

Mirk/dyrk1B kinase is upregulated following inhibition of mTOR

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The PI3K/PTEN/Akt/mTOR/p70S6K pathway is one of the most frequently deregulated signaling pathways in solid tumors and has a functional role in drug resistance. However, targeting this pathway leads to compensatory activation of several mediators of cell survival. Expression of the reactive oxygen species-controlling kinase Mirk/dyrk1B was increased severalfold by the mammalian target of rapamycin (mTOR) inhibitors RAD001, WYE354 and rapamycin, with less effect by the Akt inhibitors AZD5363 and MK-2206. Upregulation of Mirk messenger RNA (mRNA) expression was mediated by cyclic AMP response element binding protein (CREB) binding to two sites in the Mirk promoter upstream of the transcription start site and one site within exon 4. Depletion of CREB reduced Mirk expression, whereas depletion of mTOR increased it. Moreover, hydroxytamoxifen activation of an Akt-estrogen receptor construct blocked an increase in Mirk mRNA and protein. Addition of a Mirk/dyrk1B kinase inhibitor increased the sensitivity of Panc1 pancreatic cancer cells and three different ovarian cancer cell lines to the mTOR inhibitor RAD001. Targeting Mirk kinase could improve the utility of mTOR inhibitors and so presents an attractive drug target.

Introduction

The PI3K/PTEN/Akt/mTOR/p70S6K signaling pathway is frequently deregulated in solid tumors, as compiled in the Cancer Genome Atlas, and has a functional role in drug resistance. Elevated levels of activated p70S6K were found in ovarian cancers that had become non-responsive to chemotherapy, suggesting that the PI3K pathway was responsible for this chemoresistance and that targeting this pathway could have therapeutic benefit (1). However, inhibition of mTOR by allosteric inhibitors (2) leads to compensatory activation of several mediators of cell survival, including Akt, IGF1R, and Erk signaling (3–7), which limits the efficacy of such treatments (8–10). The results of the current study suggest that an additional mediator of cell survival is Mirk/dyrk1B, a kinase with reactive oxygen species (ROS)-suppressing functions in pancreatic, ovarian and colon cancers (11–13). Mirk/dyrk1B was expressed in 21 of 28 (75%) resected human ovarian cancers, primarily papillary serous cystadenocarcinomas, with upregulation in 60% of the cancers (14). In a larger clinical screen of 76 patient samples, Mirk protein was detected in 75% of the cancers and overexpressed in 41%, with lower incidence in the benign tumors and none in the non-neoplastic ovarian cysts (15). Similarly, Mirk/dyrk1B is expressed in ~90% of resected pancreatic adenocarcinomas (16) and is amplified in a subset within the 19q13 amplicon. Mirk/dyrk1B is localized at 19q13.1 (17). Akt2 is amplified in some pancreatic cancers near this region. However, the Mirk gene was among 16 genes within the consistently amplified 660kb subregion of the 19q13 amplicon in pancreatic cancers, whereas the nearby gene Akt2 was not (18), making it more likely that the 19q13 amplicon was selected for because of Mirk than Akt2.

Mirk activity is not increased by mutation in tumors. However, Mirk activity and abundance increases severalfold when cells leave the cell

cycle and become quiescent in G₀ because of poor growth conditions (13). Mirk activity also increases following exposure to chemotherapeutic drugs like 5-FU or cisplatin (12,19) through stress signaling to the Mirk kinase activator MKK3 (20). Mirk controls, in part, residence in a G₀ quiescent state. For example, ~50% of Panc1 pancreatic cancer cells accumulate in G₀ when they are serum starved, whereas only 14% of serum-starved Panc1 cells are found in G₀ if Mirk kinase is inhibited (21). Also, 86% of serum-starved HD6 colon carcinoma cells accumulated in G₀ compared with 14% when Mirk was depleted (19). Suboptimal growth conditions would normally signal entry of many cancer cells into G₀ if Mirk was active, and cells cycled out of G₀ when normal serum levels were restored, showing the entry into G₀ was reversible (11,13,14). However, if Mirk was depleted or inactivated, many serum-starved TOV21G or SKOV3 ovarian cancer cells or Panc1 or SU86.86 pancreatic cancer cells underwent apoptosis instead of remaining viable in G₀. Thus, Mirk/dyrk1B is a kinase active in quiescent ovarian, colon or pancreatic cancer cells, so presents an attractive drug target in these cells. Mirk levels vary up to 10-fold during the cell cycle (16,22), reaching their peak when cells become quiescent in response to energy limitation caused by nutrient or serum starvation (14,21), but the mechanisms that upregulate Mirk expression in quiescent cells are unknown. Signaling from mTOR (mTORC1) activates steps in translation and metabolism essential for cell growth. Moreover, proliferating cells often have active PI3K/Akt/mTOR signaling pathways. In this study, the hypothesis was examined that inhibition of mTOR or its upstream activating kinases PI3K and Akt might provide a permissive condition to upregulate Mirk expression.

Materials and methods

Materials

PLUS reagent, Lipofectamine and Lipofectamine 2000 were from Invitrogen. Polyvinylidene difluoride transfer paper Immobilon-P was purchased from Millipore. All enhanced chemiluminescence reagents were from Amersham. Rabbit polyclonal antibodies were raised to unique sequences at the C-terminus of Mirk and affinity purified. Antibodies to phospho-cyclic AMP response element binding protein (CREB) (Ser133; 87G3), phospho-Akt (ser473) and phospho-mTOR (S2448) were from Cell Signaling, and other antibodies were from Santa Cruz. LY294002 was from Calbiochem. The other PI3K, Akt and mTOR inhibitors were from Selleck. Mirk inhibitors EHT 6840 and EHT 5372 were from Diaxonhit SA (Paris, France). EHT 6840 and EHT 5372 bind to the adenosine triphosphate binding site of Mirk/dyrk1B and had IC₅₀ values on the synthetic polypeptide Dyrktide of 0.59 nM and 0.28 nM, respectively, and were highly selective within a screen of 400 kinases (23). All pancreatic ductal adenocarcinoma cell lines and ovarian cancer cell lines were obtained from the American-Type Culture Collection and confirmed negative for mycoplasma in 2011. Short tandem repeat profiling of 14 and 15 loci, respectively, was used to authenticate the SKOV3 and TOV21G cell lines in 2012. This laboratory has never grown HeLa cells. Tissue culture reagents were obtained from Mediatech. Other reagents were obtained from Sigma. Myr-Akt:estrogen receptor (ER)* HD3 colon carcinoma cells were generated by transfecting pWZL-blast3 Akt:ER*(Myr+) (a kind gift of Dr Martin McMahon) into HD3 colon cancer cells, a cloned subline of HT29. The Akt construct was created by fusing a conditionally active form of Akt containing a src myristoylation sequence to the hormone binding domain of a mutant ER that selectively binds 4-hydroxytamoxifen (4-HT) (24). Stable clones containing Myr-Akt:ER were obtained after selection in blasticidin and maintained in phenol red-free Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The Mirk promoter constructs were described previously (25), and western blotting, northern analysis for Mirk mRNA and Mirk reporter assays were done as described (25).

Results

Inhibitors of mTOR increase protein levels of the ROS-controlling kinase Mirk/dyrk1B

PI3-kinase/Akt signaling has been reported to be activated in most if not all pancreatic cancer cell lines examined because of mutation, aberrant

Abbreviations: 4-HT, 4-hydroxytamoxifen; CREB, cyclic AMP response element binding protein; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ER, estrogen receptor; FBS, fetal bovine serum; mTOR, mammalian target of rapamycin; RNAi, RNA interference; ROS, reactive oxygen species, rRNA, ribosomal RNAs.

expression of the PTEN phosphatase or recruitment by receptor kinases like IGFR1, which are commonly activated in this cancer (26) through overexpression of IRS-1 and IRS-2 (27–29). Pharmacological inhibition of PI3-kinase/Akt/mTOR signaling by LY294002 was found to increase Mirk expression 2- to 6-fold in a series of pancreatic cancer cell lines: Panc1, AsPc1, SU86.86, CAPAN2 and BxPc3 (Figure 1A). LY294002 blocked PI3K/Akt signaling because 30 min of treatment decreased Akt activation in each line (Figure 1A). However, LY294002 has many substrates in addition to PI3K, so other inhibitors were tested. A 2- to 6-fold upregulation of Mirk levels was seen after treatment with rapamycin, an allosteric inhibitor of mTORC1. The drug blocked mTOR signaling because 30 min of treatment increased Akt activation in each line but one because of complex feedback mechanisms described by others (3). MiaPaCa cells do not express Mirk, and Mirk was not induced by LY294002 or rapamycin.

RAD001 (everolimus) is an allosteric rapamycin analog, whereas WYE354 is an adenosine triphosphate-competitive mTOR inhibitor

(30). Both of these mTOR inhibitors increased Mirk protein levels in each of three pancreatic cancer cell lines, AsPc1, BxPC3 and Panc1 (Figure 1B), showing the generality of the increase in Mirk levels following mTOR inhibition.

A series of inhibitors of mTOR, Akt or PI3K were each studied at a range of concentrations to determine whether the increase in Mirk levels was due to drug toxicity inducing a stress response or growth arrest. Mirk levels increase severalfold because of poor growth conditions (13), and Mirk is activated through stress signaling to the Mirk kinase activator MKK3 (20). The mTOR inhibitor RAD001 was the most active through a wide range of concentrations. Duplicate measurements showed that Mirk levels were increased ~6-fold over the 0.1–1 μ M range and up to 9-fold at 5 μ M (Figure 1C and D). At 5 μ M, RAD001 was not toxic to Panc1 cells (Figure 1E). Significantly, RAD001 increased Mirk levels severalfold at the lower concentrations of 1, 10 or 100 nM (Figure 1C, lower panel). The mTOR inhibitor WYE354 at 0.1–1 μ M also increased Mirk levels ~6-fold over

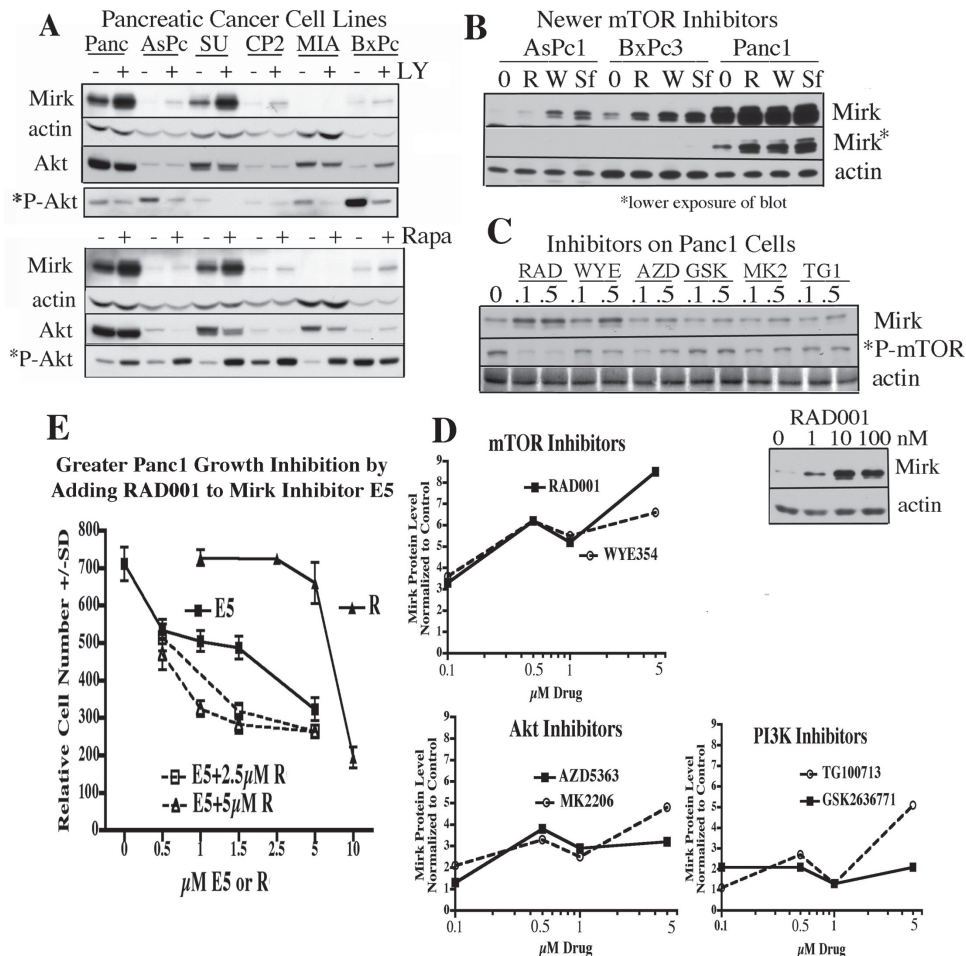


Fig. 1. mTOR and Akt kinase inhibitors increase Mirk protein levels. (A) Panc1 (Panc), AsPc1 (AsPc), SU86.86 (SU), Capan 2 (CP2), MiaPaCa and BxPc3 (BxPc) pancreatic cancer cells were treated with 1 nM rapamycin, 20 μ M LY294002 or the dimethyl sulfoxide (DMSO) diluent either for 30 min (for Akt blots) or for 1 day (MirK and actin blots). Lysates were examined by western blotting for Mirk, active phosphorylated Akt (*P-Akt), total Akt or actin. MiaPaCa cells do not express Mirk and are the negative control. LY294002 blocked PI3K/Akt signaling as shown by decreased Akt phosphorylation, whereas rapamycin blocked mTOR signaling, leading to increased Akt activation in each line but one, because of complex feedback mechanisms described by others. One of duplicate experiments shown. (B) AsPc1, BxPc3 and Panc1 cells in growth medium were treated for 24 h with 10 nM RAD001 or 5 μ M WYE354 or the DMSO diluent before lysis and western blotting for Mirk and actin. Elimination of the serum growth factor IGF-1 by serum-free culture to reduce Akt activation also increased Mirk levels. A shorter exposure of the Mirk blot is also shown for better quantitation of the Panc1 results. (C, upper panel) Panc1 cells were treated 1 day with 0.1, 0.5, 1 or 5 μ M concentrations of the mTOR inhibitors RAD001 and WYE354, Akt inhibitors AZD5363 and MK2206, the PI3K β -selective inhibitor GSK2636771, the pan-PI3K inhibitor TG100713, or the DMSO diluent before lysis and western blotting for Mirk and actin. One of four similar studies shown. Lower panel: lower concentrations (nM) of RAD001 were tested on Panc1 cells before lysis and western blotting as above. One of two experiments with similar results. (D) Graphs summarizing response of Panc1 cells to 0.1–5 μ M drugs, mean of two data points. Mirk protein levels were normalized to actin, then normalized to the amount of Mirk/actin in untreated cells. The y-axis is set to 9-fold in each graph to enable comparisons. (E) Panc1 cells were treated for 1 day with a range of concentrations of the Mirk kinase inhibitor EHT 5372 (E5), RAD001 or both inhibitors, and relative cell numbers were estimated by metabolism of 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide ($n = 3$). Mean \pm SD shown if SD > 5%.

controls (Figure 1C and D). Other investigators have shown that WYE354 at concentrations $<1 \mu\text{M}$ did not show any off-target effects (31). The Akt inhibitors AZD5363 and MK-2206 followed a similar pattern, with both increasing Mirk protein levels 3- to 4-fold between 0.1 and $1 \mu\text{M}$ (Figure 1C and D). Other investigators had shown that MK-2206 had selective *in vitro* activity at micromolar concentrations (32). The pan-PI3K inhibitor TG100713 and GSK2636771, a PI3K β -selective inhibitor, had less stimulatory effects, only up to 2-fold between 0.1 and $1 \mu\text{M}$, and only increased Mirk levels more at 5–10 μM , near toxic levels (Figure 1C and D; data not shown). Possibly, Mirk upregulation is blocked only by mTOR, so inhibition of mTOR increases Mirk levels. Akt inhibitors have less effect on Mirk since they function through mTOR, and PI3K inhibitors have the least effect as they are even more upstream of mTOR than Akt.

Each of these inhibitors had some effect on mTOR signaling. The mTORC1 complex is a key regulator of nutrient signaling. The mTORC1 complex must be intact for mTOR phosphorylation at S2448 (33) by p70S6 kinase (34), the best characterized downstream effector of the mTORC1 complex. S2448 phosphorylation is the predominant phosphorylation site for mTOR and is markedly reduced by rapamycin (33). RAD001 at 0.1–5 μM inhibited mTOR phosphorylation at S2448 ~5-fold, whereas the other inhibitors reduced S2448 phosphorylation less effectively (Figure 1C; data not shown).

Mirk kinase inhibition enhances toxicity of mTOR inhibitor RAD001 toward Panc1 cells

In earlier studies Mirk kinase depletion or pharmacological inhibition of Mirk kinase initiated apoptosis in Panc1 and AsPc1 pancreatic cancer cells (21). Possibly, an mTOR inhibitor, because it elevated Mirk levels, would be more toxic if Mirk kinase was also inhibited.

To test this hypothesis, the Mirk kinase inhibitor EHT 5372 (E5) was added to the mTOR inhibitor RAD001 and Panc1 cell growth was monitored (Figure 1E). The Mirk inhibitor reduced cell numbers in a dose-dependent manner, whereas RAD001 up to 5 μM had little effect on Panc1 cell numbers. However, addition of low levels of the Mirk inhibitor ($1\text{--}1.5 \mu\text{M}$) to RAD001 reduced cell numbers more than either inhibitor alone, up to 60%, showing that inhibiting Mirk could increase the toxicity of RAD001 toward pancreatic cancer cells.

Mirk kinase inhibitors enhance the toxicity of mTOR inhibitors toward ovarian cancer cells

Since Mirk is expressed in the majority of serous ovarian adenocarcinomas and is amplified in a subset, the effect of inhibiting PI3K/Akt/mTOR inhibitors on Mirk expression was examined in ovarian cancer cells. The mTOR inhibitors RAD001 and WYE354 increased Mirk levels in both TOV21G and SKOV3 ovarian cancer cells (Figure 2A and C, insets). In earlier studies, Mirk kinase depletion enhanced the toxicity of low levels of cisplatin toward ovarian cancer cells (12), whereas pharmacological inhibition of Mirk kinase induced apoptosis in ovarian cancer cells (13). Since Mirk kinase inhibition alone will induce toxicity in ovarian cancer cells, we tested whether the combination of a Mirk kinase inhibitor and mTOR inhibitor would be more effective than either agent alone. The Mirk kinase inhibitors EHT 6840 and EHT 5372 were used at their EC₅₀ levels. RAD001, when tested alone at 0.5–10 μM , reduced SKOV3 cell numbers by ~20%, EHT 6840 alone 37%, but together markedly reduced cell numbers up to 97% (Figure 2A), more than additive effects. The Mirk inhibitor EHT 5372 plus RAD001 similarly reduced cell numbers up to 90%.

On OVCAR3 ovarian cancer cells, RAD001 alone reduced cell numbers ~17% and EHT 6840 alone 45%, whereas the combination

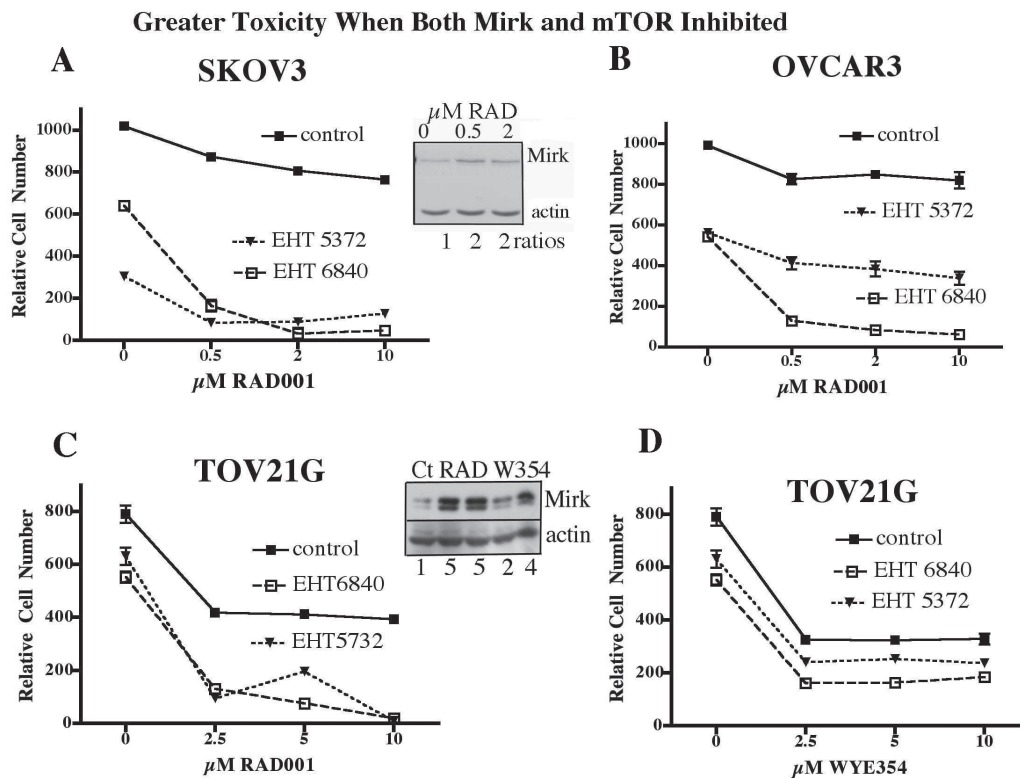


Fig. 2. Mirk kinase inhibitors enhance the toxicity of mTOR inhibitors toward three ovarian cancer cell lines. In all experiments, relative cell number was measured by 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide metabolism, mean \pm SD shown if SD $> 5\%$. Cells were treated for 3 days in serum-free Dulbecco's modified Eagle's medium (DMEM) with the Mirk/dyrk1B inhibitors 2.5 μM EHT 6840 and 5 μM EHT 5372 and with increasing concentrations of the mTOR inhibitor RAD001 or the mTOR inhibitor WYE354. (A) SKOV3 cells tested. Inset: western blot of RAD001 induced increase in Mirk protein levels, with ratios of Mirk to actin given below lanes. (B) OVCAR3 cells tested. (C) TOV21G cells tested. *P* values of control versus Mirk kinase inhibitors were <0.001 as determined by two-tailed *t*-test. Inset: TOV21G cells were treated with 0.1 and $1 \mu\text{M}$ RAD001 and 1 and $10 \mu\text{M}$ WYE354 before western blotting for Mirk and actin; ratios are shown below lanes. (D) TOV21G cells tested. *P* values of control versus Mirk kinase inhibitors were <0.001 and determined by two-tailed *t*-test.

reduced cell numbers up to 94% (Figure 2B), again more than additive effects. The combination of the other Mirk inhibitor EHT 5372 with RAD001 was slightly less effective. RAD001 at 2.5–10 μ M decreased TOV21G cell numbers by ~50%, EHT 5372 or EHT 6840 decreased TOV21G cell numbers 20–30%, but either Mirk inhibitor plus 10 μ M RAD001 almost completely eliminated all TOV21G cells, with $P < 0.001$ (Figure 2C). The mTOR inhibitor WYE354 at 2.5–10 μ M reduced TOV21G cell numbers ~60%, but the addition of either EHT 6840 or EHT 5372 further reduced cell numbers 80% or 70%, respectively, with $P < 0.001$ (Figure 2D). Thus, the toxicity of mTOR inhibitors was enhanced by either of two Mirk kinase inhibitors in three ovarian cancer cell lines.

Akt signaling led to decreased Mirk mRNA levels

Mirk is widely expressed in colon cancer, as well as in pancreatic and ovarian cancer, and stable overexpression of wild-type Mirk was shown to increase the survival of HD3 colon cancer cells, whereas overexpression of kinase-dead Mirk had no such effect (17). Since Mirk had been shown to mediate survival in HD3 colon cancer cells, these cells were treated with the PI3K inhibitor LY294002. This drug increased Mirk mRNA and protein levels (Figure 3A), as it did in a series of pancreatic cancer cell lines (Figure 1A). However, LY294002 inhibits several kinases in addition to PI3K. To probe the mechanism

of the increase in Mirk expression, an HD3 colon carcinoma sub-line was generated, which stably expresses a conditionally active form of Akt (Myr+Akt:ER cells). Within 30 min of addition of 4-HT to these cells there was a dramatic increase in activated phosphorylated exogenous Myr+Akt, which remained elevated for at least 24 h (Figure 3B, upper). The construct had little effect on endogenous Akt, which was activated by treatment with insulin-like growth factor 1 (IGF-1) in parallel wells (I lanes). Conditional activation of Akt in this system allowed measurement of the effects of Akt activation without extraneous effects that would result from activation of endogenous Akt signaling by growth factors, for example.

In order to determine whether Akt inhibits transcription of the Mirk gene, Myr+Akt:ER cells were cultured for 24 h in serum-free medium to allow Mirk transcription without the confounding influence of IGF-1 in serum on endogenous Akt (Figure 3B lower, lane 1) and in serum-free medium with 4-HT to activate the exogenous Akt construct (Figure 3B lower, lane 2). Activation of the exogenous Akt construct by 4-HT decreased Mirk levels by half, demonstrating that Akt blocked Mirk transcription (Figure 3B lower, compare lanes 1 and 2 for Mirk/18S ratios). These changes in Mirk mRNA levels led to changes in Mirk protein levels. Activation of Akt by 4-HT in the HD3-Myr+Akt:ER cells blocked the 5-fold increase in Mirk protein levels

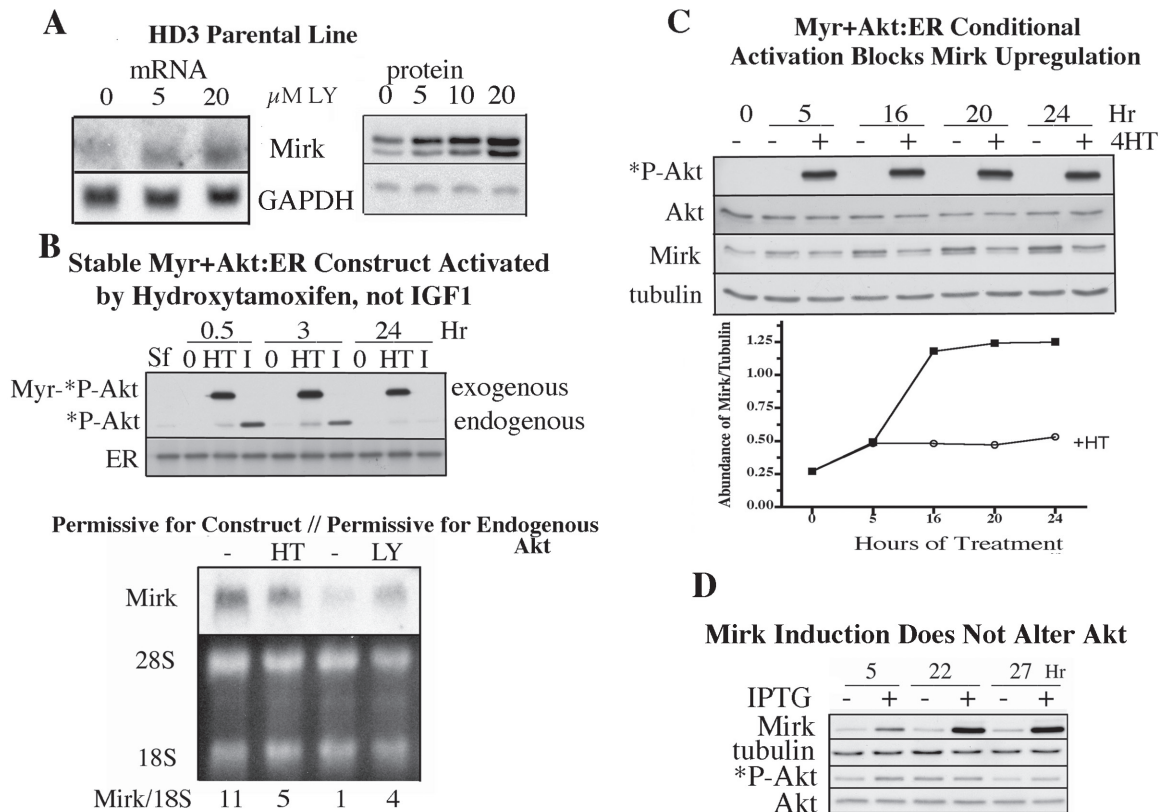


Fig. 3. Akt signaling leads to decreased Mirk mRNA levels. (A) HD3 colon cancer cells (parental line) were treated for 24 h with 0–20 μ M LY294002 and lysates were analyzed for Mirk and glyceraldehyde 3-phosphate dehydrogenase mRNA levels or Mirk protein levels (right). (B) HD3 colon carcinoma cells stably expressing a conditionally active form of Akt (Myr+Akt:ER cells) were cultured for 24 h in serum-free medium to eliminate IGF-1, then treated for 0.5, 3 or 24 h with 1 μ M HT to activate the exogenous Akt construct, 20 ng/ml IGF-1 (I) to activate the endogenous Akt isoforms that migrated more rapidly than the construct, or left untreated (0) before immunoblotting for activated Akt (*P-Akt). Lower panel: lanes 1 and 2: Myr+Akt:ER cells were cultured for 24 h in serum-free medium with 1 μ M 4-HT, a permissive condition to activate only the exogenous Akt construct not endogenous Akt, or lanes 3 and 4 in growth medium, a permissive condition for endogenous Akt as serum IGF-1 is present, or in growth medium with 20 μ M LY294002, to block serum growth factor/PI3K/Akt signaling. Total RNA was analyzed for Mirk mRNA levels by northern blotting. The 28S and 18S ribosomal RNAs (rRNAs) visualized by ethidium bromide staining are shown to document the quality and loading of the RNAs. The ratio of Mirk mRNA/18S rRNA in each culture is given below the appropriate lane. (C) Myr+Akt:ER cells were treated in serum-free medium with 4-HT (1 μ M) to activate the stably transfected Akt construct for 0–24 h before lysis and western blotting for Mirk and tubulin. Lower panel, graph of data points of Mirk normalized to tubulin. (D) The C9 stable Mirk-inducible subline of Mv1Lu lung epithelial cells was treated with 200 μ M isopropyl- β -D-thiogalactopyranoside for 27 h to release the lacI repressor before western blotting of lysates for Mirk, total Akt, Akt isoforms activated by phosphorylation and tubulin as a blotting control.

seen by culture in the permissive condition of serum-free medium (Figure 3C).

As controls, Myr+Akt:ER cells were cultured for 24 h without 4-HT but in medium containing serum and thus the serum mitogen IGF-1 to activate endogenous PI3-kinase/Akt signaling, resulting in an 11-fold decrease in Mirk mRNA (Figure 3B lower, compare lanes 1 and 3). In contrast, when Myr+Akt:ER cells were cultured in serum-containing medium plus LY294002 to block endogenous PI3-kinase/Akt/mTOR signaling, Mirk mRNA levels were increased 4-fold (Figure 3B lower, compare lanes 3 and 4 for Mirk/18S ratios). Therefore, conditionally activated Akt directly inhibits expression of Mirk. These data are consistent with a model in which activated Akt in turn activates mTOR, which inhibits expression of Mirk.

To confirm that Mirk transcription was altered by active Akt, a Mirk promoter construct was expressed in Myr+Akt:ER cells that were treated with 4-HT to activate the exogenous Akt construct, or with LY294002 to inactivate Akt. Activation of Akt by 4-HT decreased Mirk promoter activity by half, whereas pharmacological inhibition of endogenous Akt increased Mirk promoter activity 2-fold (data not shown). The results of these studies, taken together, demonstrate that Mirk mRNA levels are reduced when Akt signaling is activated.

The effect of Mirk overexpression on activation of Akt was determined. A stable Mirk-inducible subline of Mv1Lu lung epithelial cells had been established in earlier studies (35). Treatment of these cells with isopropyl- β -D-thiogalactopyranoside for 27 h to release repression of the stably incorporated Mirk promoter construct led to a 7-fold increase in Mirk protein levels but had no effect on the abundance or

activation state of Akt (Figure 3D). Thus, there appears to be no feedback from Mirk to Akt.

CREB activation is permissive for Mirk expression

The mechanisms that control Mirk/dyrk1b expression are poorly understood. However, Mirk kinase expression and activity are highest when cells are out of cycle in a quiescent state (14,22), when cAMP levels are elevated. Given that the Mirk promoter has potential CREB transcription factor binding sites, the cyclic adenosine monophosphate (cAMP) response element binding protein CREB was investigated. Treatment of Panc1 cells with the mTOR inhibitors RAD001 or WYE354 increased Mirk protein levels (Figures 1B and 4A) and activated a Mirk promoter-luciferase reporter (Figure 4D). This increase in Mirk was correlated with increased activation of CREB by phosphorylation at S133 (Figure 4A).

Since AMP-activated protein kinase (AMPK) inhibits mTOR signaling, allowing more Mirk expression, inhibition of AMPK should decrease Mirk expression. This was so, as the AMPK inhibitor compound C blocked CREB activation (Figure 4A and C) and decreased Mirk mRNA levels, as shown by a dose-response study (Figure 4B). Mirk levels were increased severalfold in Panc1 cells, as in other cell types (11,22), by culture in low-serum medium to eliminate serum growth factors like IGF-1 that activate Akt/mTOR signaling. Compound C blocked the Mirk level increase initiated by serum starvation (Figure 4C and E). Elevated activation of CREB in serum-starved cells correlated with increased Mirk expression (Figure 4C,

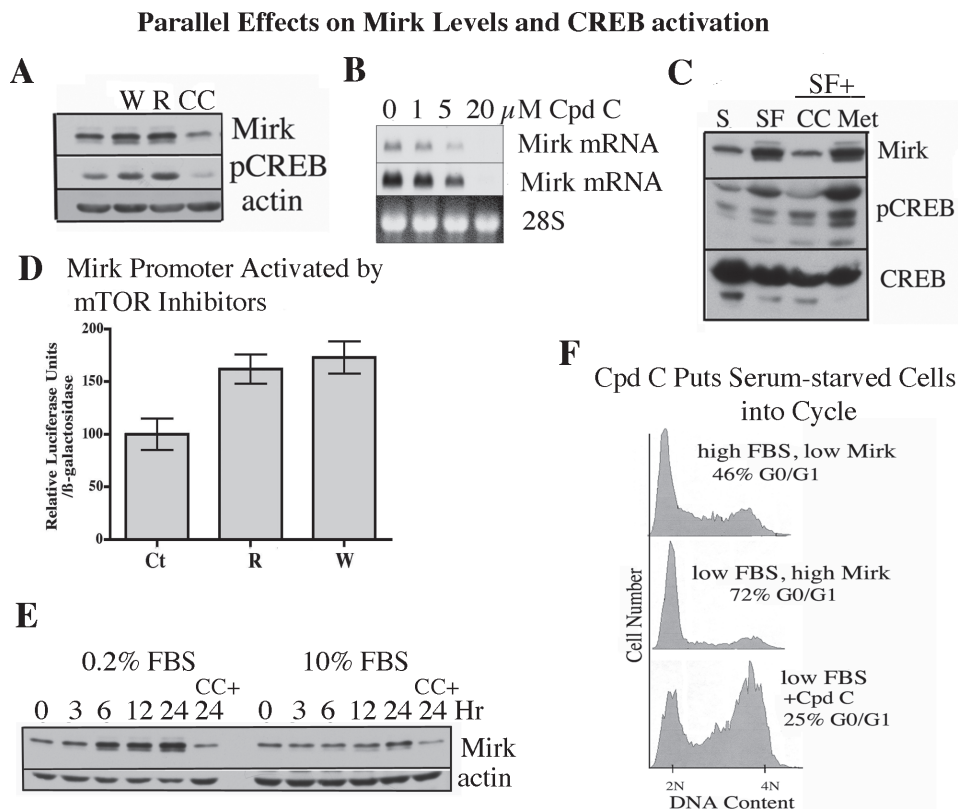


Fig. 4. Parallel effects seen on Mirk levels and CREB activation. (A) Panc1 cells were treated for 1 day with the mTOR inhibitors 5 μ M WYE354 (W) or 100 nM RAD001 (R), or the AMPK inhibitor 5 μ M compound C, before analysis of activated CREB phosphorylated at S133, Mirk and actin by western blotting. (B) Panc1 cells were cultured in growth medium [DMEM + 10% fetal bovine serum (FBS)] for 1 day with 0–20 μ M compound C, then mRNA levels for Mirk (2 exposures shown) analyzed by northern blotting with 28S rRNA as blotting control. (C) Panc1 cells were cultured in growth medium, then for 1 day in fresh DMEM containing either no FBS (serum-free) or 7% FBS (S) alone or with 10 μ M Compound C or 2 mM metformin (Met) with analysis by western blotting as in panel A. (D) Activation of Mirk promoter-luciferase reporter by 10 μ M RAD001 or WYE354. Panc1 cells were transfected with 0.5 μ g pMp (Nhe1-EcoRV-luciferase 9657 kb) Mirk promoter-reporter and 0.25 μ g β -galactosidase reporter plus the mTOR inhibitors. Cells were cultured 2 days in growth medium before lysis and assay. Data are mean \pm SD ($n = 3$). The means are statistically different by two-tailed t -test, $P = 0.047$. (E) Panc1 cells were treated for 0–24 h in DMEM with 0.2% or 10% FBS and 5 μ M compound C, as noted, before analysis by western blotting. All experiments repeated at least twice. (F) Parallel cultures to panel E analyzed for cell cycle position by flow cytometry after propidium iodide staining.

compare first 2 lanes), whereas decreased levels of Mirk and CREB activation coincided when these cells were treated with compound C (Figure 4C, lane3). Metformin is an antidiabetic drug that activates AMPK and inhibits mTOR. Metformin led to more activated CREB and higher Mirk levels (Figure 4C). Thus, in several experimental conditions, Mirk levels were higher when CREB was activated.

Possibly, any block in signaling that leads to growth arrest will create a permissive condition for Mirk upregulation. To test this possibility, the role of the AMPK signaling inhibitor compound C on cell cycling was examined. Compound C not only blocks CREB activation and Mirk expression but also blocks the growth arrest caused by serum starvation. By shown by flow cytometry, 72% of Panc1 cells arrested in G₀/G₁ when serum starved, but only 25% arrested in G₀/G₁ when treated with compound C during serum starvation (Figure 4F). Thus, the growth inhibition induced by either AMPK or by a pharmacological block of mTOR kinase led to a state permissive for upregulation of Mirk, which included activation of CREB.

Mirk promoter analysis

Examination of the genomic Mirk sequence revealed several putative CREB binding sites, some upstream of the transcription start site, and others within exons and introns. A 9697bp genomic fragment was excised, which encompassed ~5000bp upstream of the Mirk transcription start site with two canonical CREB binding sites, with the remaining 4700bp encompassing Mirk exons 1, 2, 3 and 4 and the intervening introns, including one putative CREB binding site within exon 4. This DNA fragment was coupled to a luciferase reporter. Since mTOR inhibitors activated a Mirk promoter construct in Panc1 cells (Figure 4D), Panc 1 cells were utilized to study the modulation of this promoter construct by CREB. Expression of this reporter in Panc1 cells led to a minimal activity signal that was increased up to 22-fold in a dose-dependent manner by a transfected CREB expression plasmid, showing CREB functionality in Mirk expression (Figure 5A). This increase occurred in growth medium as well as in serum-free medium, showing that excess exogenous CREB obviated the inhibition of Mirk expression by serum growth factors. Such factors, like IGF-1, activate Akt/mTOR signaling (Figure 3B).

To further define CREB sites within the Mirk promoter, deletion mutants were made, the 5887 bp NheI-SmaI fragment containing the two putative upstream CREB sites and the 4830 bp HincII-ScaI fragment containing the putative CREB site in exon 4 starting at bp 8690. Each was linked to the luciferase reporter and expressed in Panc1 cells either with wild-type CREB or inactive mutant CREB-S133A. The mutant CREB was used to eliminate non-specific increases in exogenous promoter activity. In Panc1 cells, the construct containing all three putative CREB binding sites was 4.2-fold more active with wild-type CREB than inactive mutant CREB (Figure 5B). When the construct was deleted to only contain the two upstream CREB sites, the promoter-reporter was only increased in activity 1.4-fold by wild-type CREB compared with mutant CREB. In contrast, the promoter construct containing only the CREB site in exon 4 was activated 3.6-fold by coexpressed wild-type CREB compared with mutant CREB. Thus, the CREB site within exon 4 had the most control of CREB-mediated Mirk expression, with only minor contributions from the CREB sites upstream of the transcription start site.

To confirm that the control of Mirk expression by a signaling cascade ending at a CREB binding site in exon 4 was a general finding, this experiment was expanded in C2C12 myoblasts (Figure 5C). In C2C12 cells, the construct containing all three putative CREB binding sites was activated 6.4-fold more by wild-type CREB than inactive mutant CREB. The construct containing only the two upstream CREB sites was activated only 2.4-fold by wild-type CREB compared with mutant CREB. The promoter construct containing only the CREB site in exon 4 was activated 5.9-fold by coexpressed wild-type CREB compared with mutant CREB, almost as much as the construct containing all three CREB binding sites. Wild-type CREB still increased the activity of either construct severalfold more than mutant CREB. Thus, in both Panc1 pancreatic cancer cells and in C2C12 myoblasts,

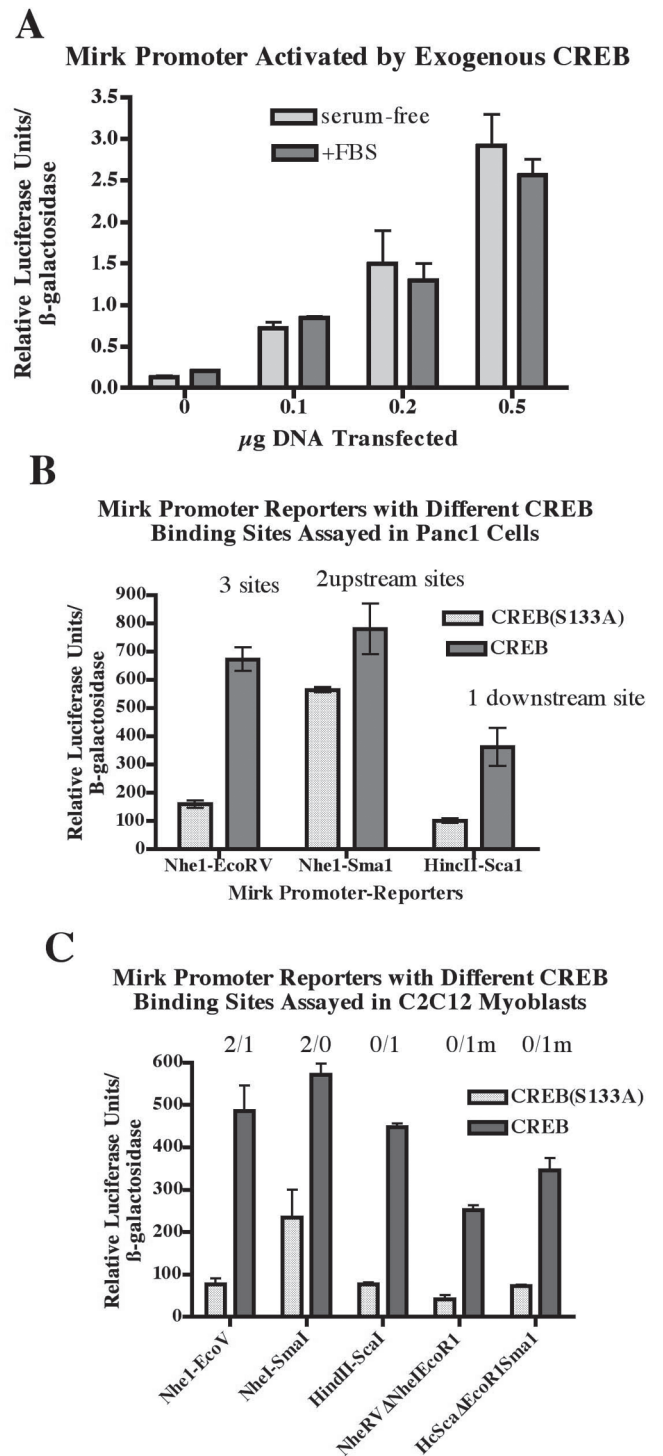


Fig. 5. Mirk promoter activated through CREB binding sites. (A) Dose-dependent activation of Mirk promoter-luciferase reporter by increasing amounts of transfected CREB. Panc1 cells were transfected with 0.5 μ g pMp (NheI-EcoRv-luciferase 9657 kb) Mirk promoter-reporter and 0.25 μ g β -galactosidase reporter plus increasing amounts of the CREB expression plasmid. Cells were cultured overnight either in serum-free DMEM or serum-containing growth medium before lysis and assay. Data are mean \pm SD ($n = 3$). (B) Panc1 cells were transfected with 0.5 μ g of either the 9600 bp NheI-EcoRv-luciferase Mirk promoter-reporter containing two upstream putative CREB binding sites and one site in exon 4, the 5887 bp NheI-SmaI Mirk promoter-reporter containing two upstream putative CREB binding sites or the 4830 bp HincII-ScaI Mirk promoter-reporter containing only the putative CRE binding site in exon 4. Cotransfected were 0.25 μ g β -galactosidase reporter plus 0.5 μ g of either the wild-type or S133A mutant CREB expression plasmid. Cells were cultured overnight in serum-free

Mirk expression was controlled by a signaling cascade to CREB binding sites. Myoblasts express three Mirk splice variants (36). The construct containing only the CREB site in exon 4 was further mutated by deletion, one construct deleting promoter A before the transcription start site upstream of exon 1A and another construct deleting promoter B upstream of the alternative splice variant start site (36). Each still contained the CREB binding site within exon 4, and was activated by wild-type CREB 6.0-fold and 4.7-fold, respectively (Figure 5C). Thus, three Mirk promoter constructs, each only containing the exon 4 CREB binding site, were activated ~6-fold by wild-type CREB compared with mutant CREB.

mTOR depletion and CREB depletion modulate Mirk mRNA levels

The mTOR inhibitor WYE354 caused an increase in Mirk/dyrk1B mRNA levels up to 3-fold in a dose-dependent manner (Figure 6A and C), consistent with WYE354's activation of a Mirk promoter construct seen earlier (Figure 4D). Therefore, the increase in Mirk protein levels by pharmacological inhibition of mTOR was due to increased transcription of the Mirk gene. Mirk mRNA levels were also increased following mTOR depletion. Two RNA interference duplexes to mTOR, T1 and T3, each targeting a different sequence, led to a 40% or 70%, respectively, reduction in mTOR protein (Figure 6B) and increased Mirk/dyrk1B mRNA levels 1.2- or 1.8-fold, respectively (Figure 6A).

The decrease in Mirk levels following loss of CREB activation (Figure 4) was due to altered transcription of the Mirk gene and was confirmed by CREB depletion. Two RNA interference duplexes each targeting a different sequence, C90 and C91, reduced CREB protein ~60% (Figure 6B), leading to a 30–50% decrease in Mirk/dyrk1B mRNA levels (Figure 6A). Depletion of both mTOR and CREB at the same time cancelled each other and left Mirk mRNA levels unchanged (summary in Figure 6D). Thus, mTOR depletion or pharmacological inhibition of mTOR by WYE354 led to upregulation of Mirk expression, whereas CREB depletion reduced Mirk mRNA levels.

Discussion

New types of treatment are urgently needed as the 5-year survival rate for pancreatic cancer is <5% and for aggressive ovarian cancer is <27%. Aberrant activation of PI3K/Akt/mTOR (mTORC1) signaling is seen in many pancreatic and ovarian cancers, making mTOR an attractive drug target. Unfortunately, mTOR inhibition is mitigated by negative feedback loops. The mTOR prooncogenic feedback loops have been extensively studied using different types of mTOR inhibitors. For example, the active site inhibitor KU63794 blocks mTOR and dampens the negative feedback loop leading to Akt activation, but still activates Erks in Panc1 cells (37). In contrast, the diabetes drug metformin, which indirectly inhibits mTOR through AMPK signaling, did not cause a compensatory activation of Akt or Erk and inhibited Panc1 cell growth (37). This regulatory picture is further complicated by the current findings that mTOR inhibitors RAD001, WYE354 and rapamycin and two Akt inhibitors increased expression of Mirk kinase. In prior observations, depletion of Mirk decreased the colony forming ability of Panc1 cells 10-fold (38), whereas inhibition of Mirk's kinase activity by RO5454948 or Mirk kinase depletion increased ROS levels in Panc1 cells and in each of four ovarian cancer cell lines and initiated their apoptosis (13,14,21). In this study,

the Mirk kinase inhibitor EHT 5372 reduced the growth of pancreatic cancer cells and ovarian cancer cells in a dose-dependent manner, as expected from the earlier studies with another Mirk inhibitor. Significantly, EHT 5372 enhanced the toxicity of the mTOR inhibitor RAD001 (everolimus). Thus, some of the resistance to mTOR inhibitors seen clinically may be due to upregulation of Mirk kinase, which has prosurvival functions in pancreatic, colon and ovarian cancers (13,39). Mirk/dyrk1B is a kinase active in quiescent ovarian cancer cells and quiescent pancreatic cancer cells so presents an attractive drug target in these cells, perhaps paired with an mTOR inhibitor.

Activation of the mTORC1/S6K/4E-BP1 (40) signaling pathway mediates cell growth, so it is not a surprise that its inhibition leads to Mirk/dyrk1B upregulation. Mirk expression appears to be inversely related to mTOR/Akt activities in tumor cells, as the latter are highest in growing cells, for example after serum stimulation. In contrast, Mirk levels vary up to 10-fold during the cell cycle (16,22), reaching their peak when cells become quiescent in response to energy limitation caused by nutrient or serum starvation and being at their lowest in S phase cells where Akt should be activated (14,21). In addition, activation of Akt in the conditionally inducible Myr+Akt:ER cell line blocked transcriptional upregulation of Mirk (Figure 3B and C). These studies were done under serum-free conditions to block activation of endogenous Akt by serum growth factors (Figure 3B).

Quiescent G₀ cells downregulate their polyribosomes, reducing their total RNA levels, so they can be distinguished from G₁ cells by two parameter flow cytometry. This assay showed that quiescent pancreatic cancer cells and ovarian cancer cells comprised 50–80% of pancreatic or ovarian cancer cell cultures grown under serum-limited conditions (14,21). Unexpectedly, some G₀ cells were even found in rapidly proliferating cultures of pancreatic cancer cells (21% of Panc1 cells, 5% of SU86.86 cells) and growing cultures of ovarian cancer cells (20% of SKOV3, 12% of TOV21G, 20% of OVCAR4, 33% of OVCAR3 cells) (11,14,21). Possibly cells damaged during normal cycling through mitosis accumulate high levels of ROS and stop in G₀ to repair. Most G₀ cells are not permanently arrested as a few day G₀ arrest was found to be reversible when culture conditions improved (11,14,21). Significantly, a minority of cancer cell lines could not undergo a reversible arrest in G₀, and these cells were over twice as likely to die in poor growth conditions (14). Thus, arrest in G₀ contributed to cancer cell survival (14), whereas Mirk overexpression enabled cancer cells to survive such adverse conditions (17).

Quiescent tumor cells are found *in vivo* and can be detected by lack of Ki67 expression. Nuclear Ki67 antigen is expressed in all phases of the cell cycle except G₀ and early G₁ and was found by immunohistochemical surveys of patient material in only 28% of pancreatic cancer cells (41) and about a third of ovarian cancer cells (42). By entering a quiescent state, tumor cells can resist the nutrient deficiencies, hypoxic and acidic conditions within the tumor mass. Mirk maintains the viability of quiescent cancer cells by increasing the expression of several antioxidant genes that reduce intracellular levels of ROS (11,12). Factors that allow the prolonged survival of quiescent tumor cells in patients *in vivo* are of clinical relevance, and include Mirk, as tumors with high levels of Mirk tended to rapidly progress compared with tumors with low Mirk levels by Kaplan–Meier analysis (43). Significantly, quiescent cancer stem cells have been identified, which are exceptionally refractory to cytotoxic treatments (44) and oxidative stress damage (45,46) and are slow-cycling, label-retaining cells with high clonogenic capacity and tumorigenicity (47). Most current therapies have been optimized to target dividing tumor cells, not the quiescent tumor cells in which Mirk levels are elevated. Thus, strategies that target Mirk may allow quiescent tumor cells including cancer stem cells to be killed.

Mirk mRNA regulation is poorly understood. In this study, mTOR inhibitors RAD001 and WYE354 were shown to increase Mirk expression through activation of CREB, the cAMP response element binding protein. However, dibutyryl cAMP treatment in cycling cells

DMEM to allow Mirk expression before lysis and assay. Data are shown as mean ± SD (*n* = 3). (C) C2C12 myoblasts in 12-well plates were transfected for 4 h with 2 μl Lipofectamine Plus and the 0.25 μg β-galactosidase reporter, 0.5 μg of either the wild-type or S133A mutant CREB expression plasmid and 5 μg of the three Mp-luciferase constructs listed in panel B plus 2 mutant constructs. The cells were cultured overnight, in DMEM + 10% FBS, then washed and placed in differentiation medium (DMEM plus 2% horse serum) for 24 h before assay. Data are shown as mean ± SD (*n* = 3). Results are one of three similar experiments. The numbers above the bars indicate whether the construct has one or two upstream CREB sites/downstream site, either wild-type or mutant (m).

Mirk mRNA Levels Increased by mTOR Inhibitor and mTOR RNAi's T1&T3, and Decreased by CREB RNAi's C90&C91

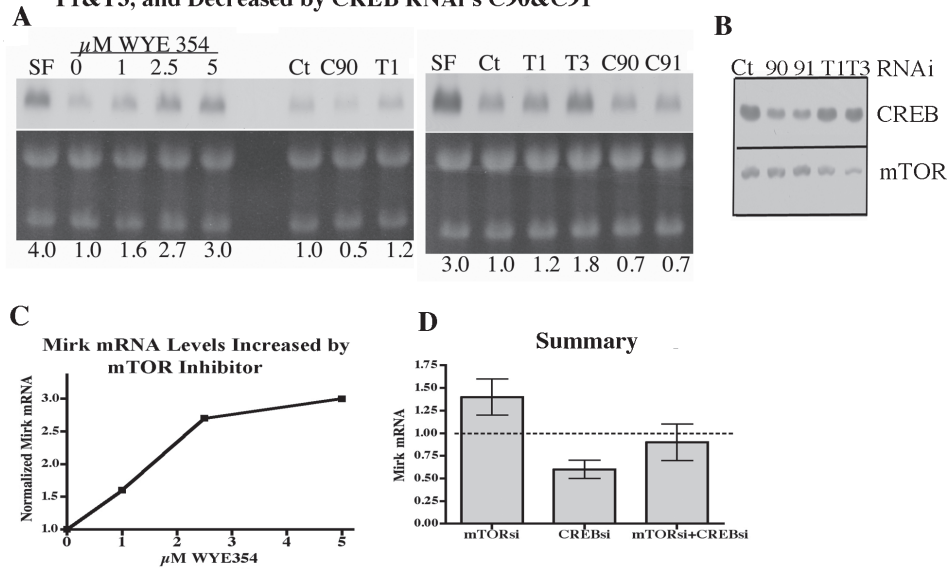


Fig. 6. Mirk mRNA levels increased by either depletion or inhibition of mTOR and decreased following CREB depletion. (A) Northern analysis of Mirk mRNA levels. Left: Panc1 cells treated with increasing concentrations of WYE354 for 1 day before northern analysis (lanes 2–5). Panc1 cells were transfected (5 μ l RNA interference (RNAi)/5 μ l Lipofectamine 2000) for 1 day in complete medium with either RNAi FRAP1HSS103825 (T1) to mTOR/FRAP, RNAi C90 to CREB or a GC-matched negative control RNAi (Ct) then switched for 1 day serum-free DMEM to allow Mirk expression before analysis, lanes 6–8. Positive control for high Mirk expression, 1 day serum-free culture. Right: Panc1 cells were transfected either RNAi's FRAP1HSS103825 (T1) or FRAP1HSS103827 (T3) to mTOR/FRAP, RNAi's C90 or C91 to CREB or a GC-matched negative control RNAi (Ct), then switched for 1 day to serum-free DMEM to allow Mirk expression before analysis. One of the duplicate experiments with similar results. Below each northern blot is an ethidium bromide-stained total RNA panel showing 28s and 18s rRNAs. (B) Western blot of CREB and mTOR protein levels after Panc1 cell treatment with RNAi's in panel A. C90 and C91 reduced CREB levels 60%, whereas T1 and T3 reduced mTOR levels 40% and 70%, respectively (Uni-Scan densitometry). (C) Graph showing increase in Mirk mRNA with increasing WYE354 concentration. (D) Summary of northern analysis of Mirk mRNA levels. Data on mRNA expression from panel A pooled, together with five samples treated with mixtures of RNAi's to CREB and to mTOR, mean \pm SE shown. Dotted line indicates control level. The mean of the mTORSi and the mean of the CREBsi were statistically different, $P = 0.022$ by two-tailed t -test. CREBsi, small interfering RNA to CREB; FRAP, FKBP12-rapamycin complex-associated protein; PTEN, phosphatase and tensin homolog; mTORSi, small interfering RNA to mTOR.

did not increase Mirk expression (data not shown), so CREB activation was not sufficient to increase Mirk transcription but was a permissive condition. Other agents that control Mirk expression include Rho family members and microRNA-880. Mirk is abundant in normal skeletal muscle and in C2C12 myotubes, and is induced when C2C12 myoblasts or primary cultured muscle satellite cells arrest and differentiate (25). In C2C12 myoblasts, a Mirk promoter construct was activated by the Rho family members RhoA, Cdc42 and Rac1, and inhibited by dominant negative RhoA-N19 (25). Inhibition of microRNA-880 increased the expression of Dyrk1b in murine embryonic stem cells (48). Thus, microRNA-880 might inhibit Mirk/dyrk1B in cycling cells, whereas arrest of cells is an essential permissive condition that allows Rho family members and CREB activation to increase Mirk expression.

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