Targeting Translation Initiation Bypasses Signaling Crosstalk Mechanisms That Maintain High MYC Levels in Colorectal Cancer

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ABSTRACT

Deregulated expression of MYC is a driver of colorectal carcinogenesis, suggesting that inhibiting MYC may have significant therapeutic value. The PI3K and mTOR pathways control MYC turnover and translation, respectively, providing a rationale to target both pathways to inhibit MYC. Surprisingly, inhibition of PI3K does not promote MYC turnover in colon carcinoma cells, but enhances MYC expression because it promotes FOXO-dependent expression of growth factor receptors and MAPK-dependent transcription of MYC. Inhibition of mTOR fails to inhibit translation of MYC, because levels of 4EBPs are insufficient to fully sequester eIF4E and because an internal ribosomal entry site element in the 5′-untranslated region of the MYC mRNA permits translation independent of eIF4E. A small-molecule inhibitor of the translation factor eIF4A, silvestrol, bypasses the signaling feedbacks, reduces MYC translation, and inhibits tumor growth in a mouse model of colorectal tumorigenesis. We propose that targeting translation initiation is a promising strategy to limit MYC expression in colorectal tumors.

SIGNIFICANCE: Inhibiting MYC function is likely to have a significant therapeutic impact in colorectal cancers. Here, we explore several strategies to target translation initiation in order to block MYC expression. We show that a small-molecule inhibitor of eIF4A inhibits MYC expression and suppresses tumor growth in vivo. Cancer Discov; 5(7); 1–14. © 2015 AACR.

See related commentary by Castell and Larsson, p. 701.

INTRODUCTION

With more than 1.2 million newly diagnosed cases per year, colorectal cancer is the most common gastrointestinal malignancy (1). Sequence analysis shows that each tumor genome carries multiple mutations that deregulate major signaling pathways that control growth and survival of colon epithelial cells (2). Despite their genomic heterogeneity, enhanced expression of MYC proteins is universally observed in colon cancers, and gene expression analyses show that a signature of activated and repressed MYC target genes is present in a vast majority of colorectal cancers (2). Deletion of the MYC gene ablates tumorigenesis in mouse models that faithfully mimic the human disease (3). Collectively, these data argue that targeting MYC might achieve significant therapeutic efficacy in colorectal cancers.

MYC is a transcription factor that binds broadly to thousands of promoters and enhances and activates or represses its target genes as part of several DNA-binding protein complexes

(4). Both direct and indirect strategies have been proposed to inhibit MYC function and expression (5–7). The MYC protein is highly unstable in nontumor cells and is constantly degraded by the proteasome system (8). Several ubiquitin ligases are known that ubiquitinate MYC and ubiquitination by FBXW7 targets MYC for proteasomal degradation (8). FBXW7 is frequently mutated in human colorectal cancer enhancing the stability of MYC (9). Furthermore, colorectal cancers express high levels of USP28, an ubiquitin protease that binds to FBXW7 and antagonizes its function; deletion of USP28 reduces MYC levels and extends life span in colon tumor models (10). Enhancing MYC turnover therefore may be a valid strategy to inhibit MYC function in colorectal cancer.

Degradation of MYC by FBXW7 is initiated by phosphorylation at S62, which primes subsequent phosphorylation at T58 by GSK3 (8). Subsequent dephosphorylation at S62 by PP2A allows recognition and ubiquitination of T58-phosphorylated MYC by FBXW7 (8). GSK3 itself is inhibited by PI3K/AKT-dependent phosphorylation at S9, and inhibitors of PI3K or dual mTOR/PI3K inhibitors enhance N-MYC turnover in pediatric tumors (11). Conversely, ectopic expression of MYC confers resistance of mammary tumor cells to PI3K inhibition (12). A second rationale to target the PI3K-mTOR pathway is provided by its ability to enhance cap-dependent translation initiation. mTORC1 and the downstream S6 kinase promote translation because they phosphorylate and thereby inactivate the 4EBP and PDCD4 proteins that inhibit the eIF4F translation initiation complex (13, 14). As a consequence, inhibition of mTORC1 blocks MYC expression in myeloma cells, and targeting protein translation limits the growth of MYC-driven hematopoietic tumors (15).

Here, we have explored whether targeting signaling pathways that control MYC turnover and translation can be used to eliminate MYC expression in colorectal cancer, using the dual mTOR/PI3K inhibitor BEZ235 and the eIF4A helicase...
inhibitor silvestrol as tools (16, 17). We show that targeting PI3K and mTOR fails to increase MYC turnover and instead enhances MYC expression and functionality. In contrast, directly targeting translation initiation bypasses the feedback mechanisms that cause this surprising response, reduces MYC expression, and inhibits tumor growth in mouse models of colorectal carcinoma.

RESULTS

FBXW7 Pathway Is Active in Colon Carcinoma Cells

In many cells, MYC proteins turn over with a half-life of approximately 20 minutes (8). To determine the stability of MYC in colorectal cancer, we added cycloheximide to block new protein synthesis and determined the amount of MYC by immunoblotting at several time points afterwards (Fig. 1A–C; Supplementary Fig. S1A and S1B; and see Supplementary Table S1 for all antibodies and primer sequences). MYC turned over with a half-life that was between 41 to approximately 60 minutes in SW480, SW620, and HCT116 cells, respectively (Fig. 1A–C and Supplementary Fig. S1A and S1B). MYC that is phosphorylated at T58, the recognition site for FBXW7, turned over with a slightly longer half-life in all three cell lines. This is consistent with the notion that FBXW7 is actively degrading a large fraction, but not all, of the MYC in these cells. In support of this notion, both MYC and phosphorylated T58 MYC turned over with a greatly extended half-life in HCT116 cells, in which the endogenous FBXW7 gene has been disrupted (Fig. 1C; Supplementary Fig. S1A; ref. 9).

The critical kinase that phosphorylates T58 and promotes degradation of MYC and N-MYC proteins is GSK3 (8). Because GSK3 in turn is inhibited by PI3K/AKT-dependent phosphorylation, we tested the effect of BEZ235, a dual mTOR/PI3K inhibitor, which destabilizes N-MYC in neuroblastoma cells (11). BEZ235 was used at a concentration of 200 nmol/L, which is sufficient to inhibit both PI3K and mTOR activity (16). Immunoblotting showed an altered migration of 4EBP1 and confirmed dephosphorylation of 4EBP1 at T70 and of S6 at S240/244, downstream targets of mTOR, in response to BEZ235 (Fig. 1D and Supplementary Fig. S1C). Furthermore, BEZ235 inhibited the AKT-dependent phosphorylation of FOXO3A, demonstrating that PI3K is also inhibited (see below). Consistent with previous observations, exposure of several human colon cancer cell lines to 200 nmol/L BEZ235 suppressed proliferation and led to a moderate accumulation in the G1 phase of the cell cycle, but did not induce apoptosis (Supplementary Fig. S1D–S1F; ref. 16). Cells resumed proliferation after withdrawal of BEZ235, suggesting that the BEZ235-induced cell cycle arrest is reversible (Supplementary Fig. S1F).

Surprisingly, the analysis also showed that exposure to BEZ235 increased rather than decreased MYC levels in SW480 cells (Fig. 1A and D). Similarly, exposure to BEZ235 led to a robust increase in MYC levels in SW620 and CACO2 cells and a weaker increase in LS174T and HCT116 cells (Fig. 1E). Titration of BEZ235 revealed an IC_{50} value of around 20 nmol/L for this increase, consistent with an on-target effect for either mTOR or PI3K (see below). Using rapamycin, a specific inhibitor of mTORC1, and LY294002, an inhibitor of PI3K, showed that inhibition of PI3K was sufficient to induce expression of MYC (Fig. 1F). Consistent with this interpretation, siRNA-mediated depletion of the catalytic subunit of PI3Kα (p110α) enhanced MYC levels in both SW620 and CACO2 cells (Fig. 1G).

Cycloheximide treatment revealed that treatment with BEZ235 did not accelerate MYC turnover (Fig. 1A and B and Supplementary Fig. S1A and S1B). To understand this result, we analyzed phosphorylation of serine 9 of GSK3. Inhibition of either PI3K or AKT, using specific inhibitors, reduced phosphorylation at this site in HeLa cells, which were used as a positive control (Fig. 1H). In contrast, inhibition of neither AKT nor PI3K decreased phosphorylation of GSK3 S9 in colorectal tumor cells, arguing that AKT activity is not rate limiting for phosphorylation of this site in colorectal tumor cells (see Discussion). Consistently, exposure of colon cancer cells to BEZ235 did not strongly alter the relative fraction of MYC that is phosphorylated at T58 (Fig. 1D). We concluded that PI3K/AKT activity is not critical for stabilizing MYC proteins in colon carcinoma cells.

MYC Protein Is Functional after Inhibition of PI3K and mTOR

To test whether MYC is functional in cells exposed to BEZ235, we performed microarray analyses of SW620 cells exposed to 200 nmol/L BEZ235 for 24 hours relative to control cells. To ascertain which changes depend on MYC, we compared control siRNA-treated cells with cells in which endogenous MYC had been depleted by a specific siRNA (Supplementary Fig. S2A). Consistent with the arrest in proliferation observed upon exposure to BEZ235, multiple gene sets encoding proteins involved in cell proliferation were robustly downregulated upon exposure to BEZ235, multiple gene sets encoding proteins involved in cell proliferation were robustly downregulated upon exposure to BEZ235 (Supplementary Fig. S2B–S2D). Many of the genes encoding proteins in translation are direct target genes of MYC (18). Consistent with this observation, siRNA-mediated depletion of MYC reduced both their basal and BEZ235-increased expression (Supplementary Fig. S2C). Several well-characterized sets of MYC target genes contain genes involved both in proliferation and in ribosome function and translation; consistent with these data, depletion of MYC reduced expression of such gene sets even in the presence of BEZ235 (Supplementary Fig. S2C, left). We concluded that the MYC protein present in BEZ235-treated cells is capable of activating MYC target genes and that genes involved in cell-cycle progression are downregulated in an MYC-independent manner upon BEZ235 treatment.

FOXO-Dependent MAPK Signaling Increases MYC Levels upon PI3K Inhibition

Incubation of SW620 cells with BEZ235 induced a dose- and time-dependent increase in MYC mRNA levels that paralleled the increase in MYC protein (Fig. 2A and B). Transcription of MYC is under control of the MAPK pathway via a joint ETS/E2F site in the MYC promoter (19). Consistent with a role for MAPK activation, incubation with BEZ235 enhanced phosphorylation of ERK in several colorectal cancer cell lines tested (Fig. 2C). Surprisingly, this increase was
Figure 1. Effect of PI3K/mTORC inhibition on MYC expression and stability in colorectal cancer cells. A, immunoblots documenting MYC and phosphorylated (p) T58 MYC stability. SW480 cells were treated with 200 nmol/L BEZ235 or solvent control for 24 hours and cycloheximide (50 μg/mL) and harvested at the indicated time points. Vinculin was used as loading control. Exposures of MYC and p T58 MYC blots were adjusted to equalize exposure at 0 minutes (n = 3; unless otherwise indicated, n indicates the number of independent biologic repeat experiments in the following legends). B, calculated half-life of total MYC and p T58 MYC. Immunoblots shown in A. C, immunoblots show MYC and pT58 MYC stability in wild-type (WT) and FBXW7-deficient (KO) HCT116 cells (n = 1). D, SW480 cells were incubated with 200 nmol/L BEZ235 for 24 hours. Left, effect on mTOR targets S6 and 4EBP1; right, effect on MYC and GSK3 (n = 2). E, immunoblots of four colorectal cell lines upon treatment with BEZ235 (500 nmol/L; 24 hours) or solvent control (n = 3). F, SW620 cells were treated for 24 hours with rapamycin (100 nmol/L), LY294002 (50 μmol/L), or both and analyzed by immunoblotting. G, the indicated cell lines were transfected with siRNA targeting the p110α subunit of PI3K or control siRNA; 72 hours after transfection, protein levels were determined by immunoblotting (n = 2). H, immunoblot of cells treated for 24 hours with indicated inhibitors or solvent control (rapamycin 100 nmol/L, LY294002 50 μmol/L, BEZ235 500 nmol/L, Akti 1/2 1 μmol/L).
**Figure 2.** BEZ235 induces MAPK signaling in a FOXO3A-dependent manner. **A,** SW620 cells were treated with indicated concentrations of BEZ235 for 24 hours. Cell lysates were probed with indicated antibodies (left). MYC mRNA levels were assessed by real-time quantitative polymerase chain reaction (RQ-PCR; right; \(n = 3\)). **B,** SW620 cells were treated with 200 nmol/L BEZ235 and harvested at indicated time points. Cell lysates were probed with indicated antibodies (left). MYC mRNA levels were assessed by RQ-PCR (right; \(n = 2\)). **C,** cell lines were treated with BEZ235 (200 nmol/L, 24 hours) or solvent control. Immunoblots of lysates were probed with the indicated antibodies ( \(n = 3\)). **D,** SW620 cells were incubated with BEZ235 (500 nmol/L), UO126 (20 \(\mu\)mol/L), or both for 24 hours. Protein levels were determined by immunoblotting (left). MYC mRNA levels were assessed by RQ-PCR analysis (right; \(n = 3\)). **E,** Ls174T cells were treated with BEZ235 (200 nmol/L, 24 hours). Immunoblots of cell lysates were probed with the indicated antibodies ( \(n = 2\)). **F,** Ls174T cells were transfected with siRNA targeting FOXO3A or control siRNA for 48 hours followed by treatment with BEZ235 (200 nmol/L) or solvent control for 24 hours ( \(n = 2\)). WT, wild-type.
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induces MAPK activity, which in turn leads to enhanced MYC expression of growth factor receptors at the cell surface in response to PI3K activity, demonstrating that activation of FOXO3A is critical for activation of MYC upon inhibition of PI3K (Fig. 2D). Arguing that an increase in MAPK activity is critical for induction of MYC protein and attenuated induction of MYC mRNA, these data suggest that enhanced growth factor signaling (Supplementary Fig. S3D). FOXO3A at T32, one of the sites phosphorylated by AKT (Fig. 2E), as well as in its nuclear translocation (Fig. 2F) and activation of known FOXO target genes (Supplementary Fig. S3C; ref. 22). Depletion of FOXO3A, a member of the FOXO family that is strongly expressed in colorectal tumor cells, attenuated both induction of MYC expression and activation of MAPK signaling, demonstrating that activation of FOXO3A is critical for activation of MYC upon inhibition of PI3K (Fig. 2G). FOXO proteins are part of an evolutionarily conserved feedback loop that limits expression of growth factor receptors at the cell surface in response to PI3K activity (20). AS a result, treatment with BEZ235 strongly induced expression of HER3 (ERBB3), of the insulin receptor (INSR), and of the insulin-like growth factor receptor (IGFR) mRNAs in a FOXO3A-dependent manner (Supplementary Fig. S3D). These data suggest that enhanced growth factor signaling induces MAPK activity, which in turn leads to enhanced MYC expression in colorectal cancer cells upon BEZ235 treatment.

Targeting eIF4F Activity Restricts MYC Expression in Colon Carcinoma Cells

Upregulation of MYC after treatment with BEZ235 is also surprising because inhibition of mTORC1 is expected to inhibit the eIF4F translation initiation factor complex (see Introduction). Treatment with BEZ235 did not alter the expression of eIF4A or eIF4E, subunits of the eIF4F complex (Fig. 3A). Antibodies directed against the four different mTORC1-dependent phosphorylation sites in 4EBP1 (Thr37/46; Ser65; Thr70) confirmed that these sites were dephosphorylated upon exposure to BEZ235 (Fig. 3B). To test whether BEZ235 inhibits translation initiation, we isolated cap-binding complexes using m7G-oligo affinity chromatography. Exposure of cells to BEZ235 did not interfere with binding of eIF4E, but reduced cap binding of eIF4A and eIF4G, which are recruited by eIF4E (Fig. 3C); this is consistent with previous observations (24). The result is compatible with two interpretations: Either translation of MYC, like that of the insulin receptor, does not depend on either eIF4A or eIF4E (25), or the amount of 4EBPs is insufficient to fully sequester eIF4E in colon carcinoma even when dephosphorylated.

To test whether expression of MYC depends on eIF4E, we expressed a doxycycline-inducible allele of 4EBP1 that carries alanine substitutions at four serine/threonine residues that are targets for mTORC1-dependent phosphorylation and that acts as a dominant inhibitor of eIF4E (ref. 26; Fig. 3D). Induction of 4EBP1(4A) by the addition of doxycycline in Ls174T and SW480 cells inhibited expression of MYC, in both the absence and the presence of BEZ235 (Fig. 3D and E). Induction of 4EBP1(4A) blocked cap binding of eIF4A and eIF4G; for both proteins, inhibition by 4EBP1(4A) was stronger than observed for BEZ235 (Fig. 3C). Because levels of 4EBP1 in the presence of doxycycline exceeded those of endogenous 4EBP, this suggested that endogenous levels of 4EBP are insufficient to sequester eIF4E in colon carcinoma cells. Consistent with this suggestion, titration of doxycycline showed that suppression of MYC expression by 4EBP1(4A) required protein levels that exceeded those found in BEZ235-treated cells (Fig. 3F and Supplementary Fig. S4A). Inhibition of MYC expression by 4EBP1(4A) correlated with an accumulation of cells in the G1 phase of the cell cycle and suppression of cell proliferation (Fig. 3G and Supplementary Fig. S4B). We concluded that efficient translation of MYC requires active eIF4E, but that endogenous levels of 4EBPs are insufficient to fully inhibit expression of MYC in colon carcinoma cells (27).

As an alternative means to inhibit eIF4F activity, we used silvestrol, a small-molecule inhibitor of the eIF4A helicase (17). Incubation of SW480 cells with nanomolar concentrations of silvestrol suppressed both basal MYC protein expression and the increase in MYC levels observed in response to treatment with BEZ235 (Fig. 4A). Identical results were obtained with rocaglamide, a structurally related compound that also inhibits the eIF4A helicase (ref. 28; Supplementary Fig. S4C). Neither compound decreased ERK or mTOR activity (Fig. 4A and Supplementary Fig. S4C). Furthermore, silvestrol did not suppress MYC mRNA levels; on the contrary, MYC mRNA levels slightly increased upon exposure to low concentrations of silvestrol, possibly because high levels of MYC repress transcription from its own promoter (Fig. 4B). Incubation with silvestrol also suppressed MYC protein levels in HCT116, Ls174T, SW480, and SW620 cells (Fig. 4C) and led to an increase in CDKN1A (encoding p21, Cip1) and MUC2 (encoding Mucin2, which is a marker of terminal differentiation of colon cancer cells) mRNA levels, both of which are repressed by MYC (Fig. 4D; ref. 29). Incubation with silvestrol suppressed proliferation of colon carcinoma cells but did not induce a significant degree of apoptosis (Fig. 4E and Supplementary Fig. S4D and S4E). FACS analysis showed that silvestrol did not induce major changes in the cell-cycle distribution, arguing that silvestrol arrests proliferation in all phases of the cell cycle (Supplementary Fig. S4E). FACS analyses showed that siRNA-mediated knockdown of MYC, in contrast with silvestrol, led to an accumulation of cells in the G1 phase; combining depletion of MYC with treatment with silvestrol further increased the accumulation of cells in the G1 phase (Supplementary Fig. S4F). Both observations suggest that silvestrol suppresses translation of proteins that are critical for progression through the cell cycle in addition to MYC. We concluded that inhibition of eIF4A helicase activity is a valid approach to inhibit proliferation and to suppress MYC expression in colon carcinoma cells.

Silvestrol Targets Both Cap- and IRES-Dependent Translation of MYC

Measurements of 35S-methionine incorporation showed that incubation of colon carcinoma cells with BEZ235 or silvestrol or induction of 4EBP1(4A) reduced global protein synthesis to a similar extent (Fig. 5A), raising the question of
Figure 3. Effect of eIF4F inhibition on MYC protein levels. **A**, the indicated cell lines were incubated with BEZ235 (200 nmol/L, 24 hours). Immunoblots of cell lysate were probed with the indicated antibodies (n = 2). **B**, SW480 cells were incubated with BEZ235 (200 nmol/L, 24 hours) and immunoblots probed for the indicated proteins (n = 2). **C**, m7GTP-cap pull-down assay was performed in SW480 cells after treatment with BEZ235 (200 nmol/L, 24 hours), doxycycline (DOX; 24 hours), silvestrol (25 nmol/L, 24 hours), or solvent control. Cell lysates were incubated with m7GTP beads and bound proteins immunoblotted for indicated proteins. Left plot demonstrates input of cell lysate, and right plot the m7GTP-bound protein fraction (n = 2). **D**, SW480 and Ls174T cells were infected with a lentivirus expressing 4EBP1(4A) under the control of a doxycycline-inducible promoter. 4EBP1(4A) harbors four mutations on mTOR phosphosites (T37A, T46A, S65A, and T70A). Cells were incubated for 24 hours with doxycycline (1 µg/mL) or ethanol as control. Protein levels were determined by immunoblotting (n = 2). **E**, SW480 cells expressing doxycycline-inducible 4EBP1(4A) were incubated for 24 hours with doxycycline, BEZ235 (200 nmol/L), or the combination of both, and cell lysates were probed for the indicated proteins (n = 2). **F**, SW480 cells expressing doxycycline-inducible 4EBP1(4A) were incubated with BEZ235 (200 nmol/L), low doxycycline (0.001 µg/mL), or high doxycycline (1 µg/mL) concentrations for 24 hours. Cell lysates were immunoblotted with the indicated antibodies (n = 2). **G**, SW480 cells described in **C** were incubated with doxycycline (1 µg/mL). Left, FACS analysis in response to doxycycline (24 hours) or solvent control. Error bars indicate SD of biologic triplicates from one representative experiment (n = 3). Right, a colony assay stained with crystal violet after 5 days of doxycycline treatment.
why MYC protein levels are differentially affected. To address this question, we performed polysome profiling from control and inhibitor-treated cells and measured the association of different mRNAs with polysomes by real-time quantitative polymerase chain reaction (RQ-PCR). Consistent with their effects on cap-binding complexes, induction of 4EBP1(4A) strongly inhibited association of two control mRNAs, ACTB and TUBB3, with polysomes, whereas BEZ235 had moderate effects (Fig. 5B). In contrast, induction of 4EBP1(4A) had only moderate effects and BEZ235 had no effects on association of MYC mRNA with polysomes, suggesting that MYC mRNA remains associated with polysomes even when cap recognition is strongly impaired (Fig. 5B).

The 5′-untranslated region (5′-UTR) of the MYC mRNA contains an internal ribosome entry site (IRES), and therefore MYC is translated in both a cap- and an IRES-dependent
Figure 5. Effects of silvestrol and BEZ235 on translation of MYC. A, incorporation of 35S-labeled methionine in SW480 cells treated with BEZ235 (200 nmol/L), doxycycline (DOX), or solvent control for 24 hours. Shown are mean ± SD (n = 3). B, polysome fractionation of SW480 cells (top left), treated with BEZ235 (200 nmol/L), doxycycline, silvestrol (25 nmol/L), or solvent control for 24 hours. RNA was isolated from the indicated fractions, and relative mRNA content per fraction was measured by RQ-PCR. Top right, MYC mRNA distribution; bottom left, ACTB mRNA; and bottom right, TUBB3 mRNA distribution (n = 2). C, schematic illustration describing the luciferase reporter systems used in D, E, and F. The pmF reporter construct contains the MYC 5′-UTR inserted into the control vector pGL3 (Promega) proximal to firefly luciferase coding sequence. The bicistronic pRmF and pRhcvF reporter constructs contain the MYC or the hepatitis C virus (HCV) IRES sequence distal to renilla and proximal to firefly luciferase gene. D, SW480 cells were transfected with pmF luciferase reporter and treated with BEZ235 (200 nmol/L), doxycycline, silvestrol (25 nmol/L), cymarin (100 nmol/L), or solvent control for 24 hours. Luciferase activity is shown relative to a cotransfected β-gal reporter (n = 3). E, SW480 cells were transfected with pmF luciferase reporter and treated with silvestrol (25 nmol/L) or solvent control. Relative firefly luciferase activity was calculated using the ratio of firefly to renilla luciferase (n = 3). F, SW480 cells were transfected with pRhcvF luciferase reporter and analyzed as in E (n = 3).
manner; translation from the IRES element depends on eIF4A, but is independent of eIF4E (27). We therefore tested how inhibition of eIF4A affects polysome profiles. Similar to the induction of 4EBP1(4A), incubation with silvestrol blocked polysome association of the ACTB and TUBB3 mRNAs. In contrast with 4EBP1(4A), silvestrol also strongly affected association of the MYC mRNA with polysomes. The dependence on eIF4A correlates with a complex secondary structure in the 5'-UTR of an mRNA, and association of some mRNAs, such as PFN2, with polysomes shows little dependence on eIF4A (30); this was also observed in SW480 cells (Supplementary Fig. S5A).

These data argue that the eIF4A-dependent, but eIF4E-independent, translation from the IRES maintains polysome association of MYC mRNA when cap-dependent translation is inhibited. Relative to the cap-dependent translation of a control mRNA, incubation with silvestrol reduced translation of a luciferase under the control of the MYC 5'-UTR (Fig. 5C and D). Furthermore, silvestrol inhibited translation under the control of the MYC IRES, which depends on eIF4A (27), but did not inhibit translation under the control of an eIF4A-independent IRES element that is present in the hepatitis C virus genome (Fig. 5C, E, and F). Collectively, these data argue that the presence of an IRES element facilitates translation of MYC when cap-dependent translation is inhibited.

To confirm these data, we used a second inhibitor of translation initiation, cymarin, that has been identified as an inhibitor of MYC IRES-dependent translation by high-throughput screening (31). Reporter assays showed that incubation with 100 nmol/L cymarin inhibited translation under the control of the 5'-UTR of MYC relative to a control reporter (Fig. 5D). Consistently, incubation of SW480 cells with cymarin mimicked the effect of silvestrol on MYC expression (Supplementary Fig. S5B).

Silvestrol Suppresses MYC Expression and Proliferation of Colon Cancers In Vivo

To test whether targeting translation initiation may open a therapeutic window in colon carcinoma, we initially analyzed publicly available gene expression databases. These analyses showed that expression of the mRNA encoding PDCD4 is strongly suppressed in colon carcinoma relative to normal tissue (Supplementary Fig. S5C). In contrast, expression of mRNAs encoding eIF4A, eIF4E, 4EBP1, and 4EBP2 showed minor changes in tumor relative to normal tissue. Histopathologic analysis of 10 human colorectal cancer samples confirmed the downregulation of PDCD4 in colon tumor relative to normal mucosa; these data are consistent with previous findings (Supplementary Fig. S5D; ref. 32). In contrast, we did not observe significant differences in expression of eIF4E, eIF4A, and 4EBP's proteins between normal mucosa and colorectal cancers in the same tumor samples (Supplementary Fig. S5D). This suggested that eIF4A activity may be enhanced due to silencing of its negative regulator PDCD4 in colon carcinoma and that targeting eIF4A may therefore be suitable for targeting MYC expression in vivo.

To explore this possibility, we assessed the ability of silvestrol to suppress the proliferation induced by acute APC deletion in the intestine. Our previous experiments have shown the “crypt progenitor phenotype” induced by Apc loss to be MYC dependent (3). To achieve APC deletion in the murine intestine, we intercrossed mice carrying the VillinCreER transgene to mice bearing the conditional knockout Apciso/iso allele to generate VillinCreER Apciso/iso mice (labeled “APCfox” in Fig. 6). To achieve high penetrant deletion of the Apc tumor suppressor, mice were given a daily injection of 2 mg of tamoxifen for 2 days (33). Mice were then given a 1 mg/kg (i.p.) injection of either silvestrol or vehicle on days 2 and 3 after induction, and harvested on day 4. For BEZ235 treatment, mice were gavaged with 45 mg/kg of BEZ235 on days 2 and 3 after induction. Four days after induction, mice were euthanized and the intestinal crypt hyperproliferative phenotype was examined. Exposure to BEZ235 had no significant impact on hyperproliferation following Apc loss, with similar crypt size, BrdUrd incorporation, and Ki67 and MYC levels (Fig. 6A and Supplementary Fig. S6A-S6D). BEZ235 was active because it led to a significant increase in expression of CDKN1A (P < 0.01; Supplementary Fig. S6E).

In marked contrast, exposure of mice to silvestrol robustly suppressed the hyperproliferation following Apc loss (Fig. 6A-D). Upon treatment with silvestrol, intestinal crypts from VillinCreER Apciso/iso mice were significantly smaller and showed a marked reduction in both BrdUrd incorporation and Ki67 positivity. Importantly, there was a clear reduction in MYC positivity via immunohistochemistry within Apc-deficient crypts (P = 0.04, Fig. 6A-E). In situ hybridization showed that silvestrol did not reduce levels of Myc mRNA (Supplementary Fig. S7A), demonstrating that it reduces MYC expression posttranscriptionally. Furthermore, quantitative evaluation documented that silvestrol led to a reduction in MYC protein levels in the lower half of the crypts (Supplementary Fig. S7B), arguing that the reduction in MYC level is not due to an indirect effect of silvestrol on crypt/villus differentiation. No impact on proliferation or MYC levels was observed in wild-type intestinal crypts (Fig. 6A-E), suggesting a clear therapeutic window exists between Apc-deficient and wild-type intestinal enterocytes.

DISCUSSION

Deregulated and enhanced expression of MYC is a driver of colorectal tumorigenesis, necessitating strategies to inhibit MYC function or expression for tumor therapy. Here, we have explored the possibility to target protein turnover and translation initiation to inhibit MYC expression. As a tool to dissect the regulatory circuits that maintain elevated MYC expression, we used BEZ235 and silvestrol, well-characterized inhibitors of the PI3K-mTOR pathway and of eIF4A helicase, respectively (16).

We expected that BEZ235 would decrease MYC expression via promoting FBXW7-dependent turnover and via inhibition of eIF4E-dependent translation of MYC. We confirmed that turnover of MYC proteins in colorectal cancer cells depends on FBXW7 (8). Inhibition of PI3K or AKT can increase MYC turnover because AKT phosphorylates and inhibits GSK3 at S9 (34). Hence, AKT inhibition can increase phosphorylation of MYC at T58 by GSK3. Surprisingly, phosphorylation of GSK3 at S9 does not depend on PI3K and AKT activity in colon carcinoma cells, suggesting that one of several AKT-independent kinases that can phosphorylate this site (e.g., Aurora-A, ref. 35; or p90RSK, ref. 36)
Figure 6. Silvestrol reduces proliferation and MYC levels in Apc-deficient intestinal enterocytes but not in wild-type cells. A, graph documenting number of proliferating cells (shown for BrdUrd incorporation; left) and number of cells staining positive for MYC (right) in silvestrol, BEZ235, or vehicle-treated wild-type or Apc-deficient intestines. The number of BrdUrd or MYC-positive nuclei per crypt-villus axis was scored in 30 full crypts in at least 3 mice. Data are presented as Box and Whisker plots. B, representative hematoxylin and eosin (H&E)–stained sections showing effects of silvestrol on wild-type (WT) and Apc-deficient crypts. Note that crypts are enlarged due to Apc loss and that this is reduced following silvestrol treatment. C, representative BrdUrd staining showing that silvestrol reduced proliferation in Apc-deficient intestines. D, representative Ki67-stained sections showing a reduction in proliferation in Apc-deficient crypts following silvestrol treatment. E, representative MYC staining showing reduction by silvestrol in Apc-deficient but not wild-type intestines.

maintains GSK3 phosphorylation upon inhibition of PI3K or AKT.

Instead of promoting degradation, inhibition of PI3K increased MYC levels in several colon cancer cell lines due to a FOXO-dependent transcriptional upregulation of growth factor receptor genes and, downstream of receptor activity, to a MAPK-dependent increase in MYC mRNA levels (see Fig. 7). A similar crosstalk between the PI3K–AKT pathway and MAPK activity has been identified previously in breast cancer cells (20). Most likely, it reflects an evolutionarily conserved
regulatory circuit that couples expression of cell surface receptor genes to PI3K activity (23).

Inhibiting protein translation has emerged as a therapeutic strategy to target MYC-dependent tumor growth, because translation initiation is deregulated in MYC-driven lymphomas, and supraphysiologic protein synthesis rates are required for their growth (37). In MYC-driven lymphomas, targeting protein translation via inhibition of mTORC1 and mTORC2 has therapeutic efficacy, because two inhibitors of eIF4F-dependent translation initiation, 4EBP and PDCD4, are inactivated via mTORC1-dependent phosphorylation and, in the case of PDCD4, subsequent ubiquitin-dependent degradation (14, 38, 39). Expression of PDCD4 is strongly downregulated in colorectal cancer. In response to BEZ235, 4EBP1 is dephosphorylated on mTORC1-dependent sites, but this does not inhibit translation of MYC. We identify two causes for this effect: First, cap binding of eIF4A and eIF4G in response to BEZ235 is only partially inhibited, arguing that the amount of 4EBPs is insufficient to fully sequester eIF4E in colorectal cancer cells. Second, MYC mRNA remains associated with polysomes even when cap binding is fully inhibited by a non-phosphorylatable allele of 4EBP1. Most likely, this is due to the presence of an IRES in the 5′-UTR of MYC, which is known to be independent of elf4E (27). Our findings are consistent with recent observations that the 4EBP proteins are not the critical targets of the mTORC1 inhibitor rapamycin, and that even genetic ablation of mTORC1 activity does not inhibit MYC expression in a mouse model of colorectal cancer (40).

Our data also show that dual PI3K/mTOR inhibition is not an effective therapeutic strategy for colorectal cancers because BEZ235 has only a small effect on MYC levels and no effect on proliferation and cellularity in a mouse model of colorectal cancer that is driven by deletion of the Apc tumor-suppressor gene. In contrast, selective targeting of mTORC1 by rapamycin, while not targeting MYC, is effective in suppressing growth of colon carcinoma (40). We suggest that the BEZ235-dependent, but not rapamycin-dependent, inhibition of PI3K and subsequent FOXO-dependent activation of MAPK limits the therapeutic efficacy of BEZ235 in this model.

In contrast with BEZ235, silvestrol inhibited expression of MYC in colorectal tumor cell lines at nanomolar concentrations. At the same time, silvestrol reduced proliferation and cellularity of colon tumors in vivo, arguing that inhibition of the eIF4A helicase is effective to inhibit MYC expression in colorectal cancer and extending similar observations made in a NOTCH-driven model of T-cell acute lymphoblastic leukemia lymphomas (41). Surprisingly, concentrations of silvestrol that strongly reduce MYC levels and proliferation in colorectal tumor cells are well tolerated without apparent toxicity; this correlates with the observation that the effects of silvestrol on MYC levels, proliferation, and cellularity of normal colon are small. Furthermore, translation of MYC is not affected by mTOR inhibition in murine fibroblasts, arguing that the dependence of MYC translation on elf4A is mediated by the presence of G-quadruplexes in the 5′-UTR (41). Because other RNA helicases, such as RHAU (42), can target G-quadruplexes, it is possible that the dependence of colon carcinoma cells on elf4A for translation of MYC opens a therapeutic window, because other helicases carry out this function in normal colon cells.

Figure 7. Model summarizing our findings. Treatment with BEZ235 upregulates MYC via a FOXO/MAPK-dependent pathway (black blunt line, top left part). Negative regulation of MYC levels via GSK3 or inhibitors of translation like PDCD4 and 4EBPs is lost in colorectal cancer (dashed lines). Treatment with silvestrol reduces MYC expression by inhibition of eIF4A (bottom right).
Methods

Reagents

BE235 (Lclabs), Rapa, Synkinase, UO126 (Promega), Akt1/2 (Sigma), Silvestrol (MedChemexpress), Cymarin (Sigma), and RecaGlamid (Sigma) were dissolved in DMSO. Doxycycline and cycloheximide (both Sigma) were dissolved in ethanol.

Cell Culture and Transfection

Caco2, HCT116, SW480, SW620, and HeLa cells were cultured in DMEM, and LS174T cells were cultured in RPMI-1640 medium containing 10% FCS and 1% penicillin/streptomycin. Cell lines were purchased from the ATCC (Caco2 in 2012; SW620 in 2012), Cell Line Services (SW480 in 2013), or the German Collection of Microorganisms and Cell Cultures (HCT116 in 2012) and were maintained according to company recommendations. LS174T and HeLa cells were kind gifts of Hans Clevers (University Medical Centre Utrecht, Utrecht, the Netherlands) and Michael Bishop (University of California, San Francisco), respectively. All cell lines were authenticated via short tandem repeat analysis in 2014.

For depletion experiments, cells were transfected with siRNAs (Dharmacon) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. Expression plasmid encoding 4EBP1(4A) was provided by David Sabatini (via Addgene). Translation reporter constructs for luciferase assays were a kind gift of Anne Willis (University of Leicester, Leicester, UK; ref. 27). FACS analysis was performed using FACS Canto II (Becton Dickinson). Antibodies are listed in Supplementary Table S1. Global translation was measured using 

Gene Expression Analysis

Agilent Human Genome Microarray 4 × 44 K v2 was used. The complete data set can be seen at Array express (E-MTAB-2882). Human PDCD4, elf4A, elf4E, 4EBP1, and 4EBP2 expression data from the Skrzypczak Colorectal 2 dataset were downloaded from Oncomine (43). Statistical evaluation was performed by 2-tailed Student unpaired t test or with Mann–Whitney test. Data are presented as mean ± SD.

Polysome Fractionation and Cap Pull-Down Assays

SW480 cells (12 × 10⁶) were plated on 3 × 15 cm cell culture dishes for 16 hours and then treated as indicated for 24 hours. Cell lysis was performed on ice in 100 μL gradient buffer (1 mmol/L DTT, 100 mmol/L KCl, 20 mmol/L Tris-HCl, pH 7.5, 5 mmol/L MgCl₂, 0.5% NP40, 20 μL 0.1 mg/mL cyclohexamide containing protease and RNase inhibitors). Lysates were cleared by centrifugation. Lysates were layered on top of 5% sucrose gradients and centrifuged in an SW41 Ti rotor at 34,500 rpm for 1 hour at 4°C. Profiles were fractionated using a Piston gradient fractionator (BioComp). RNA was analyzed by quantitative RQ-PCR. Cap pull-down assays were performed as described (44).

Animal Experiments

All animal experiments were performed under UK Home Office guidelines using the project license 60–4183. Experimental protocols underwent local ethical review at Glasgow University. VillinCreAPE1:fl/fl mice have been described previously (38). Silvestrol (MedChemexpress; H-13251) was dissolved in 20% (v/v) 2-hydroxypropyl beta-cyclodextrin vehicle (Sigma; H107) at a concentration of 125 μg/mL and injected into mice i.p. BEZ235 (Synkisnase; SYN-1018) was dissolved in 10% (v/v) 1 methyl-2-pyrrolidinone and 90% (v/v) poly(ethylene glycol), SigmaP3265, at a concentration of 4.5 mg/mL, and mice gavaged. Sample size was decided using National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs) guidelines, the smallest number of animal to yield a significant difference.

Immunohistochemistry

Antibodies are listed in Supplementary Table S1. Intestinal scoring was performed in a blinded manner. Thirty full crypt–villus axes were scored for BRDURd, CDKN1A, and MYC positivity. For quantification of the histoscore, 25 crypts from each MYC-stained section at 200× magnification were scored. Per crypt, each nucleus was scored as follows: 0 = no stain; 1 = weak stain; 2 = moderate stain; 3 = strong stain. The number assigned to each category were multiplied by the relevant multiplication factor for that score. The average histoscore of 25 crypts per mouse section was used. Statistical analysis was performed by nonparametric Mann–Whitney using Minitab version 17.

RNAscope

RNAscope images were developed from paraffin-embedded formalin-fixed samples using the Advanced Cell Diagnostics’ RNAscope 2.0 HD (brown) Kit (#310035), following the manufacturer’s instructions. Probes were as follows: Mm Myc (#413451); DapB [RNA negative control (#310043)]; Mm Ppib [RNA positive control (#313911)]. RNA control data were not shown.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: A. Wiegering, S. Herold, O.J. Sansom, M. Eilers Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Wiegering, F.W. Utke, T. Jamieson, Y. Ruoss, M. Hüttenthaler, M. Köspert, C. Pfann, S. Herold, L. Taranet, A. Rosenwald, O.J. Sansom Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Wiegering, F.W. Utke, T. Jamieson, Y. Ruoss, C. Pfann, S. Herold, S. Walz, A. Rosenwald, O.J. Sansom, M. Eilers Writing, review, and/or revision of the manuscript: A. Wiegering, C.-T. Germer, A. Rosenwald, O.J. Sansom, M. Eilers Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Jamieson, C. Nixon, M. Eilers Study supervision: C.-T. Germer, O.J. Sansom, M. Eilers

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Targeting Translation Initiation Bypasses Signaling Crosstalk Mechanisms That Maintain High MYC Levels in Colorectal Cancer

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