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Oncogene. Author manuscript; available in PMC 2018 January 10.

Published in final edited form as:

Oncogene. 2017 November 09; 36(45): 6225–6234. doi:10.1038/onc.2017.228.

Tumor suppressor Pdcd4attenuates Sin1 translation to inhibit invasion in colon carcinoma

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Abstract

Programmed cell death 4 (Pdcd4), a tumor invasion suppressor, is frequently down-regulated in colorectal cancer and other cancers. In this study, we find that loss of Pdcd4 increases the activity of mammalian target of rapamycin complex 2 (mTORC2) and thereby upregulates Snail expression. Examining the components of mTORC2 showed that Pdcd4 knockdown increased the protein but not mRNA level of stress-activated-protein kinase interacting protein 1 (Sin1), which resulted from enhanced Sin1 translation. To understand how Pdcd4 regulates Sin1 translation, the *SIN1* 5' untranslated region (5'UTR) was fused with luciferase reporter and named as 5'Sin1-Luc. Pdcd4 knockdown/knockout significantly increased the translation of 5'Sin1-Luc but not the control luciferase without the *SIN1* 5'UTR, suggesting that Sin1 5'UTR is necessary for Pdcd4 to inhibit Sin1 translation. Ectopic expression of wild type Pdcd4 and Pdcd4(157–469), a deletion mutant that binds to translation initiation factor 4A (eIF4A), sufficiently inhibited Sin1 translation, and thus suppressed mTORC2 kinase activity and invasion in colon tumor cells. By contrast, Pdcd4(157–469)(D253A,D418A), a mutant that does not bind to eIF4A, failed to inhibit Sin1 translation, and consequently failed to repress mTORC2 activity and invasion. In addition, directly inhibiting eIF4A with silvestrol significantly suppressed Sin1 translation and attenuated invasion. These results indicate that Pdcd4-inhibited Sin1 translation is through suppressing eIF4A, and functionally important for suppression of mTORC2 activity and invasion. Moreover, in colorectal cancer tissues, the Sin1 protein but not mRNA was significantly up-regulated while Pdcd4 protein

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

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was down-regulated, suggesting that loss of Pdcd4 might correlate with Sin1 protein level but not mRNA level in colorectal cancer patients. Taken together, our work reveals a novel mechanism by which Pdcd4 inhibits Sin1 translation to attenuatemTORC2 activity and thereby suppresses invasion.

Keywords

Pdcd4; Sin1; eIF4A; Akt; mTORC2; invasion

INTRODUCTION

Programmed cell death 4 (Pdcd4) is a tumor suppressor, whose expression decreases in many types of cancers including colorectal cancer.[1] Over-expression of Pdcd4 has been shown to inhibit cell proliferation,[2] while Pdcd4 knockdown promotes cell proliferation,[3] suggesting an inhibitory role of Pdcd4 in regulating cell proliferation. Besides suppressing proliferation, several studies also demonstrated that Pdcd4 is a regulator of tumor invasion and metastasis. For instance, over-expression of *PDCD4* cDNA attenuates invasion in colon and breast cancer cells.[4–6] Conversely, Pdcd4 knockdown promotes invasion.[7–9] Consistent with these *in vitro* findings, knockdown of Pdcd4 expression in colon tumor cells stimulates metastasis to lymph node and liver in nude mice,[10] and knockout of Pdcd4 in mice induces lymphomas with frequent metastasis.[11] We previously reported that Pdcd4 knockdown results in inhibition of E-cadherin expression and thereby activation of β-catenin and AP-1 dependent transcriptions.[7, 12] Suppression of E-cadherin expression in Pdcd4 knockdown cells is due to the stimulation of Snail expression since knockdown of Snail expression in Pdcd4 knockdown cells restored the expression of E-cadherin.[7] However, how Snail expression is regulated by Pdcd4 remains unknown.

Pdcd4 also functions as a protein translation inhibitor. Biochemical and crystal structural analyses demonstrated that Pdcd4 binds with translation initiation factor 4A (eIF4A) and inhibits its helicase activity.[13–15] The function of eIF4A, an ATP-dependent RNA helicase, is believed to unwind the mRNAs with secondary structure at 5’ untranslated region (5’UTR) at the stage of translation initiation.[16] Since Pdcd4 inhibits eIF4A’s helicase activity, Pdcd4 is anticipated to preferentially suppress translation of mRNAs with secondary structure at 5’UTR. Indeed, by fusing a synthetic stem-loop structure at 5’UTR of luciferase, we demonstrated that Pdcd4 suppresses translation of this stem-loop structured luciferase greater than the one without it. Although Pdcd4 functions as an inhibitor for invasion and protein translation, the mechanism by which Pdcd4 inhibits translation to control tumor invasion is still unknown, and the Pdcd4 translational targets involved in tumor invasion have not been identified yet.

We and others have found that over-expression of *PDCD4* cDNA inhibits phosphorylation of Akt at Ser473 while Pdcd4 knockdown activates Akt kinase activity and increases phosphorylation of Ser473,[3, 17, 18] suggesting that Pdcd4 regulates Akt activity. Akt is frequently activated in many types of human cancers, which mediates numerous cellular functions including invasion and metastasis.[19] The Akt activity is mainly regulated by 3-

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phosphoinositide-dependent kinase 1 (PDK1) and mammalian target of rapamycin (mTOR) complex 2 (mTORC2). Phosphorylation of Thr308 by PDK1 increases Akt kinase activity, but the maximal activity requires phosphorylation of Ser473 by mTORC2.[20] mTOR associates with different subunits to form two distinct complexes, mTORC1 (mTOR complex 1) and mTORC2. mTORC1, which is rapamycin sensitive, enhances cell growth and proliferation.[21] In contrast, mTORC2 is rapamycin insensitive and its biological functions remain understudied. mTORC2 is comprised of mTOR, rapamycin-insensitive companion of mTOR (Rictor), G protein beta subunit-like (GβL), stress-activated-protein kinase interacting protein 1 (Sin1), Protor-1, and Deptor.[22] Recent studies suggest that mTORC2 is a critical regulator for cell motility, invasion, and metastasis. For instance, suppression of mTORC2 activity by knockdown of Rictor, attenuates colon tumor cell proliferation and invasion/metastasis in cultured cells as well as in nude mice,[23, 24] while overexpression of Rictor elevates mTORC2 activity resulting in increased cell motility.[25] Sin1 is known as a unique component of mTORC2, and thought to stabilize the mTORC2 complex by preventing it from undergoing lysosomal degradation.[26] In addition, phosphorylation of Sin1 at Thr86 and Thr398 by S6K disrupts the binding between Sin1 and other mTORC2 components, resulting in decreased mTORC2 activity.[27] Immunohsitochemical staining also showed that Sin1 is up-regulated in the thyroid carcinomas and hepatocellular carcinoma.[28, 29] Xu *et al.* demonstrate that depletion of Sin1 expression reduces migration and invasion in hepatocellular carcinoma cells.[29] However, little is known about the regulation of mTORC2 activity.

In this study, we identify that Sin1 is translationally regulated by Pdcd4 through eIF4A in colorectal cancer cells. We also demonstrate that Pdcd4-inhibited Sin1 translation results in suppression of mTORC2-Akt-Snail axis and thereby attenuation of colon tumor cell invasion. Manipulating eIF4A activity with pharmacological agent silvestrol also effectively inhibits Sin1 translation and tumor cell invasion. These findings suggest a novel mechanism of regulating invasion by Pdcd4 in colon tumor cells and provide a new direction for altering mTORC2 activity.

RESULTS

Loss of Pdcd4 activates mTORC2-Akt axis and enhances Snail expression

Previously, we demonstrated that Pdcd4 knockdown in colon carcinoma activates Akt kinase activity and concomitantly increases Snail expression to suppress E-cadherin expression and thereby promotes tumor invasion.[3, 8, 12] To find out whether reduction of Pdcd4 expression in non-cancerous cells also activates Akt, we isolated the mouse embryonic fibroblast (MEF) cells from the embryos of wild-type and Pdcd4 knockout mice.[30] The morphology of Pdcd4 null MEF ($Pdcd4^{-/-}$) cells appeared slightly longer and thinner comparing to the wild-type MEF ($Pdcd4^{+/+}$) cells (Figure 1a), suggesting that Pdcd4 knockout triggered cytoskeleton rearrangement and might increase the cell motility. In agreement with the observation that Pdcd4 knockdown stimulates proliferation, the $Pdcd4^{-/-}$ MEF cells exhibited faster proliferation than $Pdcd4^{+/+}$ MEF cells (Figure 1b). As expected, the phosphorylation of Akt at Ser473 was dramatically increased in $Pdcd4^{-/-}$ MEF cells as seen in Pdcd4 knockdown cells (HT29-P, HT29 cells expressing *PDCD4* shRNA[8]) (Figure 1c). These findings suggest

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that loss of Pdcd4 expression activates Akt. Next, we tested whether the Akt activation induced by Pdcd4 knockdown affected Snail expression. Akt has 3 isoforms, among which Akt1 and Akt2 are expressed in most tissues while Akt3 expression is restricted in brain and testis.[31] Hence, we took Pdcd4 knockdown cells (HT29-P) and knocked down Akt1 and Akt2. Both significantly reduced Snail protein level, and consequently, upregulated its target E-cadherin (Figure 1d), suggesting that Pdcd4 knockdown activates Akt to increase Snail expression. In line with these results, activation of Akt by Pdcd4 knockdown or knockout also increased Snail expression (Figure 1c). It is known that mTORC2 can phosphorylate Akt at Ser473 to activate Akt.[20] We, therefore, tested whether Pdcd4 knockdown activates mTORC2 kinase activity. As shown in Figure 1d, the mTORC2 kinase activity was approximately 3.5-fold higher in HT29-P cells than that in control cells (HT29-L, HT29 cells expressing *lacZ* shRNA[8]), and in Pdcd4$^{-/-}$ MEF cells compared to wild type Pdcd4$^{+/+}$ MEF cells (Figure 1e). These results indicate that loss of Pdcd4 enhances mTORC2-Akt axis, leading to the increased Snail expression.

Knockdown of Pdcd4 stimulates Sin1 translation

To explore the mechanism by which Pdcd4 affects mTORC2 activity, we examined the expression levels of individual components in mTORC2. The Sin1 protein in HT29-P cells was approximately 2.9-fold of that in HT29-L cells, while the protein levels of mTOR, Rictor, and GβL were alike in HT29-L and HT29-P cells (Figure 2a). Similar results were observed in Pdcd4$^{-/-}$ MEF cells in which Sin1 protein increased (Supplementary Figure S1). However, Pdcd4 knockdown did not affect either *SIN1* mRNA level (Figure 2b) or Sin1 protein stability (Figure 2c). In agreement with these results, over-expression of *PDCD4* cDNA in HCT116 and RKO cells decreased the protein level of Sin1 but not mTOR, Rictor, and GβL (Figure2d). These findings suggest that Pdcd4's inhibition on Sin1 occurs at the posttranscriptional level and does not involve protein stability, presumably at the level of translation. To confirm this notion, we performed sucrose gradient fractionation to separate ribonucleoproteins (RNPs), monosomes, and polysomes. Knockdown of Pdcd4 resulted in a small reduction in free ribosomal subunits (Figure 2e). Polysomal fractions contain the actively translated mRNA bound with numerous ribosomes during protein translation. On the other hand, mRNA that is not translated or barely translated is distributed in RNPs and monosomal fractions. As shown in Figure 2f, *SIN1* mRNA level increased 2 to 4-fold in heavier polysomal fractions (fractions #10–13) in HT29-P cells as compared to HT29-L cells. By contrast, there was no significant change in the distribution of *RICTOR* mRNA in polysomal fractions between HT29-P and HT29-L cells. In summary, these findings indicate that loss of Pdcd4 enhances Sin1 translation. Notably, Sin1 appeared as a single band in HCT116 cells but showed as two bands in RKO cells (Figure 2d). This discrepancy might be due to the existence of different isoforms in different type of cells.

The *SIN1* 5'UTR is essential for Pdcd4 to inhibit Sin1 translation

We have demonstrated that Pdcd4 directly binds with eIF4A and inhibits its helicase activity.[15] eIF4A is a RNA helicase whose activity is required for translation of mRNAs with structured 5'UTR.[32] Since the 5'UTR *of SIN1* mRNA contains 330 nucleotides with high GC content which can form a stable secondary structure, translation of this type of mRNA is expected to be preferentially inhibited by Pdcd4. To test whether *SIN1* 5'UTR is an essential

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element for inhibiting Sin1 translation by Pdcd4, we fused *SIN1* 5'UTR with luciferase reporter to generate 5'Sin1-Luc (Figure 3a) and transfected it into HT29-L and HT29-P cells. The luciferase activity in HT29-P cells was 1.8-fold of that in HT29-L cells (Figure 3b), whereas the mRNA levels were about the same in both HT29-L and HT29-P cells (Figure 3c). Similar results were obtained when 5'Sin1-Luc was transfected into Pdcd4$^{+/+}$ and Pdcd4$^{-/-}$ MEF cells, in which Pdcd4 knockout increased the luciferase activity about 3.5-fold (Supplementary Figure S2). As expected, the luciferase activity is about the same in HT29-L and HT29-P cells when non-structured luciferase reporter (control) was transfected (Figure 3b), suggesting that Pdcd4 knockdown has no effect on luciferase enzymatic activity per se. Since the luciferase mRNA levels were similar between HT29-L and HT29-P cells (Figure 3c), the increase of luciferase activity should not be due to the increase in synthesis or stability of the luciferase mRNA but reflects translational enhancement. Moreover, transfection of the Pdcd4 expression plasmid along with 5'Sin1-Luc into HCT116 cells inhibited 5'Sin1-Luc translation in a concentration dependent manner, and the decrease in luciferase translation in 5'Sin1-Luc transfected cells was greater than that in control transfected cells (Figure 3d). These results suggest that *SIN1* 5'UTR is necessary for Pdcd4 to inhibit Sin1 translation.

Pdcd4 suppresses Sin1 translation through inhibition of eIF4A

If Pdcd4 knockdown increases Sin1 translation through relieving the inhibition of eIF4A activity, then directly inhibiting eIF4A activity should reduce Sin1 translation. To test this, HT29-P cells were transfected with 5'Sin1-Luc and subsequently treated with eIF4A inhibitor, silvestrol, at a final concentration of 0 to 20 nM. The 5'Sin1-Luc translation in HT29-P cells treated with silvestrol decreased in a concentration dependent manner (Figure 4a). Silvestrol at 20 nM inhibited approximately 50% of 5'Sin1-Luc translation, but did not affect translation of control luciferase reporter (Figure 4a). In addition, HT29-P cells treated with 20 or 50 nM of silvestrol showed a dramatic decrease in Sin1 and Snail abundances (Figure 4b). Similarly, exposing HCT116 cells to 20 or 50 nM of silvestrol also displayed a decrease in Sin1 and Snail protein levels. In concert with the result of polysomal fractionation, 20 or 50 nM of silvestrol did not affect the Rictor protein level (Figure 4b). These findings suggest that directly inhibiting eIF4A activity reverses Pdcd4 knockdown-induced Sin1 translation. To further demonstrate that Pdcd4 inhibits Sin1 translation mediated by eIF4A, we transfected Pdcd4(WT), Pdcd4(157–469), and Pdcd4(157–469)(D253A,D418A) along with 5'Sin1-Luc into HCT116 cells. Pdcd4(157–469), a deletion mutant containing amino acid residues 157 to 469, binds to eIF4A and inhibits translation, while Pdcd4(157–469)(D253A,D418A), the above deletion mutant with Asp residues substituted by Ala at 253 and 418, does not bind to eIF4A and fails to inhibit translation.[33] The full length Pdcd4(WT) and Pdcd4(157–469) inhibited 5'Sin1-Luc translation about 40% and 60%, respectively, comparing to empty vector (control, Figure 4c). By contrast, the cells expressing Pdcd4(157–469)(D253A, D418A) (Mut) had similar level of 5'Sin1-Luc translation as control cells. In addition, cells transfected with Pdcd4(WT) and Pdcd4(157–469) showed a decrease in Sin1 and Snail abundances (Figure 4d). These results collectively suggest that Pdcd4 inhibits Sin1 translation by suppressing eIF4A activity and possibly impede the unwinding of the secondary structure in *SIN1* 5'UTR. It also indicates that Pdcd4 inhibits Snail abundance through suppression of Sin1 expression.

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Pdcd4-inhibited Sin1 translation leads to suppression of mTORC2 activity and invasion

Next, we asked whether up-regulation of Sin1 translation by Pdcd4 knockdown is functionally significant in promoting colon tumor cell invasion. We knocked down Sin1 expression by two commercial *SIN1* siRNAs, which reduced Sin1 protein levels about 80–90%, and in consequence, phosphorylation of Akt at Ser473 and Snail expression were decreased (Figure 5a). As shown in Figure 5b, the invasive capacity of HT29-P cells transfected with *SIN1* siRNA was approximately 40–50% of that in HT29-P cells transfected with scrambled siRNA (control), suggesting that Sin1 up-regulation triggered by Pdcd4 knockdown is important for promoting colon tumor cell invasion. To confirm that Pdcd4-inhibited Sin1 translation is sufficient for suppressing mTORC2 activity and invasion, we assayed the mTORC2 kinase activity and invasive capacity with the cells expressing Pdcd4 (WT), Pdcd4(157–469), and Pdcd4(157–469)(D253A, D418A). The Pdcd4(WT) significantly inhibited both mTORC2 kinase activity and invasive ability (Figures 5c and 5d) as well as migration ability (Supplementary Figure S3). The mTORC2 kinase activity and invasive capacity in cells expressing Pdcd4(157–469) were approximately 30% and 50% of that in cells expressing empty vector (control), respectively (Figures 5c and 5d). By contrast, the cells expressing Pdcd4(157–469)(D253A, D418A) had mTORC2 kinase activity and invasive capacity similar to that observed in control cells (Figures 5c and 5d). Together, these findings suggest that Pdcd4-inhibited Sin1 translation is critical for inhibition of mTORC2 activity and invasion.

To delineate the role of eIF4A in regulating cell invasion, we assayed the effects of silvestrol on invasion. HT29-P and HCT116 cells were serum starved for 24 h and subsequently seeded on the upper Boyden chamber with or without 20 nM of silvestrol. As shown in Figure 5e, silvestrol treatment markedly reduced cell ability to penetrate the Matrigel barrier, suggesting that inhibition of eIF4A activity suppresses invasion. In addition, 20 nM silvestrol sufficiently suppressed HT29-P and HCT116 cell migration (Supplementary Figure S4). It is noteworthy that HT29-P and HCT116 cells treated with 20 nM of silvestrol did not induce cell death (Figure 5f and Supplementary Figure S5) as observed in MDA-MB-231 and PC-3 cells,[34] indicating that the reduction in invasion was not due to silvestrol-induced apoptosis.

Sin1 protein but not mRNA is up-regulated in the colorectal cancer tissues

To find out the expression status of Sin1 in human colorectal cancer, we examined the protein levels of both Sin1 and Pdcd4 in malignant colorectal tissues and adjacent normal colon tissues from 21 patients (Supplementary Table 1). The protein levels of Sin1 and Pdcd4 were assessed using immunoblot analysis. As shown in Figure 6a and Supplementary Figure S6, the Sin1 protein level was significantly elevated in the cancerous tissues compared to that in adjacent normal tissues. However, the *SIN1* mRNA level was similar in cancerous and normal tissues (Figure 6b), indicating that elevation of Sin1 protein in colorectal cancer patients is not due to the increased Sin1 transcription. In contrast to up-regulation of Sin1 protein, Pdcd4 protein level was down-regulated in the cancerous tissues (Figure 6c). These results suggest that loss of Pdcd4 might increase Sin1 protein but not mRNA in the colorectal cancer tissues as observed in cultured cells.

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DISCUSSION

The role of Pdcd4 in suppressing tumor invasion and metastasis has been established, but the molecular mechanism by which Pdcd4 inhibits invasion is not fully understood. In this study, we demonstrated that Pdcd4 suppressed Sin1 translation through inhibition of eIF4A mediated by *SIN1* 5'UTR, and Pdcd4-inhibited Sin1 translation is functionally significant in suppressing mTORC2-Akt-Snail axis and invasion. In agreement with these *in vitro* findings, the Sin1 protein but not mRNA increased and Pdcd4 protein decreased in colorectal cancer tissues. Thus, our results reveal a new mechanism of how Pdcd4 attenuates mTORC2 via inhibiting Sin1 translation to suppress colon tumor invasion (Figure 7).

Protein translational control is a critical step for regulation of cancer development. During translation initiation, the translation initiation complex binds to mRNA at 5' end and scan mRNA to locate the translation initiation codon AUG. If the mRNA forms stable secondary structure at 5'UTR, it requires the eIF4A's helicase activity to unwind such a structure allowing the translation initiation complex to pass through the secondary structure.[32] Inserting an artificial stem-loop structure at 5'UTR of luciferase, we previously found that Pdcd4 inhibited translation of luciferase with the stem-loop structure greater than the one without it. It thus implied that Pdcd4 preferentially inhibited translation of structured mRNAs at 5'UTR.[33] This concept was applied to a natural mRNA in this study, in which we showed that Pdcd4 and Pdcd4(157–469) but not Pdcd4(157–469)(D253A,D418A) inhibited translation of 5'Sin1-Luc, and suppressing eIF4A by silvestrol inhibited 5'Sin1-Luc translation and Sin1 expression as well (Figure 4). These findings provide evidence that Pdcd4 represses the translation of its natural target, *SIN1* mRNA, through *SIN1* 5'UTR.

Recent studies using genome-wide ribosome profiling revealed that eIF4A regulates a set of so-called "eIF4A-dependent transcripts".[35] Since the products of many eIF4A-dependent transcripts are transcription factors and oncogenes, inhibition of eIF4A is expected to suppress tumorigenesis. Indeed, inhibition of eIF4A activity by silvestrol suppresses tumor proliferation and induces apoptosis in various types of cancers.[34, 36, 37] Although silvestrol has been demonstrated to effectively inhibit tumor growth, its role in tumor invasion is still unknown. Our findings that silvestrol efficiently inhibits eIF4A activity (Fig. 4) to suppress invasion in HCT116 and HT29-P cells (Figure 5) provide the evidence for silvestrol as an inhibitor for tumor invasion. Our results also showed that Pdcd4 inhibited invasion through inhibition of eIF4A, which is achieved, at least in part, by suppression of Sin1 translation since Pdcd4(157–469) but not Pdcd4(157–469)(D253A,D418A) sufficiently attenuated Sin1 translation, mTORC2 activity, and invasion (Figures 3–5). In addition to inhibiting eIF4A activity, Pdcd4 has been reported to inhibit translation through an eIF4A-independent mechanism. Liwak*et al.* showed that Pdcd4 directly binds mRNAs of X-linked inhibitor of apoptosis and Bcl-x(L), and repressed their translations by preventing the formation of translation initiation complex.[38] Fehler *et al.* also demonstrated that interaction between Pdcd4 and poly(A) binding protein is required for binding to *C-MYB* mRNA to suppress its translation.[39] It is unknown whether Pdcd4 binds to mRNA through a specific motif or secondary structure, which needs further investigation.

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In colorectal cancer, the expression of mTOR, Rictor, and phospho-Akt (Ser473) are frequently up-regulated.[40] Here, we showed that Sin1 protein level was also up-regulated in colorectal cancer tissues (Figure 6 and Supplementary Figure S6). Inactivation of mTORC2 by Rictor knockdown[23] or Sin1 knockdown (Figure 5) reduces colon tumor cell invasion, implying that mTORC2 activation is critical for promoting colorectal cancer cell invasion. In addition, our findings revealed that Pdcd4 inhibits mTORC2 activity and thereby suppresses Akt activation and Snail expression (Figure 1), suggesting that repression of mTORC2-Akt-Snail axis by Pdcd4 contributes to invasion suppression (Figure 5). How does Akt regulate Snail expression? One potential path is through activation of NF-κB to up-regulate Snail expression.[41] Akt induces phosphorylation of IκB, resulting in IκB degradation and thereby NF-kB activation. The other possible mechanism is that Akt inactivates GSK3 by phosphorylation of Ser21 in α subunit and Ser9 in β subunit, resulting in stabilization of Snail (Figure 7).[42] Further investigation is needed to determine the downstream pathway of Pdcd4-mTOR2-Akt that regulates Snail expression. Dorrello *et al.* have shown that Pdcd4 can be phosphorylated at Ser67 by p70S6K and subsequently undergoes proteasome degradation.[43] Interestingly, Pdcd4 may inhibit p70S6K phosphorylation in turn.[44] Here, we further demonstrated that Pdcd4 inhibits Akt activity by controlling mTORC2 (Figure 5), suggesting that Pdcd4 inhibits p70S6K phosphorylation by suppression of mTORC2-Akt axis.

Although mTORC2 has been implicated to be important for tumorigenesis, the regulation of mTORC2 activity is still not clearly known, especially in colorectal cancer. Recent studies suggested that p300-mediated acetylation of Rictor stimulates mTORC2 activity.[45] In addition to Rictor, Sin1 also play an important role in regulating mTORC2 activity. Wei and colleagues reported that Sin1 binds to Ptdlns(3,4,5)P_3 to translocate mTORC2 to plasma membrane and activate its activity.[46] The same group also found that phosphorylation of Sin1 at Thr86 and Thr398 by either S6K or Akt interferes the binding of Sin1 to other mTORC2 components, resulting in inhibition of growth factor-induced mTORC2 activation.[27] However, James and colleagues reported that phosphorylation of Sin1 at Thr86 activates mTORC2-Akt signalling and enhances mTORC2 kinase activity.[47] It is not clear whether the discrepancy of these findings is due to the different cell-type or the kinase-substrate specificity. Our findings that Pdcd4 inhibits Sin1 translation (Figure 2) and thus suppresses mTORC2 activation and invasion in colorectal cancer cells (Figure 5) provide a new regulatory mechanism of mTORC2 activity. This mechanism is further supported by the observations that Sin1 protein but not mRNA is up-regulated whereas Pdcd4 protein is down-regulated in human colorectal cancer tissues (Figure 6).

In conclusion, these results suggest that Pdcd4 inhibits Sin1 translation through eIF4A inhibition, which may repress the unwinding of secondary structure at *SIN1* 5'UTR. Inhibition of Sin1 translation leads to attenuation of mTORC2-Akt-Snail and invasion. These findings identify a new path to suppress mTORC2 via Pdcd4-inhibited Sin1 translation, which points out a novel direction for development of mTORC2-specific inhibitors for cancer therapeutics.

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MATERIALS AND METHODS

Chemicals and reagents

Silvestrol was purchased from MedChem Express (Monmouth Junction, NJ) and dissolved in DMSO and stored at −80°C.

Cell lines and patient specimens

HT29, HCT116, and RKO cells were purchased from ATCC, and cultured in McCoy's 5A medium as described previously.[8] All cell lines were routinely tested for mycoplasma contamination. Tumor and adjacent normal tissues were obtained from 21 colorectal cancer patients at Qilu Hospital, Shandong University, China. Informed consent was obtained from all of patients. Both study protocol and informed consent were approved by the Ethical Committee of Qilu Hospital. Immediately after surgical removal, tumor and adjacent normal tissues were frozen in liquid nitrogen. Frozen tissues were grinded to fine powder and subsequently lysed in lysis buffer[8] containing 1X protease inhibitor and phosphatase inhibitor cocktail with Dounce homogenizer for protein extraction. For RNA isolation, the tissue powder was incubated with Trizol (Invitrogen, Carlsbad, CA, USA) for 30 min with occasional vortex.

Isolation of MEF cells

The Pdcd4$^{+/+}$ and Pdcd4$^{-/-}$ MEF cell lines were isolated from the embryos (day 13 or 14 p.c.) of wild-type and Pdcd4 knockout mice,[30] respectively. The embryos from placentas were passed through an 18G/1½" needle and cultured in 10 ml DMEM medium on a gelatin coated plate and incubated at 37°C until the cells reached confluence. The MEF are the only cells that attach and proliferate on the gelatin coated plate. At confluence, cells (Passage 0) were split at the ratio of 1:3. The primary MEF cells with passage 3 to 6 were used for the experiments.

Immunoblot analysis

Immunoblot analysis and quantification of band intensity were performed as described previously.[8] The following primary antibodies were used for immunoblotting: anti-Akt1 (#2938), anti-Akt2(#3063), anti-mTOR (#2972), anti-phospho-Akt (Ser473) (#4060), anti-pan Akt (#4691), anti-Rictor (#2114), anti-Sin1 (NB110–40424), anti-GβL (#3274), anti-Pdcd4, and anti-GAPDH (sc-47724). Antibody for Sin1 and GAPDH was purchased from Novus Biologicals (Littleton, CO, USA) and Santa Cruz (Santa Cruz, CA, USA), respectively. Akt1 and Akt2 antibodies were purchased from Pierce (Rockford, IL, USA). Pdcd4 antibody was generated previously.[8] The rest of antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

Quantitative real-time PCR

The total RNA was isolated from cells using Trizol reagent (Invitrogen). After synthesis first strand cDNA, the mRNA levels were quantified by quantitative PCR as described previously.[8]

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Sucrose gradient fractionation

Cells were treated with cyclohexamide (10 μg/ml) for 10 min prior to cell harvesting and lysed in the lysis buffer[48] for 30 min on ice. After centrifugation, the supernatants were layered onto 10–45% (w/w) sucrose gradient containing 10 mM Tris-HCl (pH7.2), 60 mM KCl, 10 mM MgCl, 1 mM DTT, and 0.1 mg/ml of heparin. After centrifugation at 36,000 rpm using SW41Ti rotor for 2 h, fractions were collected with Piston Gradient Fractionator (Biocomp, Fredericton, NB Canada) and monitored at 245 nm. The RNAs in each fraction were extracted using Trizol (Invitrogen)and subsequently analyzed using RT-qPCR.

mTORC2 kinase assay

Cells were lysed in CHAPS buffer[49] containing protease inhibitors (Pierce) on ice for 30 min. The lysate was incubated with mTOR antibody (Cell Signaling, #2972) and protein G magnetic beads (Pierce) at room temperature for 1h with constant rotary agitation. After wash with CHAPS buffer, the purified recombinant Akt1 (1 μl of 0.5 μg/ml, ATGen, Korea) and ATP (1 μl of 10 mM) and 18 μl of the mTORC2 kinase buffer[49] were added to the beads, and incubated at 37°C for 15 min. The reaction was terminated by adding 20 μl of SDS sample buffer and boiled for 5 min to release the bound mTOR into the supernatant.

Migration and invasion assay

Migration and invasion assays were performed as described previously.[8, 10]

Statistical analysis

The levels of Sin1 and Pdcd4 protein, and *SIN1* mRNAin malignant and adjacent normal tissues were analyzed by Wilcoxon signed rank test. For kinase activity assays, the one-sample *t*-test was used to compare control and treated groups by examining whether the mean relative fold change is equal to one. For other experiments, the Bartlett's test was first used to test for equal variances between groups. The Student's *t*-test assuming equal variances was used to compare experimental groups if the Bartlett's test was not significant, and the Welch's unequal variances t-test was used otherwise. Data were expressed as mean ± SD. $P < 0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the NIH/NCI grant to H.-S. Yang (R01CA129015 and R03CA187839),NCI/NIH center core support grant to C. Wang (P30CA177558), and National Natural Science Foundation of China grant to J Zhu (81402192). The Flow Cytometry facility is supported in part by NCI Center Core Support Grant (P30CA177558).

We thank Dr. Jian-Li Wang (Shandong University) for experimental supports and Ms. Donna Gilbreath and Ms. Catherine Anthony (Markey Cancer Center) for editing the manuscript.

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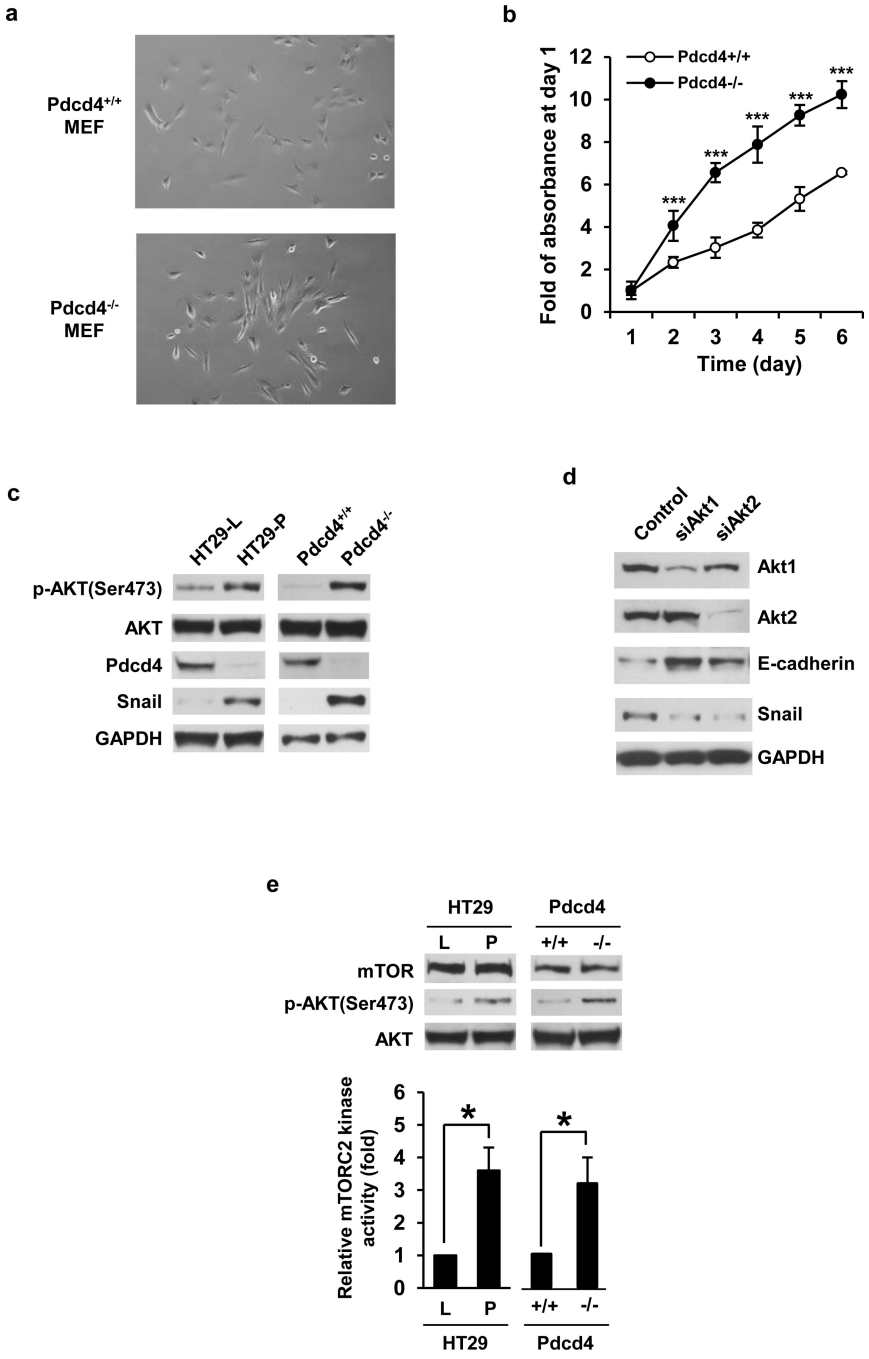

Figure 1.

Loss of Pdcd4 upregulates Snail expression through mTORC2-Akt-Snail axis. (**a**) Images of wild type (Pdcd4$^{+/+}$) and Pdcd4 null (Pdcd4$^{-/-}$) MEF cells. (**b**) Pdcd4 knockout promotes cell proliferation. Pdcd4$^{+/+}$ and Pdcd4$^{-/-}$ MEF cells were seeded onto 96-well plates at day 0 (1.5×10^3 cells/well). The rate of cell growth was measured using XTT from day 1 to day 6. The value recorded at day 1 is designated as 1. Data were analyzed by Student t-test (n=4, mean±SD, ***P<0.001 vs Pdcd4$^{+/+}$ at the same day). (**c**) Pdcd4 knockdown or knockout increases the phosphorylation of AKT at Ser473. Immunoblot analysis was performed using

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indicated antibodies. (**d**) Knockdown of Akt1 and Akt2 inhibits Snail expression induced by Pdcd4 knockdown. HT29-P cells were transfected with scrambled siRNA, *AKT1* siRNA, and *AKT2* siRNA. Forty-eight hours post-transfection, lysates were collected and immunoblotted with the indicated antibodies. (**e**) Pdcd4 knockdown or knockout increases mTORC2 activation. Cell lysates from the indicated cells were subjected to immunoprecipitation with mTOR antibody and the precipitated complex was used for mTORC2 kinase assay followed by immunoblot to examine the level of phospho-AKT(Ser473). Top, Image of representative mTORC2 kinase assay. Bottom, quantification of mTORC2 kinase activity with the ratio of phospho-Akt/precipitated mTOR in HT29-L and Pdcd4$^{+/+}$ cells designated as 1. Data were analyzed by one-sample *t*-test (n=3; mean ±SD; *P<0.05).

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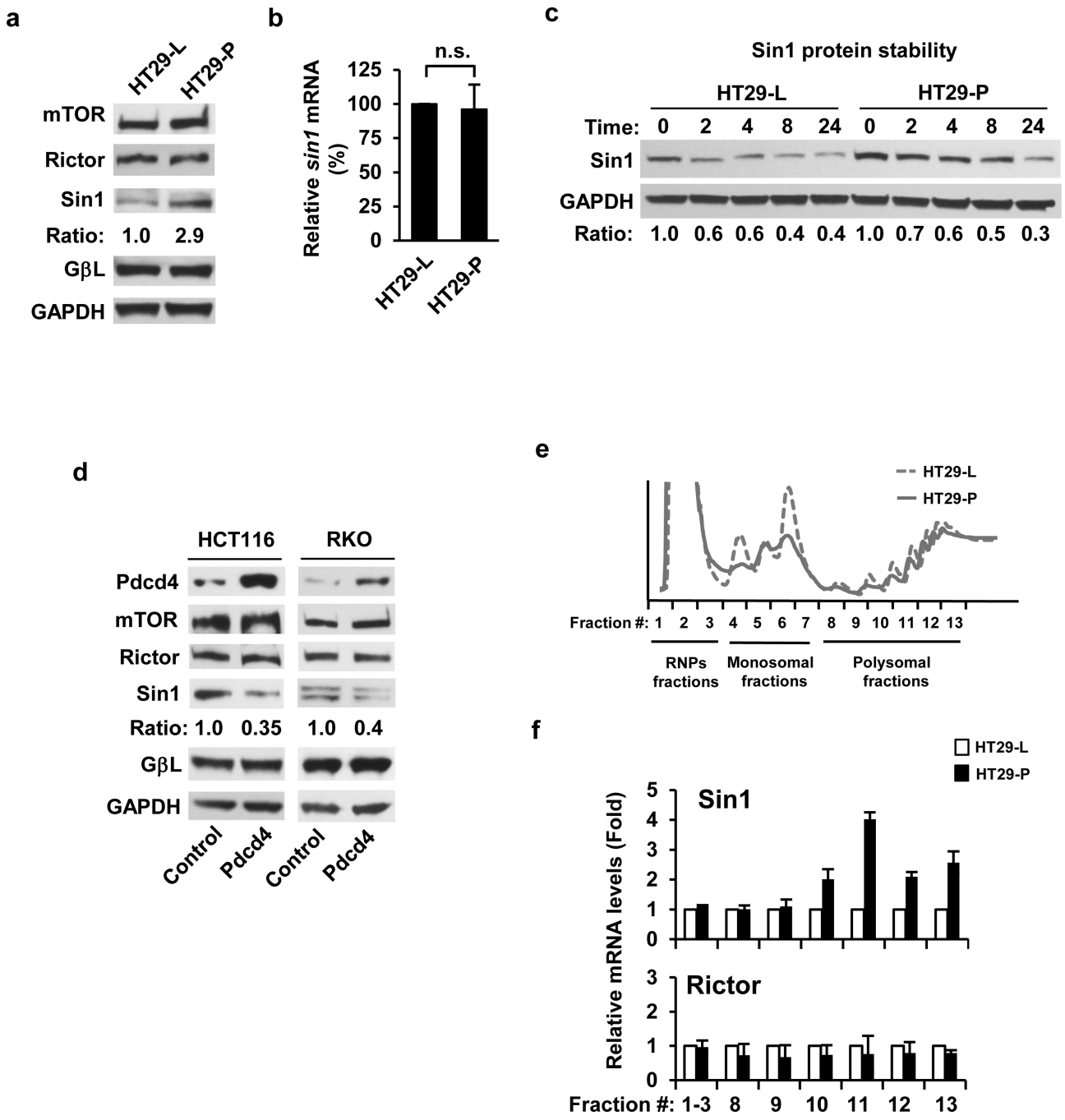

Figure 2.

Pdcd4 knockdown enhances Sin1 translation. (**a**) Pdcd4 knockdown increases Sin1 protein expression. Cell lysates were probed with indicated antibodies in immunoblot analysis. The ratio of Sin1/GAPDH protein level in HT29-L cell is designated as 1.0. (**b**) Pdcd4 knockdown has no impact on *SIN1* mRNA level. The *SIN1* mRNA levels were determined by RT-qPCR. The ratio of Sin1/GAPDH mRNA level in HT29-L cell is designated as 100%. Data were analyzed by one-sample *t*-test (n=3; mean±SD; n.s.= non-significant). (**c**) Pdcd4 knockdown has no impact on Sin1 protein stability. Cells were treated with cycloheximide

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from 0 to 24 h, and Sin1 protein levels were analyzed with immunoblot analysis. The level of Sin1/GAPDH in HT29-L or HT29-P at 0 h is designated as 1.0. (**d**) Overexpression ofPdcd4 inhibits Sin1 expression. HCT116 and RKO cells (5×10^5 per dish) were transfected with 5.0 μg of Pdcd4 expressing plasmid (pcDNA-Pdcd4). Cell lysates were used for immunoblot assay with antibodies against subunits of mTORC2. The ratio of Sin1/GAPDH in control HCT116 or RKO cells is designated as 1.0. (**e**) Polysomal profiles of sucrose gradient assay using HT29-L and HT29-P cells. (**f**) Pdcd4 knockdown increases distribution of *SIN1* mRNA (top) but not *RICTOR* mRNA (bottom) in polysomal fractions. The mRNA level of Sin1/GAPDH and Rictor/GAPDH in HT29-L cells in each fraction is designated as 1.

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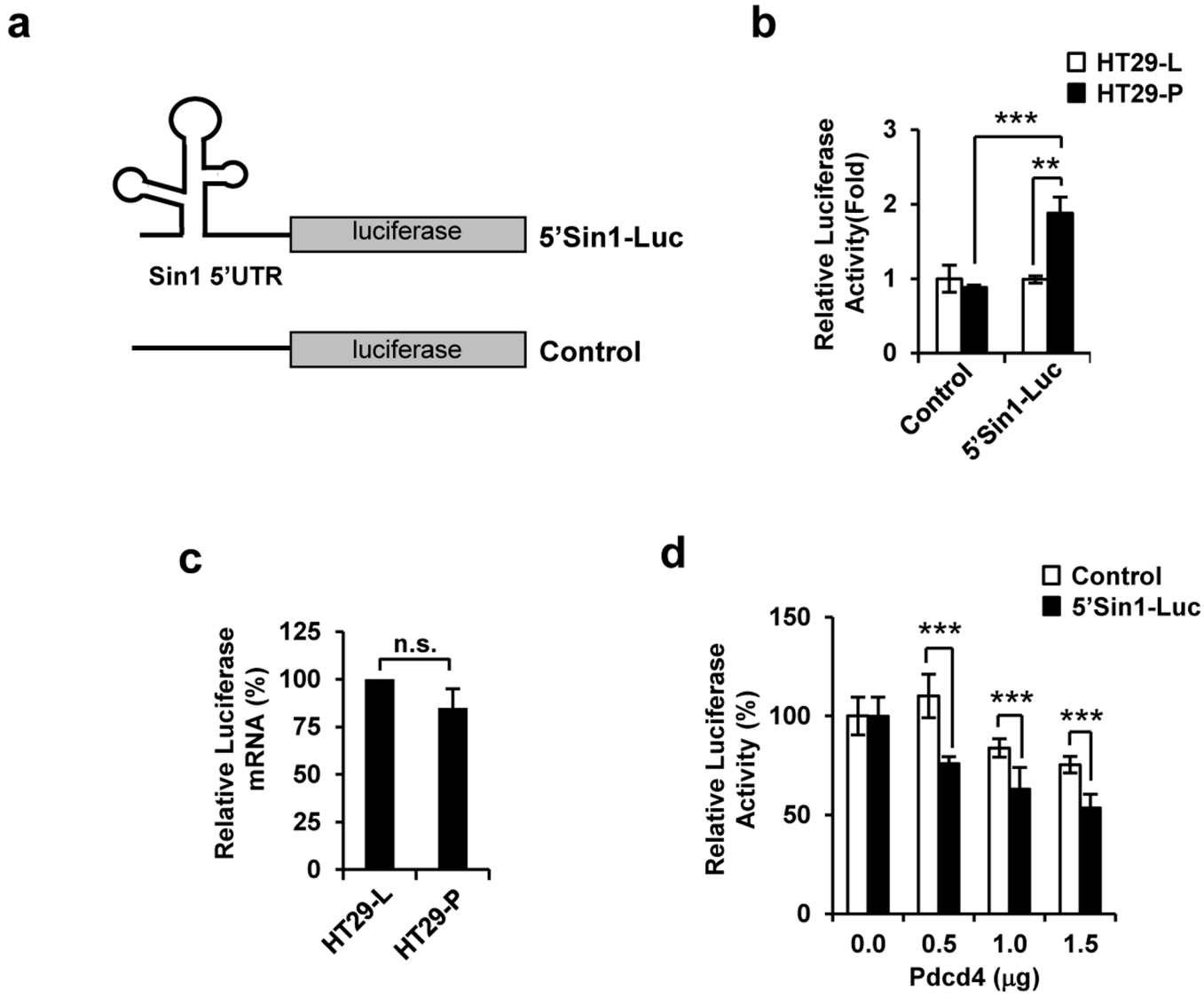

Figure 3.

The 5'UTRon *sin1* mRNA is required for Pdcd4 to inhibit Sin1 translation. (**a**) Illustration of the 5'Sin1-Luc construct. (**b** and **c**) Pdcd4 knockdown increases *SIN1* 5'UTR luciferase activity (**b**) but not luciferase mRNA (**c**). HT29-L and HT29-P cells (5×10^4 per well) were transfected with 0.2 μg of 5'Sin1-Luc or pCMA-Luc (control) along with 20 ng of pRL-SV40 (Promega). The normalized luciferase activity (firefly luciferase/Rinilla luciferase) in HT29-L cells is designated as 1. Data were analyzed by Welch's t-test (n=5; mean ± SD; ** P<0.01; *** P<0.001) for (**b**). The 5'Sin1-Luc mRNA levels were determined by RT-qPCR and the level in HT29-L cells is designated as 100% (n=3; mean±SD; n.s.= non-significant by one-sample t-test) in (**c**). (**d**) Pdcd4 inhibits 5'Sin1-Luc activity in a dose dependent manner. HCT116 cells (5×10^4 per well) were transfected with 0–1.5 μg of pcDNA-Pdcd4, 0.2 μg of 5'Sin1-LUC or pCMA-LUC (control), along with 20 ng of pRL-SV40 (Promega). The total DNA for each transfection was maintained at 1.7 μg by adding pcDNA 3.1+ vector DNA. The normalized luciferase activity (firefly luciferase/Rinilla

luciferase) in cells transfected with 0 μg of Pdcd4 is designated as 100%. Data were analyzed by Student's *t*-test (n=5; mean±SD; ***$P<0.001$).

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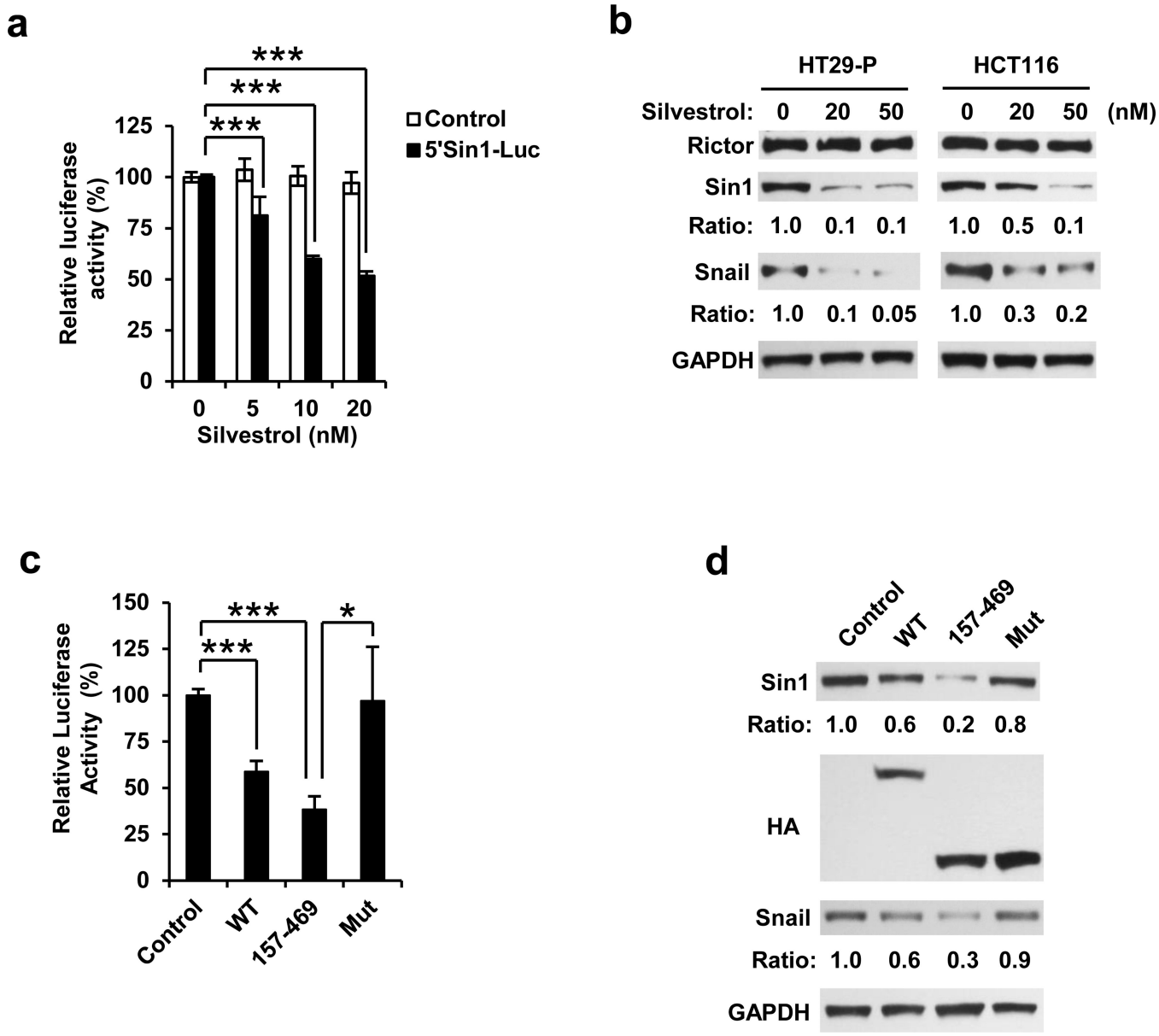

Figure 4.

Pdcd4 suppresses Sin1 translation through inhibition of eIF4A. (**a**) Silvestrol inhibits the 5'Sin1-Luc translation in a dose-dependent manner. The luciferase activity in cells treated with DMSO (0 nM silvestrol) is designated as 100%. Data were analyzed by Welch's t-test (n=5; mean±SD; ***P<0.001). (**b**) Sin1 protein level decreases in cells treated with silvestrol. HT29-P or HCT116 cells were treated with indicated concentration of silvestrol for 24 h. The ratio of Sin1/GAPDH and Snail/GAPDH in DMSO (0 nM silvestrol) treated cells is designated as 1.0. (**c**) Pdcd4(157–469)(D253A,D418A) does not inhibit 5'Sin1-Luc activity. HCT116 cells (1×10^5 per dish) were transfected with 1.0 μg pCMV-HA (control) or plasmid expressing HA tagged WT or mutant Pdcd4, 0.2 μg of 5'Sin1-LUC, and 20 ng of pRL-SV40. The normalized luciferase (firefly luciferase/Rinilla luciferase) activity in cells transfected with pCMV-HA (control) is designated as 100%. Data were analyzed by Welch's

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t-test (n=5; mean±SD; *P<0.05; ***P<0.001). Mut: Pdcd4(157–469)(D253A,D418A). (**d**) Pdcd4(157–469)(D253A,D418A) does not inhibit Sin1 protein expression. HCT116 cells (6 × 10^5) were transfected with 5.0 μg pCMV-HA (control) or plasmid expressing HA tagged WT or mutant Pdcd4. Seventy-two hours post-transfection, cell lysates were collected and subsequently immunoblotted with indicated antibodies. The ratio of Sin1/GAPDH and Snail/GAPDH in pCMV-HA transfected cells (control) is designated as 1.0.

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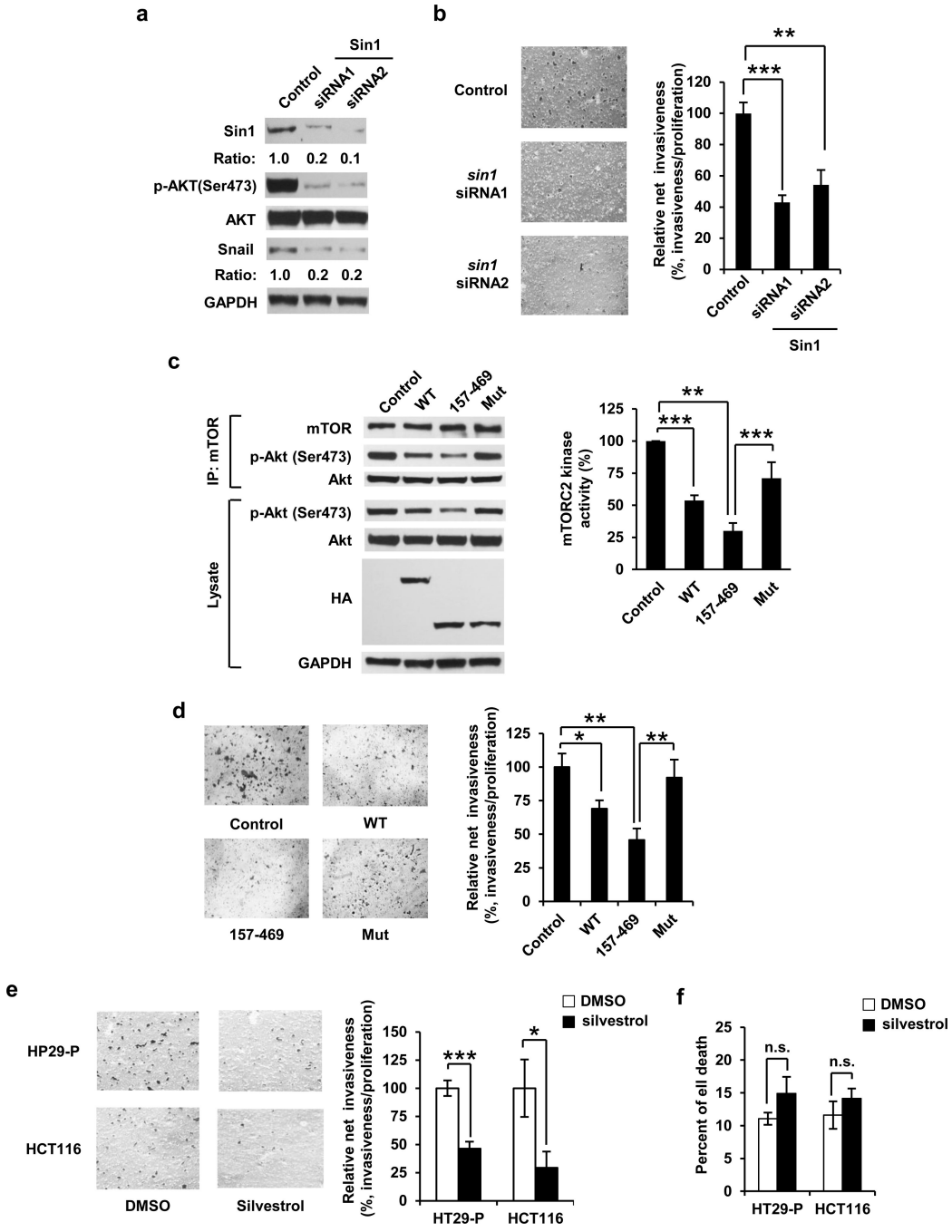

Figure 5.

Pdcd4 suppresses mTORC2 activity and invasion through inhibition of Sin1 translation. (**a**) Sin1 knockdown inhibits Akt phosphorylation in HT29-P cells. The Sin1 expression was knocked down by two commercial *SIN1* siRNAs in HT29-P cells and the cell lysates were subjected to immunoblot analysis. The ratio of phospho-Akt/GAPDH and Snail/GAPDH in scrambled siRNA transfected cells (control) is designed as 1.0. (**b**) Sin1 knockdown in HT29-P cells decreases invasion. The HT29-P cells with Sin1 knockdown were serum starved for 24 h and subsequently assayed for invasive capacity. Left, representative images

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of Matrigel invasion. Right, quantification of invasion. Cell proliferation was determined under the same conditions of invasion assay. Net invasiveness was measured as the ratio of invasion/proliferation with scrambled siRNA transfected cells as 100%. Data were analyzed by Student's *t*-test (n=3; mean±SD; ***P*<0.01; ****P*<0.001). (**c** and **d**) Pdcd4 inhibits mTORC2 kinase activity (c) or invasion (d) via inhibition of Sin1 translation. HCT116 cells (6×10^5 per dish) were transfected with 5.0 μg of pCMV-HA (control) or plasmid expressing HA tagged WT or mutant Pdcd4. Seventy-two hours post-transfection, cells were used for mTORC2 kinase and invasion assays. (c) Left, Image of representative mTORC2 kinase assay. Right, quantification of mTORC2 kinase activity. The ratio of p-Akt/ precipitated mTOR in control cells is designated as 100%. Data were analyzed by one-sample *t*-test (n=3; mean±SD; ***P*<0.01; ****P*<0.001). (d) Left, the representative images of Matrigel invasion. Right, quantification of invasion. The net invasiveness was measured as the ratio of invasion/proliferation with control cells as 100%. Data were analyzed by Student's *t*-test (n=3; mean±SD; **P*< 0.05; ***P*<0.01). Mut: Pdcd4(157–469)(D253A, D418A). (**e**) Silvestrol treatment decreases the invasion capacity of tumor cells. Cells (2×10^5 per well) were seeded on the Matrigel coated Boyden chamber and silvestrol was added to the upper and lower chambers to a final concentration of 20 nM. After 18h, the invasive cells were stained with 0.5% (v/w) crystal violet. Left, Representative images of the lower membrane surface. Right, quantification of invasion. The net invasiveness was measured as the ratio of invasion/proliferation with cells treated with DMSO as 100%. Data were analyzed by Student's *t*-test (n=3; mean±SD; **P*< 0.05; ****P*<0.001). (**f**) No change of apoptosis rate in cells treated with silverstrol. Cells were treated with 20 nM of silvestrol for 24 h and subsequently assayed for apoptosis using annexin V-FITC. Data were analyzed by student's *t*-test (n=3; mean±SD; n.s.= non-significant).

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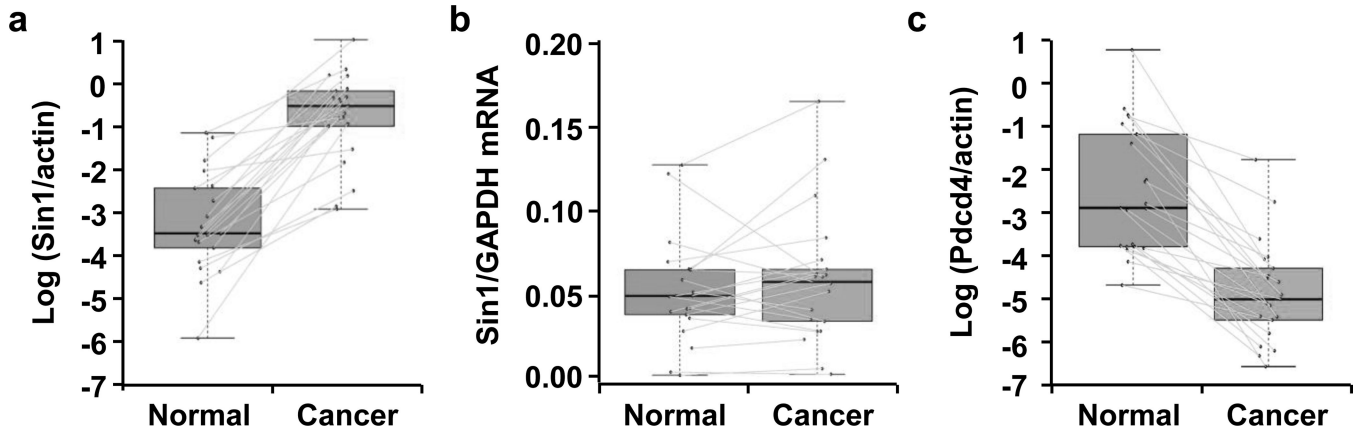

Figure 6.

Sin1 protein but not mRNA is up-regulated in the human colon cancer tissues. (**a** and **c**) The Sin1 protein level is up-regulated (a) and Pdcd4 protein level is down-regulated (c) in malignant tissues compared to the adjacent normal tissues. Protein levels of Sin1 and Pdcd4 in 21-patient specimens were analyzed with immunoblot analysis. The value of Sin1/actin (a) and Pdcd4/actin (c) was used for statistical comparison by Wilcoxon signed rank test (Sin1, P=9.5×10^{-7}; Pdcd4, P=9.5×10^{-7}). (**b**) The level of *SIN1* mRNA is similar in normal and malignant tissues .The SIN1 mRNA level in 21-patient specimens was analyzed by RT-qPCR. The value of SIN1/GAPDH in each sample was used for statistical comparison (Wilcoxon signed rank test, P=0.24).

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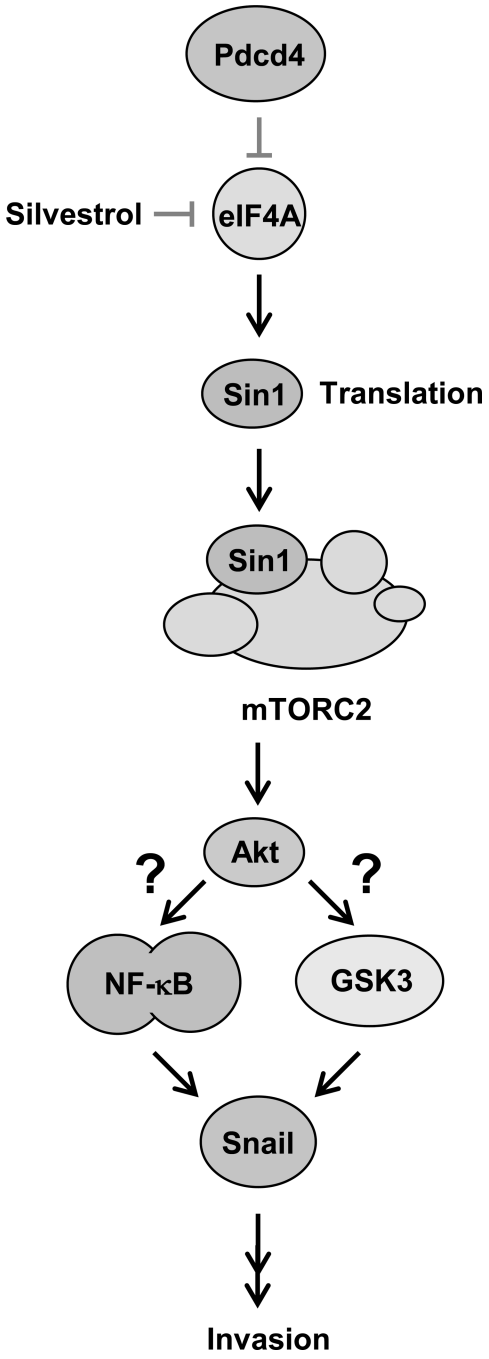

Figure 7.

Schematic diagram showing that Pdcd4 suppresses Sin1 translation to inhibit mTORC2 activity leading to suppression of invasion. Pdcd4 suppresses Sin1 translation via inhibition of eIF4A. Inhibition of eIF4A by silvestrol also suppresses Sin1 translation. The inactivation of mTORC2 by Pdcd4 or silvestrol leads to Akt inhibition, then either inhibits NF-κB or GSK3, and thus reduces Snail expression, which ultimately suppresses invasion.