Vanishin is a novel ubiquitinylated death-effector domain protein that blocks ERK activation

Runa SUR* and Joe W. RAMOS†[1]

*Department of Cell Biology and Neuroscience, Rutgers, The State University of New Jersey, 604 Allison Road, Piscataway, NJ 08854, U.S.A., and †Cancer Research Center of Hawaii, University of Hawaii at Manoa, 1236 Lauhala Street, Honolulu, HI 96813, U.S.A.

The ERK (extracellular-signal regulated-kinase)/MAPK (mitogen-activated protein kinase) pathway can regulate transcription, proliferation, migration and apoptosis. The small DED (death-effector domain) protein PEA-15 (phosphoprotein enriched in astrocytes-15) binds ERK and targets it to the cytoplasm. Other DED-containing proteins including cFLIP and DEDD can also regulate signal transduction events and transcription in addition to apoptosis. In the present study, we report the identification of a novel DED-containing protein called Vanishin. The amino acid sequence of Vanishin is closest in similarly to PEA-15 (61 % identical). Vanishin mRNA is expressed in several mouse tissues and in both mouse and human cell lines. Interestingly, Vanishin is regulated by ubiquitinylation and subsequent degradation by the 26 S proteasome. The ubiquitinylation is complex and occurs at both the internal lysine residues and the N-terminus. We further show that Vanishin binds ERK/MAPK but not the DED proteins Fas-associated death domain, caspase 8 or PEA-15. Vanishin is present in both the nucleus and Golgi on overexpression and forces increased ERK accumulation in the nucleus in the absence of ERK stimulation. Moreover, Vanishin expression inhibits ERK activation and ERK-dependent transcription in cells, but does not alter MAPK/ERK activity. Therefore Vanishin is a novel regulator of ERK that is controlled by ubiquitinylation.

Key words: apoptosis, death-effector domain, extracellular-signal-regulated kinase (ERK), immunofluorescence, ubiquitinylation, Vanishin.

INTRODUCTION

The ERK (extracellular-signal-regulated kinase)/MAPK (mitogen-activated protein kinase) pathway is involved in cellular responses to several extracellular stimuli including growth factors and adhesion to extracellular matrices. Activated ERK can alter transcription, proliferation, apoptosis and migration [1,2]. The canonical pathway is stimulated by a growth factor such as epidermal growth factor. In response to binding to the growth factor, epidermal growth factor receptors dimerize, leading to the autophosphorylation of their cytoplasmic domains on tyrosine. The phosphorylated tyrosine residues bind to linker proteins such as Grb-2 that in turn recruit guanosine nucleotide exchange factors such as Son of Sevenless. Son of Sevenless catalyses the exchange of GDP for GTP on the small GTPase Ras. GTP-loaded Ras then recruits the MEK (MAPK/ERK kinase) kinase Raf to the membrane where it is activated. Activated Raf phosphorylates and activates MEK, which then phosphorylates and activates the MAPK/ERK, which phosphorylates a number of substrates including both kinases and structural proteins (reviewed in [3,4]). ERK activity is carefully controlled in cells by a number of accessory proteins including KSR [5], MP-1 [6] and PEA-15 (phosphoprotein enriched in astrocytes-15) [7] that act at various points in the pathway. The outcome of ERK activation is also affected by the duration of the ERK signal, the localization of ERK and the integration of multiple signalling pathways.

One function of MAPK signalling pathways is to modulate apoptosis (reviewed in [8]). Apoptosis is activated by several conserved pathways [9]. Death-receptor mediated apoptosis through the tumour necrosis factor receptor 1 and Fas (CD95/APO-1) receptors depends on conserved structural modules such as death domains and DEDs (death-effector domains) that transmit and regulate the death signal [10,11]. Stimulation of the Fas receptor results in homotypic interactions between the death domains of FADD (Fas-associated death domain) with that of the Fas receptor followed by the interaction of the DEDs of FADD and pro-caspase 8 (FLICE) or pro-caspase 10 leading to the formation of a protein complex called the DISC (death-inducing signalling complex). This triggers a cascade of events resulting in apoptosis [12,13].

Proteins with DED domains also antagonize cell death. Anti-apoptotic DED-containing proteins include the FLIPs (FLICE-inhibitory proteins) that inhibit TNF-α (tumour necrosis factor-α) and Fas-induced apoptosis by blocking caspase activation [14–16]. PEA-15, another DED-containing protein, has also been found to have an anti-apoptotic function by inhibiting apoptosis induced by either Fas or TNF-α in astrocytes and some tumour cells [17–19]. Phosphorylated PEA-15 inhibits apoptosis mediated by TRAIL (TNF-related apoptosis inducing ligand) in glioma cells by being recruited to the DISC and thus blocking caspase recruitment and activation [19]. Interestingly, both PEA-15 and FLIP are also reported to regulate ERK signal transduction. PEA-15 binds directly to ERK and promotes the accumulation of active ERK in the cytoplasm [7], whereas FLIP interacts with Raf and causes increased ERK activity [20]. Other DED proteins including DEDD and Flame-3 can alter transcription by binding

Abbreviations used: BrdU, bromodeoxyuridine; CHO, Chinese-hamster ovary; CHX, cycloheximide; DAPI, 4′,6-diamidine-2′-phenylindole dihydrochloride; DED, death-effector domain; DISC, death-inducing signalling complex; ERK, extracellular-signal-regulated kinase; FADD, Fas-associated death domain; FLIP, FLICE inhibitory protein; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, haemagglutinin; JNK-1, c-Jun N-terminal kinase-1; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; NES, nuclear export sequence; PEA-15, phosphoprotein enriched in astrocytes-15; TNF-α, tumour necrosis factor-α; TRAIL, TNF-related apoptosis inducing ligand.

[1] To whom correspondence should be addressed (email jramos@crch.hawaii.edu).

directly to transcription factors in the nucleus [21,22]. Hence, in addition to apoptosis, DED-containing proteins can also regulate proliferation and transcription.

The steady-state expression levels of many proteins are regulated by ubiquitinylation. In most cases, ubiquitinylation of the target protein signals its degradation by the 26 S proteasome. Degradation of a protein by the ubiquitin–proteasome pathway involves two discrete and successive steps: (i) covalent attachment of multiple ubiquitin molecules to the protein substrate either with the ε-amino group of a protein lysine residue or in certain cases with the α-amino group at the N-terminus of the target protein; and (ii) degradation of the tagged protein by the 26 S proteasome. The initial conjugation of ubiquitin to the substrate protein involves the sequential action of three classes of enzymes, known as the ubiquitin-activating enzyme E1, the ubiquitin-conjugating enzyme E2 and the ubiquitin ligase E3 (see [23–25] for recent reviews). Ubiquitinylation regulates both pro-apoptotic and anti-apoptotic proteins and hence the 26 S proteasome governs both proliferation and apoptosis, opposite functions that determine the fate of a cell (reviewed in [26]).

To understand what the outcome of ERK activation will be, we must fully understand all of the regulatory proteins involved in a given cell and the molecular mechanism of their activity. In the present study, we describe a novel DED-containing protein called Vanishin that binds ERK. Vanishin has 61 % amino acid sequence similarly with PEA-15 but has very different effects on ERK. Vanishin translocates to the nucleus and retains ERK in the nucleus in the absence of stimulation of the ERK/MAPK pathway. Vanishin modulates ERK signalling by inhibiting ERK activation and ERK-dependent transcription without altering MEK activity. Vanishin expression was also found to induce apoptosis in cells by approx. 2-fold. Finally, we demonstrate that Vanishin is regulated by ubiquitinylation and is subject to degradation by the 26 S proteasome. Thus Vanishin is a novel ubiquitinylated DED-containing protein that regulates ERK function by altering its location and activity.

EXPERIMENTAL

Cloning of Vanishin cDNA and plasmid construction

BLASTP searches of the nr database using the amino acid sequence of PEA-15 as query resulted in the identification of a PEA-15-like protein located on chromosome 5C3.3 of the mouse genome. This protein that we refer to as Vanishin is a 127 amino acid protein similar to PEA-15. The GenBank® accession no. of Vanishin is AY487415.

The open reading frame encoding Vanishin was amplified by PCR from NIH3T3 cells using the forward primer, 5′-GGAGGATCCGGAGGCGTCATGGGCAGAAGCAGC-3′ and the reverse primer 5′-CCGGAATTCCGGCTAGAGATACTGCTTAGC-3′. The PCR product was digested with *Bam*HI and *Eco*RI and cloned into the respective sites of pcDNA3 (Invitrogen Life Technologies). Epitope-tagged expression plasmids were constructed by adding a HA (haemagglutinin) tag sequence in the forward primer and cloning the PCR product into the *Bam*HI and *Eco*RI sites of pcDNA3. GFP (green fluorescent protein)–Vanishin was generated by subcloning Vanishin into *Hin*dIII and *Eco*RI sites of the vector pEGFP-C1 (ClonTech). His-tagged Vanishin was constructed by subcloning into the *Bam*HI and *Eco*RI sites of the vector pcDNA3-HisC (Invitrogen Life Technologies). Vanishin was subcloned into the *Bam*HI and *Eco*RI sites of the vector pGEX-4T-1 (Amersham Biosciences, Piscataway, NJ, U.S.A.) and expressed in the strain BL21 (DE3) pLysS to get a GST (glutathione S-transferase) fusion protein. KR mutants were produced by replacing the lysine residues of Vanishin with arginine residues with site-directed mutagenesis using the Quik Change™ site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) according to the manufacturer's instructions. All the constructs were confirmed by sequencing.

Cell culture, transfections, reagents and antibodies

NIH3T3, Cos-7, HeLa, CHO (Chinese-hamster ovary)-K1 and all other cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10 % (v/v) fetal bovine serum (Gemini Bioproducts, Calabassas, CA, U.S.A.), 1 % penicillin-streptomycin (Invitrogen) and non-essential amino acids (Sigma). NIH3T3 and Cos-7 cells were transfected using LIPOFECTAMINE™ Plus reagent (Invitrogen) according to the manufacturer's instructions. CHO-K1 cells were transfected with LIPOFECTAMINE™ 2000 reagent (Invitrogen) and HeLa cells were transfected with Polyfect (Qiagen). MG132 was obtained from Peptides International (Osaka, Japan). 12CA5 antibody was obtained from Roche Applied Science (Indianapolis, IN, U.S.A.). Myc-tag, His-tag, pMEK1/2 and MEK1/2 antibodies were from Cell Signaling Technology (Beverly, MA, U.S.A.). ERK1/2, p38, JNK-1 (c-jun N-terminal kinase-1), p-ERK1/2 and FADD antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Human recombinant TNF-α was purchased from Sigma and the anti-Fas CH11 antibody was from MBL (Watertown, MA, U.S.A.). BrdU (bromodeoxyuridine), MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] and PI reagents were purchased from Sigma.

Reverse transcriptase–PCR analysis

RNA was isolated from different cell lines using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. RNA was isolated from different mouse tissues using the RNAlater RNA stabilization reagent (Qiagen) followed by the RNeasy mini kit (Qiagen). First-strand cDNA synthesis was performed using SUPERSCRIPT II (Invitrogen) and the PCR was performed using the Vanishin forward and reverse primers used for cDNA cloning and glyceraldehyde-3-phosphate dehydrogenase primers.

Immunofluorescence assays

Cos-7 cells were grown on coverslips in six-well plates and transfected with GFP and GFP–Vanishin. After 48 h, cells were fixed using 4 % (w/v) paraformaldehyde for 10 min followed by washing with PBS. Cells were then stained with BODIPY® TR ceramide (Molecular Probes) to stain the Golgi network, according to the manufacturer's instructions, and the nuclei were stained with DAPI (4′,6-diamidine-2′-phenylindole dihydrochloride). For the ERK co-localization studies, cells were serum-starved by growing in 0.2 % serum for 24 h or serum-stimulated with 20 % serum for 3 h. Cells were washed twice with PBS, fixed with 4 % paraformaldehyde and permeabilized with 0.2 % Triton X-100 in PBS. Cells were incubated overnight at 4 °C with the ERK2 antibody (Santa Cruz Biotechnology) at 1:500 dilution in PBS containing 0.2 % Triton X-100 and 3 % BSA. After washing with PBS, cells were incubated for 1 h at room temperature with Texas red-conjugated anti-rabbit antibody (Molecular Probes). Nuclei were stained with DAPI. The coverslips were then mounted on slides using Fluoromount-G (Southern Biotechnology Associates) mounting media and cells were imaged in a Zeiss Axiovert 100 M fluorescence microscope using a × 100 oil immersion objective.

Protein stabilization studies

Cos-7 cells were grown in six-well plates and transfected with HA-tagged Vanishin. After 24 h, fresh medium was added to the

cells followed by the addition of 1 μM MG132 or equal volume of DMSO as required. After an additional 24 h, protein synthesis was blocked by adding 10 μg/ml CHX (cycloheximide) and at the indicated time points cells were lysed with sample buffer and subjected to SDS/PAGE. Protein (20 μg) was loaded unless otherwise indicated.

His-tagged protein precipitations

Cos-7 cells in 10 cm dishes were transfected with His_6-tagged Vanishin or His_6-pcDNA3 as control along with the respective proteins using LIPOFECTAMINE™ Plus reagent (Invitrogen). After 24 h, cultures were treated with 1 μM MG132 for an additional 24 h. The cells were then lysed using 800 μl Nonidet P40 lysis buffer followed by centrifugation at 18 110 ***g*** for 10 min at 4 °C. Supernatant was passed over 50 μl Ni^{2+} Probond beads, which were pretreated by washing with distilled water and Nonidet P40 buffer. Lysates were incubated with the Ni^{2+} beads for 60 min at 4 °C. The beads with the bound protein were washed several times with Nonidet P40 buffer and then resuspended in 50 μl sample buffer. At times, 10 mM imidazole was included in the wash buffer to omit non-specific binding. Proteins were subjected to SDS/PAGE followed by Western blotting with respective antibodies.

Immunoprecipitations and GST pull-down assay

CHO cells were co-transfected with vectors encoding myc-tagged Vanishin and HA-tagged ERK2 or p38. Cells were washed with PBS buffer and lysed 36–40 h after transfection. The lysate was centrifuged at 13 306 ***g*** for 10 min, and cleared lysates were separately immunoprecipitated by incubation with 5 μl of anti-myc antibody (Vanishin) or control mouse serum. Immune complexes were captured with Protein A–Sepharose beads (50 μl) overnight at 4 °C. After immunoprecipitation, the beads were washed four times with lysis buffer, eluted with SDS sample buffer, resolved by SDS/PAGE and visualized by immunoblottting with anti-HA, anti-p38, anti-JNK-1 and anti-myc antibodies.

For GST pull-down assays, cells grown on 10 cm dishes were lysed with MLB buffer (25 mM Hepes, 150 mM NaCl, 1 % Nonidet P40, 0.25 % sodium deoxycholate, 10 % glycerol, 10 mM $MgCl_2$, 1 mM EDTA and protease inhibitor) and then incubated with equal amounts of purified GST or GST–Vanishin immobilized on agarose beads for 60 min at 4 °C. Since GST–Vanishin yield was really low due to the formation of inclusion bodies, empty beads were added to the GST control to account for equal bead volume. This was followed by washing the beads extensively three times with MLB buffer and resuspending them in sample buffer. The pulldowns were subjected to SDS/PAGE followed by Western blotting using the ERK1/2 antibodies.

Transcription assays

Transcription assays were performed using the Pathdetect *in vivo* signal transduction pathway Elk-1 trans-reporting systems kit according to the manufacturer's instructions (Stratagene, CA, U.S.A.). Briefly, CHO-K1 cells were co-transfected with 50 ng of pFA2-Elk-1, 500 ng of pFR-luciferase and 500 ng of Vanishin or vector-alone plasmids. pFA2-Elk-1 contains the DNA-binding domain of GAL4 fused to the transactivation domain of Elk-1. pFR-luc has the luciferase reporter gene under the control of a synthetic promoter containing five tandem repeats of the yeast GAL4-binding sites. Cells were grown, 24 h post-transfection, in 0.2 % serum (serum-starved) overnight, and stimulated with 20 % serum for 1 h as indicated.

Cell viability and proliferation assays

Cell viability was determined by MTT assay. CHO-K1 cells transfected with myc-Vanishin or vector alone were seeded in 12-well plates at 1000, 10 000 and 100 000 cells per well. After the indicated times of 48 and 72 h, cells were washed once with PBS and treated with 500 μl of 0.5 mg/ml of MTT in PBS at 37 °C for 4 h. The converted dye was then solubilized with 500 μl of acidic propan-2-ol (0.04 M HCl in absolute propan-2-ol). Absorbance of the converted dye was measured at a wavelength of 570 nm in a microplate reader.

For the cell proliferation assay, 48 h post-transfection cells grown in 10 cm dishes were treated with 10 μM BrdU for 1 h at 37 °C. Cells were then trypsinized, washed with PBS and fixed in 70 % ethanol on ice for 30 min. After fixation, the chromatin was rendered accessible by a 30 min treatment with 2 M HCl/0.5 % Triton X-100, followed by a neutralizing step with 0.1 M borate buffer (pH 8.5). Cells were counted to take equal number of cells and the incorporated BrdU was detected by incubating the cells for 30 min with anti-BrdU FITC antibody (2 μl/4 $\times$ 10^6 cells; eBiosciences, CA, U.S.A.). The cells were then stained with propidium iodide (Sigma) in the presence of RNase A (Sigma) for 30 min. Samples were analysed on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) using the CELL Quest program. A doublet discrimination was performed during acquisition of results to exclude the doublet population of cells. Plotting the anti-BrdU FITC fluorescence versus the PI fluorescence showed us the % of cells in the different stages of the cell cycle. The subdiploid peak obtained from the DNA histogram was considered to be apoptotic cells.

Apoptosis assays using flow cytometry

For death receptor-induced apoptosis, HeLa and NIH3T3 cells were transfected with Vanishin and vector control plasmids. After 48 h, apoptosis was induced in HeLa and 3T3 cells by incubating them for 16 h with anti-FasR antibody (100 ng/ml) and TNF-α (100 ng/ml) respectively along with CHX (10 μg/ml). HeLa and 3T3 cells were harvested in PBS and the cell pellet (1×10^6) was gently resuspended in 1 ml of staining solution (50 μg/ml propidium iodide, 0.2 % saponin and 100 μg/ml RNase A) and incubated at 37 °C for 30 min in the dark. Propidium iodide stained cells were analysed on a FACScan flow cytometer (Becton Dickinson) using the CELL Quest program. The subdiploid peak obtained from the DNA histogram was considered to be apoptotic cells.

RESULTS

Identification and sequence analysis of Vanishin

A BLASTP search of the mouse genome database using the amino acid sequence of PEA-15 as query revealed the existence of a similar protein, which we now call Vanishin. Vanishin is located on mouse chromosome 5 (map 5C3.3). In contrast, the PEA-15 gene is located on mouse chromosome 1. cDNA clones of Vanishin were obtained by amplifying cDNA from NIH3T3 cells. The open reading frame corresponds to a 127 amino acid protein including a DED spanning amino acids 30–103 (Figure 1A). Mouse Vanishin shares 61 % amino acid sequence similarity to mouse PEA-15 with maximum similarity lying in the DED regions of the two proteins. Vanishin lacks the NES (nuclear export sequence) of PEA-15 (underlined in Figure 1B). However, the amino acid Asp^{74} required for ERK binding in PEA-15, is conserved in Vanishin (marked by an asterisk in Figure 1B). Amino acid sequence alignment of mouse Vanishin with PEA-15 is represented in

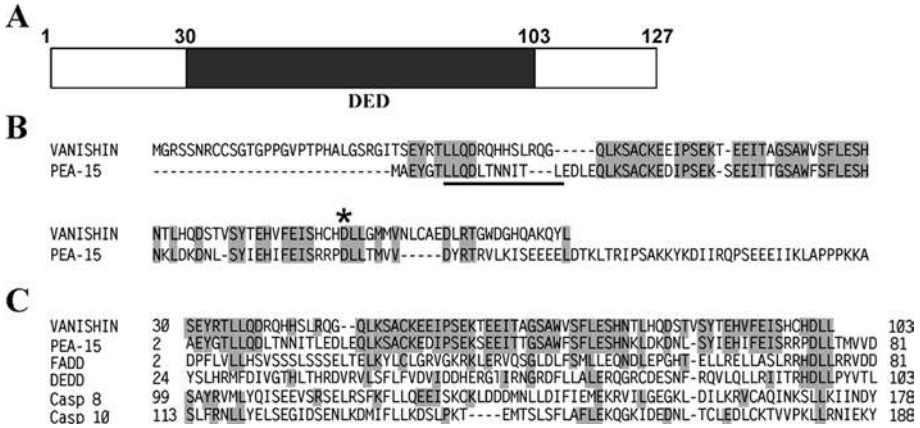

Figure 1 Sequence analysis of Vanishin

(**A**) Schematic representation of Vanishin. Boxed region indicates the DED. (**B**) Protein sequence alignment of mouse Vanishin with mouse PEA-15. Underlined sequence represents the NES of PEA-15. *, the conserved amino acid required for ERK binding in PEA-15. (**C**) Alignment of the DED region of mouse Vanishin with other DED-containing proteins. Regions sharing two or more similar residues are shaded.

Figure 1(B). The DED of Vanishin is similar to that of other DED-containing proteins and the conserved residues are highlighted in Figure 1(C). The sequences outside the DED are not similar to any known conserved domains. We have not identified the human orthologue of Vanishin in the genome, however, using the mouse derived Vanishin primers we were able to amplify Vanishin from human cells (see below). This sequence was nearly identical (98 %) at the nucleotide level to the mouse sequence. This is comparable with PEA-15, which is 94 % identical with mouse and human coding sequences.

Expression of Vanishin in mouse tissues and cell lines

Northern blotting showed the existence of Vanishin as two transcripts of length approx. 2 and 3.2 kb in several mouse tissues (results not shown). However, the message was present in very low quantities. Therefore we performed reverse transcriptase–PCR assays using primers specific to Vanishin on different mouse tissues. In these experiments, Vanishin mRNA was expressed in various mouse tissues with the highest levels of expression in the mouse spleen and thymus (Figure 2A). All the cell lines tested, which included both mouse and human lines, were positive for Vanishin mRNA (Figure 2B).

Vanishin is present in both the nucleus and the Golgi

Evaluation of subcellular localization of GFP–Vanishin by fluorescence microscopy revealed that exogenously expressed Vanishin was in the nucleus and perinuclear area (Figure 3). To determine the nature of the perinuclear compartment, cells were treated with BODIPY® TR ceramide, a red fluorescent probe that specifically labelled the Golgi network. GFP–Vanishin showed co-localization with the Golgi marker (Figures 3C and 3D). Thus Vanishin localizes to the nucleus and the Golgi. GFP localization was also monitored as a control and GFP was found throughout the cell (Figures 3E–3H).

Vanishin expression is regulated by the 26 S proteasome

Ectopic expression of HA-tagged mouse Vanishin in both NIH3T3 cells (results not shown) and Cos-7 cells (Figure 4A) produced very little protein. One explanation for this would be that the protein is rapidly degraded in these cells. Many proteins are degraded by the ubiquitin–proteasome pathway. We therefore tested

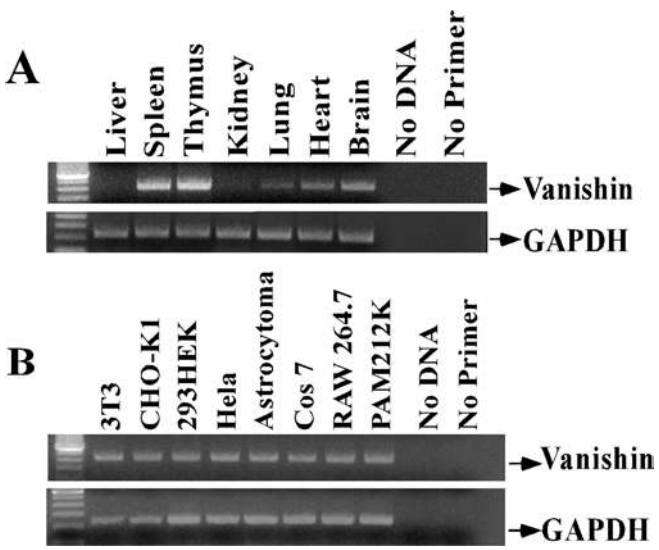

Figure 2 Expression of Vanishin in mouse tissues and cell lines

First-strand cDNA from (**A**) various adult mouse tissues and (**B**) various cell lines was amplified by PCR using primers specific to mouse Vanishin. Primers to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as control. Note that bands of the appropriate size were detected in human, monkey and mouse derived cell lines.

whether a proteasome pathway regulates Vanishin expression by inhibiting the 26 S proteasome with increasing concentrations of MG132. We found that Vanishin was stabilized under these conditions suggesting that its expression is regulated by the 26 S proteasome (Figure 4A). MG132 (1–5 μM) was sufficient to stabilize the protein completely. The higher molecular mass band observed in the MG132 treated lanes (represented by asterisk in Figure 4A) may correspond to a single ubiquitin-conjugated Vanishin that migrates approx. 23 kDa after conjugation to an approx. 7.8 kDa endogenous ubiquitin moiety. These bands and others of very high molecular mass corresponding to polyubiquitinylated protein (results not shown) became more evident in the presence of MG132, indicating that when proteasome function is abrogated, ubiquitinylated species of Vanishin accumulate. α-Tubulin was used as a control and was not affected by MG132 (Figure 4A).

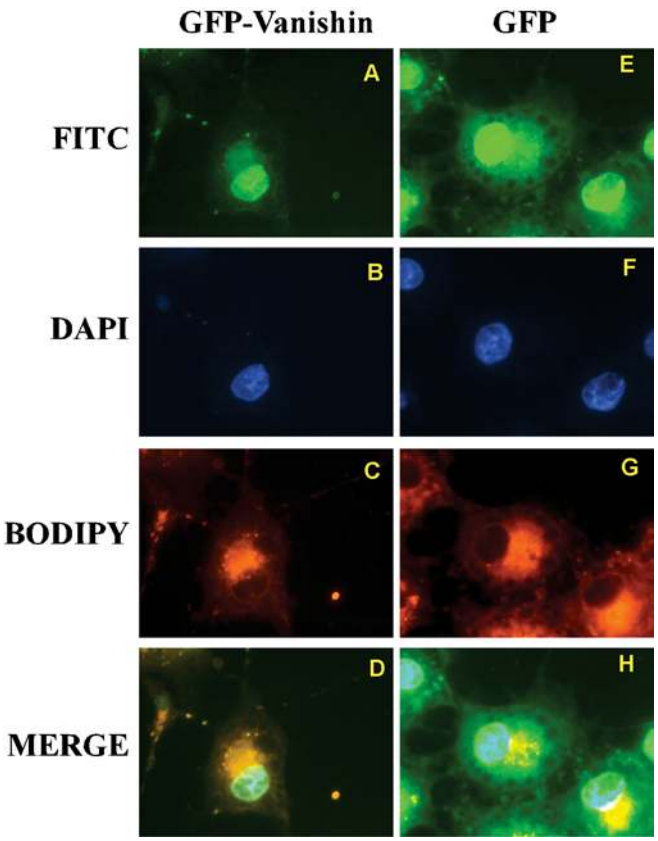

Figure 3 Vanishin is in the nucleus and the Golgi

Cos-7 cells were transfected with pEGFP-C1–Vanishin (**A**–**D**) or GFP alone as control (**E**–**H**) and subjected to immunofluorescence after 48 h. (**A**, **E**) Localization of the indicated proteins in the cell. (**B**, **F**) Nuclear staining with DAPI. (**C**, **G**) Golgi staining with BODIPY® TR ceramide. (**D**–**H**) Co-localization of GFP and GFP–Vanishin in the nucleus and the Golgi.

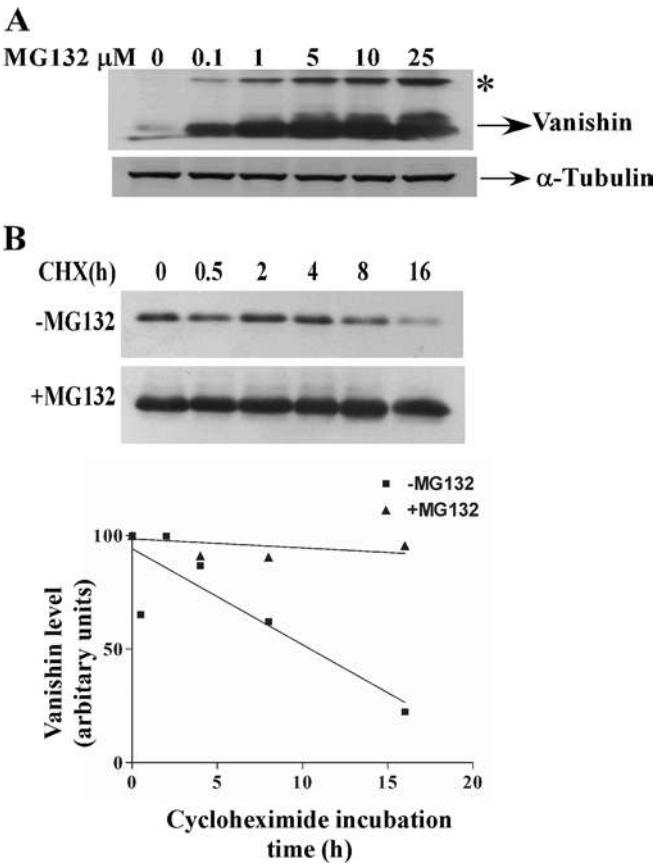

Figure 4 Vanishin is regulated by the 26 S proteasome

(**A**) Cos-7 cells were transfected with HA-tagged Vanishin and PEA-15 and treated with different concentrations of MG132 as indicated. The samples were subjected to Western blot and probed with the HA antibody. Antibody to α-Tubulin was used to verify equal protein loading. The higher molecular mass Vanishin band is indicated by an asterisk. (**B**) To determine the half-life of Vanishin, Cos-7 cells were transfected with HA–Vanishin. After 48 h, cells were treated with 10 μg/ml CHX. The expression level of Vanishin was studied for 16 h by Western blotting with anti-HA antibody in the presence and absence of 1 μM MG132. Protein (40 μg) was loaded for each sample. Lower panel represents a spot densitometry of the band intensities plotted against the incubation time of CHX in hours.

To determine how rapidly Vanishin is degraded by the proteasome, we examined the half-life of Vanishin in both Cos-7 (Figure 4B) and NIH3T3 cells (results not shown). Protein expression was inhibited by the addition of CHX and Vanishin protein levels were then followed by Western blotting. In both cell lines, Vanishin half-life in the absence of MG132 was approx. 8 h. As expected, addition of MG132 prevented Vanishin degradation in the presence of CHX indicating that it is degraded by the proteasome (Figure 4B).

Efficient recognition of proteins that are targeted for degradation by the 26 S proteasome requires many ubiquitin molecules to be ligated to the substrate in the form of a polyubiquitin chain. Target proteins are commonly ubiquitin-conjugated at internal lysine residues. Vanishin has four lysine residues at amino acid positions 50, 54, 61 and 124. To identify the sites responsible for the ubiquitinylation and subsequent degradation of Vanishin the lysine residues were mutated to arginine by site-directed mutagenesis. The mutant KR1 has lysine residues 50, 54 and 61 mutated; KR2 has the residue 124 mutated and KR0 has all four lysine residues mutated. Maximum stabilization of Vanishin was by KR0 (Figure 5A). Moreover, the higher molecular mass band, which corresponds in size to the monoubiquitin-conjugated Vanishin band, is also absent in this mutant. These results indicate that the lysine residues are involved in the ubiquitinylation of Vanishin. The monoubiquitinylated Vanishin band may result from the addition of a single ubiquitin moiety at an internal lysine residue.

In proteins like MyoD, LMP1, E7 and p21, lysine-independent ubiquitinylation occurs [27–30]. In each case, ubiquitinylation of the N-terminus occurs and attachment of a Myc_6-tag to the N-terminus stabilizes the proteins. We therefore examined whether N-terminal tagging of Vanishin had any effect on its stability. In addition, we compared the myc-tag with HA and FLAG-tagged versions of Vanishin. The myc-tagged protein was substantially more stable in the absence of MG132 than the other two tags (Figure 5B). Moreover, the addition of MG132 stabilized the protein further. This indicates that the myc-tag can confer stability to Vanishin independent of the ubiquitinylated lysine residues. It also suggests that the myc-tag is doing more than simply blocking ubiquitinylation of the N-terminus as neither the HA nor the FLAG tags had comparable effects. Myc-tagging of the lysine-less KR0 mutant did not stabilize Vanishin further (Figure 5B).

Vanishin is ubiquitinylated *in vivo*

To determine if Vanishin is directly ubiquitinylated in cells and whether Vanishin lacking the lysine residues could still be ubiquitinylated at other sites, we followed conjugation of an HA-tagged ubiquitin to Vanishin *in vivo*. We co-transfected Cos-7 cells

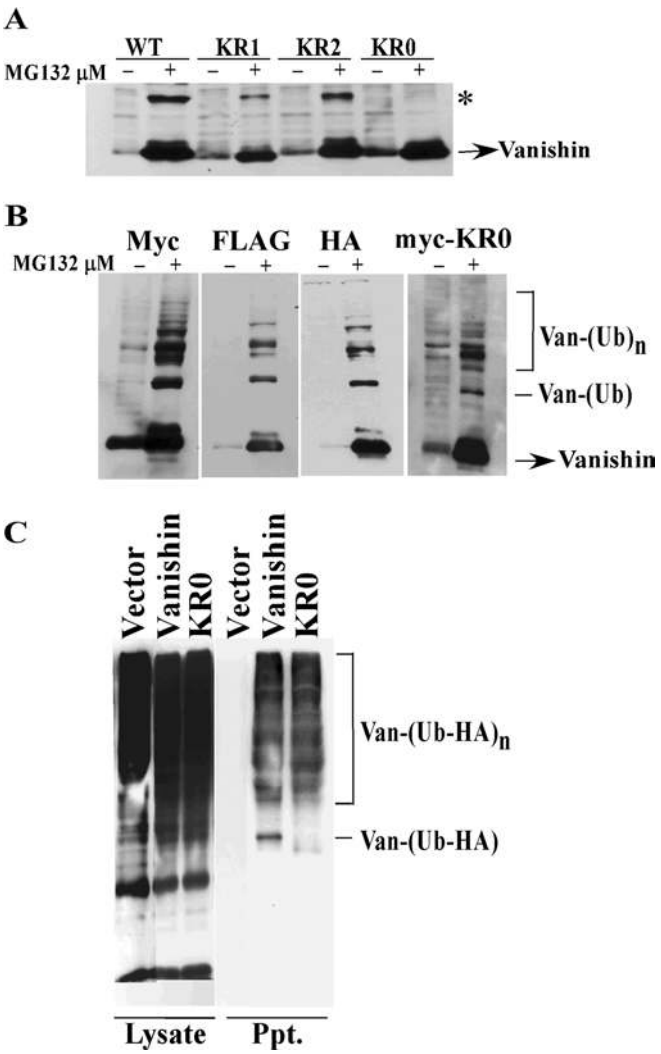

Figure 5 Ubiquitinylation of Vanishin

(**A**) Expression of wild-type mouse Vanishin and the lysine mutants KR1, KR2 and KR0 in the presence and absence of 1 μM MG132. *, the higher molecular mass ubiquitinylated Vanishin band. (**B**) Stability levels of the Myc, FLAG, HA-tagged and myc-KR0 Vanishin are shown in the presence and absence of 1 μM MG132. The higher molecular mass ubiquitinylated bands are presented. (**C**) *In vivo* ubiquitinylation of Vanishin. Cos-7 cells were cultured in 10 cm dishes and co-transfected with His_6-tagged Vanishin or the vector control or KR0, along with HA-tagged Ubiquitin. Cells were treated with 1 μM MG132 for 24 h. After 48 h, the His-tagged proteins were precipitated using Probond Ni^{2+} columns, washed extensively, and analysed by SDS/PAGE and Western blotting with the HA antibody to detect ubiquitinylated Vanishin. Ppt., precipitate.

with HA-tagged ubiquitin along with His-tagged Vanishin, the lysine-less mutant KR0 or the vector alone. The cells were treated with MG132 and the overexpressed His-tagged proteins were isolated by precipitation on a Ni^{2+} column. The purified proteins showed multiple higher molecular mass forms corresponding to ubiquitinylated Vanishin, which were absent in the vector control (Figure 5C). The lysine-less KR0 mutant was also ubiquitinylated. However, the monoubiquitin-conjugated Vanishin band is absent in this mutant in agreement with the Western-blot result shown in Figure 5(A). Thus Vanishin is ubiquitinylated *in vivo* at both lysine and non-lysine residues.

Vanishin does not bind PEA-15, FADD or caspase 8

Since Vanishin has a DED domain we investigated the possibility of the interaction of Vanishin with other DED-containing proteins.

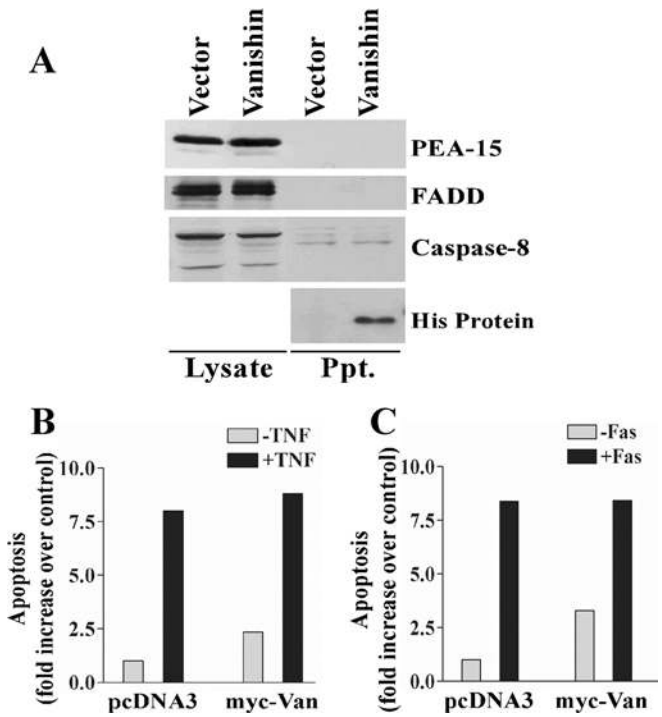

Figure 6 Interaction of Vanishin with other DED-containing proteins and effect on apoptosis

(**A**) Cos-7 cells were transiently transfected with His_6-tagged Vanishin or the vector control along with HA-tagged mouse PEA-15, HA-tagged FADD and HA-tagged caspase 8. Cells were treated with 1 μM MG132. The His-tagged proteins were precipitated using Probond Ni^{2+} columns, washed extensively, and analysed by SDS/PAGE and Western blotting with specific antibodies to see the interaction with Vanishin. The blot was probed with the His antibody to see His–Vanishin bound to the beads. Ppt., precipitate. (**B**) NIH3T3 cells were transfected with pcDNA3 and myc-tagged Vanishin (Van) and incubated for 16 h with TNF-α. (**C**) Effect of Vanishin on Fas-mediated apoptosis. HeLa cells were transfected with the respective plasmids and apoptosis was induced by Fas for 16 h. Apoptotic cells were obtained by FACS analysis. Apoptosis is represented as fold increase in apoptosis over control pcDNA3 transfected cells which was set as 1. Representative results from one of three independent experiments.

To this end, Cos-7 cells were co-transfected with His-tagged Vanishin and other DED-containing proteins such as HA-tagged PEA-15, FADD and HA-tagged caspase 8. In these experiments, Vanishin did not bind PEA-15, FADD or caspase 8 (Figure 6A).

Since DED-containing proteins are involved in death-receptor-mediated apoptosis, we next assessed whether Vanishin played any role in apoptosis. To this end, we tested whether Vanishin could either block or enhance apoptosis activated by Fas, TNF-α or TRAIL. Interestingly, we found that Vanishin expression in the absence of TNF-α or Fas induced apoptosis by approx. 2-fold in both 3T3 and HeLa cells (Figures 6B and 6C). However, Vanishin did not augment or inhibit TNF-α-induced apoptosis in 3T3 cells (Figure 6B) or Fas-induced apoptosis in HeLa cells (Figure 6C). Finally, Vanishin did not affect TRAIL-induced apoptosis of 3T3 cells (results not shown). Hence Vanishin does not enhance or block death receptor-mediated apoptosis although its expression alone increases apoptosis.

Vanishin binds ERK/MAPK

Since the DED-containing protein PEA-15 binds ERK1/2 and regulates ERK/MAPK signalling [7], we investigated whether Vanishin, which shares significant sequence similarity with PEA-15, interacted with ERK. Ectopically expressed His-tagged

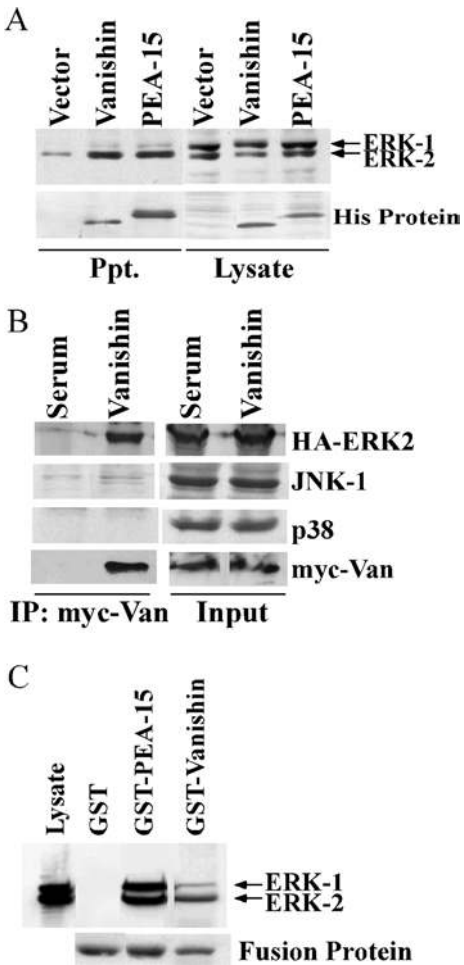

Figure 7 Interaction of Vanishin with ERK1/2

(**A**) Cos-7 cells were transiently transfected with His_6-tagged Vanishin or PEA-15 or the vector control. Cells were treated with 1 μM MG132 for 24 h. His-tagged proteins were precipitated using Probond Ni^{2+} columns, washed extensively, and analysed by SDS/PAGE. Western blotting was performed with the ERK1 and ERK2 antibodies and with the anti-His tag antibody to see the precipitated protein. (**B**) CHO cells were co-transfected with vectors encoding myc-tagged Vanishin and HA-tagged ERK or p38. The lysates were separately immunoprecipitated by incubation with anti-myc antibody or serum as control as described in the Experimental section. Note the presence of ERK2 but not p38 and JNK in the lysates immunoprecipitated with anti myc-antibody but not with the control serum. Blots were reprobed with the myc antibody to see the amount of Vanishin (Van) immunoprecipitated. (**C**) Cells lysates were incubated with equal amounts of purified GST or GST–Vanishin or GST–PEA-15. The pull downs were subjected to SDS/PAGE followed by Western blotting using the ERK1/2 antibodies. The amount of fusion protein in the pull downs is shown below by Coomassie staining.

Vanishin bound both ERK1 and ERK2 in Cos-7 cells (Figure 7A). Furthermore, ERK2 and Vanishin were also co-immunoprecipitated (Figure 7B). In comparison, Vanishin did not bind the MAPKs p38 or JNK-1 (Figure 7B). Vanishin also bound ERK1 and ERK2 in a GST pull-down assay, with a stronger interaction for ERK2 (Figure 7C).

Since Vanishin binds ERK, we determined whether the two proteins co-localize in the cell. We found that whereas GFP–Vanishin co-localized with a subset of ERK in the nucleus and the perinuclear region, control GFP expressing cells did not show a similar pattern (Figure 8A). Importantly, we also found that Vanishin altered localization of ERKs in serum-starved cells. ERK was largely localized to the nucleus in the presence of Vanishin in contrast with GFP (Figures 8A and 8B).

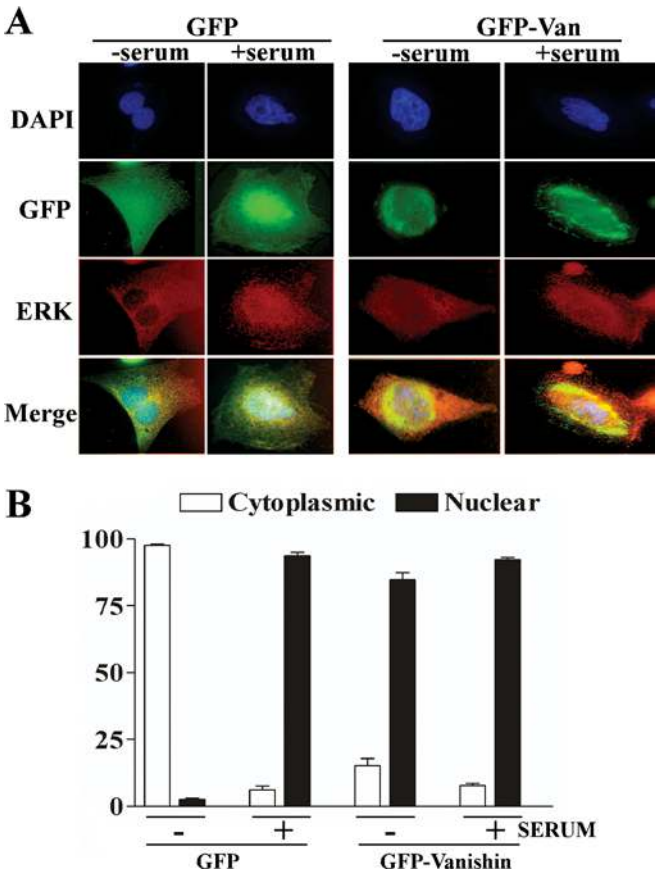

Figure 8 Vanishin co-localizes with ERK

(**A**) CHO-K1 cells were transfected with pEGFP-C1–Vanishin (GFP-Van) or GFP alone as control. Cells were cultured in 0.2 % serum for 24 h (− serum) and subsequently stimulated with 20 % serum for 3 h (+ serum) and then stained for ERK2. The nucleus was stained with DAPI. (**B**) Cells in which the nucleus was less (open bars) or more (filled bars) stained for ERK than the cytoplasm were counted. Means ± S.E.M. for three independent experiments are shown ($n \geqslant 600$). Note that Vanishin expressing cells manifest nuclear staining for ERK even in 0.2 % serum.

Vanishin down-regulates ERK signalling

We previously reported that the DED-containing protein, PEA-15, activates ERK MAPK and modifies ERK signalling by excluding ERK from the nucleus [7,31]. Since Vanishin interacted and co-localized with ERK we looked at the effect of Vanishin on ERK activation. We found that, in contrast with PEA-15, Vanishin did not activate ERK; however, it blocked ERK activity in the presence of serum by 40 % (Figure 9A). To determine whether Vanishin was directly altering activation of ERKs or was doing it indirectly through MEK1/2, which activates ERK, we looked at MEK phosphorylation. We found that Vanishin did not affect the activation of MEK1/2 in these cells (Figure 9A).

To determine whether Vanishin affected ERK nuclear signalling *in vivo*, we looked at Elk-1-dependent transcription. Serum induced Elk-1-dependent transcription through the activation of ERK in control pcDNA3 transfected cells. However, Vanishin expression inhibited Elk-1-dependent transcription *in vivo* by 50 % (Figure 9B).

Since ERK plays an important role in regulating cell survival and proliferation, we next asked whether Vanishin altered these outcomes. We performed an MTT assay on CHO-K1 cells after transfecting them with myc-tagged Vanishin for 48 or 72 h. Vanishin expression reduced live cell numbers both at 48 and 72 h post-transfection by approx. 35 % (Figure 10A). To

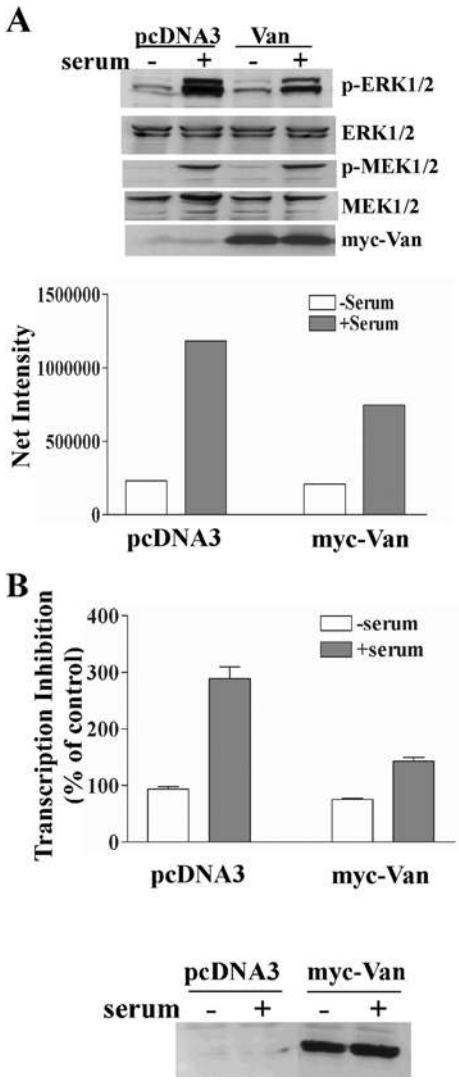

Figure 9 Vanishin down-regulates ERK signalling

(**A**) CHO-K1 cells grown in six-well dishes were transfected with pcDNA3 or myc-tagged Vanishin vectors. After 24 h, cells were serum-starved by leaving them overnight in 0.2 % serum-containing medium. The respective wells were then serum-stimulated by adding 20 % serum for 30 min. Cells were lysed in M2 buffer [0.5 % Nonidet P40, 20 mM Tris, pH 7.6, 250 mM NaCl, 5 mM EDTA, 3 mM ethylene glycol-bis(β-aminoethyl ester)-*N*,*N*,*N*′,*N*′-tetra-acetic acid, 20 mM sodium phosphate, 20 mM sodium pyrophosphate, 3 mM β-glycero-phosphate, 1 mM sodium orthovanadate, 10 mM NaF and 1× protease inhibitor cocktail] and spun down at 12 000 rev./min for 10 min. Samples were analysed by SDS/PAGE followed by blotting with the p-ERK1/2, ERK1/2, p-MEK1/2, MEK1/2 and myc antibodies. Lower panel represents a spot densitometry of the pERK1/2 bands. Note the 40 % decrease in ERK activation with Vanishin. (**B**) CHO-K1 cells grown in six-well dishes were transfected with pcDNA3 or myc-tagged Vanishin vectors. Transcription assays were performed as described in the Experimental section. Elk-1-dependent transcriptional inhibition is represented as percentage of control pcDNA3 transfected cells which was set at 100 %. Vanishin expression inhibited Elk-1-dependent transcription by 50 %. The lower panel shows exogenous Vanishin protein expression.

determine whether the decrease in cell number was specifically due to a decrease in cell proliferation or an increase in apoptosis, we examined BrdU incorporation followed by PI staining of the DNA. Flow cytometric analysis of PI-stained cells showed that Vanishin expression brought about a 100 % or 2-fold induction of apoptosis (Figure 10B), which is also apparent from the dot plot showing the subdiploid apoptotic cells (Figure 10C). The BrdU incorporation assay shows that there is no significant difference in the proportion of cells cycling through the different stages of the cell cycle suggesting that Vanishin expression did not have any significant effect on cell proliferation (Figure 10C).

DISCUSSION

We have identified and characterized a new DED-containing protein, Vanishin, that binds to ERK and regulates its activity. Vanishin is similar to PEA-15 but is functionally distinct. Whereas PEA-15 is exclusively localized to the cytosol, Vanishin was predominantly in the nucleus and the Golgi. Vanishin modulates ERK signalling by inhibiting ERK activation and ERK-dependent nuclear transcription without affecting MEK activity. Importantly, Vanishin is the first DED protein reported to be regulated by the 26 S proteasome. The DED of Vanishin is also unusual in that it is in the middle of the protein rather than at the N-terminus. Finally, ectopic expression of Vanishin inhibits cell viability by promoting apoptosis. Thus Vanishin is a novel ERK binding partner that is regulated by ubiquitinylation.

We show that Vanishin expression levels are regulated by proteasome degradation. Vanishin is ubiquitinylated *in vivo*. Proteins that are targeted for degradation by the 26 S proteasome are commonly ubiquitin-conjugated at internal lysine residues. Mutating the four internal lysine residues of Vanishin to arginine failed to stabilize the protein completely. Moreover, the lysine-less mutant KR0 continued to undergo ubiquitinylation *in vivo*. Hence other non-lysine residues may also play a role in Vanishin ubiquitinylation and subsequent degradation. In other proteins, including MyoD, LMP1, E7 and p21, lysine-independent ubiquitinylation is reported to occur [27–30]. In each case, ubiquitinylation of the N-terminus occurs and attachment of a Myc_6-tag to the N-terminus stabilizes the protein. It has been suggested that the Myc_6-tag blocks the recognition site for ubiquitin ligases or ubiquitin or both. N-terminal tagging of Vanishin with the myc-tag similarly stabilized the protein independent of the lysine mutations. Hence Vanishin may also be ubiquitinylated at the N-terminus in a manner similar to that reported for p21. However, when we placed either the HA- or FLAG-tag at the N-terminus of Vanishin, no stability was conferred. Hence there is something distinct about the myc-tag sequence that is involved in stabilizing Vanishin and perhaps the other proteins as well. It is not simply a matter of blocking the N-terminus.

Vanishin is a DED-containing protein. Homotypic interactions occur between the DEDs of proteins like FADD with that of pro-caspase 8 or pro-caspase 10 leading to the formation of the DISC. This subsequently triggers apoptosis [12,13]. Hence we looked at the interaction of Vanishin with other DED-containing proteins. Vanishin did not interact with FADD, caspase 8 or a similar protein PEA-15. Vanishin did not affect Fas, TNF-α or TRAIL-induced apoptosis. This is in contrast with PEA-15, which inhibits apoptosis induced by either Fas or TNF-α in astrocytes and some tumour cells [17–19]. Phosphorylated PEA-15 also inhibits apoptosis mediated by TRAIL in glioma cells [19]. The DEDs of both PEA-15 and Vanishin are very similar differing primarily in the initial 20 amino acids. It will be of interest to determine if the residues that are not conserved are crucial in the anti-apoptotic function of PEA-15.

Vanishin did not augment or inhibit death receptor activated apoptosis; however, we found that ectopic expression of Vanishin in cells induced apoptosis by 2-fold. ERK/MAPK can regulate apoptosis by causing the phosphorylation of the pro-apoptotic protein Bad by Rsk2 [32]. Phosphorylated Bad is retained in an inactive complex with 14-3-3 in the cytoplasm [33]. Vanishin-bound ERK, blocked ERK activation by MEK and retained the ERK in the nucleus. Cells overexpressing Vanishin would have

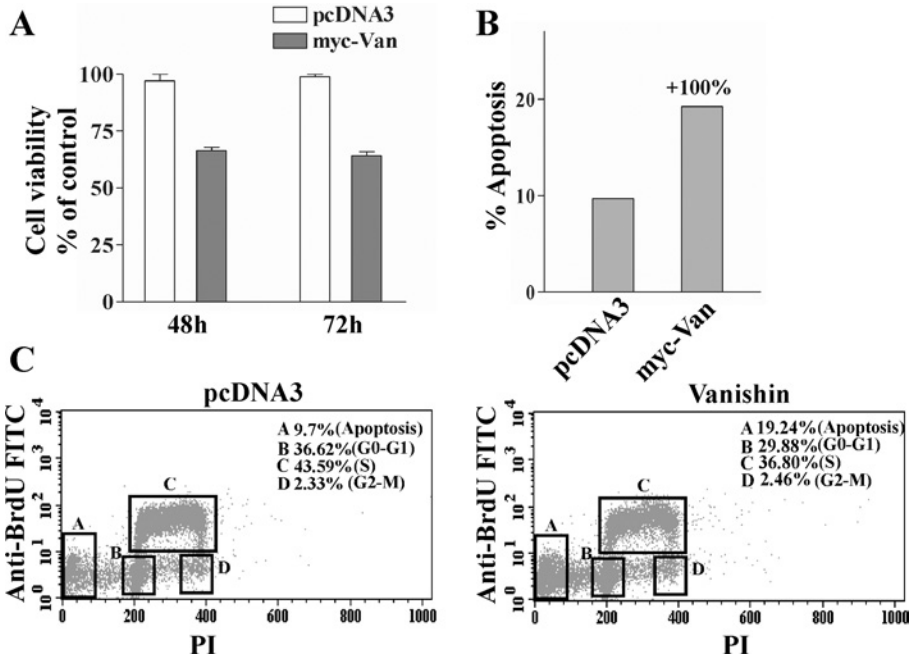

Figure 10 Vanishin decreases cell viability by inducing apoptosis in cells

(**A**) Cell viability was determined by MTT assay. CHO-K1 cells transfected with myc–Vanishin or vector alone were seeded in twelve-well plates at 100000 cells per well. After the indicated times of 48 and 72 h, cells were treated with 0.5 mg/ml of MTT at 37 °C for 4 h. The converted dye was then solubilized with acidic propan-2-ol and the absorbance measured at a wavelength of 570 nm. Cell viability is represented as percentage of control pcDNA3 transfected cells which was set at 100 %. (**B**, **C**) For the cell proliferation and apoptotic assays, CHO cells transfected with pcDNA3 and myc-tagged Vanishin were treated with 10 μM BrdU for 1 h at 37 °C. Cells were then stained with the anti-BrdU FITC antibody and propidium iodide for 30 min as described in the Experimental section. Samples were analysed on a FACScan flow cytometer. (**B**) Percentage of apoptotic cells was obtained from the subdiploid peak of the DNA histogram. (**C**) Percentages of cells in different stages of the cell cycle are indicated by plotting the BrdU fluorescence versus the PI fluorescence.

less-active ERK in the cytoplasm, where Bad is found. Bad that is not phosphorylated translocates to the mitochondria, where it promotes the release of cytochrome *c* and apoptosis. Hence Vanishin may promote apoptosis by blocking ERK-mediated phosphorylation of Bad.

Vanishin promotes nuclear accumulation of ERK even in unstimulated serum-starved cells. In contrast, PEA-15 contains an NES and localizes ERK to the cytoplasm by promoting ERK export out of the nucleus [7]. MEK is also reported to contain an NES and to export inactive ERK from the nucleus [34]. Vanishin differs from PEA-15 in the DED exactly at the NES. Vanishin does not appear to have an NES elsewhere in its sequence. Hence Vanishin would not be expected to promote nuclear export of ERK. Several sequences in PEA-15 that are required for ERK binding (e.g. Asp^{74}) are conserved in Vanishin. Therefore these residues might also be important in Vanishin binding to ERK. If similar sequences are necessary for binding ERK then it is probable that both Vanishin and PEA-15 bind to the same site on ERK. It is thus possible that Vanishin might compete for ERK binding with PEA-15, and perhaps MEK, and in this way promote accumulation of ERK in the nucleus. Competition with MEK might also explain how Vanishin blocks MEK phosphorylation and subsequent activation of ERK. This must be further investigated.

Vanishin promotes accumulation of inactive ERK in the nucleus and Golgi. Vanishin inhibits ERK activity and is itself regulated by ubiquitinylation and proteasomal degradation. Therefore controlled expression of Vanishin could provide the cell with a mechanism to inhibit ERK activity at specific sites in the cell at specific times. As an example, ERK activity in the Golgi at mitosis is associated with Golgi breakdown [35]. Blockage of ERK activity in the Golgi by Vanishin might therefore provide the cell with a mechanism whereby it could control the breakdown of the Golgi in co-ordination with other events in the cell cycle. On the basis of our results, we conclude that Vanishin is the first DED protein reported to be regulated by ubiquitinylation and one of the first ubiquitinylated proteins known to regulate a MAPK. This study therefore provides a new model for the regulation of MAPK pathways.

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