ONLINE SUPPORTING INFORMATION

Regulation of autophagic activity by 14-3-3ζ proteins associated with class III phosphatidylinositol-3‑kinase

Mercedes Pozuelo Rubio

Centro Andaluz de Biología Molecular y Medicina Regenerativa, Consejo Superior de Investigaciones Científicas. Sevilla. Spain.

Correspondence to Mercedes Pozuelo Rubio, CABIMER (SC4). Av. Américo Vespucio s/n, Sevilla-41092, Spain. TEL: (34)-663719656; FAX: (34)-954-461664 E-mail: merce_pozo@yahoo.es

Supplementary Figure S1. C2-ceramide 10 μM for 24 h does not induce cell death. Hela cells were left untreated or stimulated with C2-ceramide (10 μM for 24 h) or TRAIL (1 μg/ml) (generously provided by Dr. Lopez-Rivas) as a control of cell death. Cells were analyzed for the DNA content by flow cytometry. The percentage of death cells with sub-G1 DNA content is shown and the values represent mean ± SD for three independent experiments.

Supplementary Figure S2. Over expression of 14-3-3ζ proteins block C2-ceramide cell death with autophagy. HeLa and HEK293T cells left untransfected or HeLa cells stably expressing GFP-14-3-3ζ and HEK293T cells transiently expressing GFP-14-3-3ζ were stimulated with C2-ceramide (C2) (10 μM) for 3 or 5 days. The percentage of living cells (Crystal violet) was expressed according to untreated cells and quantified (Quantification: N = 3, * P=0.0148, ** P=0.00033, ** P=0.0019, ** P=0.0082, Student t-test).

Supplementary Figure S3. Downregulation of endogenous 14-3-3ζ sensitizes cells to autophagy.

(A) MCF-7 cells stably expressing GFP-LC3 fusion protein were transfected either with siRNA oligonucleotide targeting 14-3-3ζ or with a scrambled RNA oligonucleotide as described in Material ad Methods. After 48 hours, untransfected cells (Control) or cells transfected with either with siRNA 14-3-3ζ (14-3-3ζ) or scrambled siRNA (SC) were analysed using green fluorescence GFP-LC3 by microscopy**.** Bars = 10 μm. Quantification of number of GFP-LC3 punctae per cell was performed by counting in a blinded experiment. Columns, average of three different experiments, set up at duplicated samples and counting 6 x 100 cells per sample (N=3, * *P*=0.0004, ** *P*=0.0006 Student *t*-test). **(B)** As in (A) extracts from untransfected cells (Control) or transfected either with siRNA 14-3-3ζ (14-3-3ζ) or scrambled siRNA (SC) were harvested (after 48 hours) for immunoblot analysis of LC3-I processing into LC3-II, GFP-LC3 processing into GFP, p62/SQSTM1 and endogenous 14-3-3ζ isoform. Tubulin was used as a protein loading control. Endogenous LC3-II/Tubulin levels were quantified and the ratio presented as arbitrary units (N=3, * *P*=0.0026, ** *P*=0.0072, Student *t*-test).

Supplementary Figure S4. Downregulation of endogenous 14-3-3ζ does not affect levels of expression of autophagy-related proteins. Extracts of HeLa cells (30 μg) untransfected (C) or transfected either with siRNA 14-3-3ζ (14-3-3ζ) or scrambled siRNA (SC) for 48 hours were run on SDS/PAGE transferred to nitrocellulose membranes and probed with indicated antibodies.

Supplementary Figure S5. Downregulation of endogenous 14-3-3σ and 14-3-3θ sensitizes cells to autophagy. (A) Extract of HeLa, MCF-7 and HEK293T cells (30 μg) were run on SDS/PAGE transferred to nitrocellulose membranes and probed with indicated antibodies against different 14-3-3 isoforms. The 14-3-3 pan antibody recognized all of the different isoforms. GAPDH was used as a proteins loading control. **(B)** MCF-7 cells expressing GFP-LC3 were left untransfected (Control) or transfected either with siRNA oligonucleotide targeting 14-3-3σ (14-3-3σ) or with a scrambled RNA oligonucleotide (SC) as described in Material and Methods. After 48 hours, MCF-7/GFP-LC3 cells were analysed using green fluorescence GFP-LC3 by microscopy. Quantification of number of GFP-LC3 punctae per cell was performed by counting in a blinded experiment. Columns, average of

three different experiments, set up at duplicated samples and counting 6 x 100 cells per sample (N=3, * *P*=0,0018, ** *P*=0,0026, Student *t*-test). **(C)** As in (B) MCF-7 cells expressing GFP-LC3 were left untransfected (Control) or transfected either with siRNA oligonucleotide targeting 14-3-3θ (14-3-3θ) or with a scrambled RNA oligonucleotide (SC). After 48 hours, cells were analysed using green fluorescence GFP-LC3 by microscopy**.** Quantification of number of GFP-LC3 punctae per cell was performed by counting in a blinded experiment. Columns, average of three different experiments, set up at duplicated samples and counting 6 x 100 cells per sample (N=3, * *P*==0,0085, ** *P*=0,0031, Student *t*-test). **(D)** As in (B) extracts from MCF-7/GFP-LC3 untransfected cells (C) or transfected either with siRNA 14-3-3σ (14-3-3σ) or scrambled siRNA (SC) for 48 hours were harvested for immunoblot analysis of LC3-I processing into LC3-II, GFP-LC3 processing into GFP, p62/SQSTM1 and endogenous 14-3-3 isoforms. GAPDH was used as a protein loading control. Endogenous LC3-II/GAPDH levels were quantified and the ratio presented as arbitrary units (N=3, * *P*=0.00031, ** *P*=0.04, Student *t*-test). **(E)** As in (C) extracts from MCF-7/GFP-LC3 untransfected cells (C) or transfected either with siRNA 14-3-3θ (14-3-3θ) or scrambled siRNA (SC) for 48 hours were harvested for immunoblot analysis of LC3-I processing into LC3-II, GFP-LC3 processing into GFP, p62/SQSTM1 and endogenous 14-3-3 isoforms. GAPDH was used as a protein loading control. Endogenous LC3-II/GAPDH levels were quantified and the ratio presented as arbitrary units (N=3, * *P*=0.00039, ** *P*=0.0065, Student *t*-test).

Supplementary Figure S6. hVps34 dissociates from 14-3-3 and is activated during C2-ceramide-induced autophagy in HEK293T cell. HEK293T continuously growing in serum were left untreated or stimulated with C2-ceramide (10 μM) for 24 h to promote autophagy. **(A)** Extracts (2 mg) from untreated (U) or C2-ceramide (10 μM for 24 h) (C2) stimulated HEK293T cells were incubated with 1 μg of Beclin-1 (Santa Cruz) antibody bound to Protein-G-Sepharose. The washed immunoprecipitates were resolved using SDS-PAGE, transferred to nitrocellulose and probed with DIG-14-3-3 (14-3-3 overlay), Beclin-1 (BD Biosciences) and hVps34 (Zymed) antibodies. The washed immunoprecipitates were also used for hVps34 *in vitro* kinase assay. Autoradiography shows levels of $PI3P^{32}$. (Quantification of hVps34 *in vitro* kinase assay expressed as PI(3) fold change, N = 3, * *P*=0.0005, Student *t*-test). **(B)** Extracts (1 mg) from untreated (U) or C2-ceramide (10 μM for 24 h) (C2) stimulated HEK293T cells were incubated with 1 μg of

hVps34 (Echelon Biosciences) antibody bound to Protein-G-Sepharose. The washed immunoprecipitates were used for hVps34 *in vitro* kinase assay and also to analyse interaction with 14-3-3 (DIG-14-3-3) and presence of Beclin-1 and hVps34 in the immunoprecipitates (Quantification of hVps34 *in vitro* kinase assay, N = 3, * *P*=0.0064, Student *t*-test). A piece of the DIG-14-3-3 shown corresponds to hVps34 size. **(C)** Extracts (30 μg) from HEK293T cells continuously grown in serum or stimulated with C2-ceramide (10 μM) for 24 h to promote autophagy were run on SDS-PAGE, transferred to nitrocellulose membranes and probed with indicated antibodies**.**

Supplementary Figure S7. Overexpression of 14-3-3ζ decreases hVps34 activity meanwhile blocking C2-ceramide-dependent autophagy in HEK293T cells. HEK293T cells were transfected with GFP-control (C) or GFP-14-3-3ζ. **(A)** Continuously serum-grown cell extracts (1 mg) were incubated with 1 μg of hVps34 (Echelon Biosciences) antibody bound to Protein-G-Sepharose. The washed immunoprecipitates were used for hVps34 *in vitro* kinase assay and also resolved to detect Beclin-1 and hVps34 proteins. Cell extracts (30 μg) from both samples were run on SDS-PAGE, transferred to nitrocellulose membranes and probed with indicated antibodies (bottom panel) (Top panel: Quantification of hVps34 *in vitro* kinase assay expressed as PI(3) fold change, N = 3, * *P*=0.006 Student *t*-test). **(B)** Cell extracts (30 μg) from left untreated (U) or C2-ceramide (10 μM for 24h) stimulated (C2) were run on SDS-PAGE, transferred to nitrocellulose membranes and probed with indicated antibodies. Endogenous LC3-II/Tubulin levels were quantified and the ratio presented as arbitrary units (N=3, * *P*=0.0045 Student *t*-test). **(C)** Cell extracts (2 mg) from left untreated (U) or C2-ceramide (10 μM for 24h) stimulated (C2) cells were incubated with 1 μg of Belin-1 (Santa Cruz) antibody bound to Protein-G-Sepharose. The washed immunoprecipitates were used for hVps34 *in vitro* kinase assay and to analyse presence of Beclin-1 and hVps34 proteins in immonoprecipitates. (On the top: Quantifications of hVps34 *in vitro* kinase assays, N = 3, * *P*=0.0059, Student *t*-test).

Supplementary Figure S8. Downregulation of endogenous 14-3-3ζ increase hVps34 activity in MCF-7 cells. MCF-7 cells were transfected either with siRNA oligonucleotide targeting 14-3-3ζ or with a scrambled RNA oligonucleotide as described in Material ad Methods. After 48 hours, extract of untransfected (Control) or transfected either with siRNA 14-3-3ζ (14-3-3ζ) or scrambled siRNA (SC) MCF-7 cells were incubated with

1 μg of hVps34 (Echelon Biosciences) antibody bound to Protein-G-Sepharose. The washed immunoprecipitates were used for hVps34 *in vitro* kinase assay and to analyse presence of hVps34 protein in immonoprecipitates (Top panel: Quantification of hVps34 *in vitro* kinase assay, N = 3, * P=0.025, ** P=0.011, Student t-test). Extracts from untransfected cells (C) or transfected either with siRNA 14-3-3ζ (14-3-3ζ) or scrambled siRNA (SC) were harvested for immunoblot analysis of 14-3-3ζ isoform. Tubulin was used as a protein loading control (bottom panel).

Supplementary Figure S9. hVps34 dissociates from 14-3-3 and is activated during Starvation-induced autophagy in MCF-7 cells. (A) MCF-7 cells expressing GFP-LC3 fusion protein and continuously growing in serum were left untreated or starved during 6h. Cells were analysed using green fluorescence GFP-LC3 by microscopy**.** Bars = 10 μm. Quantification of number of GFP-LC3 punctae per cell was performed by counting in a blinded experiment. Columns, average of three different experiments, set up at duplicated samples and counting 6 x 100 cells per sample (N=3, * P=0.00027, Student t-test) (top panel). Untreated and starved MCF-7/GFP-LC3 cells were harvested for immunoblot analysis indicated proteins. Tubulin was used as a protein loading control (bottom panel). **(B)** MCF-7/GFP-LC3 cells were treated as in (A) and extracts from untreated and starved cells were incubated with 1 μg of hVps34 (Echelon Biosciences) antibody bound to Protein-G-Sepharose. The washed immunoprecipitates were used for hVps34 *in vitro* kinase assay and test presence of hVps34 protein in immonoprecipitates and 14-3-3-binding (DIG-14-3-3) (Top panel: Quantification of hVps34 *in vitro* kinase assay, N = 3, * P=0.0035, Student t-test).

Supplementary Figure S10. hVps34 dissociates from 14-3-3 during Etoposide and Rapamycin-induced autophagy. (A) HeLa continuously grown in serum (C) were left untreated or incubated in presence or absence of 3-methyladenine (3MA) (10 mM for 1 h) and then stimulated with Etoposide (20 μM for 24 h) as indicated. Cell extracts were incubated with 1 μg of hVps34 (Echelon Biosciences) antibody bound to Protein-G-Sepharose. The washed immunoprecipitates were used to test presence of hVps34 protein in immonoprecipitates and 14-3-3-binding (DIG-14-3-3) (Quantification of DIG-14-3-3 overlay signal, N = 3, * P=0.00081, ** P=0.00014, Student t-test). **(B)** HeLa/GFP-LC3 cells treated as in (A) were analysed using green fluorescence GFP-LC3 by microscopy.

Quantification of number of GFP-LC3 punctae per cell was performed by counting in a blinded experiment. Columns, average of three different experiments, set up at duplicated samples and counting 6 x 100 cells per sample (N=3, * *P*=0,0021, ** *P*=0,009, Student *t*-test). **(C)** HeLa cells treated as in (A) were harvested for immunoblot analysis of indicated proteins. GAPDH was used as a protein loading control. (Endogenous LC3-II/GAPDH levels were quantified and the ratio presented as arbitrary units (N=3, * *P*=0.00097, ** *P*=0.00072, Student *t*-test). **(D)** MCF-7/GFP-LC3 cells continuously grown in serum (C) were left untreated or incubated in presence or absence of 3-methyladenine (3MA) (10 mM for 1 h) and then stimulated with Rapamycin (5 μM for 24 h) as indicated. Cell extracts were incubated with 1 μg of hVps34 (Echelon Biosciences) antibody bound to Protein-G-Sepharose. The washed immunoprecipitates were used to test presence of hVps34 protein in immonoprecipitates and 14-3-3-binding (DIG-14-3-3) (Quantification of DIG-14-3-3 overlay signal, N = 3, * *P*=0.0018, ** *P*=0.002, Student *t*-test). **(E)** MCF-7/GFP-LC-3 cells treated as in (D) were analysed using green fluorescence GFP-LC3 by microscopy. Quantification of number of GFP-LC3 punctae per cell was performed by counting in a blinded experiment. Columns, average of three different experiments, set up at duplicated samples and counting 6 x 100 cells per sample (N=3, * *P*=0,00014, ** *P*=0,00031, Student *t*-test). **(F)** MCF-7/GFP-LC-3 cells treated as in (D) were harvested for immunoblot analysis of indicated proteins. GAPDH was used as a protein loading control. (Endogenous LC3-II/GAPDH levels were quantified and the ratio presented as arbitrary units (N=3, * *P*=0.00045, ** *P*=0.00092, Student *t*-test).

Supplementary Figure S11. hVps34 from lung mouse tissue binds 14-3-3 proteins. (A) Extract of lung mouse tissue and HeLa cells continuously growing in serum (30 μg) were run on SDS-PAGE, transferred to nitrocellulose membranes and probed with indicated antibodies. GAPDH was used as a protein loading control. **(B)** Extract of lung mouse tissue (3-5 mg of protein) were incubated for 2 h with 50 μl of 14-3-3–Sepharose, pellets were washed (C) and extracted with SDS-sample buffer (Pull down), run on SDS/PAGE and probed with antibodies against hVps34. Beside, lung mouse tissue extract (Ext) were run in term of comparison. **(C)** Extract of lung mouse tissue (2 mg of protein) was incubated with 1 μg of hVps34 (Echelon Biosciences) antibody bound to Protein-G-Sepharose. The washed immunoprecipitates, from extract (IP) or control (C), were resolved

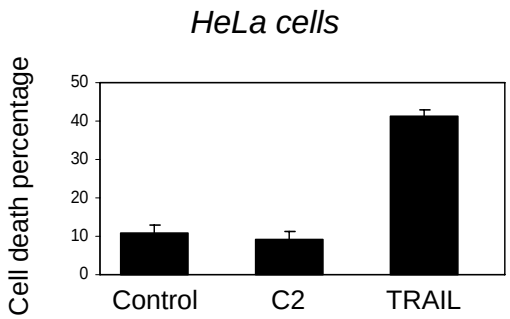
in SDS-PAGE, transferred to nitrocellulose and probed with DIG-14-3-3 (14-3-3 overlay) and hVps34 (Zymed) antibodies.

Supplementary Figure S12. Serum-free medium increases hVps34 lipid kinase activity. Continuously serum-grown HeLa cells were left untreated (U) or serum-starved (-S) for 16 h. Cells extracts (1 mg) from both samples were incubated with 1 μg of hVps34 antibody bound to Protein-G-Sepharose. The washed immunoprecipitates were used for hVps34 *in vitro* kinase assay and also resolved to detect hVps34 protein (Top panel: Quantification of hVps34 *in vitro* kinase assay expressed as PI(3) fold change, N = 3, * P=0.0029, Student t-test). Additionally, cell extract (30 μg) was used for immunoblot analysis of indicated proteins. Tubulin was used as a protein loading control.

Supplementary Figure S13. hVps34 binds 14-3-3 proteins by a PMA-phosphorylation-dependent mechanism in HEK293T cells. HEK293T cells growing in medium containing serum were treated with C2-ceramide (10 μM, 24 h) (C2) or serum-starved for 16 h and where indicated, cells were incubated with H-7 (100 μM), PD 184352 (2 μM) or no inhibitor for 1 h before stimulation with PMA (400 ng/ml) for a further 15 min. Extract from HEK293T cells were incubated with 1 μg of hVps34 (Echelon Biosciences) antibody bound to Protein-G-Sepharose. The washed immunoprecipitates were used to detect hVps34 protein and for interaction with 14-3-3 proteins by analysing DIG-14-3-3. Cell extracts (30 μg) from both samples were run on SDS-PAGE, transferred to nitrocellulose membranes and probed with indicated antibodies (bottom panel).

Supplementary Figure S14. Amino acid sequence of hVps34 with the 14-3-3-binding domains. The amino acid sequence of hVps34 with the 14-3-3-binding domains is shown. Length of deletion mutants MT1 (1-680 aa) and MT2 (1-390 aa) can be distinguish by asterisk (*). Serines or Threonines mutated to Alanine by site-directed mutagenesis are shown in bold. Underlined sequences show Thr197 and S212 domains whose change to Alanine reduces its 14-3-3-binding status.

Supplementary Figure S1



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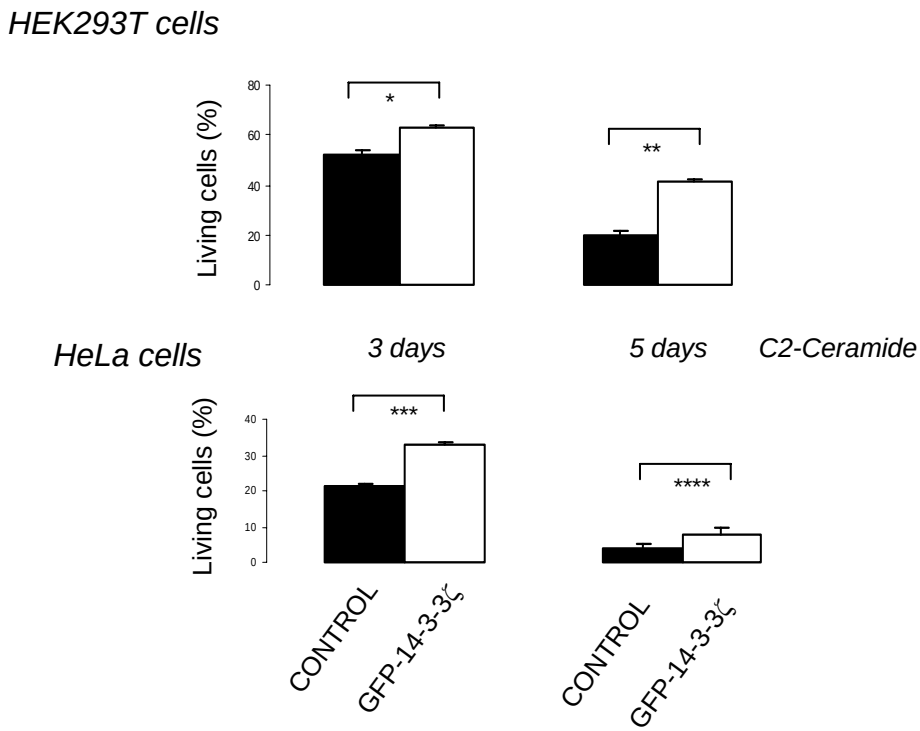

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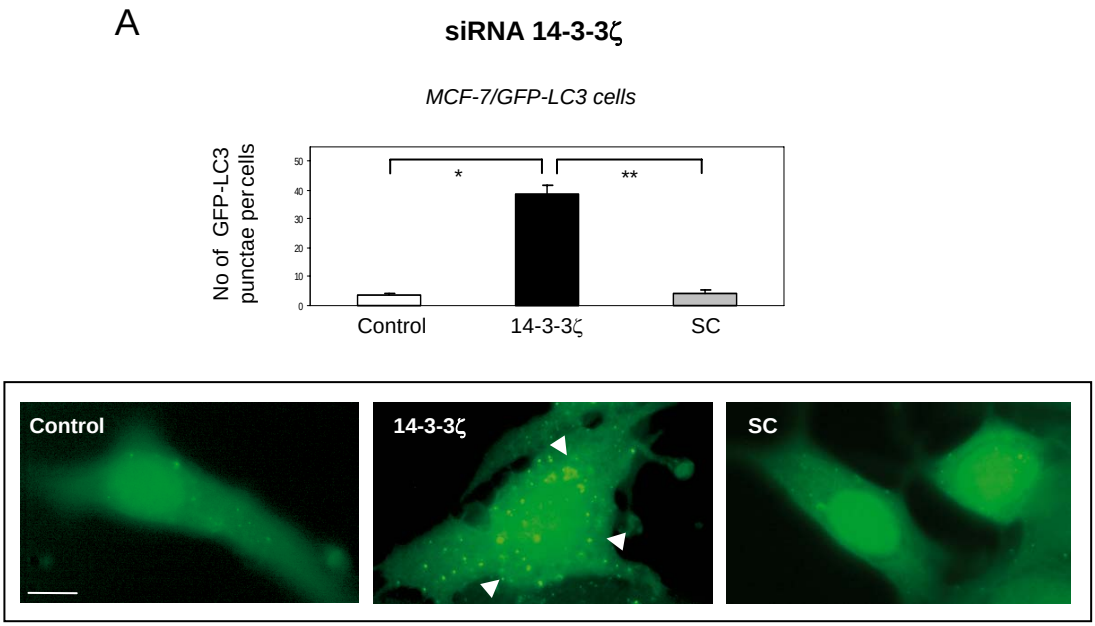

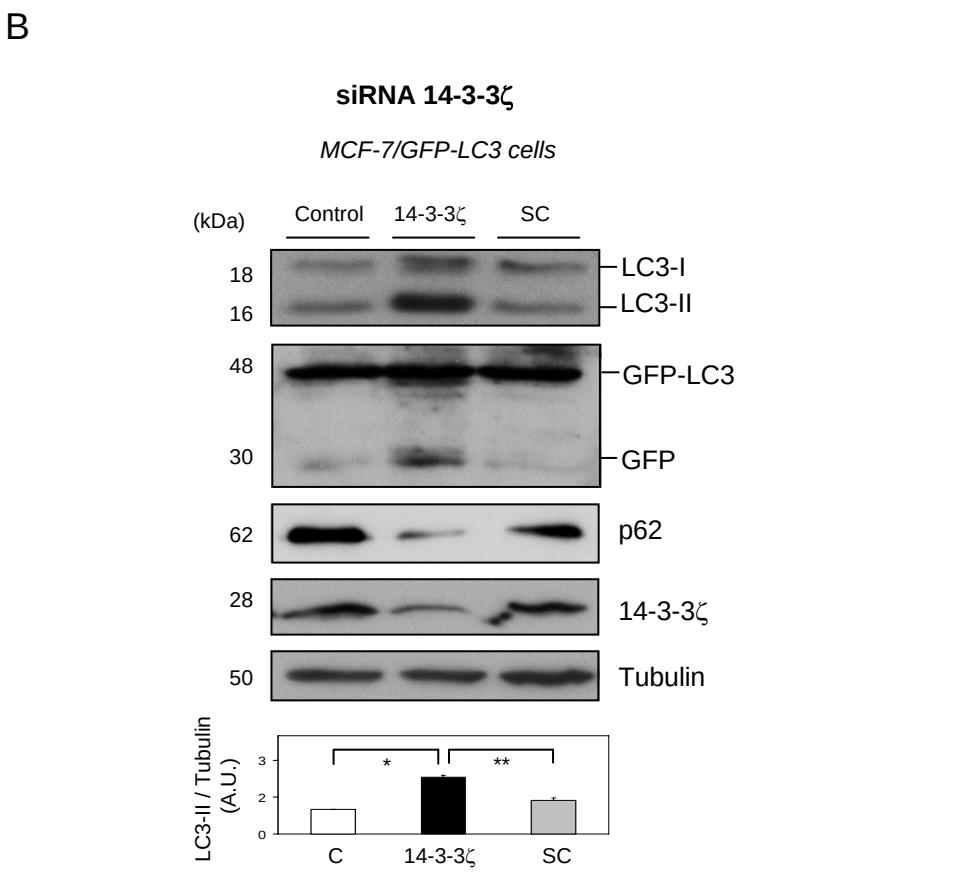

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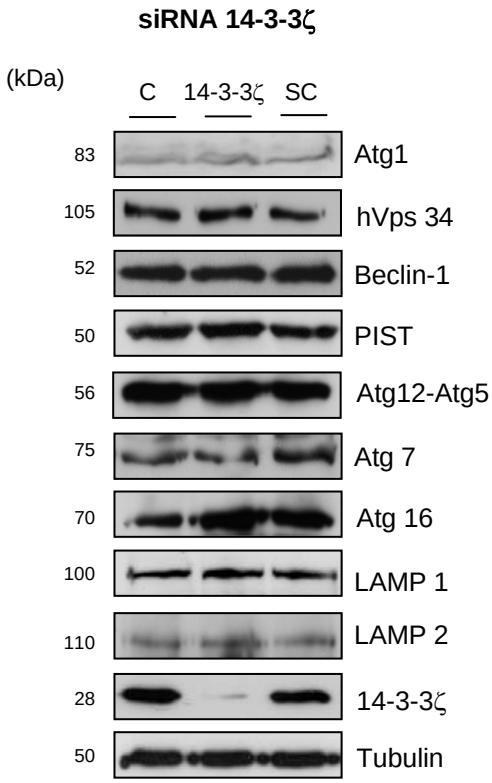

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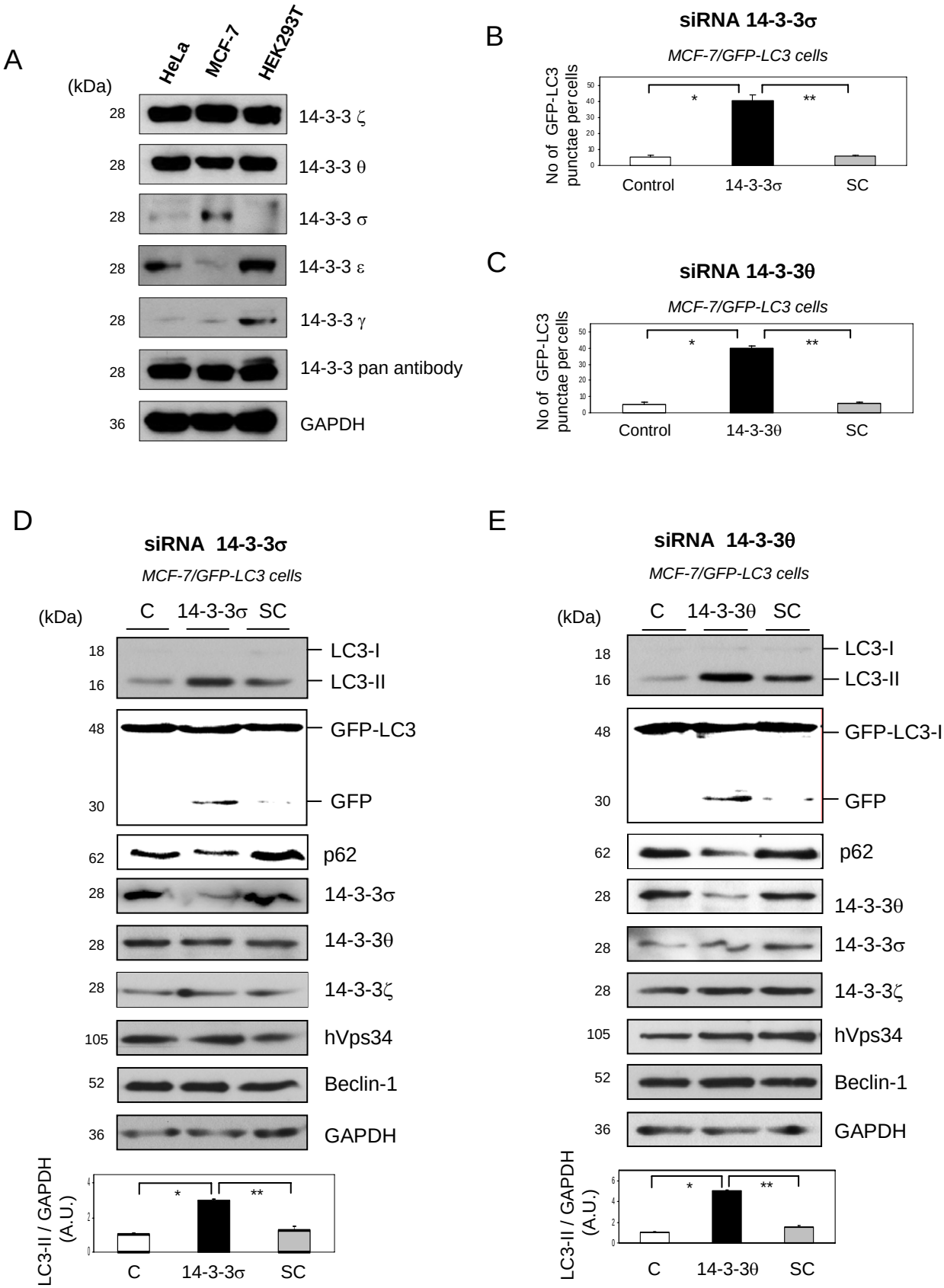

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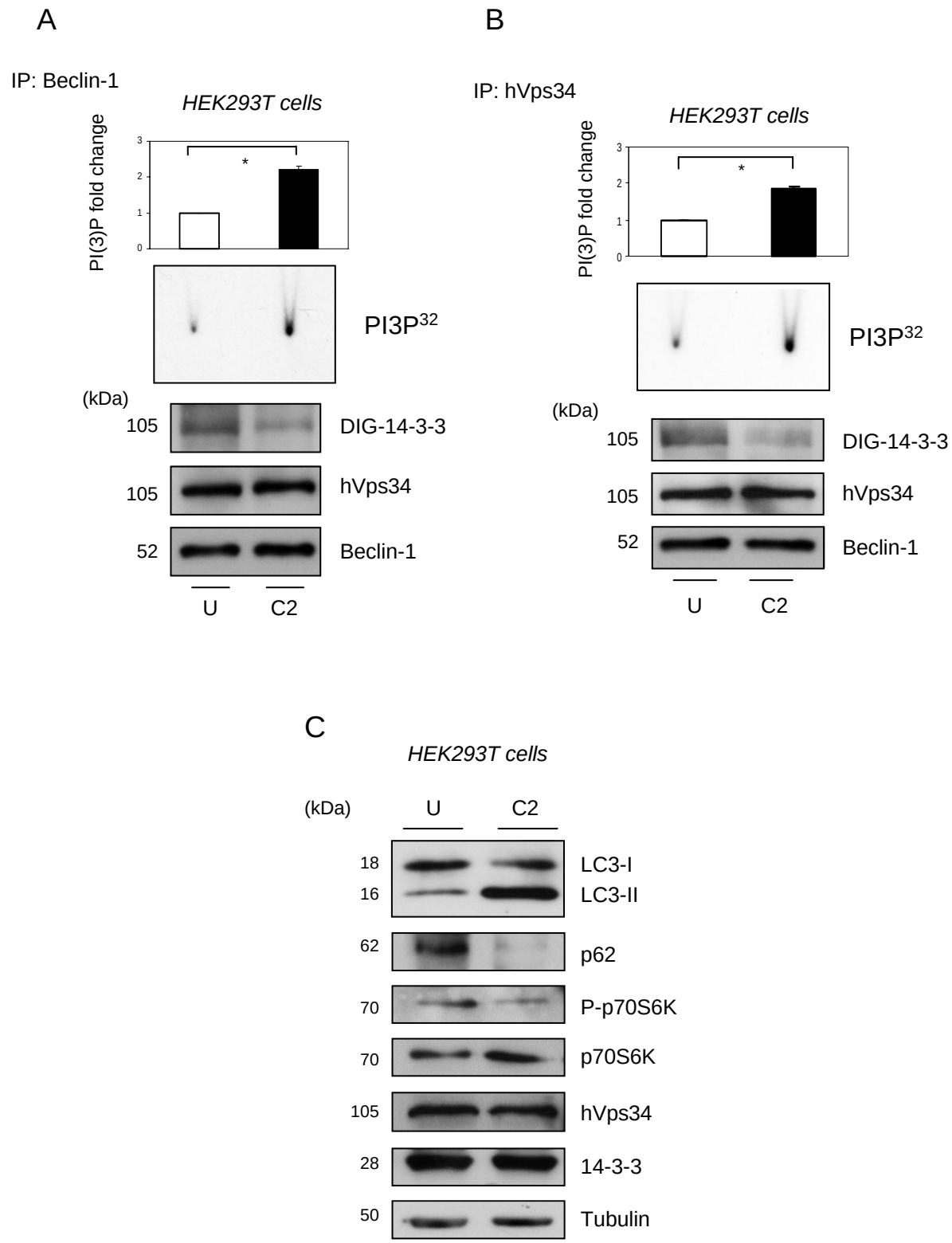

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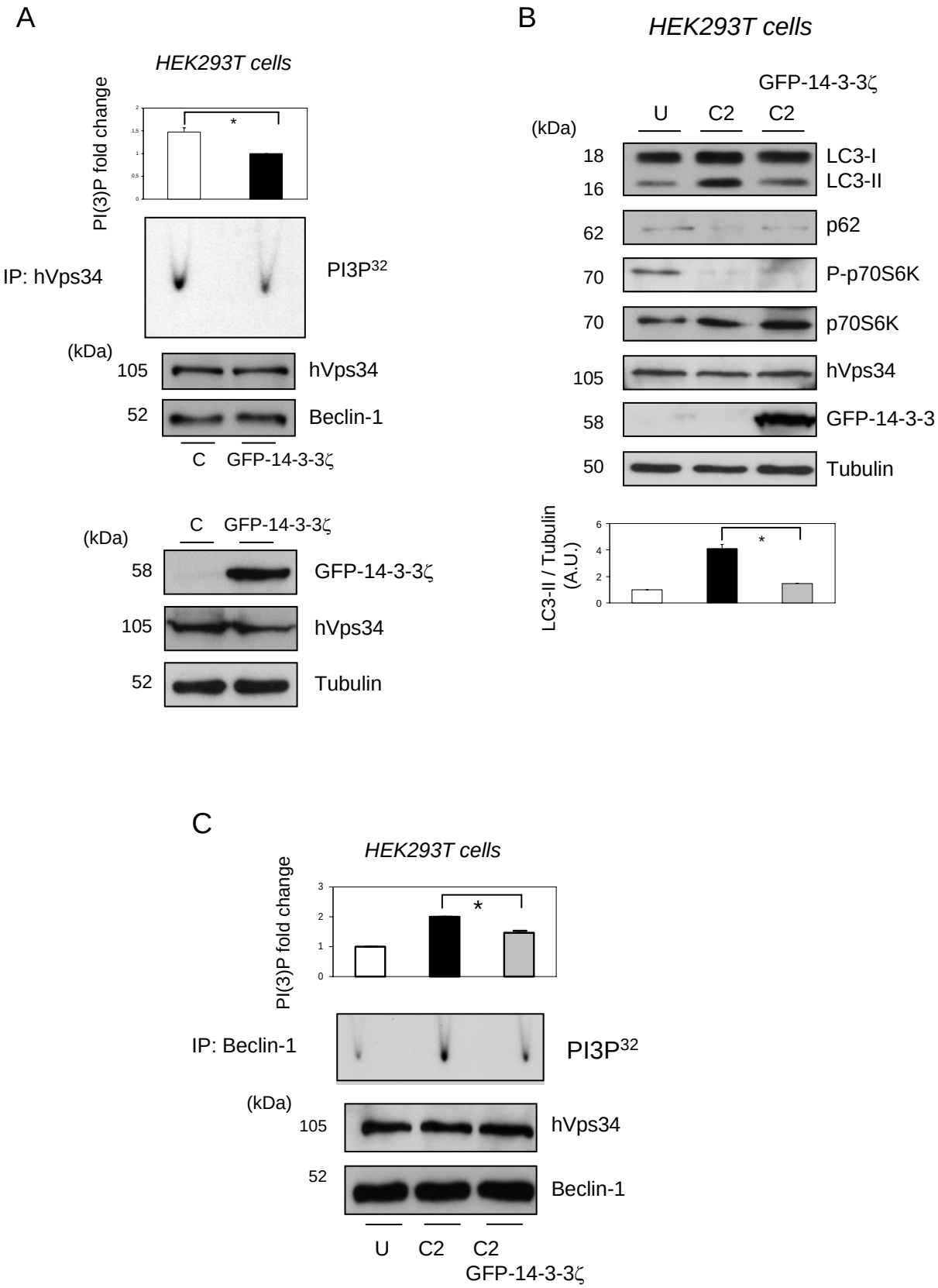

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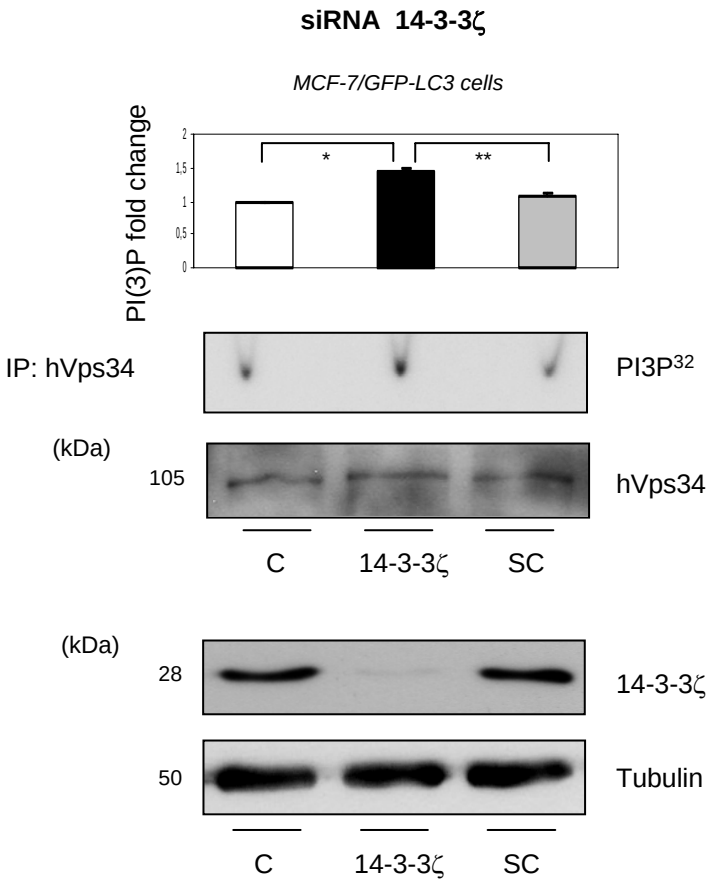

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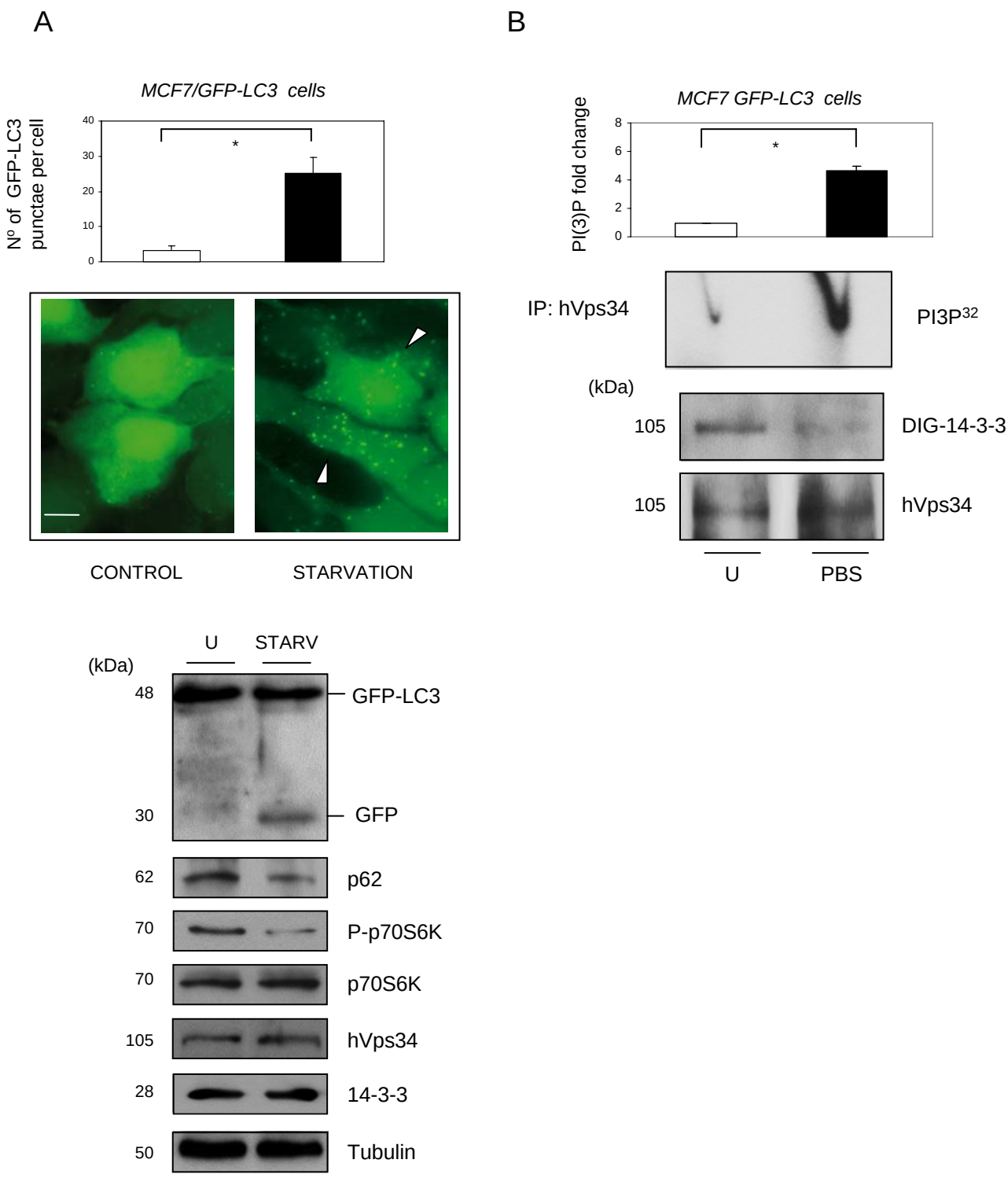

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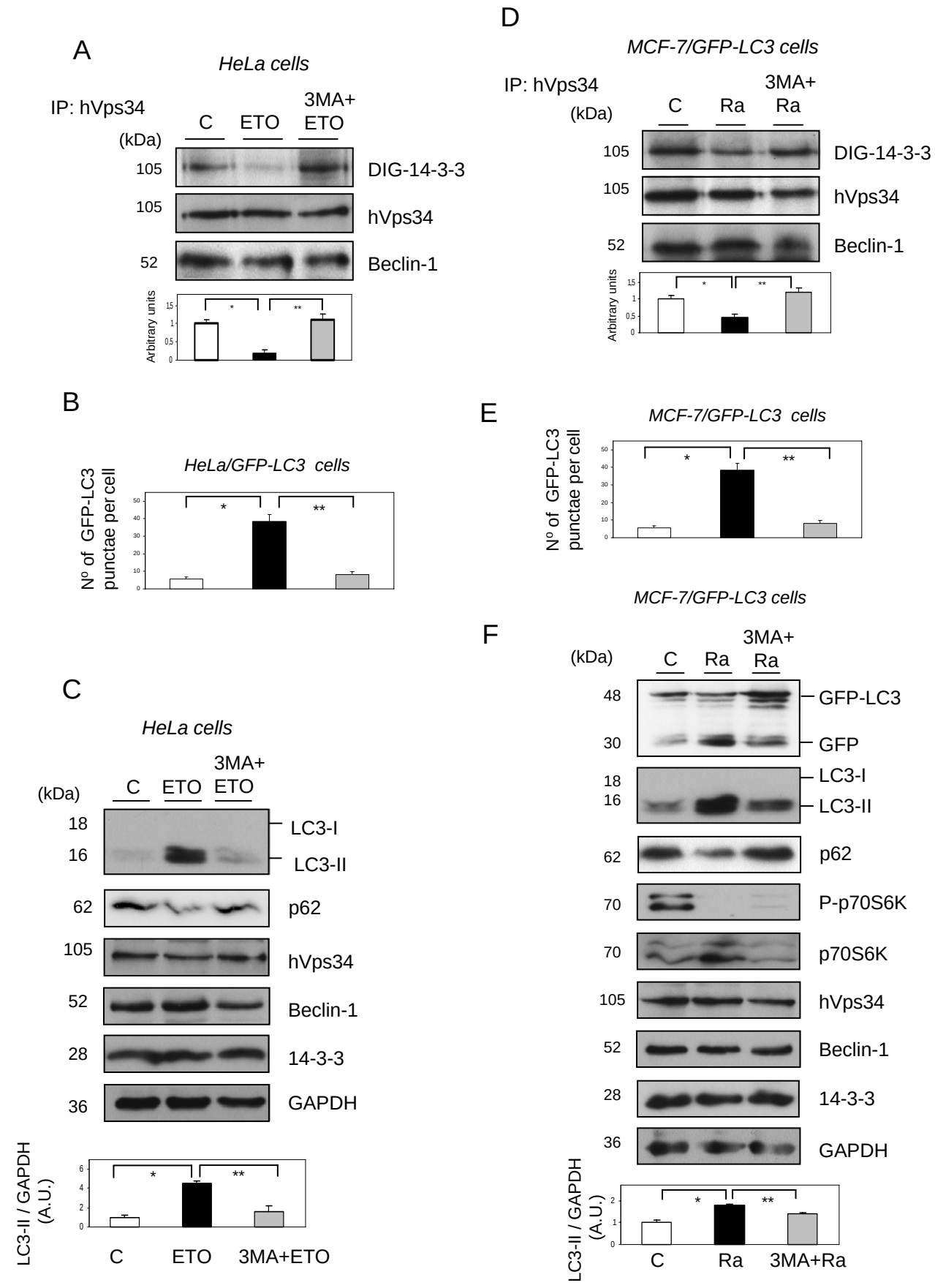

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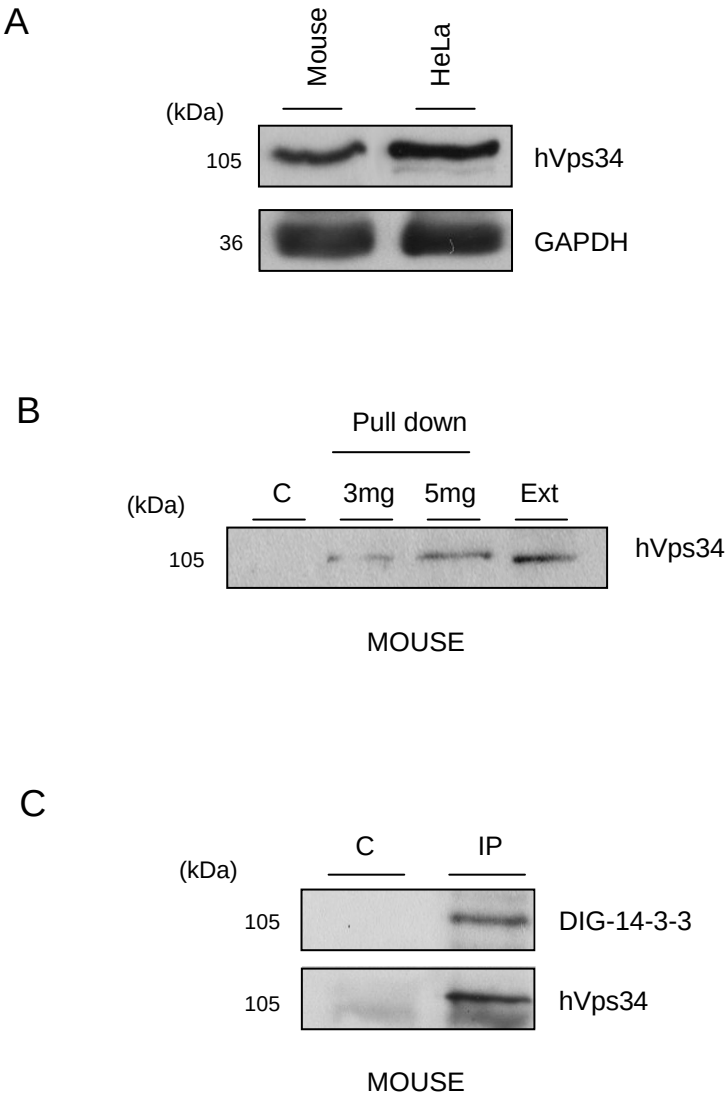

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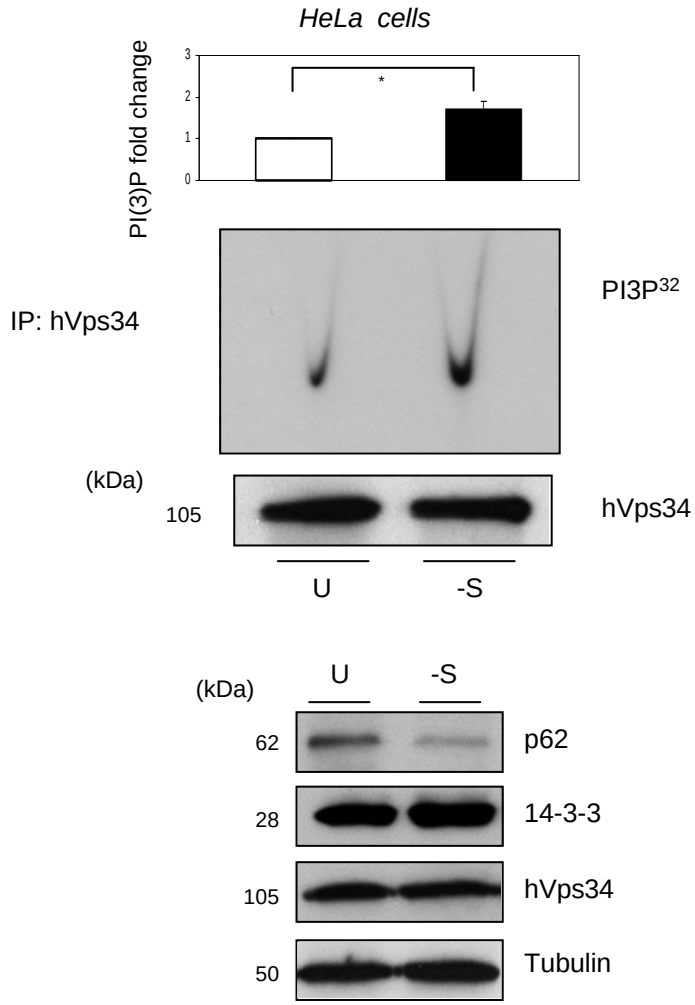

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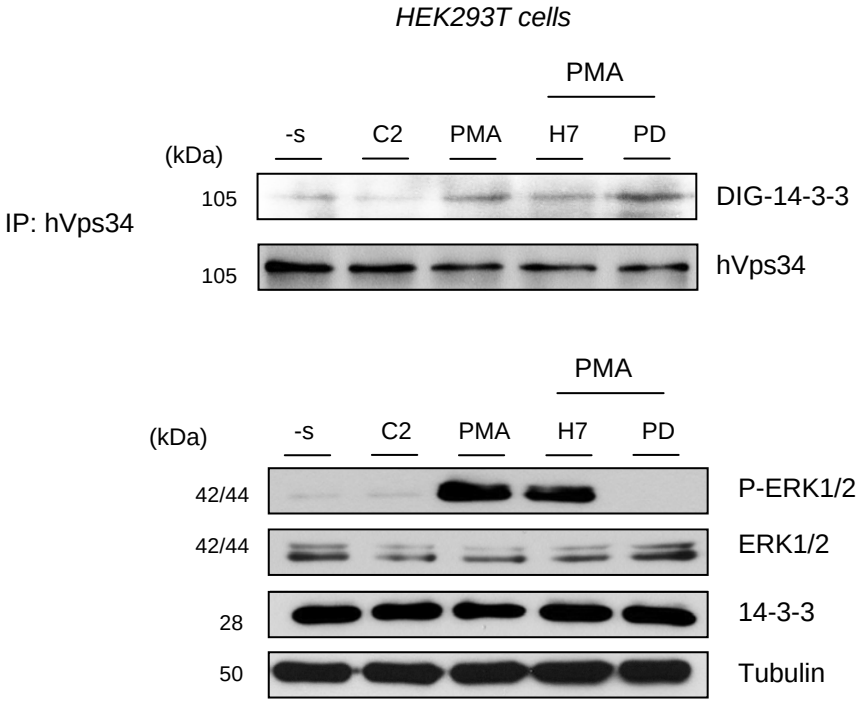

Supplementary Figure S14