Interactions Between Scaffold Proteins and Ras-ERK Pathway as Antitumoral Targets

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Abstract

Signals transmited by Ras-ERK pathway regulate the activation of multiple substrates present in the nucleus and in the cytoplasm. This is a critical step in translating extracellular stimuli into intracellular responses by phosphorylation and subsequent activation of multiple substrates, through which mechanisms essential for proliferation, differentiation and cell survival are regulated. Upregulation of ERK1/2 MAPK cascade occurs in a high percentage of cancers, often through mutational activation of receptor tyrosine kinases or other upstream genes, including *KRAS* and *BRAF*. Efforts to target endogenous MAPKs are challenged by the fact that these kinases are required for viability in mammals.
ERK signals are optimized by scaffold proteins that modulate their intensity and spatial fidelity. MAPK scaffolds, such as IQ motif-containing GTPase activating protein IQGAP1 and IQGAP2 (White, C.D. et *al*., 2009) and kinase suppressor of Ras (KSR) assemble pathway kinases to affect signal transmission (Kolch, W., 2005), and modulating scaffold function therefore offers a novel approach to MAPK cascade inhibition. We hypothesized that Differences in scaffold usage may underlie the biochemical, biological and clinical divergences displayed by tumors of the same type but driven by different oncogenic lesions within the Ras-ERK pathway, such as those elicited by mutant K-Ras or B-Raf. The main objective is to prove this postulate by investigating ERK1/2 scaffolding in tumor cells displaying a defined oncogenic signature with respect to the Ras-ERK pathway, with the purpose of identifying scaffolds essential for the maintenance of the oncogenic phenotype, which may serve as targets for future therapeutic agents directed to modulate scaffold-ERK complexes.
Scaffold-kinase interaction modulation acts by a mechanism distinct from direct kinase inhibition and may be a novel strategy to target overactive oncogenic kinase cascade in cancer for antitumoral therapeutic intervention.

Materials and Methods

Cell Culture and Transfection
HEK293T and RKO cells were grown in Dulbecco modified Eagle medium (DMEM) – 10% fetal calf serum (FCS). Subconfluent HEK293T cells were transiently transfected with Lipofectamine LTX (Invitrogen) according to the manufacture's instructions. RKO stable lines were generated by transfection with Lipofectamine LTX (Invitrogen) according to the manufacture's recommendations and selected with Puromicine 2,5 µg/ml. siRNAs against the different scaffold proteins were from Sigma-Aldrich.

Cellular Proliferation Assays
Cells were plated at low density in six-well plates (30.000 cells/per plate) and grown as indicated above. Cells were detached and scored by standard cell counting techniques during 96 hours every 24 hours.

Immunoblotting and Imunoprecipitations
Lysates were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF (Millipore) filters. Immunocomplexes were visualized by ECL detection (Amersham) with horseradish peroxidase-conjugated secondary antibodies (Cappel). The antibodies used were mouse monoclonal anti-BRaf, anti-ERK2, anti-IQGAP2, anti-phospho-ERK (Santa Cruz) anti-IQGAP1 (BDBioscience), anti-Pan-Ras (Calbiochem), rabbit monoclonal anti-cleaved caspase 3 (Cell Signalling) and rabbit polyclonals anti-EKR2 (Santa Cruz) and anti-KSR2 (ABCAM). Immunoprecipitations were performed as described previously (Crespo, P. et *al*., 1994),

Tumor Formation in Chick Embryo CAM
Fertilized eggs (Gibert Farm Tarragona, Spain) were incubated in a rotary incubator at 38° C with 60% humidity for 10 days. At this time, the CAM was dropped as described (Zijlstra, A. et *al*., 2002). Single-cell suspensions of RKO tumor cells were prepared at 1 × 10^6 cells in 25 µL of serum-free DMEM and inoculated on the dropped CAM. The embryos were incubated for 7 days in a humidified stationary incubator at 38° C. Experiments were terminated on day 7, when primary tumors were excised and weighed.

Ras-ERK Pathway and Associated Scaffolds

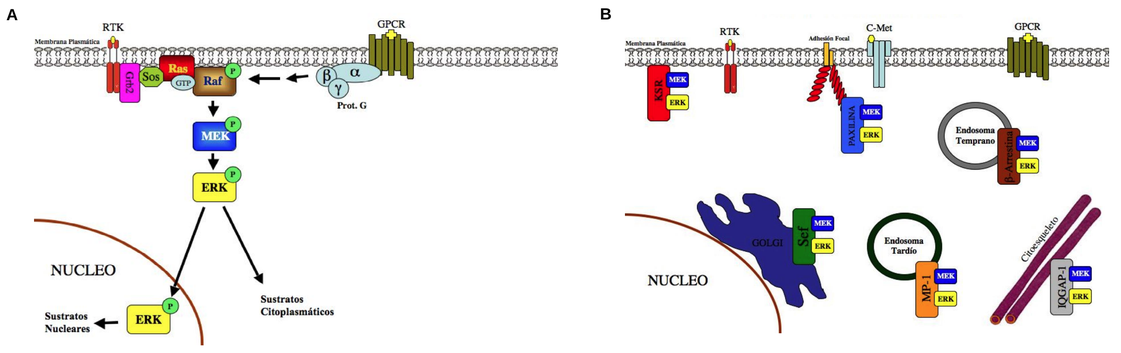


Figure 1. Ras-ERK pathway and its associated scaffold proteins
A: ERK cascade activation from cell surface. MAP kinase cascade activation is triggered by binding a particular ligand to a tyrosine kinase receptor (TKR) or G coupled-protein (GCPR). After this, ERK may perform its action on nuclear or cytoplasmic substrates.
B: Ras-ERK pathway is modulated by adapter molecules or scaffold proteins. Scaffolds form signaling modules with other signal pathway components and direct them to specific cellular compartments.
(Images were given by Adán Pinto Fernández)

IQGAP1, IQGAP2 and KSR2 Expression in Colorectal Tumors and Colorectal Tumor Cell Lines

IQGAP1 and IQGAP2 are downexpressed in colorectal tumors

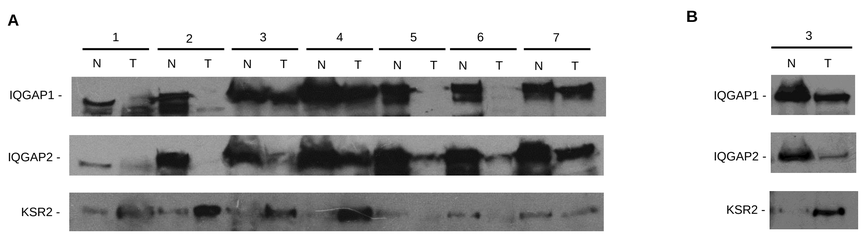
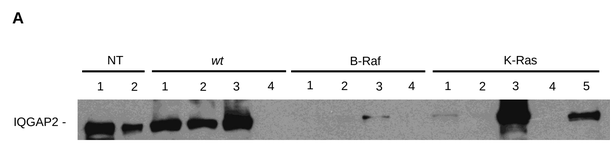


Figure 2. ERK1/2 scaffold proteins expression pattern in normal and tumoral tissue is different.
A: Western blot analysis for IQGAP1, IQGAP2 and KSR2 in normal (N) and tumoral (T) human colorectal tissue from seven different patients (1 to 7). IQGAP1 and IQGAP2 protein levels are lower in tumoral tissue, while KSR2 protein levels are higher in some tumors and lower in others.
B: Western blot analysis for IQGAP1, IQGAP2 and KSR2 in normal (N) and tumoral (T) human colorectal tissue from patient number 3. IQGAP1 and IQGAP2 protein levels are lower in tumoral tissue, while KSR2 protein levels are higher.
(Images given by Ana Herrero Mier)

IQGAP2 is downregulated in B-Raf mutated colorectal tumor cell lines



B

	wt	B-Raf (V600E)	K-Ras (V12)
1	CACO2	COLO205	DLD-1
2	COLO320	COLO829	HCT-116
3	KM12	HT29	SW 480
4	SW48	SW1417	SW 620
5			SW116

Figure 3. IQGAP2 is downregulated in B-Raf mutated colorectal tumor cell lines
A: Western blot analysis for IQGAP2 in normal colorectal tissue (NT), wild type colorectal tumor cell lines (*wt*) and B-Raf/K-Ras mutated colorectal tumor cell lines (B-Raf/K-Ras). IQGAP2 protein levels are lower in B-Raf mutated colorectal cell lines and higher in normal tissue and wild type cell lines.
B: Summary table of used cell lines. Wild type colorectal tumor cell lines (*wt*): CACO2, COLO320, KM12 and SW48. B-Raf mutated colorectal tumor cell lines (B-Raf): COLO205, COLO829, HT29 and SW1417. K-Ras mutated colorectal tumor cell lines (K-Ras):DLD-1, HCT-116, SW480, SW620, SW116.
(Image was given by Ana Herrero Mier)

IQGAP1, IQGAP2 and KSR2 Expression in K-Ras and B-Raf Transfected Cells

Scaffold expression pattern in K-Ras and B-Raf transfected HEK293T cells is similar

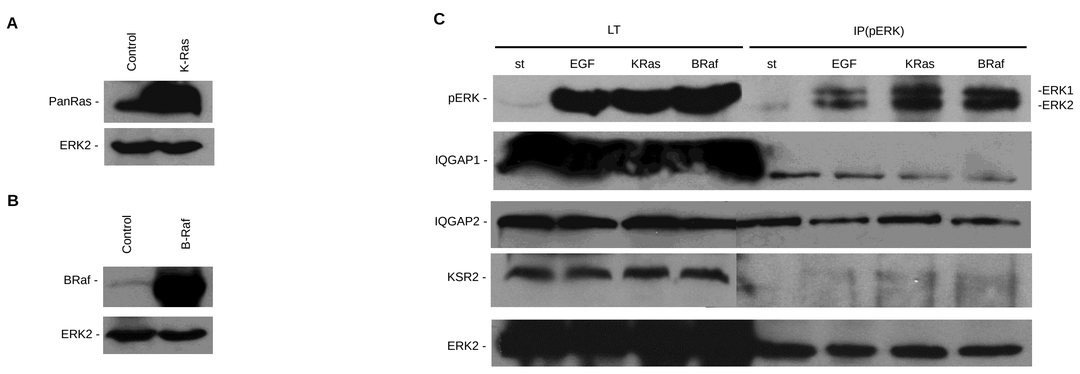


Figure 4. ERK1/2 scaffold proteins expression pattern in K-Ras and B-Raf transfected HEK293T cells is similar.
A: Western blot analysis for K-Ras and ERK2 in HEK293T and pCEFL HA KRasV12 transfected HEK293T cell lysates. K-Ras protein levels are higher on transfected HEK293T cells than on control cells.
B: Western blot analysis for B-Raf and ERK2 in HEK293T and pCDNA FLAG BRafV600E transfected HEK293T cell lysates. B-Raf protein levels are higher on transfected HEK293T cells than on control cells.
C: pERK was immunoprecipitated in HEK293T cells (starved, EGF-estimulated and KRas/BRaf-transfected) and the associated IQGAP1, IQGAP2 and KSR2 scaffolds were examined by immunoblotting.

Stable RKO shIQGAP1, shIQGAP2 and shKSR2 Lines Proliferation Assays

Silencing IQGAP1 increases cell proliferation

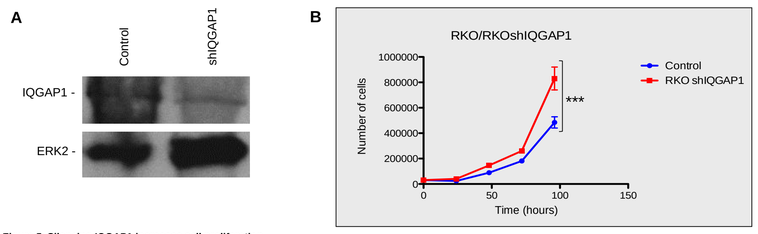


Figure 5. Silencing IQGAP1 increases cell proliferation.
A: Western blot analysis for IQGAP1 and ERK2 in RKO and stable RKOshIQGAP1 cell lysates. IQGAP1 protein levels are lower on RKOshIQGAP1 cells than on un-transfected RKO cells.
B: *In vitro* cell proliferation assay. RKO and RKO-shIQGAP1 cells were grown for 96h and detached and scored by standard cell counting techniques every 24 hours. The absence of IQGAP1 promoted RKO-shQGAP1 cell proliferation compared to non-transfected cells (RKO). Data expressed as mean ± SD (n = 2). Significance was determined by two-way ANOVA followed by Bonferroni's post-test; ***p<0,001 vs control was considered significant.

Silencing IQGAP2 increases cell proliferation and its overexpression induces apoptosis

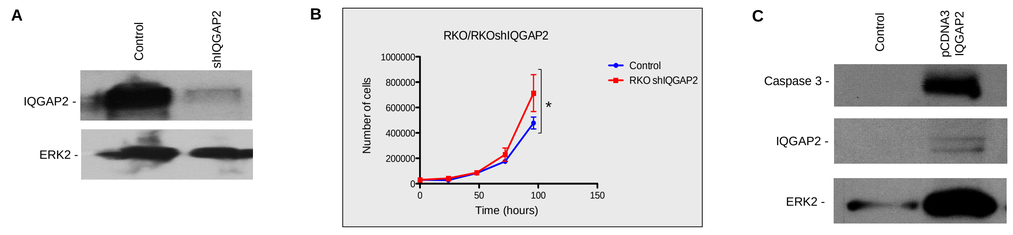


Figure 6. Silencing IQGAP2 increases cell proliferation and its overexpression induces apoptosis
A: Western blot analysis for IQGAP2 and ERK2 in RKO and stable RKOshIQGAP2 cell lysates. IQGAP2 protein levels are lower on RKOshIQGAP2 cells than on un-transfected RKO cells.
B: *In vitro* cell proliferation assay. RKO and RKO-shIQGAP2 cells were grown for 96h and detached and scored by standard cell counting techniques every 24 hours. The absence of IQGAP2 promoted the proliferation of RKO-shIQGAP2 cells compared to non-transfected cells (RKO). Data expressed as mean ± SD (n = 2). Significance was determined by two-way ANOVA followed by Bonferroni's post-test; *p<0,05 vs control was considered significant.
C: Western blot analysis for cleaved Caspase 3, IQGAP2 and total ERK ½ in RKO cell lysates transfected with pCEFL (Control) and pCDNA3IQGAP2. Cleaved Caspase 3 protein levels are higher on pCDNA3IQGAP2 transfected RKO cells than on control cells.

Silencing KSR2 increases cell proliferation

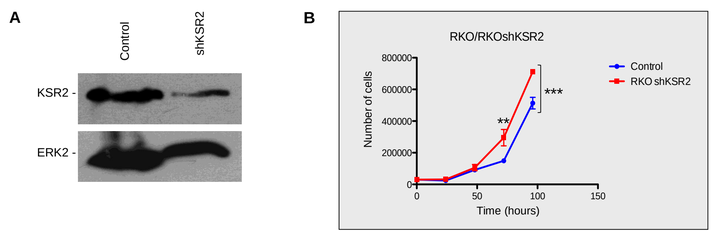


Figure 7. Silencing KSR2 increases cell proliferation
A: Western blot analysis for KSR2 and ERK2 in RKO and stable RKOshKSR2 cell lysates. KSR2 protein levels in RKOshKSR2 cells are lower than on un-transfected RKO cells.
B: *In vitro* cell proliferation assay. RKO and RKO-shKSR2 cells were grown for 96h and detached and scored by standard cell counting techniques every 24 hours. The absence of KSR2 promoted the proliferation of RKO-shKSR2 cells compared to non-transfected cells (RKO). Data expressed as mean ± SD (n = 2). Significance was determined by two-way ANOVA followed by Bonferroni's post-test; ***p<0,001, **p<0,001 vs control were considered significant.

Chick Embryo CAM Model for Tumor Development

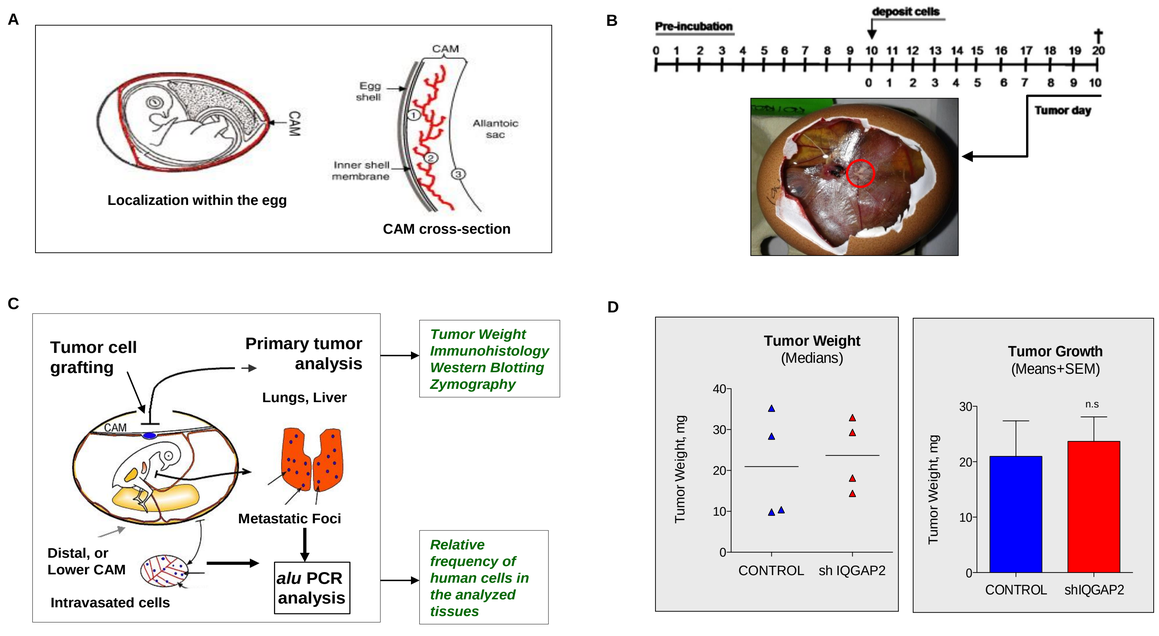


Figure 8. Chick embryo CAM model for tumor development
A: Chick embryo chorioallantoic membrane (CAM). Left image: Localization of the CAM (in red) around the embryo and in direct contact with the eggshell at day 12 of incubation. Right image: Illustration of a cross-section of the CAM at day 10–12 of incubation. 1: Chorionic epithelium; 2: Mesoderm with blood vessels depicted in red; 3: Allantoic epithelium (Vargas, A. et *al*., 2007).
B: CAM tumor development assay, overview of the procedure. The development of the chick embryo lasts 21 days after incubation at 37ºC. A hole is made in the shell after 10 days and human tumor cells are seeded on the CAM. Embryos are euthanized at EDD20.
C: Chick embryo spontaneous metastasis model. At embryonic day 17, the primary tumor and secondary organs such as lung and liver are harvested. Genomic DNA is extracted from the secondary organs and analyzed by qPCR for the presence of human cells (Adapted from Zijlstra, A. et *al*., 2002).
D: Primary tumors were excised and weighted. Data expressed as mean ± SD (n = 1). Significance was determined by two-way ANOVA followed by Bonferroni's post-test; p>0,05 vs control was considered no significant (n.s).

Conclusions

1. **ERK1/2 scaffold proteins expression pattern in normal and tumoral colorectal tissues is different. IQGAP1 and IQGAP2 levels are lower in tumoral tissue, while KSR2 levels are higher in some tumors and lower in others.**
2. **IQGAP2 is downexpressed in B-Raf mutated colorectal cell lines.**
3. **ERK1/2 scaffold proteins expression pattern in EGF stimulated and K-Ras/B-Raf transfected HEK293T cells is similar.**
4. **Silencing IQGAP1, IQGAP2 and KSR2 increases cell proliferation.**
5. **IQGAP2 overexpression induces apoptosis.**
6. **CAM shIQGAP2 RKO cells inoculation develop bigger tumors than RKO, but differences are not significative**

Future Perspectives

1. **To genotype colorectal tumors so as to determine if they are *wt* or K-Ras/B-Raf mutated. This could help to establish a differenced ERK1/2 scaffold proteins expression pattern according to the presence/absence of these mutations.**
2. **To repeat new RKOshIQGAP2 cell proliferation assays so as to reduce the mean standard error.**
3. **To perform new proliferation assays with *wt* and K-Ras mutated stable shIQGAP1, shIQGAP2 and shKSR2 colorectal tumor cell lines.**
4. **To develop IQGAP1, IQGAP2 and KSR2 overexpressed stable cell lines in order to make a comparative proliferation assay with shIQGAP1, shIQGAP2 and shKSR2 stable cell lines.**
5. **To inoculate new fertilized eggs with RKO/RKOshIQGAP2 cell suspensions so as to find if there are significant differences.**
6. **To analyze by qPCR if there is any metastasis in other chick tissues (liver and distal CAM mainly).**
7. **To develop stable mutated WW IQGAP2 domain cell lines disrupting IQGAP2-ERK1/2 interaction. This will allow us to see if this is the mechanism by which IQGAP2 inhibits cell proliferation and increases apoptosis.**

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