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AATF, a novel transcription factor that interacts with Dlk/ZIP kinase and interferes with apoptosis[1]

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Abstract Dlk, also known as ZIP kinase, is a serine/threonine kinase that is tightly associated with nuclear structures. Under certain conditions, which require cytoplasmic localization, Dlk can induce apoptosis. In search for interaction partners that might serve as regulators or targets of this kinase we identified apoptosis antagonizing transcription factor (AATF), a nuclear phosphoprotein of 523 amino acids. The 1.8 kb mRNA seems to be ubiquitously expressed. AATF contains an extremely acidic domain and a putative leucine zipper characteristic of transcription factors. Indeed, a Gal4-BD-AATF fusion protein exhibited strong transactivation activity. Interestingly, AATF interfered with Dlk-induced apoptosis.

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Key words: Dlk/ZIP kinase; Two-hybrid screen; Transcription factor; Acidic domain; Apoptosis; Par-4

1. Introduction

Phosphorylation of proteins is a regulatory tool perhaps most commonly used in eukaryotic cells. The number of protein kinases that are recognized as members of signal transduction cascades or as key regulators of processes such as cell cycle progression, transcription, replication, mitosis, apoptosis, metabolic processes, etc. is steadily increasing. We have recently cloned a novel kinase gene which, due to its homology to DAP kinase was named Dlk, for DAP like kinase [1]. Dlk was independently isolated as an interaction partner of ATF-4 [2], a transcription factor of the ATF/CREB family [3]. The new kinase contains a leucine zipper, that mediates homo- and hetero-dimerization, therefore, it was named zipper interacting protein (ZIP) kinase [2]. Dlk (ZIP kinase) is tightly associated with speckle like nuclear structures some of which overlap with PML bodies [4]. These latter structures have been implicated in transcription and regulation of apoptosis (reviewed in [5]). Dlk exhibits autophosphorylation and phosphorylates histones H3 and H4 as well as myosin light chain in vitro. Together, these findings suggested a role of Dlk in transcription. On the other hand, the homology to DAP kinase, which is involved in interferon γ-induced cell death [6] suggested a role in apoptosis. Indeed, under certain conditions which require cytoplasmic localization and association with actin filaments Dlk can induce apoptosis [2,4,7]. This can be achieved by interaction with Par-4 (prostate apoptosis response gene 4) [8], or by C-terminal truncation [4]. However, both the function of Dlk in the nucleus and the condition and mechanism by which it induces apoptosis remain to be elucidated.

Apoptosis plays an important role in morphogenesis, tissue homeostasis, and protection against pathological agents and stress conditions. Apoptosis can be induced by extracellular signals such as Fas ligand, TNFα, interferon, etc. or by intracellular signals arising from DNA damage, hypoxia or oxidative stress, oncogenic activation, and other stress situations. These signals are either translated into genetic activity, by inducing or repressing pro- or anti-apoptotic genes, respectively, or they are directly translated into apoptotic activity resulting in activation of caspases and nucleases, membrane blebbing, chromatin condensation and fragmentation, etc. (reviewed in [9]). The decision over cell survival or death appears to result from a sensitive balance between antagonistically acting factors such as Bcl-2 and Bax [10].

In search for interaction partners of Dlk that might function as upstream regulators or as downstream targets we employed the yeast two-hybrid system [11] and succeeded in isolating several cDNA clones. Four of these encoded known proteins, ATF-4, Par-4, tropomyosin α and Rabin3 [7]. One clone encoded an unknown protein of 73 kDa which is a nuclear phosphoprotein and exhibits strong transactivation activity. Interestingly, this protein can antagonize Dlk-induced apoptosis. Therefore, it was named AATF, for apoptosis antagonizing transcription factor.

2. Materials and methods

2.1. Cell lines

Rat embryo fibroblasts line REF52.2 were used for expression studies. Cells were grown as monolayers in Dulbecco's minimal essential medium (Gibco-BRL, Eggenstein, Germany) supplemented with 10% fetal bovine serum (Biochrome Seromed, Berlin, Germany) and antibiotics; insect SF9 (*Spodoptera frugiperda*) cells were used for the baculovirus expression system and grown in serum free SF900 medium containing 50μg/ml gentamicin (Gibco-BRL); *Saccharomyces cerevisiae* strain Y190 was employed in the two-hybrid system.

2.2. Expression plasmids

Plasmid pEGFP-C1-Dlk and pKEX-HA-Dlk encoding GFP- or HA-tagged Dlk fusion proteins or deletion mutants thereof were previously described [1,4]. The corresponding AATF expression constructs pEGFP-C1-AATF and deletion mutants thereof were generated accordingly. Molecular biological techniques followed standard protocols [12,13].

2.3. Two-hybrid screening

The HybriZAP version (Stratagene, La Jolla, CA, USA) of the two-hybrid-system [11,14] has been employed as described [7]. Briefly, the coding region of Dlk [1] was fused to the Gal4 DNA binding domain (BD) to be used as bait, and a cDNA library from SV40-transformed rat cells was fused to the GAL4 transactivation domain (AD) to be

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[1] Accession no. for rat AATF nucleotide sequence at the EMBL GenBank database is RNO238717.

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screened for interaction partners. Yeast Y190 cells were sequentially transformed with pBD-GAL4 Dlk and the pAD-GAL4 cDNA library plasmids, His^+ clones were isolated and further assayed for β-galactosidase activity. Positive clones were confirmed by retransformation, the pAD-GAL4 cDNA plasmids were recovered, amplified in *E. coli* and sequenced using the T7 Sequencing kit (Amersham Pharmacia Biotech, Freiburg, Germany). Complete sequence analysis was performed on both strands by MWG Biotech (Munich, Germany).

2.4. In vitro translation

For in vitro translation the cDNAs isolated as pAD-GAL4 fusion gene were PCR-amplified with primers 5′-CCA AAC CCA AAA ATG GAG ATC GAA TTC-3′and 5′-CTA GAG TCG ACC CGG GCT CGA-3′, thereby providing a start codon 5′ of the open reading frame, and cloned into the *Sma*I-site of pBluescript SK+. In vitro translation was performed in a coupled transcription/translation system (TNT-T7/T3 Coupled Reticulocyte Translation System, Promega, Heidelberg, Germany).

2.5. Expression and purification of His-tagged recombinant proteins from baculovirus-infected SF9 cells

Recombinant baculoviruses encoding histidine-tagged wild-type or mutant forms of Dlk or AATF were generated and propagated as described [15]. Cell extracts were prepared by sequential extraction with isotonic lysis buffer (PLB) and high salt RIPA buffer [1]; His-tagged proteins were purified by affinity chromatography on Ni-NTA agarose (Qiagen, Hilden, Germany) essentially as described [1]. For binding studies, His-Dlk was first bound to Ni-NTA-agarose, the beads were washed five times with IMAC-50 (20 mM Tris-HCl pH 8, 0.5 M NaCl, 10% glycerol, 1 mM PMSF, 5 mM β-ME, containing 50 mM imidazole) and once with PLB, in vitro translated and [^{35}S]methionine-labeled AATF was added and incubated at 4°C for 2 h. The beads were further washed, bound proteins were eluted and analyzed by SDS-PAGE and detected by fluorography.

2.6. Kinase assays

Kinase assays were performed with purified His-tagged Dlk or AATF in kinase buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM $MnCl_2$, 1 mM DTT, 1 mM PMSF, and 1 μCi [^{32}P]γATP or [^{32}P]γGTP (specific activity 3000 Ci/mmol, Amersham Pharmacia Biotech) in 10 μl reactions at 37°C for 30 min [1]. Reactions were stopped by 2×SDS-PAGE sample buffer containing 10 mM EDTA and subjected to SDS-PAGE and autoradiography.

2.7. Transfection and immunofluorescence analyses

REF52.2 cells were plated on coverslips and transfected with 300 ng of respective expression vectors with Lipofectamine (Gibco BRL) for 2 h. In co-transfections one of the protein partners was expressed as GFP-fusion protein and the other one as HA-tagged protein. The cells were incubated for 24 h and inspected directly for GFP fluorescence or stained with anti-Par-4 [8] or anti-HA antibodies (Roche Diagnostics, Mannheim, Germany) for indirect immunofluorescence. Briefly, cells were washed two times with phosphate buffered saline (PBS), fixed with 2% paraformaldehyde in PBS for 15 min, washed again 2 times with PBS and permeabilized with 0.2% Triton X-100 in PBS for 5 min. Cells were washed again, blocked for 30 min with 5% dry milk, 0.1% Triton X-100 in PBS and then incubated with monoclonal anti-HA antibody (1:500) for 1 h and, after washing, with goat anti-mouse Cy3-conjugated secondary antibody (Dianova, Hamburg, Germany) (1:500) for 30 min. DAPI staining of nuclei was performed after fixation and permeabilization of cells with 1 μg/ml of 4,6-diamidino-2-phenylindole (DAPI) for 15 min and subsequently washed as described above. The subcellular distribution of expressed proteins or morphological changes of nuclei were analyzed by fluorescence microscopy with an Axioplan fluorescence microscope (Zeiss).

3. Results

A two-hybrid screen for Dlk interaction partners revealed four clones encoding known proteins [7] and one clone representing an unknown cDNA. Sequence analysis of this cDNA revealed 1796 bp with a continuous open reading frame of 1692 bp translating into a polypeptide of 564 residues. There

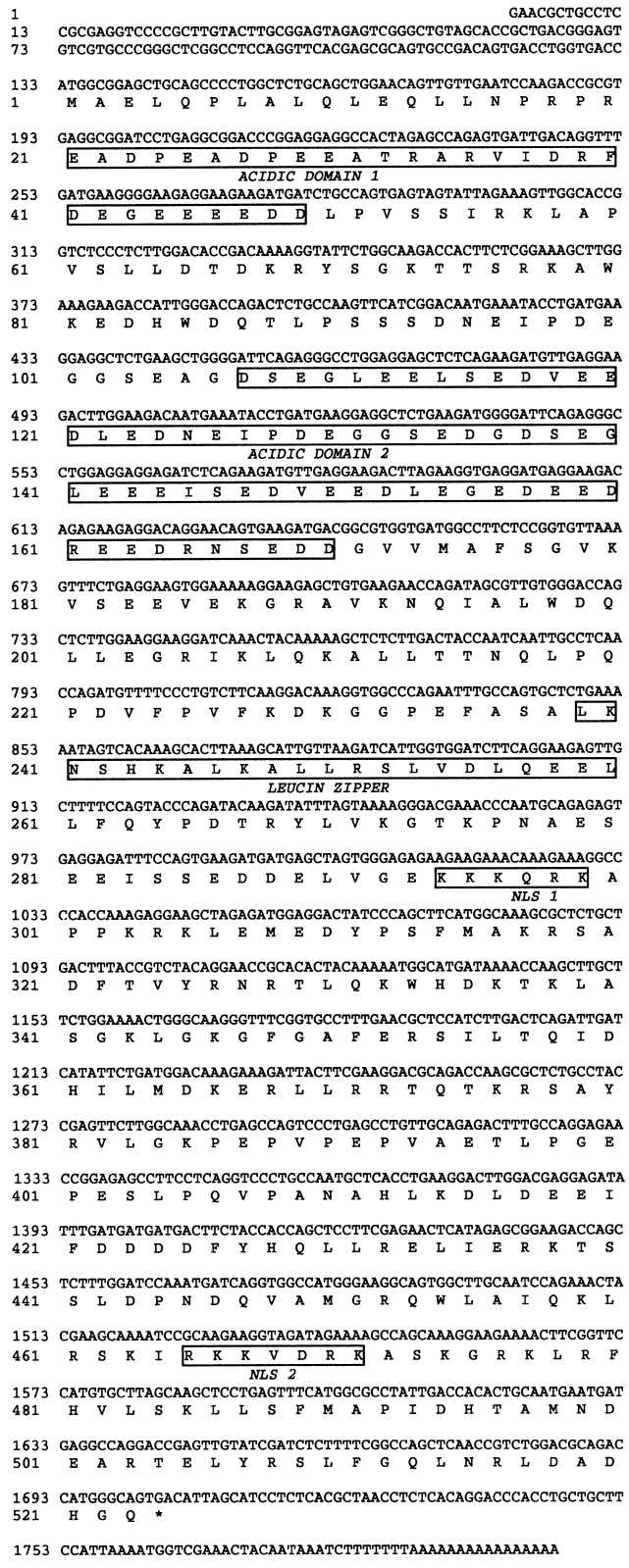

Fig. 1. Nucleotide and amino acid sequences of AATF. The following features are indicated: acidic domains 1 and 2, leucine zipper, NLS 1 and 2.

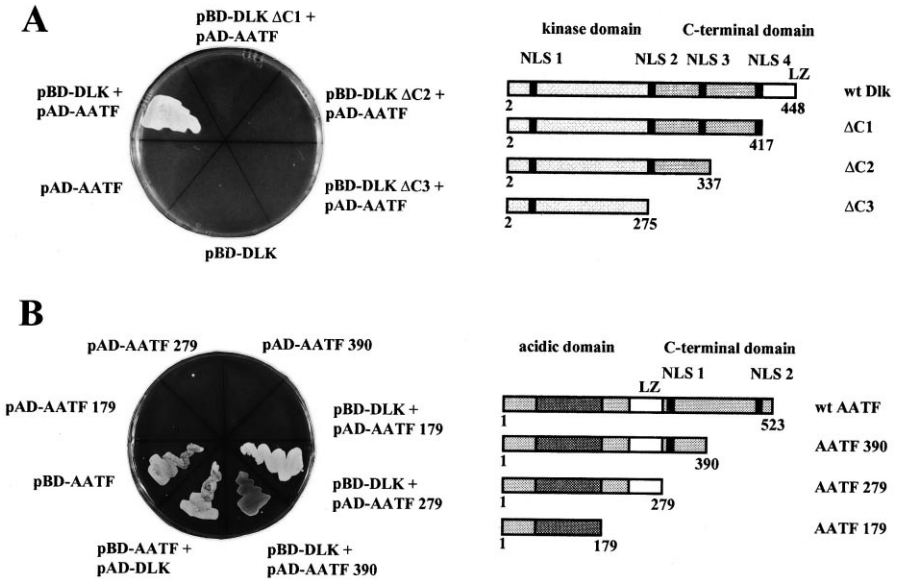

Fig. 2. Deletion mapping of the interaction domains between Dlk and AATF and test for transactivation using the two-hybrid system. Circles on the left show segments where yeast cells transfected with the indicated expression constructs were plated. A: Full length Gal4-AD-AATF was cotransfected with Gal4-BD-Dlk variants; B: Full length Dlk was co-expressed with AATF variants; the segment with pBD-AATF transformed cells demonstrates transactivation activity of AATF. Schemes on the right show the extensions of the deletion mutants used.

were two small stretches showing partial homology to human zinc finger protein ZNF76 [16] (33 of 44 bp) and neural zinc finger factor 1 (NZF-1 [17]) (82 of 134 bp). The residual sequence did not exhibit significant homology to known proteins.

As a matter of principle, the two-hybrid system reveals cDNAs that are translated in frame with the Gal4-activation domain. Thus, we assumed that the 5′ end of the cDNA including the initiation codon was missing. Northern blot analysis with a cDNA probe revealed a single mRNA of about 1800 bp (not shown) indicating that our cDNA clone was almost complete. Screening of a λ-ZAP cDNA library revealed several positive plaques with identical inserts of 1800 bp. Sequence analysis revealed a 5′ extension of only 9 bp with respect to the two-hybrid cDNA, thereby extending the open reading frame to the very 5′ end, without an initiation codon, however. Rather, there was an in frame methionine codon at nucleotide +133, corresponding to triplet 45 (with respect to the λ cDNA clone), which fulfilled the requirements of an eukaryotic initiation codon. In vitro translation of the cDNA with and without an artificial initiation codon at the 5′ end (see Section 2) revealed products of 76 and 73 kDa, respectively (not shown) indicating that naturally, initiation occurred indeed at codon 45 of the open reading frame. Accordingly, the protein product consists of 523 residues. We called this protein AATF. The complete amino acid sequence is shown in Fig. 1. The protein can be roughly divided into two domains, an N-terminal acidic domain and a relatively basic domain in the C-terminus. The acidic domain contains two highly acidic stretches from residues 20–49 and 107–170 which are separated by a Ser/Thr-rich domain. Secondary structure calculations predict mostly α helical structures. There is a putative leucine zipper between residues 239 and 260, putative nuclear localization signals (NLS) around residues 300 and 467, and several putative phosphorylation sites for protein kinases CKII, PKA and PKC.

To determine the interaction domains of Dlk and AATF a series of deletion mutants was generated and employed as Gal4-fusion proteins in the two-hybrid interaction assay. The Dlk deletion mutants included deletion of the leucine zipper (ΔC1), deletion of an Arg-rich domain (ΔC2) and deletion of the complete extra kinase domain (ΔC3) (see Fig. 2A). Likewise, in AATF we sequentially deleted from the carboxy terminus NLS2, NLS1, and the putative leucine zipper (see Fig. 2B). Full length AATF exhibited a functional interaction only with full length Dlk, but not with mutants lacking the leucine zipper (Fig. 2A). On the other hand, full length Dlk interacted with full length AATF as well as with deletion mutants terminating at residue 390 or 279 but not at 179 lacking the putative leucine zipper (Fig. 2B). These results

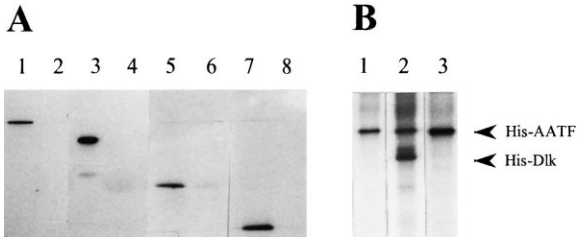

Fig. 3. A: In vitro binding of AATF to Dlk. cDNAs encoding full length or deletion mutants of AATF were translated and labeled with [^{35}S]methionine in vitro and allowed to bind to His-tagged Dlk immobilized to Ni-NTA agarose as described under Section 2. Please note: the doublets 1, 2 and 3, 4 show binding of full length AATF or deletion mutant AATF390 to Dlk versus control beads, respectively, while the doublets 5, 6 and 7, 8 show translation products AATF279 and AATF179 prior and after binding to Dlk-Ni-NTA agarose, respectively (see Fig. 2B for deletion constructs). B: Phosphorylation of AATF in vitro. His-tagged AATF was expressed in the baculovirus system, purified via Ni-NTA agarose and subjected to a kinase reaction in the absence (lanes 1 and 3) or presence (lane 2) of Dlk and [^{32}P]γATP (lanes 1 and 2) or [^{32}P]γGTP (lane 3) as phosphate donor.

suggested that interaction of these proteins occurred via their leucine zippers.

Complex formation between Dlk and AATF was verified by in vitro binding studies. To this end, the cDNA of full length and truncated forms of AATF were translated in vitro and tested for binding to His-tagged Dlk that was bound to Ni-NTA agarose and used as affinity matrix. As shown in Fig. 3A, full length and deletion mutant AATF390 bound specifically to Dlk (lanes 1 and 3) but not to control beads (lanes 2 and 4). Mutant AATF179 did not bind, as expected (lane 8). The lack of binding of mutant 1–279 (lane 6) (which was positive in the two-hybrid assay) may be explained by conformational restraints that may not be present in the Gal4-fusion proteins.

To test whether AATF was a substrate of Dlk, His-tagged AATF was expressed in the baculovirus system, purified on Ni-NTA agarose and employed in kinase reactions using purified His-tagged Dlk as kinase. Surprisingly, AATF was phosphorylated to a similar extent whether Dlk was present or not (Fig. 3B, lanes 1 and 2) suggesting that AATF was phosphorylated by an associated kinase rather than by Dlk. Since AATF is a rather acidic protein and contains eight potential CKII phosphorylation sites we considered that it was phosphorylated by CKII. Indeed, when the kinase reaction was carried out in the presence of GTP, which is indicative of CKII activity, AATF was phosphorylated to the same extent as with ATP (Fig. 3B, lane 3).

Expression analyses may reveal hints as to whether a protein might perform tissue-specific functions. A multiple tissue mRNA blot containing transcripts from brain, heart, skeletal muscle, lung, liver, spleen, kidney and testis of adult rat was hybridized with labeled cDNA probe. This experiment revealed ubiquitous expression of AATF with little quantitative variation (data not shown). Thus, AATF may perform a general function.

The acidic domain and the putative NLS sequences suggested that AATF might act as transcription factor. Expression as GFP-AATF fusion protein revealed a diffuse distribution in the nucleus in virtually all of the cells. (not shown).

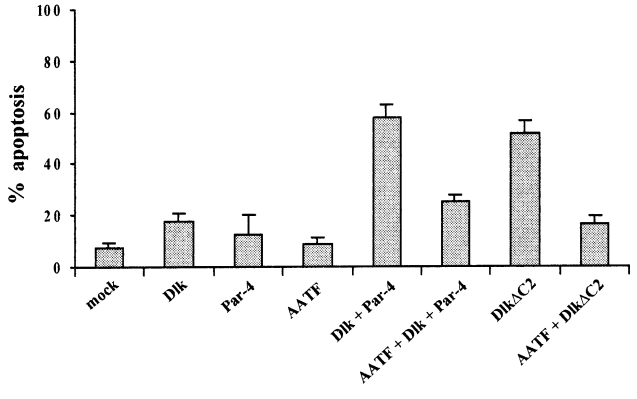

Fig. 4. AATF interferes with Dlk/Par-4-induced apoptosis. REF52 cells were transfected with expression plasmids encoding GFP-Dlk, GFP-DlkΔC2, Par-4, and/or GFP-AATF, as indicated. In co-expression experiments, GFP- or HA-tagged versions were employed to allow discrimination of the respective proteins. After 24 h, 100 GFP-positive cells were stained as described under Section 2 and scored for characteristic apoptotic alterations as judged by membrane blebbing, chromatin condensation and nuclear fragmentation. All transfections were performed in triplicate.

Only rarely did we observe AATF in speckles as seen for Dlk [1,4]. Surprisingly, co-expression of AATF with Dlk resulted in a diffuse distribution of Dlk rather than relocation of AATF to speckles suggesting that AATF interfered with speckle association of Dlk.

To test for transcriptional activity, AATF was fused to the Gal4 DNA binding domain and tested for activation of Gal4 responsive promoters (His and β-galactosidase) in yeast cells. The His auxotroph yeast strain employed in the two-hybrid system was transformed with the Gal4-BD-AATF fusion construct and assayed for His autotrophy and β-galactosidase activity. Indeed, this fusion construct behaved as a potent transactivator (see Fig. 2B).

We next asked whether AATF would influence one of the known functions of Dlk, induction of apoptosis. As mentioned above, Dlk and its interaction partner Par-4 seem to cooperate in apoptosis. Neither protein can induce apoptosis on its own. But co-expression of Dlk and Par-4 causes cytoplasmic retention of Dlk, association with actin filaments, and efficient induction of apoptosis [7]. However, when Dlk and Par-4 were co-expressed with AATF, apoptosis was greatly reduced (Fig. 4). Similarly, when the Dlk mutant ΔC2, which due to its cytoplasmic localization is a potent inducer of apoptosis [4], was co-expressed with AATF, apoptosis was greatly inhibited. Thus, AATF seems to antagonize the apoptotic function of Dlk.

4. Discussion

In search for interaction partners of Dlk we identified a novel transcription factor, AATF. The interaction with Dlk was verified by in vitro binding and the interaction domains were mapped to a small region containing the leucine zipper. Known transcription factors with leucine zippers appear to act as homo- or hetero-dimers. Thus, AATF might interact with other transcription factors, too. However, whether AATF contains a bona fide leucine zipper has to be experimentally confirmed. One peculiarity of AATF is its highly acidic domain consisting of two stretches with 50 or even 59% acidic residues. This high degree of acidity is surpassed only by NZF-1 which has an acidic domain of 85 residues with 80% Glu or Asp [17]. It is this acidic domain which is homologous between AATF and NZF-1. Such acidic domains of transcription factors might participate in chromatin remodelling as shown recently for VP16 [18] and BRCA1 [19] and thereby facilitate transcription. The classification of AATF as a transcription factor is so far based on its ability as Gal-4 fusion protein to transactivate Gal-4 responsive promoters. Several transcription factors such as VP16 [20], p53 [21], or BRCA-1 [22] have been identified this way. Clearly, the genes and their response elements targeted by AATF have to be elucidated.

Interestingly, all identified interaction partners of Dlk, including AATF, are either transcription factors (ATF4 and Par-4) and/or modulators of apoptosis (Par-4), suggesting that Dlk participates in a particular apoptotic pathway, perhaps through regulating transcription of specific target genes. On the other hand, Par-4 appears to be a regulator of Dlk as it causes relocation of Dlk to the cytoplasm and thereby induction of apoptosis [7]. Since AATF was not phosphorylated by Dlk, at least not in vitro, yet appeared to antagonize Dlk-mediated apoptosis, it might as well be a regulator rather than

a target of Dlk. It might do so by competing for binding of pro-apoptotic factors. Alternatively, AATF might act as transcription factor by enhancing expression of anti-apoptotic genes like *bcl-2* or repressing pro-apoptotic genes like *bax*. In this case, AATF might be linked to the anti-apoptotic Akt kinase pathway [23]. Akt inactivates pro-apoptotic transcription factor forkhead [24]. It might as well activate AATF as an anti-apoptotic transcription factor. Another question is, whether AATF is specific for Dlk-mediated apoptosis or whether it acts in a more general way. Investigating these issues will provide further insights into the complex network regulating cell death and survival.

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