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c-FLIP_L enhances anti-apoptotic Akt functions by modulation of Gsk3 β activity.

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Abstract

Akt is a serine threonine kinase that plays an important role in transducing survival signals. Akt also regulates a number of proteins involved in the apoptotic process. To find new Akt interactors, we performed a two-hybrid screening in yeast using full length Akt c-DNA as bait and a human c-DNA heart library as prey. Among 200 clones obtained, two of them were identified as coding for the c-FLIP_L protein. c-FLIP_L is an endogenous inhibitor of death receptor-induced apoptosis through the caspase 8 pathway. By co-immunoprecipitation experiments of either transfected or endogenous proteins, we confirmed the interaction between Akt and c-FLIP_L. Further, we found that c-FLIP_L overexpression interferes with Gsk3- β phosphorylation levels. Moreover, through its effects on Gsk3 β , c-FLIP_L overexpression in cancer cells induced resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). This effect was mediated by the regulation of p27^{Kip1} and caspase 3 expression. These results indicate the existence of a novel mechanism of resistance to TRAIL in cancer cells, and unexpected functions of c-FLIP_L.

INTRODUCTION

Apoptosis, or programmed cell death, is an evolutionarily conserved mechanism of elimination of unwanted cells. This endogenous death machinery is triggered via two principal signaling pathways (1). The extrinsic pathway is activated by the engagement of death receptors on the cell surface. The binding of ligands such as Fas, tumor necrosis factor (TNF), or TNF-related apoptosis-inducing ligand (TRAIL) to cognate death receptors (DR) induces the formation of the death-induced signaling complex (DISC). The DISC in turn recruits caspase 8 and promotes the cascade of procaspase activation (2). The intrinsic pathway is triggered by various intracellular and extracellular stresses, whose signals converge mainly to the mitochondria (2, 3). The balance between pro-and anti-apoptotic members of apoptosis is crucial for the regulation of survival and cell death. Aberrant resistance to apoptosis may lead to the development of cancer.

Cellular FLICE-inhibitory protein (c-FLIP) is a Death Effector Domain (DED) containing-family member that inhibits one of the most proximal steps of DR-mediated apoptosis. Two isoforms of c-FLIP are commonly detected in human cells: a long form (c-FLIP_L) and a short form (c-FLIP_S). c-FLIP_L, a 55 kDa protein, contains two DEDs and a caspase-like domain, whereas c-FLIP_S, a 26 kDa protein consists only of two DEDs (4). Both isoforms are recruited to the DISC, prevent procaspase-8 activation and block DR-mediated apoptosis, although with different mechanisms (5, 6). c-FLIP_L is overexpressed in a number of different tumors and its overexpression is related to TRAIL resistance (7, 8). Beside cell death, c-FLIP_L might also regulate other DR-mediated signals that may be important for tumor-promoting functions such as proliferation, migration, inflammation or metastasis (9-11). Activation of the transcription factor NF- κ B, the PKB/Akt pathway and mitogen-activated protein kinases (MAPK) such as cJun N-terminal kinase (JNK), ERK and p38 have been demonstrated to be a consequence of DR triggering (9). Akt is a serine threonine kinase that regulates the expression and the function of a number of proteins involved in the apoptotic process (12). Akt interaction or phosphorylation of different signaling molecules may regulate their function by different mechanisms, including increased protein stability, cellular localization, or binding to a different cellular partner. Akt interacts with a number of proteins involved in apoptotic signaling cascades, including BAD (13), caspase 9 (14), the Forkhead transcription factor FOXO3 (15) and Bcl-w (16). Interaction of Akt with one of these proteins prevents apoptosis through

several different mechanisms (13). One major Akt substrate is the serine/threonine kinase Gsk3 (17). Originally studied for its role in glycogen metabolism and insulin action, Gsk3, present in the cells in two isoforms, α and β , has subsequently been shown to have central functions in many cellular process including transcription, cell cycle division, cell fate determination, and stem cell maintenance, as well as apoptosis (17, 18). Gsk3 is constitutively active in resting cells, and is functionally inactivated after phosphorylation in response to different stimuli.

In this study, we set out to find and investigate new possible partners of Akt that may participate in the regulation of the apoptosis pathway. In this paper, we give evidence that Akt directly interacts with c-FLIP_L. Further, we demonstrate that c-FLIP_L modulates the activation of Gsk3 β . We also provide evidence that this interaction is important for the regulation of TRAIL sensitivity, through the regulation of p27 and caspase 3 levels.

Methods

Materials- Media, sera, and antibiotics for cell culture were from Life Technologies, Inc. (Grand Island, NY, USA). Protein electrophoresis reagents were from Bio-Rad (Richmond, VA, USA), and Western blotting and ECL reagents were from GE Health care. All other chemicals were from Sigma (St. Louis, MO, USA). The antibodies were: anti-caspase 8 antibody (1C12), anti-Akt, anti-P-Akt, anti-P-Gsk3 β , anti-Gsk3 β and anti-p27^{Kip1} from Cell Signalling Technology, Inc. (Danvers, MA, USA); anti-caspase 3 antibody from Abcam (Cambridge, USA); anti-c-FLIP (NF6) antibody from Alexis (Lausen, Switzerland); anti-Flag M2 and anti- β -actin antibody from Sigma (St. Louis, MO, USA); anti-HA from Covance (Berkeley, CA USA). SuperKiller TRAIL was purchased from Alexis (Alexis, Lausen, Switzerland).

Plasmids. pcDNA3 Flag(hs)FLIP_L and FLIP_s were kindly provided by Prof. Pasquale Vito and Henning Walczack respectively. Akt wild type, Akt E40K (constitutively active, HA-Akt-D+) and Akt K179M (dominant negative HA-Akt D-) with an HA-tag were a kind gift of Prof. Gianluigi Condorelli. Gsk3 β WT and Gsk3 β -kinase inactive (KI) cDNAs were kindly provided by Prof. Junichi Sadoshima. p27 cDNA was kindly provided by Prof. Alfredo Fusco. [pRetroSuper vectors expressing RNAi for c-FLIP were obtained by Prof. Simone Fulda.](#)

Cell culture- Human HeLa, HEK-293, K562 and A459 cell lines were grown in DMEM containing 10% heat-inactivated FBS with 2mM L-glutamine and 100U/ml penicillin-streptomycin. DU145 and H460 cell lines were grown in RPMI containing 10% heat-inactivated FBS with 2mM L-glutamine and 100U/ml penicillin-streptomycin.

Yeast Two-hybrid System- All experiments were performed in the yeast reporter MaV203. The human heart cDNA library was from Invitrogen. Screening of the library was performed essentially following instructions for the ProQuest two-hybrid system (Life Technologies) and has been previously described (19). The GAL4 DNA-binding domain/human Akt fusion was obtained from Dr. Alfonso Bellacosa (Fox Chase Cancer Center, Philadelphia, Pennsylvania, USA). Subsequently, yeast pLEx4-Akt plasmid was transformed with the pPC86AD-cDNA library and plated onto plates lacking histidine in the presence of 3AT (aminotriazole; 10 mM). Approximately 1.2×10^6 individual clones were plated, and about 200 grew on the selective medium. Resistant colonies were grown on a master plate and then replica-plated onto selection plates to determine their ability to induce three independent reporters (*HIS3*, *URA3*, and *lacZ*). Eighty independent clones were isolated after this first screening. DNA was isolated from each positive clone and sequenced to identify the inserts. Independent clones were retransformed into yeast and tested for interaction with a fresh Akt clone.

c-FLIP_L deletion mutants generation- We generated by PCR three deletion mutants of c-FLIP_L, using as template the plasmid pcDNA3-3XFlag-FLIP_L. c-FLIP I-DED mutant, encoding amino acids 81-480 that lacks the first DED, was generated using the primers 5'- cccaagcttaccacctgctcaggaaccct-3' and 5'- gctctagattatgtgtaggagaggata -3'; c-FLIP-II-DED, encoding amino acids 1-93 and 178-480 that lacks the second DED, was generated using the primers 5'-cccaagcttatgtctgctgaagtcacat-3' and 5'-tgtccctgcatagtcgaaacaaggtgagg-3' for amino acids 1-93 and 5'-tcggactatgcagggacaagttacaggaat-3' and 5'- gctctagattatgtgtaggagaggata-3' for amino acids 178-480; FLIP_L-ΔDED, encoding amino acids 178-488 that lacks both DEDs, was generated using the primers 5'-cccaagcttgaggacaagttacaggaat-3' and 5'-gctctagattatgtgtaggagaggata-3'. The amplified sequences were cloned in p3X-Flag-CMV

previously linearized with the restriction enzymes HINDIII and XbaI. The following deletion mutants were generated: c-FLIP_L-F1, encoding aa 1-253, was generated using the primers 5'-tgacgataaagaattcatgtctgc-3' and 5'-gattcctaggggcttctt-3'; c-FLIP_L-F3, encoding aa 1-339, was generated using the primers 5'-tgacgataaagaattcatgtctgc-3' and 5'-catcctcctgatgtgatgca-3'; cFLIP_L-F5, encoding aa 1-434, was generated using the primers 5'-tgacgataaagaattcatgtctgc-3' and 5'-ttctgtctcagtttctggg-3'. c-FLIP_L-R0, encoding aa 1-177, was generated using the primers 5'-tgacgataaagaattcatgtctgc-3' and 5'-gccctcgagttatccagttgatctggggcaac-3'; c-FLIP_L-R1, encoding aa 1-182, was generated using the primers 5'-tgacgataaagaattcatgtctgc-3' and 5'-gccctcgagttactgtaactgtccctgctcc-3'. Temperature cycles were the following: 95°C 1 minute; 95°C 50 seconds, 60°C 50 seconds, 68°C 7 minutes for 35 cycles; 68°C 2 minutes.

Production of retroviral particles and infection of HeLa and HEK-293 cells. c-FLIP_L cDNA was cloned in PINCO vector (20). The amphotropic packaging cell line Phoenix was transfected by standard calcium phosphate/chloroquine method, and culture supernatants containing retroviral particles were collected 48 hours after transfection. Transduction was done by culturing thrice 5×10^5 cells in 1 mL of 0.45 mmol/L filtered supernatant containing viral particles. Gene transfer efficiency was evaluated by flow cytometry analysis based on the expression of the GFP reporter. The levels of c-FLIP expression were evaluated by immunoblot analysis using lysates of cells infected with the empty tween vector (HeLa Tween and 293 Tween) for comparison.

Western blotting- Total proteins from cells was extracted with RIPA buffer (0.15mM NaCl, 0.05mM Tris-HCl, pH 7.5, 1% Triton, 0.1% SDS, 0.1% Sodium deoxycolate and 1% Nonidet P40). Fifty µg of sample extract were resolved on 7.5-12% SDS-polyacrylamide gels using a mini-gel apparatus (Bio-Rad Laboratories, Richmond, CA) and transferred to Hybond-C extra nitrocellulose. Membranes were blocked for 1hr with 5% non-fat dry milk in TBS containing 0.05% Tween-20, incubated for 2h with primary antibody, washed and incubated with secondary antibody, and visualized by chemiluminescence.

Phosphorylation experiments- HeLa cells were transiently transfected with different cDNAs as indicated. After 24h, the cells were incubated in serum-free culture medium for 16h at 37°C. Insulin (final concentration, 100 nM) was then added, and the cells were

rapidly rinsed with ice-cold saline followed by solubilization with 0.5 ml of RIPA buffer per dish for 1 hr at 4°C. Lysates were centrifuged at 5,000 xg for 20 min, and solubilized proteins were precipitated with the indicated antibodies, separated by SDS-PAGE, and revealed by western blot with antibodies recognizing the phosphorylated proteins.

Immunoprecipitation- Cells were cultured at a final concentration of 90% in p100 plates. The cells were harvested with RIPA Buffer on a shaker for 30 minutes. 1 mg of total extract was immunoprecipitated using the indicated antibodies (5 µg/ml Anti-Flag, 2 µg/ml Anti-HA, 3 µg/ml anti-Akt, 3 µg/ml anti-Gsk3β), for 16 hrs on shaker. Then, A/G beads (Santa Cruz, Santa Cruz, CA USA) were added for two hrs. The beads were washed for three times with washing buffer (50mM Tris Hcl pH 7.5, 150 mM NaCl, 0.1% Triton, 10% glycerol), and then 20 µl of sample buffer was added; the samples were boiled at 100 °C for 5 minutes and then the supernatants resolved by SDS-PAGE.

Caspase Assay- The assay was performed using the Colorimetric CaspACE™ Assay System, (Promega) as reported in the instruction manual. Briefly, HeLa cells were transfected with lipofectamine 2000; 48hr after transfection cells were harvest in Caspase assay buffer and protein were quantified by Braedford Assay. 50 µg of protein were used.

Cell death and cell proliferation quantification- Cells were plated in 96-well plates in triplicate and incubated at 37°C in a 5%CO₂ incubator. To induce apoptosis, Superkiller TRAIL (Alexis Biochemicals, Lausen, Switzerland) was used for 24 h at 500ng/ml. Cell viability was evaluated with the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA), according to the manufacturer's protocol. Metabolically active cells were detected by adding 20 µl of MTT to each well. After 30 min of incubation, the plates were analyzed in a Multilabel Counter (Bio-Rad, Richmond, VA, USA). Apoptosis was also assessed using annexin V–FITC Apoptosis Detection Kits followed by flow cytometric analysis. Cells were seeded at 1.8 10⁶ cells per 100-mm dish, grown overnight in 10% FBS/RPMI, washed with PBS, then treated for 24 hours with 200 ng TRAIL. Following incubation, cells were washed with cold PBS and removed from the plates by very mild trypsinization conditions (0.01 % trypsin/EDTA). The resuspended cells were washed with cold PBS and stained with FITC-conjugated annexin V antibody and propidium iodide (PI) according to the instructions provided by

the manufacturer (Roche Applied Science, Indianapolis, IN). Cells (50,000 per sample) were then subjected to flow cytometric analysis. Propidium iodide staining and flow cytometry analysis were done as described (16). In order to quantify caspase activation, cells were transfected with the indicated cDNA or treated with Lithium chloride (20mM) and then incubated with superkiller TRAIL for the indicated times. Lysates were examined by Western blotting with anti-caspase 8 antibodies.

siRNA transfection- HeLa cells were cultured to 80% confluence, kept in antibiotic-free, serum-containing medium, and transiently transfected using LIPOFECTAMINE 2000 with 150 nMol anti-Gsk3- β siRNA (Invitrogen), a pool of two target specific 20-25 nt siRNAs, or with siCONTROL oligonucleotides, as indicated. Cells were incubated with siRNAs for the indicated times.

The siRNAs were transfected with 6 μ l transfection reagent, as described in the manufacturer's protocol. Anti p27^{Kip1} siRNA was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). siCONTROL Non-Targeting siRNA Pool #2 (D-001206-14-05) was from Dharmacon (Lafayette, CO, USA) and comprised four siCONTROL Non-Targeting siRNAs. Each individual siRNA within this pool was characterized by genome-wide microarray analysis and found to have minimal off-target signatures.

c-FLIP_L Knockdown- Stable knockdown of c-FLIP_L in HeLa cells was obtained with siRNAs (complementary sense and antisense oligonucleotides): FLIP-909, (GGAGCAGGGACAAGTTACA), and FLIP-1003 (GTAAAGAACAAAGACTTAA) or scrambled oligonucleotide, were cloned in the pRSC retroviral vector as described (21). Cells were selected with 10 μ g/ml puromycin.

RNA isolation and real-time PCR analysis- RNA was extracted with TRIzol solution (Invitrogen, Carlsbad, CA, USA) followed by DNase treatment (DNA-free, Ambion). The quality and quantity of RNA was determined by measuring the absorbance of the total RNA at 260 and 280 nm, and by 1% agarose electrophoresis under reducing conditions and visualized with ethidium bromide. For mRNA profiling, reverse transcription (RT) was performed by using Superscript II first strand synthesis kit (Invitrogen). Real time PCR to assay mRNA level was performed in an iQTM Real Time PCR detection System (BIO-RAD, Hercules, CA, USA) with iQTM SYBR[®] Green Supermix (BIO-RAD, Hercules,

CA, USA). All primers were synthesized commercially (PRIMM, Milan, Italy). PCRs were performed in triplicate and fold changes were calculated with the following formula: $2^{-(\text{sample 1 } \Delta\text{Ct} - \text{sample 2 } \Delta\text{Ct})}$ where ΔCt is the difference between the amplification fluorescent thresholds of the mRNA of interest and the mRNA of β -Actin used as an internal reference. All reactions were performed according to manufacturer's protocol.

Results

Akt interacts with c-FLIP_L- In order to find new Akt interactors, we performed a yeast two-hybrid screening. We used the full length human Akt c-DNA sequence as bait and a human c-DNA heart library as prey. Among the 200 clones obtained, two were identified to code for the anti-apoptotic protein, c-FLIP_L. To prove the interaction between Akt and c-FLIP_L, we performed immunoprecipitation experiments on endogenous proteins as well as on protein extracts from cells transfected with Akt and c-FLIP_L cDNAs. We were able to confirm the Akt/c-FLIP_L interaction in extracts from transfected cells (Figure 1A), as well as in endogenous proteins from different cell lines (Figure 1B). To verify whether Akt activity has a role on Akt/c-FLIP_L interaction, HeLa cells were transfected with either wild type Akt cDNA or with two different Akt mutants: kinase-dead Akt (Akt D-) and constitutively active Akt (Akt D+). Protein extracts were immunoprecipitated using a monoclonal anti-Flag antibody and subsequently blotted using an anti-HA antibody. As shown in Figure 1A, c-FLIP_L interacted at comparable levels with both the activated kinase and the kinase-dead Akt.

c-FLIP_L is characterized by two DED effector Domains (DED), which are important for interaction with members of the apoptosis cascade. We examined whether these DED domains were important for the interaction with Akt. For this purpose, we generated three different mutants: cFLIP_L I-DED, missing the first DED; cFLIP_L II-DED, missing the second DED; and cFLIP Δ-DED, missing both DEDs. The three mutants were transfected together with HA-Akt cDNA into HeLa cells. Extracts were immunoprecipitated with anti-Flag antibody and blotted with an anti-HA antibody. As shown in Figure 1C, all the c-FLIP_L deletion mutants interacted with Akt, indicating that neither DED domain is necessary for the interaction with Akt.

We next investigated whether the carboxy terminal of c-FLIP_L was the region of interaction with Akt. For this purpose, we generated different c-FLIP_L carboxy-terminal mutants named: c-FLIP_L F1 (aa 1-253), c-FLIP_L F3 (aa 1-339), c-FLIP_L F5 (aa 1-434), c-FLIP_L R0 (aa 1-149), and c-FLIP_L R1 (aa 1-182). Each mutant was transfected together with HA-Akt cDNA in HEK-293 cells. Extracts were immunoprecipitated after 48 hr with anti-Flag antibody and blotted with anti-HA-antibody. Akt interacted with F3 and F5 mutants but not with F1, R0 or R1 mutants (Figure 1D). The interaction of AKT with the short c-FLIP isoform (FLIPs) was barely detectable (Figure 1E). This suggests that the

Akt-c-FLIP interacting region is located between aa 253 and aa 339, within the caspase-like domain.

Role of c-FLIP_L on growth factor-mediated Akt signaling- Beside cell death, c-FLIP_L regulates also other DR-mediated signals. Thus, we set out to verify whether Akt-c-FLIP_L interaction might modulate Akt activation. For this purpose, we first transfected increasing amounts of c-FLIP_L cDNA and assessed the levels of the activated Akt, with specific phospho-Akt antibodies. c-FLIP_L overexpression did not induce significant differences in insulin-induced Akt phosphorylation (Figure 2A) even though modified the phosphorylation of Gsk3 β . As shown in Figure 2B, c-FLIP expression induced a reduction of endogenous Gsk3 β basal phosphorylation, in a dose-dependent fashion. A similar inhibition of Gsk3 β phosphorylation both basal and upon insulin stimulation, was observed cotransfecting the HA-Gsk3 β together with c-FLIP (Figure 2C). In not observed in presence of c-FLIP_s (Figure 2F).

This inhibition was not observed in HeLa cells transfected with c-FLIP_L mutants that do not interact with Akt, suggesting that Akt-FLIP interaction is necessary for this effect (Figure 2 D-E).

Role of c-FLIP modulation of Gsk3 β pathway on TRAIL induced cell death- While it has been clearly shown that c-FLIP_L overexpression may cause resistance to TRAIL, the effects of Gsk3 β on cell death are more controversial (22). However, recently it was described that Gsk3 β is involved in resistance to TRAIL-induced apoptosis. Therefore, we investigated whether c-FLIP_L-induced apoptosis resistance upon extrinsic pathway activation was at least in part mediated by its effects on Gsk3 β activation.

For this purpose, HeLa cells were transfected with Flag c-FLIP_L cDNAs alone or in the presence of the inhibitor of Gsk3 activity lithium chloride (23). The cells were subsequently incubated with TRAIL, and cell death was assessed with a cell viability assay or with propidium iodide staining followed by FACS analysis. As shown in Figure 3A and B, c-FLIP_L overexpression decreased the sensitivity of HeLa to TRAIL-induced apoptosis. However, treatment with LiCl completely counteracted the protective effect of c-FLIP on cell death (Figure 3A, B). In order to exclude unspecific effects of LiCl on cell death, the role of the Gsk3 β pathway in the anti-apoptotic effect of c-FLIP was further evaluated using a specific Gsk3 β kinase-inactive cDNA (Gsk3 β -KI) and measuring

caspace 8 activation. As shown in Figure 3C and D, c-FLIP_L overexpression reduced TRAIL-induced caspace 8 activation, and this effect was counteracted by both LiCl and Gsk3 β -KI cDNA. LiCl and Gsk3 β -KI or GSK3 β wt cDNA did not produce any effects on endogenous c-FLIP_L levels (supplemental figure 1).

Effects of c-FLIP_L on p27^{Kip1} expression- Recently, Gsk3 β inhibition has been suggested to regulate the cell cycle through regulation of p27^{Kip1} levels (24). In addition, we have recently shown that miRNAs regulates p27 expression and TRAIL sensitivity (25). Therefore, we addressed the question of whether the effect of c-FLIP_L on TRAIL resistance was mediated through Gsk3 β activity and thus on p27 expression levels.

As shown in Figure 4A, we found that the levels of p27^{Kip1} were drastically reduced in 293 cells stably overexpressing c-FLIP_L. A similar result was observed also in HeLa cells stably (HeLa Tween FLIP) or transiently overexpressing c-FLIP_L (Flag FLIP) (Figure 4B). On the other end, overexpression of c-FLIP_L deletion mutants of the Akt interaction site did not induce reduction of p27^{Kip1} levels (Figure 4C). Moreover, this effect was not observed in presence of c-FLIP_S (Figure 4F).

Down-regulation of Gsk3 β , by a specific siRNA or inactivation with LiCl, induced an increase of p27^{Kip1} levels in HeLa tween-FLIP_L compared to cells transfected with a scrambled siRNA (Figure 4D). Taken together these results indicate that the effect of c-FLIP on p27^{Kip1} is mediated by Gsk3 β activity. We next investigated whether c-FLIP_L-Gsk3 regulate p27^{Kip1} at mRNA levels. To asses this point, HeLa cells were transfected with 5 μ g of Flag c-FLIP_L cDNA or a control vector for 48 hr, and p27^{Kip1} cDNA levels were evaluated by Real time PCR. Interestingly, we found a significant reduction of p27^{Kip1} mRNA levels in HeLa cells transfected with c-FLIP_L but not with its mutant (Figure 4E), suggesting that the c-FLIP_L-Gsk3 pathway regulates p27^{Kip1} expression levels through a transcriptional mechanism.

The effect of p27^{Kip1} on TRAIL-mediated apoptotic signaling- We recently provided evidence that p27^{Kip1} is involved in TRAIL resistance in non small cell lung cancer (NSCLC) (25). We demonstrated that in TRAIL resistant CALU-1 cells, miRs-222 and -221 are overexpressed and target p27^{Kip1}, inducing its down-regulation. On the other hand, TRAIL sensitive H460 cells exhibited reduced levels of miR-222 and -221 and increased p27^{Kip1} expression. We therefore investigated whether p27^{Kip1} modulated sensitivity to TRAIL-mediated cell death through the regulation of the apoptotic

machinery molecules. To this aim, HeLa tween FLIP cells, that express very low p27^{Kip1} levels, were transfected with HA-p27 cDNA, and caspase 3 levels were investigated by western blot. We observed a significant increase of caspase 3 levels (Figure 5A). Furthermore silencing of p27^{Kip1} with a specific siRNA in H460 cells, that express high level of p27^{Kip1}, resulted in reduction of caspase 3 (Figure 5B).

To further confirm the role of the FLIP-Gsk3 pathway on TRAIL apoptotic machinery, we evaluated caspase 3 levels in HeLa c-FLIP overexpressing cells whose Gsk3 pathway has been inhibited either by Gsk3 siRNA or by LiCl treatment. Both, inhibitions resulted in an increase of caspase 3 expression levels, whereas no differences were observed in FADD levels (Figure 5C).

We investigated whether c-FLIP_L modulated caspase 3 transcript levels through a transcriptional mechanism, by Real Time-PCR. Interestingly, we found a significant reduction of caspase 3 mRNA levels in HeLa cells transfected with c-FLIP_L compared to controls, while this effect was not observed in FLIP R1 mutant (Figure 5D).

Finally, we examined also the activity of caspase 3 by the colorimetric CaspACETM assay in HeLa cells transfected with an empty vector or with c-FLIP_L cDNA. The expression of c-FLIP_L induced a reduction of caspase 3 activity (Figure 5E). [All these effects were reverted when c-FLIP_L endogenous levels were down-regulated by a specific c-FLIP_L siRNA \(Figure 5F\).](#)

DISCUSSION

In this report, we give evidence for a novel role of c-FLIP_L. c-FLIP_L has been identified as an inhibitor of apoptosis triggered by engagement of death receptors, such as Fas or TRAIL (26, 27). c-FLIP_L has been implicated also in other cellular functions, such as control of gene expression by extracellular signal-regulated kinase (ERK) and NF-κB (9, 28).

We demonstrate for the first time that Akt interacts with c-FLIP_L, and that this interaction functionally regulates Gsk3β activation and apoptosis. Recently, Giampietri et al (29) described that in c-FLIP transgenic mice, the phosphorylation of Akt and Gsk3β were reduced compared to control animals, even though caspase 3 activity was unchanged, highlighting an apoptosis-independent role of c-FLIP on pressure overload-mediated cardiac hypertrophy. The role of c-FLIP in heart development has been previously

described in c-FLIP ko mice that, similarly to FADD ko mice, developed severe defects of heart development (30, 31). These studies identify c-FLIP as a novel regulator of heart development and the hypertrophic response, possibly through Gsk3 signaling.

In this report, by genetic and biochemical methodologies, we have demonstrated that Akt is able to interact with c-FLIP_L in the region stretching from amino acid 253 to 339 of the c-FLIP_L protein. We found that overexpression of c-FLIP_L albeit does not interfere with insulin-induced Akt activation, almost abolishes Gsk3 β phosphorylation. The effects on Gsk3 β were abrogated when we overexpressed c-FLIP_L mutants that do not bind Akt. This may mean that, by binding to Akt, c-FLIP_L relegates the kinase in a different cellular compartment, and abolishes its ability to bind and phosphorylate its substrates. It is interesting that the phosphorylation of other Akt substrates besides Gsk3 β , such as BAD, was reduced in c-FLIP_L overexpressing cells (data not shown).

It has been reported that Gsk3 β contributes both to cell death and cell survival, depending on the cellular system and the appropriate stimuli (22). Several studies indicated that inhibition of Gsk3 β activity in cancer cells potentiates apoptosis stimulated by death receptor (32-35). Furthermore, knocking out Gsk3 β or inhibiting Gsk3 β with lithium, potentiates TNF-induced apoptosis, indicating an antiapoptotic role for Gsk3 β (33). Therefore we asked whether c-FLIP_L-mediated reduction of Gsk3 β phosphorylation, and thus increase of its kinase activity, might be necessary for the antiapoptotic function of c-FLIP_L. Interestingly, when we interfered with Gsk3 β activity, either with LiCl or with overexpression of a kinase inactive form of Gsk3, anti-apoptotic c-FLIP_L effects were significantly reduced. Thus, Gsk3 β may act as an important mediator that participates in FLIP's antiapoptotic function in human cancer.

We have recently demonstrated that p27 expression is linked to TRAIL resistance in non small cell lung cancer (NSCLC) cells overexpressing miR-222 (25). We therefore investigated the level of p27 in different cells overexpressing c-FLIP_L. Interestingly, we found an inverse correlation between the c-FLIP_L and p27 expression levels as well as Gsk3 β phosphorylation. This was also true in forced c-FLIP_L expressing cells (293 and HeLa). We then investigated whether c-FLIP could affect p27 levels through the activation of Gsk3 β . For this purpose, we interfered with Gsk3 β expression levels or activity in c-FLIP_L overexpressing cells and evaluated p27 levels. We found that Gsk3 β inhibition increased protein and mRNA p27 levels. The effects of FLIP_L on p27 depend on its interaction with Akt, since c-FLIP_L wild type overexpression but not its Akt-binding

site deletion mutants, was able to reduce p27 mRNA. Recently, Wang et al. described that Gsk3 β negatively regulates p27 protein in MLL leukemia cells (24), being thus critical for maintenance of MLL leukemia, and prospecting Gsk3 as an interesting target for this form of cancer. In the MLL cellular system, the effects were mainly at the protein level since the inhibition of Gsk3 β did not affect mRNA levels. Therefore, although the final effect is similar, the functional relationships of Gsk3 β with p27 seem cell type-dependent. Gsk3 β is a negative regulator of heart hypertrophy (36). Interestingly, Hauck recently described that silencing p27 induced cardiomyocyte hypertrophic growth in the absence of growth factor stimulation (37). It is interesting to speculate that Gsk3 β mediates negative regulation of hypertrophic growth through its effects on p27 expression levels.

Finally, we investigated the mechanisms of c-FLIP-Gsk3 β -p27-mediated inhibition of cell death, by the evaluation of protein and mRNA levels of apoptosis signaling molecules.

We showed that the absence of p27 induces a reduction of caspase 3 levels. This effect was mediated by Gsk3 β since its inactivation induced an increase of caspase 3. The effect was specific on caspase 3 because other apoptosis signaling molecules, such as FADD, were not affected. This effect occurred at the transcriptional level because c-FLIP_L overexpression, but not its mutants, was able to reduce caspase 3 mRNA as assessed by RT-PCR. Overexpression of c-FLIP_L induces also a significant reduction of the amount of the active caspase in untreated cells. Thus, taken together these data depict a model in which in c-FLIP overexpressing cells, the activation of Gsk3 β induces a reduction of p27^{Kip1} and caspase 3 expression and activity levels and thus a reduction of TRAIL-induced cell death (figure 7).

Recently, Gsk3 β has been described as a protein complex associated with death receptors, DDX3, and cellular inhibitor of apoptosis protein-1 (cIAP-1) (32). In that study, Gsk3 β inhibited apoptosis by interfering with DISC formation and caspase 8 activation. Our data reveal another possible mechanisms through which Gsk3 might inhibit apoptosis, that is regulation of p27 expression and, downstream of that, caspase 3 (Figure 7).

Our data show that c-FLIP overexpression strongly reduces AKT-mediated Gsk3 β phosphorylation. Furthermore, its down-regulation by a specific FLIPsi RNA, resulted in an increase of Gsk3 β phosphorylation, as well of p27 and caspase 3 levels.

In conclusion, this work demonstrates that anti-apoptotic functions of c-FLIP_L are mediated by its effects on Gsk3 β activity, and p27 and caspase 3 levels. These findings may be of importance in optimizing a strategy for the treatment of TRAIL-resistant human cancer.

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Figure legends

Figure 1- Akt interacts with c-FLIP_L. (A) HeLa cells were co-transfected with HA-Akt wt, Akt D+, or Akt D- cDNAs and c-FLIP_L for 48hrs, as indicated. Protein extracts were immunoprecipitated (Ip) with anti-Flag antibody and blotted with an anti-HA antibody. As negative control, proteins were incubated with beads without antibody. Total lysates were immunoblotted with the indicated antibody. (B) Co-immunoprecipitation of endogenous proteins. Equal amounts of total cell proteins from HeLa, K562, DU145, and A459 cell lines were immunoprecipitated with anti-Akt antibody and blotted with anti- c-FLIP_L antibody. Total lysates (50 µg) were immunoblotted with anti- c-FLIP_L or anti β-actin antibodies. (C) and (D) Identification of FLIP/Akt interaction site: HEK-293 cells were transfected with 2µg of either wt c-FLIP_L cDNA or the N-terminal deletion mutants, c-FLIP_L I-DED, c-FLIP_L II-DED, c- FLIP_L Δ-DED (C), or the C-terminal deletion mutants c-FLIP_L-F1, -F3, -F5, -R0, -R1 (D), as indicated. Protein extracts were immunoprecipitated with anti-Flag antibody and blotted with anti-Akt antibody. Total extracts were loaded as control, and blotted with anti Akt or anti Flag antibodies. Akt was not able to interact with F1, R0 and R1 mutants, indicating that Akt-c-FLIP interacting region is included from aa 253 to aa 339. (E) 293T cells were transfected with c-FLIP_L or c-FLIP_S cDNA. Total lysates were immunoprecipitated with an anti Flag antibody and then blotted with an anti Akt antibody.

Figure 2- Role of c-FLIP_L on Akt/Gsk3β signaling pathway. (A) c-FLIP effects on Akt activation. HeLa cells were transfected with c-FLIP_L cDNA for 24 hr, serum-starved for 12 hr and then treated with insulin (100 nM) for 15 min. Total cellular extracts were resolved by western blot and analyzed with the indicated antibodies. FLIP overexpression does not affect Akt phosphorylation. (B) Western blot analysis of p-Gsk3β and Gsk3β expression in HeLa wt or transfected with different concentrations of c-FLIP_L c-DNA (500 ng, 1 µg, and 2 µg) for 48 hr. We observed a strong reduction of Gsk3β phosphorylation. (C) HeLa cells were transfected with HA-Gsk3β c-DNA, and c-FLIP_L cDNA or control vector, as indicated for 24 hr. Cells were starved for 12 hr and then treated with insulin (100nM) for 15 min. Cell lysates were immunoprecipitated with anti-HA antibody and blotted with phospho-Gsk3β antibody. (D) Western blot analysis of

HeLa cells transfected with c-FLIP-wt, -F1, -R0, or -R1 cDNA. Total lysates were analyzed with anti-phospho-Gsk3 β , Gsk3, Flag and β -actin antibodies. c-FLIP mutants were not able to decrease phospho Gsk3 β levels. **(E)** HeLa cells were transfected with HA-Gsk3 β and c-FLIP-R1 c-DNA or with a control vector, treated with Insulin (100nM) for 15 min, immunoprecipitated with anti-HA antibodies, and blotted with P-Gsk3 β antibodies. Total lysates were analyzed with the indicated antibodies. c-FLIP mutant overexpression did not reduce Gsk3 β phosphorylation. **(F)** HeLa cells were transfected with HA-Gsk3 β c-DNA, and c-FLIP_L cDNA, c-FLIP_S cDNA or control vector, as indicated for 24 hr. Cells were serum starved for 12 hr and then treated with insulin (100nM) for 15 min. Cell lysates were immunoprecipitated with anti-HA antibody and blotted with anti-phospho-Gsk3 β antibody.

Figure 3- Role of c-FLIP_L and Gsk3 β signaling pathway on TRAIL-induced cell death- Cell Death quantification- HeLa cells were transfected with 2 μ g of c-FLIP_L cDNA for 24hrs, plated in 96 well plates in triplicate and then treated with SuperKiller TRAIL (500 ng/ml) and lithium chloride (20mmol) for 48hr, as indicated. Cell viability was assessed by Cell Vitality assay **(A)** or by propidium iodine staining and FACS analysis **(B)**. **(C, D)** Western blot analysis of caspase 8 activation. The inhibition of Gsk3 β was obtained by transfection of HeLa cells with kinase inactive Gsk3 β cDNA or by treatment with 20mMol lithium chloride for 24 h. Cells were incubated with 500 ng/ml TRAIL for 1 or 3 hrs. The inhibition of Gsk3 β induced an increase of caspase 8 activation in c-FLIP overexpressing cells.

Figure 4- c-FLIP overexpression regulates p27^{Kip1} levels through Gsk3 β . Western blot analysis of Gsk3 β , p-Gsk3 β and p27^{Kip1} levels in different cell lines. **(A)** HEK293-tween GFP and tween-GFP c-FLIP_L; **(B)** HeLa cells transfected with c-FLIP_L cDNA and HeLa tween c-FLIP_L. There is an inverse correlation between FLIP and p-Gsk3 levels. **(C)** Western blot analysis of p27^{Kip1} levels in HeLa cells transfected with c-FLIP_L mutants F1, R0, and R1 cDNA. **(D)** Effects of inhibition of Gsk3 β on p27^{Kip1} expression. HeLa tween FLIP cells were transfected with Gsk3 β -siRNA or scrambled-siRNA or treated with 20 mMol lithium chloride for 24 hr. Levels of p27^{Kip1} and Gsk3 β were analyzed by immunoblotting. **(E)** Real time PCR analysis of p27^{Kip1} mRNA with transfection of FLIP_L cDNA in HeLa cells. c-FLIP reduces p27^{Kip1} levels through Gsk3 β . c-FLIP deletion

mutants were not able to reduce p27^{Kip1} levels. **(F)** Total lysates of HeLa cells transfected with c-FLIP_L, c-FLIP_S or control vector were analyzed for p27 expression.

Figure 5- Role of p27^{Kip1} on caspase 3 expression. **(A)** HeLa tween-FLIP cells transfected with HA p27^{Kip1} cDNA were analyzed by western blot for caspase 3. Loading and transfection control were analyzed with p27^{Kip1} and β -actin antibodies. p27 overexpression induced an increase of caspase 3 expression levels. **(B)** Western blot analysis of caspase 3 and 8 in H460 cells upon transfection of p27^{Kip1} siRNA. The inhibition of p27 decreases caspase 3 levels **(C)** Immunoblotting of HeLa Tween-FLIP cells upon transfection of scrambled siRNA (sc-si), Gsk3 β siRNA or treatment with 20mM lithium chloride for 24 hr with caspase 3, FADD or Gsk3 β antibodies. The inhibition of Gsk3 pathway, resulted in a increase of expression levels of Caspase 3 while no differences were observed in FADD and caspase 8 expression. **(D)** Real time PCR of caspase 3 mRNA upon transfection in HeLa cells with Flag-FLIP_L cDNA. FLIP overexpression induced a significant reduction of caspase 3 mRNA levels. **(E)** Quantification of caspase 3 activity by Colorimetric CaspACETM Assay System in HeLa cells transfected with an empty vector or Flag-FLIP_L cDNA. **(F)** Total lysate of HeLa cells expressing RNAi constructs for c-FLIP (FLIP 1003) or with a scrambled control. 40 μ g of proteins were loaded and blotted with the indicated antibodies. β -actin was used as the loading control. FLIP down-regulation induced an increase of GSK3 β phosphorylation, p27 and caspase 3 levels. Representative experiment done in triplicates.

Figure 6- Role of cFLIP -Gsk3 signaling pathway in the regulation of cell death. In FLIP overexpressing cells, activation of Gsk3 β induces a reduction of p27^{Kip1} and caspase 3 expression levels and a reduction of TRAIL-induced cell death.

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A

cDNA HA-AKT - wt D+ D-
 cDNA Flag FLIP_L - + + +

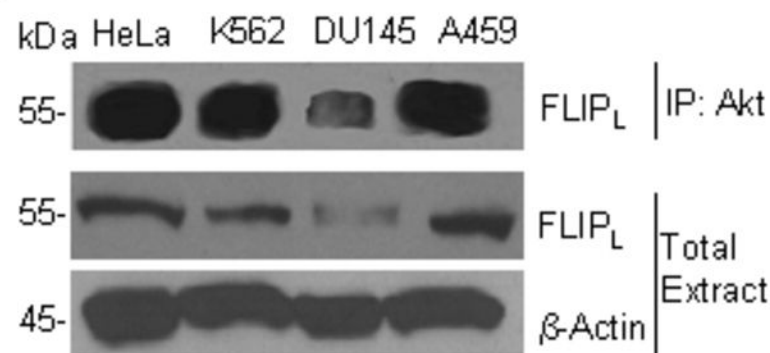
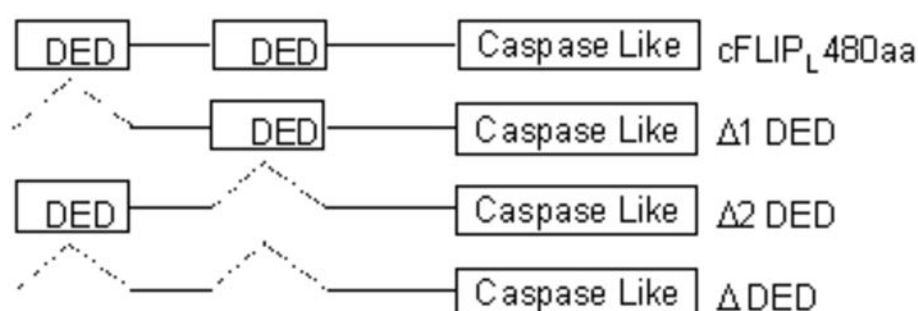
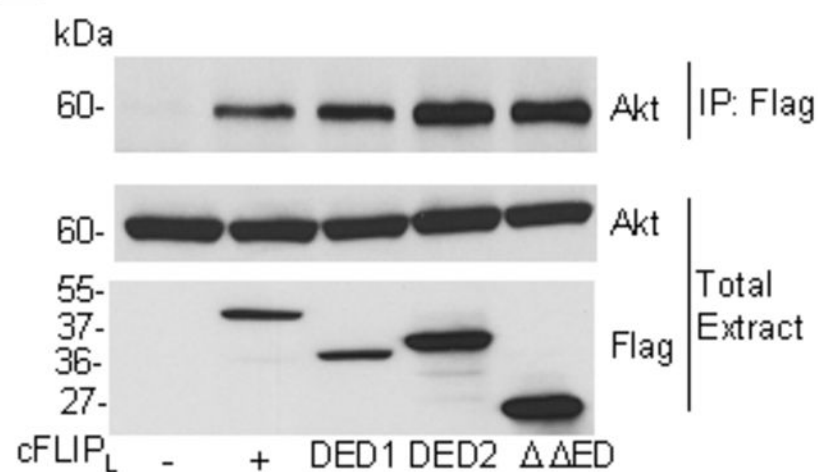
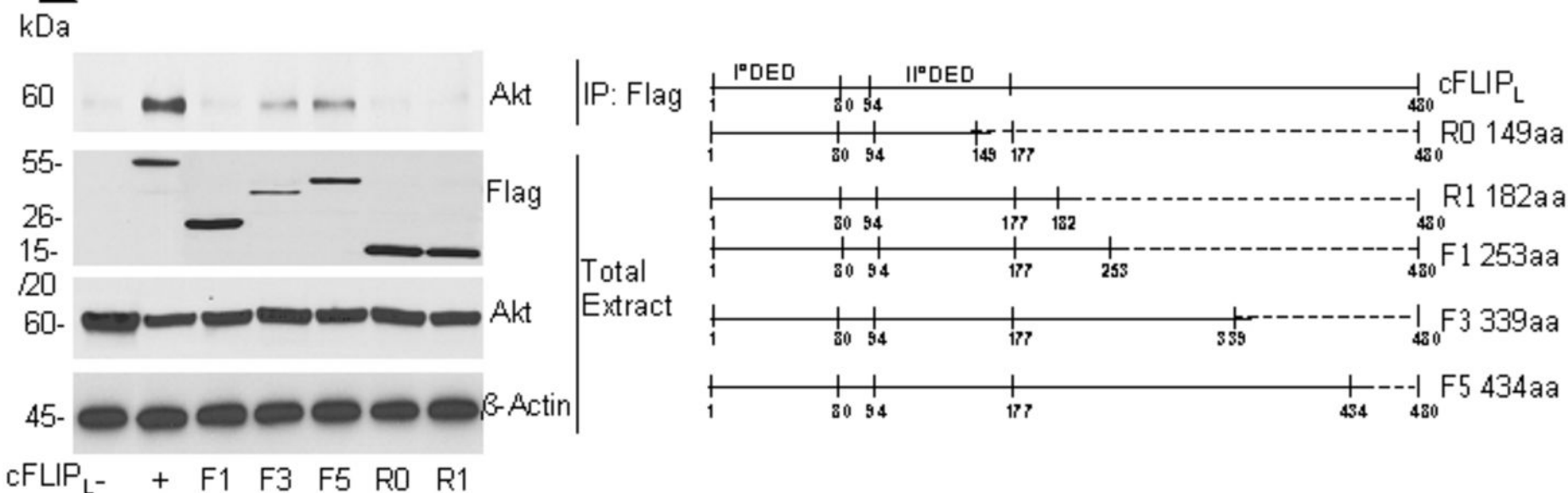
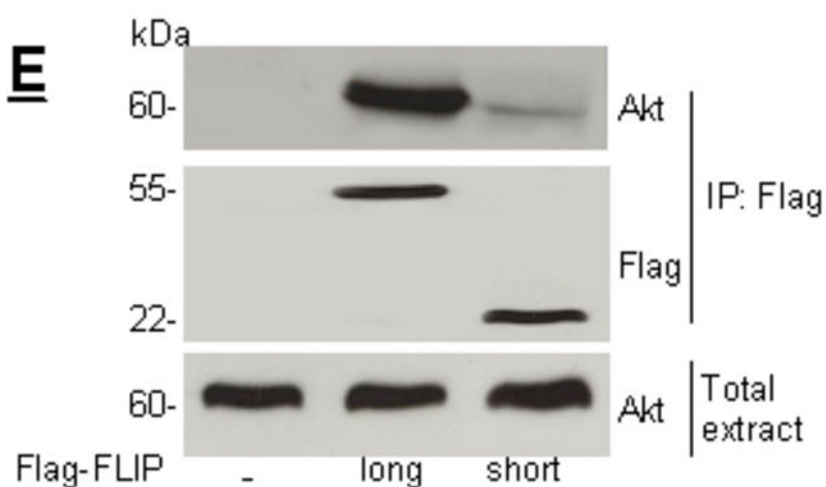
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Figure 1

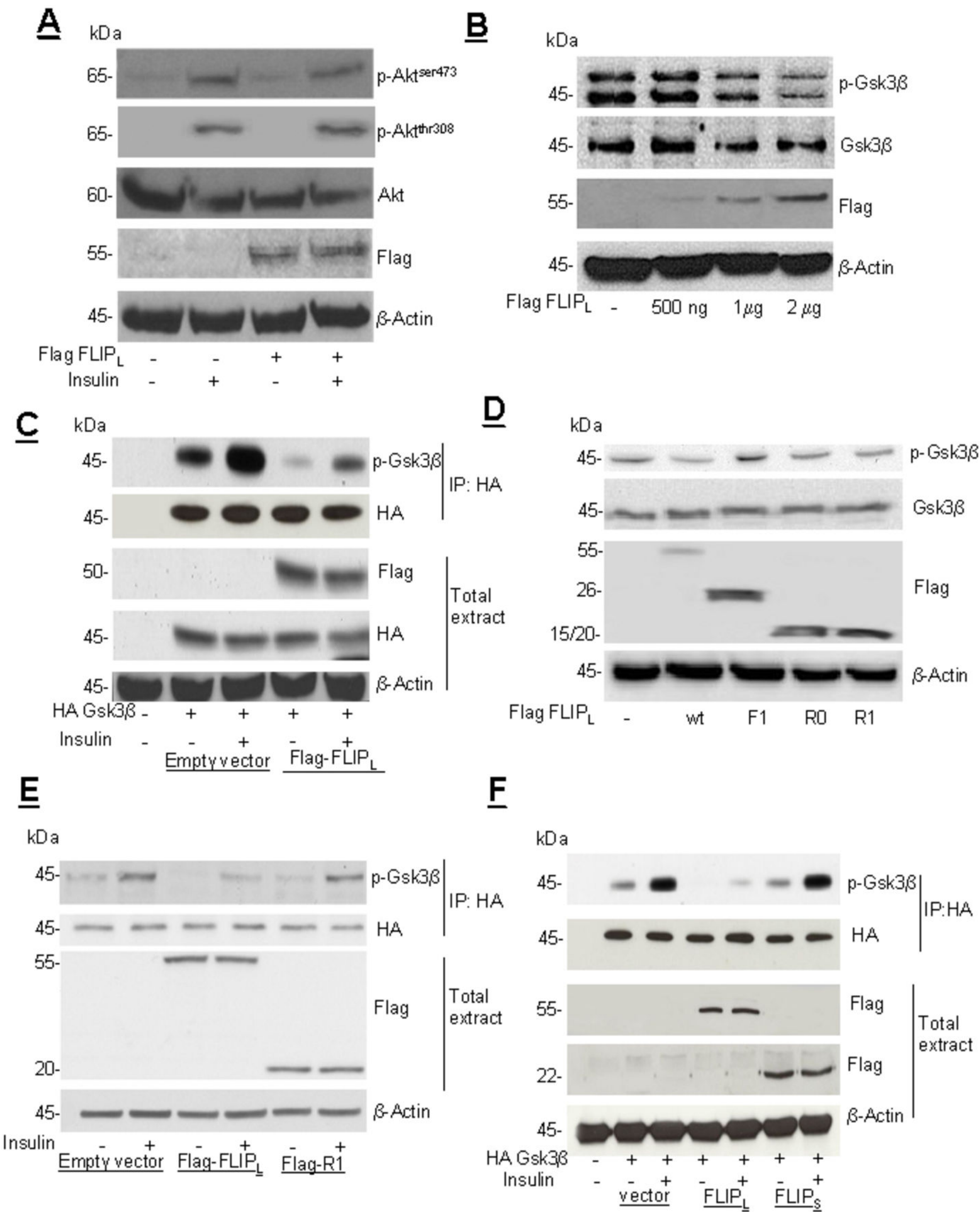


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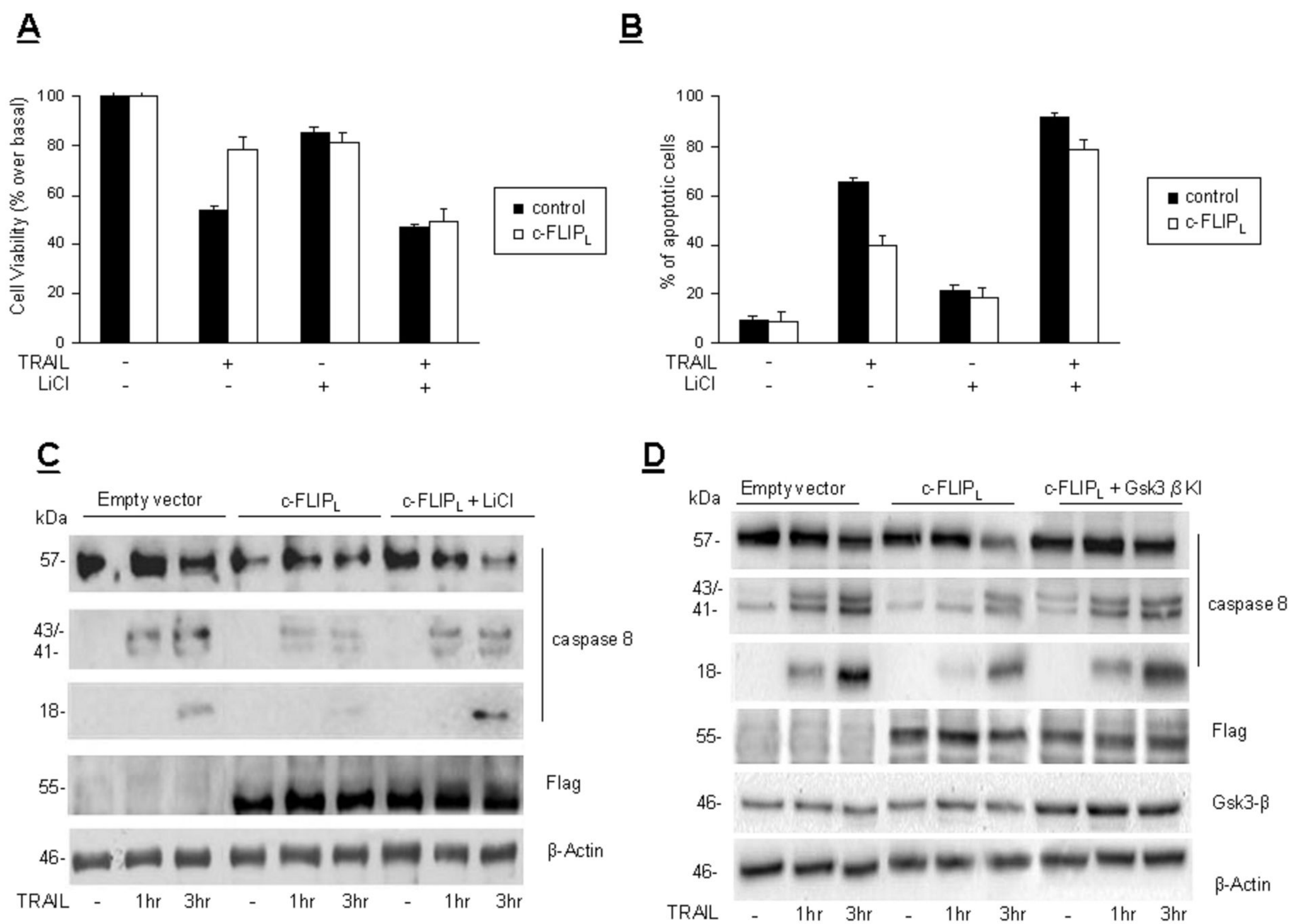


Figure 3

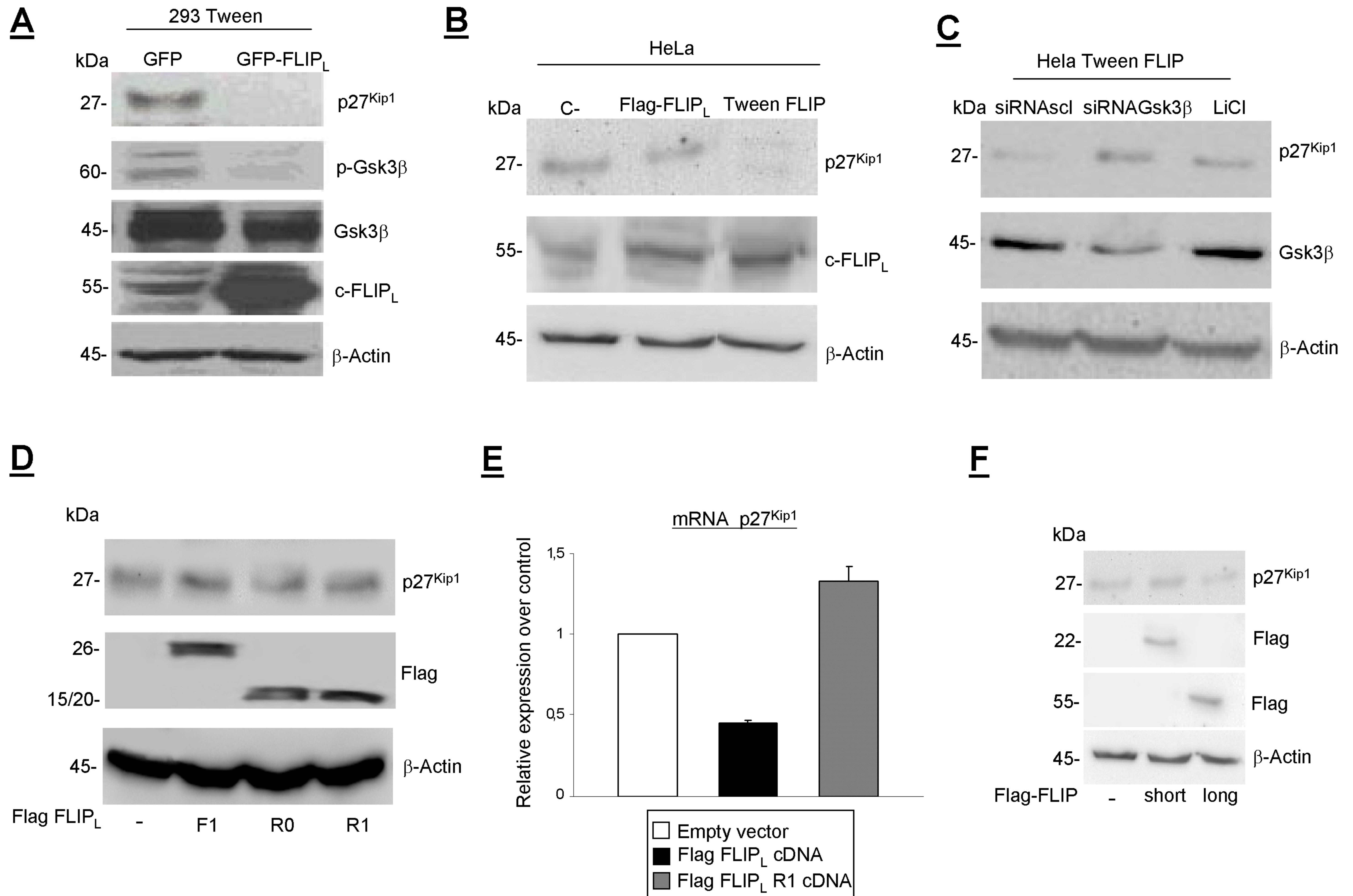


Figure 4

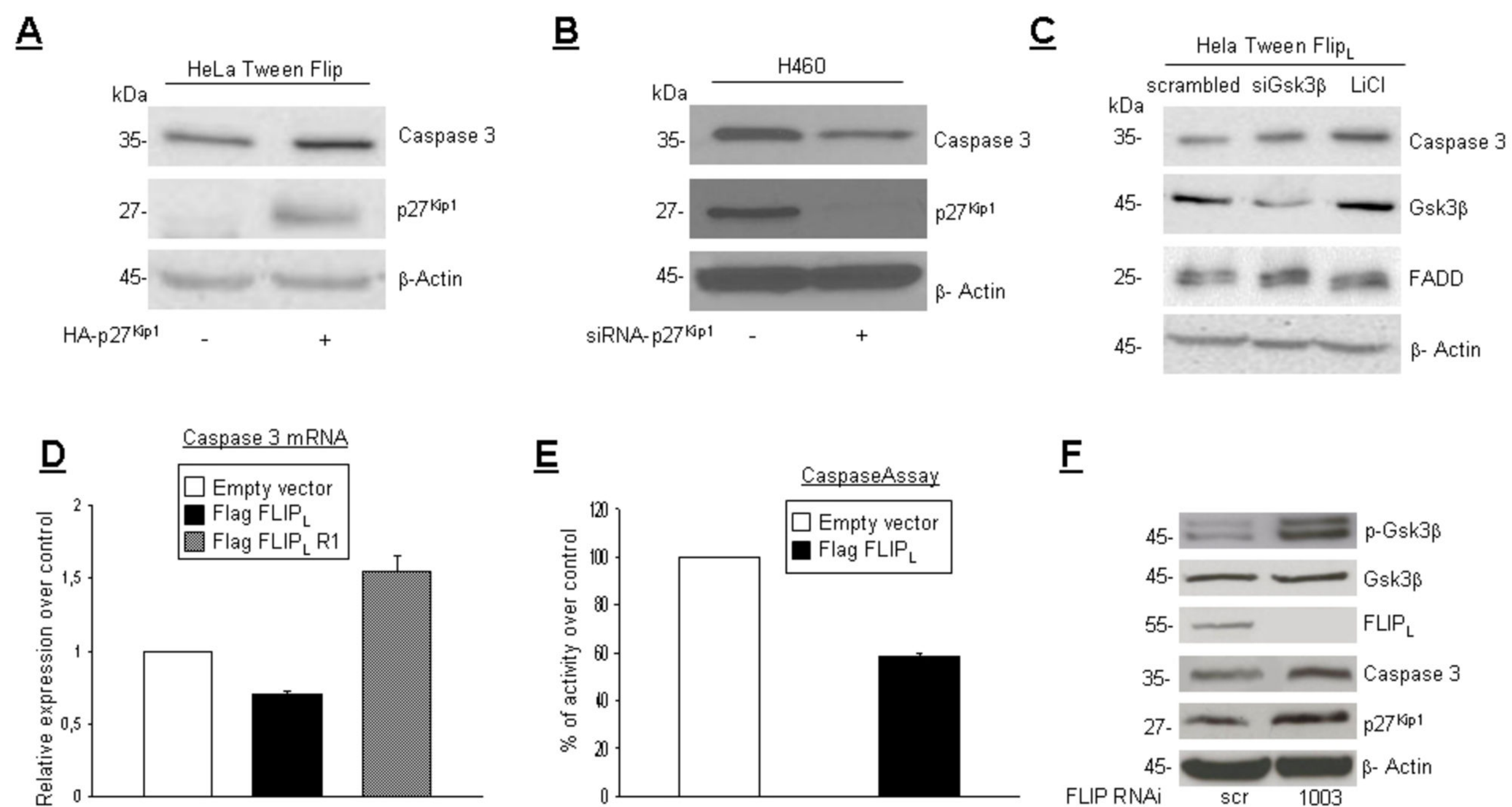


Figure 5

