

Extra View

The translation initiation factor DAP5 is a regulator of cell survival during mitosis

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Initiation of protein translation is tightly regulated by various physiological signals and involves cap-dependent and independent mechanisms. DAP5 protein is an eIF4G family member previously implicated in mediating cap-independent IRES driven translation in response to various cellular stresses. Unexpectedly, we have recently found that DAP5 is also essential for continuous cell survival in non-stressed cells. We reported in this respect that the knock down of endogenous DAP5 by RNA-interference induces M-phase specific caspase-dependent cell death. Bcl-2 and CDK1 were identified as DAP5 mRNA targets, the translation of which was selectively reduced in the DAP5 knock down cells. They each possess a functional IRES element in their 5'UTR. Here we review the major results of this study and present new data on the link of DAP5 to additional Bcl-2 family members. In addition we discuss other possible cellular phenotypes resulting from the knock down of DAP5 in these cells.

Introduction

Protein synthesis is a highly regulated process which may determine the response of cells to different stresses and cellular conditions.¹ It also participates in the execution and maintenance of fundamental processes in the adult organism such as learning and memory.^{2,3} Initiation of protein synthesis in the eukaryotic cell proceeds by two major mechanisms: cap-dependent, which involves the assembly of the pre-initiation complex at the 5'cap m⁷GpppX structure in the mRNA, and cap-independent, which enables recruitment of the ribosome directly to the mRNA. The cap-dependent mode of initiation involves formation of the eIF4F complex which consists of three subunits, eIF4A, eIF4E and eIF4G, the latter acting as a scaffold to bridge between eIF4E and eIF4A.^{4,5} The second mode of initiation requires the presence of an IRES (Internal Ribosome Entry Site) element within the mRNA's 5'UTR, which by directly interacting with the translation machinery enables initiation of translation independently of the 5'cap structure.^{6,7}

The balance between two fundamental processes in eukaryotic cells, i.e., cell cycle progression and programmed cell death, determines whether a tissue or an organ will grow, remain constant or regress in size. Cell cycle progression is driven by a tightly regulated and ordered process that ensures proper DNA replication during S-phase and segregation during M-phase into daughter cells. The cell cycle events are regulated by the activation and inactivation of a conserved family of serine/threonine kinases, the cyclin dependent kinases (CDKs) and their regulatory subunits, the cyclins. Each particular CDK/cyclin complex controls a specific transition along the cell cycle phases.⁸⁻¹⁰ Apoptosis, one of the well studied forms of programmed cell death—is also a tightly regulated process, leading to the self elimination and demise of damaged, redundant or unwanted cells. Two evolutionary conserved gene families regulate/execute the apoptotic process, including the Bcl-2 family members, which are the regulators of the process and the caspases, which are the executioner cysteine proteases.¹¹⁻¹³ Notably, cell cycle progression and programmed cell death are two intimately linked processes.¹⁴ Failure to traverse specific stages of the cell cycle phases will lead to cell cycle arrest or cell death. Therefore, these processes are coordinated with respect to each other to assure proper development and functioning of the tissue, the organ and the organism. This understanding has led to the discovery that several cell cycle regulators participate in programmed cell death while several regulators of programmed cell death are involved in cell cycle progression.¹⁵⁻²⁰

Notably, previous research on the regulation of cell cycle progression and cell death/survival pathways has focused mainly on the post-translational mechanisms, such as phosphorylation and dephosphorylation, proteolytic cleavage, changes in the cellular localization of proteins, protein ubiquitination and more.^{9,21-23} Recently however, we have demonstrated an intricate connection between cell cycle and cell death which is based on the regulation of protein translation. We reported a mechanism by which DAP5, an eIF4G family member and a mediator of cap-independent translation, impacts on cell survival during mitosis. Knock down of DAP5 from cells by RNA-interference resulted in substantial loss of cell viability, in particular during the mitotic phase, via activation of caspase-dependent pathways. The molecular basis for this apoptotic phenotype is provided by identifying two mRNA targets, Bcl-2 and CDK1, the translation of which is modulated by DAP5.²⁴ In this article we will discuss the roles and the regulation of CDK1 and Bcl-2, in cell cycle progression and programmed cell death. We will

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also provide some new data on two additional Bcl-2 family members in this context, Bcl-x_L and Bax.

DAP5 Protein Function Is Required for Cell Survival during Mitosis²⁴

Transfection of HeLa cells with DAP5 shRNA led to an overall reduction in the number of viable cells due to increased cell death. A careful analysis, including flow cytometry analysis, western blotting, microscopic observations and treatment with zVAD (a broad caspase inhibitor) revealed that this cell death has typical apoptotic hallmarks and is fully dependent on the activity of caspases. In addition, the observation that DAP5 knock down leads to apoptotic cell death, without the addition of an external trigger to the cell culture, prompted a more detailed analysis of this phenotype along the cell cycle. The cells were arrested either at S-phase with thymidine, or at M-phase with sub-lethal doses of Taxol (a microtubule-stabilizing agent which arrests cells at the boundary between prophase and metaphase). Synchronized cell populations proceeding towards mitosis were also examined at various time points after release from thymidine block. From these experiments we were able to detect a substantial increase in cell death during the M-phase of the cell cycle. These results were validated with a second method to knock down DAP5, using the SMARTpool (Dharmacon) siRNA against DAP5. Thus, DAP5 function is required to maintain cell survival during mitosis. Polysomal profiles of DAP5 knock down cells indicated that the overall protein translation was not affected and further suggested that DAP5 would rather affect the translation of specific target proteins. Therefore, the next step was to discover which proteins are regulated by DAP5 function leading to the above mentioned phenotype.

CDK1 Is a Target of Translation Regulation Mediated by DAP5²⁴

CDK1 was identified as a putative DAP5 translation target by performing a gene array-based search for mRNAs which specifically and physically bind, directly or indirectly, to DAP5 protein. Further analysis revealed that expression of endogenous CDK1 during mitosis is regulated at the level of translation by DAP5. In addition, the 5'UTR of CDK1 mRNA was capable of driving translation independently of a 5'cap structure, and therefore functions as an IRES element. These data demonstrate the existence of a layer of regulation of CDK1 expression during mitosis which functions in addition to the well studied post-translational modifications and other molecular processes that serve to regulate the kinase activity. Impaired translation of CDK1 during mitosis in DAP5 knock down cells resulted in a significant decrease in the phosphorylation of its target proteins within cells. Notably, the mitotic cell death which developed in response to DAP5 knock down could be partially rescued by the ectopic expression of CDK1. Therefore, de novo synthesis of CDK1 during mitosis is critical for its cellular functions and for maintaining cell survival during mitosis.

There has been some controversy around the contribution of CDK1 activity to cell death/survival during mitosis and specifically with respect to the spindle assembly checkpoint. On the one hand, evidence has brought forward the notion that CDK1 activity is required for cell death and that premature activation of the complex

leads to apoptosis.¹⁷⁻¹⁹ On the other hand, studies involving genetic inactivation of the kinase have suggested the opposite, i.e., that loss of CDK1 activity leads to cell death and apoptosis.^{18,25} Experiments using inhibitors of CDK1 have led to conflicting results in which the same inhibitor under different cellular settings may cause any one of these phenotypes.^{18,19} In our study the data indicated toward a role for CDK1 in protecting the cells from cell death. Notably, entry into mitosis was not affected by DAP5 knock down suggesting that each cellular outcome (i.e., G₂ to M-phase progression versus cell survival during mitosis) relies on a different threshold of the active CDK1 kinase. The newly synthesized molecules during mitosis which are regulated by DAP5 may have a functional advantage over the pre-existing CDK1 in which inhibitory phosphorylations need to be removed for the kinase to be activated. In addition, we observed reductions in the steady state levels of Survivin, a target of CDK1 activity. Survivin is a member of the inhibitor of apoptosis (IAP) gene family. It is selectively expressed at the M-phase and is localized to the mitotic spindle microtubules. Disruption of Survivin-microtubule interactions results in increased caspase3 activity and antisense targeting of Survivin results in increased caspase3 activity at mitosis followed by apoptosis. It was shown that CDK1/cyclinB phosphorylates Survivin on Thr34 and that this phosphorylation acts to stabilize Survivin for its proper activity during the assembly of the bipolar metaphase spindle.²⁶⁻²⁸ We showed that knock down of DAP5 resulted in reduced steady-state levels of endogenous Survivin during mitosis as compared to the control (HcRed shRNA) cell population. This implied that DAP5 is required for the stabilization of Survivin during mitosis, most probably through CDK1. In addition, it was recently published that caspase9 is phosphorylated at Thr125 during mitosis by CDK1 and that this phosphorylation is important in maintaining cell survival during mitosis.²⁹ Thus, the phosphorylation of Survivin and of caspase9 during mitosis, both support the concept that CDK1 restrains apoptosis during mitosis. Future work may reveal additional CDK1 mediated phosphorylation events which are cell death protective. The identification of CDK1 as a target of DAP5 highlights the important role that DAP5 has in driving cap-independent initiation during mitosis.

Bcl-2 Is a Target of Translation Regulation Mediated by DAP5²⁴

Bcl-2 was identified as a putative DAP5 target by performing a biased search for changes in the steady-state levels of proteins previously shown to be linked to cell survival. Further analysis revealed that Bcl-2 steady state expression levels were significantly reduced in DAP5 knock down cells. While no effect on the overall polysomal profile was detected in DAP5 knock down cells, there was a selective shift of Bcl-2 mRNA towards the light polysomal fractions upon analysis on sucrose gradients, reflecting its reduced efficiency of translation initiation in response to DAP5 depletion. Furthermore, the activity of the Bcl-2 IRES as assessed in the classical bi-cistronic vectors was significantly reduced in DAP5 knock down cells, indicating that DAP5 regulates Bcl-2 expression through the IRES. Notably, the mitotic cell death which developed in response to DAP5 knock down could be partially rescued by the ectopic expression of Bcl-2.

The central role of Bcl-2 in cell survival, cell growth and tumorigenesis has been extensively discussed in many publications over the years.^{16,30-32} The Bcl-2 protein family plays a key role in the

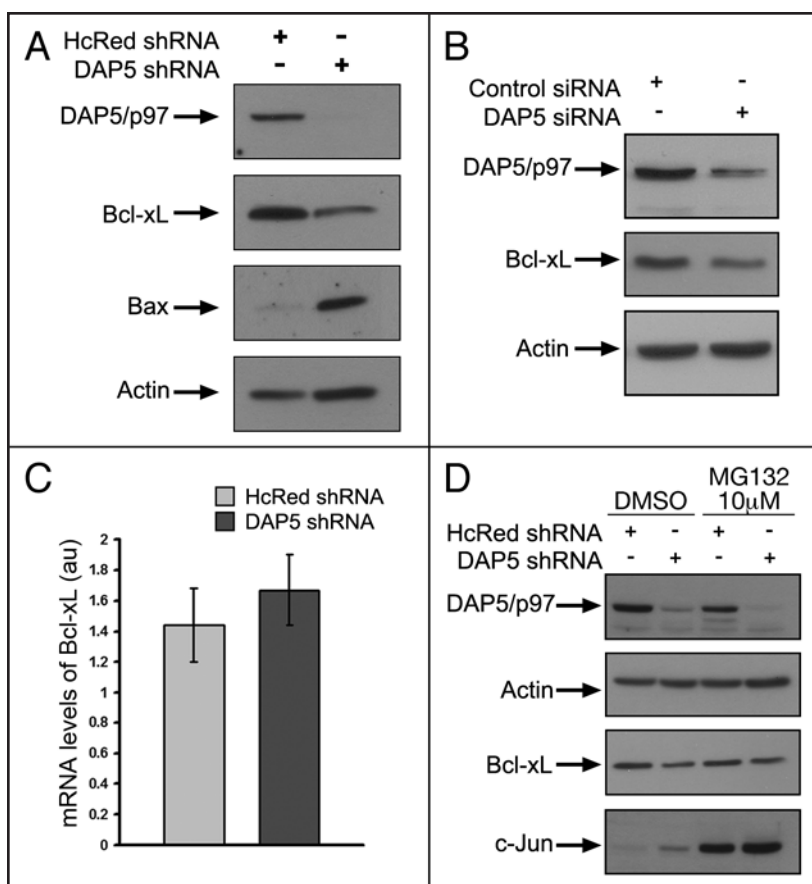


Figure 1. Bcl-x_L and Bax expression levels are modulated by DAP5. (A) HeLa cells were plated at the concentration of 2×10^5 cells per 9 cm culture dish and transiently transfected with 10 μ g shRNA against DAP5 or HcRed by means of the standard calcium-phosphate precipitation method. Cells were kept in culture for four days and then re-plated at the concentration of 5×10^5 cells per 9 cm culture dish. 24 hours following re-plating cell extracts were prepared and examined on immunoblots. Steady state levels of DAP5, Bcl-x_L, Bax and Actin were determined with specific antibodies. (B) HeLa cells were transfected with SMARTpool siRNA against DAP5 or siCONTROL (Dharmacon), using the Transit-IT1 + Transit-TKO transfection kits (Mirus), for 72 hours. Steady state levels of DAP5, Bcl-x_L and Actin were assessed by immunoblotting. (C) Total RNA was isolated from HeLa cells treated as in (A), and subjected to reverse transcription. Real-Time PCR was performed on the cDNA samples using SYBR Green dye. Relative amounts of Bcl-x_L mRNA in each sample normalized to HPRT mRNA are presented as the mean \pm SD of triplicates. The experiment was repeated three times with reproducible results. (D) Cells treated as in (A), were exposed for 16 hours to 10 μ M MG132. Steady state levels of the DAP5, Bcl-x_L, Actin and c-Jun were assessed by immunoblotting. Additional protocol details are found in.²⁴

regulation of cell death/survival and the status of the Bcl-2 family members largely influences the decisions which determine whether an individual cell will live or die. The original family member, Bcl-2, was cloned as a deregulated oncogene at the chromosomal translocation breakpoint of t(14;18) in follicular lymphomas. This was the first example of an oncogene promoting tumorigenesis by prolonging survival/preventing cell death rather than accelerating cell proliferation.³³⁻³⁷ The ultimate vulnerability of cells to diverse apoptotic stimuli is determined by the relative ratio of various pro-apoptotic and anti-apoptotic members of the family. Therefore, a reduction of Bcl-2 expression level may change the balance in DAP5 knock down cells causing the cells to be more susceptible to a stress or death stimuli. In addition, Bcl-2 is known to undergo

phosphorylation during G₂/M-phase in normal cycling cells and also under the treatment with microtubule targeting drugs, such as Taxol.^{15,16,38-40} Some controversy has aroused around the function of this phosphorylation, however the leading indication is that the phosphorylation reduces the anti-apoptotic function of Bcl-2.^{15,16,31} A model has been proposed that hyper-phosphorylation of Bcl-2 leads to the loss of function and dissociation from its dimeric pro-apoptotic partner Bax. This reduction in Bcl-2/Bax heterodimers, when reaching a certain threshold, might drive the cells towards apoptosis.^{15,40-42} From a different direction, inhibition of Bcl-2 expression by antisense oligonucleotides was shown to facilitate and amplify a process called "mitotic catastrophe", a type of cell death occurring during mitosis or resulting from mitotic failure, characterized by certain key molecular events characteristic of apoptosis.¹⁷ Therefore, Bcl-2 may also act during mitosis as a guardian of microtubule integrity. From this point of view, the M-phase specific cell death may be the result of impaired expression of both CDK1 and Bcl-2.

Are Additional Bcl-2 Family Members Regulated by DAP5?

The discovery of Bcl-2 as a target for DAP5 regulation led us to examine here the steady state expression levels of additional Bcl-2 family members. We decided to examine two opposing family members, the anti-apoptotic Bcl-x_L and the pro-apoptotic Bax. Bcl-x_L, is both a structural and functional homologue of Bcl-2.^{30,43,44} However, their functions in the cell do not overlap entirely as portrayed in the knock out mice studies.^{31,32,45} Interestingly, it was recently reported that Bcl-x_L mRNA also possesses a bona fide IRES element.⁴⁶ We found that the steady state levels of endogenous Bcl-x_L protein were significantly reduced in DAP5 knock down cells, as compared to the control cells (Fig. 1A). The selective reduction in Bcl-x_L protein levels was also confirmed by the use of the siRNA SMARTpool against DAP5 (Fig. 1B). Measurement of Bcl-x_L mRNA levels by Real-Time PCR revealed equal levels of expression in cells transfected with control or DAP5 shRNA (Fig. 1C). In addition, the decrease in Bcl-x_L protein expression was not affected by treatment with the proteasome inhibitor MG132 (Fig. 1D). Taken together, these data suggest that Bcl-x_L protein translation is positively regulated by DAP5.

Bax is a pro-apoptotic member of the family known to interact with anti apoptotic Bcl-2 and Bcl-x_L.^{30,43,44} As mentioned before, the ratio between Bax and Bcl-2/Bcl-x_L is considered to predetermine the life or death response of a cell under different circumstances. When examining the steady state levels of Bax, we found that Bax protein expression is significantly increased in DAP5 knock down cells (Fig. 1A). The molecular mechanisms through which the knock down of DAP5 increases Bax levels are not known and raise a challenging question as to how a translation factor which so far was identified as a positive regulator of translation displays such opposite effects. The simple answer would be that DAP5 might positively regulate a

repressor which shuts down the expression of Bax, thus exerting an indirect effect. The second possibility is that DAP5 has another unknown yet function with direct inhibitory effects on the translation of some mRNAs, leading to their elevated expression when DAP5 is knocked down in the cells. This is an aspect of DAP5 function that needs to be further investigated in the future. However, from the functional point of view the increase in Bax protein levels complements the decrease in the steady state levels of Bcl-2 and Bcl-x_L, further increasing the above mentioned critical ratio between the apoptotic and anti-apoptotic family members, and constituting at least part of the cell death pathway in DAP5 knock down cells.

Looking at the Data from an Additional Point of View

In our experiments conducted in HeLa cells we noticed that the DAP5 knock down cells were consistently morphologically different than the control cells. These differences observed by light microscopy could be interpreted as differences in cell size, DAP5 knock down cells being smaller or as differences in attachment and spreading out, DAP5 knock down cells being less spread as compared to the control cells (Fig. 2). This led to the notion that DAP5 may have additional effects, apart from maintaining cell survival during mitosis. Several options may be suggested to explain these morphological differences between control and DAP5 knock down cells, among them the possibility that DAP5 regulates a set of genes involved in cell size regulation or a set of genes involved in the cytoskeleton organization and cellular adhesion. An additional difference between the cell cultures was observed while analyzing the cell cycle distribution, by flow cytometry, after release from double thymidine block. As can be seen in Figure 3—Upper panel and middle panel, most of the control cells are arrested at the boundary of G₁/S, while the DAP5 knock down cells seem to be arrested more into the S-phase. This means that in the DAP5 knock down cells more cells are arrested with a higher DNA content compared to the control cells. Consistent with this observation, when both cultures are released from the thymidine arrest, the DAP5 knock down cells progress one step ahead of the control cells and reach mitosis and the subsequent cycling G₁-phase faster. This observation is reinforced by the Western Blot analysis which shows a shift in the electrophoretic pattern of Bcl-2, corresponding to the entry into mitosis at an earlier time point following the release, in DAP5 knock down cells (Fig. 3, lower panel).

What can we speculate about these different patterns of cell cycle distribution and do they correlate with the above mentioned changes in expression of the Bcl-2 family members as the death phenotype does? It is well known that Bcl-2 family members participate not only in cell death/survival decisions but also function in cell cycle progression. For example, Bcl-2 and Bcl-x_L are known to exert anti-proliferative functions by facilitating G₀ and delaying G₀/G₁ to S transition.^{16,20,30,47} On the other hand, Bax is known to have pro-proliferative functions by facilitating the S-phase transition.²⁰ Bax expression has been correlated with higher S-phase fraction, increased

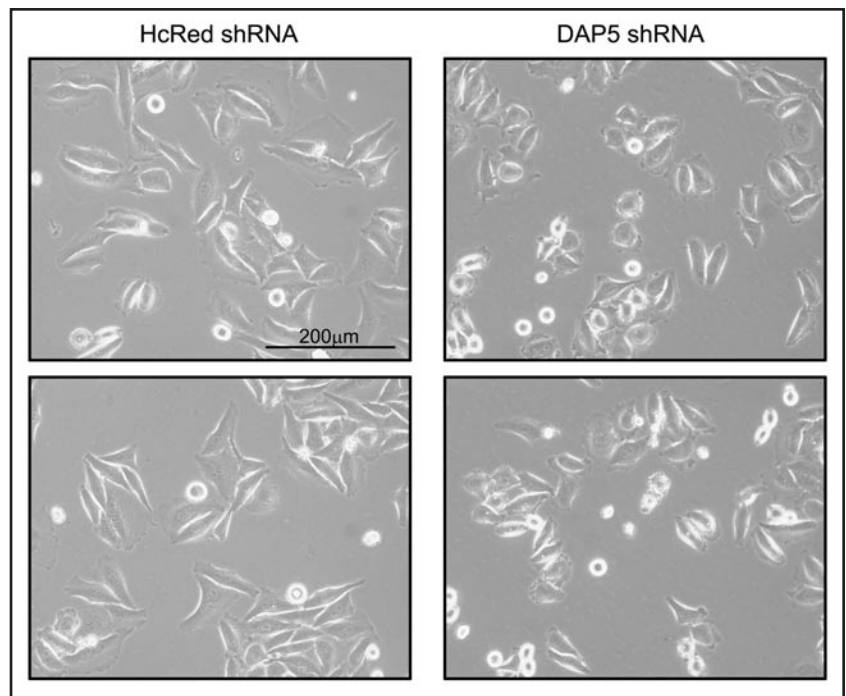


Figure 2. Morphological distinctions between DAP5 knock down and control cells. HeLa cells transiently transfected with shRNA against DAP5 or HcRed for 5 days were plated at similar cell densities and visualized by light microscopy using an inverted IX70 microscope (Olympus) equipped with a LCPlanFl 20x objective (N.A. 0.4) and a DVC digital camera (Digital Video Camera Co.). Images were captured using the C-View V2.1 imaging program and processed with Adobe Photoshop. Additional protocol details are found in ref. 24

BrdU uptake and Bax expressing cells were shown to re-enter cell cycle faster and enter S-phase faster than wild type cells.^{20,30,48} Hence, it is tempting to speculate that these other functions of the Bcl-2 family members may impose the cell cycle phenotype conveyed by the knock down of DAP5, capable of increasing the ratio between Bax and Bcl-2/Bcl-x_L. According to this model the cells that reach mitosis may have two fates. This will depend on the degree of DAP5 knock down and the degree of the changes in the levels of its target proteins in the cell. On the one hand, the status of the proteins may be such that the cell will not be able to survive mitosis and will enter the process of apoptosis. On the other hand, the decrease in protein levels might not reach the critical threshold required to evoke apoptosis and the cell is able to continue the cell cycle. At this point DAP5 knock down and the changes in the levels of the Bcl-2 family members may affect the progression of the cells through the cell cycle. This model awaits further and more detailed investigation and obviously may only be part of the causal relationship between DAP5 knock down and the cellular phenotype.

Conclusions and Perspectives

It is clear that DAP5 and its function as a regulator of protein translation have a substantial role in maintaining cell survival during mitosis. This fundamental process joins the well established role of DAP5 in cell death under stress conditions (reviewed in ref. 49). Thus, the major goal in the field will be to understand at the molecular level what directs the function of this factor towards these two apparently different directions. One way will be to continue

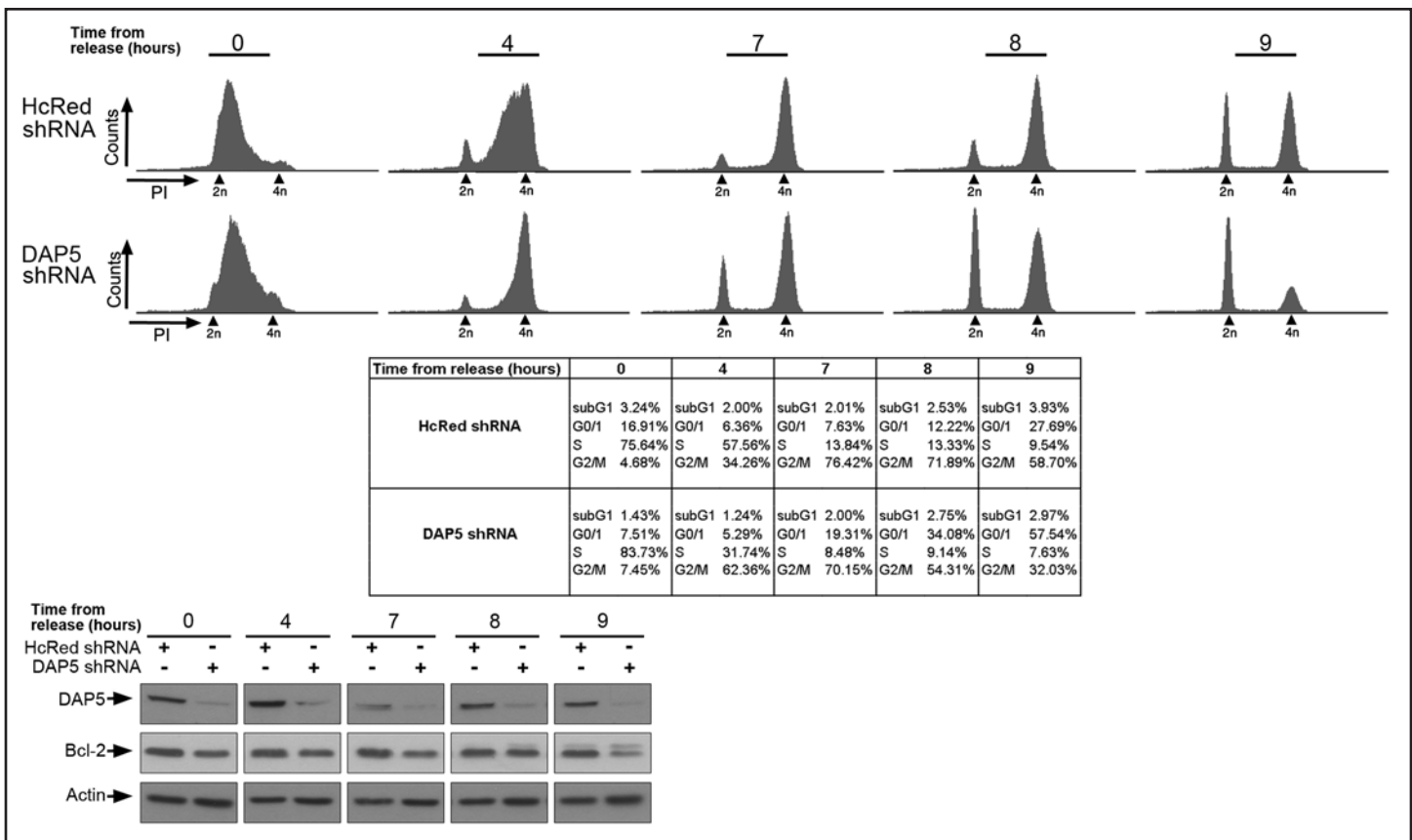


Figure 3. DAP5 knock down affects in the pattern of S-phase arrest induced by thymidine block and the subsequent cell cycle progression upon release from block. HeLa cells transiently transfected with shRNA against DAP5 or HcRed for 5 days were plated at similar cell densities and were synchronized by a double thymidine block (2 mM thymidine-Sigma), 14 hours incubation each time separated by a 9 hour release. Cells were collected 0, 4, 7, 8, 9 hours after release from block. Upper: Flow cytometry analysis. Cells were fixed with 70% Ethanol, and were stained with 40 $\mu\text{g/ml}$ Propidium Iodide (PI). Histograms represent cell population distribution according to their DNA content. 2n and 4n DNA content is indicated. Middle: Percentages of cells at different phases of the cell cycle. Lower: Cell extracts were prepared and separated with SDS-PAGE. Steady state levels of DAP5, Bcl-2 and Actin were assessed by Western blotting with specific antibodies. Additional protocol details are found in ref. 24.

unraveling the targets of DAP5 translation regulation, in the hope of understanding in a more global fashion the machinery of the cap-independent translation and its importance to the cell.

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