

DAP-5 is involved in MycN/IFN γ -induced apoptosis in human neuroblastoma cells

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Abstract

Death associated protein-5 (DAP-5) is a ubiquitously expressed member of the translation initiation factor eIF4G family that lacks the eIF4E binding site. A dominant negative fragment of DAP-5 protects HeLa cells from IFN γ -induced cell death. By employing a functional approach we examined the role of DAP-5 in human neuroblastoma cells that are sensitized for IFN γ -induced apoptosis by tetracycline controlled *MYCN* expression. DAP-5 fragment transcribed at high levels and translated into a functional miniprotein of 28 kDa protected neuroblastoma cells from IFN γ -induced apoptosis. Reduced serum levels were toxic to cells constitutively expressing DAP-5 fragment suggesting that DAP-5 protein is essential for both viability and death of human neuroblastoma cells. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Apoptosis plays a central role in the development and homeostasis of multicellular organisms [1]. Cell suicide can be triggered by a wide range of challenges such as DNA damage, the loss of contact from neighboring cells that normally provide survival signals, the conflict of growth and differentiation signals or the activation of cell surface receptors that promote cell death [2]. Among the ligands of death-inducing receptors are CD95L (also called FasL or Apo1L), TNF and other cytokines [3].

The identification of apoptosis-related genes has been pursued by a number of experimental approaches,

one of which consists of a functional gene selection in HeLa cells that are continuously exposed to the cytokine IFN γ [4]. This ‘technical knock out’ (TKO) approach is based on the random inactivation of gene expression and results in a dominant selectable phenotype. Cells with reduced susceptibility to the death-inducing IFN γ signal survive and grow out into colonies [5]. Downregulation of death-promoting genes is achieved by transfecting the cells with a cDNA library that directs either a high level of antisense RNA expression or the expression of truncated sense fragments that can block the function or activation of corresponding proteins. The cDNA fragments from surviving cells can easily be rescued as the cDNA library is cloned into an Epstein–Barr virus (EBV)-based episomal shuttle vector that does not integrate into the host genome. Previous studies had resulted in the isolation of five novel genes, called DAP (death associated protein)

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genes. DAP proteins act in a diverse spectrum of biochemical pathways and include a cytoskeleton-associated calcium/calmodulin-dependent kinase, a small cytoplasmatic protein with so far unidentified function, a nucleotide binding protein and the novel translation initiation regulator DAP-5 [6]. *DAP-5* (synonym *p97*, *NAT-1*) encodes a 97 kDa protein that is highly homologous to translation initiation factor 4G (eIF4G1; p220), with an overall 27% identity and 48% similarity at the amino acid level [7].

Neuroblastoma is the most common solid tumor in young children [8]. A fascinating feature is the unusually high incidence of spontaneous regression in up to 10% of the tumors, in spite of metastatic tumor spread. It has been suggested that this clinical phenotype results from apoptosis [9,10]. The *MYCN* proto-oncogene is overexpressed in 20–25% of neuroblastoma, either consequent to amplification or by deregulation of gene expression or RNA/protein stabilization [11]. Previous studies had shown that *MYCN* under the control of the bacterial tetracycline repressor sensitizes human neuroblastoma cells for IFN γ - or cytostatic drug-mediated apoptosis [12,13]. Although programmed cell death is accompanied by changes in the expression pattern of a number of known apoptosis-related genes, little is known about the molecular mechanisms dictating apoptosis in human neuroblastoma cells. To examine the possible role of DAP-5 in apoptosis in neuroblastoma we have used the human neuroblastoma cell line Tet21N harboring the tetracycline inducible *MycN* gene [14].

2. Materials and methods

2.1. Plasmids

pPUR-EBNA-1 was generated through the insertion of a CMV-EBNA-1 gene (3.7 kb *PstI*-*Bam*HI fragment of p1327) into *EcoRI/NotI* digested pPUR selection vector (Clontech). To obtain pTKO-1 derivative pTKO-CZ- β Gal both the original hygromycin resistance gene and the SV40 promoter were replaced by a PCR-generated *HindIII-EcoRI* fragment of bleomycin gene (*ble^r*) and CMV promoter. The 763 bp DAP-5 fragment from pTKO-260 [7] was cloned into *HindIII/BglIII* digested pTKO-CZ- β Gal, resulting in plasmid pTKO-CZ-DAP-5.

2.2. Cell culture, transfections and selection procedures

Tet21N human neuroblastoma cells were cultured in RPMI1640 supplemented with 1, 5 or 10% fetal calf serum (FCS), 4 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 0.5 mg/ml amphotericin. Purified pPUR-EBNA-1 was transfected with Effectene Reagent (QIAGEN) into Tet21N cells [15]; the cells were selected with 2 μ g/ml puromycin (Clontech). Single clones were obtained by limiting dilution and tested for Epstein–Barr virus nuclear antigen-1 (EBNA-1) expression by Western blotting.

The resulting clone Tet21N-EBNA-1-3 and the original Tet21N cells were subsequently transiently transfected with pTKO-CZ- β Gal reporter plasmid, and the percentage of transfected cells was determined by 4-Cl-5-Br-3-indolyl- β -galactosidase (X-Gal) staining.

To generate polyclonal Tet21N-EBNA-1-3 populations expressing the 763 bp DAP-5 fragment or the β Gal reporter, subconfluent monolayers of 1×10^5 Tet21N-EBNA-1-3 cells were transfected with 7 μ g pTKO-CZ-DAP-5 or pTKO-CZ- β Gal (Effectene Reagent, QIAGEN). Stable clones were selected for bleomycin resistance the presence of 250 μ g/ml Zeocin TM (Invitrogen) and analyzed for expression of DAP-5 fragment by Northern and Western blotting. Cells were treated for 24 h with 1 μ g/ml tetracycline (Sigma) to switch exogenous *MycN* off (–*MycN*) or stimulated with recombinant human IFN γ at 1000 U/ml (Roche) when indicated (+IFN γ). The IFN γ -resistant phenotype of stably transfected DAP-5 or β Gal Tet21N-EBNA-1-3 cells was tested in media supplemented with 1, 5 and 10% FCS. While transiently DAP-5 fragment or β Gal transfected cells were split 1:4 2 days after transfection in six-well plates and selected with IFN γ (1000 U/ml, Roche) and Zeocin TM (250 μ g/ml, Invitrogen) over a period of 4 weeks in RPMI1640 supplemented with 10% FCS. Surviving cells were fixed with methanol and stained with Giemsa.

2.3. X-Gal staining

Forty-eight hours after transfection with pTKO-CZ- β Gal cells were fixed for 10 min in 3.7% formaldehyde, washed three times with phosphate-buffered

saline (PBS) and overlaid for 2 h with a histochemical reaction mixture containing 1 mg/ml X-Gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM MgCl₂ in PBS. Photography was done under phase-contrast and without phase-contrast microscopy with Kodak Ektachrome EPY 64T film.

2.4. Western blotting

Cell lysates were prepared [14], separated by 10% SDS-PAGE and blotted onto nitrocellulose

membranes (Schleicher and Schuell). Membranes were incubated with anti-EBNA-1 rat monoclonal antibody 1H1-1-4, kindly provided by W. Hammerschmidt (GSF, München), anti-MycN monoclonal antibody [16], or anti-DAP-5 rabbit polyclonal antibody [7]. Proteins were visualized using the ECL detection system (Amersham).

2.5. RNA preparation and Northern blotting

RNA was extracted by guanidinium thiocyanate

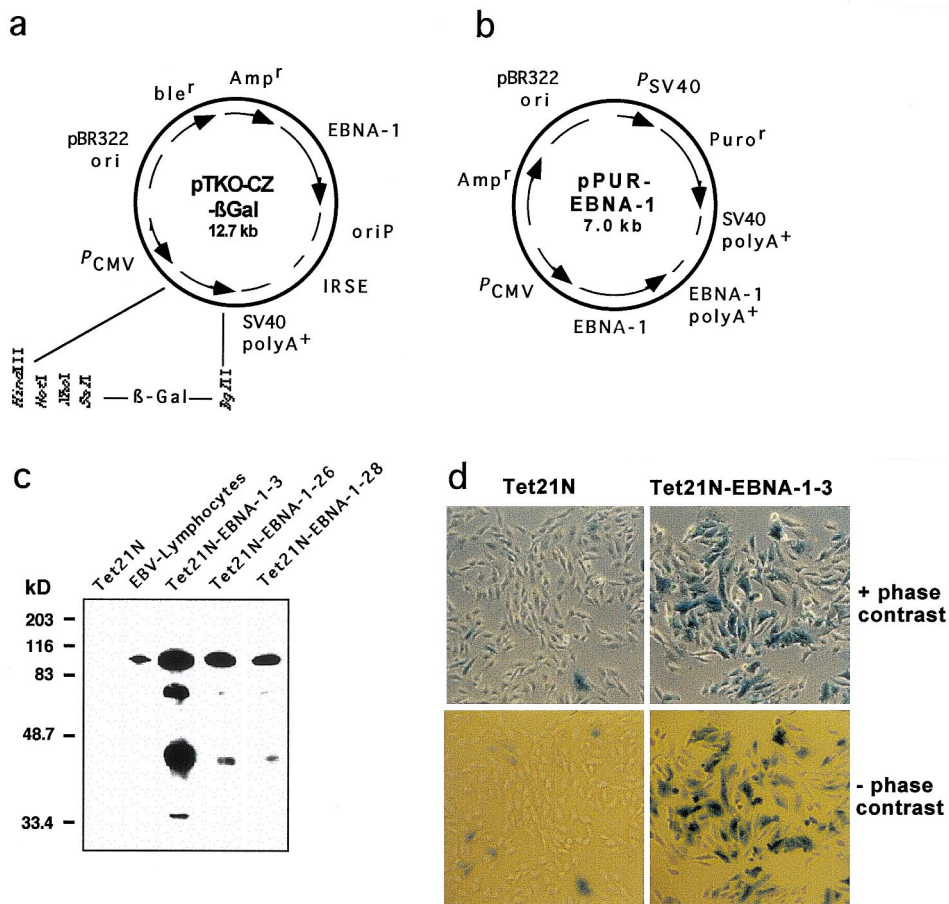


Fig. 1. High-efficiency transfection of episomal pTKO-CZ into Tet21N neuroblastoma cells. (a) pTKO-1 derivative pTKO-CZ-βGal. Both the original hygromycin resistance gene and the SV40 promoter were replaced by the bleomycin selection marker (ble^r) and CMV promoter, respectively. (b) Helper plasmid pPUR-EBNA-1. (c) Western Blot with lysates of Tet21N neuroblastoma cells stably transfected with pPUR-EBNA-1. Protein extracts (50 μg) from Tet21N-EBNA-1 clones were separated by 10% SDS-PAGE, blotted onto nitrocellulose and probed with monoclonal anti-EBNA-1 antibody. Tet21N cells served as a negative and EBV-transformed lymphocytes as a positive control for EBNA-1 expression. (d) Highly increased transfection efficiency of episomal pTKO-CZ-βGal in stably EBNA-1 expressing Tet21N-EBNA-1-3 cells. Tet21N and Tet21N-EBNA-1-3 cells were transfected with pTKO-CZ-βGal reporter, fixed and stained for βGal-activity 48 h after transfection. The percentage of transfected cells was determined by counting blue versus non-stained cells. (Upper) Phase-contrast photography of transfected cells; (lower) same picture without phase. Comparable results were obtained in three independent experiments.

[17]. Total RNA (20 μg) was fractionated through a 1% agarose-formaldehyde gel, blotted onto Hybond N⁺ nylon filters (Amersham) and hybridized with randomly primed ³²P-labeled DAP-5 fragment and β -actin probes. Hybridization and washing conditions were as according to the protocol of the manufacturer (Hybond N⁺, Amersham).

3. Results and discussion

3.1. High-efficiency transfection of episomal pTKO-CZ- β Gal in stably EBNA-1 expressing neuroblastoma cells

To adapt the functional TKO system to the neuroblastoma cell line Tet21N the cDNA vector governing insert expression [5] was modified. The SV40 promoter was replaced by a CMV promoter and the hygromycin selection gene was substituted by a bleomycin gene (pTKO-CZ- β Gal, Fig. 1a). Transient transfection with a β -galactosidase reporter (pTKO-CZ- β Gal) revealed an efficiency of only 5–10% of the cells. This would not be sufficient to transfer an entire cDNA library into a manageable population of cells.

To increase the transfection efficiency we made use of the observation that cells constitutively expressing EBNA-1 are highly permissive for transfection with EBV-derived episomal vectors [18,19]. For the generation of EBNA-1 expressing cells, the CMV-EBNA-1 expression cassette from p1327 was subcloned into pPUR (Fig. 1b). The resulting pPUR-EBNA-1 was introduced into Tet21N cells; stable clones were selected and examined by Western blot for expression of EBNA-1 protein. Clone Tet21N-EBNA-1-3 expressed EBNA-1 at the highest level (Fig. 1c) and was chosen for further experiments. Transient transfection efficiency, as determined with pTKO-CZ- β Gal, was 70% in these EBNA-1 expressing cells (Fig. 1d).

3.2. DAP-5 fragment is expressed at high levels and protects Tet21N-EBNA-1-3 neuroblastoma cells from IFN γ -induced apoptosis

To examine the possible role of DAP-5 in programmed cell death in neuroblastoma a sense fragment was cloned into pTKO-CZ. The fragment consists of 763 bp from 3.8 kb full-length DAP-5 and protects HeLa cells weakly from apoptosis through the

translation of a functional mini-protein [7]. Tet21N-EBNA-1-3 cells were transfected with pTKO-CZ-DAP-5 and selected from day 2 till day 30 for bleomycin resistance in the presence of IFN γ . Staining of colonies revealed 60% more clones for DAP-5 transfected cells compared to controls (Fig. 2), showing a protective effect of DAP-5 fragment from apoptosis.

The level of protection is in agreement with that in HeLa cells where expression of the same cDNA fragment has dual effects: while low expression levels weakly protected the cells from programmed cell death, high expression levels were toxic and prohibited continuous cell growth [20]. The transfected Tet21N-EBNA-1 cells express high levels of both endogenous full-length and exogenous DAP-5 fragment (Fig. 3a).

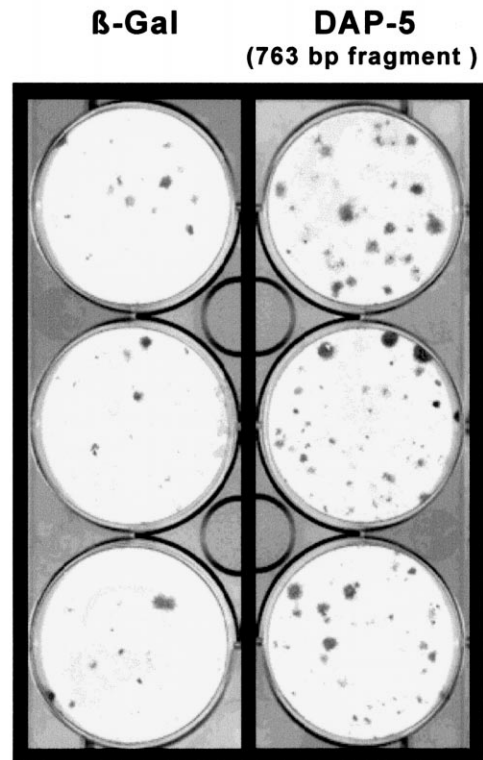


Fig. 2. pTKO-CZ-DAP-5 fragment confers protection against apoptosis. Tet21N-EBNA-1 cells were transfected with either TKO-CZ-DAP-5 (DAP-5) or pTKO-CZ- β Gal (β Gal). Two days after transfection cells were split in six-well plates and selected with IFN γ (1000 U/ml) and Zeocin TM (250 $\mu\text{g}/\text{ml}$) over a period of 4 weeks in RPMI1640 supplemented with 10% FCS. Surviving cells were fixed with methanol and stained with Giemsa. The figure shows representative data of four independent experiments.

The lack of toxicity of high DAP-5 fragment expression in neuroblastoma cells growing in medium with 10% FCS is in contrast to HeLa cells. Under reduced serum concentrations (1–5% FCS), however, DAP-5 fragment stably transfected cells were less viable than control cells expressing β -galactosidase (Fig. 4), suggesting that under stress conditions cell growth in neuroblastoma also depends on the expression level of DAP-5. In agreement, the cells were protected from apoptosis only when IFN γ was present immediately after transfection of DAP-5 fragment but not if already stable clones were selected (Fig. 4). Because copy numbers of episomal vectors vary directly after transfection and are stabilized during selection for stable clones [21–23], immediately after transfection the cells show a broad range of gene expression. As only a well-defined expression level of DAP-5 fragment

appears to protect cells from IFN γ -induced apoptosis, the selection of survival-promoting clones is more probable in the heterogeneous population of transiently transfected cells.

Approximately 0.1% of Tet21N cells transfected with DAP-5 fragment survived long-term IFN γ treatment (Fig. 2), which is comparable to the 0.1–1.0% surviving clones in HeLa [7]. Thus, DAP-5 fragment can protect neuroblastoma as well as HeLa cells from IFN γ -induced apoptosis. Although the IFN γ -resistant phenotype in neuroblastoma is more complex, the mechanism that protects cells from IFN γ -induced apoptosis seems to be similar to that in HeLa cells and is mediated by the translation of a functional mini-protein which probably competes directly with full-length DAP-5 protein [20]. This exogenous miniprotein of 28 kDa was translated as well as the endogenous

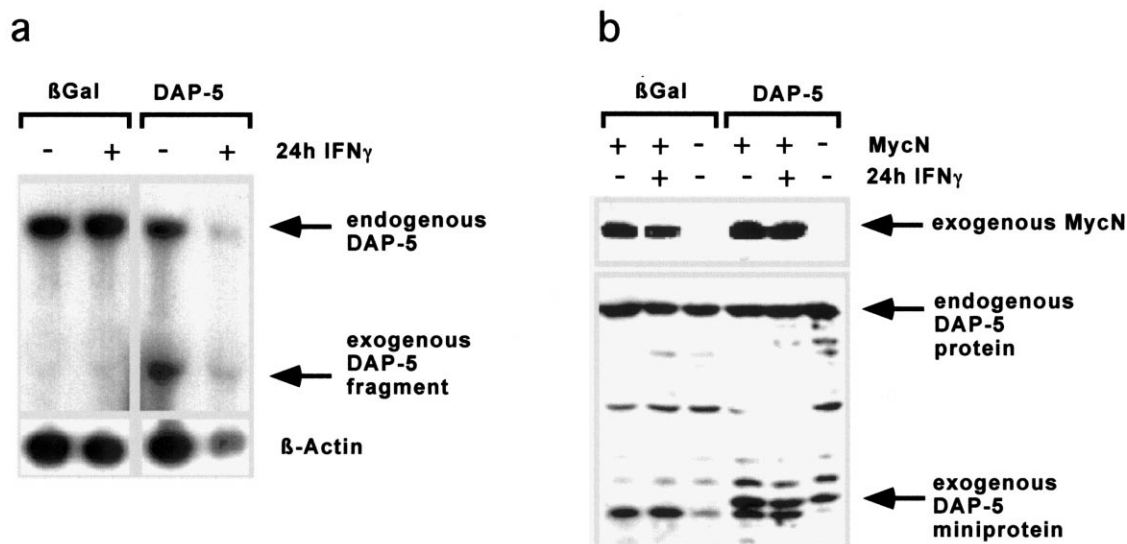


Fig. 3. High expression of DAP-5 fragment in stably transfected neuroblastoma cells. The Tet21N-EBNA-1-3 cell line was transfected with either pTKO-CZ-DAP-5 (DAP-5) or pTKO-CZ- β Gal control plasmid (β Gal). (a) Northern blot monitoring high exogenous DAP-5 expression exclusively in DAP-5 fragment but not in β Gal transfected cells. Total RNA was extracted prior to (-) or after (+) 24 h IFN γ selection (1000 U/ml) from stably transfected cells that were cultured in medium containing 10% FCS. Total RNA (20 μ g) was fractionated through a formaldehyde-containing agarose gel, blotted onto nylon filters and hybridized with 32 P-labelled DAP-5 fragment and β -actin probe as loading control. Endogenous and exogenous DAP-5 expression was not further influenced by IFN γ treatment as, judging by the β -actin signal, differences in expression are caused by a loading artifact. (b) Immunoblot with lysates of DAP-5 fragment (DAP-5) or control plasmid (β Gal) stably transfected Tet21N neuroblastoma cells. Lysates of untreated (+MycN, -IFN γ), IFN γ (+MycN, +IFN γ) or tetracycline (-MycN, -IFN γ) stimulated cells were analyzed for the presence of MycN and DAP-5 expression. Protein (30 μ g) was separated by 10% SDS-PAGE and blotted onto nitrocellulose membranes, which were incubated with a 1:500 dilution of polyclonal anti-DAP-5 antibody or a 1:1000 dilution of monoclonal anti-MycN-antibody. Exogenous DAP-5 miniprotein of 28 kDa is exclusively translated in DAP-5 fragment transfected cells while endogenous 97 kDa full-length protein is abundant in both cell populations. Additional bands are caused by non-specific binding of polyclonal anti-DAP-5 antibody. Expression of endogenous DAP-5 and DAP-5 miniprotein was not affected by downregulation of MycN expression (-MycN, -IFN γ).

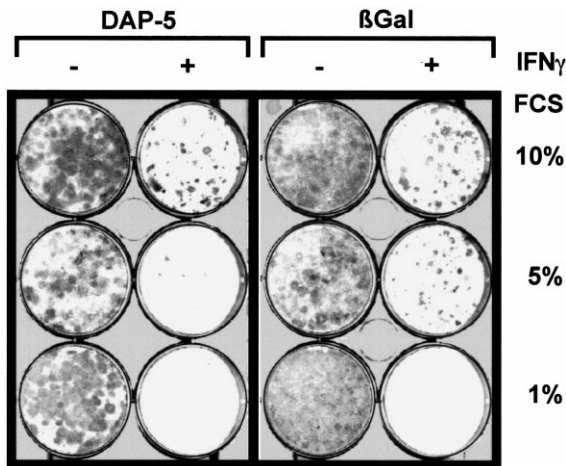


Fig. 4. High-level expression of DAP-5 fragment prohibits well growth in low serum concentrations. Tet21N-EBNA-1 cells were transfected with pTKO-CZ-DAP-5 (DAP-5) or pTKO-CZ- β Gal (β Gal); stable clones were selected over a period of 2 weeks in RPMI1640 containing 250 μ g/ml Zeocin TM and 10% FCS. Stable clones were subsequently cultured in RPMI1640 supplemented with 1, 5 or 10% FCS and 1000 U/ml IFN γ (+) or without cytokine treatment (-).

full-length protein at high levels in DAP-5 fragment stably transfected Tet21N neuroblastoma cells (Fig. 3b).

3.3. DAP-5 protein is not cleaved into a truncated 86 kDa form during IFN γ -induced apoptosis

Endogenous DAP-5 protein consists of 907 amino acids and is highly homologous to translation initiation factor eIF4G. eIF4G is the central organizing protein that directs ribosomes to the cap-site of mRNA by interacting with several translation initiation factors. A binding site in the N-terminal part of eIF4G recruits the cap-binding protein eIF4E while binding sites in the central part assemble the bidirectional helicase eIF4A and the component of the 43s initiation complex eIF3 [24,25]. DAP-5 protein lacks the N-terminal part of eIF4G that is responsible for the association with the cap-binding protein eIF4E while it binds to eIF4A and eIF3 [24].

Opposing biochemical functions of DAP-5 have been predicted. One model suggests that DAP-5 functions as a general repressor of translation by titrating eIF4A and eIF3 out from cells. This repressor hypoth-

esis is based on the finding that DAP-5 overexpression suppressed cap-dependent and -independent translation in HeLa cells [24]. Another model suggests that DAP-5, which was reported to be ubiquitously and abundantly expressed in normal tissues and cell lines [7,24,26], is modified during apoptosis or other stress conditions in an active form that promotes cell death [6]. Recently, two mechanisms regulating DAP-5 on post-transcriptional levels have been reported: in response to Fas- or p53-mediated apoptosis DAP-5 was shown to be cleaved at a caspase cleavage site, yielding a C-terminal truncated 86 kDa protein that is capable of generating complexes with eIF4A and eIF3 translation initiation factors. DAP-5 and its truncated 86 kDa form were further shown to mediate cap-independent translation from an internal ribosome entry site (IRES) element that was identified in the 5'-untranslated region (5'-UTR) of the DAP-5 gene [27]. These data suggest that DAP-5 can serve as a caspase-activated translation factor which mediates cap-independent translation at least from its own IRES. But even if DAP-5 is translationally and post-translationally modified during Fas- and p53-induced apoptosis, so far no post-transcriptional activation of the protein has been demonstrated in IFN γ -induced apoptosis. Although the protein is essential for both cell viability and IFN γ -induced apoptosis in the HeLa and the Tet21N neuroblastoma in vitro system, DAP-5 cleavage was not detectable in IFN γ -stimulated neuroblastoma cells (Fig. 3b). This suggests an alternative regulatory mechanism of DAP-5 function besides the activation by caspase cleavage.

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After this manuscript had been submitted Yamanaka et al. published that DAP-5 plays an essential role in embryogenesis and that DAP-5 $^{-/-}$ embryonic stem cells are resistant to differentiation induced by retinoic

acid (RA) [28]. RA is known to affect cell growth of most neuroblastoma by inducing different degrees of either apoptosis or differentiation [29]. Further studies may elucidate if the RA and IFN γ induced maturation of neuroblastoma is mediated through DAP-5 activation.

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