

FIST/HIPK3: a Fas/FADD-interacting Serine/Threonine Kinase that Induces FADD Phosphorylation and Inhibits Fas-mediated Jun NH₂-terminal Kinase Activation

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Abstract

Fas is a cell surface death receptor that signals apoptosis. Several proteins have been identified that bind to the cytoplasmic death domain of Fas. Fas-associated death domain (FADD), which couples Fas to procaspase-8, and Daxx, which couples Fas to the Jun NH₂-terminal kinase pathway, bind independently to the Fas death domain. We have identified a 130-kD kinase designated Fas-interacting serine/threonine kinase/homeodomain-interacting protein kinase (FIST/HIPK3) as a novel Fas-interacting protein. Binding to Fas is mediated by a conserved sequence in the COOH terminus of the protein. FIST/HIPK3 is widely expressed in mammalian tissues and is localized both in the nucleus and in the cytoplasm. In transfected cell lines, FIST/HIPK3 causes FADD phosphorylation, thereby promoting FIST/HIPK3–FADD–Fas interaction. Although Fas ligand-induced activation of Jun NH₂-terminal kinase is impaired by overexpressed active FIST/HIPK3, cell death is not affected. These results suggest that Fas-associated FIST/HIPK3 modulates one of the two major signaling pathways of Fas.

Key words: Fas/CD95 • apoptosis • kinase • Jun NH₂-terminal kinase • signal transduction

Introduction

Fas (APO-1/CD95) is a member of the TNFR family and induces apoptosis when cross-linked with Fas ligand (FasL)¹ (1). The Fas–FasL system has an important role in the immune system, where it is involved in the effector function of cytolytic T cells and in the downregulation of immune responses (2). Clustering of Fas upon binding of the membrane-bound form of FasL recruits the bipartite molecule Fas-associated death domain (FADD), composed of an NH₂-terminal death effector domain (DED) and a COOH-terminal DD (death domain). FADD binds to Fas via homophilic DD–DD interactions and recruits the upstream DED-containing caspase-8 to the receptor via DED–DED interactions. Neighboring caspase-8 molecules within this newly formed death-inducing signaling complex then proteolytically activate each other and ini-

tiate apoptosis by subsequent cleavage of downstream effector caspases (caspase-3, -6, and -7).

An alternative pathway of Fas signaling involves the Fas-binding protein Daxx (3), although these data have been recently challenged (4). It was proposed that upon Fas activation, Daxx interacts with and activates a mitogen-activated protein kinase kinase kinase termed ASK1 (apoptosis signal-regulating kinase 1), leading to the activation of the Jun NH₂-terminal kinase (JNK) and p38 mitogen-activated protein kinase pathways (5, 6). JNK has been implicated in activating apoptosis *in vitro* and *in vivo* (7–9), but its functional role in death receptor signaling, as assayed by expressing dominant negative proteins, has been controversial (10, 11). JNK activation has also been reported to occur downstream of caspases via the cleavage and activation of MEKK1, a JNK kinase kinase (12).

Aside from FADD and Daxx, the intracellular domain of Fas was reported to interact with several other proteins, including FAF-1, FAP-1, SUMO-1/Sentrin, and UBC9. FAF-1 augments apoptosis when overexpressed in mammalian cells (13), in contrast to the phosphatase FAP-1, which has been proposed to act as an inhibitor of cell death (14). The ubiquitin-like protein SUMO (15) and its ligase

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¹Abbreviations used in this paper: aa, amino acid(s); DED, death effector domain; FADD, Fas-associated death domain; FasL, Fas ligand; FIST, Fas-interacting serine/threonine kinase; HIPK, homeodomain-interacting protein kinase; JNK, Jun NH₂-terminal kinase.

UBC9 (16, 17) are involved in the modification of a number of proteins, thereby targeting them to the nuclear membrane and certain intranuclear structures (18).

Here we report the identification of a novel Fas-interacting protein that we have named Fas-interacting serine/threonine (Ser/Thr) kinase (FIST)3. FIST3, which is identical to homeodomain-interacting protein kinase (HIPK)3, is a Ser/Thr kinase that is capable of inducing FADD phosphorylation. Moreover, FIST/HIPK3 modulates FasL-induced JNK activation.

Materials and Methods

Antibodies. Antibodies used for immunoprecipitation, JNK activation assay, immunofluorescence and Western blot include: anti-Flag M2 (Sigma-Aldrich), anti-FADD (Signal Transduction Labs.), anti-Fas C-20 (Santa Cruz Biotechnology, Inc.), anti-PML (promyelocytic leukemia protein) C7 (a gift of H. de Thé, Hopital St. Louis, Paris, France), anti-phospho-JNK (New England Biolabs), peroxidase- or Cy5-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories), peroxidase-conjugated anti-IgG1 secondary antibody (Southern Biotechnology Associates, Inc.), and Alexa-conjugated secondary antibodies (Molecular Probes). Rabbit polyclonal antibodies were raised against amino acids (aa) 775–878 (clone 25 isolated from the two-hybrid screen) of murine (m)FIST/HIPK3 and aa 766–920 of the rat homologue (a gift of J. Palvimo, Institute of Biomedicine, Helsinki, Finland).

Two-Hybrid Screen. A mouse embryo cDNA library cloned into the NotI site of the vector pVP16 (19), which codes for fusion proteins of the viral transcription activation domain VP16, was provided by S. Hollenberg (Vollum Institute, Portland, Oregon). The two-hybrid screen and the generation of cytoplasmic mutants of Fas were previously described (16).

Cloning of FIST/HIPK3 cDNA. A cDNA clone for mFIST/HIPK3 was isolated from a mouse testis cDNA library (Stratagene) using a ³²P-labeled DNA fragment (random-primed DNA labeling kit; Boehringer Mannheim) encoding aa 775–878. Double-stranded sequencing revealed an open reading frame of 3,576 nucleotides encoding a protein of 1,192 aa with a putative Ser/Thr kinase domain. EST clones encoding the human homologue of murine FIST/HIPK3 were identified in the dbEST database at the National Center for Biotechnology Information (EST clones H37893 and R57262) by performing a database search using mFIST/HIPK3 (<http://ncbi.nlm.nih.gov>). The accession numbers (available from EMBL/GenBank/DBJ) for the human and murine FIST/HIPK3 cDNA sequences reported in this paper are AF305238 and AF305239, respectively.

Northern Blot Analysis. Northern blot analysis was performed using human and mouse multiple tissue and human cancer cell Northern blots (CLONTECH Laboratories, Inc.) with a ³²P-labeled antisense RNA probe encoding aa 531–658 of hFIST/HIPK3 or a cDNA probe encoding aa 333–591 of mFIST/HIPK3 according to the manufacturer's instructions.

Expression Vectors. mFIST/HIPK3 isolated from the mouse testis cDNA library or fragments containing the kinase domain (aa 142–608) or the COOH-terminal domain of mFIST/HIPK3 (aa 592–1192) were subcloned into a derivative of the pCR3 expression vector (Invitrogen), in frame with an NH₂-terminal Flag tag. Mutations of the kinase domain of mFIST/HIPK3 (Lys226 → Ser, K226S or Asp322 → Asn, D322N) were achieved by site directed mutagenesis with two sequential rounds of PCR, using

oligonucleotides containing the target mutation. PCR products were cloned into pCR-blunt (Invitrogen) and subcloned into the pCR3-derived expression vector, in frame with an NH₂-terminal Flag. Human Fas cDNA was cloned into pCR3 with or without an NH₂-terminal Flag tag. Vesicular stomatitis virus (VSV)-tagged mutant FADD lacking aa 202–208 or 191–208, respectively, were generated as described above. The following expression plasmids were obtained from the indicated sources: Flag-JNK (C. Widmann, Institute of Cell Biology and Morphology, Lausanne, Switzerland) and PML (H. de Thé, Hopital St. Louis, Paris, France).

Immunoprecipitation. 293T cells were seeded in 6-cm plates and transfected for 8–15 h with the calcium phosphate precipitation method (20). Cells were harvested 24–36 h after the onset of transfection and lysed in lysis buffer containing 0.1% NP-40, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and protease inhibitors (Complete™; Boehringer Mannheim), followed by three freeze-thaw cycles. After preclearing for at least 1 h on Sepharose 6B (Amersham Pharmacia Biotech), Flag-tagged proteins were immunoprecipitated for at least 2 h with anti-Flag agarose. Immunoprecipitates were washed three times in lysis buffer without protease inhibitors before analysis by SDS-PAGE and Western blotting.

Sample fractionation with nonionic NP-40 detergent was performed as described (21). In brief, the NP-40-soluble fraction was separated from the insoluble fraction by centrifugation. The pellet was washed twice in PBS and after sonication, both the washed pellet and the initial supernatant were solubilized in SDS sample buffer containing 100 mM dithiothreitol. For the induction of apoptosis, Jurkat cells were treated with 100 ng/ml SuperFasL (Alexis) for 5 h in the presence and the absence of z-VAD-fmk (50 μM).

In Vitro Kinase Assay and Phosphoamino Acid Analysis. Expression plasmids for wild-type or mutant Flag-tagged mFIST/HIPK3 (full length or kinase domain) were transfected in 293T cells in 10-cm plates. After cell lysis in buffer containing 1% Brij-96, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM MgCl₂, 50 mM NaF, 1 mM NaVO₄, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 250 μg/ml pepablock, and 10 mM Na₄P₂O₇, immunoprecipitated Flag-tagged proteins were subjected to in vitro kinase assay in 0.05% Brij-96, 1 mM Tris-HCl, pH 7.5, 7.5 mM NaCl, 25 mM Hepes, pH 7.3, 10 μM MgCl₂ in the presence of 10 μCi γ-[³²P]ATP as previously described (22). The presence of Flag-tagged proteins was confirmed by Western blotting.

For phosphoamino acid analysis, phosphorylated proteins were separated by SDS-PAGE. Dried bands containing phosphorylated FIST/HIPK3 were excised and proteins were extracted from the gel by incubation in 50 mM NH₄HCO₃, 0.1% SDS, 1% β-mercaptoethanol for 10 min at 100°C and 2 h at room temperature. Proteins were precipitated in presence of 20 μg of RNase A, 15% TCA for 90 min at 4°C and washed once in 10% TCA and once in ethanol/ether (1:1) before drying. Proteins were lysed in 6 M HCl for 70 min at 110°C and 10 min on ice, dried, and resuspended in acetic acid/formic acid/H₂O (156:44:1,800), pH 1.9, containing cold phospho-serine, phospho-threonine, and phospho-tyrosine (250 μg/ml each). Samples were analysed by two-dimensional electrophoresis on cellulose TLC plates (Merck) at 1,600 V in acetic acid/formic acid/H₂O (156:44:1,800), pH 1.9, for the first dimension and at 2,000 V in 5% acetic acid, 0.5% pyridine for the second dimension. Standards were revealed with 0.2% ninhydrine in acetone.

JNK Activation Assays. For JNK activation assays, transfected 293T cells were treated for 4 h with SuperFasL (Alexis) and lysed

as described before. Cell lysates were analysed by Western blotting for protein expression and the presence of activated (phosphorylated) JNK.

Confocal Immunofluorescence Microscopy. 293T cells seeded on glass coverslips in 6-cm plates were transfected. 2 d after transfection, cells were washed with PBS, fixed with 4% paraformaldehyde in PBS, and permeabilized in 0.1% saponin in PBS. After washing, cells were blocked in PBS containing 0.1% saponin and 2% skim milk, incubated with appropriate primary and secondary antibodies in blocking buffer, and mounted in flouresave reagent (Calbiochem). Confocal microscopy was performed on a ZEISS Axiovert 100 microscope (ZEISS Laser Scanning Microscope 410) with a 63× oil objective. To detect Cy5 fluorochrome, a helium laser was filtered at 633 nm. An argon laser at 488 nm was used to detect Alexa fluorochrome. Under standard imaging conditions, no signal from one fluorochrome could be detected on

the other filter set. Standardized conditions for the pinhole size, for gain and offset (brightness and contrast), were used for image capture, and each image was the average of 16 scans.

Results and Discussion

To identify novel proteins interacting with Fas, we used the yeast two-hybrid system to screen for proteins that bind to the cytosolic domain of murine Fas (Ser183 → Glu306). A library of murine embryo cDNA fused to the transcription activation domain VP16 (23) was screened for possible interaction with the Fas bait. 15 positive clones were obtained from 5×10^6 clones screened. Sequencing of the inserts revealed that four inserts corresponded to the murine SUMO-E2 ligase UBC9 as previously reported (16, 17,

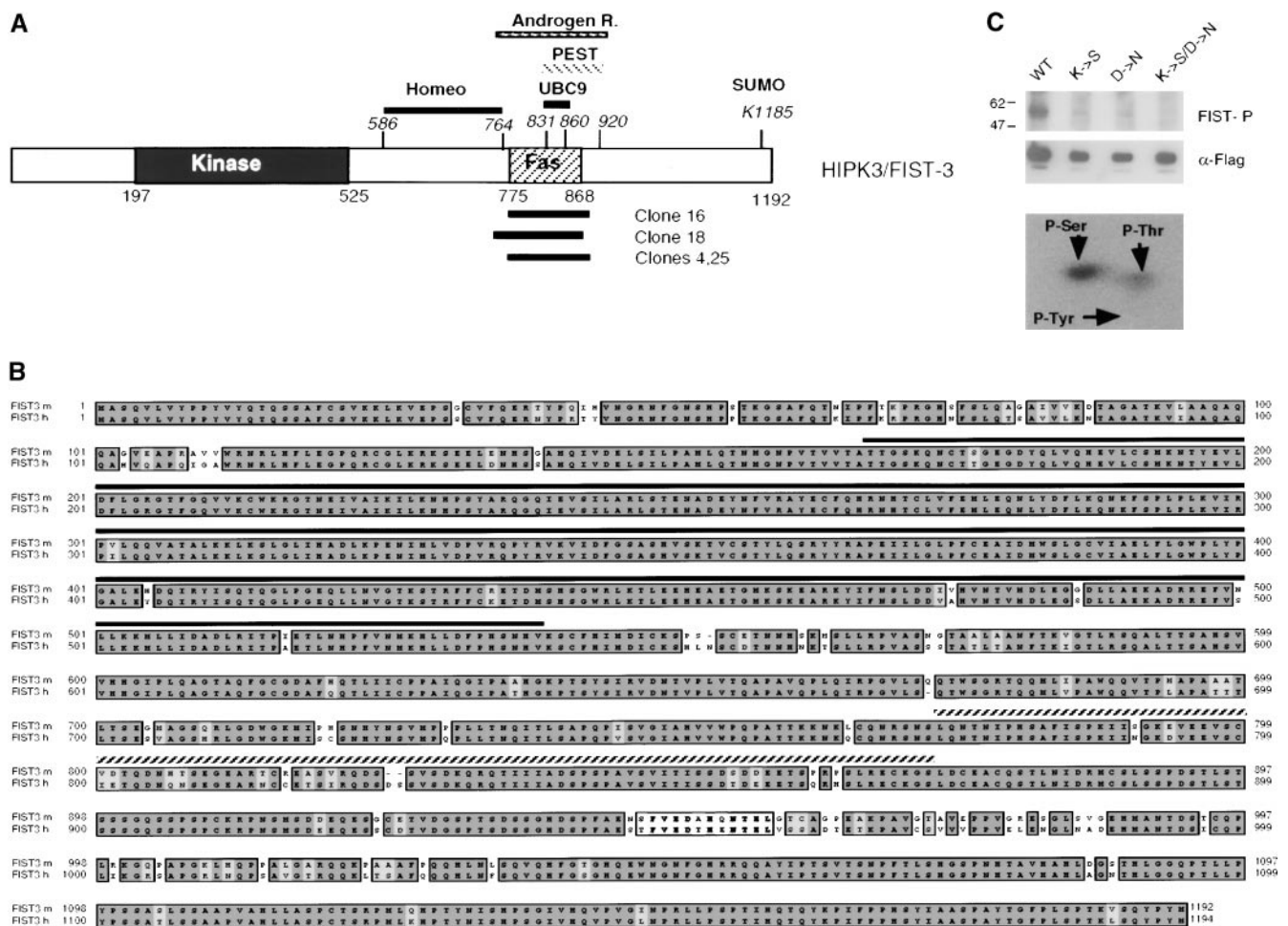


Figure 1. Structure, expression and enzymatic activity of FIST/HIPK3. (A and B) Predicted aa sequence and structural organization of human and murine FIST/HIPK3. FIST/HIPK3 contains a kinase domain and a PEST sequence, which are linked by a region of ~200 aa that was identified to interact with the homeoproteins. The PEST domain overlaps with the interaction site for Fas, UBC9, and the androgen receptor (28). Lys1,185 is predicted to be an acceptor site for SUMO. For each block of aligned sequences, black boxes indicate aa sequence identity, and gray shading indicates sequence similarity through conservative aa substitutions. The kinase domain and the Fas interaction domain are indicated by a solid and hatched line, respectively. (C) Kinase activity of FIST/HIPK3. Flag-tagged FIST/HIPK3 kinase was immunoprecipitated from 293T cells expressing the kinase domain of wild-type or mutated FIST/HIPK3 (Lys226Ser or Asp322Asn) and from control transfectants. The immunoprecipitates were subjected to an in vitro kinase assay, followed by SDS-PAGE and transfer to nitrocellulose, and the membrane was revealed by autoradiography (top, FIST/HIPK3-P) or by immunoblot analysis with an anti-Flag-antibody. Bottom panel, phosphorylated FIST/HIPK3 kinase was subjected to SDS-PAGE, and radioactive bands corresponding to phosphorylated proteins were excised and hydrolyzed as described in Materials and Methods. Phosphoamino acids were resolved by two-dimensional electrophoresis. Positions of unlabeled internal phosphoamino acid standards are indicated by arrowheads.

24). Another group of 6 clones contained identical (4 clones) or highly homologous sequences (2 clones) (Fig. 1 A). In the two-hybrid system, the protein fragments (we named the protein FIST for Fas-interacting Ser/Thr kinase) encoded by the insert of the various clones all strongly interacted with the complete cytoplasmic domain of Fas but not with TNFR1, lamin A, LDL receptor, or poly Ig receptor (not shown). An EMBL/GenBank/DDBJ search revealed that the sequences of clones 4, 16, 18, and 25 were contained in a murine gene recently identified as HIPK3, whereas clones 10 and 12 corresponded to murine HIPK1 (25). We cloned the full-length murine and human homologue of FIST/HIPK3, whose aa sequences were found to be more than 90% identical (Fig. 1 B). FIST/HIPK3 contains an NH₂-terminal kinase domain, followed by the domain that interacts with homeoproteins and a PEST sequence. For HIPK2, it was demonstrated that it binds to the SUMO-E2 ligase UBC9 through the PEST domain and that the protein is consequently modified on a lysine close to the COOH terminus by the ubiquitin-like SUMO-1 (21). The Fas-interacting domain of FIST/HIPK3 (aa 775–868), which was deduced from the original yeast two-hybrid clones, partly overlaps with the UBC9 binding domain and is highly conserved. Interestingly, this sequence (and the kinase sequence but not the remainder) is highly conserved in a sequence found in the *Caenorhabditis elegans* genome (F20B6). The kinase domain of FIST/HIPK3 shows extensive sequence homology with the Ser/Thr kinase YAK1 from *Saccharomyces cerevisiae* (26) and the minibrain gene that is implicated in learning defects associated with Down's syndrome (27).

We tested whether the predicted Ser/Thr kinase domain of murine FIST/HIPK3 was functionally active. The kinase-containing region of FIST/HIPK3 (FIST/HIPK3kin; aa 142–608), tagged at the NH₂ terminus with a Flag epitope, was expressed in 293T cells, immunoprecipitated with an anti-Flag antibody, and subjected to an in vitro kinase assay. SDS-PAGE analysis revealed a ³²P-labeled 50-kD band, indicating that FIST/HIPK3 is a protein kinase that becomes autophosphorylated, in agreement with a recent report on the rat homologue of FIST/HIPK3 (28). Similar results were obtained with full-length FIST/HIPK3 (data not shown). The kinase activity was impaired when either of the conserved residues, Lys226 or Asp322, two invariant residues essential for the enzymatic activity of protein kinases (29), were mutated to Ser and Asn, respectively (Fig. 1 C). Two-dimensional gel electrophoresis revealed that phosphorylation occurred on Thr and Ser residues (Fig. 1 C).

FIST/HIPK3 mRNA is widely expressed. Northern blot analysis revealed that FIST/HIPK3 is constitutively expressed as a transcript of ~7.5 kb in most human and murine tissues (Fig. 2, A and C) and in a panel of human cell lines (Fig. 2 B). In murine (and human, detectable after longer exposure) testis, an additional transcript of 4.4 kb was detected. The human FIST/HIPK3 gene is localized on chromosome 7 (30).

Subcellular localization of FIST/HIPK3 was assessed in 293T cells transiently transfected with either FIST/HIPK3 or kinase-defective FIST/HIPK3 (Fig. 3 A). In agreement with previous reports on the subcellular localization of HIPK2, a large part of FIST/HIPK3 was localized to nu-

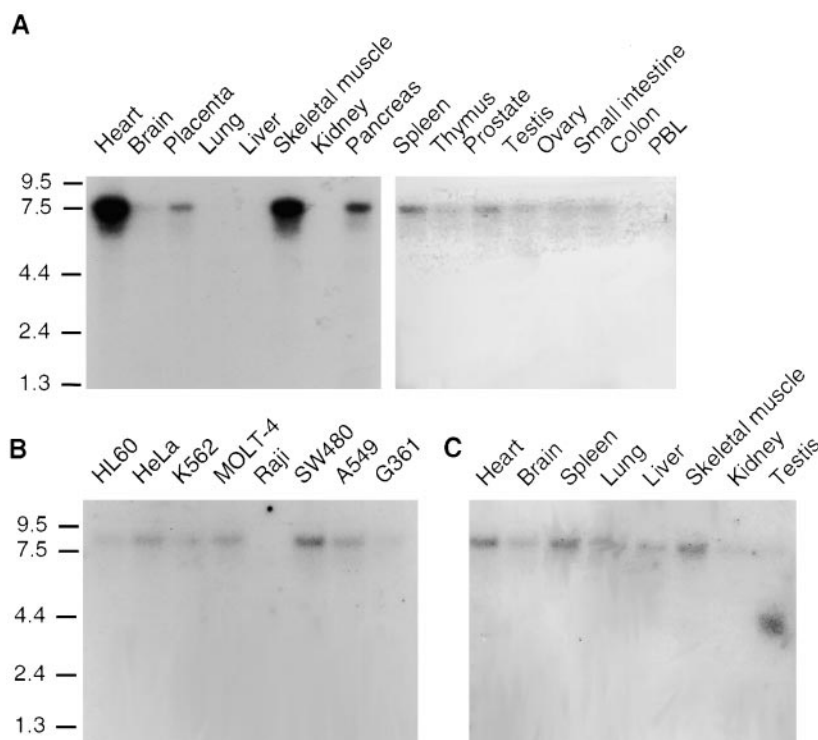


Figure 2. Expression of FIST/HIPK3. Tissue distribution of the FIST/HIPK3 transcripts. Northern blots of various human tissues and tumor cell lines (A and B) and mouse tissues (C) (CLONTECH Laboratories, Inc.) were hybridized with a radioactive probe of FIST/HIPK3.

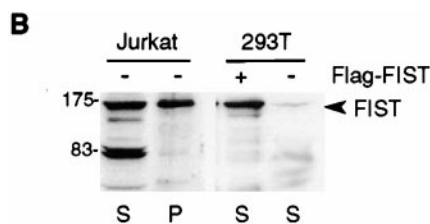
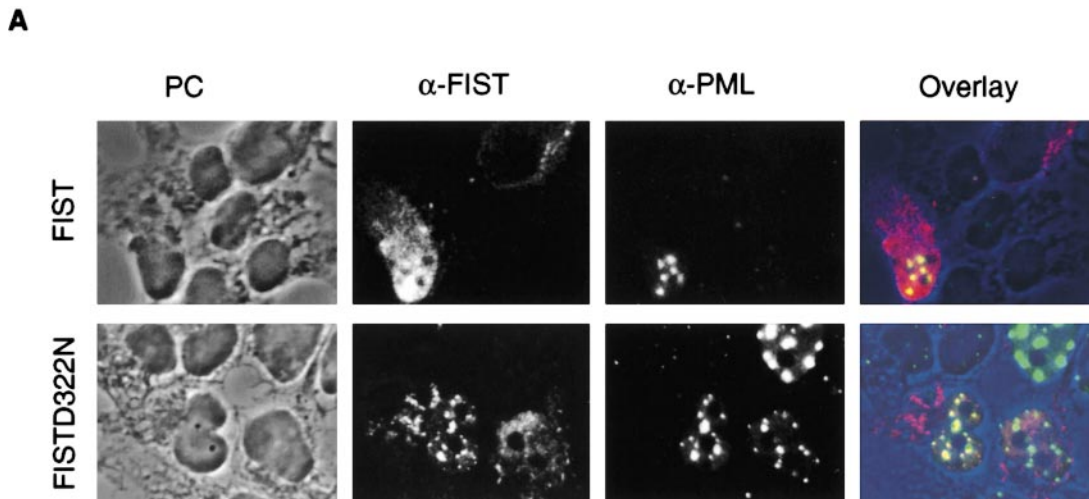


Figure 3. FIST/HIPK3 is localized in nuclear and cytoplasmic compartments. (A) 293T cells were transfected with FIST/HIPK3 or kinase-defective FIST/HIPK3 and PML, and their subcellular localization was determined 48 h after transfection by confocal microscopy using the respective antibodies. The overlay of PML fluorescence (green) and FIST/HIPK3 fluorescence (red) is shown in yellow. The cellular localization of FIST/HIPK3 was identical in the absence of cotransfected PML (data not shown). PC, phase contrast. (B) Fractionation of endogenous FIST/HIPK3. Jurkat T cells were lysed in NP-40. Cellular extracts were then separated into the NP-40-insoluble (P) and -soluble (S) fractions and analyzed by Western blot using an anti-FIST/HIPK3 antibody. The specificity of the antibody was verified in 293T cells transfected with a FIST/HIPK3 expression vector or a vector control, respectively (two right lanes). The weak band in nontransfected cells corresponds to endogenous FIST/HIPK3.

clear bodies, also referred to as PML bodies or nuclear domain (ND)10, where it colocalized with PML (21). However, FIST/HIPK3 also showed diffuse cytoplasmic staining. The kinase-defective mutant of FIST/HIPK3 displayed an identical subcellular localization. This localization pattern was confirmed using cell fractionation studies of Jurkat T cells where a substantial amount of the kinase was recovered in the detergent-soluble, nonnuclear fraction (Fig. 3 B).

To map FIST/HIPK3 and Fas interaction more precisely, several truncated versions of the Fas cytosolic domain (Fig. 4 A) were constructed. In yeast, clone 25/FIST/HIPK3 (Fig. 1) interacted strongly with the entire cytosolic domain of Fas (Arg166–Glu306) and with the fragments that lacked either 17 aa adjacent to the membrane-interacting segment (Ser183–Glu306) or the COOH-terminal 18 aa (Arg166–Asp288) (Fig. 4 A). Weak interaction was observed with Fas containing the *lpr^{cs}* mutation in the DD that prevents FADD recruitment and thus renders Fas inactive (31). No binding at all was observed with the fragment of Fas corresponding to the DD (Ile207–Asp288) alone or with the COOH-terminal 18 aa (Leu289–Glu306). Thus, efficient binding of FIST/HIPK3 to the cytosolic domain of Fas requires both a functional DD and part of the membrane proximal segment.

To determine whether FIST/HIPK3 also interacts with Fas in mammalian cells, both proteins were expressed in 293T cells. Transfection with Flag-tagged FIST/HIPK3 constructs and subsequent immunoprecipitation revealed that full-length FIST/HIPK3 and its kinase-inactive ver-

sion interacted with Fas. Moreover, the Flag-FIST/HIPK3 COOH-terminal domain (FIST/HIPK3-C; aa 592–1,192), which includes the segment that interacts with Fas in yeast (Fig. 1), was able to pull down Fas (Fig. 4 B). In contrast, no interaction was detectable with the region containing the kinase domain only. Interestingly, no FIST/HIPK3 was detected when Fas was immunoprecipitated, suggesting that overexpression of Fas which triggers signaling leads to a weakening of the Fas–FIST/HIPK3 interaction.

We next investigated whether FADD would compete with the binding of FIST/HIPK3 to Fas, as both FADD and FIST/HIPK3 interact with overlapping segments of Fas. FADD, Fas, and FIST/HIPK3 were therefore expressed in 293T cells and while analyzing the data, we made an interesting observation (Fig. 4 C). In SDS-PAGE, endogenous or overexpressed FADD always migrates as two molecular species, which correspond respectively to the phosphorylated and unphosphorylated protein (32). Phosphorylation occurs on Ser194 close to the COOH terminus of human FADD (33, 34). The role of FADD phosphorylation is not known, and the kinase responsible for this modification has not been identified. Whenever full-length FIST/HIPK3 or the kinase domain of FIST/HIPK3 were coexpressed, the phosphorylated FADD species was predominantly detected, suggesting that FADD was a substrate of FIST/HIPK3. In contrast, no induction of FADD phosphorylation was seen with kinase-dead FIST/HIPK3. Phosphorylation occurred at or close to the natural phosphorylation site (Ser194), as a mutant FADD lacking aa residues 191–208 (Fig. 4 D) was not phosphorylated. In an in

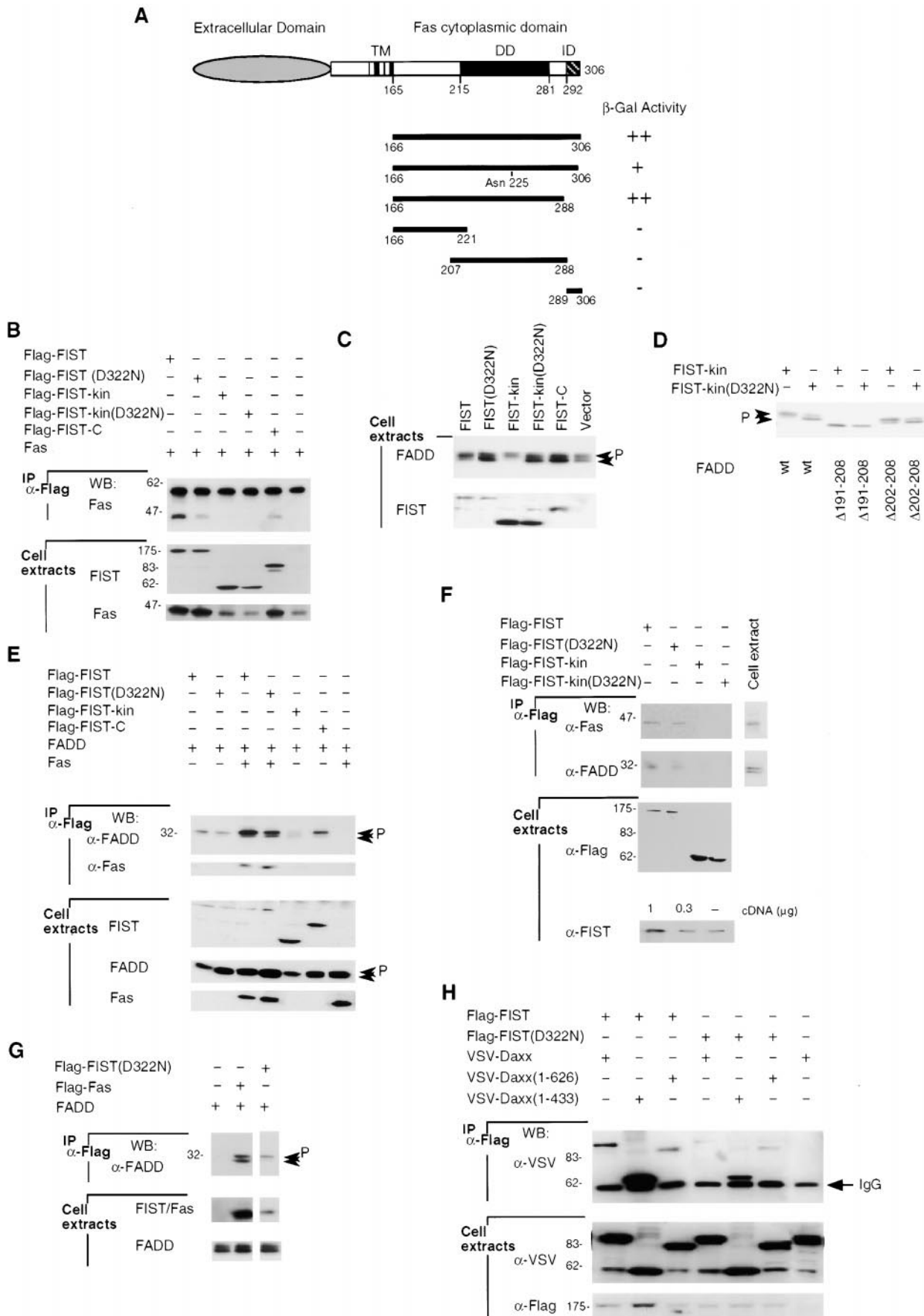


Figure 4.

vitro kinase assay, where immunoprecipitated FIST/HIPK3 was added to purified glutathione *S*-transferase–FADD, incorporation of ³²P into FADD was very weak (data not shown). Thus, phosphorylation of FADD by FIST/HIPK3 may be indirect via a FIST/HIPK3-activated kinase. The fact that full-length FIST/HIPK3 was less active than the FIST/HIPK3 kinase domain alone can be explained by different expression levels (full-length FIST/HIPK3 is usually expressed poorly as compared with the kinase domain alone; Fig. 4, C and E).

These experiments raised the possibility that FADD is directly interacting with FIST/HIPK3, and indeed, when FADD and Flag–FIST/HIPK3 constructs were overexpressed in 293T cells, FADD was detected in anti-Flag immunoprecipitates (Fig. 4 E). Similar to the FIST/HIPK3–Fas interaction, the COOH-terminal segment but not the kinase domain appeared to mediate the FIST/HIPK3–FADD interaction. FIST/HIPK3 associated only weakly with FADD in the absence of Fas, suggesting that Fas was required for the stabilization of the FIST/HIPK3–FADD complex (Fig. 4 E).

FIST/HIPK3–Fas–FADD interaction was also observed in cells in which Fas and FADD were not overexpressed (Fig. 4 F). The formation of the trimolecular complex composed of transfected Flag-tagged FIST/HIPK3 and endogenous FADD and Fas was only detected with FIST/HIPK3, which contained the COOH-terminal part. No FIST/HIPK3 was found to coimmunoprecipitate with Fas when the complex was precipitated with antibodies to Fas or via Flag-tagged soluble FasL, in agreement with the data obtained with cells that overexpress these proteins (data not shown). Using anti-FIST/HIPK3 antibodies to immunoprecipitate endogenous FIST/HIPK3, no complex formation was observed, suggesting that the epitopes recognized by the antibodies in Western blots are masked in the FIST–FADD–Fas complex. To ascertain that the observed Fas–FADD–Flag–FIST/HIPK3 interaction was of physiological significance, we adjusted the levels of exogenous Flag–

FIST/HIPK3 so that they were only slightly higher than that of endogenous FIST/HIPK3 (Fig. 4 F).

It is well known that phosphorylated and unphosphorylated FADD interact equally well with Fas (Fig. 4 G; reference 32). In contrast, under conditions where FADD was not completely phosphorylated by FIST/HIPK3, the phosphorylated form was predominant in FIST/HIPK3 immunoprecipitates, suggesting that phosphorylation of FADD induced by FIST/HIPK3 leads to an increased association of the kinase with FADD (Fig. 4 G).

From the above results, it appears that FIST/HIPK3 is a protein capable of interacting with a surface receptor but at the same time also functions as a nuclear protein. This is reminiscent of Daxx (3), and we therefore investigated whether the two proteins interact. Daxx and FIST/HIPK3 were coexpressed in 293T cells, and strong binding between full-length FIST/HIPK3 and the NH₂-terminal portion (aa 1–433) was indeed detected (Fig. 4 H). By contrast, the COOH-terminal, Fas-interacting segment of Daxx (aa 628–740) (3) failed to bind FIST/HIPK3 (data not shown). Interestingly, binding of FIST/HIPK3 to Daxx was dependent on the kinase activity of FIST/HIPK3, as the kinase-dead version of FIST/HIPK3 exhibited a greatly diminished binding activity (Fig. 4 H). The significance of this observation is presently unclear.

Transient transfection assays were used to examine the consequences of FIST/HIPK3 overexpression and FADD phosphorylation on Fas-induced cell death. We found that death signals were transmitted normally in the presence of FIST/HIPK3. A second signaling pathway triggered by Fas leads to the activation of JNK, whose activation can ultimately lead to caspase-8-independent apoptosis (3). This second activity has been attributed to Daxx, which interacts with the cytoplasmic domain of Fas and whose overexpression results in an ASK1-dependent JNK activation (5). We activated JNK by adding FasL to 293T cells and found that under these conditions, JNK activation was maximal 4 h after stimulation and was mostly independent of caspase

Figure 4. Interaction of FIST/HIPK3 with Fas in yeast and mammals. (A) Several deletion mutations of the cytosolic domain of Fas (TM, transmembrane segment of Fas; ID, inhibitory domain, sequence proposed to interact with FAP-1) were cloned as fusion proteins with the DNA binding domain of Gal4. Interaction of these with the VP16 fusion protein of FIST/HIPK3 (aa 775–878, clone 25; Fig. 1) was tested in a two-hybrid system. β -galactosidase activity is indicated. ++, strong reaction (dark blue color); +, weak interaction (faint blue color); –, no reaction. (B) Interaction of FIST/HIPK3 with Fas. 293T cells were cotransfected with expression vectors for Flag-tagged FIST/HIPK3 and Fas as indicated, and anti-Flag immunoprecipitates (IP) were analyzed for the presence of Fas by immunoblotting using anti-Fas antibody. In this and the subsequent figures (Fig. 4, E–H), the expression levels of the transfected proteins in cell extracts are shown in the lower panels. (C) FIST/HIPK3 overexpression results in FADD phosphorylation. 293T cells were transfected with expression vectors for Flag-tagged FIST/HIPK3, and phosphorylation of endogenous FADD was analyzed by Western blotting. The upper of the two bands corresponds to phosphorylated FADD. (D) FIST/HIPK3 induces FADD phosphorylation between aa residues 191 and 201. 293T cells were cotransfected with expression vectors for Flag-tagged kinase domains of FIST/HIPK3, wild-type FADD, FADD lacking the COOH-terminal residues 192–208, or FADD lacking residues 202–208. FADD was analyzed by Western blotting. Note that mutant FADD Δ 192–208 yields only one phosphospecies. (E) Interaction of FIST/HIPK3 with phosphorylated FADD. 293T cells were cotransfected with expression vectors for Flag-tagged FIST/HIPK3, FADD, and Fas as indicated, and anti-Flag immunoprecipitates (IP) were analyzed for the presence of FADD or Fas by immunoblotting using an anti-FADD or anti-Fas antibody. (F) Interaction of FIST/HIPK3 with endogenous FADD and Fas. 293T cells were transfected with the various FIST/HIPK3 constructs and anti-Flag immunoprecipitates analyzed as described in E. 1 μ g of FIST/HIPK3-encoding plasmids was used to transfect 293T cells, which resulted in protein concentrations that were comparable to that of the endogenous protein (lower panel). (G) Interaction of Fas and FIST/HIPK3 with FADD. Expression vectors for Flag-tagged Fas and FADD were cotransfected into 293T cells, and anti-Flag immunoprecipitates were analyzed for the presence of FADD by immunoblotting using anti-FADD antibody. Note that phosphorylated and unphosphorylated FADD is associated with Fas, whereas only the phosphorylated form is found in the IP of FIST/HIPK3. (H) Interaction of FIST/HIPK3 with Daxx requires the kinase activity of FIST/HIPK3. Binding experiments were done as described above, by overexpressing Flag-tagged FIST/HIPK3 and VSV-tagged Daxx in 293T cells.

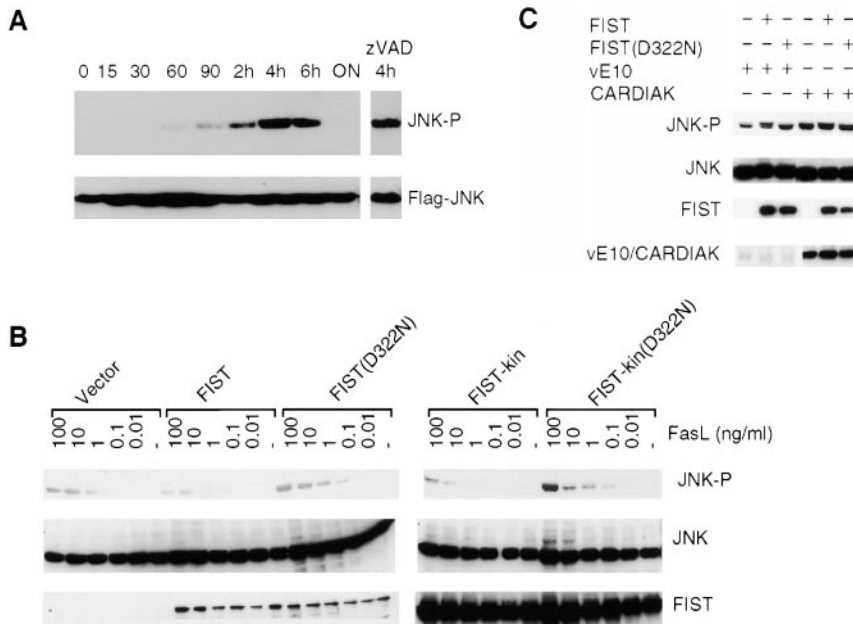


Figure 5. Activation of JNK by FIST/HIPK3. (A) Kinetics of FasL-induced JNK activation. 293T cells (2×10^5) were transfected with 3 μ g of a JNK expression construct. 48 h after transfection, FasL (100 ng/ml) was added and JNK activation was determined after various time periods by Western blotting using an antibody specifically detecting active, phosphorylated JNK. JNK activation measured after 4 h is only partly inhibited by the general caspase inhibitor z-VAD-fmk (right panel). The expression levels of JNK were determined using anti-Flag. (B) As in A, but the JNK expression construct was transfected in combination with plasmids encoding full-length FIST/HIPK3 or its kinase domain alone. The kinase domain was either wild-type (wt) or mutated (D322N). JNK activation induced by increasing concentrations of FasL was determined 4 h after the addition of the ligand by Western blotting using an antibody specifically detecting active, phosphorylated JNK. (C) FIST does not suppress JNK activation induced by viral E10 or CARDIAK/RIP2. JNK activation was determined as in B.

activation, as the general caspase inhibitor z-VAD had little effect (Fig. 5 A). When FIST/HIPK3 was overexpressed, JNK activation was severely impaired, but only when the catalytic site was intact, suggesting that phosphorylation of an unknown target protein negatively regulates signals that lead to FasL-induced JNK activation (Fig. 5 B). When overexpressed, CARDIAK/RIP2 and viral E10 are both potent inducers of JNK activation (22, 35). The presence of FIST/HIPK3, however, did not modulate JNK activation under these conditions (Fig. 5 C), indicating that FIST/HIPK3 does not modulate all pathways leading to JNK activation.

In summary, we have identified FIST/HIPK3 as a kinase that interacts with Fas and FADD. FIST/HIPK3 appears to belong to a growing family of proteins that shuttle between the cytoplasm and nuclear PML bodies in a SUMO-dependent manner (18). For HIPK2 it has been shown that SUMO modification occurs on a lysine close to the COOH terminus (21) that is conserved in all three HIPKs. It is therefore likely that such posttranscriptional modification also occurs in the FIST/HIPK3 protein, considering also the physical presence of the molecules required for SUMO modification (the cytoplasmic domain of Fas has been previously shown to interact with both the SUMO ligase UBC9 and its substrate SUMO). One of several plausible models would predict that Fas-associated FIST/HIPK3 becomes SUMO modified by UBC9 upon Fas activation and subsequently translocates to PML bodies. Although the role of PML bodies remains elusive despite links to oncogenesis and viral replication, it is noteworthy that inhibition of PML body formation caused by the deletion of the PML protein suppresses Fas-induced apoptosis (36). Considering that FIST/HIPKs are also potent repressors of transcription (25), it is tempting to speculate that FIST/HIPK3 may participate in a Fas-dependent nuclear

response. This function is similar to the role suggested for Daxx, which interestingly is also modified by SUMO and proposed to shuttle from Fas to the nucleus (3, 37, 38). Moreover, Daxx interacts with FIST/HIPK3, at least upon overexpression, and it is therefore likely that the two proteins modulate each other's activities in a yet undefined manner.

Additional work is also required to determine the exact functional significance of the Fas-FADD-FIST/HIPK3 interaction. FADD becomes phosphorylated in the presence of FIST/HIPK3, and this phosphorylation is important for FADD-FIST/HIPK3 interaction to occur. In agreement with other studies, FADD phosphorylation does not appear to regulate apoptosis, as FasL-induced apoptosis is not affected by FIST/HIPK3 overexpression. Recently, several uncharacterized Fas-associated Ser/Thr kinase activities were described that phosphorylate Fas and FADD (39). One of these kinases exhibits a molecular mass of ~ 120 kD and could therefore correspond to FIST/HIPK3. We found that FasL-mediated JNK activation is impaired by active FIST/HIPK3, but whether this is due to FADD phosphorylation remains to be established in future studies.

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References

- Ashkenazi, A., and V.M. Dixit. 1998. Death receptors: signaling and modulation. *Science*. 281:1305-1308.

2. Nagata, S. 1997. Apoptosis by death factor. *Cell*. 88:355–365.
3. Yang, X., R. Khosravi-Far, H.Y. Chang, and D. Baltimore. 1997. Daxx, a novel Fas-binding protein that activates JNK and apoptosis. *Cell*. 89:1067–1076.
4. Torii, S., D.A. Egan, R.A. Evans, and J.C. Reed. 1999. Human Daxx regulates Fas-induced apoptosis from nuclear PML oncogenic domains (PODs). *EMBO (Eur. Mol. Biol. Organ.) J.* 18:6037–6049.
5. Chang, H.Y., H. Nishitoh, X. Yang, H. Ichijo, and D. Baltimore. 1998. Activation of apoptosis signal-regulating kinase 1 (ASK1) by the adapter protein Daxx. *Science*. 281:1860–1863.
6. Kyriakis, J.M., and J. Avruch. 1996. Sounding the alarm: protein kinase cascades activated by stress and inflammation. *J. Biol. Chem.* 271:24313–24316.
7. Yang, D.D., C.Y. Kuan, A.J. Whitmarsh, M. Rincon, T.S. Zheng, R.J. Davis, P. Rakic, and R.A. Flavell. 1997. Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. *Nature*. 389:865–870.
8. Xia, Z., M. Dickens, J. Raingeaud, R.J. Davis, and M.E. Greenberg. 1995. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science*. 270:1326–1331.
9. Zanke, B.W., K. Boudreau, E. Rubie, E. Winnett, L.A. Tibbles, L. Zon, J. Kyriakis, F.F. Liu, and J.R. Woodgett. 1996. The stress-activated protein kinase pathway mediates cell death following injury induced by cis-platinum, UV irradiation or heat. *Curr. Biol.* 6:606–613.
10. Verheij, M., R. Bose, X.H. Lin, B. Yao, W.D. Jarvis, S. Grant, M.J. Birrer, E. Szabo, L.I. Zon, J.M. Kyriakis, et al. 1996. Requirement for ceramide-initiated Sapk/Jnk signaling in stress-induced apoptosis. *Nature*. 380:75–79.
11. Goillot, E., J. Raingeaud, A. Ranger, R.I. Tepper, R.J. Davis, E. Harlow, and I. Sanchez. 1997. Mitogen-activated protein kinase-mediated Fas apoptotic signaling pathway. *Proc. Natl. Acad. Sci. USA*. 94:3302–3307.
12. Deak, J.C., J.V. Cross, M. Lewis, Y. Qian, L.A. Parrott, C.W. Distelhorst, and D.J. Templeton. 1998. Fas-induced proteolytic activation and intracellular redistribution of the stress-signaling kinase MEKK1. *Proc. Natl. Acad. Sci. USA*. 95:5595–5600.
13. Chu, K.T., X.H. Niu, and L.T. Williams. 1995. A Fas-associated protein factor, FAF1, potentiates Fas-mediated apoptosis. *Proc. Natl. Acad. Sci. USA*. 92:11894–11898.
14. Sato, T., S. Irie, S. Kitada, and J.C. Reed. 1995. FAP-1: a protein tyrosine phosphatase that associates with Fas. *Science*. 268:411–414.
15. Okura, T., L. Gong, T. Kamitani, T. Wada, I. Okura, C.F. Wei, H.M. Chang, and E.T. Yeh. 1996. Protection against Fas/APO-1- and tumor necrosis factor-mediated cell death by a novel protein, sentrin. *J. Immunol.* 157:4277–4281.
16. Becker, K., P. Schneider, K. Hofmann, C. Mattmann, and J. Tschopp. 1997. Interaction of Fas(Apo-1/CD95) with proteins implicated in the ubiquitination pathway. *FEBS Lett.* 412:102–106.
17. Wright, D.A., B. Futcher, P. Ghosh, and R.S. Geha. 1996. Association of human Fas (CD95) with a ubiquitin-conjugating enzyme (UBC-FAP). *J. Biol. Chem.* 271:31037–31043.
18. Kretz-Remy, C., and R.M. Tanguay. 1999. SUMO/sentrin: protein modifiers regulating important cellular functions. *Biochem. Cell Biol.* 77:299–309.
19. Vojtek, A.B., S.M. Hollenberg, and J.A. Cooper. 1993. Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell*. 74:205–214.
20. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Vol. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 1632–1636.
21. Kim, Y.H., C.Y. Choi, and Y. Kim. 1999. Covalent modification of the homeodomain-interacting protein kinase 2 (HIPK2) by the ubiquitin-like protein SUMO-1. *Proc. Natl. Acad. Sci. USA*. 96:12350–12355.
22. Thome, M., K. Hofmann, K. Burns, F. Martinon, J.L. Bodmer, C. Mattmann, and J. Tschopp. 1998. Identification of CARDIAK, a RIP-like kinase that associates with caspase-1. *Curr. Biol.* 8:885–888.
23. Rothe, M., M.G. Pan, W.J. Henzel, T.M. Ayres, and D.V. Goeddel. 1995. The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral-inhibitor of apoptosis proteins. *Cell*. 83:1243–1252.
24. Gong, L., T. Kamitani, K. Fujise, L.S. Caskey, and E.T.H. Yeh. 1997. Preferential interaction of sentrin with a ubiquitin-conjugating enzyme, UBC9. *J. Biol. Chem.* 272:28198–28201.
25. Kim, Y.H., C.Y. Choi, S.J. Lee, M.A. Conti, and Y. Kim. 1998. Homeodomain-interacting protein kinases, a novel family of co-repressors for homeodomain transcription factors. *J. Biol. Chem.* 273:25875–25879.
26. Garrett, S., and J. Broach. 1989. Loss of Ras activity in *Saccharomyces cerevisiae* is suppressed by disruptions of a new kinase gene, YAKI, whose product may act downstream of the cAMP-dependent protein kinase. *Gen. Dev.* 3:1336–1348.
27. Smith, D.J., and E.M. Rubin. 1997. Functional screening and complex traits: human 21q22.2 sequences affecting learning in mice. *Hum. Mol. Genet.* 6:1729–1733.
28. Moilanen, A.M., U. Karvonen, H. Poukka, O.A. Janne, and J.J. Palvimo. 1998. Activation of androgen receptor function by a novel nuclear protein kinase. *Mol. Biol. Cell.* 9:2527–2543.
29. Hanks, S.K., and T. Hunter. 1995. Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J.* 9:576–596.
30. Touchman, J.W., G.G. Bouffard, L.A. Weintraub, J.R. Idol, L. Wang, C.M. Robbins, J.C. Nussbaum, M. Lovett, and E.D. Green. 1997. 2006 expressed-sequence tags derived from human chromosome 7-enriched cDNA libraries. *Genome Res.* 7:281–292.
31. Kimura, M., and A. Matsuzawa. 1994. Autoimmunity in mice bearing lprcg: a novel mutant gene. *Int. Rev. Immunol.* 11:193–210.
32. Kischkel, F.C., S. Hellbardt, I. Behrmann, M. Germer, M. Pawlita, P.H. Krammer, and M.E. Peter. 1995. Cytotoxicity-dependent Apo-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:5579–5588.
33. Scaffidi, C., J. Volkland, I. Blomberg, I. Hoffmann, P.H. Krammer, and M.E. Peter. 2000. Phosphorylation of FADD/MORT1 at serine 194 and association with a 70-kDa cell cycle-regulated protein kinase. *J. Immunol.* 164:1236–1242.
34. Zhang, J., and A. Winoto. 1996. A mouse Fas-associated protein with homology to the human Mort1/Fadd protein is essential for Fas-induced apoptosis. *Mol. Cell Biol.* 16:2756–2763.
35. Thome, M., F. Martinon, K. Hofmann, V. Rubio, V. Steiner, P. Schneider, C. Mattmann, and J. Tschopp. 1999. Equine herpesvirus-2 E10 gene product, but not its cellular homologue, activates NF-kappaB transcription factor and c-Jun N-terminal kinase. *J. Biol. Chem.* 274:9962–9968.
36. Wang, Z.G., D. Ruggiero, S. Ronchetti, S. Zhong, M.

- Gaboli, R. Rivi, and P.P. Pandolfi. 1998. PML is essential for multiple apoptotic pathways. *Nat. Genet.* 20:266–272.
37. Ishov, A.M., A.G. Sotnikov, D. Negorev, O.V. Vladimirova, N. Neff, T. Kamitani, E.T. Yeh, J.F. Strauss 3rd, and G.G. Maul. 1999. PML is critical for ND10 formation and recruits the PML-interacting protein daxx to this nuclear structure when modified by SUMO-1. *J. Cell Biol.* 147:221–234.
38. Reed, J.C. 1999. Dysregulation of apoptosis in cancer. *J. Clin. Oncol.* 17:2941–2953.
39. Kennedy, N.J., and R.C. Budd. 1998. Phosphorylation of FADD/MORT1 and Fas by kinases that associate with the membrane-proximal cytoplasmic domain of Fas. *J. Immunol.* 160:4881–4888.