Ran is a potential therapeutic target for cancer cells with molecular changes associated with activation of the PI3K/Akt/mTORC1 and Ras/MEK/ERK pathways

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Abstract

Purpose—Cancer cells have been shown to be more susceptible to Ran knockdown compared to normal cells. We now investigate whether Ran is a potential therapeutic target of cancers with frequently found mutations that lead to higher Ras/MEK/ERK and PI3K/Akt/mTORC1 activities.

Experimental Design—Apoptosis was measured by flow cytometry (PI and Annexin V staining) and MTT assay in cancer cells grown under different conditions after knockdown of Ran.. The correlations between Ran expression and patient survival were examined in breast and lung cancers.

Results—Cancer cells with their PI3K/Akt/mTORC1 and Ras/MEK/ERK pathways inhibited are less susceptible to Ran silencing-induced apoptosis. KRas mutated, c-Met amplified and Pten-deleted cancer cells are also more susceptible to Ran silencing-induced apoptosis than their wild-type counterparts and this effect is reduced by inhibitors of the PI3K/Akt/mTORC1 and MEK/ERK pathways. Overexpression of Ran in clinical specimens is significantly associated with poor patient outcome in both breast and lung cancers. This association is dramatically enhanced in cancers with increased c-Met or osteopontin expression, or with oncogenic mutations of KRas or PIK3CA, all of which are mutations that potentially correlate with activation of the PI3K/Akt/mTORC1 and/or Ras/MEK/ERK pathways. Silencing Ran also results in dysregulation of nucleocytoplasmic transport of transcription factors and downregulation of Mcl-1 expression, at the transcriptional level, which are reversed by inhibitors of the PI3K/Akt/mTORC1 and MEK/ERK pathways.

Conclusion—Ran is a potential therapeutic target for treatment of cancers with mutations/changes of expression in protooncogenes that lead to activation of the PI3K/Akt/mTORC1 and Ras/MEK/ERK pathways.

Keywords

RanGTPase; Akt; ERK; mTORC1; nucleocytoplasmic transportation

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Introduction

Normal cells acquire mutations to activate/suppress various pathways to fulfill certain functions to become cancerous (1). Targeting these cancer hallmarks may exert a selective killing effect on cancer cells. Cancer cells are addicted to oncogenes or oncogenic pathways (2), which are usually uniquely present or hyperactivated, and are essential for tumor cell growth and survival. The two most frequently dysregulated signaling pathways in cancers are the PI3K/Akt/mTORC1 and Ras/MEK/ERK pathways (3). Due to the presence of parallel pathways in cancer cells, targeting these pathways in cancer therapy leads to limited success (3).

These growth signaling pathways usually exert their ultimate effect by regulating the translocation of transcription factors into or out of the nucleus, thereby altering the transcriptome and consequently, the expressome (4). The activity of several oncogenic or tumor suppressive transcription factors, such as NFκB, FOXO1, p53 and β-catenin (4), and non-transcription factors, such as survivin (5), are regulated by their subcellular localization. Therefore, the uncoupling of nucleocytoplasmic transport from growth and survival signaling pathways has been suggested to be a potential target for cancer therapy (6).

Ran GTPase (Ran), a Ras-related G protein, is involved in several important cellular processes, the best known being nucleocytoplasmic transportation (7). Ran guanine nucleotide exchange factor, RCC1, is retained in the nucleus and Ran GTPase activating protein, RanGAP, is retained in the cytoplasm. Their cellular compartmentalization creates a RanGTP gradient, which regulates the transport of macromolecules between nucleus and cytoplasm (8). Ran also plays an important role in cancer development and progression. It is overexpressed in various cancers with prognostic significance (9, 10) and its overexpression is correlated with increased aggressiveness of the cancer cells *in vitro* and *in vivo* (11-13). Ran has been shown to be a promising cancer therapeutic target; Silencing Ran expression induces more apoptosis in cancer cells compared to normal cells (14) as well as in activated K-Ras mutant cells compared to their isogenic K-Ras wildtype counterparts (15). However, the reasons for these selective killing effects are far from clear.

There are reports showing that Ran may be a mediator between growth signaling and nucleocytoplasmic transport. Ran is up-regulated by the PI3K/Akt pathway in H_2O_2-induced mitosis (16) and is also activated by growth factors (13). Ran binding protein 3 (RanBP3) is phosphorylated in response to Ras/MEK/ERK and PI3K/Akt activation, while the phosphorylation of RanBP3 modulates Ran-dependent nucleocytoplasmic transport (17, 18). Taken together, these findings suggest that the expression level and the activity of Ran, and thereby the capacity of nucleocytoplasmic transportation, are regulated by growth and survival signaling pathways. Here, we demonstrate that Ran silencing results in a selective killing effect on cancer cells with stronger activation of the PI3K/Akt/mTORC1 and MEK/ERK pathways probably through dysregulation of nucleocytoplasmic transportation and down-regulation of Mcl-1.

Materials and Methods

Cell culture conditions

See details in Supplementary Materials and Methods.

Plasmids

pLKO.1-shRan1, −2, −3, −4 and −5 (clone IDs were NM_006325.2-697s1c1, NM_006325.2-198s1c1, NM_006325.2-484s1c1, NM_006325.2-625s1c1 and

NM_006325.2-142s1c1, respectively) were obtained from Sigma-Aldrich. pLKO.1-shScramble (Scr, #1864), pCMV-dR8.2 dvpr (#8455) and pCMV-VSV-G (#8454) were obtained from Addgene (Cambridge, MA).

Transfection and infection

Transfection was performed using GeneJuice® according to the manufacturer's instructions. Viral particles were harvested 48 hours post-transfection and were applied to the target cells with 6 µg/ml polyprene supplement. The target cells were allowed to be infected for 4 hours and medium was refreshed. The same amount and same batch of viral particles were used for any comparison made in the present study.

Culture conditions

Cells were cultured in their normal medium until 24 hours post-infection. At 24 hours post-infection, the medium was changed to the desired medium with different drugs (the concentrations of the drugs used are listed in Supplementary Table S1, unless otherwise specified). Cells were harvested at 72 hours post-infection for protein and apoptosis analyses, unless otherwise specified.

Western Blot

Western Blotting was performed as previously described (19). Nuclear/cytoplasmic fractionation was performed by using N-Per kit from Pierce. The details of the antibodies used in the present study are listed in Supplementary Table S2.

Flow cytometric analysis

The extent of apoptosis was estimated by the percentage of sub-G1 phase cells by Propidium iodide (PI) staining. Annexin V staining was performed using Annexin V-Fluos staining Kit (Roche, Welwyn Garden City, UK) according to the manufacturer's instructions. Stained cells were analyzed by using a BD LSRII Flow cytometer.

Patients and specimens

Breast cancer patient specimens were obtained from University of Liverpool, UK (20) while lung cancers patient specimens were obtained from St. James Hospital, Dublin, Ireland. See details of patient information in Supplementary Materials and Methods.

Immunohistochemical staining, evaluation and statistical analysis

Immunohistochemical staining and evaluation were performed as previously described (20). See details in Supplementary Materials and Methods.

Analysis of breast and lung cancer microarray data

A total of four breast cancer data sets (GSE1378, GSE1379, GSE2034 and GSE3143), consisting of 564 patients and a total of six lung cancer data sets (GSE6253, GSE3141, GSE8894, GSE4573, GSE5123 and GSE13213) consisting of 601 patients with their corresponding microarray and survival data available in Gene Expression Omnibus were included in this study. The data sets were pre-processed as previously described using R and Bioconductor for normalization (21). See details in Supplementary Materials and Methods.

Results

Ran silencing results in higher levels of apoptosis in cancer cells than in immortalized cells

Since infection of shRan1 and shRan2 led to the lowest expression of Ran protein and highest apoptotic responses among the five shRan sequences (Supplementary Fig. 1), these two shRNAs were used in this study. Ran silencing induced significantly fewer cell deaths and lower activation of apoptotic pathways in the immortalized human embryonic kidney 293 (HEK 293) cell line compared to cancer cell lines from breast (MDA-MB-231), lung (A549), prostate (DU145), esophageal (EC109) and colon (HCT116 and DLD-1) ($p < 0.01$; Supplementary Fig. 2A-B). Ran silencing also resulted in a significantly higher apoptotic response in breast cancer MDA-MB-231 than immortalized MCF10a cells (Supplementary Fig. 3), in line with a previous study[14].

Ran silencing induces apoptosis in cancer cells and is reversed by inhibitors of PI3K/Akt/mTROC1 or MEK/ERK pathways

Growth signaling pathways are usually aberrantly activated in cancers. Therefore, we investigated whether uncoupling of the activated growth signal and Ran expression underpins the differential response to Ran silencing in immortalized and cancer cells. Significantly more apoptotic sub-G1 phase and annexin V positive cells were detected upon Ran silencing when MDA-MB-231 breast and A549 lung cancer cells were grown in the presence of FBS, which stimulates the growth signaling pathways, compared to serum-free conditions ($p < 0.01$; Fig. 1A and B). The lowest apoptosis induced by Ran silencing was detected when cells were grown in Hank's buffered salt solution (HBSS), where the growth signal is inactive because of the absence of growth factors and amino acids ($p < 0.01$; Fig. 1A and B). PI3K/Akt/mTORC1 and Ras/MEK/ERK are two of the most frequently hyperactivated pathways in cancer cells, we therefore investigated whether inhibiting these two pathways by using mTORC1 inhibitor, rapamycin, Class I PI3K/mTORC1 dual inhibitor, PI103 and MEK1/2 inhibitor, PD184352 may affect the sensitivity of cancer cells to Ran silencing-induced apoptosis. As expected, treatment of the MDA-MB-231 and A549 cells with these drugs inhibited phosphorylation of their corresponding downstream targets (Supplementary Fig. 4) and reduced cell proliferation (Supplementary Fig. 5). These inhibitors of the PI3K/Akt/mTORC1 or MEK/ERK pathways significantly reduced Ran silencing-induced apoptosis in MDA-MB-231 and A549 cells ($p < 0.05$; Fig.1C and D). Similar results were obtained using MTT cell survival assay (Supplementary Fig. 6). These results suggest that Ran may be preferentially required for survival in cancer cells with higher activities of the PI3K/Akt/mTORC1 and MEK/ERK pathways.

Mutant K-Ras cells are more susceptible to Ran silencing-induced apoptosis

Ran silencing resulted in a significantly higher apoptotic response in the mutant K-Ras transformed cells compared to the wild-type (WT) K-Ras counterparts in the DLD-1/DKO-3 colon cancer isogenic pair ($p < 0.01$; Fig. 2A-C). This result suggests that cancer cells with Ras activation are more susceptible to Ran silencing-induced apoptosis. Mutant K-Ras activates the MEK/ERK and PI3K/Akt/mTORC1 pathways to increase cell proliferation and cell survival [22]. Inhibitors of PI3K or MEK, but not mTORC1, reduced the mutant K-Ras mediated sensitization of the cells to Ran-silencing induced apoptosis (Fig. 2D). The results were confirmed by Western blots of cleaved PARP, caspase 3 and 9 (Fig. 2E-G). Similar results were obtained using the HCT116 and Hkh-2 colon cancer isogenic pair, where inhibitors of PI3K/Akt or MEK/ERK pathways abolished mutant K-Ras mediated sensitization to Ran silencing-induced apoptosis (Supplementary Fig. 7). The results were further confirmed using MTT cell survival assay showing that cancer cells with mutant K-Ras were more susceptible to Ran silencing-induced apoptosis compared to their wild-type

counterparts (Supplementary Fig. 8A-B). Our results show that isogenic cancer cells with activated K-Ras are more susceptible to Ran silencing-induced apoptosis, and this may be due to the higher activities of the PI3K/Akt and MEK/ERK pathways.

Cancer cells with c-Met amplification are more susceptible to Ran silencing-induced apoptosis

c-Met amplification has been shown to promote resistance to the EGFR inhibitor, gefitinib, in lung cancer cells through persistent activation of the PI3K/Akt and MEK/ERK pathways (23). The effect of Ran knockdown on apoptotic induction in a pair of lung cancer cell lines, HCC827 parental cells and HCC827 GR5 c-Met amplified cells was also tested. HCC827 parental cells have a significant level of c-Met phosphorylation, while HCC827 GR5 cells, generated by prolonged exposure of gefitinib, have increased levels of total c-Met and phosphorylated c-Met (23). Upon treatment of gefitinib, the pAkt and pERK levels were greatly decreased in HCC827 parental cells, whereas their levels are persistent in GR5 cells (23). Ran silencing resulted in a significantly higher apoptotic response in GR5 than in HCC827 cells ($p < 0.01$; Fig. 3A and Supplementary Fig. 8C), suggesting that cells with c-Met amplification are more susceptible to Ran silencing-induced apoptosis. Similar to mutant K-Ras, inhibitors of the PI3K/Akt and Ras/MEK/ERK pathways significantly reduced c-Met amplification-mediated sensitization to Ran silencing-induced apoptosis ($p < 0.01$; Fig. 3B). Interestingly, Ran silencing induced a significant apoptotic response in GR5 cells in the presence of gefitinib but not in HCC827 parental cells ($p < 0.01$; Fig. 3B), suggesting that cells that are resistant to gefitinib may maintain their sensitivity to Ran silencing-induced apoptosis. Our results demonstrate that isogenic cancer cells with c-Met amplification are more susceptible to Ran silencing-induced apoptosis and this effect is probably dependent on the PI3K/Akt and MEK/ERK pathway.

Cancer cells with pten deletion are more susceptible to Ran silencing-induced apoptosis

In another colon cancer isogenic cell pair, the HCT116 WT and Pten-null cell lines, the activation of the PI3K/Akt pathway through deletion in Pten sensitizes the cancer cells to Ran silencing-induced apoptosis also potentially in a PI3K/Akt/mTORC1 pathway-dependent manner (Fig. 3C-D).

Ran protein expression is correlated with reduced survival in breast and lung cancer patients

We investigated whether a high level of immunohistochemical detectable Ran is associated with aggressive breast and lung cancers. Breast cancer patients with a higher percentage of malignant cell nuclei stained for Ran in their primary tumors had a shorter median survival time than those with < 1% of malignant cell nuclei stained for Ran in their primary tumors (Figure 4A and B, $p < 0.001$). When analyzed with the other prognostic factors of tumor size, grade and lymph node involvement in the Cox-regression analysis, staining for nuclear Ran was independently associated with patient survival time (Relative Risk (RR) = 12.53, 95% CI = 3.95 – 39.758, $p < 0.001$; Table S3A). Similar results were obtained for cytoplasmic staining for Ran. Thus patients with a higher cytoplasmic staining for Ran had a significantly shorter survival time than those with < 1% of cytoplasmic staining for Ran in their primary tumors ($p < 0.001$; Figure 4A and C). When analyzed with the other prognostic factors, staining for cytoplasmic Ran was independently associated with patient survival time (RR = 2.50, 95% CI = 1.59 – 3.93, $p = 0.001$; Table S3B). Higher levels of staining for Ran were also significantly correlated with a shorter patient survival time in lung cancer patients ($p = 0.001$; Supplementary Figure 9A-B). When analyzed with other prognostic factors of clinical stage, lymph node involvement and histology, this overall staining for Ran was independently associated with patient survival times in the Cox-regression analysis (RR = 1.63, 95% CI = 1.08 – 2.44, $p = 0.019$; Table S3C). Results were substantiated using

patient data available in Gene Expression Omnibus database. A high level of Ran mRNA expression was associated with poorer prognosis in both breast (RR = 1.57, 95% CI = 1.06 – 2.32, p = 0.022; Fig. 4D) and lung (RR = 1.591, 95% CI = 1.139 – 2.222, p = 0.006; Supplementary Fig. 9C) cancer patients.

Ran mRNA expression is correlated with reduced survival in cancers with PIK3CA activation

We have also analyzed the GEO breast cancer dataset with more than 200 patients (GSE2034, Fig. 4E), where we have sufficient information to identify patients with PIK3CA activation mutations that lead to an activation of the PI3K/Akt/mTORC1 pathway based on the gene signature described previously (24). In this dataset, a high level of Ran was significantly correlated with shorter survival time in patients with PIK3CA mutation gene signature (Fig. 4F, p = 0.018), but not in those with PIK3CA wild-type gene signature (Fig. 4G, p = 0.186).

Ran protein expression is correlated with survival in breast cancer patients with increased expression of c-Met or OPN

There was a much greater difference in patient survival time between high and low level of Ran in c-Met positive tumors (median survival 58.1 months, final proportion surviving 18% vs >216 months, 81%, respectively; Wilcoxon-Gehan χ^2 = 16.5, $p < 0.001$; Fig. 4H) than in c-Met negative tumors (> 216 months, 78% vs > 216 months, 100%, respectively; Wilcoxon-Gehan χ^2 = 9, p = 0.003; Fig. 4I). Similar results were obtained when using a metastasis-promoting protein, OPN, which has been shown to activate the PI3K/Akt and MEK/ERK pathways and is available in the same cohort (20, 25, 26), to stratify the patients. The difference in patient survival time was much greater between high and low Ran in OPN positive tumors (57.2 months, 16% vs > 216 months, 79%, respectively; Wilcoxon-Gehan χ^2 = 16.7, $p < 0.001$; Fig. 4J) than in OPN negative tumors (> 216 months, 86% vs > 228 months, 100%, respectively; Wilcoxon-Gehan χ^2 = 6.58, p = 0.01; Fig. 4K).

Ran mRNA expression is correlated with reduced survival in cancers with either PIK3CA or KRas activating mutations

A colorectal cancer dataset (GSE16125) that records K-Ras mutation, survival data and Ran mRNA expression was also analyzed. In patients whose tumors did not contain mutated KRas, 71% and 73% patients died with high and low levels of Ran, respectively (Fisher Exact test, p = 1.000). In contrast, in patients with K-Ras activating mutation and a high level of Ran, the median survival time was 10 months (7 out of 7 patients died), while for those with K-Ras activating mutation and a low level of Ran, the median survival time was 62 months (6 out of 12 patients died; Fisher Exact test, p = 0.044). Taken together, these results suggest that a high level of Ran is specifically required for human cancers with mutations that correlate with activation of the PI3K/Akt/mTORC1 or Ras/MEK/ERK pathways.

Ran silencing results in dysregulation of nucleocytoplasmic transportation

We also investigated the role of nucleocytoplasmic transport on Ran silencing-induced apoptosis. Rapamycin significantly inhibited Ran silencing-induced apoptosis in MDA-MB-231 breast cancer cells ($p < 0.001$) and this was reversed by Leptomycin B (LMB) ($p < 0.001$; Fig. 5A-B), which impairs nuclear export by covalently binding to CRM1 (27). LMB treatment also suppressed the inhibitory effect of PD184352 on Ran silencing-induced apoptosis in MDA-MB-231 cells ($p < 0.01$; Fig. 5C-D). LMB also reversed the Rapamycin and PI103-mediated suppression on Ran silencing-induced apoptosis in A549 lung cancer cells (Supplementary Fig. 10A-D). In addition, nuclear localization of β-catenin and NFκB

was decreased and that of p53 and p27 was increased in MDA-MB-231 cells (Fig. 5E); similarly, nuclear localization of β-catenin was decreased and that of p53, p27 and c-jun was increased in A549 lung cancer cells upon Ran silencing (Supplementary Fig. 10E). In MDA-MB-231 cells, the decrease in β-catenin and increase in p27, but not that of p53 and NFκB, in the nucleus were diminished by treatment with rapamycin or PD184352 (Fig. 5F-G). Similarly, in A549 cells, the decrease in β-catenin and increases in p53 and p27 in the nucleus were diminished by treatment with rapamycin or PI103 (Supplementary Fig. 10F-G). These results suggest that the dysregulation of nuclear localization of transcription factors upon Ran silencing requires active PI3K/Akt/mTORC1 and MEK/ERK pathways and that nucleocytoplasmic transportation plays an important role in governing PI3K/Akt/mTORC1 and MEK/ERK pathway-mediated sensitization of cancer cells to Ran silencing-induced apoptosis.

Ran silencing results in down-regulation of Mcl-1

In addition to the above changes in subcellular localization of transcription factors, the protein level of a prosurvival upstream regulator of caspase 9, Mcl-1, was decreased upon Ran silencing, but not that of other prosurvival and proapoptotic factors tested (data not shown). Indeed, Mcl-1 mRNA was significantly down-regulated in both MDA-MB-231 breast (Fig. 5H) and A549 lung (Supplementary Fig. 10H) cancer cells upon Ran knockdown. This result suggests that the observed reduction in Mcl-1 occurs at the transcriptional level, and is probably due to the dysregulation of nucleocytoplasmic transportation upon Ran silencing. Most importantly, Ran silencing-mediated Mcl-1 downregulation was reversed by inhibitors of the PI3K/Akt/mTORC1 and Ras/MEK/ERK pathways in both MDA-MB-231 (Fig. 5I-L) and A549 cells (Supplementary Fig. 10I-L).

Discussion

Activation of the PI3K/Akt/mTORC1 and Ras/MEK/ERK pathways is common in different types of cancer. In addition to cell survival, these two pathways promote cell proliferation, angiogenesis, metastasis as well as other important cellular responses (28, 29). In this report, we show that cancer cells with KRas activating mutation, c-Met amplification or Pten-deletion are more sensitive to Ran silencing-induced apoptosis. Since they can be inhibited by relatively specific inhibitors of the PI3K/Akt/mTORC1 and MEK/ERK pathways, our results, therefore, demonstrate that Ran silencing may be potentially dependent on these two pathways. Thus Ran silencing-induced apoptosis is likely to be specific for cancers with mutations that correlate with activation of these two pathways. Activating mutations of PIK3CA and K-Ras, c-Met activation, and OPN overexpression have been previously shown to correlate with activation of the PI3K/Akt/mTORC1 and MEK/ERK pathways (3, 24-26, 30, 31). Since Ran expression is associated with reduced survival of patients with mutation of PIK3CA or K-Ras, or increased expression of c-Met or OPN, our results suggest that Ran may be a potential therapeutic target for cancers with hyperactivation of the PI3K/Akt/mTORC1 and MEK/ERK pathways induced by various mutations or overexpression of specific protooncogenes.

Ran is involved in regulating the rate of nucleocytoplasmic shuttling, both nuclear import as well as nuclear export (7, 32). Growth signaling pathways transmit their signals by regulating the subcellular location of transcription factors, which finally convey the signals to modulate the transcriptome for an appropriate response (4). mTORC1 has been shown to affect translation of different transcription factors in response to growth and stress signals (33, 34). Akt is regulated through its nuclear export signal (35), whilst PI3K/Akt pathway also regulates expression and localization of growth, apoptosis and stress response-mediated transcription factors (36-39). Similarly, ERK is regulated by nucleocytoplasmic transport (40, 41) and itself also regulates localization of transcription factors in response to various

stimuli (42, 43). Ran is an important mediator in nucleocytoplasmic transportation, Ran activity, which itself is regulated by growth signaling (13, 17, 18), may be required to fulfill nucleocytoplasmic transport requirements for proper response to various stimuli (4, 6). We suggest that Ran silencing may result in an impaired or decreased rate of nucleocytoplasmic transportation, leading to the uncoupling of the growth stimuli and the required translocation of transcription factors. Indeed, we have found that the nuclear localization of β-catenin, NFκB, p53, p27 and c-jun is altered upon Ran silencing and the Mcl-1 transcript is down-regulated. Interestingly, p53 has been shown to transcriptionally repress (44), while NFκB, the PI3K/Akt and Ras/MEK/ERK pathways have been shown to transcriptionally stimulate, Mcl-1 (45-47). Thus, the overall effect of the alteration of the nuclear levels of transcription factors upon Ran silencing may lead to altered transcription of the survival/apoptotic effectors thereby resulting in apoptosis. Further analysis, probably through DNA microarray and 2-D gel electrophoresis of nuclear/cytoplasmic fractionated proteins, is required for a clearer picture of the overall effect of Ran-silencing on nucleocytoplasmic transportation and gene expression.

Another possible explanation is that Ran silencing may induce apoptosis in cancer cells with a higher proliferation rate. As serum withdrawal, inhibitors of the PI3K/Akt/mTORC1 and Ras/MEK/ERK pathways and removal of the active KRas allele in the isogenic cells result in decreased cell proliferation, which also leads to a reduced sensitivity to Ran silencing-induced apoptosis.

The GR5 cells is different from its parental HCC827 cells, in which c-Met amplification in GR5 cells sustains the PI3K/Akt signaling in the presence of EGFR inhibitor (23). We found that knockdown of Ran leads to a significant increase in apoptotic response in GR5 cells, but not in the HCC827 parental cells, in the presence of gefitinib. The persistent activation of PI3K/Akt and MEK/ERK pathways in GR5 cells treated with gefitinib may contribute to the Ran silencing-induced apoptosis. Our results therefore suggest that targeting Ran can also be used in combination with other therapeutic agents for cancer treatment in the future.

In conclusion, the present study shows that Ran silencing results in apoptosis preferentially in cancer cells with mutations that correlate with activation of the PI3K/Akt/mTORC1 and Ras/MEK/ERK pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Statement of Translational Relevance

Hyperactivation of the PI3K/Akt/mTORC1 and Ras/MEK/ERK pathways is a very common event in cancer progression, and this can lead to development of resistance to conventional therapies. In the present study, using chemical inhibitors and isogenic cell lines, we show that cancer cells with mutations that potentially correlate with activation of these two pathways are more susceptible to Ran silencing. In addition, we also show that Ran silencing results in dysregulation of nucleocytoplasmic transportation and Mcl-1 downregulation. We further demonstrate that a high level of Ran expression correlates with poorer survival in cancer patients with PIK3CA activation, osteopontin (OPN) or c-Met overexpression, which are mutations that potentially correlate with activation of these two pathways. Our results suggest that Ran may be a novel prognostic marker and a therapeutic target for cancers with mutations/changes in expression of protooncogenes/suppressors that are associated with activation of these two signaling pathways.
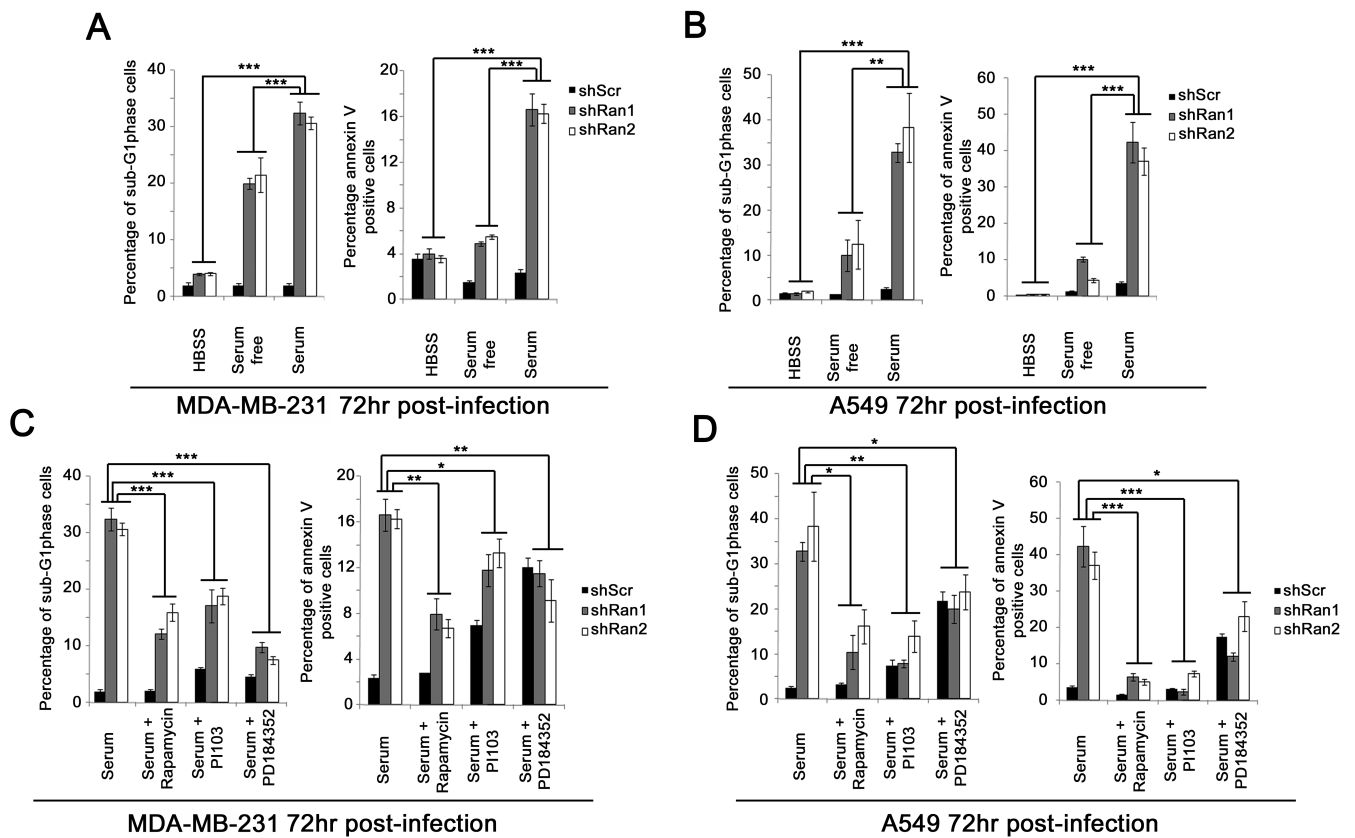
Figure 1. Ran silencing induces apoptosis to a different extent in different conditions

Percentage of sub-G1 apoptotic phase cells (right panel) and Annexin V positive apoptotic cells (left panel). (A-B) Significantly more apoptotic sub-G1 phase and annexin V positive cells were detected when MDA-MB-231 breast and A549 lung cancer cells were grown in the presence of FBS, which stimulates the growth signaling pathways, compared to serum-free conditions. When these cancer cells were grown in Hank's buffered salt solution (HBSS), where the growth signal would be mostly inactive because of the absence of growth factors and amino acids, the apoptotic-inducing effect of Ran silencing was significantly reduced compared to cancer cells grown in FBS-supplemented or serum-free medium. (C-D) Inhibition of mTORC1 by Rapamycin, PI3K by PI103 or MEK by PD184352 significantly reduced Ran-silencing induced apoptosis in (C) MDA-MB-231 and (D) A549 cells. Cancer cells with active PI3K/Akt/mTORC1 and MEK/ERK pathways, therefore, probably require more Ran for survival. Results are plotted as histograms showing the mean ± SD from three independent experiments. Key; *, ** and *** represent $p < 0.05$, < 0.01 and < 0.001, respectively
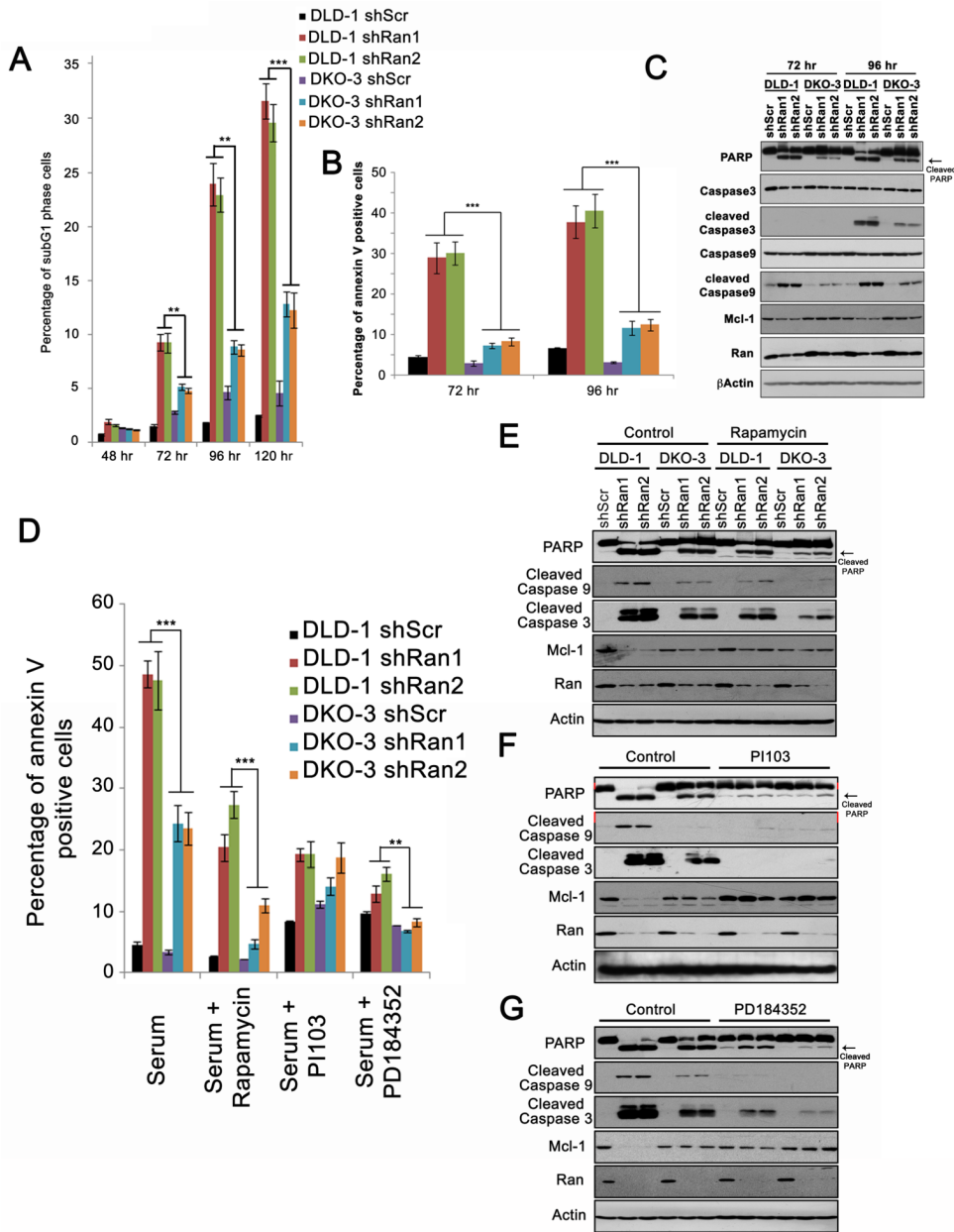
Figure 2. Cancer cells with K-Ras activated mutation are more susceptible to Ran silencing-induced apoptosis

(A) Percentage of sub-G1 apoptotic cells and (B) annexin V positive apoptotic cells in colon cancer DLD-1 (Ras activating mutation) and DKO-3 (WT) infected with Scr or Ran shRNAs. (C) Western blot analysis for DLD-1 and DKO-3 cells after Ran silencing. (D) Percentage of annexin V positive cells, and (E-G) Western blots of DLD-1 and DKO-3 cells infected with shScr/shRan1/shRan2, and treated with inhibitors for PI3K/Akt/mTORC1 or Ras/MEK/ERK pathways at 24 hours post-infection. Cells were harvested at 72 hours post-infection. Results are plotted as histograms showing the mean ± SD from three independent experiments. Key; *, ** and *** represent $p < 0.05$, < 0.01 and < 0.001, respectively. Representative blots from three experiments are shown.

Wild-type / c-Met amplified
HCC827 / HCC827 GR5

Wild-type / Pten-null
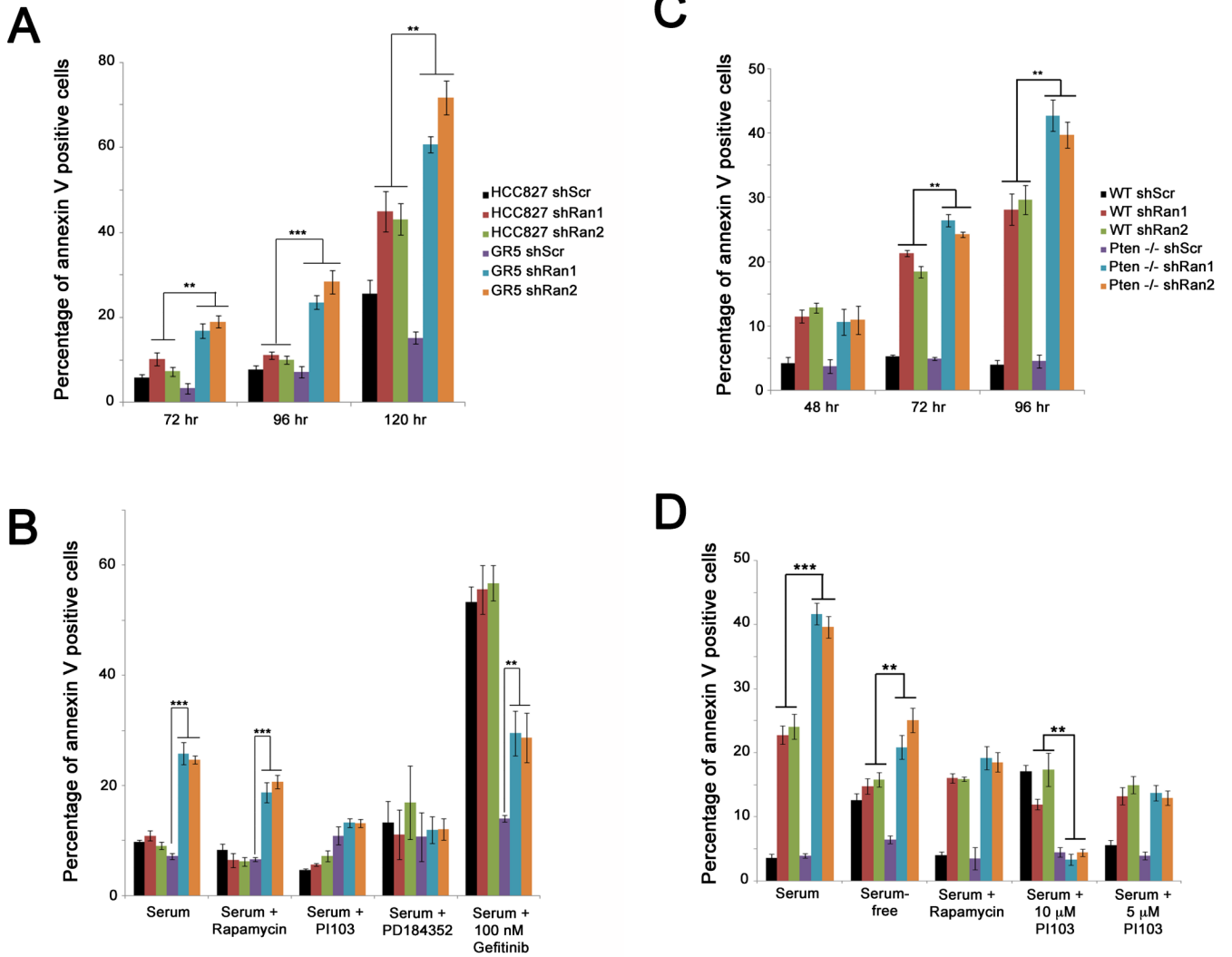## HCT116 / HCT116 Pten -/-

Figure 3. c-Met amplification and Pten deletion sensitize cells to Ran silencing-induced apoptosis
Percentage of annexin V positive apoptotic cells of (A) the lung cancer HCC827 parental and c-Met amplified gefitinib-resistant GR5 cell lines after Ran silencing, (B) HCC827 and GR5 cell lines infected with shScr/shRan1/shRan2 and treated with inhibitors for PI3K/Akt/mTORC1 or Ras/MEK/ERK pathways at 24 hours post-infection. Cells were harvested at 72 hours post-infection, (C) colon cancer HCT116 parental and Pten-null HCT116 cell lines after Ran silencing, and (D) HCT116 and HCT116 Pten-null cell lines infected with shScr/shRan1/shRan2 and treated with inhibitors for PI3K/Akt/mTORC1 pathways at 24 hours post-infection. Cells were harvested at 72 hours post-infection. Results are plotted as histograms showing the mean ± SD from three independent experiments. Key; *, ** and *** represent $p < 0.05$, < 0.01 and < 0.001, respectively.
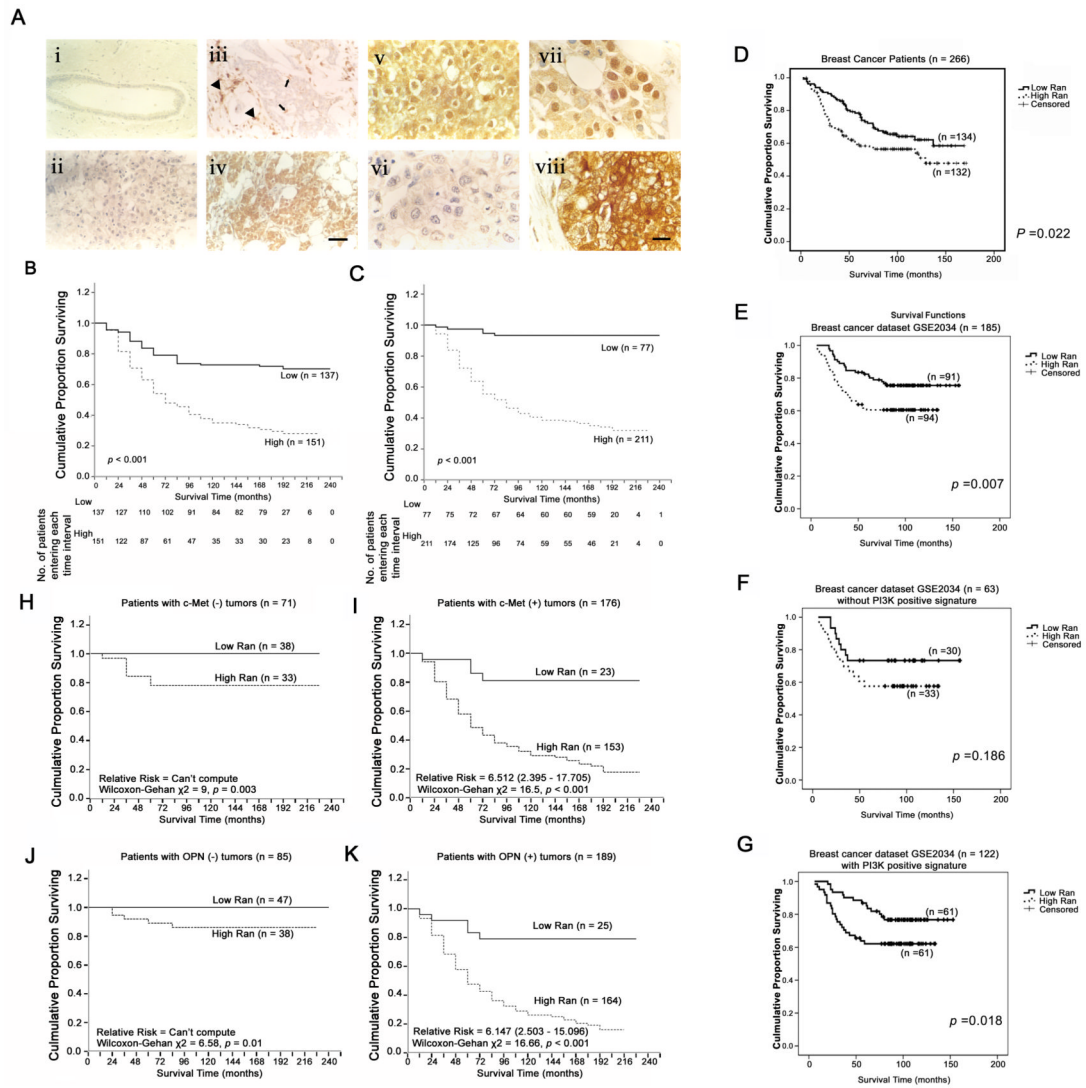
Figure 4. High level of Ran is correlated with shorter survival time of breast cancer patients
(A) Representative images for IHC staining in breast cancer specimens. (i) Normal breast
tissue showing no staining for Ran, (ii) Invasive carcinoma showing no staining for Ran,
(iii) Invasive carcinoma showing borderline staining for Ran (1-5% carcinoma cells stained)
(arrows). The surrounding host myofibroblastic-like cells are well stained (arrowheads), (iv)
Invasive carcinoma showing nuclear and cytoplasmic staining for Ran, (v) Higher power of
another invasive carcinoma showing both nuclear and cytoplasmic staining for Ran, (vi)
Blocked control of a similar section to E above showing no staining, Invasive carcinoma
showing (vii) predominantly nuclear and (viii) predominantly cytoplasmic staining for Ran.
Original magnification A-D × 190; E-H × 480. Bars A-D = 50 μm, E-H 20 μm. (B&C)
Kaplan-Meier plots of survival of breast cancer patients with (B) cytoplasmic and (C)
nuclear staining for Ran. Cumulative proportion of breast cancer patients surviving with
time is plotted for high and low staining groups using 1% stained carcinoma cells as cut-off
point. Kaplan-Meier plots of the breast cancer patients stratified by (D) high and (E) low c-
Met, (F) high and (G) low OPN expression levels for nuclear staining of Ran. (H) Kaplan-
Meier plot of survival of breast cancer patients from four microarray datasets available in
GEO database for Ran mRNA. Cumulative proportion of breast cancer patients surviving

with time is plotted for high (top 25% of patients expressing a high level of Ran) and low (bottom 25% of patients expressing a low level of Ran) Ran groups. (I-K) Kaplan-Meier plots of a breast cancer patient cohort from the GSE2034 dataset, where PIK3CA mutation gene signature, Ran mRNA expression and survival data are available. (I) The whole cohort with top and bottom 25% expressors included, (J) patients with PIK3CA mutation gene signature and (K) patients with PIK3CA wild-type gene signature.
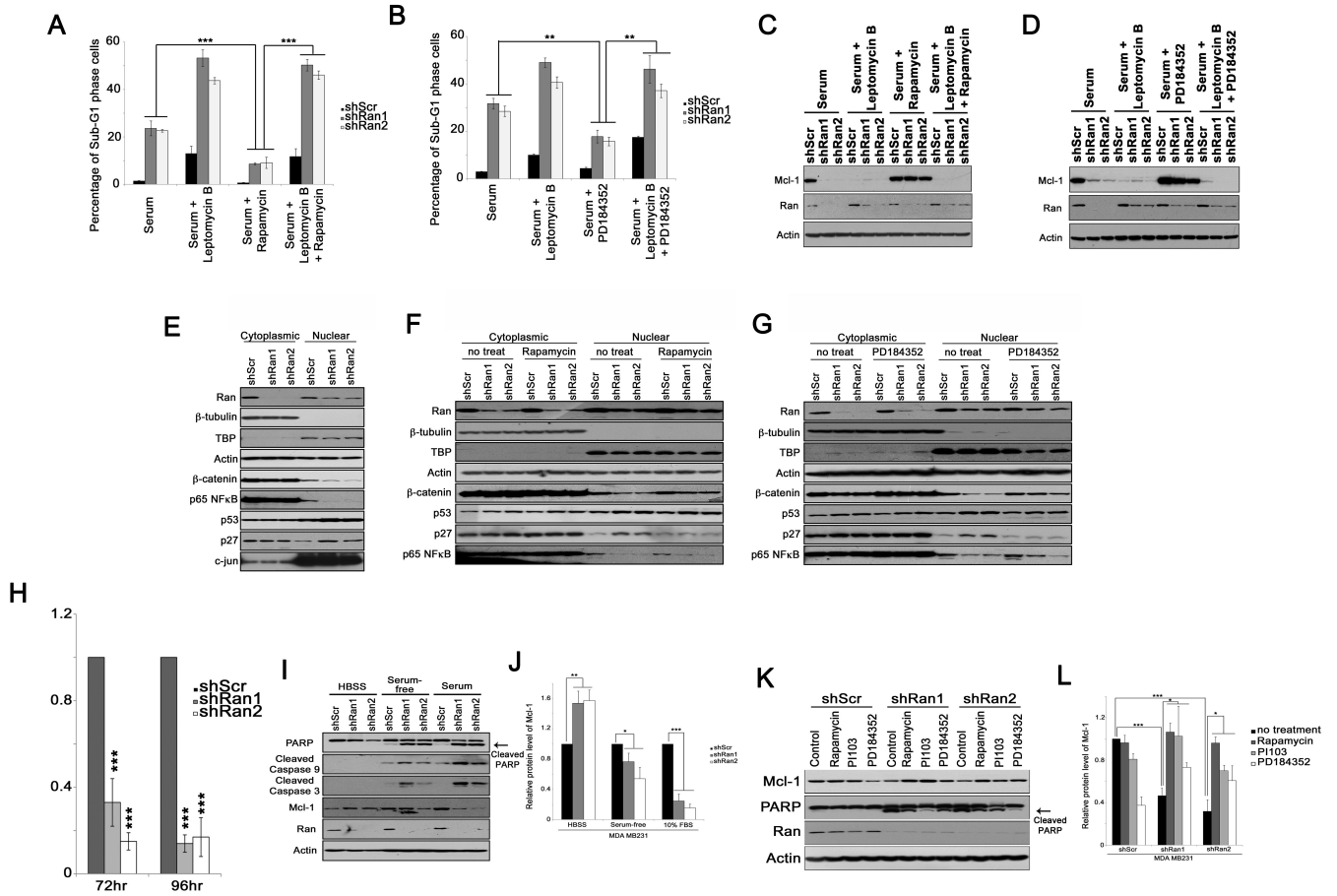
Figure 5. The role of nucleocytoplasmic transportation and Mcl-1 expression in Ran silencing-induced apoptosis

Cells were grown in different conditions at 24 hours post-infection and were then harvested at 72 hours post-infection. Percentage of sub-G1 phase apoptotic cells are shown in MDA-MB-231 breast cancer cells treated with (A) rapamycin and/or LMB, and (B) PD184352 and/or LMB. Western blots of PARP, Mcl-1 and Ran, in MDA-MB-231 cells expressing various levels of Ran treated with (C) rapamycin and/or LMB, and (D) PD184352 and/or LMB. Western blots of various transcription factors in cytoplasmic and nuclear fraction of scramble (Scr) and Ran knockdown (E) MDA-MB-231 cells, (F) MDA-MB-231 cells with or without rapamycin, (G) MDA-MB-231 cells with or without PD184352. (H) Quantitative PCR of Mcl-1 transcript in MDA-MB-231 cells infected with Scr and Ran shRNAs. (I) Western blots of Mcl-1, PARP and Ran at 72hr post-infection of Ran shRNAs in MDA-MB-231 cells grown in Hank's buffered salt solution (HBSS), serum-free and serum-supplemented conditions. (J) Quantification of Mcl-1 protein levels from figure (I). (K) Western blots of PARP, Mcl-1 and Bcl-2 in MDA-MB-231 cells upon Ran silencing in the presence of different inhibitors. (L) Quantification of Mcl-1 protein levels from figure (K).