinct reduction of cyclin D1 protein expression was detected 72 h after the transfection of the HMGA1-specific siRNA in MiaPaCa2 and DanG cells (Fig. 3*A*) compared with untransfected or control siRNA-transfected cells. In addition, 96 h after the transfection of the HMGA1-specific siRNA, cyclin D1 protein abundance was reduced (Supplementary Fig. S1*A*). These data propose that HMGA1 controls G_1-phase progression by regulating the protein level of cyclin D1 and, therefore, also the RB-dependent G_1-phase checkpoint.

The regulation of cyclin D1 protein abundance can be controlled at the transcriptional, the translational, and the degradation level. Because HMGA1 is known to act as a transcriptional regulator, we investigated the cyclin D1 mRNA level. About 72 and 96 h after transfection with the HMGA1-specific siRNA, no significant down-regulation of cyclin D1 mRNA was observed in MiaPaCa2 cells (Fig. 3*B* and Supplementary Fig. S1*B*). In DanG cells, we observed up-regulation of cyclin D1 mRNA expression after the HMGA1 knockdown (Fig. 3*B* and Supplementary Fig. S1*B*). Because we observed no down-regulation of cyclin D1 mRNA abundance in both cell lines investigated, HMGA1-dependent transcription of the cyclin D1 gene in PDAC cells is unlikely to explain impaired cyclin D1 protein expression. To determine whether the decrease of cyclin D1 protein is caused by enhanced degradation, we used the proteasome inhibitor MG132. Because the down-regulation of cyclin D1 protein level induced by HMGA1 siRNA transfection was independent of MG132 treatment in MiaPaCa2 and DanG cells (Fig. 3*C*), a translational mechanism is likely to cause the observed cyclin D1 regulation. Translational control of cyclin D1 occurs via the eIF4E binding protein (4E-BP1). To analyze whether the reduction of cyclin D1 proteins involves 4E-BP1, Western blots were done. In MiaPaCa2 cells, hypophosphorylated, active 4E-BP1 was detected 72 and 96 h after the knockdown of HMGA1 compared with untransfected or control siRNA-transfected cells (Fig. 3*D*). Similar, hypophosphorylated 4E-BP1 was found in DanG cells 72 and 96 h after the transfection of the HMGA1-specific siRNA (Fig. 3*D*). These experiments show that the HMGA1 knockdown results in an impaired phosphorylation of 4E-BP1.

HMGA1 controls transcription of the IR. The phosphorylation status of 4E-BP1 is known to be regulated by the IR (22). Additionally, HMGA1 proteins have been shown to control *IR* gene transcription in concert with the C/EBPβ transcription factor (23, 24). To evaluate whether the *IR* is also controlled by HMGA1 proteins in PDAC cells, we analyzed IR mRNA and protein expression levels after HMGA1 siRNA transfection. Real-time PCR revealed that *IR* mRNA expression was reduced to 41.3% in MiaPaCa2 cells and 25.8% in DanG cells (Fig. 4*A*) compared with untransfected and control siRNA-transfected cells. Furthermore, as

shown by Western blot analysis, IR protein levels were reduced in MiaPaCa2 and DanG cells (Fig. 4*B*) 72 and 96 h after the transfection of the HMGA1-specific siRNA. To detect binding of HMGA1 to the *IR* gene promoter, we did ChIP experiments using primers specific for the E3 region, known to be bound by HMGA1 and C/EBPβ. As shown in Fig. 4*C*, binding of HMGA1 to the E3 *IR* gene promoter region was significantly reduced 72 h after the transfection of MiaPaCa2 and DanG cells (Fig. 4*C*) with the HMGA1-specific siRNA. Furthermore, C/EBPβ binding to the E3 region of the *IR* gene promoter was significantly reduced after the HMGA1 knockdown in MiaPaCa2 and DanG cells (Fig. 4*C*). These data suggest that HMGA1 recruits C/EBPβ to the *IR* promoter to control *IR* gene transcription.

The IR controls cyclin D1 translation. To show whether cyclin D1 translation is downstream of the IR in PDAC cells, we used an IR-specific siRNA. Seventy-two hours after the transfection of the IR-specific siRNA, IR mRNA levels were reduced to 33% in MiaPaCa2 cells and to 9.4% in DanG cells (Fig. 5*A*) compared with untransfected and control-transfected cells. In agreement with the observed mRNA decline, IR protein levels were considerably reduced after the IR knockdown in MiaPaCa2 and DanG cells (Fig. 5*D*). In line with the HMGA1 knockdown, impaired proliferation after the transfection of the IR-specific siRNA was detected. BrdUrd incorporation after IR knockdown was reduced to 52.9% in MiaPaCa2 cells and to 59.1% in DanG cells (Fig. 5*B*) compared with untransfected and control siRNA-transfected cells. Furthermore, we detected reduced cyclin D1 protein levels 72 h after transfection of the IR siRNA into MiaPaCa2 and DanG cells (Fig. 5*D*). In contrast, only a marginal decrease of cyclin D1 mRNA abundance after the IR siRNA transfection was observed in MiaPaCa2 (Fig. 5*C*). In DanG cells, cyclin D1 mRNA levels were slightly increased after IR knockdown (Fig. 5*C*). 4E-BP1 is hypophosphorylated and active after HMGA1 siRNA transfection. Consequently, we examined whether the IR knockdown also influences the phosphorylation status of 4E-BP1. In agreement with our previous observations, the IR knockdown inhibited the phosphorylation of 4E-BP1 in MiaPaCa2 and DanG cells (Fig. 5*D*). These data suggest that IR signaling controls cyclin D1 translation in PDAC cells.

Discussion

HMGA1 proteins are involved in the transcriptional regulation of several genes important in carcinogenesis. Interestingly, they do not directly act as transcription factors, but are able to induce allosteric changes in the regulated promoter regions. In this virtue, they facilitate the assembly and the binding of corresponding transcription factors to form a multiprotein complex named the enhanceosome. Using their three independent acting basic AT-hook motifs, they can stabilize protein-protein as well as protein-DNA interactions (27). HMGA1 overexpression has become a diagnostic marker of neoplastic transformation and increased

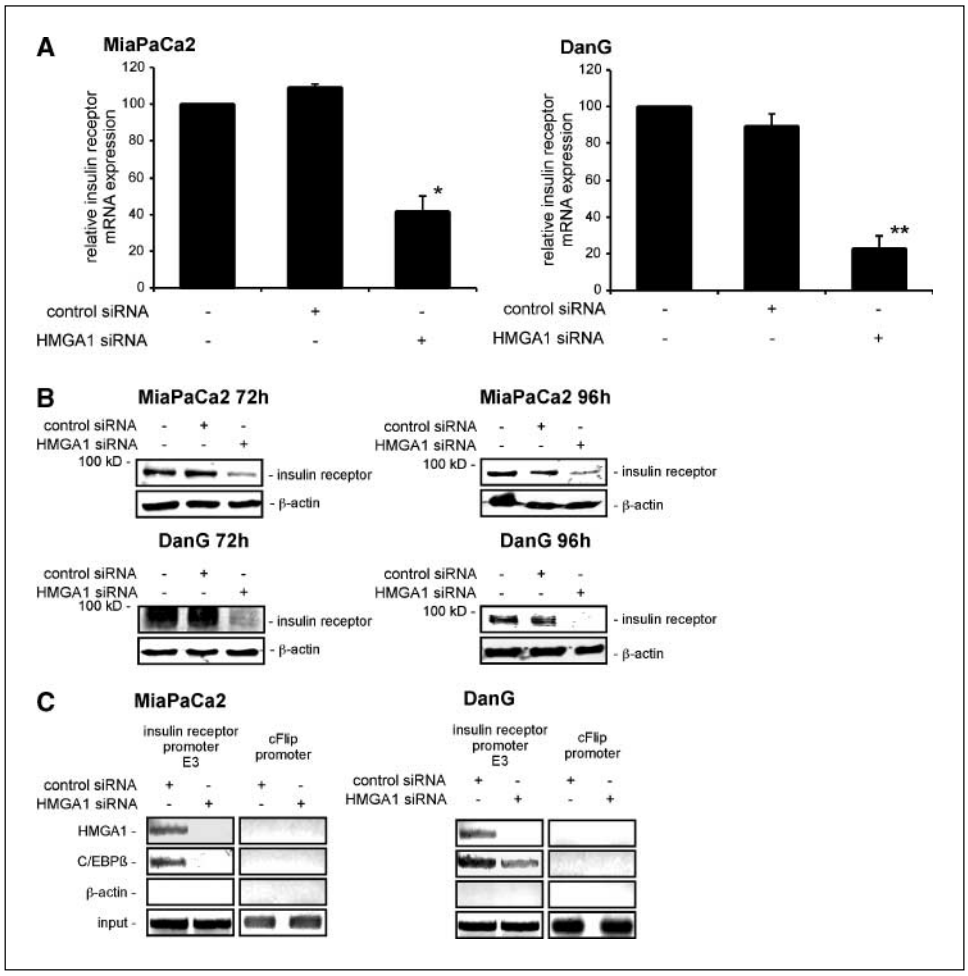

Figure 4. HMGA1 controls IR transcription. *A,* MiaPaCa2 and DanG cells were transfected as indicated. Seventy-two hours after the transfection, IR mRNA levels were quantified using real-time PCR analysis and normalized to cyclophilin expression levels. *, $P < 0.05$; **, $P < 0.001$ versus controls by Student's *t* test. *B,* MiaPaCa2 and DanG cells were transfected as indicated. About 72 and 96 h after the transfection, Western blot analysis shows the expression of the IR. β-Actin control equals protein loading. *C,* MiaPaCa2 and DanG cells were transfected as indicated. Seventy-two hours after the transfection, chromatin was immunoprecipitated with a HMGA1-specific antibody, a C/EBPβ-specific antibody, or a β-actin antibody as a negative control. Precipitated DNA or 10% of the chromatin input was amplified with gene-specific primers for the E3 region of the *IR* promoter or the c-Flip promoter.

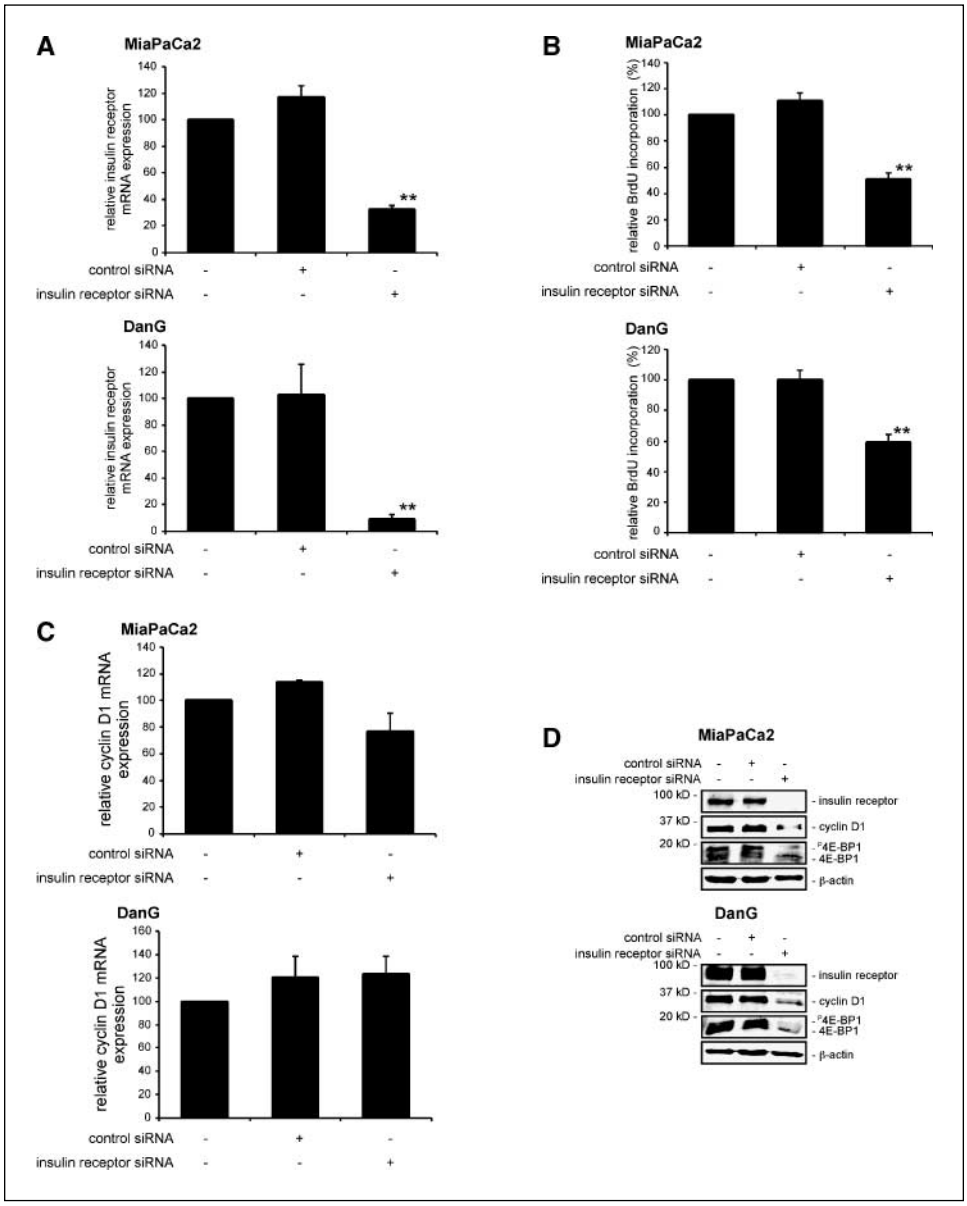

Figure 5. Insulin receptor and HMGA1 knockdown display similar phenotypes. *A,* MiaPaCa2 and DanG cells were transfected as indicated. Seventy-two hours after the transfection, IR mRNA levels were quantified using real-time PCR analysis and normalized to cyclophilin expression levels. *, $P < 0.001$ versus controls by Student's *t* test. *B,* MiaPaCa2 and DanG cells were transfected as indicated. BrdUrd incorporation was determined 72 h after the transfection. *, $P < 0.001$ versus controls by Student's *t* test. *C,* MiaPaCa2 and DanG cells were transfected as indicated. Cyclin D1 levels were quantified using real-time PCR analysis and normalized to cyclophilin expression levels. $P > 0.05$ versus controls by Student's *t* test. *D,* MiaPaCa2 and DanG cells were transfected as indicated. Seventy-two hours after the transfection, Western blot analysis displays the expression of the IR, cyclin D1, and the phosphorylation status of 4E-BP1. β-Actin control equals protein loading.

metastatic potential (2, 28). HMGA1 proteins are also overexpressed in PDAC (8, 9). Although Trapasso et al. reported that treatment of PDAC cells with HMGA1-specific antisense phosphorothioate oligodeoxynucleotides reduced proliferation, the exact molecular mechanism regulated by HMGA1 to control proliferation of PDAC cells is unknown. Herein, we show that the siRNA-mediated knockdown of HMGA1 leads to a significant reduction of cell proliferation of PDAC cells due to a G_1-phase arrest. Furthermore, we show that HMGA1 controls cyclin D1 translation indirectly by transcriptional regulation of the IR gene. Because titration of the HMGA1 siRNA shows dose dependency of the molecular events regulated by HMGA1 (Supplementary Fig. S2) and two different HMGA1-specific siRNA provoke identical molecular changes (Supplementary Fig. S3), we describe a HMGA1-specific pathway.

Progression through the G_1 phase of the cell cycle is mediated by several proteins, including cyclins, CDK, CDK inhibitors, and pocket proteins. Cyclin D1 has been found to be frequently overexpressed in pancreatic cancer, and this overexpression was shown to be associated with poor prognosis (12–14, 29). Microarray analyses of human breast epithelial cells, overexpressing HMGA1, revealed that this overexpression results in a 3.1-fold increase in cyclin D1 mRNA content, suggesting a transcriptional regulation of cyclin D1 (28). Although we found a HMGA1 binding site in the cyclin D1 promoter, bound by HMGA1 with high affinity in electrophoretic mobility shift assay experiments (data not shown), no down-regulation of cyclin D1 mRNA after the HMGA1 knockdown was detected. These findings suggest that the down-regulation of cyclin D1 protein abundance after the HMGA1 knockdown is not due to transcription in our experimental model. In addition to transcriptional control, cyclin D1 turnover is regulated by the ubiquitin-proteasome pathway (16). Because cyclin D1 is down-regulated by the HMGA1 knockdown independently of the proteasome inhibition, HMGA1 does not seem to regulate protein turnover. Inhibition of the proteasome induces apoptosis starting after 12 h of treatment in PDAC cells (data not

shown). Due to this experimental limitation, we investigated only one time point in the proteasome inhibitor experiment, and therefore, we cannot rule out changes of the cyclin D1 degradation pathway after the HMGA1 knockdown completely.

HMGA1 was found to be specifically phosphorylated by the cdc2 kinase in late G_2 phase of the cell cycle, arguing for contribution of HMGA1 in the control of G_2- to M-phase progression (30). Because we observed the accumulation of cells in the G_2-M phase of the cell cycle after the HMGA1 knockdown only in DanG cells, HMGA1-dependent G_2-M-phase control seems to be cell type specific.

Translation control of most eukaryotic mRNAs occurs in a cap-dependent manner. Formation of the eukaryotic initiation factor 4F (eIF4F) complex is rate limited by the cap structure binding eIF4E (31–33). Cyclin D1 mRNA translation is also mediated by eIF4E (20, 21). Binding of eIF4E binding proteins (4E-BP, also known as PHAS-I for phosphorylated heat and acid-soluble protein stimulated by insulin) to eIF4E inhibit cap-dependent translation (34). This binding is regulated through phosphorylation in a PI3K/Akt-dependent pathway: Hypophosphorylated 4E-BPs are strongly attached to eIF4E, whereas hyperphosphorylation leads to a reduction of the binding affinity (35). We show in this study that the siRNA-mediated knockdown of HMGA1 protein synthesis leads to a reduced phosphorylation of 4E-BP1. Therefore, eIF4E is bound to 4E-BP1, becomes inactivated, and leads to a translational inhibition of cyclin D1 expression.

Induction of a proliferative response through the IR has been shown in various transformed cells. Furthermore, the IR is overexpressed in epithelial tumors, like breast cancers, colon cancers, or ovarian cancers (36–39). Also, in PDAC cells, insulin induces a proliferative response (40). In addition, insulin signaling induces PI3K activity in PDAC cells (41). Interestingly, the IR substrate 1 (IRS-1) is overexpressed in and contributes to the unrestrained growth of PDAC cells (42). Whereas Asano et al. (43) recently reported that IRS-1 mediates PI3K activation in PDAC cells, the receptor responsible for the IRS-1 phosphorylation is not known. Because we observed that the IR is involved in the control of proliferation of pancreatic cancer cells and the insulin-like growth factor-I receptor was not immunoprecipitated with IRS-1 in the study of Asano et al., it is attractive to speculate that the IR contributes to PI3K activation in pancreatic cancer cells (43).

Insulin stimulates phosphorylation of 4E-BP1 and, thus, induces translation (44). Several studies have shown that HMGA1 proteins are involved in the transcriptional regulation of the *IR* (23, 24, 45, 46). Together with C/EBPβ and SP1, HMGA1 proteins form a nucleoprotein complex that binds to two distinct regions (E3 and C2) of the IR promoter (23). In our work, we showed that the knockdown of HMGA1 proteins results in significant decrease of both IR mRNA and protein expression. Furthermore, using ChIP techniques, we showed that HMGA1 proteins as well as C/EBPβ bind to the E3 promoter region of the *IR* gene in PDAC cells. HMGA1 seems to increase C/EBPβ recruitment to the *IR* promoter because HMGA1 knockdown impairs C/EBPβ binding to the E3 region of the *IR* promoter, thereby contributing to *IR* transcription.

In summary, our results revealed that HMGA1 promote cell cycle progression of PDAC cells by a rather indirect mechanism. Activating the *IR* promoter leads to an increase of cyclin D1 expression and inactivation of an RB-dependent G_1-phase checkpoint. According to the fact that the overexpression of both HMGA1 and cyclin D1 are hallmarks of PDAC, lowering HMGA1 abundance is a potential therapeutic strategy.

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References

1. Reeves R, Beckerbauer LM. HMGA proteins as therapeutic drug targets. Prog Cell Cycle Res 2003;5:279–86.
2. Sgarra R, Rustighi A, Tessari MA, et al. Nuclear phosphoproteins HMGA and their relationship with chromatin structure and cancer. FEBS Lett 2004;574:1–8.
3. Reeves R. Molecular biology of HMGA proteins: hubs of nuclear function. Gene 2001;277:63–81.
4. Wood LJ, Maher JF, Bunton TE, Resar LM. The oncogenic properties of the HMG-I gene family. Cancer Res 2000;60:4256–61.
5. Treff NR, Pouchnik D, Dement GA, Britt RL, Reeves R. High-mobility group A1a protein regulates Ras/ERK signaling in MCF-7 human breast cancer cells. Oncogene 2004;23:777–85.
6. Xu Y, Sumter TF, Bhattacharya R, et al. The HMG-I oncogene causes highly penetrant, aggressive lymphoid malignancy in transgenic mice and is overexpressed in human leukemia. Cancer Res 2004;64:3371–5.
7. Fedele M, Pentimalli F, Baldassarre G, et al. Transgenic mice overexpressing the wild-type form of the HMGA1 gene develop mixed growth hormone/prolactin cell pituitary adenomas and natural killer cell lymphomas. Oncogene 2005;24:3427–35.
8. Abe N, Watanabe T, Masaki T, et al. Pancreatic duct cell carcinomas express high levels of high mobility group I(Y) proteins. Cancer Res 2000;60:3117–22.
9. Tarbe N, Evtimova V, Burtscher H, Jarsch M, Alves F, Weidle UH. Transcriptional profiling of cell lines derived from an orthotopic pancreatic tumor model reveals metastasis-associated genes. Anticancer Res 2001;21:3221–8.
10. Trapasso F, Sarti M, Cesari R, et al. Therapy of human pancreatic carcinoma based on suppression of HMGA1 protein synthesis in preclinical models. Cancer Gene Ther 2004;11:633–41.
11. Rozenblum E, Schutte M, Goggins M, et al. Tumor-suppressive pathways in pancreatic carcinoma. Cancer Res 1997;57:1731–4.
12. Huang L, Lang D, Geradts J, et al. Molecular and immunochemical analyses of RB1 and cyclin D1 in human ductal pancreatic carcinomas and cell lines. Mol Carcinog 1996;15:85–95.
13. Gansauge S, Gansauge F, Ramadani M, et al. Overexpression of cyclin D1 in human pancreatic carcinoma is associated with poor prognosis. Cancer Res 1997;57:1634–7.
14. Kornmann M, Ishiwata T, Itakura J, Tangvoranuntakul P, Beger HG, Korc M. Increased cyclin D1 in human pancreatic cancer is associated with decreased postoperative survival. Oncology 1998;55:363–9.
15. Alt JR, Cleveland JL, Hannink M, Diehl JA. Phosphorylation-dependent regulation of cyclin D1 nuclear export and cyclin D1-dependent cellular transformation. Genes Dev 2000;14:3102–14.
16. Diehl JA, Zindy F, Sherr CJ. Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. Genes Dev 1997;11:957–72.
17. Diehl JA, Cheng M, Roussel MF, Sherr CJ. Glycogen synthase kinase-3β regulates cyclin D1 proteolysis and subcellular localization. Genes Dev 1998;12:3499–511.
18. Muise-Helmericks RC, Grimes HL, Bellacosa A, Malstrom SE, Tsichlis P, Rosen N. Cyclin D expression is controlled post-transcriptionally via a phosphatidylinositol 3-kinase/Akt-dependent pathway. J Biol Chem 1998;273:29864–72.
19. Koziczak M, Hynes NE. Cooperation between fibroblast growth factor receptor-4 and ErbB2 in regulation of cyclin D1 translation. J Biol Chem 2004;279:50004–11.
20. Rosenwald IB, Kaspar R, Rousseau D, et al. Eukaryotic translation initiation factor 4E regulates expression of cyclin D1 at transcriptional and post-transcriptional levels. J Biol Chem 1995;270:21176–80.
21. Rousseau D, Kaspar R, Rosenwald I, Gehrke L, Sonenberg N. Translation initiation of ornithine decarboxylase and nucleocytoplasmic transport of cyclin D1 mRNA are increased in cells overexpressing eukaryotic initiation factor 4E. Proc Natl Acad Sci U S A 1996;93:1065–70.
22. Hay N, Sonenberg N. Upstream and downstream of mTOR. Genes Dev 2004;18:1926–45.
23. Foti D, Iuliano R, Chiefari E, Brunetti A. A nucleoprotein complex containing Sp1, C/EBP β, and HMGI-Y controls human insulin receptor gene transcription. Mol Cell Biol 2003;23:2720–32.
24. Paonessa F, Foti D, Costa V, et al. Activator protein-2 overexpression accounts for increased insulin receptor expression in human breast cancer. Cancer Res 2006;66:5085–93.
25. Schneider G, Saur D, Siveke JT, Fritsch R, Greten FR, Schmid RM. IKKα controls p52/RelB at the skp2 gene

promoter to regulate G_1- to S-phase progression. EMBO J 2006;25:3801–12.
26. Johnson KR, Disney JE, Wyatt CR, Reeves R. Expression of mRNAs encoding mammalian chromosomal proteins HMG-I and HMG-Y during cellular proliferation. Exp Cell Res 1990;187:69–76.
27. Thanos D, Du W, Maniatis T. The high mobility group protein HMG I(Y) is an essential structural component of a virus-inducible enhancer complex. Cold Spring Harb Symp Quant Biol 1993;58:73–81.
28. Reeves R, Edberg DD, Li Y. Architectural transcription factor HMGI(Y) promotes tumor progression and mesenchymal transition of human epithelial cells. Mol Cell Biol 2001;21:575–94.
29. Diehl JA, Benzeno S. Cyclin D1 and pancreatic carcinoma: a proliferative agonist and chemotherapeutic antagonist. Clin Cancer Res 2005;11:5665–7.
30. Nissen MS, Langan TA, Reeves R. Phosphorylation by cdc2 kinase modulates DNA binding activity of high mobility group I nonhistone chromatin protein. J Biol Chem 1991;266:19945–52.
31. Gingras AC, Raught B, Sonenberg N. eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annu Rev Biochem 1999; 68:913–63.
32. Dever TE. Gene-specific regulation by general translation factors. Cell 2002;108:545–56.
33. Gingras AC, Raught B, Sonenberg N. Regulation of translation initiation by FRAP/mTOR. Genes Dev 2001; 15:807–26.
34. Mamane Y, Petroulakis E, Rong L, Yoshida K, Ler LW, Sonenberg N. eIF4E-from translation to transformation. Oncogene 2004;23:3172–9.
35. Gingras AC, Gygi SP, Raught B, et al. Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. Genes Dev 1999;13:1422–37.
36. Massague J, Blinderman LA, Czech MP. The high affinity insulin receptor mediates growth stimulation in rat hepatoma cells. J Biol Chem 1982;257:13958–63.
37. Straus DS. Growth-stimulatory actions of insulin *in vitro* and *in vivo*. Endocr Rev 1984;5:356–69.
38. Giorgino F, Belfiore A, Milazzo G, et al. Overexpression of insulin receptors in fibroblast and ovary cells induces a ligand-mediated transformed phenotype. Mol Endocrinol 1991;5:452–9.
39. Milazzo G, Giorgino F, Damante G, et al. Insulin receptor expression and function in human breast cancer cell lines. Cancer Res 1992;52:3924–30.
40. Fisher WE, Boros LG, Schirmer WJ. Insulin promotes pancreatic cancer: evidence for endocrine influence on exocrine pancreatic tumors. J Surg Res 1996;63:310–3.
41. Kornmann M, Maruyama H, Bergmann U, et al. Enhanced expression of the insulin receptor substrate-2 docking protein in human pancreatic cancer. Cancer Res 1998;58:4250–4.
42. Bergmann U, Funatomi H, Kornmann M, Beger HG, Korc M. Increased expression of insulin receptor substrate-1 in human pancreatic cancer. Biochem Biophys Res Commun 1996;220:886–90.
43. Asano T, Yao Y, Shin S, McCubrey J, Abbruzzese JL, Reddy SA. Insulin receptor substrate is a mediator of phosphoinositide 3-kinase activation in quiescent pancreatic cancer cells. Cancer Res 2005;65:9164–8.
44. Lawrence JC, Jr., Abraham RT. PHAS/4E-BPs as regulators of mRNA translation and cell proliferation. Trends Biochem Sci 1997;22:345–9.
45. Brunetti A, Manfioletti G, Chiefari E, Goldfine ID, Foti D. Transcriptional regulation of human insulin receptor gene by the high-mobility group protein HMGI(Y). FASEB J 2001;15:492–500.
46. Foti D, Chiefari E, Fedele M, et al. Lack of the architectural factor HMGA1 causes insulin resistance and diabetes in humans and mice. Nat Med 2005;11: 765–73.

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