

Original Article

Ectopic expression of new alternative splice variant of Smac/DIABLO increases mammospheres formation

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Abstract: Smac- α is a mitochondrial protein that, during apoptosis, is translocated to the cytoplasm, where it negatively regulates members of the inhibitor of apoptosis (IAP) family via the IAP-binding motif (IBM) contained within its amino-terminus. Here, we describe a new alternative splice variant from Smac gene, which we have named Smac- ϵ . Smac- ϵ lacks both an IBM and a mitochondrial-targeting signal (MTS) element. Smac- ϵ mRNA exhibits a tissue-specific expression pattern in healthy human tissues as well as in several cancer cell lines. The steady-state levels of endogenous Smac- ϵ protein is regulated by the proteasomal pathway. When ectopically expressed, this isoform presents a cytosolic localization and is unable to associate with or to regulate the expression of X-linked Inhibitor of apoptosis protein, the best-studied member of IAP family. Nevertheless, over-expression of Smac- ϵ increases mammosphere formation. Whole genome expression analyses from these mammospheres show activation of several pro-survival and growth pathways, including Estrogen-Receptor signaling. In conclusion, our results support the functionality of this new Smac isoform.

Keywords: Smac- ϵ , tumorigenicity, cancer, breast

Introduction

Apoptosis is a type of cell death that occurs during both physiological and pathological conditions. In healthy cells, apoptosis is activated by the ligand-stimulated engagement of death receptors or by the cytoplasmic presence of certain mitochondrial proteins (Cytochrome-C and Smac- α). These events trigger the activation of effector caspases that degrade their target molecules. The inhibitor of apoptosis proteins (IAPs) block the activity of caspases via physical interactions. This family of proteins is characterized by the presence of one to three baculovirus IAP repeat (BIR) domains. XIAP, the most extensively studied IAP family member, interacts with and inhibits caspase-3, caspase-7, and caspase-9 by direct interaction via its BIR domains. Smac- α , the main IAP inhibitor, is located within the mitochondrial intermembrane space, from which it is released during apoptosis [1]. Upon release, Smac- α interacts with IAPs, de-repressing caspases. The

first four amino acids (AVPI, also called the IAP-binding motif or IBM) in the NH₂-terminal segment of mature Smac- α are responsible for the interaction with IAPs [2-4]. Although it has been established that mutating the AVPI sequence of Smac- α is sufficient to abrogate its interaction with IAPs, a Smac- α protein with a mutated AVPI still elicits biological effects. This mutant is unable to bind to XIAP and Livin, but it nonetheless induces their ubiquitination [5] and decreases cell survival [6]. Smac-75 is a truncated mutant protein that lacks the first 20 amino acids from the NH₂-terminus of mature Smac- α . This mutant is unable to associate with IAPs, but it nevertheless sensitizes cells to apoptosis by different stimuli [7, 8]. New Smac- α variants generated by alternative splicing have also been described. Among them is Smac- β , which, despite harboring neither an IBM nor a mitochondrial targeting sequence (MTS), can sensitize cells to apoptosis by different stimuli when this isoform is over-expressed [7]. Interestingly, TRAIL-induced apoptosis has

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been shown to be increased by the ectopic co-expression of both nerve growth factor receptor associated protein-1 (NGFRAP1) and mature Smac-α. Mature Smac-α is able to associate with NGFRAP1 in an IBM-independent way, inhibiting its ubiquitination by XIAP [9]. These results highlight the IBM-independent cellular functions of Smac-α, although information about its cellular impact remains limited.

After an initial PCR screening, we discovered a new Smac isoform that is generated by alternative splicing, which we have named Smac-ε. Smac-ε was detected at both RNA and, to a lesser extent, protein levels. In basal conditions, the protein derived from this new isoform is tightly regulated by the proteasomal pathway. In contrast to canonical Smac-α, this new isoform lacks both IBM and MTS elements. The absence of these structural motifs allowed us to seek IBM-independent functions without using an artificial Smac mutant. In breast cancer cells, ectopic Smac-ε expression promoted the generation of larger mammospheres, pointing toward a pro-tumorigenic role in cancer. Microarray assays of these mammospheres indicated that the expression of this new Smac isoform results in the activation of several oncogenic pathways not attributable to any direct or indirect regulation of IAPs.

Materials and methods

Cell culture and reagents

HeLa (CCL2) and MCF-7 (HTB22) cells were acquired from the American Type Culture Collection (ATCC), and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% and 10% fetal bovine serum (FBS), respectively. The cells were grown in a humidified incubator that was maintained at 37°C with 5% CO₂. Mammospheres were obtained from MCF-7 cells that were seeded in non-adherent culture flasks in Leibovitz L-15 medium with 10% FBS and then transferred into a rotatory incubator. The medium was changed every two days to avoid cytotoxicity. After 8 days of culture in those conditions, pictures were taken at 10× magnification. Mammospheres were assumed to have an ellipsoidal shape, we measured two perpendicular diameters and the area was determined. Transfection of constructs was performed using Lipofectamine 2000 (Invitrogen), and stable cell lines

were selected using puromycin (1 µg/ml for 15 days). Anti-Smac-α, anti-XIAP, anti-Myc and anti-Cox-IV were purchased from Cell Signaling Technology. Anti-c-IAP1 and anti-tubulin were purchased from Santa Cruz Biotechnology. MG132 was purchased from Calbiochem. TNF-α was purchased from R&D. For soft agar clonogenic assays, 10000 cells of each population were resuspended in DMEM containing 10% SFB and 0.3% agarose. Cells were then seeded into six-well plates over a bottom layer containing a solution of 0.5% agarose and DMEM 1%. Plates were incubated at 37°C for 5 weeks. Viable colonies were visualized by 0.005% crystal violet staining and counted.

Immunoblotting

Protein fractions were subjected to either 15% or 18% SDS-PAGE and were transferred to Immobilon P membranes (Millipore). Next, the membranes were incubated with the indicated antibodies overnight and the blots were visualized using the Immobilon Western kit (Millipore) with a peroxidase-labeled secondary antibody, according to the manufacturer's protocols.

Reverse transcription-polymerase chain reaction

Total RNA was extracted from monolayer cultures and from mammospheres using TRIzol reagent (Invitrogen) and then subjected to reverse transcription using the Superscript III kit (Invitrogen) following the manufacturer's protocols. Using Amplitaq Gold (Applied Biosystems), cDNA was amplified with the primers described in [Table S1](#) in Supporting Information.

Immunoprecipitation assay

Briefly, the cells were washed with PBS, scraped and centrifuged at 2,000 rpm for 3 min. Cells were lysed using 1 mL of TNTE-5 buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.5% Triton, 1 mM EDTA and 1X protease inhibitor cocktail) and incubated for 15 minutes on ice. The lysates were centrifuged at 14,000 rpm for 5 min. The supernatants were incubated with 10 µL of recombinant protein G (Life Technologies) for 1 hour. After incubation, the lysates were centrifuged at 14000 rpm for 10 sec. The supernatants were incubated overnight at 4°C with 3 µL of primary antibody with constant agitation.

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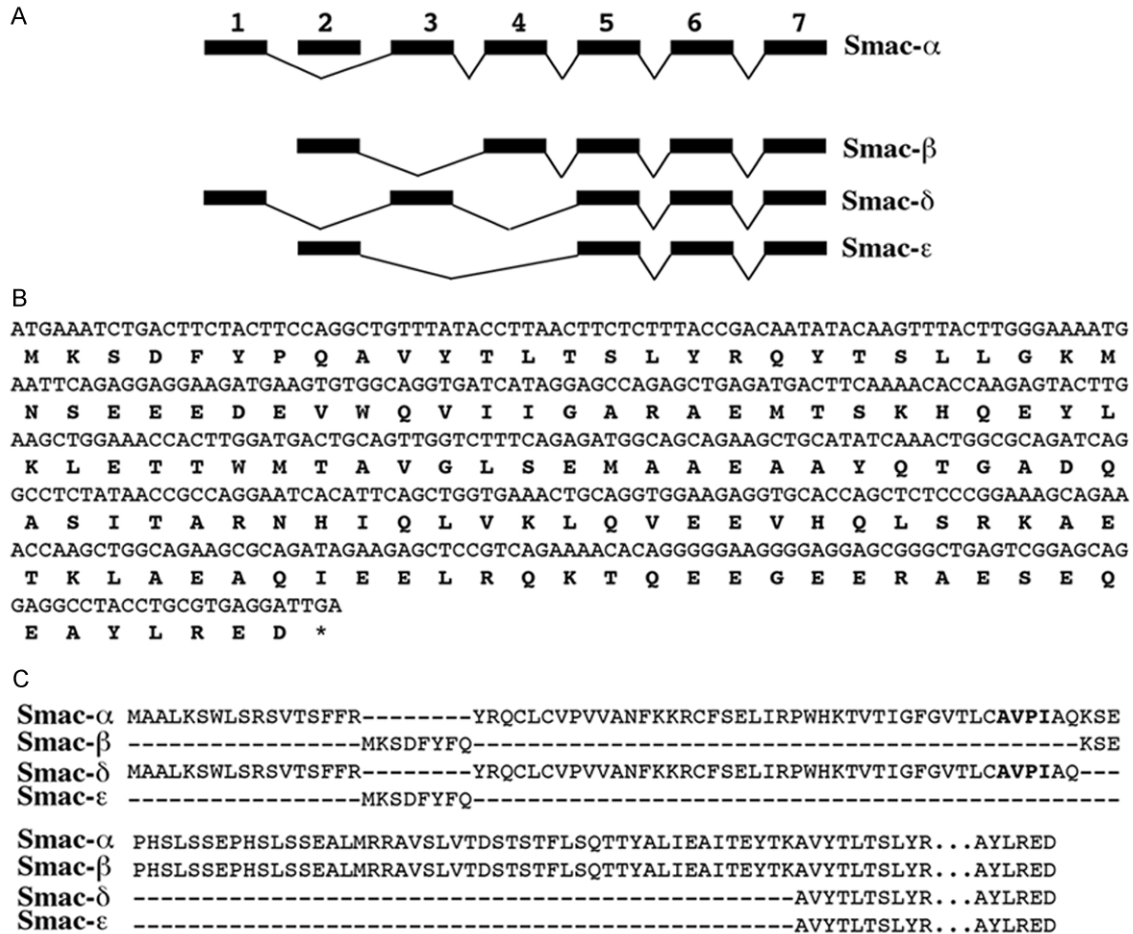


Figure 1. Smac-ε, a new alternative splice variant from Smac/DIABLO gene. A. Schematic representation of Smac/DIABLO isoforms generated by alternative splicing. Exons are shown as black boxes; B. Nucleotides and amino acids sequences of Smac-ε; C. The alignment shows the differences between Smac-α, Smac-β, Smac-δ and Smac-ε. The COOH-terminus is the same for all these isoforms. Boldface type shows the IAP-Binding-Motif (IBM).

Next, 10 μL of recombinant protein G was added to each lysate, and the lysates were then incubated with constant agitation for 1 hour on ice. The lysates were next centrifuged at 14,000 rpm for 10 sec. The resulting pellets were washed twice with TNTE-1 buffer (50 mM Tris at pH 7.4, 150 mM NaCl, 0.1% Triton, 1 mM EDTA and 1X protease inhibitor cocktail), followed by boiling in Laemmli sample solution (100 mM Tris pH 6.8, 20% Glycerol, 2% SDS, 0.05% Bromophenol blue and 100 mM DTT) for further analysis.

Oligo nucleotide microarray analysis

Affymetrix Human Gene 1.0 ST array GeneChips were used for all of the experiments. Total RNA from mammospheres was isolated using the

TRIzol reagent (Life Technologies) following the manufacturers' protocols. 200 ng of total RNA with a RNA Integrity Number (RIN) of up to 8.8 was used as starting material. Processed arrays were scanned using the GeneChip Scanner 7 G, and the resulting image data were managed with the Affymetrix Expression Console Version 1.1 using the RMA algorithm for the quality control data.

Data analysis

Analysis of the microarray data was performed using Bioconductor packages. The Oligo package was used for background subtraction, quantile normalization and summarization via median polish. The Limma package was used to detect differentially expressed probes, taking into account technical replication data sets

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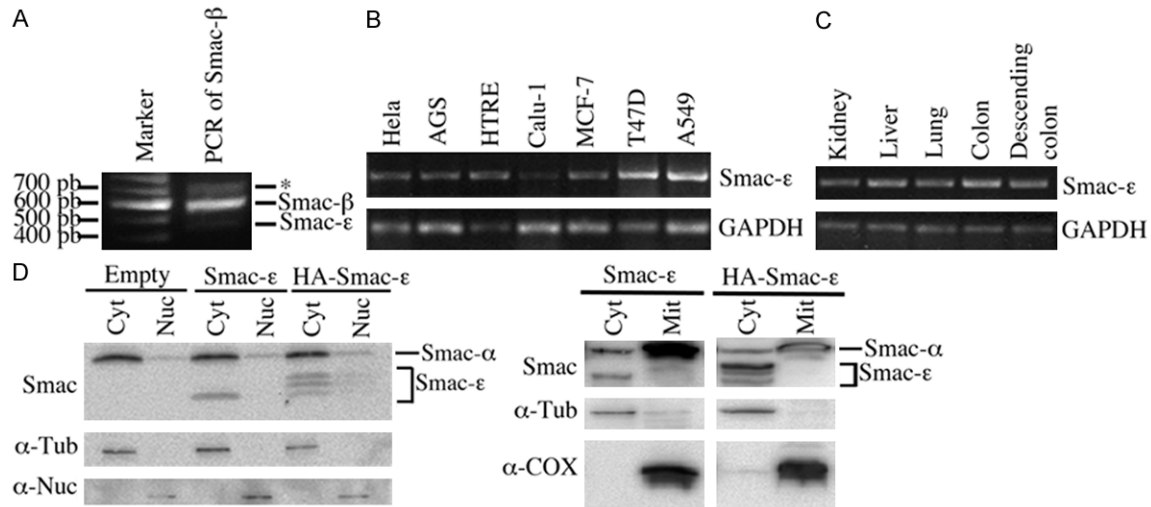


Figure 2. Characterization of Smac-ε. (A) RT-PCR from HeLa cells was done with specific primers anchored in the 5' and 3'-ends of the complete Smac-β isoform. From the three bands detected, the two lowest mRNAs signals were identified by capillary sequencing as Smac-β and Smac-ε. *, uncharacterized band. RT-PCR analysis of Smac-ε and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in healthy tissues and in (C) cancer cell lines (B). (D) HeLa cells were stably transfected with Smac-ε, HA-Smac-ε or an empty vector. Cellular fractionation was performed, and equal amounts of protein from each fraction were subjected to western blot analyses with the indicated antibodies. Smac-ε and HA-Smac-ε present cytosolic localization. Cyt, cytoplasm; Nuc, nucleus; Mit, mitochondria; Tub, tubulin; Nuc, nucleolin; COX, COX-IV.

using Benjamini-Hochberg correction for multiple testing [10].

Gene interaction network analysis

Ingenuity® Pathway Analysis (IPA; Ingenuity Systems) software was used to determine which functional processes were differentially represented in the gene lists obtained from data analysis. The Ingenuity Pathways Knowledgebase was used to build gene interaction networks derived from the provider's data. The statistical significance of interaction networks was determined by the Fisher's exact test.

Cytoplasmic and nuclear protein fractionation

Cells were scraped into PBS (phosphate-buffered saline) and centrifuged at 2,000 rpm for 3 min. The pellets were resuspended in 300 μL of Buffer A (10 mM HEPES pH 7.5, 2 mM MgCl₂, 15 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA and 1X protease inhibitor cocktail (Sigma)). The cells were then incubated in lysis buffer for 20 minutes on ice. Fifty microliters of NP-40 (10%) was added and incubated for 2 min. The lysates were centrifuged at 5,000 rpm for 5 min. The supernatants were recovered (cytosolic frac-

tion) for further analysis. The residual pellets were resuspended vigorously in 200 μL of Buffer C (25 mM HEPES at pH 7.5, 400 mM NaCl, 1 mM EDTA and 20% glycerol) and incubated for 30 min on ice with sporadic agitation. Next, the lysates were centrifuged at 8000 rpm for 5 min. The supernatants (nuclear extract) were recovered for further analysis.

Mitochondrial and cytoplasmic fractionation

Mitochondrial and cytoplasmic fractions were obtained as previously described [11]. Briefly, 1×10⁶ HeLa cells were sonicated in isolation buffer (0.25 M sucrose and 10 mM HEPES; pH 7.5) using a probe sonicator (ultrasonic processor, Cole Palmer) for 10 sec/10 cycles on ice. The samples were centrifuged at 8,000 rpm for 10 min, and the resulting supernatants were collected and then centrifuged at 12,000 rpm for 25 min (cytoplasmic fraction). The pellets were retained, washed once with fresh isolation buffer, and then centrifuged at 12,000 rpm for 25 min. The pellets were resuspended in mitochondrial buffer (7 M urea, 2 M thiourea, 4% CHAPS, 120 mM dithiothreitol, and 40 mM Tris-HCl), incubated at 4°C for 30 min, and centrifuged at 12,000 rpm for 5 min. The superna-

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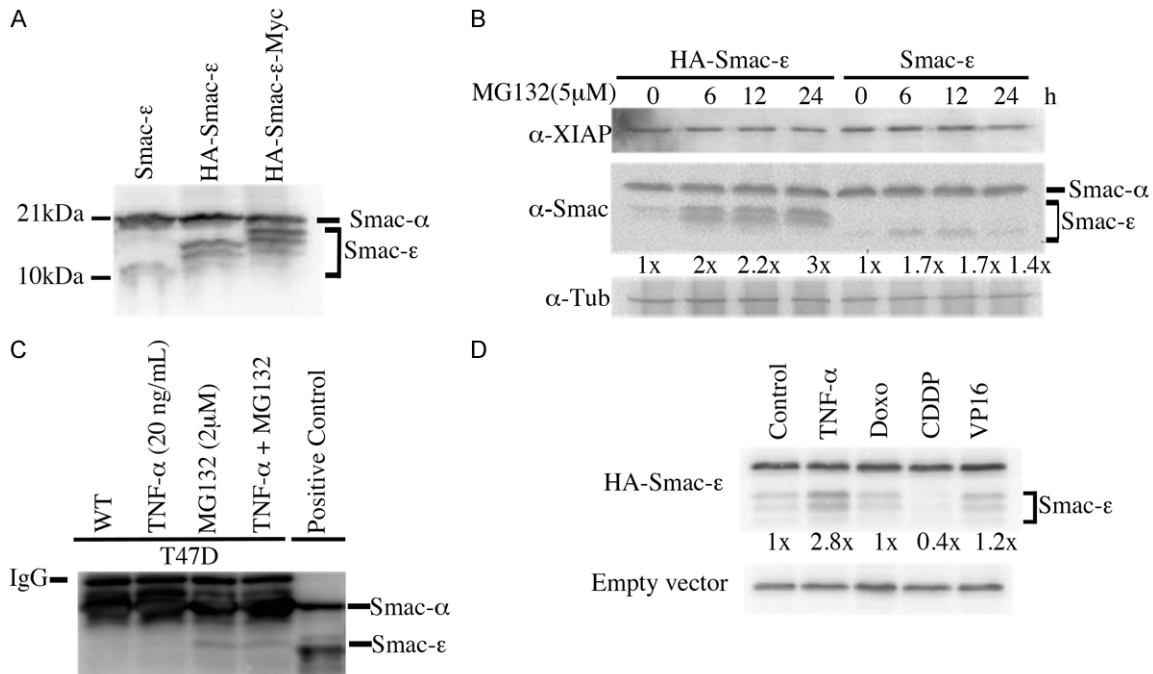


Figure 3. Smac-ε has a short life. A. MCF-7 cells were transfected with Smac-ε, HA-Smac-ε or HA-Smac-ε-Myc. Forty-eight hours posttransfection, lysates were generated and immunoblotted with an anti-Smac-α antibody; B. HeLa cells stably transfected with Smac-ε and HA-Smac-ε were treated with 5 μM MG132 for the indicated times and analyzed by western blot using the indicated antibodies. Tubulin was used as a loading control; C. T47D cells were stimulated with TNF-α or MG132 or both at the indicated concentrations for 24 h. Equal amounts of total proteins were subject to immunoprecipitation with an anti-Smac antibody, this antibody recognizes both Smac-α and Smac-ε proteins, followed by immunoblotting using the same antibody. Immunoprecipitation from cells ectopically expressing Smac-ε was included as a control. D. HeLa cells transfected with HA-Smac-ε or an empty vector were treated with TNF-α at 20 ng/mL for 4 h, doxorubicin at 1.3 μM for 12 h, cisplatin at 12.5 μM for 12 h or etoposide at 100 μM for 12 h. Equal amounts of total proteins from each lysate were subject to western blot analysis with an anti-Smac-α antibody. Doxo, doxorubicin; CDDP, cisplatin; VP16, etoposide; Tub, tubulin.

tants (mitochondrial extracts) were retained for further analysis.

Plasmid constructions

Smac-ε was amplified from cDNA using Pfu polymerase (Stratagene) and cloned into pTZ-57RT (Fermentas). It was then subcloned into the pQCXIP (Clontech) expression vector, producing the pQCXIP-Smac-ε construct. To generate a Smac-ε with a NH₂-terminal hemagglutinin (HA) tag (HA-Smac-ε), PCR amplification was performed using pQCXIP-Smac-ε as a template with the following primers: Forward, 5'-CATACGACGTCCCAGACTACGCTATGAAATCTGACT-3'; Reverse, 5'-CGGATCCGTCAATCCTCACGCA-GGTAG-3'. The PCR product was cloned into pTZ57RT (Fermentas). Re-amplification was performed using the same reverse primer and the following primer: 5'-CGCGGCCGCATGTACC-CATACGACGT-3', which contains a Not1 site

(underlined). The PCR product was cloned into pQCXIP, generating pQCXIP-HA-Smac-ε. HA-Smac-ε with a COOH-terminal Myc tag (HA-Smac-ε-Myc) was created by PCR-amplifying HA-Smac-ε using pQCXIP-HA-Smac-ε as a template with the following primers: Forward, 5'-CGCGGCCGCATGTACCATACGACGT-3' and Reverse, 5'-TGCTCGAGGTCATCCTCAGCAGG-3', each of which contains a Xho1 site (underlined). The product was cloned into the pCVM-tag5C vector (pCVM-Tag5C-HA-Smac-ε-Myc). Subcloning of HA-Smac-ε-Myc was performed from pCMV-Tag5C to pQCXIP, generating pQCXIP-HA-Smac-ε-Myc. cDNA encoding Smac-75 was amplified by PCR and cloned into pQCXIP to generate pQCXIP-Smac-75 with the following primers: Forward 5'-CGCGGCCGCAT-GAGAAGAGC-3', which contains a Not1 site (underlined), and Reverse 5'-CGGATCCGTCA-ATCCTCAGCAGGTAG-3', which contains a BamH1 site (underlined). Smac-α was amplified

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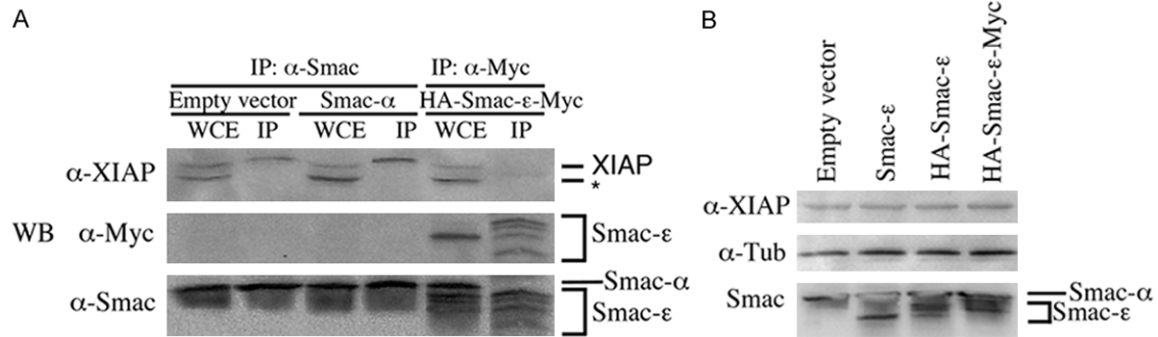


Figure 4. Smac-ε neither associates with nor regulates the protein levels of XIAP. **A.** MCF-7 cells were transiently transfected with empty vector, Smac-α or HA-Smac-ε-Myc. Forty-eight hours after transfection, lysates from cells transfected with HA-Smac-ε-Myc was subjected to immunoprecipitation with an anti-Myc antibody, followed by immunoblotting using the indicated antibodies. We included a positive control of (Smac-α)-XIAP interaction, immunoprecipitation with an anti-Smac-α antibody were performed in lysates from cells transfected with empty vector or Smac-α; **B.** MCF-7 cells transfected with Smac-ε, HA-Smac-ε, HA-Smac-ε-Myc or empty vectors were subjected to immunoblotting analyses with the indicated antibodies. WCE, whole cell extract; IP, immunoprecipitation; Tub, Tubulin; *, non-specific band.

from cDNA using Pfu polymerase (Stratagene) with the following primers: Forward, 5'-GCGC GGATCCATGGCGGCTCTGAAGAGTTGGCT-3'; Reverse, 5'-AGCTCTCTAGACTCAGGCCCTCAATCCTCACGC-3' and cloned into PCR2.1-TOPO (Invitrogen). It was then subcloned into the pLXSN (Clontech) expression vector, producing the pLXSN-Smac-α construct. All of the constructs were verified by capillary sequencing.

Statistical analysis

GraphPad Prism version 5.0 for Windows (La Jolla, CA) was used to perform statistical analyses. One-way analysis of variance was performed, and the Bonferroni post-test was used at 95% or 99% confidence intervals to determine significant differences.

Results

Identification of Smac-ε as a novel splice variant with cytosolic distribution

The Smac gene is composed of 7 exons. Exons 1 and 3 encode the mitochondrial targeting signal (MTS) and the IBM elements. Using HeLa cells, we performed a screen using primers anchored in the 5'- and 3'-ends of the putative complete Smac-β mRNA. We found two new variants and further analyzed the shortest isoform (**Figure 2A**). The novel variant described here is generated by alternative splicing that results in the exclusion of exons 1, 3 and 4 (**Figure 1**) To assess the mRNA expression and

distribution of this isoform, specific primers were designed and used to analyze the ratio between Smac-ε and GAPDH in several human tissues and cell lines. Tissue-specific expression was detected in five different normal human tissue samples and in seven different cancer cell lines (**Figure 2B** and **2C**). Smac-ε was anticipated to exhibit cytosolic localization as it lacks a MTS. Cellular fractionating was performed to verify this prediction using HeLa cells stably expressing Smac-ε. As expected, Smac-ε exhibited exclusively cytosolic localization (**Figure 2D**).

Smac-ε undergoes constitutively proteasome-dependent degradation and proteolytic cleavage

The expected translated sequence of Smac-ε comprises 146 amino acids, with an expected molecular weight of 16.3 kDa. Under denaturing conditions, Smac-ε exhibited an approximate molecular weight of 10 kDa, which is lower than expected. Taking into consideration that an over-expressed Smac-β isoform undergoes NH₂-terminal cleavage producing truncated protein [7] and that Smac-ε and Smac-β are largely homologous within their NH₂-termini, we explored the possibility of NH₂-terminal cleavage in the new isoform. It has been shown that adding peptide tags to short-lived proteins stabilizes them and increases effectively their half-life [12, 13]. For this purpose, we protected the NH₂-terminus of Smac-ε by adding a hemagglu-

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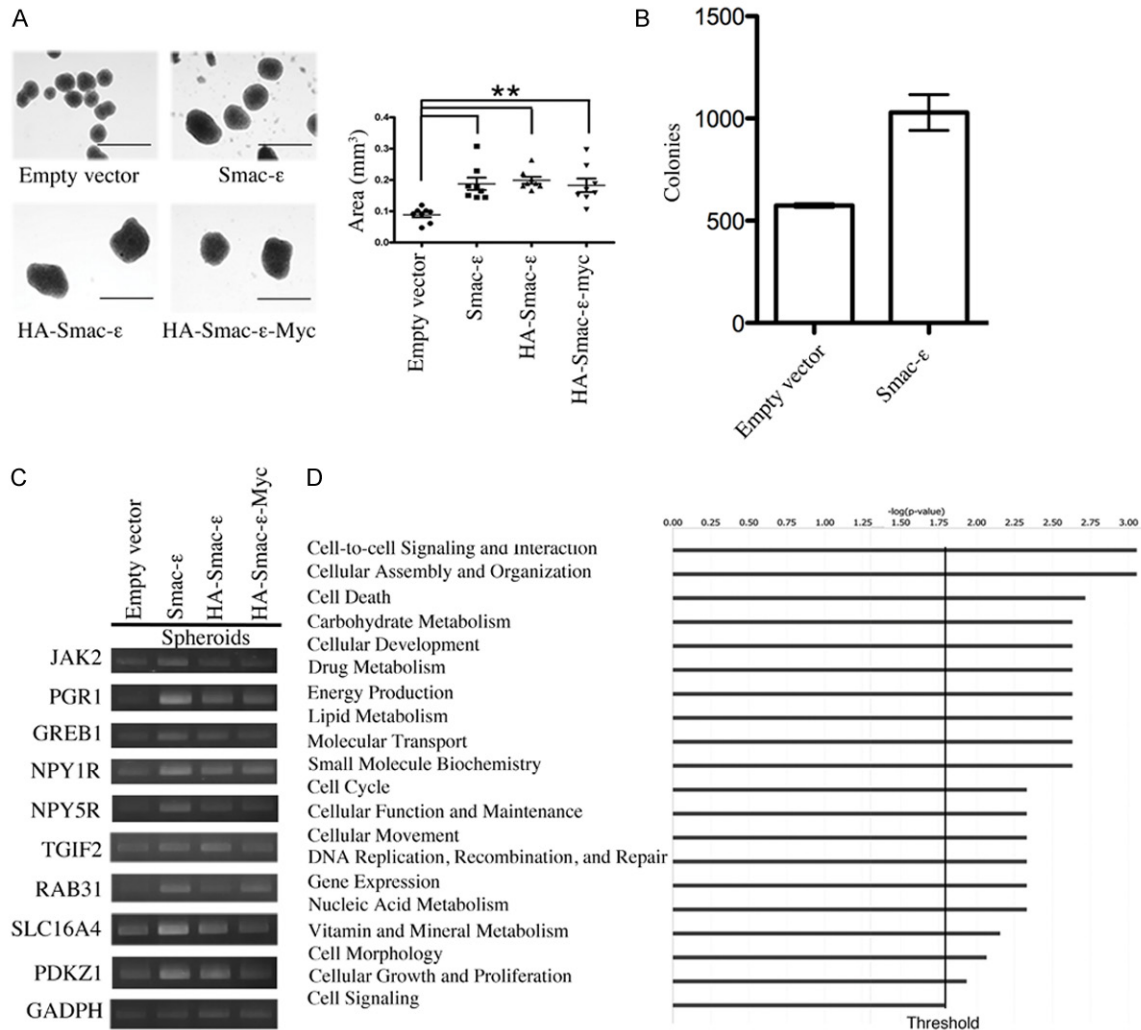


Figure 5. Smac-ε enhances mammosphere formation. A. MCF-7 cells stably transfected with Smac-ε, HA-Smac-ε, HA-Smac-ε-Myc or an empty vector were subjected to 3D culture conditions (described in the Methods section). Left, representative images from two independent experiments were selected, scale bar=1 mm. Right, the graph indicates the dimension of spheres generated from each transfection, $**P < 0.01$; B. Soft agar clonogenic assays of MCF-7 cells overexpressing Smac-ε. Graph shows number of colonies after six weeks in triplicate independent assays; C. Semi-quantitative RT-PCR validation analysis of nine differentially expressed genes of mammospheres expressing Smac-ε, HA-Smac-ε or HA-Smac-ε-Myc; D. Bio-function analysis of differentially expressed genes in Smac-ε mammospheres. Ingenuity Pathway Analysis (IPA) software was used to generate the molecular and cellular functions histogram. The significance referred in the histograms as $-\log(P\text{-value})$ was determined using the Fisher's exact test. The threshold was set at $P=0.05$.

tinin (HA) epitope coding sequence (HA-Smac-ε) and the COOH-termini of the isoform with a Myc tag (HA-Smac-ε-Myc). Transient expression of HA-Smac-ε produced a protein with increased molecular weight compared to over-expressed Smac-ε (Figure 3A). Carboxyl tagging further increased the molecular weight of the isoform (Figure 3A). Protecting both NH₂- and CO₂H-terminal ends of Smac-ε was enough to detect

this new isoform in its expected weight. The presence of additional shorter bands after tag addition could indicate us that Smac-ε was only partially protected from proteolytic cleavage. Furthermore, the incorporation of the HA-epitope sequence did not change the cellular localization of Smac-ε (Figure 2D). The above findings support the idea that Smac-ε undergoes proteolytic cleavage at both termini.

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Table 1. Differentially-expressed genes in Smac-ε-expressing cells

Symbol	Name	Fold change
LYZ	Lysozyme	4.580
IGSF1	Immunoglobulin-superfamily, member 1	2.733
RAB31	RAB31, member RAS oncogene family	2.635
NPY5R	Neuropeptide Y receptor Y5	2.315
PGR	Progesterone receptor	2.269
SLC5A8	Solute carrier family 5 (iodide transporter), member 8	2.070
DOK5	Docking protein 5	1.758
PDZK1	PDZ domain containing 1	1.348
NPY1R	Neuropeptide Y receptor Y1	1.218
FRK	Fyn-related kinase	1.263
UNC5C	Unc-5 homolog C (C elegans)	1.218
RHOBTB1	Rho-related BTB domain containing 1	1.122
BCL2	B cell CLL/lymphoma 2	0.974
SLC16A4	Solute carrier family 16, member 4	0.918
ZNF185	Zinc finger protein 185 (LIM domain)	0.915
TSPAN5	Tetraspanin 5	0.893
DSCAM	Down syndrome cell adhesion molecule	0.889
JAK2	Janus kinase 2	0.880
CACNA2D1	Calcium channel, voltage-dependent, alpha 2/delta subunit 1	0.878
SHC4	SHC (Src homology 2 domain containing) family, member 4	0.837
COX6C	Cytochrome c oxidase subunit VI C	0.779
GFRA1	GDNF family receptor alpha 1	0.735
MOCS2	Molybdenum cofactor synthesis 2	0.703
STC1	Stanniocalcin 1	0.702
MGAM	Maltose-glucoamylase 8alpha-glucoamidase)	0.696
GREB1	Growth regulation by estrogen in breast cancer 1	0.637
TGIF2	TGFB-induced factor homeobox 2	0.585
KCNJ3	Potassium unwardly rectifying channel subfamily J, member 3	0.577
FLNB	Filamin B, beta	0.546

Symbol and name are HUGO Gene Nomenclature Committee approved. We selected an up-regulation cut-off ratio of 0.5 or more and a down-regulation cut-off of 0.5 or less and a false discovery rate of 0.5%.

We observed low protein expression levels of Smac-ε, regardless of the type of transfection (transitory or permanent). Since it has been demonstrated that proteasomal activity is the main mechanism for the regulation of short-lived proteins [14, 15], we sought to determine whether the proteasome might be implicated in the degradation of Smac-ε. For this purpose, cells stably transfected with plasmids encoding either Smac-ε or HA-Smac-ε were treated with a proteasome inhibitor (MG132). Proteasomal inhibition by MG132 caused an accumulation of both Smac-ε and HA-Smac-ε proteins (**Figure 3B**). In addition, endogenous Smac-ε protein was also stabilized by MG132 (**Figure 3C**).

These results strongly suggest that proteasomal activity is implicated in the degradation of Smac-ε in basal conditions.

Since ectopically-expressed Smac-ε protein is expressed at low levels due to mentioned mechanisms, we explored the possibility that specific stimuli might stabilize the protein to promote a specific function. For this purpose, Smac-ε expressing cells were exposed to different stimuli, and its levels were analyzed by western blotting. As shown in **Figure 3D**, exposure to TNF-α increased the expression of HA-Smac-ε, in the absence of mRNA regulation (results not shown).

Smac-ε neither interacts with nor modulates the protein expression of XIAP

Since mature Smac-α mediates its biological actions by interacting with IAP family members, we sought to

determine whether Smac-ε was able to interact with them. For this, we tested a well-characterized Smac-α partner, XIAP [3, 5, 16]. Whereas Smac-α clearly interacted with XIAP (**Figure 4A**), HA-Smac-ε-Myc was unable to interact with it (**Figure 4A**). Interestingly, HA-Smac-ε-Myc associated with its cleaved variants but not with Smac-α (**Figure 4A**). The same pattern of interaction was showed by HA-Smac-ε (data not shown). The inability of Smac-ε to interact with Smac-α has also been described for another Smac isoform, Smac Smac δ [17]. Since it has been previously shown that Smac isoforms are able to increase the auto-ubiquitination and degradation of XIAP and c-IAP-1 [5,

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18], we next analyzed whether Smac- ϵ could be altering the expression levels of these IAP family members. As shown in **Figure 4B**, the expression of Smac- ϵ or its tagged variants did not change XIAP expression. In accordance with this result, XIAP protein levels were not affected during the stabilization of HA-Smac- ϵ induced by the treatment with MG132 (**Figure 3B**). Additionally, we evaluated if Smac- ϵ or its tagged variants could induce changes in c-IAP1 protein levels. We could not detect any significant c-IAP1 protein regulation (data not shown). The above findings indicate that Smac- ϵ is unable to regulate the IAP family members as canonical Smac- α does.

Smac- ϵ increase mammosphere size

To assess a possible role of this new isoform in tumorigenicity, we used mammosphere assays. These assays provide a model for tumor development, cancer stem cell enrichment and, more interestingly, an accurate predictor of breast cancer cells tumorigenicity [19]. The inner layers of cells growing in three-dimensional spheres are exposed to hypoxic and nutrient-deprived conditions [20]. As the spheres continue to grow, hostile conditions at the center worsen and eventually lead to a limit to the sphere size. When we analyzed mammospheres derived from MCF-7 cells transfected with Smac- ϵ , we found a significant increase in their size (**Figure 5A**), pointing toward a more aggressive phenotype. In addition, we found that overexpression of the new Smac isoform induced an important increase in the clonogenic ability of MCF-7 cells (**Figure 5B**). Altogether, these results point toward a possible pro-tumorigenic effect of Smac- ϵ .

Smac- ϵ regulates distinct transcriptional networks

To further explore the mechanism by which the overexpression of Smac- ϵ regulates the growth of mammospheres, we performed whole-genome expression analyses of MCF-7 spheres derived from cells overexpressing the new isoform. In these assays, we identified 45 differentially expressed genes (**Table 1**). To validate these microarray results, semiquantitative RT-PCR was performed from randomly selected genes. We found a 100% concordant rate (**Figure 5C**). Additionally, we also confirmed the mRNA up-regulation of these genes in spheres

generated from HA-Smac- ϵ and HA-Smac- ϵ -Myc-transfected cells (**Figure 5C**). We found 9 enriched signaling networks, with the top two including cell survival, growth and proliferation (**Figure S1** in supplemental data). In addition, we found 20 overrepresented GO cellular processes, including cell-to-cell signaling, cell death and growth and proliferation, among others (**Figure 5D**). These results provide support to our previous mammosphere results. In the first of the two signaling networks, eleven molecules showed an increased expression: Janus kinase (JAK2), Filamin B (FLNB), neuropeptide Y Y1 receptor (NPY1R), NPY5R (neuropeptide Y Y5 receptor), SCL548 (sodium-coupled transporter 548), PGR (progesterone receptor), PAG1 (Associated RAS oncogene phosphoprotein family), and RHOBTB1 (Rho - related BTB domain containing 1) (**Figure S1** in supplemental data). The latter two are members of the RAS family, a crucial transduction pathway regulating cellular growth and carcinogenesis. The second network is also associated with cell growth and proliferation and contains four molecular loci deregulated in the microarray analysis: Fyn-related kinase (FRK), Growth Regulation by estrogen in breast cancer 1 (GREB 1), Growth Regulation by estrogen in breast cancer 1 (SHC4) and SHC (Src homology 2 domain containing) family, member 4 (**Figure S1** in supplemental data). Both networks are related to important transduction pathways such as NFkappaB, TGF β 1, EGFR and ERKs, which together may favor cell growth. Interestingly, Smac- ϵ induced the transcriptional activation of estrogen-responsive genes (PGR, GREB1, PDZK1 and RAB31) (**Figure 5C**). The activation of the estrogen receptor (ER)-mediated transcriptional program has been associated with proliferation, metastasis and invasion [21, 22].

Discussion

In this study, we describe Smac- ϵ , a new alternatively spliced isoform of Smac, which lacks both IBM and MTS elements. As expected by this, we have experimentally determined that Smac- ϵ is a cytosolic protein. The mRNA of this new isoform is ubiquitously expressed in normal human tissues and cancer cell lines (**Figure 2B** and **2C**). Intriguingly, endogenous Smac- ϵ protein has a very short half-life, due to the activity of the proteasomal degradation pathway.

Smac- ϵ increases mammosphere formation

Cytosolic Smac- α relieves the negative regulation of caspases via its IBM [1-3, 5]. Previous studies assumed that the IBM was the only element in Smac- α that exerts biological effects. Molecules mimicking this IBM have been designed and are now being tested for clinical activity. Smac- α mimetics exert their functions by interacting with XIAP and other IAPs [23, 24]. Consistent with the findings described above, Smac- β and Smac- α mutant proteins lacking an IBM are unable to interact with IAPs. However, functional experiments demonstrated that IBM is dispensable for the induction of apoptosis [7, 8, 17, 25], pointing toward the presence of an unknown mechanism residing on Smac- α 's COOH-terminus and/or IBM-proximal aminoacids. As expected, we demonstrated that Smac- ϵ is unable to associate with a classical member of the IAP family (**Figure 4A**). Our results are in concordance with previous reports indicating that mutation of IBM is enough to loss that ability [2, 3]. Interestingly, Smac- α with a mutated IBM is able to induce ubiquitination of IAPs independently of any physical interaction [5]. In that study, an artificial Smac mutant did not modify the half-life of ubiquitinated IAPs, although the biological relevance of this finding was not studied. These results are in accordance with our findings (**Figure 4B**).

Mammosphere culture has been widely used as an assay for propagating and analyzing cancer stem cells. Besides the utility for determining stem phenotype, it has been shown that the size of the spheres correlates with the ability of freshly-derived patient cancer cells to induce tumors in immunodeficient mice [19]. To study the biological effects of this novel Smac isoform, Smac- ϵ -transfected cells were grown as mammospheres. Unexpectedly, independently of any proteolytic cleavage, Smac- ϵ and its tagged variants were able to enhance the mammosphere formation ability of breast cancer cells, probably due to an increase in proliferation at higher cell density. To explain this phenomenon, we performed whole-genome microarray experiments from mammospheres expressing Smac- ϵ . Several pathways related to growth and proliferation were induced by the new Smac isoform. Interestingly, Smac- ϵ induced the transcriptional activation of estrogen-responsive genes. The activation of the estrogen receptor (ER)-mediated transcriptional program has been associated with prolifera-

tion, metastasis and invasion [21, 22]. It is notable that estrogen increases breast cancer cell survival via an IAP-dependent mechanism that can be inhibited by Smac- α [26], supporting the importance of the contribution of this gene. Analyzing all of the interacting proteins of Smac- ϵ might provide an insight into the molecular mechanisms responsible for these effects.

In the current study, we describe a new alternative splice variant from the Smac gene. Ectopic expression of this isoform was able to increase the growth of mammospheres by activating several transduction pathways in an IAP-independent process. Since it has been previously shown that mammosphere size was a better indicator of tumorigenicity than the content of cancer stem cells [19], the effect of Smac- ϵ is particularly interesting. Notwithstanding the results presented, the undetectable expression of the Smac- ϵ protein in non-stimulated cell lines raises the question of the exact relevance of this new isoform protein product during basal or physiological conditions. We also cannot exclude a possible additional non-coding function of the Smac- ϵ messenger RNA, as recent evidence has pointed toward a plethora of new biological functions for the recently discovered RNA universe [27]. Additional studies are required to resolve this issue. Nonetheless, our results could be used to infer a possible IBM-independent mechanism present in Smac- α and, in addition, it raises the question of a possible activity of Smac- ϵ well-expressed messenger RNA, even in the absence of a protein product.

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Disclosure of conflict of interest

None.

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Table S1. Primers used in this work

Gene name	Forward primer	Reverse primer	Product length
PDZK1	CGGCGAGTCTGTGGAACCC	GAGCCTGGCAGACCCCGAAT	327
RAB31	GCCATCGCTGGAACAAGTGCG	GCGGCTGGCTTGCATGGTTG	234
PGR1	TTGAGGAGGAGGATGGCTCTGAGT	ATAAGGCGTGATTGAGAGGCAGGATA	285
NPY1R	CAGTCTGCTGGCTCCCTTAC	ATGCGACTGGGCTTGCTTG	297
NPY5R	GGTCCCACCTTGCTTTGAGA	ACAGGACATCATGCCCAACA	259
SLC5A8	AGGATTTGATCTGTGGGGCG	CCAAATACGCTGAGTGCTGC	779
SLC16A4	ATTACCACAAGTCTTACCTCATCCTC	AGAAAGGACAGCCATCCCAGC	221
JAK2	GGGAATGGCCTGCCTTACGA	ATCATGCCGCACTGAGCAA	478
GREB1	ATCCCTGCGGTCCAACAACCT	GCACACCAGGAGATGGTCCGG	331
TGIF2	AACGCCTACCCTCAGAGCAGG	CGGCATGGAGCACACAGACAG	273
GAPDH	CCCCTTATTGACCTCAACT	TTGTCATGGATGACCTTGGC	396
SMAC-ε	CTTCTACTTCCAGGCTGTTTA	CCTTCTCTGTGTTTTCTGAC	369

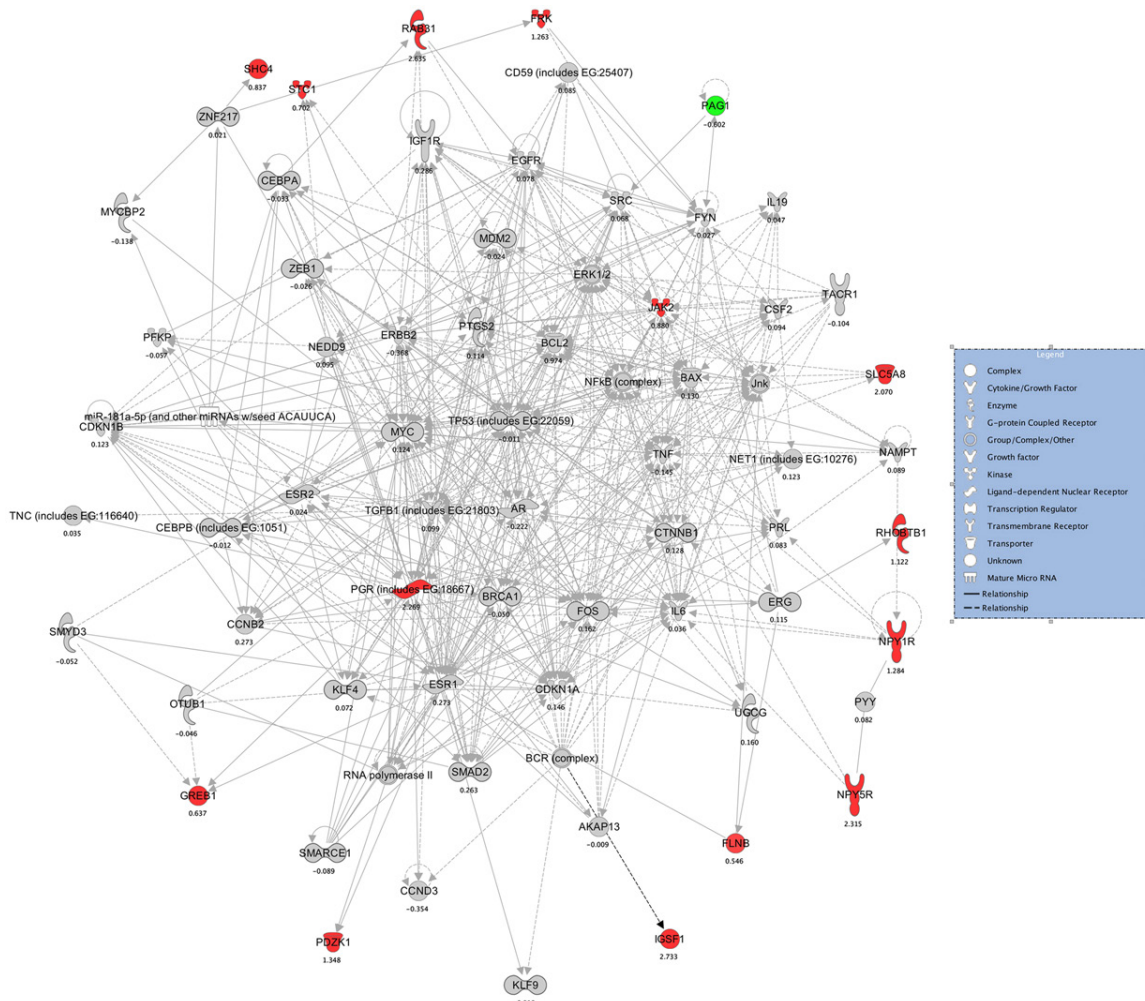


Figure S1. Gene network analysis by Ingenuity Pathway Analysis of a comparison between Smac-ε and control spheres. The two top pathways were merged. Direct or indirect interactions are shown by complete or dashed lines, respectively. Fold changes in log scales are shown below each symbol. The color scales goes from green (under-regulated) to red (up-regulated).