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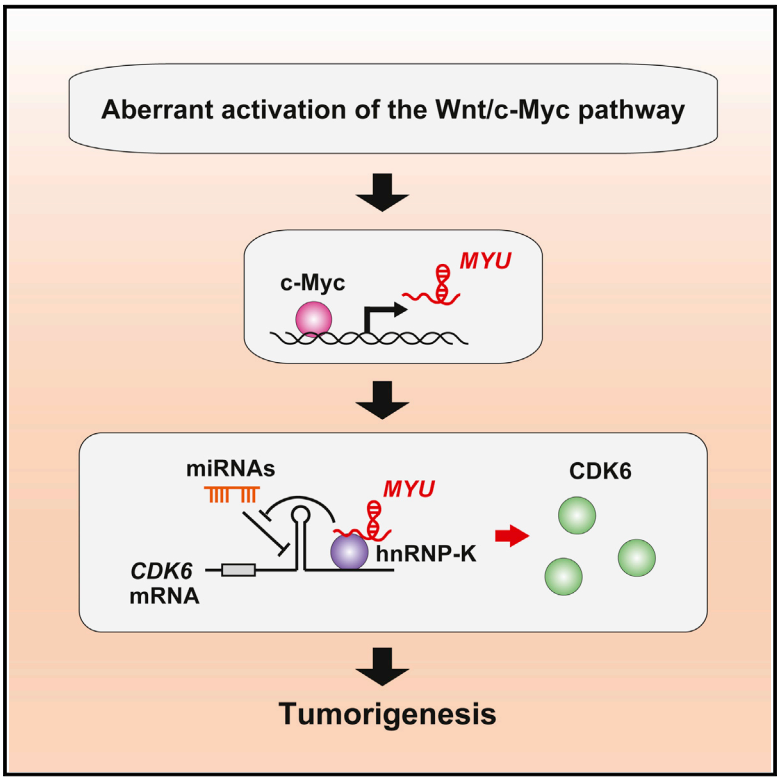
MYU, a Target lncRNA for Wnt/c-Myc Signaling, Mediates Induction of CDK6 to Promote Cell Cycle Progression

Graphical Abstract



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In Brief

Kawasaki et al. identify a direct target of the Wnt/c-Myc pathway, *MYU* (c-Myc-upregulated long non-coding RNA) and show that Wnt/c-Myc/*MYU*-mediated upregulation of CDK6 is essential for the proliferation of colon cancer cells.

Highlights

- *MYU* is identified as a direct target lncRNA of the Wnt/c-Myc pathway
- *MYU* is required for the tumorigenicity of colon cancer cells
- *MYU* associates with the RNA-binding protein hnRNP-K to stabilize CDK6 expression
- *MYU*-mediated stabilization of CDK6 is critical for the growth of colon cancer cells

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MYU, a Target lncRNA for Wnt/c-Myc Signaling, Mediates Induction of CDK6 to Promote Cell Cycle Progression

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SUMMARY

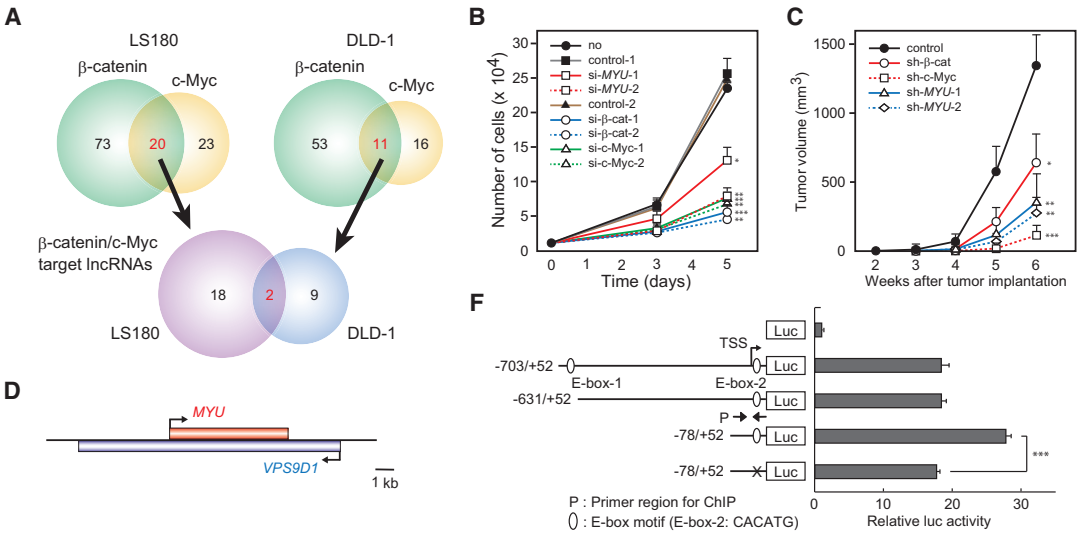
Aberrant activation of Wnt/β-catenin signaling is a major driving force in colon cancer. Wnt/β-catenin signaling induces the expression of the transcription factor c-Myc, leading to cell proliferation and tumorigenesis. c-Myc regulates multiple biological processes through its ability to directly modulate gene expression. Here, we identify a direct target of c-Myc, termed *MYU*, and show that *MYU* is upregulated in most colon cancers and required for the tumorigenicity of colon cancer cells. Furthermore, we demonstrate that *MYU* associates with the RNA binding protein hnRNP-K to stabilize CDK6 expression and thereby promotes the G1-S transition of the cell cycle. These results suggest that the *MYU*/hnRNP-K/CDK6 pathway functions downstream of Wnt/c-Myc signaling and plays a critical role in the proliferation and tumorigenicity of colon cancer cells.

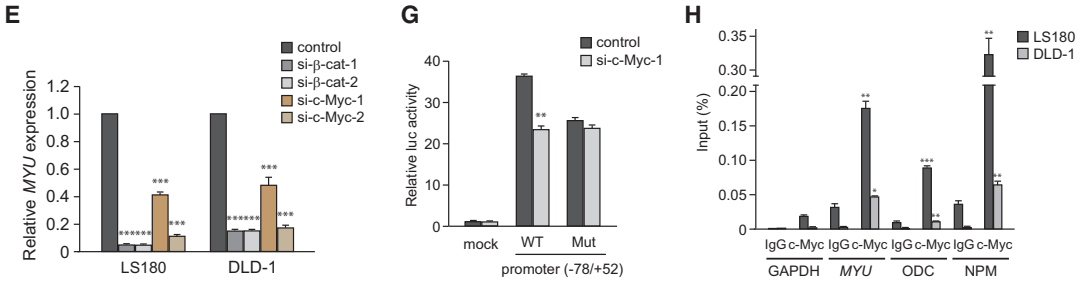
INTRODUCTION

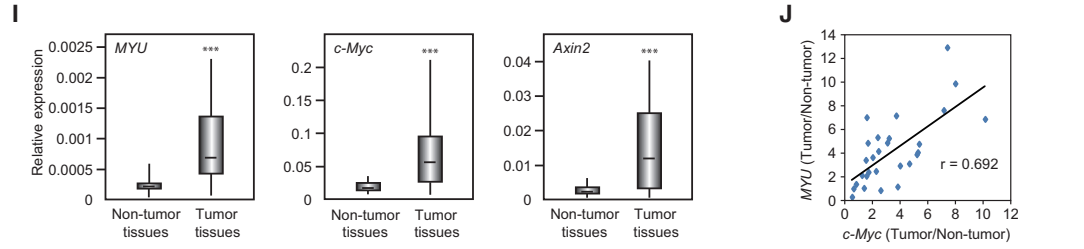
The canonical Wnt signaling pathway has been implicated in development and tumorigenesis (Bienz, 2005; Clevers and Nusse 2012; Pronobis and Peifer, 2012). β-Catenin, a key component of this pathway, is normally phosphorylated and targeted for degradation by the destruction complex, which contains Axin and the tumor suppressor adenomatous polyposis coli (APC). Wnt stimulation releases β-catenin from the destruction complex, allowing it to accumulate and migrate to the nucleus, where it interacts with T cell factor (TCF)/lymphoid enhancer factor (LEF) transcription factors to stimulate the transcription of Wnt target genes. The vast majority of colorectal cancers are caused by mutations in APC or β-catenin, characterized by the stabilization of β-catenin and constitutive activation of Wnt signaling (Anastas and Moon, 2013; Kinzler and Vogelstein, 1996; Polakis, 2012).

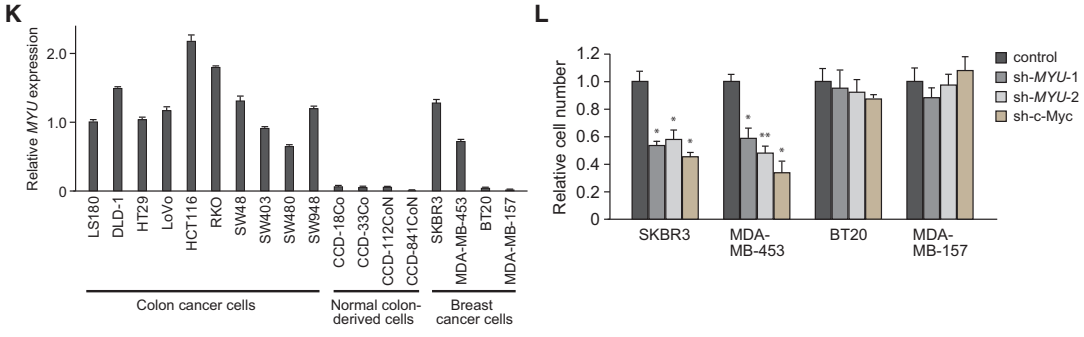
c-Myc is one of the Wnt target genes that play a critical role in the development of colorectal cancer. c-Myc is also known to be upregulated in multiple cancers by a variety of mechanisms, including amplification and translocation. c-Myc is a basic helix-loop-helix leucine zipper (bHLH/LZ) transcription factor that controls the expression of up to 15% of genes in the genome and regulates various cellular processes such as proliferation, differentiation, and apoptosis (Bretones et al., 2015; Cole and Cowling, 2008; Dang, 2012; Eilers and Eisenman, 2008; Kress et al., 2015; Meyer and Penn, 2008; Pelengaris et al., 2002; Rahl and Young, 2014; Sabò and Amati, 2014; Schmitz et al., 2014; van Riggelen et al., 2010; Zeller et al., 2006). Activation of transcription by c-Myc depends on heterodimerization with Max, which also belongs to the bHLH/LZ family. c-Myc/Max heterodimers bind to consensus (CACGTG) as well as to non-consensus E-box sequences (e.g., CACATG and CATGTG) in the regulatory regions of its targets genes (Adhikary and Eilers, 2005; Zeller et al., 2006). Furthermore, indirect recruitment of c-Myc/Max to DNA via the zinc-finger protein Miz-1 leads to E-box-independent repression of c-Myc-regulated genes (Wiese et al., 2013; Walz et al., 2014).

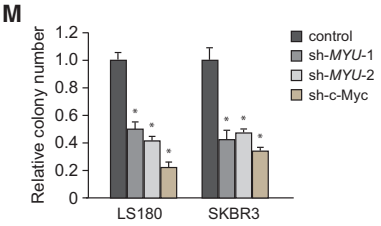
In the present study, we show that *MYU*, a direct target of the Wnt/c-Myc pathway, is upregulated in most colon cancers and required for the tumorigenicity of colon cancer cells. Furthermore, we demonstrate that *MYU* associates with the RNA-binding protein heterogeneous nuclear ribonucleoprotein-K (hnRNP-K) to stabilize cyclin-dependent kinase 6 (CDK6) expression, thereby promoting the G1-S transition of the cell cycle. Taken together, these results suggest that the *MYU*/hnRNP-K/CDK6 pathway functions downstream of Wnt/c-Myc signaling and is crucial for colorectal tumorigenesis.











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RESULTS

MYU, a Direct Target of c-Myc, Is Required for the Tumorigenicity of Colon Cancer Cells

To obtain new insights into the role of c-Myc in tumorigenesis, we attempted to identify the target genes of c-Myc that are responsible for cell growth. It has been reported that comparable numbers of c-Myc-binding sites are present in non-coding genes as well as in protein-coding genes (Cawley et al., 2004). Furthermore, it has recently been shown that a number of long non-coding RNAs (lncRNAs) play critical roles in tumor development and progression (Batista and Chang, 2013; Yanagida et al., 2013; Taniue et al., 2016). These findings raise the possibility that lncRNAs may also play key roles in c-Myc-mediated biological activities. Indeed, RNA sequencing (RNA-seq) analysis showed that knockdown of c-Myc expression by small interfering RNA (siRNA) resulted in the downregulation of 43 lncRNAs in LS180 colon cancer cells (Figure 1A; Table S1). We also found that knockdown of β-catenin led to the downregulation of 93 lncRNAs in LS180 cells. From these data, we selected 20 overlapping lncRNAs as potential targets whose genes are modulated by Wnt/c-Myc signaling. Furthermore, we performed similar experiments using DLD-1 colon cancer cells and selected 11 lncRNAs as potential Wnt/c-Myc targets (Figure 1A). Comparison of the data from LS180 and DLD-1 cells revealed that only two lncRNAs are commonly upregulated in both cell lines as potential Wnt/c-Myc targets.

Analysis of ENCODE (Encyclopedia of DNA Elements) multiple c-Myc chromatin immunoprecipitation sequencing (ChIP-seq) datasets revealed that c-Myc is associated with the promoter regions (−2 kb from the transcriptional start sites [TSS]) of 13 of 20 β-catenin/c-Myc target lncRNAs identified using LS180 cells (Table S1). To confirm this, we performed ChIP assays and observed that c-Myc binds to the promoter regions of 8 of 13 β-catenin/c-Myc target lncRNAs examined, including two lncRNAs, LOC100499227 and LC100128881, which are commonly upregulated in both LS180 and DLD-1 cells (Figure S1A; Table S1).

We then examined the potential role of these two lncRNAs in the proliferation of colon cancer cells. We found that siRNA knockdown of one of the two lncRNAs, termed *MYU* (c-Myc-upregulated lncRNA, LOC100128881, NCBI: NR_036480.1), resulted in reduced proliferation of LS180 cells (Figure 1B). We infected LS180 cells with a lentivirus expressing a short hairpin RNA (shRNA)-targeting *MYU* and transplanted these into nude mice. The growth of these tumor cells was markedly retarded compared with tumor cells infected with control virus (Figures 1C, S1B, and S1C). In parallel experiments, knockdown of either β-catenin or c-Myc also resulted in reduced proliferation and tumorigenicity of LS180 cells. These results suggest that *MYU* is required for the tumorigenicity of colon cancer cells. Thus, we focused our analysis on the function of *MYU*.

MYU is generated from the opposite strand of the *VPS9D1* gene (Figure 1D). RT-PCR analysis revealed that the majority of the *MYU* transcripts were localized in the cytoplasm (Figures S1D and S1E). We first confirmed that knockdown of β-catenin or c-Myc led to reductions in the amounts of *MYU* in LS180 and DLD-1 colon cancer cells (Figure 1E). To clarify the mechanisms underlying c-Myc-mediated upregulation of *MYU*, we performed luciferase assays with reporter constructs containing various fragments of the *MYU* promoter region. Deletion of one of the two E-boxes (E-box-1 in Figure 1F) did not have any significant effect on promoter activity, but mutations in the other E-box (E-box-2) caused a reduction in activity (Figure 1F). Inclusion of the region between −631 and −78 led to a decrease in luciferase activity, suggesting that a negative regulatory element(s) is present in this region. Furthermore, knockdown of c-Myc reduced the activity of a promoter region containing wild-type (WT) but not mutated (Mut) E-box-2 (Figure 1G). In addition, ChIP assays showed that c-Myc specifically associated with this region (Figures 1H and S1F), which is also associated with highly

Figure 1. *MYU* Is Required for the Tumorigenicity of Colon Cancer Cells

(A) Experimental layout to identify Wnt/c-Myc target lncRNAs. The top Venn diagrams represent the overlap of lncRNAs downregulated (>2-fold, FPKM >0.5) by knockdown of β-catenin (green) or c-Myc (orange) in LS180 and DLD-1 cells. The bottom Venn diagram shows target lncRNAs of β-catenin/c-Myc in LS180 (purple) and DLD-1 (blue) cells. The number of lncRNAs within each category is indicated.

(B) Proliferation of LS180 cells transfected with the indicated siRNAs or negative controls (control-1 for *MYU* siRNAs and control-2 for β-catenin [β-cat] and c-Myc siRNAs) or not transfected with any siRNA (no).

(C) LS180 cells infected with the indicated lentivirus were subcutaneously injected into nude mice (n = 8/group).

(D) Schematic of the *MYU* locus.

(E) qRT-PCR analysis of *MYU* expression in LS180 and DLD-1 cells transfected with the indicated siRNA.

(F) Left: schematic of reporter constructs used for luciferase assays. Right: LS180 cells were transfected with reporter constructs containing *MYU* promoter sequences and subjected to luciferase assays.

(G) LS180 cells that had been treated with the indicated siRNA were transfected with an *MYU* reporter construct containing either WT or Mut E-box-2, and activity was monitored by luciferase assay.

(H) ChIP assays were performed using anti-c-Myc antibody or rabbit immunoglobulin G (IgG). The promoter regions of ornithine decarboxylase (ODC) and nucleoplasmin (NPM) were used as positive controls. The promoter region of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a negative control.

(I) qRT-PCR analysis of *MYU*, *c-Myc*, and *Axin2* expression in human colon cancer tissues (n = 28 pairs). *Axin2* was used as a marker of β-catenin-TCF4-mediated activation.

(J) Relationship between the increases in *MYU* and *c-Myc* transcripts in colon cancer.

(K) qRT-PCR analysis of *MYU* expression in the indicated cell lines.

(L) Breast cancer cells infected with the indicated lentivirus were cultured for 7 days, and the number of cells was counted.

(M) LS180 and SKBR3 cells were infected with the indicated lentivirus and subjected to colony formation assays in soft agar.

All data represent mean ± SEM (n = 3–6). *p < 0.05, **p < 0.01, ***p < 0.001.

trimethylated histone H3 lysine 4 (H3K4me3) (Figures S1F and S1G). Thus, c-Myc may directly upregulate the transcription of *MYU* by binding to E-box-2, the sequence of which (CACATG) is identical to that of the non-consensus c-Myc-binding motif. Taken together, these results suggest that *MYU* is a direct target of Wnt/c-Myc signaling in colon cancer cells.

MYU Plays a Critical Role in c-Myc-Dependent Proliferation

Consistent with the above results, *MYU* expression was upregulated in human colorectal cancer specimens, similar to c-Myc and Axin2 expression (Figure 1I). We also detected a significant correlation between increased *MYU* and c-Myc transcript levels in cancers (Figure 1J), although no significant relationship was detected between *MYU* expression and histological grade/prognosis (data not shown). *MYU* was also highly expressed in colorectal cancer cell lines compared with normal colon-derived fibroblast cells (Figure 1K). We also found that knockdown of *MYU* barely affected the growth of CCD-112CoN normal colon fibroblast cells (Figures S1H and S1I). Thus, *MYU* may play an essential role in tumor cells overexpressing c-Myc.

To confirm this possibility, we examined the effect of *MYU* knockdown on the growth of two types of breast cancer cell lines that express high levels or low levels of c-Myc. It has been shown that knockdown of c-Myc results in a significant reduction in the growth of breast cancer cells overexpressing c-Myc, such as SKBR3 and MDA-MB-453 cells, but had little effect on cells expressing c-Myc at low levels, such as BT20 and MDA-MB-157 cells (Kang et al., 2014). In line with these results, we found that *MYU* as well as c-Myc were highly expressed in SKBR3 and MDA-MB-453 cells compared with BT20 and MDA-MB-157 cells (Figures 1K and S1J). Knockdown of either *MYU* or c-Myc resulted in growth inhibition of only SKBR3 and MDA-MB-453 cells (Figures 1L, S1K, and S1L). Colony formation assays with LS180 and SKBR3 cells in soft agar showed that knockdown of either *MYU* or c-Myc caused a significant reduction in their clonogenicity (Figure 1M). These results suggest that *MYU* plays a critical role in c-Myc-dependent proliferation.

Identification of CDK6 as a Target of the Wnt/c-Myc/*MYU* Pathway

To identify target molecules downstream in the Wnt/c-Myc/*MYU* pathway, we used siRNA to suppress β-catenin, c-Myc, or *MYU* expression in LS180 and DLD1 cells and then examined their gene expression profiles. DNA microarray and gene ontology analyses indicated that the target genes regulated in common by Wnt, c-Myc, and *MYU* were enriched for those involved in cell-cycle phase and cell-cycle process (Figure 2A). Notably, qRT-PCR and immunoblotting analyses revealed that the expression of CDK6 was reduced in all of these cells (Figures 2B–2E). We also observed decreased phosphorylation of the retinoblastoma (Rb) protein at Ser807/811, the target sites of CDK6, in LS180 cells in which β-catenin, c-Myc, or *MYU* had been knocked down (Figure S2A). Furthermore, we found that overexpression of *MYU* partially restored CDK6 expression in LS180 cells in which either β-catenin or c-Myc had been knocked down (Figures 2F and S2B). In addition, overexpression of c-Myc did not affect *CDK6* expression in HCT116 colon cancer cells in which *MYU* had been knocked down (Figures S2C and S2D). These results suggest that the Wnt/c-Myc/*MYU* pathway has the potential to upregulate CDK6 expression.

MYU Interacts with hnRNP-K to Stabilize CDK6 Expression

To investigate the mechanisms underlying *MYU*-mediated upregulation of CDK6, we sought to identify proteins associated with *MYU* by RNA pull-down experiments. Proteins co-precipitated with a biotinylated *MYU* transcript were separated by SDS-PAGE and analyzed by mass spectrometry (Figure 3A; Table S2). We identified eight candidate binding proteins and examined whether knockdown of each of their genes by siRNA affected CDK6 expression. Among these genes, knockdown of hnRNP-K caused the most significant decrease in CDK6 expression (Figures 3B and 3C). Moreover, knockdown of hnRNP-K or CDK6 reduced the growth and tumorigenicity of LS180 cells (Figures S3A–S3D). Immunoblotting analysis revealed that hnRNP-K was specifically associated with the sense strand of *MYU* (Figure 3D). Furthermore, RNA immunoprecipitation (RIP) analysis showed that endogenous *MYU* was associated with hnRNP-K (Figure 3E). In addition, deletion mapping experiments showed that fragments of the *MYU* transcript containing nucleotides 396–1,410 or 852–1,753 were capable of binding to hnRNP-K (Figure 3D). Consistent with this result, these regions, especially nucleotides 852–1,753, contain multiple cytidine-uridine (CU)-rich elements that are potential consensus binding sites for hnRNP-K (Matunis et al., 1992; Ostareck et al., 1997).

hnRNP-K contains multiple RNA recognition motifs and is involved in transcription, RNA splicing, mRNA stability, and translation. hnRNP-K plays an important role in the mitotic process and proliferation of colon cancer cells (Sugimasa et al., 2015). hnRNP-K is normally localized exclusively in the nucleus in normal colon tissues but is found in both the cytoplasm and nucleus in colon cancer cells (Carpenter et al., 2006). Although extracellular signal-regulated kinase (ERK)-dependent phosphorylation of hnRNP-K triggers its accumulation in the cytoplasm (Habelhah et al., 2001), we found that ectopic overexpression of *MYU* did not affect the localization of hnRNP-K in CCD-112CoN normal colon cells.

We examined whether the *MYU*-hnRNP-K complex interacts with *CDK6* mRNA and regulates its expression via a posttranscriptional mechanism. RIP analysis revealed that hnRNP-K is associated with *CDK6* mRNA in vivo (Figure 3E). RNA pull-down assays revealed that hnRNP-K could bind to nucleotides 7558–7,989 in the *CDK6* 3′ UTR (WT-3′ UTR), which contains three CU-rich elements, designated CU-rich elements 2, 3, and 4 (Figures 3F and 3G). Furthermore, mutations in CU-rich element 2 (Mut1- and Mut2-3′ UTR), but not in CU-rich element 3 (Mut3-3′ UTR) caused a reduction in binding activity. Mutations in CU-rich element 4 (Mut4-3′ UTR) led to a slight decrease in binding activity. Thus, hnRNP-K may directly interact with *CDK6* mRNA mainly through CU-rich element 2. In addition, RNA pull-down assays followed by qRT-PCR analysis revealed that *MYU* co-precipitated with the *CDK6* WT-3′ UTR but not

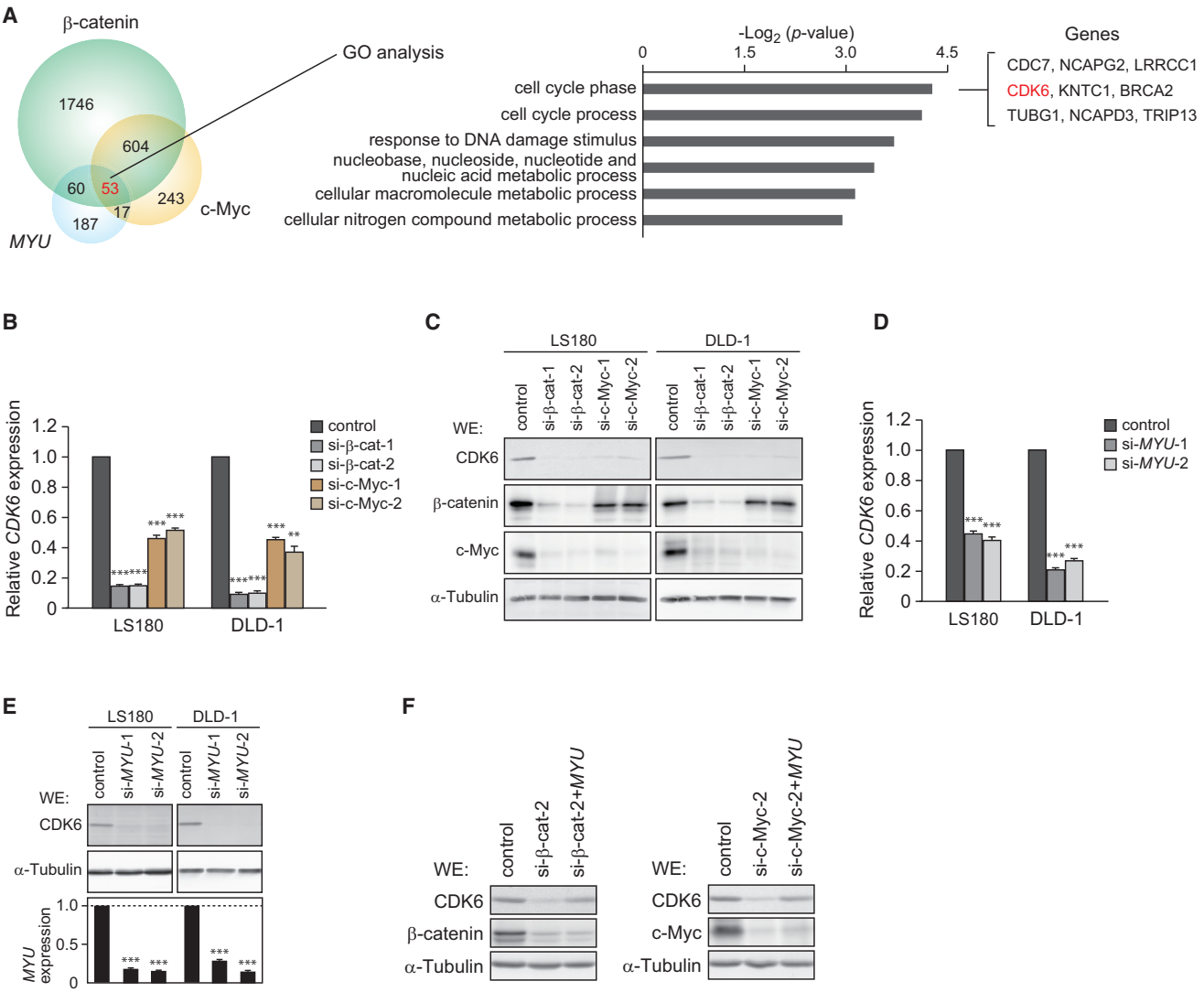

Figure 2. Identification of CDK6 as a Target of the Wnt/c-Myc/*MYU* Pathway

(A) Microarray analysis of *MYU*-, β-catenin-, and c-Myc-regulated genes in LS180 cells. Left: Venn diagram showing the overlap of genes downregulated by *MYU* (blue), β-catenin (green), or c-Myc (orange) knockdown. The number of genes within each category is indicated. Right: gene ontology analysis of the genes shared by the *MYU*, β-catenin, and c-Myc signatures.

(B–E) qRT-PCR (B, D, and E, bottom panel) and immunoblotting (C and E, top two panels) analyses of CDK6 expression in LS180 and DLD-1 cells transfected with the indicated siRNA. Results are expressed as the mean ± SEM (n = 3–4). **p < 0.01, ***p < 0.001.

(F) Immunoblotting analysis of CDK6 expression in LS180 cells. Lentiviral expression of *MYU* restores expression of CDK6 in β-catenin or c-Myc knockdown cells.

the Mut1-3′ UTR (Figure 3H). We also found that knockdown of *MYU* or c-Myc reduced the amount of *CDK6* that co-immunoprecipitated with hnRNP-K (Figures 3I–3K). By contrast, knockdown of *MYU* barely affected the binding of hnRNP-K to other target RNAs, *MYCLo-2* and *CDKN1A* (Kim et al., 2015; Figure 3I). These results suggest that *MYU* and c-Myc promote the recruitment of hnRNP-K to the *CDK6* mRNA. Comparison of the sequences of *MYU* and *Cdk6* mRNA excludes their direct base-pairing. Thus, the detailed molecular mechanisms by which the *MYU*/hnRNP-K complex recognizes the *Cdk6* mRNA remain to be investigated.

We performed qRT-PCR analysis to measure the expression levels of *MYU* and *hnRNP-K* and found that the approximate cycle numbers of *MYU, hnRNP-K*, and *Actin* in LS180 cells were ~24, ~20, and ~15, respectively. Thus, the expression level of *MYU* may be much lower than that of *hnRNP-K*. This result suggests that *MYU* could be a limiting factor for the interaction between *MYU* and hnRNP-K.

We next examined whether the seven other candidate *MYU*-binding proteins are present in the *MYU*-hnRNP-K complex. When lysates from LS180 cells were subjected to immunoprecipitation with anti-hnRNP-K antibody followed by immunoblotting

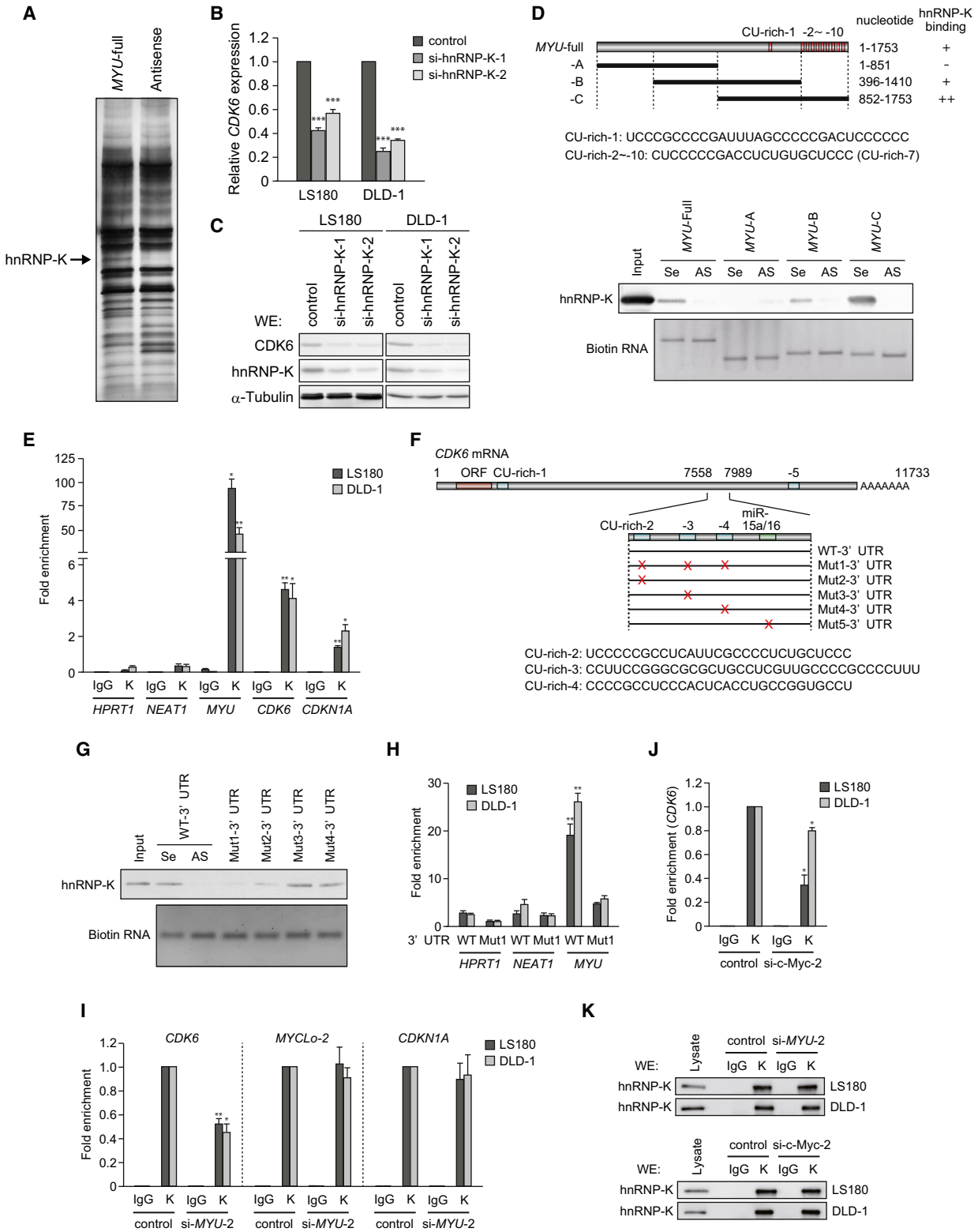

Figure 3. *MYU* Interacts with hnRNP-K to Stabilize CDK6 Expression

(A) Proteins from HT29 cell lysates were subjected to RNA pull-down assays using biotinylated full-length *MYU* (*MYU*-full) or its antisense RNA (negative control) and analyzed by SDS-PAGE followed by silver staining. The band specific to *MYU* was cut out and identified by mass spectrometry.

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with antibodies against the seven proteins, FUBP3 and IGF2BP2 were found to co-immunoprecipitate with hnRNP-K (Figure S3E; Table S2). However, knockdown of FUBP3 and IGF2BP2 as well as other candidate proteins barely affected the viability of LS180 cells (Figure S3F).

miR-15a and miR-16 share a common seed sequence and are known to have tumor suppressor activity. Overexpression of miR-15a or miR-16 mimics reduced CDK6 expression (Figure S3G), as reported previously (Liu et al., 2008). Conversely, inhibition of miR-16, but not of miR-15a, resulted in increased expression of CDK6 in SW480 colon cancer cells (Figure S3H). In contrast to a previous report (Liu et al., 2008), the miRNA target prediction algorithm TargetScan indicated that *CDK6* harbors one well conserved miR-15a/16 binding site at nucleotides 7,881–7,887 in its 3′ UTR. Overexpression of a miR-16 mimic resulted in decreased activity of a reporter construct containing the wild-type, but not mutant, miR-15a/16 seed sequence (Figures 3F and S3I). These results suggest that miR-16 has the potential to suppress CDK6 expression by directly binding to the conserved miR-15a/16 binding site in the *CDK6* 3′ UTR.

It has been reported that the expression of COX-2 and serotonin transporter (SERT) is suppressed by miR-16 and that this suppression is antagonized by hnRNP-K binding to sites adjacent to the miR-16-binding sites in the 3′ UTRs of these genes (Shanmugam et al., 2008; Yoon et al., 2013). Because the miR-15a/16 target site in the *CDK6* 3′ UTR resides adjacent to the CU-rich elements 2∼4 (Figure 3F), we examined the effect of hnRNP-K expression on the miR-16-mediated inhibition of CDK6 expression. We found that overexpression of hnRNP-K or *MYU* suppressed the inhibitory effect of miR-16 on the activity of the luciferase reporter containing the miR-15a/16 binding site (Figure S3J). These results raise the possibility that hnRNP-K and *MYU* may interfere with the interaction of the miR-16-Ago2 complex with the miR-15a/16 binding site. We therefore performed RNA-binding protein pull-down assays. When miR-16 was transfected into LS180 cells, Ago2 was found to co-precipitate with the *CDK6* WT-3′ UTR but not the Mut5-3′ UTR (Figure S3K). Furthermore, we found that knockdown of hnRNP-K led to an increase in the amount of Ago2 co-precipitated with the *CDK6* WT-3′ UTR in the presence of miR-16. These results suggest that hnRNP-K inhibits the interaction of the miR-16-Ago2 complex with the miR-16 target site in the *CDK6* 3′ UTR.

Wnt/c-Myc/*MYU*-Mediated Upregulation of CDK6 Is Essential for Cell Cycle Progression and Clonogenicity

CDK6 is known to play an important role in facilitating progression through G1 phase of the cell cycle. To address the significance of Wnt/c-Myc- and *MYU*/hnRNP-K-induced CDK6 expression in the cell cycle, LS180 cells were transfected with siRNA to knock down expression of each of several genes, cultured in the presence of nocodazole, which prevents re-entry of cells into G1 phase, and then stained with propidium iodide (PI) and analyzed by fluorescence-activated cell sorting (FACS). In cells transfected with *MYU* siRNA, there was a significant increase in the G1 cell population (Figure 4A), consistent with arrest in G1 phase. This indicates that *MYU* may be required for the G1/S transition. We obtained similar results with cells transfected with siRNA targeting β-catenin, c-Myc, hnRNP-K, or CDK6 (Figure S4A), consistent with previous reports (Sherr, 1993; Tang et al., 2014). We also found that overexpression of *MYU* could partially rescue cell-cycle progression in c-Myc knockdown cells (Figure 4B). Furthermore, although knockdown of *MYU*, β-catenin, c-Myc, or hnRNP-K by siRNA caused an increase in the fraction of LS180 cells in G1, as shown above, concomitant overexpression of CDK6 suppressed this increase and partially restored cell-cycle progression (Figures 4C, 4D, and S4B). We also performed long-term colony formation assays with the FLAG-tagged *CDK6* coding sequence fused to its 3′ UTR fragment (nucleotides 7,558–7,989, shown in Figure 3F) that contains either wild-type (WT-3′ UTR) or mutated (Mut1-3′ UTR) *MYU*/hnRNP-K binding sites. We found that overexpression of the *CDK6*-WT-3′ UTR, but not of the *CDK6*-Mut1-3′ UTR, partially restored the colony-forming capacity of LS180 cells in which c-Myc had been knocked down (Figure 4E). These results suggest that Wnt/c-Myc- and *MYU*/hnRNP-K-mediated upregulation of CDK6 is essential for cell-cycle progression and clonogenicity.

It has been shown that CDK4 is a direct target of c-Myc (Hermeking et al., 2000) and that it regulates G1 progression. Therefore, we next examined the relationship between the *MYU*/hnRNP-K/CDK6 pathway and the CDK4 pathway in c-Myc-mediated G1 progression. The effect of β-catenin or c-Myc knockdown on G1 arrest was more significant than that observed with CDK4, *MYU*, or CDK6 knockdown (Figure 4F; Figures S4C and S4D). Furthermore, double knockdown

(B and C) qRT-PCR (B) and immunoblotting (C) analyses of CDK6 expression in LS180 and DLD-1 cells transfected with the indicated siRNAs.
(D) Top: schematic of the full-length and deletion fragments of *MYU* used for the precipitation of hnRNP-K from DLD-1 cell lysates. Se, sense transcript; AS, antisense transcript. ++ and +, detectable binding activity; −, no detectable activity. Bottom: precipitated hnRNP-K and biotin-labeled fragments of *MYU* are shown.
(E) RIP assays were performed on LS180 and DLD-1 cells using anti-hnRNP-K antibody or mouse IgG. *HPRT1* and the lncRNA *NEAT1* were used as negative controls. *CDKN1A* was used as a positive control.
(F) Schematic of the *CDK6* mRNA and constructs used for RNA pull-down experiments (G and H) and luciferase assays (Figures S3I and S3J).
(G) Lysates from DLD-1 cells were incubated with biotinylated wild-type and mutant fragments of the *CDK6* 3′ UTR, and bound proteins were analyzed by immunoblotting analysis with anti-hnRNP-K antibody.
(H) Lysates from LS180 and DLD-1 cells were incubated with biotinylated wild-type and mutant fragments of the *CDK6* 3′ UTR, and bound RNAs were analyzed by qRT-PCR. *HPRT1* and *NEAT1* were negative controls.
(I and J) The amounts of the hnRNP-K-*CDK6* mRNA complexes are decreased in LS180 and DLD-1 cells in which *MYU* (I) or c-Myc (J) had been knocked down. RIP assays were performed using anti-hnRNP-K (K) antibody or mouse IgG.
(K) Immunoblotting analysis of the immunoprecipitates using an anti-hnRNPK antibody. All data represent mean ± SEM (n = 3–4). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

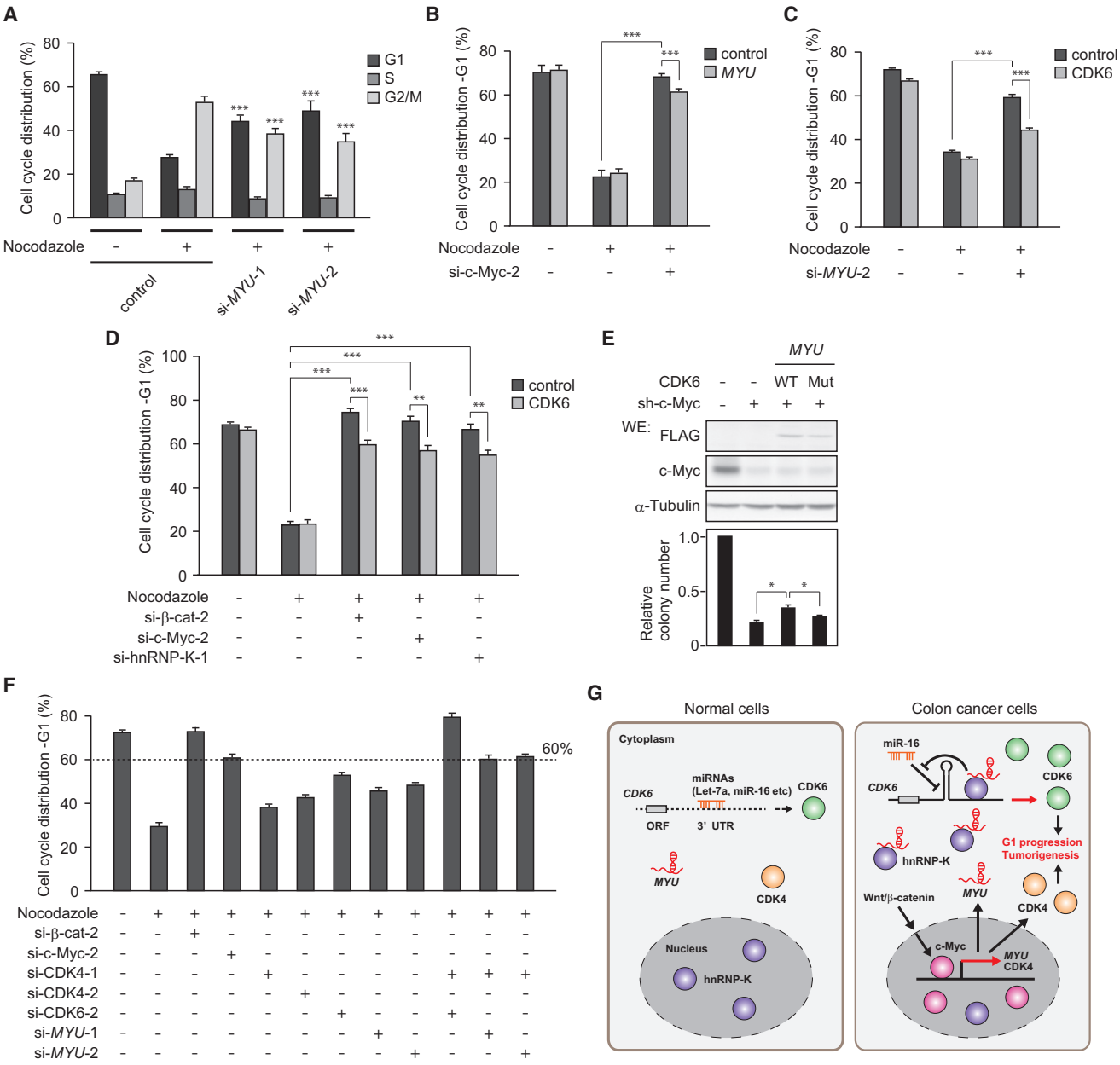

Figure 4. Wnt/c-Myc/*MYU*-Mediated Upregulation of CDK6 Is Essential for Cell-Cycle Progression

(A) LS180 cells were transfected with the indicated siRNA, and cell-cycle distribution was analyzed by FACS.

(B) Lentiviral *MYU* partially rescues G1 progression of LS180 cells in which c-Myc had been knocked down.

(C and D) Exogenous expression of CDK6 restores G1 progression of LS180 cells in which either *MYU* (C), β-catenin, c-Myc, or hnRNP-K (D) had been knocked down.

(E) Colony-forming ability of LS180 cells infected with a lentivirus expressing the FLAG-tagged *CDK6* coding sequence fused to its 3′ UTR fragment (nucleotides 7,558–7,989) that contains either a WT or Mut *MYU*/hnRNPK binding site along with a lentivirus expressing *MYU* or shRNA targeting c-Myc. Top: immunoblotting analysis of FLAG-tagged CDK6 and c-Myc expression.

(F) LS180 cells were transfected with the indicated siRNAs and subjected to cell-cycle analysis by FACS.

(G) Model of Wnt/c-Myc/*MYU*-induced tumorigenesis. Wnt/c-Myc signaling directly enhances *MYU* expression. *MYU* forms a complex with hnRNP-K and stabilizes CDK6 expression by suppressing the inhibitory effect of miR-16 and thereby facilitates cell-cycle progression. The *MYU*/hnRNP-K/CDK6 pathway and the CDK4 pathway may contribute independently to Wnt/c-Myc-induced cell-cycle progression.

All data represent mean ± SEM (n = 3–10). *p < 0.05, **p < 0.01, ***p < 0.001.

of CDK4/*MYU* led to increased G1 arrest comparable with that obtained by c-Myc knockdown; thus, the effect of knocking down CDK4 and *MYU* appears to be additive. Therefore, the *MYU*/hnRNP-K/CDK6 pathway and the CDK4 pathway may contribute independently to Wnt/c-Myc-mediated cell-cycle progression.

DISCUSSION

c-Myc is one of the most commonly activated oncogenes and is estimated to be involved in 20% of all human cancers (Dang et al., 2006). Thus, much effort has been devoted to understanding the molecular mechanisms underlying the role of c-Myc in cell-cycle control. It has been shown that c-Myc-induced cell proliferation is generally associated with an increase in CDK4 and CDK6 activities, which regulate G1 progression (Mateyak et al., 1999). It has also been reported that c-Myc induces the expression of CDK4 by directly binding to the *CDK4* promoter region (Hermeking et al., 2000). Gene profiling studies have shown that c-Myc regulates the expression of *CDK6* mRNA (Yap et al., 2011). In the present study, we identified *MYU* as a direct target gene of the Wnt/c-Myc pathway and showed that Wnt/c-Myc/*MYU*-mediated upregulation of CDK6 is essential for cell-cycle progression of colon cancer cells (Figure 4G).

Although the Wnt/β-catenin signal and c-Myc drive multiple downstream targets, we found that ectopic CDK6 is able to partially restore G1/S cell-cycle progression in LS180 cells in which β-catenin or c-Myc had been knocked down (Figure 4D). Consistent with this finding, the effect of CDK6 knockdown on G1 arrest was more significant than that observed with CDK4 knockdown (Figure 4F), suggesting that CDK6 functions as a major mediator in LS180 cells. We also observed that LS180 cells express high levels of cyclin D1 but low/moderate levels of cyclin D2 and D3, and knockdown of c-Myc did not result in critical reductions in the expression of any of the D-type cyclins (Figure S4E), although cyclin D2 has been reported to be a direct target of c-Myc (Bouchard et al., 1999). Furthermore, we found that knockdown of β-catenin did not reduce the expression of cyclin D1, a well-known Wnt/β-catenin target, in LS180 cells (Figure S4F). These results are consistent with the notion that Wnt/c-Myc/*MYU*-mediated upregulation of CDK6 may play an important role in the proliferation and tumorigenicity of some colon cancer cells.

Tumorigenesis assays showed that c-Myc knockdown cells grew more slowly than β-catenin knockdown cells. The mechanisms underlying this different tumorigenic activity between β-catenin and c-Myc remain to be elucidated. We speculate that some of the β-catenin target genes other than c-Myc may negatively affect the growth of colon cancer cells.

It is well known that c-Myc functions downstream of many signal transduction pathways, serving as a central organizer that integrates multiple intracellular and extracellular cues. In addition to cancer, previous studies have shown that c-Myc is implicated in many diseases, including inflammation (Pap et al., 2004; Weng et al., 2006) and cardiovascular diseases (Ahuja et al., 2010). It has also been reported that the role of c-Myc in pluripotency is related to its ability to regulate the cell-cycle machinery (Singh and Dalton, 2009). Thus, our findings may have implications beyond cancer and apply to other diseases and to stem cell biology. Understanding the molecular details of *MYU*/hnRNP-K cooperation may provide a basis for the rational development of novel drugs to treat some cancers and other diseases.

EXPERIMENTAL PROCEDURES

Ethical Statement

All animal experiments were carried out in accordance with the Guidelines for Proper Conduct of Animal Experiments provided by the Science Council of Japan, and were approved by the Ethics Committee of the Institute of Molecular and Cellular Biosciences, University of Tokyo.

Cell Proliferation Assays and Tumorigenesis Assays

Cells transfected with siRNAs were seeded in 48-well plates at 1.25×10^4 cells/well, and cell proliferation was measured by counting cells after 3 and 5 days in culture. Cell viability (for Figure S3F) was assessed 3 days after siRNA transfection using CellTiter-Glo (Promega). For tumorigenesis assays, LS180 cells infected with a lentivirus were suspended in PBS with an equal volume of Matrigel (BD Biosciences) and were subcutaneously injected (5×10^2 cells/mouse) into 7- to 10-week-old nude mice. Tumor appearance was evaluated using a caliper, and the tumor volume was calculated according to the formula ($V = \pi/6 \times [L \times W^2]$), where V = volume, L = length, and W = width (length is greater than width).

Statistical Analysis

Statistical analysis was performed using Mann-Whitney *U* test and Student's t test. $p < 0.05$ was considered to be statistically significant.

ACCESSION NUMBERS

The accession number for the microarray data reported in this paper is GEO: GSE64889.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.08.015.

AUTHOR CONTRIBUTIONS

Y.K., M.K., S.S., M.O., and T.O. performed the experiments. K.M. performed the array and bioinformatic analyses. L.N. performed the mass spectrometry analysis. N.Y., T.N., M.O., and K.S. performed the RNA-seq studies. M.H. and J.K. prepared the colon tumor specimen and mRNA. Y.K. and T.A. analyzed the data and wrote the paper.

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