

## News and Commentary

# DAP5 and IRES-mediated translation during programmed cell death

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DAP5 (Death Associated Protein 5), also named p97 and NAT1, is a member of the eIF4G family that lacks the eIF4E binding site. Its function was linked to programmed cell death (PCD) based on the seminal finding that a fragment of DAP5/p97 protein, which acted in a dominant-negative manner, protected against IFN- $\gamma$ -induced cell death. Subsequently, it was found that DAP5 protein is activated during cell death by caspase cleavage, yielding a C-terminal-truncated protein of 86 kDa. The p86 form promotes internal ribosome entry site (IRES)-dependent translation of several mRNAs including Apaf-1, c-myc, XIAP, HIAP2 and DAP5 itself, which carries an IRES element in its 5'UTR. The activation of DAP5 IRES creates a positive feedback loop, which results in sustained translation of DAP5 protein during PCD under conditions of reduced cap-dependent translation. DAP5 deficiency prevents embryonic stem (ES) cell differentiation, suggesting a wider range of DAP5 mRNA targets and additional mechanisms that might activate the protein.

## PCD Displays Diversity in Cellular Phenotypes and in the Underlying Molecular Mechanisms

Developmental processes and tissue homeostasis within a multicellular organism require a tight control over cell population size. This is achieved by a complicated set of genetically controlled and highly conserved processes, which lead to the death of a cell (named PCD). In general, the balance between cell death and survival signals, integrated via diverse cellular signaling pathways, dictates whether the individual cell will live or die.<sup>1</sup>

Apoptosis (type I PCD) is the most characterized example of PCD. Its cellular phenotype, as well as the molecular and genetic mechanisms underlying the process, has been extensively studied over the years. A distinguishing set of morphological events, which includes cell shrinkage, membrane blebbing, nuclear condensation, and chromatin and DNA fragmentations, characterize this process. Altogether, these subcellular changes lead to the complete disintegration

of the cell into membrane-bound apoptotic bodies, which are rapidly engulfed by neighboring cells or by professional phagocytes, avoiding development of an inflammatory response. It has been documented that type I PCD plays a central role in the maintenance of a correct equilibrium of cell number, controls various developmental processes and tissue remodeling, and eliminates cells bearing severe damage or mutations.<sup>1–5</sup>

Autophagy (type II PCD), another form of cell death, is characterized by the appearance of double- or multiple-membrane cytoplasmic vesicles engulfing bulk cytoplasm and/or cytoplasmic organelles such as mitochondria and endoplasmic reticulum.<sup>6</sup> Autophagic vesicles and their contents are destroyed by the lysosomal system of the same cell. As a consequence, the cell 'cannibalizes itself' from inside, which is in contrast to the heterophagy characteristic of type I PCD. Many physiological scenarios of cell death that lack the hallmarks of type I cell death, yet display autophagic activity, had been described. Examples are death of palatal epithelial cells during closure, regression of the Mullerian duct during male sexual development, death of the central cells of the intestine during cavity formation, and hormone deprivation induced regression of mammary gland or atrophy of prostate.<sup>7,8</sup> Furthermore, autophagy and apoptosis can be simultaneously observed in the same tissue, indicating that different types of cell death may develop concomitantly to accomplish the important tasks of tissue remodeling and other developmental processes that require genetically controlled PCD.<sup>6</sup>

Improper execution of apoptosis or of autophagy usually has quite hazardous effect upon the entire organism. Mutations affecting the key components of the cell death machineries frequently contribute to malignant transformation and tumor progression, as well as to the development of lymphoproliferative/autoimmune diseases due to the failure in selection processes during lymphocyte maturation.<sup>9</sup> On the other hand, accelerated cell death resulting in the elimination of broad cellular populations is a common hallmark of a wide variety of neurodegenerative disorders and AIDS.<sup>4,8,10,11</sup>

On the one hand, the tight regulation upon execution of PCD is achieved by a stringent requirement for specific triggers, and on the other hand, protection from inappropriate activation of cell death program in cells intended to survive. This specificity is accomplished both by *de novo* synthesis of death proteins, which takes place after the exposure to the death signal, and by post-translational modifications of pre-existing proteins, which lie dormant in the healthy cell until activated by the death signal. It has become evident that the cell death programs are composed of a dense network of genes that fulfill various functions such as stress sensors, signal transducers, executors, protectors and more. So far, most of the molecular studies have focused on type I PCD. Recently, some progress has also been achieved concerning

the identification of executors and regulators involved in autophagic cell death. The reader is referred to several reviews, which summarize the global molecular information available in both types of PCD.<sup>6,12–15</sup> In this chapter, we wish to focus upon a functional module in cell death that has only recently started to receive a lot of attention, that is, how switches in translation regulation integrate into this dense network of genes.

One of the most extensively studied groups of proapoptotic genes corresponds to a family of cysteine proteases named caspases, which cleave their substrates after conserved aspartate residues.<sup>16,17</sup> Owing to the high potency of caspases for the induction of cell death, their activity is tightly controlled at various levels. These proteases are constitutively expressed as inactive proenzymes, which are activated by processing either in an autocatalytic event or in trans by other caspases upon induction of apoptosis.<sup>16,18</sup> Importantly, cleavage by caspases may lead to either loss of function or gain of function of an individual substrate, which is determined by its anti- or proapoptotic nature, respectively.<sup>17</sup> Interestingly, various components of the translation machinery are also direct targets for caspases during apoptosis.<sup>19–21</sup> This special issue of *CDD*, which is devoted to translation control during different types of stress conditions, will highlight several scenarios where specific components of the translation initiation machinery are being modified by caspase-mediated proteolysis. In the context of the present chapter, we will focus on the translation initiation factor DAP5/p97, which has a central role during cell death and displays interesting crossinteractions with caspases.

## Switching the Initiation of Protein Synthesis from Cap-Dependent to Cap-Independent Translation under Stress Conditions

Eukaryotic translation initiation is a rate-limiting step of protein synthesis. It is a highly regulated process, which involves the coordinated assembly of a multiprotein–RNA complex that directs the ribosome to the initiation codon at the mRNA. Formation of an initiation complex is tightly regulated throughout the cell cycle, as well as under various cellular stresses such as viral infection and the different forms of PCD.<sup>22–29</sup>

The vast majority of the translation initiation events in the cell involve binding of the eIF4F complex to the 5' cap m<sup>7</sup>GpppX structure, which is present in all eukaryotic mRNAs.<sup>22–24,26,27,29</sup> Mammalian eIF4F consists of three subunits: eIF4A, eIF4E and eIF4G. eIF4E is 25 kDa protein, which specifically recognizes the cap structure.<sup>29,30</sup> eIF4A is an ATP-dependent RNA helicase of the DEAD box protein family, which, together with eIF4B and eIF4H, serves to unwind secondary structures within the 5'UTR of mRNA.<sup>29,31</sup> This helicase activity is of special importance, since it allows the 40S ribosomal subunit to bind the mRNA and to scan toward the initiation codon. eIF4G, which in mammals has two isoforms, eIF4GI and eIF4GII, is a scaffold protein, serving as a bridge between eIF4E and eIF4A, thus forming the eIF4F complex. Its N-terminal portion contains a single eIF4E

binding site, which is necessary for cap-dependent translation. In addition, eIF4G associates through its N-terminus with the poly(A)-binding protein (PABP). This step allows the circularization of mRNA, which is thought to enhance translation efficiency.<sup>27</sup> The eIF4A binding sites are located within the middle (amino acids 635–1039) and the C-terminal (amino acids 1040–1560) parts of eIF4G. The middle portion also contains a binding site for eIF3 – a multisubunit ribosome-associated translation factor. The interaction of eIF4G with eIF3 enables the recruitment of the 40S ribosomal subunit to the 5'-end of the mRNA. In addition, the C-terminal part of eIF4G encompasses a docking site for Mnk1 kinase. Mnk1 kinase belongs to the MAPK cascade and is a downstream component of the ERK and p38/stress signaling pathways. Binding of Mnk1 to eIF4G serves to bring Mnk1 into close proximity with its substrate, eIF4E. Phosphorylation of eIF4E increases its binding affinity to the cap structure and stabilizes the entire eIF4F complex, this way providing positive feedback on the initiation of protein synthesis.<sup>27,29,32–37</sup>

Another mechanism of translation initiation, which does not require the mRNA cap structure, exists as well. In this case, instead of scanning of the preinitiation complex from the 5' cap toward the first initiation codon, the ribosome lands directly on the internal AUG. This mode of initiating translation requires the presence of a functional secondary structure within the mRNA's 5'UTR. This structure is designated IRES, and by directly interacting with the translation machinery, it allows the initiation of translation independently of the mRNA cap structure. While translation through IRES elements is usually significantly less efficient than cap-dependent translation, its main advantage is that it allows translation under conditions where cap-dependent translation is severely abrogated.<sup>27,29,32,38</sup>

IRES elements were first described in picornavirus-infected cells, where they serve to initiate translation of uncapped viral mRNAs. Following infection by many picornaviruses, the cap-dependent translation of cellular mRNAs is inhibited due the cleavage of eIF4G by viral proteases. It should be emphasized that cleavage of both eIF4GI and eIF4GII is required for a complete shut-off of host protein synthesis. In fact, the cleaved form of eIF4G lacks the N-terminal part responsible for the interaction with the cap structure, and fails to mediate cap-dependent translation. Nevertheless, the eIF3 and eIF4A binding sites both remain intact after the cleavage. It was therefore hypothesized that truncated eIF4G would form an initiation complex independently of the cap structure. Indeed, eIF4G was shown to stimulate translation through some viral IRES elements.<sup>27,39</sup>

Recently, it has become clear that IRES elements are not unique to the viral systems, and cellular IRESes were subsequently discovered in eukaryotic cells. Despite the fact that the vast majority of translational events in the cell occur through the assembly of an initiation complex at the 5' cap structure, there is a growing list of cellular mRNAs that possess functional IRES elements within their 5'UTR in addition to the cap structure. Importantly, the presence of IRES element in some mRNAs, but not in the others, is far from being accidental. In fact, IRESes are usually found in mRNAs encoding various regulatory proteins whose translation requires tight regulation on the one hand, but has to

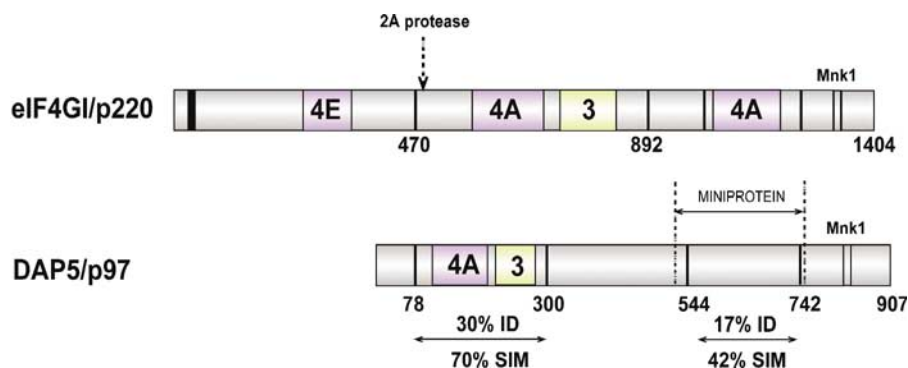
proceed when overall protein synthesis in the cell is inhibited. Among these are mRNAs that encode proteins involved in growth control, cell cycle progression as well as in PCD. Some of these cellular IRESes are utilized during different stress conditions such as hypoxia or heat shock.<sup>40–54</sup>

In light of the above, a major question that became of interest to the cell death community, and will be addressed in detail in this issue of *CDD*, is whether translational switches are part of the PCD process, and more specifically, whether a shift from cap-dependent to IRES-mediated translation occurs under certain settings of cell death. In fact, it turned out that induction of type I PCD, for example, is associated with a rapid and substantial shut-off of overall protein synthesis in the cell. This is achieved by a combination of several inhibitory processes affecting global translation initiation proceeding through the cap structure. These include caspase-mediated processing and inactivation of different initiation factors such as eIF4B, eIF3/p35, eIF2 $\alpha$ , eIF4G1 and eIF4GII.<sup>9,19,21,55</sup> In addition, it has been documented that PKR kinase is activated by caspases and further inhibits translation initiation by phosphorylating eIF2 $\alpha$ .<sup>56</sup> Also, some apoptotic settings involve changes in the phosphorylation state of eIF4E and 4E-BP1, which has a negative impact on protein synthesis.<sup>27,29</sup> Finally, massive degradation of cellular mRNAs and 28S rRNA take place and further contribute to the translational block under the cell death circumstances.<sup>57–59</sup> All these scenarios are described in detail in other chapters of this issue of *CDD*.

Although many pathways in cell death, especially those that require only a few hours from initiation to death execution of the cell, are based primarily on activation of pre-existing death-promoting proteins, for other pathways, *de novo* protein synthesis is an obligate requirement. This is of special importance in cases where death of the cell occurs a day or two after exposure to the death stimulus. One of the most intriguing questions therefore relates to the nature of these protein translation events, which proceed under conditions when cap-dependent translation is shut down. This particular question will be dealt with in this review, focusing on one of the eIF4G family members – the DAP5/p97 protein, which has been suggested to mediate IRES-driven initiation of protein translation during cell death.

## DAP5/p97/NAT1 – The Discovery of the Gene and Initial Structural Characterization of the Protein

DAP5 is one of several members of the expanding eIF4G protein family. The multiple names for this protein (i.e. DAP5/p97/NAT1) stem from the fact that the gene was identified and cloned simultaneously by four independent groups.<sup>60–63</sup> In our laboratory, DAP5 was isolated as a positive mediator of IFN- $\gamma$ -induced cell death.<sup>64</sup> It was rescued in a genetic screen, which was based on knocking down RNA expression by transfection with antisense cDNA libraries followed by positive growth selection. The underlying assumption was that the specific inactivation of a rate-limiting death gene would confer some growth advantage to cells that are continuously exposed to the death stimulus of the cytokine IFN- $\gamma$ . The cDNA fragments that conveyed selective resistance to IFN- $\gamma$ -induced cell death were recovered and served as the starting point for the cloning of the full-length genes, designated as DAP (Death Associated Proteins) genes.<sup>64</sup> In retrospect, it turned out that for some technical reasons the library also included cDNA fragments that were oriented in the expression vector in the sense direction. Thus, while most of the other rescued cell death-protective fragments were antisense cDNAs, which acted by knocking down protein expression, in the case of DAP5, a sense cDNA fragment conferred protection from cell death. It turned out that this cDNA fragment codes for a fragment of the protein, that is, a miniprotein encompassing amino acids 522–776 from the C-terminal region of the full-length protein (Figure 1). The sequence of the full-length protein shared strong homology to eIF4G (58) and placed this novel gene within this family of scaffold proteins, which has since expanded to also include the PAIPs.<sup>65,66</sup> In addition, detailed sequence alignments showed that the region corresponding to the rescued miniprotein was less conserved within the family members (Figure 1). Therefore, it was assumed that this miniprotein acted in a dominant-negative manner selectively counter-acting the function of the endogenous DAP5 (and not the other family members), thus conferring some resistance to IFN- $\gamma$  during the selection procedure. This suggests that DAP5, of



**Figure 1** Schematic representation of homology regions of eIF4G1 and DAP5/p97. eIF4G1 and DAP5/p97 are divided into regions based on the high homology of the central segment of eIF4G1 with N-terminal portion of DAP5. The colored boxes indicate binding sites for translation initiation factors: eIF4A, eIF4E and eIF3 and the Mnk1 kinase and PABP. Dashed arrow indicates the site of cleavage of eIF4G1 by 2A protease upon viral infection. Regional homologies at the amino-acid level between DAP5 and eIF4G1 are indicated. ID identity, SIM similarity. The boundaries of the mini protein are shown

all the eIFG family members, plays a rate-limiting role in PCD. Independently, expression of an antisense cDNA fragment of DAP5 was also found to exert similar death-protective effects, further establishing the role of DAP5 as a positive mediator of cell death in the IFN- $\gamma$  system, which represents a slow type of cell death.<sup>61</sup>

In parallel, Sonenberg and colleagues cloned DAP5/p97 in an effort to identify novel homologs of the eukaryotic translation initiation factor eIF4G1.<sup>60</sup> In addition, Shaughnessy *et al.*, cloned the mouse DAP5 gene based on its physical linkage to a common retroviral integration site found in myeloid leukemia of BXH2 mice. They mapped the human DAP5 within a cluster of genes on human chromosome 11p15 that harbors several unidentified tumor suppressor genes.<sup>62</sup> A fourth group, headed by TL Innerarity, identified DAP5/NAT1 as a novel target for RNA editing in transgenic mice overexpressing Apobec-1, the catalytic subunit of the editosome complex. In these mice, DAP5 mRNA was extensively edited, creating multiple stop codons. Transgenic mice and rabbits overexpressing Apobec-1 developed liver dysplasia and hepatocellular carcinoma, linking oncogenesis with the aberrant hyperediting of Apobec-1 target mRNAs. Although this artificial system of hyperediting fails to provide the precise physiological editing sites along the RNA molecule, it definitely identifies the physiologic target mRNAs of Apobec-1. Thus, the identification of DAP5 mRNA as a principal editing target in these mice suggested that RNA editing might be one of a few mechanisms that regulate DAP5 function by generating novel forms of the protein.<sup>63</sup>

In all these studies, the cloning of the gene was followed by straightforward characterization steps such as the analysis of DAP5/p97/NAT1 mRNA expression profiles, sequence alignments, phylogenetic studies and structural dissection of the protein. The latter was done by comparing the amino-acid sequence to the other eIF4G family members and then by testing basic functions of DAP5 in translation according to sequence predictions. It emerged from these initial studies that DAP5/p97/NAT1 is a 97 kDa protein, which is ubiquitously expressed at high levels in the heart, brain, lung, kidney, pancreas, and organs of the immune system and reproductive tract. The sequence of DAP5/p97/NAT1 is highly conserved among various species. Its mRNA possesses a relatively long 5'UTR and a single ORF starting at a GUG initiation codon, which resides within the consensus sequence for non-AUG initiators.<sup>60–63</sup>

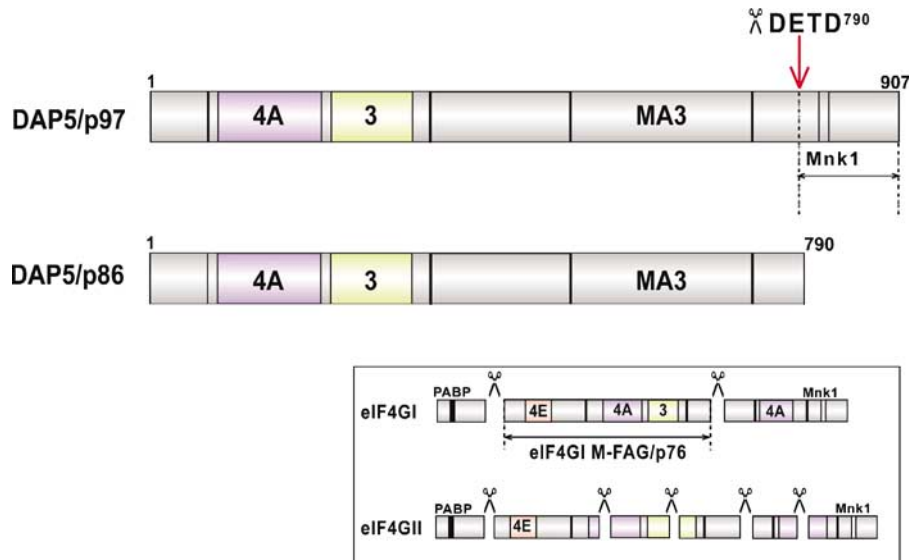
The homology to eIF4G<sup>65,66</sup> was restricted to the central segment of eIF4G1/II, which corresponds to the region that binds to eIF4A and eIF3, necessary elements of the translation initiation complex. Interestingly, the N-terminal part of eIF4G1/GII, which mediates the interaction with the mRNA cap structure by direct binding to eIF4E, is completely missing from DAP5 protein (Figure 1). Consistent with the structural predictions, it was shown by a few strategies that DAP5/p97/NAT1 indeed binds eIF3 and eIF4A, but fails to bind eIF4E, which is essential for mediating cap-dependent translation.<sup>60,61,63,65–68</sup> In this respect, DAP5 resembles the cleaved version of eIF4G1/GII that arises upon picornavirus infection. This structural resemblance raised the possibility that DAP5, like the virally cleaved eIF4G1/II that lost the cap-binding features, may maintain the capability to mediate IRES-driven translation under stress conditions.<sup>61</sup>

## DAP5 Regulation and Function in PCD

In light of the findings that the DAP5 protein is constitutively expressed in growing cells, yet is rate limiting during cell death, it became of interest to find out how its death-promoting functions are activated when necessary to fulfill its death-promoting functions. Although additional efforts in this direction are still required to resolve this issue thoroughly, there are several established findings regarding the regulation of this intriguing protein, and its putative function in PCD, which will be discussed below.

DAP5/p97-specific features, that is, its ability to interact with components of basic translation machinery such as the RNA helicase eIF4A, and the ribosome adaptor complex eIF3, on the one hand, and inability to interact with the cap structure on the other hand, implied a unique function for this protein. The first suggestion made by Imataka *et al.* was that DAP5/p97 might act as an inhibitor that represses translation. This was based on transient cotransfections of DAP5/p97 with reporter genes, which showed inhibitory effects on both cap-dependent as well as cap-independent translation from the EMCV IRES. Also, the overexpression of DAP5 in HeLa cell clones stably transfected with tetracycline-inducible DAP5/p97 caused mild reductions in overall protein synthesis. A similar inhibitory effect was observed in cell-free systems, in which the CAT reporter gene was translated in either a cap-dependent manner or from EMCV IRES.<sup>60</sup> This was consistent with findings of Innerarity's group, which indicated that DAP5/p97/NAT1 inhibited translation of coexpressed CAT reporter constructs, with or without EMCV IRES, in COS7 cells.<sup>63</sup> These data together indicated that DAP5/p97/NAT1, when introduced into cells or when supplemented to cell-free systems, acts as a general repressor of protein synthesis, presumably by forming translationally inactive complexes that include eIF4A and eIF3 but exclude eIF4E. Based on these findings, it was further speculated that a competition between endogenous DAP5/p97 and eIF4G proteins for the binding to eIF4A and eIF3 might exist within cells.<sup>60</sup> Yet, this model was never confirmed by measuring the steady-state levels of these components in the cell, or by demonstrating whether endogenous DAP5 is indeed capable of titrating out a factor necessary for eIF4G scaffold's functions.

Simplistic models often characterize the initial stages of scientific research. However, as time goes on and more details emerge, it becomes clear that the state is much more complicated. In this respect, DAP5/p97 was not exceptional. When DAP5/p97 was extensively studied as a part of the cell death process, it was found that induction of apoptosis led to the appearance of a shorter fragment recognized by anti-DAP5-specific antibodies.<sup>68</sup> Further analysis revealed that under apoptotic conditions, DAP5/p97 undergoes proteolytic cleavage, giving rise to an 86 kDa fragment (DAP5/p86) (Figure 2). By using a panel of inhibitors for various cellular proteases, the processing of DAP5 was attributed to caspases. Examination of amino-acid sequence of DAP5 protein revealed the existence of four potential caspase cleavage sites of the motif DXXD (amino acid 185, 592, 790, 824), yet only two of these sites, DETD<sup>790</sup> and DHVD<sup>824</sup>, seemed capable of yielding a cleavage product of the



**Figure 2** A diagram representing caspase-mediated processing of DAP5/p97 into DAP5/p86 and the fate of eIF4G isoforms following induction of cell death. Regions of binding to PABP, Mnk1, eIF4A, eIF4E and eIF3 are indicated by colored boxes. The position of the cleavage site on DAP5/p97 is marked in red. The region of M-FAG/p76 that corresponds to an eIF4GI caspase cleaved fragment is indicated. Scissors mark caspase cleavage sites within the proteins. The mapping of the cleavage sites is according to Clemens *et al.*<sup>20</sup> Marissen *et al.*<sup>21</sup> Craig *et al.*<sup>65</sup> and Henis-Korenblit *et al.*<sup>71</sup> Ct tail – C-terminal tail of DAP5/p97

expected molecular weight. Generating DAP5 mutants carrying single substitution of aspartate to alanine (DXXD–DXXA) and following the ability of each mutation to abolish the appearance of exogenous DAP5/p86 upon death stimulus revealed that DAP5/p97 is converted to DAP5/p86 by a single caspase cleavage event at DETD<sup>790</sup> (Figure 2).<sup>68</sup>

Tracking the protein levels of DAP5 during cell death led to an interesting observation. While induction of cell death in SKW B-cell lymphoma cells with anti-Fas agonistic antibodies caused significant reduction in the expression of most cellular proteins, protein levels of DAP5 (the sum of cleaved and uncleaved forms) remained unchanged.<sup>68</sup> Since no significant changes in DAP5 mRNA levels and its half-life were evident during cell death, the sustained levels of DAP5 protein in apoptosing cells implied that it might be continuously translated despite the general inhibition of protein synthesis. This hypothesis was confirmed by measuring the incorporation of radiolabeled methionine, which reflects active protein synthesis, into DAP5 protein following the cell death stimulus. While incorporation of radioactivity into  $\alpha$ -tubulin was almost undetectable in the dying cell, no significant reduction in DAP5 translation rate was observed.<sup>68</sup>

Close examination of the DAP5 mRNA revealed that its 5'UTR, which includes two polypyrimidine tracts, is capable of forming a stable secondary structure, which potentially may serve as an IRES element. Functional analysis performed by positioning DAP5 5'UTR between two reporter genes in a bicistronic vector revealed the existence of an IRES element within the 5'UTR of DAP5 mRNA, fulfilling the set of functional requirements defined for a *bona fide* IRES. Moreover, while cap-dependent translation from this bicistronic vector was reduced during Fas-induced apoptosis, translation via the DAP5 IRES was selectively maintained, showing differential utilization of this IRES during cell death. Based on these

findings, it was concluded that the IRES element within the DAP5 UTR is responsible for the sustained DAP5 protein expression under the apoptotic circumstances.<sup>68</sup>

The unexpected appearance of a novel DAP5 form associated with cell death, and the identification of an IRES element within the DAP5 5'UTR, raised two immediate questions: what is the impact of the caspase-mediated cleavage on DAP5 function, and how is the DAP5 IRES regulated during apoptosis? It was assumed that if DAP5 protein is involved in a cap-independent mechanism of translation, it might influence its own IRES as one of its targets, thus providing a positive feedback loop. Indeed, coexpressing DAP5/p86 with a bicistronic vector bearing the DAP5 IRES provided an answer to both these questions simultaneously. This was studied both in cell-free systems and in cotransfection experiments. The addition of recombinant DAP5/p86 to cell-free systems preferentially enhanced the translation through the DAP5 IRES.<sup>68</sup> Transfection-based experiments in which DAP5/p86 was introduced together with the bicistronic vector indicated that translation via the DAP5 IRES was stimulated, while the cap-dependent translation was not affected. Moreover, the full-length DAP5/p97 had no effect on cap-independent translation in these experiments, suggesting that the removal of the C-terminal tail relieves an inhibitory effect on the protein's ability to support cap-independent translation through the DAP5 IRES.<sup>68</sup> These findings strongly suggested that, upon induction of apoptosis, DAP5 becomes activated by the caspase-mediated removal of C-terminal tail. The truncated DAP5/p86 is a functional translation factor, which supports its own translation through the IRES element within its 5'UTR and independently of the 5' cap structure. Furthermore, it was demonstrated that stimulation of DAP5 IRES requires active DAP5 protein, since applying the DAP5 dominant-negative fragment (the

miniprotein isolated in the genetic screen) diminished IRES-mediated translation in the *in-vitro* translation system.<sup>68</sup> Thus, it was confirmed that this self-regulatory event generates a positive feedback loop on DAP5 translation in the dying cell, and allows preferential and continuous production of DAP5 protein under the stress-associated translational block.<sup>68</sup> These were the first functional assays that described a positive effect of DAP5 on cap-independent translation.

Further analysis revealed that the appearance of DAP5/p86 is a common phenomenon to many cell death stimuli that involve activation of caspases, usually those that exhibit type I PCD characteristics. However, there are striking differences in the extent of the cleavage, and therefore in the amounts of DAP5/p86, resulting from distinct cell death triggers in different cell lines. For instance, treating SKW B-cell lymphoma cells with anti-Fas agonistic antibodies leads to the full cleavage of the endogenous DAP5/p97 into DAP5/p86 as early as 5 h following treatment. p53 activation gave rise to intermediate levels of cleavage.<sup>68</sup> Induction of DNA damage by agents such as etoposide, or general inhibition of kinase activity by the broad-range kinase inhibitor staurosporine in HeLa cells, resulted in moderate cleavage of DAP5/p97, and therefore in low amounts of DAP5/p86 as compared to the endogenous full-length protein. What is the physiological significance of these differences? While this issue has yet to be resolved, two explanations can be proposed. First, it is possible that the activity of DAP5/p86 is not linear with its expression levels. Therefore, even low levels of an activated DAP5 form are enough to achieve the desired functional effect on translation machinery under the stress conditions. Alternatively, it could be that while in certain systems caspase cleavage is the major activating step of DAP5/p97, in others, there may be additional post-translational modifications that activate DAP5/p97. Of note, DAP5 was rescued as a proapoptotic gene in a setting of cell death induced by IFN- $\gamma$  that is caspase independent. Thus, additional putative types of DAP5 modifications, which still need to be identified, must be dominant, particularly in caspase-independent cell death systems such as type II PCD, which involves autophagic cell death.

## Identification of DAP5 mRNA Targets

As previously discussed, apoptosis, like other extreme cellular stresses, impacts upon the translation apparatus of the cell. In the dying cell, the vast majority of translation events (primarily cap-dependent translation) are severely abrogated. However, a subset of mRNAs manages to escape this translational block and maintain their translation mostly via the cap-independent mechanism. Extensive work over the past several years revealed that this group comprise mRNAs encoding both proapoptotic proteins such as DAP5, c-Myc and Apaf-1, and antiapoptotic proteins such as XIAP and HIAP2, all of which harbor functional IRES elements within their 5'UTR, known as 'death IRESes'.<sup>38,42,48,69,70</sup> In theory, *de novo* synthesis of proapoptotic proteins provides positive feedback, reinforcing the death cascade. This might be especially important when dealing with short-lived protein products (as in the case of c-myc). Conversely, maintaining

translation of antiapoptotic proteins in the presence of a death trigger generates a negative feedback loop, which can postpone to some extent 'the point of no return' when the cell becomes fully committed to die.

The discovery of a novel apoptotic form – the DAP5/p86 – and its ability to stimulate translation from its own IRES in a cap-independent manner opened the door for testing whether it may stimulate translation from the other 'death IRESes', thus playing a role in the sustained translation of the IRES containing RNAs which participate in cell death. In our laboratory, we addressed this issue by coexpression studies using Apaf-1, c-myc, DAP5 and XIAP IRESes cloned within bicistronic reporter vectors (the dual luciferase vectors). These constructs were individually coexpressed with DAP5/p86 and the results were compared to the outcome of cotransfections with the unmodified DAP5/p97, which was tested in parallel.<sup>71</sup> It was found that overexpressing DAP5/p86 strongly enhanced translation via DAP5, c-Myc, Apaf-1 and XIAP-IRESes, whereas the full-length DAP5/p97 failed to stimulate these 'death IRESes'.<sup>71</sup> In parallel, Holcik and colleagues found that DAP5 and Apaf1 IRESes are stimulated by DAP5/p86, while the coexpression of these IRESes with DAP5/p97 did not induce their activity. Of note, in this latter study, DAP5/p86 failed to activate the c-myc and XIAP IRESes.<sup>72</sup> A third work, also from Holcik's group, showed that HIAP2 IRES is strongly stimulated by DAP5/p86.<sup>70</sup> While it is difficult to explain why in some settings DAP5/p86 did not enhance translation via the IRESes of c-myc or XIAP, the overall picture that emerges from these three studies is that an active role in supporting several 'death-IRES'-mediated translation can be attributed to the caspase-activated DAP5/p86 form.

It is important to emphasize that DAP5 is not the only member of the eIF4G family that undergoes caspase cleavage upon induction of apoptosis. As was mentioned in the introduction, it was documented that both eIF4GI and eIF4GII are processed by the executioner caspase-3.<sup>19–21,73,74</sup> A consequence of these cleavage events is the complete elimination of the full-length proteins, which may contribute to the sharp reduction in cap-dependent translation. Yet, the appearance of a single stable protein fragment corresponding to the middle portion of eIF4GI, designated eIF4GI M-FAG/p76, was also noticed<sup>19,74</sup> (Figure 2). Intriguingly, DAP5/p86 and eIF4GI M-FAG/p76 possess more common structural features than DAP5/p97 and full-length eIF4GI. Both of them include the minimal core region with eIF4A and eIF3 binding sites required for the initiation of translation, but lack the C-terminal Mnk1 binding site. In the case of eIF4GI M-FAG/p76, also the PABP and the second eIF4A binding sites are removed by caspases, which enhances the similarity between the two proteins. However, in contrary to DAP5, eIF4GI M-FAG/p76 still encompasses the binding site for eIF4E, and therefore its cap-binding capacity remains intact (Figure 2). Based on these comparisons, it became of interest to compare in the same experimental settings the functional activities of both DAP5/p86 and the M-FAG/p76, in activating the above-mentioned 'death IRESes'. In one study, it was shown that M-FAG/p76 had no statistically significant effect on DAP5, c-Myc, Apaf-1 or XIAP-IRESes under the same conditions where DAP5/p86 significantly

enhanced translation via these 'death IRESes'.<sup>71</sup> Another study found that DAP5 and Apaf1 IRESes were stimulated by eIF4G1 M-FAG/p76, yet, DAP5/p86-mediated enhancement of these IRES activities was two-fold higher than the M-FAG/p76-mediated one.<sup>72</sup> Thus, additional studies are needed to clarify whether cleaved products of eIF4G1/II may exert under some circumstances a similar role to that shown for DAP5/p86.

In conclusion, the current emerging data suggest that DAP5/p86 can support translation of both pro- and antiapoptotic mRNAs at the same time. This excludes a simplified model according to which the DAP5/p86 is committed only to translation of proapoptotic genes. Instead, there is obviously a more complex role for DAP5 in apoptosis, which relies on feeding into the cellular system both positive and negative mediators of apoptosis. Ultimately, the stronger arm of DAP5 in a particular cellular setting will define whether it acts in a pro- or antiapoptotic manner.

### The Phenotype of DAP5 Knockout Mice – A Critical Role for IRES-Mediated Translation in Early Developmental Stages?

A different insight into DAP5 function was provided from the analysis of DAP5/p97-deficient mice.<sup>75</sup> While heterozygous animals did not exhibit any abnormal phenotype, DAP5 homozygous deficiency was lethal at the E7.5 stage. Closer examination of DAP5<sup>-/-</sup> embryos revealed that they failed to develop properly; three primary organized germ layers were not formed and the mesoderm did not emerge from the epiblast. Based on these observations, it was postulated that DAP5/p97 is essential for gastrulation. Owing to the early embryonic lethality of the knockout animals, DAP5/p97 function was further studied in the ES cell system (ES cells). Interestingly, total protein synthesis was not affected in DAP5<sup>-/-</sup> ES cells. Moreover, activity of ectopically expressed DAP5, c-myc and EMCV IRESes was indistinguishable in DAP5<sup>+/-</sup> and DAP5<sup>-/-</sup> ES cells. At the phenotypic level, these cells lost their ability to undergo differentiation, at least in response to retinoic acid, and tended to remain as multipotent cells. This failure to differentiate was attributed by the authors to impaired transcription from retinoic acid-responsive elements in DAP5<sup>-/-</sup> ES cells.<sup>75</sup>

The novel DAP5 function in ES cell early differentiation is especially intriguing if one attributes the phenotype to translation deficiencies. So far, translational switches from cap-dependent to cap-independent translation at this early developmental stage were not discovered. It is therefore surprising that no effect of DAP5 deficiency was observed on the tested protein translation machinery. Yet, it should be noted that these translation-based studies were carried out in the multipotent ES cells rather than after retinoic acid treatment when the impact of loss of DAP5 is rate limiting. Perhaps only then DAP5, which is ubiquitously expressed in the ES cells, becomes activated by some yet unknown modifications. Thus, it is still possible that translation of IRES-harboring mRNAs specific and critical for embryonic development and early differentiation have been affected in

DAP5<sup>-/-</sup> cells. Obviously, additional data are required in order to understand the precise function/s of DAP5 in differentiation and to assess the molecular basis underlying the DAP5-deficient phenotype.

### Conclusions and Perspectives

Looking toward the future, an ultimate requirement for proceeding in the understanding of DAP5 function is the identification of as many target mRNAs as possible. Two different approaches can be undertaken to further resolve the identity of DAP5 target mRNAs. One direction comprises the biased approach of an 'educated guess', and the other involves high-throughput whole genome screens. An important recent achievement in our laboratory, which further shaped the strategy behind these two approaches, is our current ability to detect specific mRNAs in RNP complexes obtained by immunoprecipitation with anti-DAP5 antibodies. Proof of principle was provided by the fact that DAP5 mRNA could be identified by PCR amplification in these immunoprecipitates, while a nonrelevant mRNA such as SOD was not present (unpublished data). Based on these findings, it will be feasible to conduct the 'educated guess' approach by subjecting the population of cellular mRNAs present in the RNP complexes to RT-PCR analysis using specific primers against genes, which may be DAP5 targets. Targets may include candidates from the growing list of IRES-containing mRNA coding for proteins that may participate in type I or type II PCD. If the guess proves correct, then functional validations in cells, as previously done in the case of DAP5, XIAP, Apaf-1 and HIAP2 IRESes, must be performed. Obviously, the biased approach is limited since it is based on the individual researcher's subjective judgment, and is restricted to the mRNA candidates whose relevance to a certain system has been already documented. The alternative approach is to screen large populations of cellular mRNAs present in the DAP5 immune complexes in an unbiased manner, using cDNA gene arrays. Such whole genome screens make this approach useful for the identification of less obvious mRNA targets. High-throughput methods for identifying DAP5 mRNA targets may also broaden the spectrum of biological processes controlled by DAP5, in addition to PCD. For instance, some of the DAP5 mRNA targets relevant to the aforementioned early developmental stages may be discovered by this manner. Also, a collection of other unpredicted mRNA targets may highlight additional biological processes in which DAP5 might be involved in regulating switches from cap-dependent to cap-independent translation. In our laboratory, we had recently performed such high-throughput search for DAP5 targets and isolated a number of putative DAP5 target mRNAs, which are currently undergoing different validation steps.

To date, it is not known whether DAP5 protein binds directly to its target mRNAs, or whether the interaction that is detected by the RT-PCR experiments, occurs via accessory protein(s). Obviously, RNA binding assays should be performed with purified DAP5 protein, and in case of indirect interactions, the protein complexes at the IRES site should be further dissected by proteomic-based approaches.

Identifying a large repertoire of DAP5 targets in PCD is especially valuable for addressing more general questions such as the relative importance of an individual 'death IRES' to the apoptotic outcome. The answer to this question clearly requires a more complete list of 'death-IRESes' and an assessment of the necessity of each of these IRESes for the proper regulation/execution/completion of the apoptotic process. For instance, the necessity of the death IRES will be more apparent for proteins with a short half-life or those that are completely absent in growing cells and must be *de novo* synthesized after the exposure to the death stimulus. While so far less than a dozen mRNAs have been demonstrated to contain a 'death IRES' that enables them to maintain their translation under apoptotic conditions, we believe that we are dealing with a much wider phenomenon, with numerous 'death IRES'-regulated proteins awaiting identification.

Only after expansion of the IRES list will we be able to answer basic questions regarding their structural and functional properties. Will all IRES-harboring mRNAs act as one homogeneous group and maintain their translation in a similar manner under stress conditions? Will all 'death IRESes' be regulated by DAP5? For instance, Nevins *et al.*<sup>72</sup> presented conditions of etoposide-induced apoptosis in which translation through the proapoptotic DAP5 and Apaf-1 IRESes was stimulated, while that of XIAP and c-myc IRESes was not. Another study concluded that phosphorylation of eIF2 $\alpha$  can stimulate translation from the Cat-1 cellular IRES, but not from others such as those of Bip and Pim-1.<sup>54</sup> Recently, Warnakulasuriyachchi *et al.* demonstrated that IRES elements of an antiapoptotic HIAP2, which appeared as a novel DAP5/p86 target, was stimulated by ER stress, whereas XIAP IRES failed to do so.<sup>70</sup> Thus, it is clear that cellular IRESes do not act as one homogeneous group in stress conditions.

Another subject that remains to be addressed concerns the mechanisms by which DAP5 is activated. So far, caspase-driven production of DAP5/p86 is the only known modification that influences DAP5 function. Future structural analysis of the DAP5 protein, such as by solving its X-ray crystal structure, may reveal how the removal of the C-terminal tail activates this function of the protein to promote IRES-mediated translation. Yet, as was discussed previously, this mode of activation is restricted to type I PCD, in which caspase activation occurs. Taking into consideration that DAP5 was originally isolated as a mediator of IFN- $\gamma$ -induced cell death, which belongs to caspase-independent type II PCD, one can predict the existence of alternative post-translational modifications of DAP5 that serve to activate its function. These putative alterations should in one way or another mimic the functional outcome of the removal of the C-terminus of the protein and may consist of phosphorylations, Apobec-1-mediated RNA editing, and more.

Last but not least, the question regarding the function of the unmodified DAP5/p97 in normally growing somatic cells should be further addressed. One possibility is that DAP5/p97 is a silent, inert protein, in terms of translation activity, unless activated by the appropriate stimulus. The activating stimulus could be restricted to the modifications that occur exclusively under specific stress signals as discussed in this chapter, or may be further broadened in the future to other

biological processes, including even the possibility of phase-specific activation during the normal progression of cells along the cell cycle. Another possibility is that unmodified DAP5/p97 is not inert, but rather displays other translation-based functions in growing cells, other than the aforementioned IRES-dependent activations. More advanced functional read outs in reconstituted *in vitro* systems with purified components should promote our understanding in these directions.

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