

SCAFFOLD PROTEINS AND CYTOSKELETAL DYNAMICS AS MODULATORS OF Ras-ERK PATHWAY IN CANCER

Dalia de la Fuente-Vivas, Berta Casar and Pedro Crespo

Department of Molecular and Cellular Signalling, Institute of Biomedicine & Biotechnology of Cantabria

ABSTRACT

An overwhelming body of data unquestionably links the Ras-ERK pathway to the upbringing and progression of human malignancies. About 40% of human cancers harbor activating mutations in proteins involved in this signaling cascade, in particular Ras and Braf. In addition to the main proteins of this signaling pathway, there are several regulatory proteins known as scaffold proteins, that modulate the intensity, amplitude and duration of ERK signals. Interestingly, it can propose the scaffold proteins as antitumoral targets. Considering the hypothesis that scaffold proteins interact and regulate the signal transduction through the Ras-ERK pathway in a coordinated manner we try to find out how these interactions are regulated. Ras proteins are distributed in different types of plasma membrane: microdomains and endomembranes. We have previously demonstrated that compartmentalization dictates Ras utilization of effectors, the intensity of its signals and the biological response of the cells. Cell migration is critical for many physiological processes and is often misregulated in developmental disorders and pathological conditions including cancer. MAPK signaling are known regulators of cytoskeletal dynamics during cellular processes such as cell adhesion and migration. Our hypothesis is that localized ERK signaling mediated through recently identified scaffold proteins and its interactions with the cytoskeleton may regulate specific cellular responses. Here we propose that associations among different scaffold proteins and cytoskeleton will add one further degree of regulation for an already tightly regulated cascade and could provide a novel means for manipulating ERK signals, even with therapeutic purposes.

OBJECTIVES

Aim 1. To study the role of microtubules between in KSR₁ and cPLA₂ interaction.

Aim 2. To investigate if the specific Ras sublocalizations regulate ERK₂ substrates activity (phosphorylation) in a different manner.

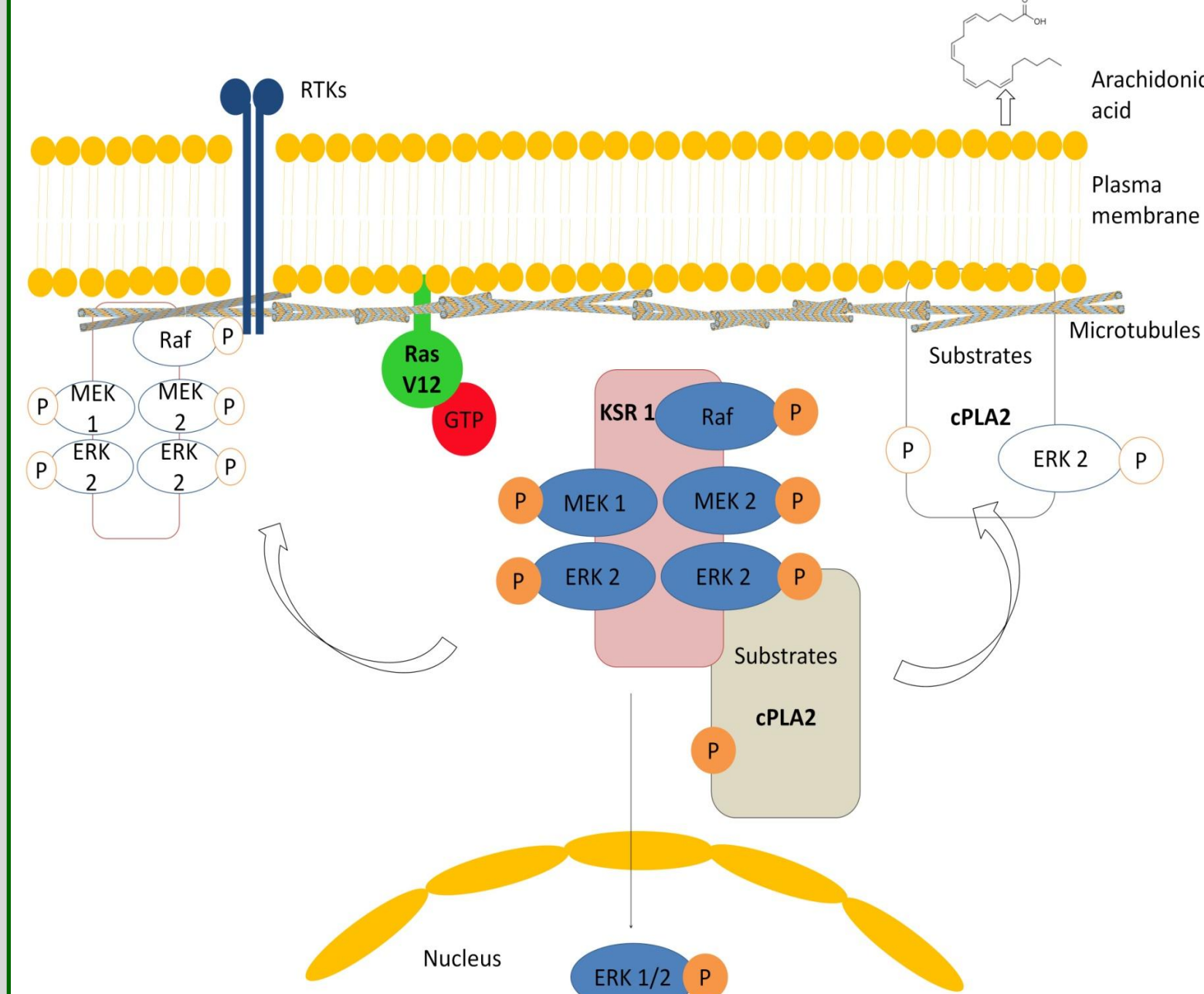


Figure 1. A model for the role of KSR₁, a scaffold protein, in ERK signaling. ERKs signals are activated depending on Ras localization and they are optimized by KSR₁ that modulate their intensity. KSR₁ is required of activating cPLA₂ mediated by microtubules.

Cell culture and transfection

293T and CHO cells culture were grown in DMEM-10% FBS, MEFs in DMEM- 10% FCS. Cells were transfected with lipofectamine following manufacturer's recommendations (Figure 5).

Immunofluorescence

Cells were serum starved overnight prior fixation with ice cold methanol for 10 seconds at -20°C, and incubated with the appropriate primary antibodies (α -Tubulin 1:200 and Flag 1:50) for 1h at room temperature in PBS. Following two washes with PBS, cells were incubated with Alexa 488 and Alexa 594 conjugated secondary antibodies (1:100) for 1h at room temperature. After two washes in PBS cells were stained with Prolong with DAPI. Fluorescence images were acquired using Leica TCS SP5 inverted multispectral confocal microscope (Figure 2). The Pearson correlation coefficient was used to estimate colocalization (Figure 3).

Immunoblotting

Cells were harvested after washing with PBS and lysate with low salt buffer on ice. Protein samples (20 μ g) were resolved by electrophoresis on 12% acrylamide gel and electrophoretically transferred to nitrocellulose membrane. The blots were probed with the appropriate primary antibodies followed by anti-rabbit IgG or anti-mouse IgG conjugated to horseradish peroxidase and visualized using enhanced chemiluminescence. (Figure 4).

Immunoprecipitation

293T cells were seed and transfected with 2 μ g DNA (1 μ g pCEFL-HRasV₁₂-CD8 HA*, pCEFL-HRasV₁₂-LCK-HA*, pCEFLH-RasV₁₂-M1 HA*, pCEFL-HRasV₁₂-KDEL HA*, pCEFL-HRasV₁₂-HA, pCMV-FLAG-KSR vectors and 1 μ g pCMV-FLAG-KSR). Transfection was carried out using lipofectamine as described above. 24h after transfection, the cells were serum starved overnight. The cells were harvested and lysed in Lysis Buffer. The lysates were centrifuged at 13,000 rpm for 10min and the supernatants were collected. Protein estimation was performed using Bradford's reagent and the supenatant was used for immunoprecipitation. Anti-Flag (1mg/mL) was added to the supernatants and incubated for 3h at 4°C on a rotating rack. Thereafter, 20 μ L of Protein G sepharose was added and incubated at 4°C on a rotating rack for 20min. The beads were pelleted by centrifugation ant 13,000rpm for 1min and washed 3 times in Wash Buffer. The proteins were analysed by immunoblotting as described above. (Figure 4).

cPLA2 activation assay

MEFs and MEFs KSR^{-/-} cells were labeled by incubation with [³H] arachidonic acid (1 μ Ci per well in a 12-well plate for 18 h). Cells were washed twice with Dulbecco modified Eagle medium, 1% fatty acid-free bovine serum albumin. Where indicated, cells were stimulated with EGF (100ng/mL) for 2h. Medium was removed and labeled arachidonic acid realeased into the medium by liquid scintillation counting (Figure 6).

METHODS and RESULTS

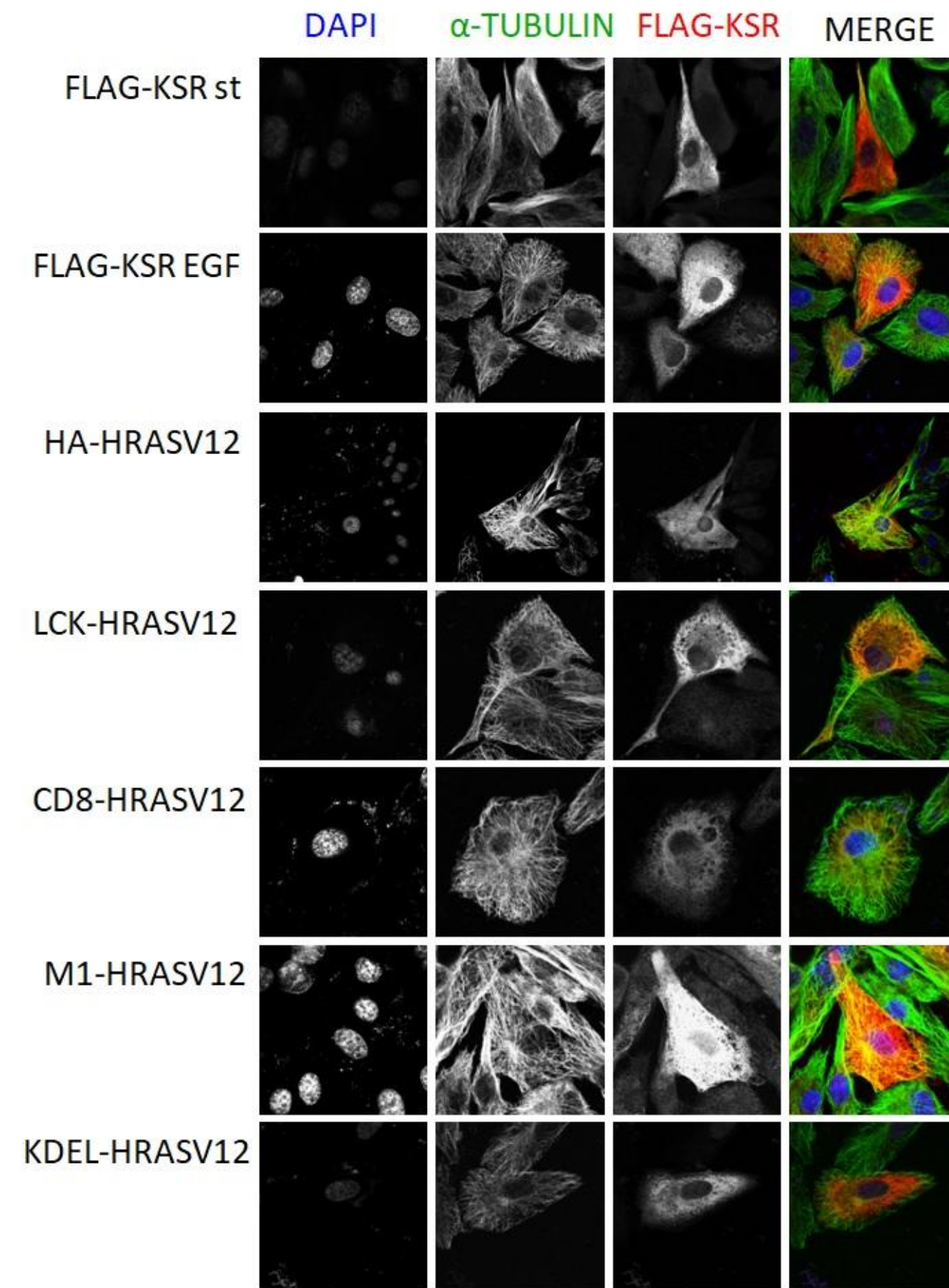


Figure 2. Immunofluorescence images. Confocal immunofluorescence images are shown for control and Ras-expressing cells immunostained for α -tubulin and KSR₁.

Colocalization tubulina vs. KSR1

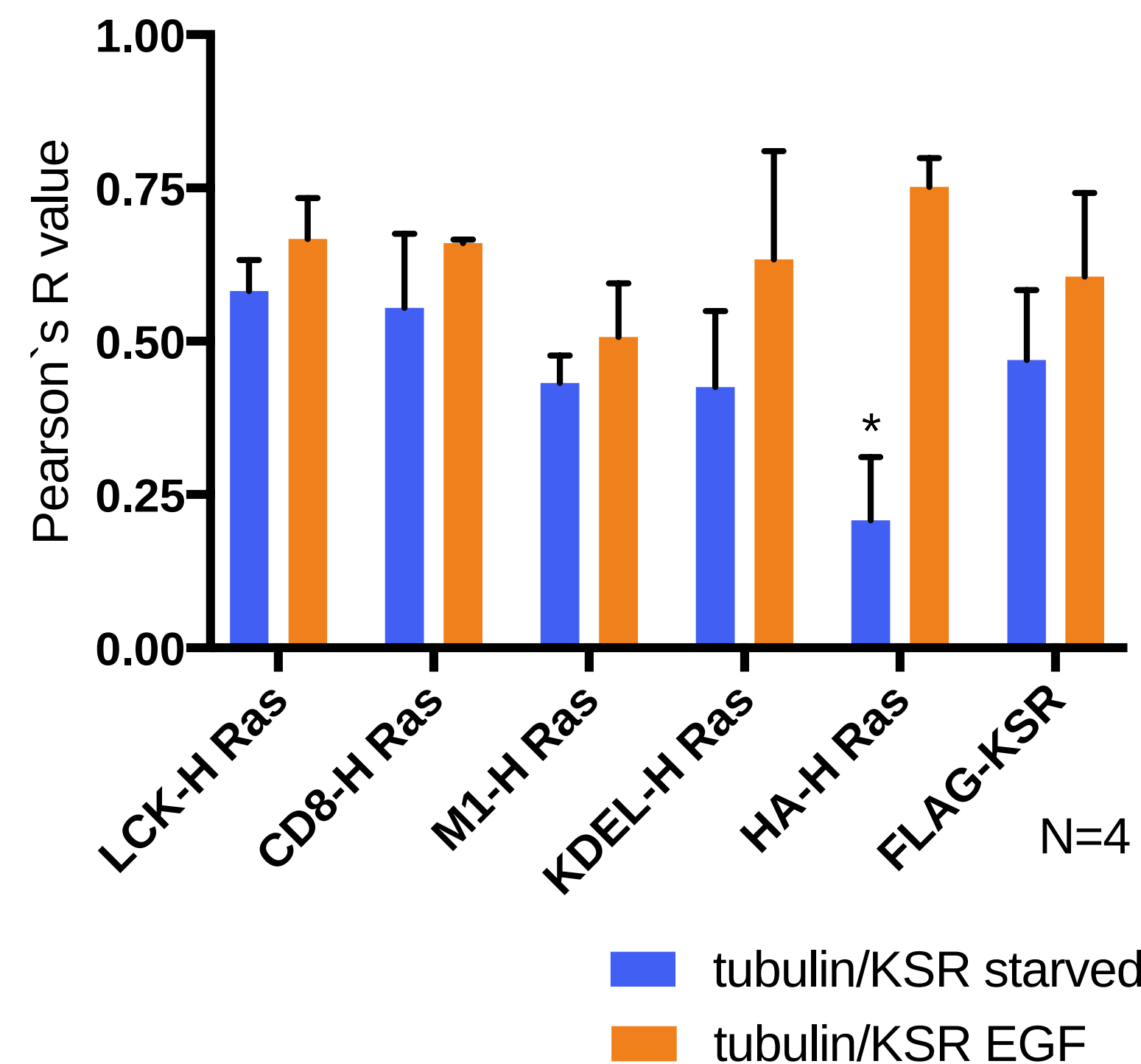


Figure 3. Colocalization was analise using Pearson's R value. Results show average $\pm$ SEM of four independent experiments relative to starved (st) cells.

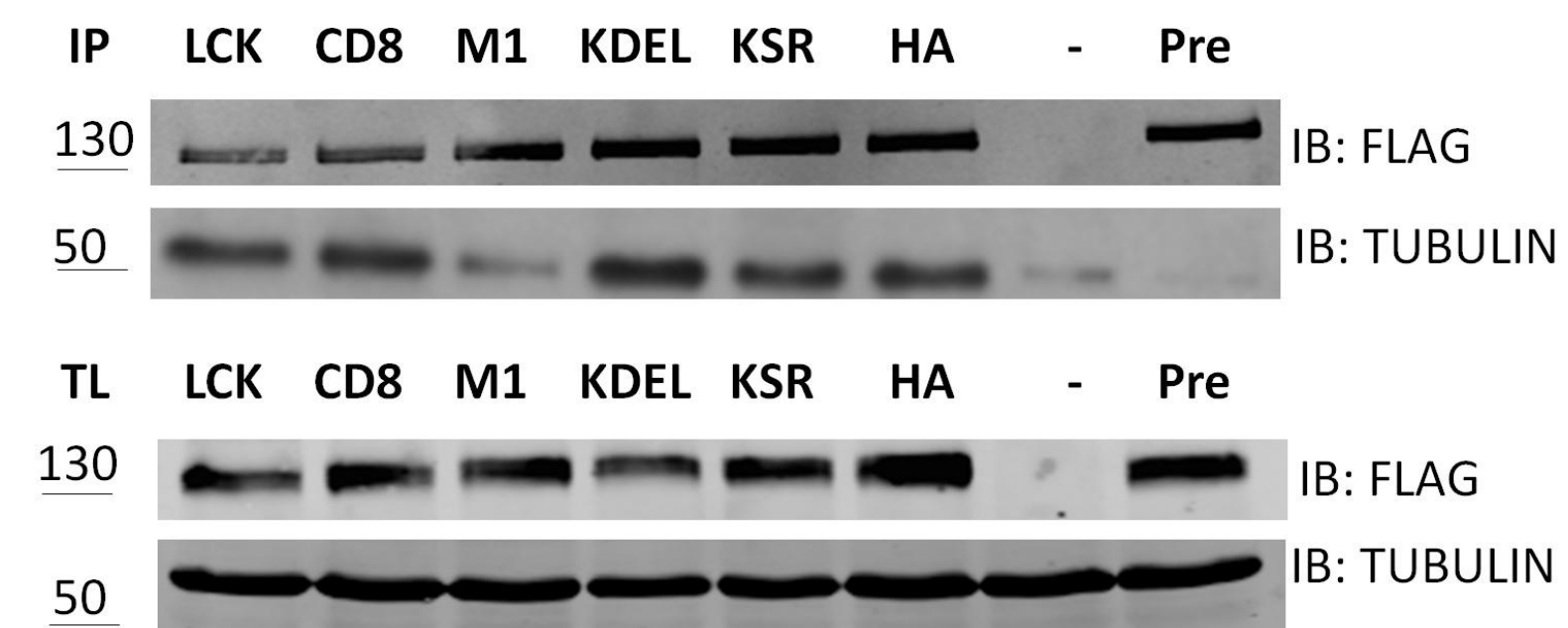


Figure4. Biochemical immunoblotting. The association of KSR₁-Flag and endogenous α -Tubulin was analysed in anti-Flag and anti-Tubulin immunoprecipitates from starved 293T cells. There are no significant results between α -Tubulin and KSR₁.

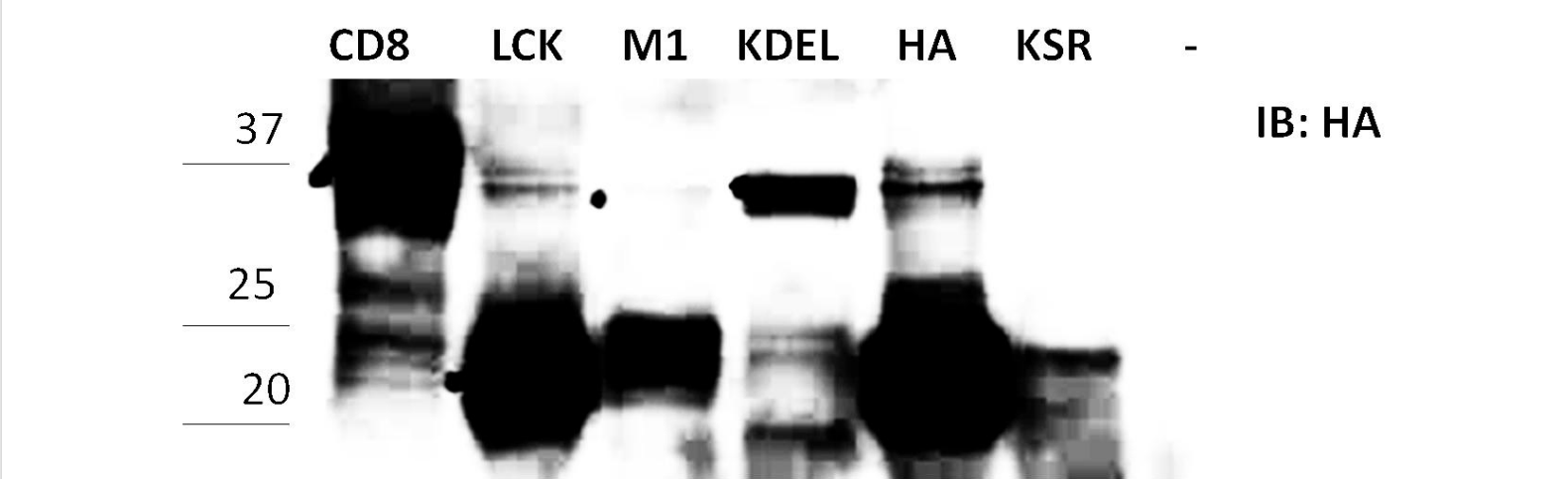


Figure 5. Transfection proof. CHO cells were transfected with HA-HRasV₁₂ and Flag-KSR constructs and total lisate were analysed by immunoblotting used HA antibody.

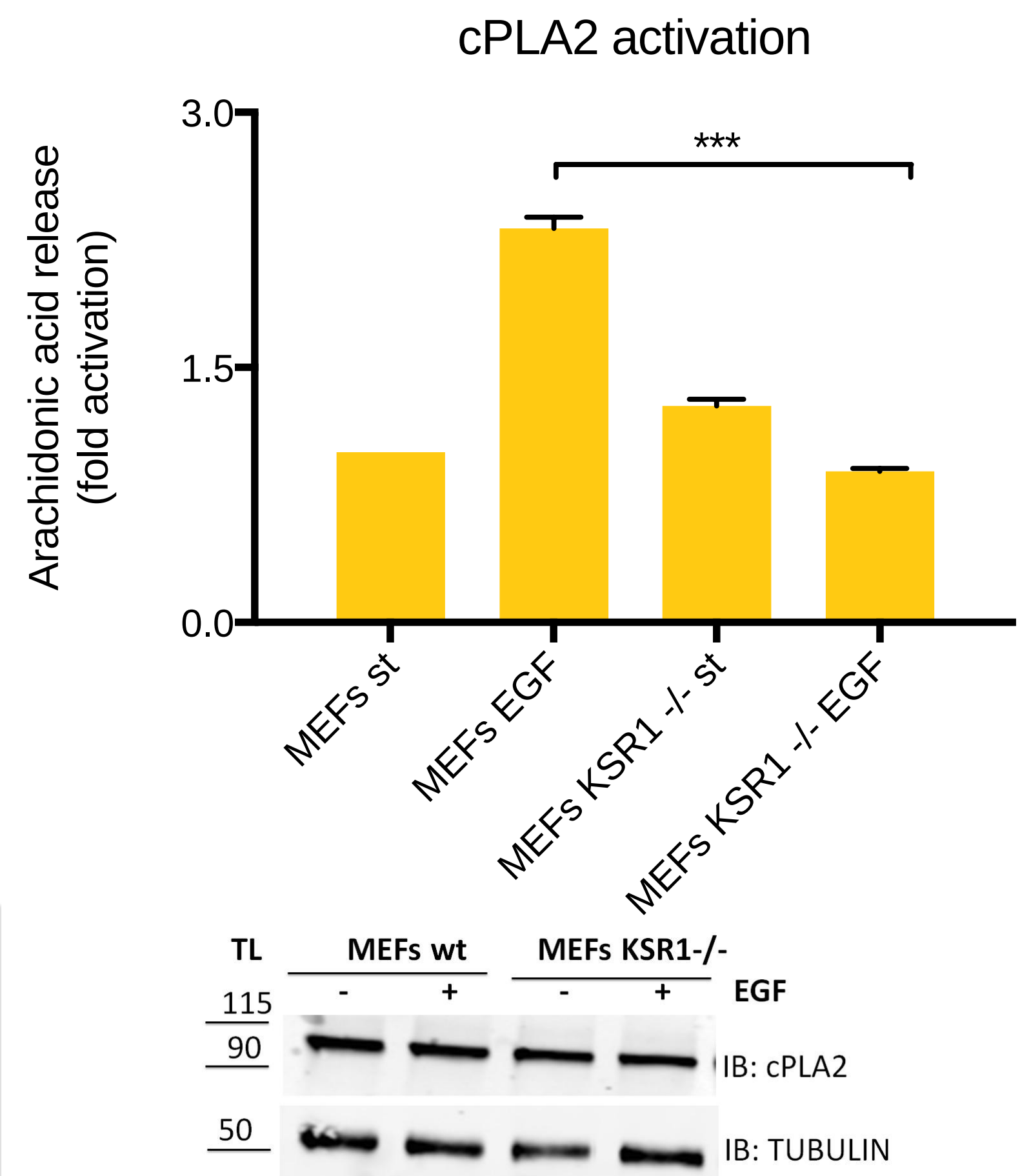


Figure 6. Endogenous cPLA2 activation determined by [³H] AA release. Results show average $\pm$ SEM of three independent experiments relative to starved (st) cells.

CONCLUSIONS

The protein scaffold KSR₁ modulte the intensity of ERK signalling in the cytoplasm binds cytoskeleton. We show the colocalization between tubulin and KSR₁ is weaker when HRasV₁₂ is expressed constitutive in endomembranes (Golgi -KDEL- and endoplasmic reticulum -M1-) than in lipid rafts (LCK) or disorder membrane (CD8) although it is not significative.

Arachidonic acid (AA) release is dependent on EGF stimulation and KSR₁, we suggest that MEFs which express LCK-HRasV₁₂ would have a higher AA release than the control (MEFs wt).

FUTURE RESEARCH:

1. The immunofluorescences will be repeated with stably cell culture which express the different sublocalizations of HRasV₁₂ (LCK, CD8, M1, KDEL) .
2. To analise the function of KSR₁ in Ras-ERK signalling we will use several KSR₁ mutants (KSR₁-C809Y does not bind MEK and KSR₁-ASAP does not bind ERK).
3. The arachidonic acid (AA) release will be repeated with MEFs cells nucleotransfected with LCK-HRasV₁₂ and KSR₁ to study the activation of cPLA₂ in lipid rafts.

REFERENCES

- Casar, B., Pinto, A., and Crespo, P. (2008). Essential role of ERK dimers in the activation of cytoplasmic but not nuclear substrates by ERK-scaffold complexes. Mol. Cell 31, 708–721
- Casar, B., Arozarena, I., ..., and Crespo, P. (2009). Ras Subcellular Localization Defines Extracellular Signal-Regulated Kinase 1 and 2 Substrate Specificity through Distinct Utilization of Scaffold Proteins. Mol. Cell 29, 1338-1353
- Casar, B., Pinto, A., and Crespo, P. (2009). ERK dimers and scaffold proteins: unexpected partners for a forgotten (cytoplasmic) task. Cell Cycle 8, 1007–1013
- Casar, B., and Crespo, P. (2016) ERK Signals: Scaffolding Scaffolds?. Front Cell Dev Biol 31; 4:49

AKNOWLEDGEMENTS

I would like to express my very great appreciation to the Piero's lab, and the IBBTEC institute.

CONTACTS:

Dalia de la Fuente: dfuente@unican.es, Berta Casar: berta.casar@unican.es, Piero Crespo: pedro.crespo@unican.es