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M-Ras Induces Ral and JNK Activation to Regulate MEK/ERK-Independent Gene Expression in MCF-7 Breast Cancer Cells

Ariel F. Castro,[1*] Tania Campos,[1] Justin T. Babcock,[3] Marisol E. Armijo,[1] Alfonso Martínez-Conde,[2] Roxana Pincheira,[1] and Lawrence A. Quilliam[3]

[1]*Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Biológicas, Universidad de Concepción, Concepción, Chile*

[2]*Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad Complutense de Madrid, Spain*

[3]*Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana 46202*

ABSTRACT

Constitutive activation of M-Ras has previously been reported to cause morphologic and growth transformation of murine cells, suggesting that M-Ras plays a role in tumorigenesis. Cell transformation by M-Ras correlated with weak activation of the Raf/MEK/ERK pathway, although contributions from other downstream effectors were suggested. Recent studies indicate that signaling events distinct from the Raf/MEK/ERK cascade are critical for human tumorigenesis. However, it is unknown what signaling events M-Ras triggers in human cells. Using constitutively active M-Ras (Q71L) containing additional mutations within its effector-binding loop, we found that M-Ras induces MEK/ERK-dependent and -independent Elk1 activation as well as phosphatidylinositol 3 kinase (PI3K)/Akt and JNK/cJun activation in human MCF-7 breast cancer cells. Among several human cell lines examined, M-Ras-induced MEK/ERK-independent Elk1 activation was only detected in MCF-7 cells, and correlated with Rlf/M-Ras interaction and Ral/JNK activation. Supporting a role for M-Ras signaling in breast cancer, EGF activated M-Ras and promoted its interaction with endogenous Rlf. In addition, constitutive activation of M-Ras induced estrogen-independent growth of MCF-7 cells that was dependent on PI3K/Akt, MEK/ERK, and JNK activation. Thus, our studies demonstrate that M-Ras signaling activity differs between human cells, highlighting the importance of defining Ras protein signaling within each cell type, especially when designing treatments for Ras-induced cancer. These findings also demonstrate that M-Ras activity may be important for progression of EGFR-dependent tumors. J. Cell. Biochem. 113: 1253–1264, 2012.

KEY WORDS: M-Ras; Elk1; Rlf/Rgl2; Ral; JNK; ESTROGEN

H-, K-, and N-Ras (Ras) proteins regulate a variety of biological processes that include cell growth, differentiation, and apoptosis. Furthermore, when these GTP-binding proteins are constitutively activated, they promote cellular transformation [Campbell et al., 1998]. Ras proteins act as molecular switches that alternate between inactive GDP-bound and active GTP-bound states. Switching between these states is controlled by guanine nucleotide exchange factors (GEFs) that promote the release of GDP, thus enabling the more abundant cellular GTP to associate with Ras and induce a conformational change that allows interaction with downstream effectors. GTPase activating proteins (GAPs) then increase the intrinsic GTPase activity of Ras, converting it back to its inactive GDP-bound state [Campbell et al., 1998; Vetter and Wittinghofer, 2001; Quilliam et al., 2002]. Mutations that inhibit intrinsic and GAP-stimulated GTP hydrolysis result in the accumulation of Ras–GTP and its

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*Correspondence to: Ariel F. Castro, Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Biológicas, Universidad de Concepción, Casilla 160-C, Correo 3, Ciudad Universitaria, Concepción, Chile.
E-mail: arcastro@udec.cl

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constitutive association with effector proteins such as Raf, phosphatidylinositol 3 kinase (PI3K), and RalGDS family members [Campbell et al., 1998].

In addition to the classic Ras proteins that are mutated in ~30% of human cancers [Bos, 1989], there are ~35 Ras-related GTPases that overlap significantly in sequence and function. These include the Rap (Rap1A, 1B, 2A, 2B, and 2C), R-Ras (R-Ras, TC21/R-Ras2, and M-Ras/R-Ras3), Ral (A and B), Rheb (1 and 2), RGK (Rad, Gem, and Kir), and Rit (Rit and Rin) sub-families of GTPases [Colicelli, 2004; Caraglia et al., 2005]. M-Ras was independently isolated by several investigators through cDNA library screens, cytokine-induced expression and Ras homology searching of the expressed sequence tag database [Kimmelman et al., 1997; Matsumoto et al., 1997; Ehrhardt et al., 1999; Louahed et al., 1999; Quilliam et al., 1999]. Like Ras and TC21, GTPase-defective mutants of M-Ras were found to promote transformation of NIH3T3 cells, differentiation of PC12 pheochromocytoma cells, dendritic development and factor-independent survival of leukocytes [Kimmelman et al., 1997, 2002; Ehrhardt et al., 1999; Louahed et al., 1999; Quilliam et al., 1999; Sun et al., 2006; Saito et al., 2009]. In addition, activated M-Ras induced hematopoietic cell transformation and participated in osteogenesis [Guo et al., 2006; Watanabe-Takano et al., 2011].

M-Ras shares approximately 50% sequence identity with Ras, including complete conservation within its effector-binding loop. However, residues immediately outside this region diverge, suggesting that M-Ras will differentially interact with known and novel effectors. Consistent with this suggestion, M-Ras has been reported to interact with Raf, PI3K, AF6, Nore1, several RalGDS family members, a complex comprised of Shoc2/Sur-8 and the catalytic subunit of protein phosphatase 1 (PP1c) [Quilliam et al., 1999; Ehrhardt et al., 2001; Rodriguez-Viciana et al., 2004, 2006], and to uniquely associate with certain Rap GEFs [Rebhun et al., 2000; Gao et al., 2001]. Preferential binding of M-Ras to B-Raf [Kimmelman et al., 2002] and Rgl3 [Ehrhardt et al., 2001] has been reported, suggesting that it signals differently than Ras. Similarly, M-Ras may be activated by a different but overlapping spectrum of GEFs [Quilliam et al., 1999; Ohba et al., 2000; Ehrhardt et al., 2004]. Although no activating mutations have so far been found in human tumor samples (LAQ, unpublished), homology with Ras and interaction with overlapping panels of GEFs, GAPs, and effectors suggested that M-Ras is likely to play key roles in both normal human physiology and cancer. However, M-Ras' contribution to human malignancies and the signaling events it triggers in human cells are unknown.

We analyzed M-Ras-mediated signal transduction in several human cell lines. M-Ras was found to activate Raf/MEK/ERK, and PI3K/Akt cascades, but surprisingly, signaling pathways activated by M-Ras differed between MCF-7 and the other cell lines examined. In mapping these events, we found that the differential activity of constitutively activated M-Ras in MCF-7 cells correlated with MEK/ERK-independent gene expression through the activation of Rlf/Ral and JNK. In addition, we showed that constitutive activation of M-Ras overcame the estrogen-dependent growth of MCF-7 cells.

MATERIALS AND METHODS

MATERIALS

B-Raf (C19), ERK1 (K-23), Elk1 (I-20), phospho-Elk1 (Ser 383) (B-4), Rlf/Rgl2 (F-30.1) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rlf/Rgl2 (4D10) was from Abnova (Taiwan). Phospho-MEK1/2 (Ser 217/221), phospho-ERK1/2 (Thr 202/Tyr 204) (E10), phospho-SAPK/JNK (Thr 183/Tyr 185) (98F2), and phospho-Akt (Ser 473) antibodies were from Cell Signaling Technology (Beverly, MA). GAPDH (Biodesign, Kennebunk, ME), hemagglutinin (HA) (Covance, Berkeley, CA), and FLAG (M2, Sigma-Aldrich, Saint Louis, MO) antibodies were purchased from the indicated suppliers. RalA specific antibody was from Upstate/Millipore (Temecula, CA). SP600125, U0126, and LY294002 inhibitors were from Calbiochem (San Diego, CA).

cDNA MANIPULATION AND PLASMID CONSTRUCTS

Cloning of M-Ras and introduction of an activating Q71L mutation was previously described [Quilliam et al., 1999]. Similarly, mutations in the effector binding domain were introduced by two-step polymerase chain reaction, and all products were sequenced prior to subcloning into the pZip-NeoSV(X)1 (pZIP) [Cepko et al., 1984], pCGN [Tanaka and Herr, 1990], pcDNA3 (Invitrogen) or pFLAG-CMV2 (Sigma) vectors. pMTZ-HA-Rlf, pcDNA3-HA-B-Raf, -myc-p110γ of PI3K, -myc-RalGDS, -myc-RIN, -myc-PLCε, -myc-NORE1, -myc-Shoc2, and -myc-Raf-1 were a generous gifts from Drs. D. Andres (University of Kentucky), A. Vojtek (University of Michigan) and P. Rodriguez-Viciana (University College London). RalA 31N and Rlf-CAAX were from Channing Der (University of North Carolina).

293T, HeLa, AND MCF-7 CELL CULTURE, TRANSFECTION, AND IMMUNOBLOT ANALYSIS

293T human embryonic kidney and HeLa cells were cultured in DMEM, and MCF-7 cells in EMEM, both supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells (293T) were transfected with calcium phosphate [Quilliam et al., 2001], but omitting glycerol shock. HeLa and MCF-7 cells were transfected using FuGENE 6 (Roche) as described by the manufacturer. After 24 h, cells were serum-starved overnight and lysed in 20 mM Tris–HCl pH 8.0, 2 mM EDTA, 50 mM p-glycerophosphate, 1 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 1 mM PMSF, 2.2 μg/ml aprotinin (lysis buffer A). Lysates were subjected to SDS–PAGE, and Western blotting with indicated antibodies. Protein expression and phosphorylation were detected by using ECL (GE Biosciences).

CO-IMMUNOPRECIPITATION STUDIES

Cells (293T) were plated at 3×10^5 per 60-mm dish and transfected the following day with 3 μg of pcDNA3-myc-Raf1, -myc-p110γ of PI3K, -myc-RalGDS or -HA-B-Raf co-transfected with 3 μg of either pFLAG-CMV2, pFLAG-CMV2- M-Ras (71L), -M-Ras (71L, 45S), -M-Ras (71L, 47G), or -M-Ras (71L, 50C). For MCF-7 cells, additional co-immunoprecipitation studies of M-Ras (71L, 50C) with Raf1, PI3K, RalGDS, B-Raf, Rlf, RIN, PLCε, Nore1, Shoc2, and

ALS2CR9 were performed. M-Ras mutants were immunoprecipitated from cell lysates using anti-FLAG antibody as described [Quilliam et al., 1999]. Co-precipitation of either effector with M-Ras proteins was visualized by immunoblotting with anti-Myc antibody or anti-HA antibody following SDS–PAGE. Expression and precipitation of M-Ras mutants were determined by FLAG antibody using ECL reagents.

TRANSCRIPTION ASSAYS

Cells were seeded at 1×10^5 per 35-mm dish and co-transfected with 0.125 μg Gal4-Elk1 or Gal4-cJun, 1.25 μg 5XGal4-Luc, and 0.5 μg plasmid encoding genes of interest as indicated in figure legends. After 24 h, cells were serum-starved overnight, and luciferase activity was determined the following day as described [Quilliam et al., 2001].

IMMUNOPRECIPITATION AND Raf PROTEIN KINASE ASSAY

Cells (3×10^5 per 60-mm dish) were transfected with 1 μg of pFLAG-CMV2, pFLAG-CMV2-MRas (71L), -MRas (71L, 45S), -MRas (71L, 47G), or -MRas (71L, 50C). After overnight serum starvation, cells were lysed in lysis buffer A and then microcentrifuged for 10 min at maximum speed to remove cellular debris. The supernatants were pre-cleared with 30 μl protein A/G agarose beads. Pre-cleared samples were then tumbled with 5 μg B-Raf antibody and 30 μl protein A/G agarose beads (Santa Cruz Biotech), 4°C for 2 h. The beads were washed 3 times with 1 ml of lysis buffer, once with 200 μl of kinase assay buffer (50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 75 mM NaCl, 5 mM EGTA), and combined with 35 ng/μl GST-MEK, 70 ng/μl GST-ERK (kinase-dead), and 0.16 mM ATP in 25 μl of kinase buffer. GST-MEK and GST-ERK fusion proteins were kindly provided by Dr. Mark Marshall (Indiana University School of Medicine). The kinase reaction mixtures were incubated at 30°C for 30 min, and the reaction was stopped by adding boiled protein sample buffer. Levels of immunoprecipitated B-Raf and ERK phosphorylation were viewed by immunoblotting with phospho-specific antibodies following SDS–PAGE.

IN VIVO PULL-DOWN Ral ACTIVATION ASSAY

MCF-7 cells were plated at 1×10^6 per 100-mm dish, and transfected with 6 μg of either pFLAG-CMV2, pFLAG-CMV2- M-Ras (71L), -M-Ras (71L, 45S), -M-Ras (71L, 47G), or -M-Ras (71L, 50C). After overnight serum starvation, cells were lysed and Ral protein was precipitated as described for other Ras proteins [Castro et al., 2005]. Ral BP1 agarose (Upstate/Millipore) was used to precipitate activated Ral. Activated RalA bound to Ral BP1 was viewed by immunoblotting with a specific antibody.

ESTROGEN-INDEPENDENT GROWTH ASSAYS

Pooled populations of MCF-7 stably expressing pCGN or pCGN-M-Ras (71L) were selected on Hygromycin B (100 μg/ml). Cells were plated at 2×10^4 cells/24-well plates in regular medium. After 24 h, cells were transferred to phenol red-free EMEM with 5% charcoal-stripped FBS plus 100 nM ICI 182780 or 10 nM estradiol (E2). The medium was changed every other day, and cells were scored for survival every day using a cell proliferation/cytotoxicity kit (Cambrex) based on the bioluminescent measurement of ATP that is present in all metabolically active cells. In parallel, the same experiments were performed to evaluate proliferation on estrogen-free media by cell counting.

Survival of pCGN-M-Ras (71L) expressing cells in the absence of estrogen and the presence of PI3K (LY294002, 20 μM), MEK (U0126, 10 μM) or JNK (SP600125, 10 μM) inhibitor was evaluated by using a CytoTox-Glo cytotoxicity assay kit (Promega).

IN VIVO PULL-DOWN M-Ras ACTIVATION ASSAY

A pooled population of MCF-7 cells stably expressing pCGN-HA-M-Ras wt was selected on 100 μg/ml Hygromycin B. Cells were plated at 1×10^6 per 100-mm dish, serum-starved overnight the following day, and then incubated in the absence or presence of EGF (50 ng/ml) for the indicated times. Cells were lysed and M-Ras protein was precipitated as described [Castro et al., 2005]. The GST-RBD of Rlf was used to precipitate activated M-Ras. Preparation of GST-RBD of Rlf was described elsewhere [Quilliam et al., 2001]. Activated HA-tagged M-Ras bound to the GST-RBD of Rlf was viewed by immunoblotting with anti-HA antibody.

RESULTS

Since most Ras-induced human cancers are carcinomas of epithelial origin, we chose to study M-Ras signaling in various human carcinoma cell lines. Using an M-Ras-specific antiserum, we found that this GTPase is expressed in MCF-7 breast, HeLa cervical, and LNCaP prostate cancer cell lines, as well as in the HEK 293T cell line (Supplementary data 1).

M-Ras INDUCES CELL-SPECIFIC RESPONSES

To determine which downstream signaling pathways are important for M-Ras function in human cells, we introduced point mutations into the M-Ras effector binding domain to correlate the biological and signaling properties of the mutant proteins. This strategy was previously used to delineate the effectors that contribute to Ras-induced cellular transformation [Khosravi-Far et al., 1996] and other biological events [Ramocki et al., 1997, 1998]. We created M-Ras T45S, E47G, or Y50C mutations in combination with activating Q71L substitutions. The M-Ras effector mutants are equivalent to previous substitutions at position 35, 37, or 40 in H-Ras [White et al., 1995]. Among the three most characterized Ras effectors, Raf, PI3K, and RalGDS, the T35S mutation in H-Ras retains binding to Raf-1, but not RalGDS or PI3K. The mutation at position 37 only retains interaction with RalGDS, whereas the Y40C mutation only interacts with PI3K [White et al., 1995; Rodriguez-Viciana et al., 1997]. The binding capacity of the M-Ras effector mutants was analyzed in HEK 293T cells, and found to differ from that described for H-Ras (Fig. 1). The E47G mutation in M-Ras did not significantly alter the binding with effector proteins, suggesting that this residue is not essential for M-Ras binding capacity. The T45S mutation retained weak

binding to PI3K and B-Raf, but not to RalGDS. The Y50C mutation showed very weak interaction with B-Raf, but no interaction with the other effectors (Fig. 1).

To determine what signaling events are triggered by M-Ras in human cells, we next examined its ability to induce Gal4-Elk1 luciferase activation in various cell lines shown in supplementary data 1 to express this GTPase. Although the M-Ras effector mutants produced a similar pattern of luciferase activation in HeLa and HEK 293T cells (Fig. 2), a unique pattern of Elk1 induction was observed in MCF-7 cells. In particular, M-Ras (71L, 50C) potently induced gene expression and this reporter gene activity correlated well with the levels of endogenous MCF-7 cell Elk1 phosphorylation (Fig. 3A). Because M-Ras mutants did not significantly affect metabolic activity (Supplementary data 2), the observed differences in luciferase activity were not due to an ability of the Y50C mutant to differentially promote cell survival in the absence of serum.

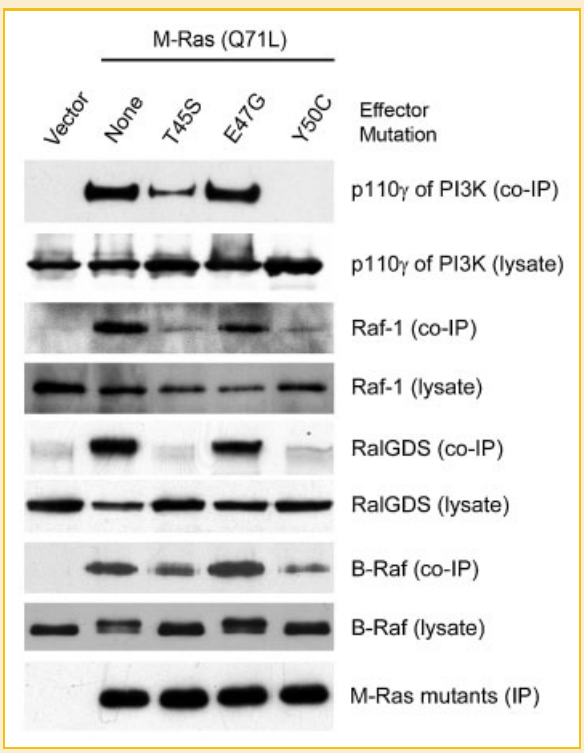

Fig. 1. Interaction between M-Ras mutants and effectors in 293T cells. 293T cells were transfected with empty vector or M-Ras Q71L mutants along with the indicated effectors. M-Ras mutants were immunoprecipitated using anti-FLAG antibody, and coprecipitation of effectors was determined by immunoblotting with anti-myc antibody (Raf1, RalGDS and p110γ) or anti-HA antibody (B-Raf). Immunoprecipitation of M-Ras mutants were confirmed by immunoblotting with anti-FLAG antibody (lower panel). Results are representative of three independent experiments.

DIFFERENTIAL M-Ras SIGNALING IN MCF7 CELLS IS DUE TO MEK/ERK-INDEPENDENT AND PI3K-INDEPENDENT ACTIVATION OF Elk1

Due to the ability of M-Ras to transform mouse mammary cells, [Ward et al., 2004] and its above described unique signaling in MCF-7 human breast cancer cells, we focused on defining signaling components downstream of M-Ras in MCF-7 cells.

The Raf/MEK/ERK pathway is the major means by which Ras induces Elk1 activation [Campbell et al., 1998]. To understand the signaling properties of M-Ras (71L, 50C) in MCF-7 cells, we asked whether activation of this pathway correlates with the pattern of Elk1 activation by M-Ras mutants. The same lysates were analyzed for MEK1/2 and ERK1/2-induced phosphorylation, as well as for Gal4-Elk1 activation. Surprisingly, M-Ras (71L, 50C) induced only weak phosphorylation of MEK1/2 or ERK1/2, which did not correlate with its ability to robustly activate Elk1-mediated gene expression (Fig. 3A). However, there was a good correlation between Elk1 activation (Fig. 2) and ERK1/2 phosphorylation induced by M-Ras mutants in HeLa cells (Supplementary data 3A). Thus, all the above results suggested that M-Ras induces MEK/ERK-dependent and -independent Elk1 activation in MCF7 cells. Furthermore, the MEK inhibitor U0126 did not completely block Elk1 activation by M-Ras (71L, 50C) under conditions where it effectively eliminated ERK1/2-induced phosphorylation in MCF7 cells (Fig. 3B). A similar result was obtained for M-Ras (Q71L), indicating that the ERK1/2-independent Elk1 activation by M-Ras is not an artifact caused by the mutation of the effector binding domain (Fig. 3B).

M-Ras induces cell survival through the activation of PI3K/Akt in PC12 cells [Kimmelman et al., 2000]. We similarly found that M-Ras (Q71L) induces strong Akt phosphorylation in MCF-7 cells that is dependent on PI3K activation (Fig. 3C). It was possible that the PI3K/Akt pathway promotes ERK1/2-independent activation of Elk1 in MCF-7 cells. However, the lack of interaction of the Y50C mutant with the p110 subunit of PI3K (Fig. 1A) indicated that this was unlikely. To confirm that PI3K/Akt was not responsible for the ERK1/2-independent activation of Elk1, we investigated the effect of the PI3K inhibitor, LY294002, on M-Ras-induced Elk1 activation. LY294002 did not significantly affect Elk1 activation by M-Ras (71L, 50C) (Fig. 3D). In contrast, LY294002 enhanced both Elk1 activation and ERK-induced phosphorylation by M-Ras in HeLa cells (Supplementary data 3B). This is consistent with M-Ras activating the ERK/Elk pathway through B-Raf [Kimmelman et al., 1997], which is negatively regulated by Akt [Guan et al., 2000]. Indeed, M-Ras (Q71L) induces endogenous B-Raf activation measured by a kinase assay in HeLa cells, and the pattern of activation by the effector mutants follows that of Elk1 activation (Supplementary data 3C). All these results indicated that M-Ras can regulate MCF-7 cell Elk1 activation independently of either Raf/MEK/ERK or PI3K pathways.

M-Ras-INDUCED ACTIVATION OF Ral IS RESPONSIBLE FOR THE MEK/ERK-INDEPENDENT ACTIVATION OF Elk1

To identify the signaling pathway responsible for the MEK/ERK-independent Elk1 activation by M-Ras, we performed a series of

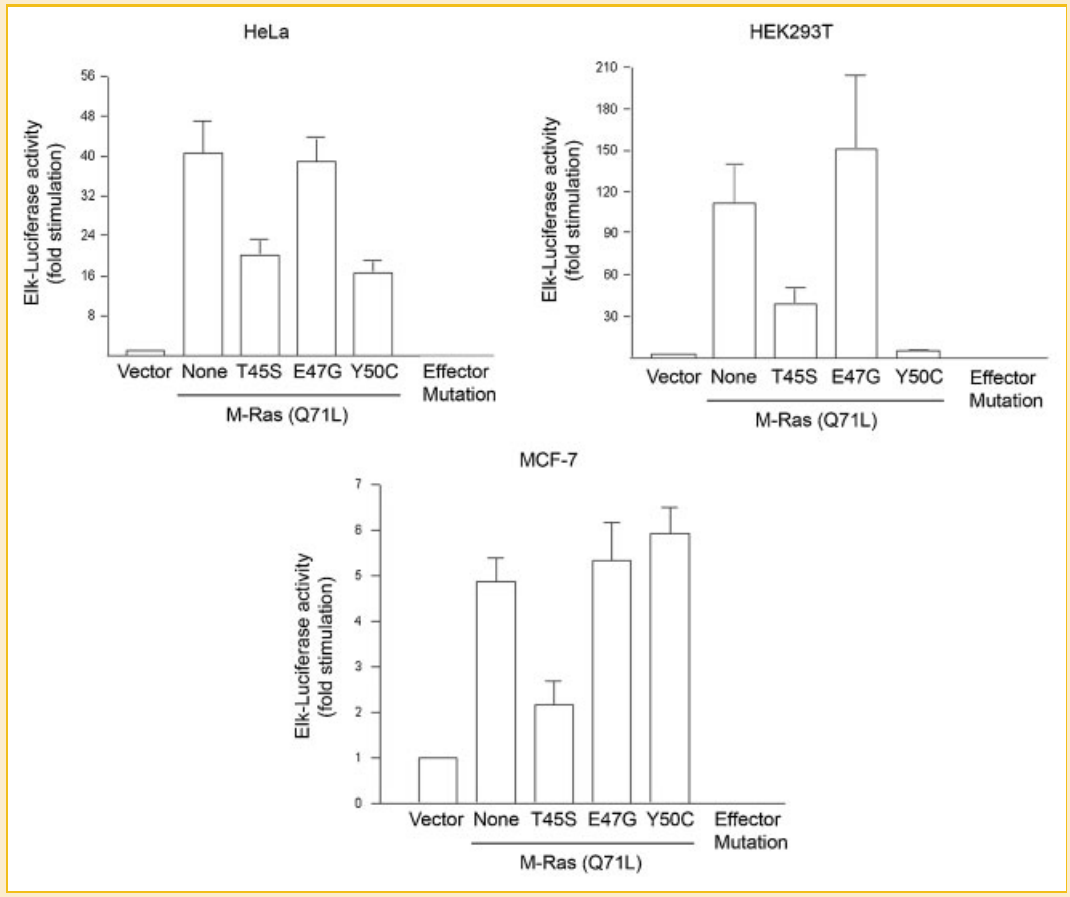

Fig. 2. M-Ras effector mutants induce a different pattern of Elk1 activation in MCF-7 cells. MCF-7, HeLa, or 293T cells were transfected with Gal4-Elk1 and 5XGal-Luc reporter plasmids along with pcDNA3 plasmids encoding the indicated proteins. After serum starvation, luciferase activity was measured from cell lysates. Data show mean ± SEM from at least three experiments performed in duplicate.

co-immunoprecipitation studies between M-Ras (71L, 50C) and a panel of known Ras effectors (RalGDS, Raf, PI3K, Rlf/Rgl2, Rin, phospholipase Cε, Nore1, and Shoc2) in MCF-7 cells. M-Ras (71L, 50C) showed strong interaction with Rlf (Fig. 4A), but undetectable interaction with other effectors (data not shown). Ras interacts with and targets Rlf to the membrane to induce activation of the Ral GTPase. M-Ras effector mutants induced activation of RalA that correlated with the pattern of Elk1 activation in MCF-7 cells (Fig. 4B). As control, an activated membrane-targeted mutant of Rlf, RlfCAAX was also shown to induce Ral activation. To address whether Ral was responsible for the MEK/ERK-independent Elk1 activation by M-Ras, we inhibited Ral activity using a dominant negative RalA 31N mutant. We found that RalA 31N reduced M-Ras-induced Elk1 but not ERK1/2 activation. Consistent with a role for Rlf/Ral in promoting ERK-independent M-Ras signaling, RlfCAAX induced Elk1 activation in the absence of detectable ERK1/2 phosphorylation (Fig. 4C). Endogenous Rlf expression was detected with two different commercial antibodies in MCF-7 cells, but only one of them revealed very faint bands in HeLa or HEK 293T cells in which we were unable to detect MEK/ERK-independent activation of Elk1 (Fig. 4D). To confirm a role for Rlf in the ERK-independent M-Ras signaling, we knockdown Rlf using two specific shRNA as in [Vigil et al., 2010]. Although this resulted in >90% loss of protein, it also impacted cell survival and phosphoMAPK levels (data not shown), compromising their use. This is consistent with depletion of various RalGEFs having deleterious effects on cytokinesis [Cascone et al., 2008] preventing us from evaluating the impact of Rlf loss on M-Ras signaling in MCF-7 cells. However, overexpression of Rlf in HeLa cells cooperated with M-Ras (71L, 50C) to induce Ral (Fig. 5 *right*) and ERK-independent Elk1 activation (Fig. 5 *left*).

In order to support the in vivo relevance of M-Ras/Rlf signaling, we tested whether a receptor-mediated activation of M-Ras promotes its interaction with Rlf. Since we have previously reported that SOS, a GEF downstream of the EGFR, induces M-Ras activation [Quilliam et al., 1999], we first determined whether M-Ras was activated downstream of the EGFR in MCF-7 cells. We found that EGF promotes M-Ras-GTP loading in a time-dependent manner (Supplementary data 4A), and this activation of M-Ras promoted interaction with endogenous Rlf (Supplementary data 4B).

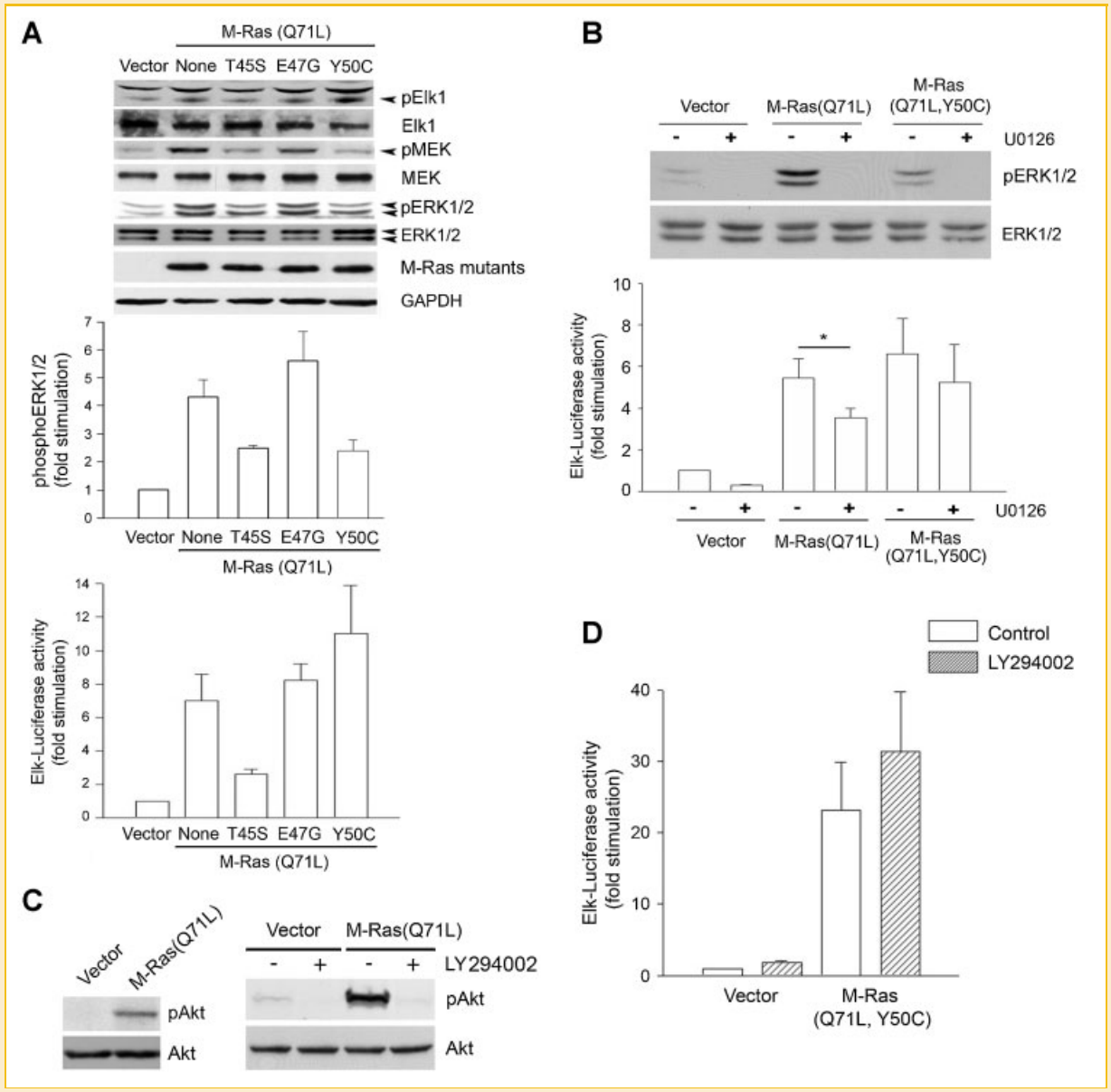

Fig. 3. M-Ras induces MEK1/2-, ERK1/2-, and PI3K-independent activation of Elk1 in MCF-7 cells. A: MCF-7 cells were transfected as in Figure 2, except pFLAG-CMV2 plasmids were used. Following serum starvation, lysates were prepared, and an aliquot was used to determine luciferase activity. Data show mean ± SEM of three experiments performed in duplicate. The remaining lysates were subjected to SDS-PAGE, and levels of ERK1/2, MEK1/2, and Elk1 phosphorylation were evaluated by Western blotting with phospho-specific antibodies. M-Ras effector mutants were detected with M2 anti-FLAG antibody. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was used to evaluate equal loading. Data are representative of three independent experiments. B: MCF-7 cells transfected with empty pcDNA3 or vector encoding M-Ras (71L, 50C), or M-Ras (71L) plus Gal4-Elk1 and 5XGal-Luc were serum-starved overnight in the presence of vehicle (DMSO) or 10 μM of the specific MEK inhibitor, U0126. Luciferase activity, levels of ERK1/2 expression and phosphorylation were determined from cell lysates. Results are representative of three independent experiments. Luciferase activity data show mean ± SEM. *Student's *t*-test ($P < 0.05$). C: MCF-7 cells were transfected with empty pFLAG-CMV2 or vector encoding M-Ras (71L). Cells were serum-starved overnight and then incubated for 1 h in the presence of vehicle or 20 μM PI3K inhibitor, LY294002. Levels of Akt phosphorylation were determined by Western blotting with specific antibody. D: MCF-7 cells were transfected with pcDNA3 or pcDNA3 encoding M-Ras (71L, 50C), along with Gal4-Elk and 5XGal-Luc. Cells were serum-starved overnight in the presence of vehicle (DMSO) or 20 μM LY294002. Luciferase activity was determined from cell lysates. Data show mean ± SEM from three pooled experiments performed in duplicate.

M-Ras INDUCES JNK ACTIVATION

Rlf/Ral activation induces JNK/c-Jun activation [de Ruiter et al., 2000]. Accordingly, M-Ras (Q71L) induced JNK phosphorylation similar to that induced by RlfCAAX (Fig. 6A) in MCF7 but not HeLa cells (Fig. 5 *right*). Using both the anti phospho-JNK antibody and a Gal4-c-Jun luciferase reporter plasmid, we observed that the pattern of JNK/c-Jun activation by the M-Ras effector mutants in MCF-7 cells correlated with that of Elk1

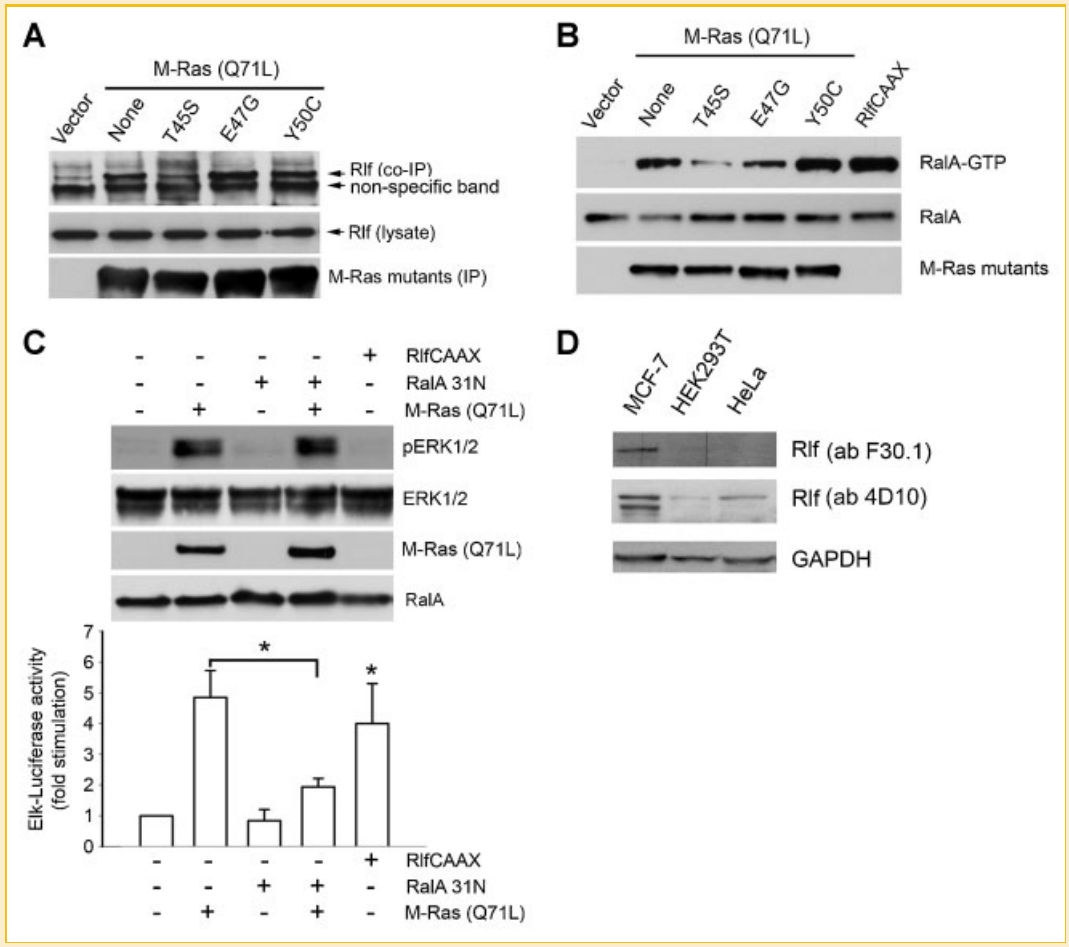

Fig. 4. M-Ras induces ERK-independent Elk1 activation through Rlf/RalA. A: MCF-7 cells were transfected with empty vector or M-Ras mutants along with Rlf. M-Ras mutants were immunoprecipitated using anti-FLAG antibody, and coprecipitation of Rlf was determined by immunoblotting with anti-HA antibody. Immunoprecipitation of M-Ras mutants were confirmed by immunoblotting with anti-FLAG antibody (lower panel). Results are representative of three independent experiments. B: MCF-7 cells were transfected with empty vector or M-Ras mutants. Cells were serum-starved overnight, and Ral BP1 agarose (5 μg) was used to precipitate activated Ral-GTP from cell lysates. Activated RalA was detected by Western blotting with a specific antibody, and M-Ras mutants with Flag antibody. Data are representative of three independent experiments. C: MCF-7 cells were transfected with Gal4-Elk1 and 5XGal-Luc reporter plasmids along with plasmids encoding the indicated proteins. After serum starvation, luciferase activity was measured from cell lysates. The same lysates were subjected to SDS–PAGE, and levels of indicated proteins were evaluated by Western blotting. Data show mean ± SEM from three experiments performed in duplicate. *Student's *t*-test ($P < 0.05$). D: Endogenous Rlf expression in MCF-7, HEK 293T, and HeLa cell lysates (50 μg of protein each) was evaluated by Western blotting.

activation (Fig. 6B). These results suggested that Rlf-dependent activation of JNK mediates ERK-independent Elk1 activation by M-Ras. However, inhibition of JNK with SP600125 unexpectedly increased Gal4-Elk1 activation by M-Ras effector mutants (data not shown), likely by release of a competitive ERK1/2-dependent Elk1 activation [Shen et al., 2003; Waetzig and Herdegen, 2005].

M-Ras INDUCES ESTROGEN-INDEPENDENT GROWTH

Breast cancer progression is frequently associated with estrogen-independent proliferation of tumor cells. To understand the relevance of the M-Ras signaling in MCF-7 cells, we determined whether M-Ras activity influenced estrogen-dependent growth. To assay this, we compared proliferation of cells stably expressing M-Ras (Q71L) in the absence of estrogen with those stably transfected with only empty vector (Fig. 7A). Cell proliferation was monitored both by measuring metabolic cell activity (Fig. 7B) and by direct cell count (Fig. 7C). Only cells stably expressing M-Ras (Q71L) could proliferate in the absence of estrogen. By using inhibitors of PI3K, MEK, and JNK, we showed that each pathway contributes to the M-Ras-induced estrogen-independent biological activity (Fig. 7D).

Although activating M-Ras mutations that could induce estrogen-independent growth in breast cancer have yet to be reported, M-Ras may still be overexpressed in breast cancer. To investigate this possibility we analyzed publicly available microarray gene expression data from 295 patients (www.oncomine.org).

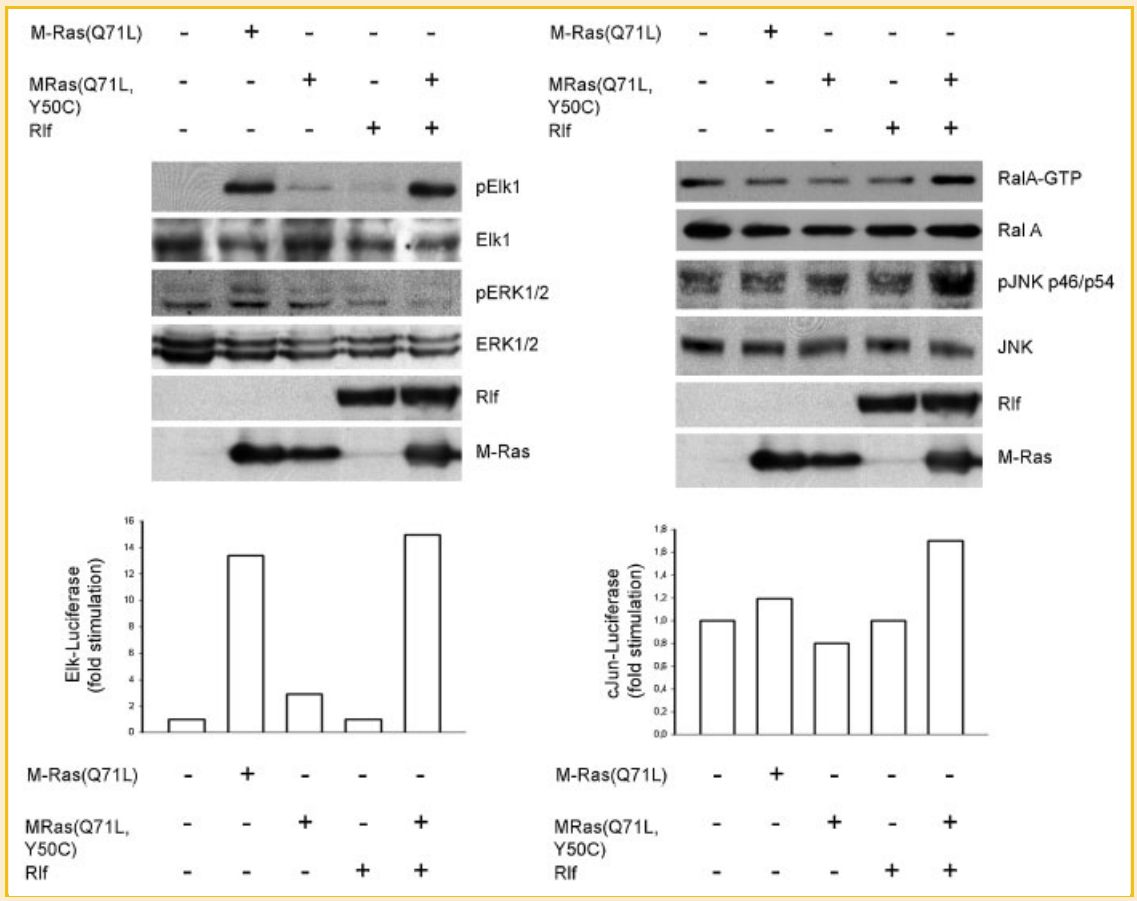

Fig. 5. Rlf overexpression cooperates with M-Ras to induce RalA, JNK, and ERK-independent Elk1 activation in HeLa cells. Cells were transfected with Gal4-Elk1 (*left*) or Gal4-Jun (right) and 5XGal-Luc reporter plasmids along with plasmids encoding the indicated proteins. After serum starvation, luciferase activity was measured from cell lysates. The same lysates were subjected to SDS–PAGE, and levels of indicated proteins were evaluated by Western blotting. For RalA activation assay, lysates were treated as in Figure 4B. Data are representative of two independent experiments performed in duplicate.

Interestingly, this analysis revealed that M-Ras message is significantly upregulated in estrogen receptor (ER)-negative versus ER-positive breast carcinomas ([van de Vijver et al., 2002], Supplementary data 5). Microarray gene expression data from two additional studies confirmed the up-regulation of M-Ras mRNA levels in ER-negative breast carcinoma ($P = 2.8\,e^{-7}$, student's *t*-test for [Hess et al., 2006] and $P = 1.1\,e^{-5}$, student's *t*-test for [Chin et al., 2006]).

DISCUSSION

Despite strong evidence implicating M-Ras in the transformation of murine cells [Kimmelman et al., 1997; Quilliam et al., 1999; Wang et al., 2000; Ward et al., 2004; Guo et al., 2005], little is known about its contribution or mechanism(s) of action in human malignancies. Studies in NIH 3T3 fibroblasts suggested that M-Ras has weaker transforming activity than other members of the Ras family due to less efficient activation of the Raf/MEK/ERK pathway [Kimmelman et al., 1997; Quilliam et al., 1999]. However, similar to other Ras proteins, M-Ras-induced transformation may involve interaction with and activation of multiple downstream effectors [Campbell et al., 1998]. Recent studies indicate that signaling via pathways other than the canonical Raf/MEK/ERK cascade may be critical for human tumor development [Repasky et al., 2004; Gonzalez-Garcia et al., 2005; Lim et al., 2005; Parsons et al., 2005]. Our current study demonstrates that the signaling events activated by M-Ras diverge between different human cell lines. Essentially, we found that M-Ras can induce gene expression independently of the canonical Raf/MEK/ERK pathway, but this activity is cell- or context-specific.

The differential response to M-Ras was demonstrated following creation of a Y50C mutation in its switch I domain, in combination with a Q71L activating mutation. While this M-Ras (71L, 50C) mutant had weak activity in other human cell lines, it induced robust Elk1 activation in MCF-7 breast cancer cells. Intriguingly, this pathway had an ERK-independent component that was activated by Ral but not PI3K and correlated with stronger expression of the Ral GEF, Rlf/Rgl2 in MCF7 cells.

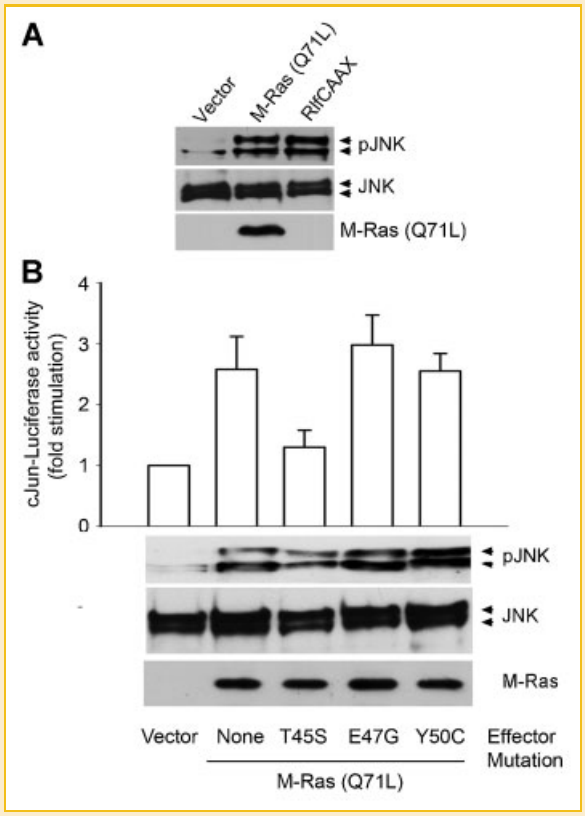

Fig. 6. M-Ras induces JNK activation. A: MCF-7 cells were transfected with empty vector, M-Ras (Q71L) or RlfCAAX. After serum starvation, cell lysates were subjected to SDS–PAGE, and levels of JNK phosphorylation were evaluated by Western blotting with a phospho-specific antibody. B: MCF-7 cells were transfected with Gal4-Jun and 5XGal-Luc reporter plasmids along with pcDNA3 plasmids encoding the indicated proteins. After serum starvation, luciferase activity was measured from cell lysates. Data show mean ± SEM from at least three experiments performed in duplicate. In parallel, lysates were subjected to SDS–PAGE, and levels of JNK phosphorylation were evaluated by Western blotting with a phospho-specific antibody.

Activation of RalA has been shown to be critical for Ras-induced tumorigenesis of human cells. The implications of the M-Ras-induced Ral-dependent activation of gene expression in breast cancer require further study. However, it is significant that M-Ras promotes epithelial–mesenchymal transition in a murine mammary epithelial cell line [Ward et al., 2004] and induces estrogen-independent growth of human mammary carcinoma cells (Fig. 7). Thus, M-Ras may play a role in the pathology of breast cancer. The observations that M-Ras mRNA levels are upregulated in ER-negative breast carcinoma (Supplementary data 5 and [van de Vijver et al., 2002; Chin et al., 2006; Hess et al., 2006]) is consistent with this possibility. Although additional studies are needed to demonstrate that upregulation of M-Ras mRNA levels associates with an increase in protein levels, M-Ras could also contribute to breast cancer by signaling downstream of EGFR. In fact, upregulation of this receptor is frequently associated with breast cancer, and we demonstrated that EGF induces M-Ras activation and thereby Rlf interaction with M-Ras in MCF-7 cells. It remains to be investigated what signaling pathway M-Ras/Rlf mediates downstream of EGFR. In addition, since M-Ras is activated by the GEFs Sos and RasGRP3 [Quilliam et al., 1999; Ohba et al., 2000], a number of additional growth-stimulating ligands may activate it in tumor cells. Indeed, ERα has been reported to couple to Sos via a Shc-Grb2 complex [Song et al., 2002]. This non-genomic estrogen signaling also induces gene expression via Elk1 downstream of the JNK and ERK MAP kinases [Song et al., 2002; Chen et al., 2004; Kim et al., 2007]. Whether the non-genomic effects of estrogen either via its classical receptors ERα and ERβ or through G-protein-coupled estrogen receptor 1 (GPER1/GPR130) involve M-Ras activation or its downstream regulation of Ral and Elk1 will require further investigation.

We also found that M-Ras activates JNK. Consistent with a role for JNK in M-Ras signaling, it was recently shown that M-Ras activation of JNK is important for M-Ras-induced osteogenesis [Watanabe-Takano et al., 2011]. Since Rlf is involved in growth factor-induced, Ras-dependent, activation of JNK [de Ruiter et al., 2000], it is likely that Rlf mediates M-Ras-induced activation of JNK. In agreement, the activated membrane-targeted mutant of Rlf, RlfCAAX induced strong JNK activation in MCF-7 cells and overexpression of Rlf correlated with M-Ras-induced JNK activation in HeLa cells. Whether Rlf-dependent JNK activation is parallel or responsible for the ERK-independent Elk1 activation in MCF7 cells is unclear. In fact, although the activation of JNK/cJun by M-Ras effector mutants followed a similar pattern to that of Elk1 activation, JNK inhibition was unable to reduce Elk1 activation. Instead, it released basal and M-Ras-induced ERK-dependent Elk1 activation indicating that a crosstalk between the ERK and JNK pathway may exist.

Since the JNK pathway has been associated with cancer progression [Saadeddin et al., 2009], the regulation of JNK activity by M-Ras may have important implications for cancer biology. Consistent with this possibility, we found that JNK contributes to M-Ras-induced estrogen-independent growth. In addition, the JNK pathway has been involved in the expression of matrix metalloproteinases that are associated with extracellular matrix degradation and tumor metastasis [Gee et al., 2000; Hauck et al., 2001; Sugioka et al., 2004; Cui et al., 2006]. An association between JNK/c-Jun activation and angiogenesis in invasive breast cancer has also been reported [Vleugel et al., 2006].

In summary, our results demonstrate that M-Ras signaling depends on cellular context. Constitutively activated M-Ras appears to activate the classical Raf/MEK/ERK cascade to induce Elk1 activation in human HeLa and HEK293T cells, but uses additional signaling components to activate Elk1 and JNK in MCF-7 cells. This may permit certain tumors to evade the effects of pharmaceuticals that block the ERK/MAPK cascade [Collisson et al., 2003] or Ras membrane binding. The Raf, PI3K, and RalGDS pathways have all been implicated in the development of human tumors [Repasky et al., 2004]. Our studies are consistent with M-Ras inducing signaling events independently of these pathways to promote gene expression in MCF-7 cells, and M-Ras activation

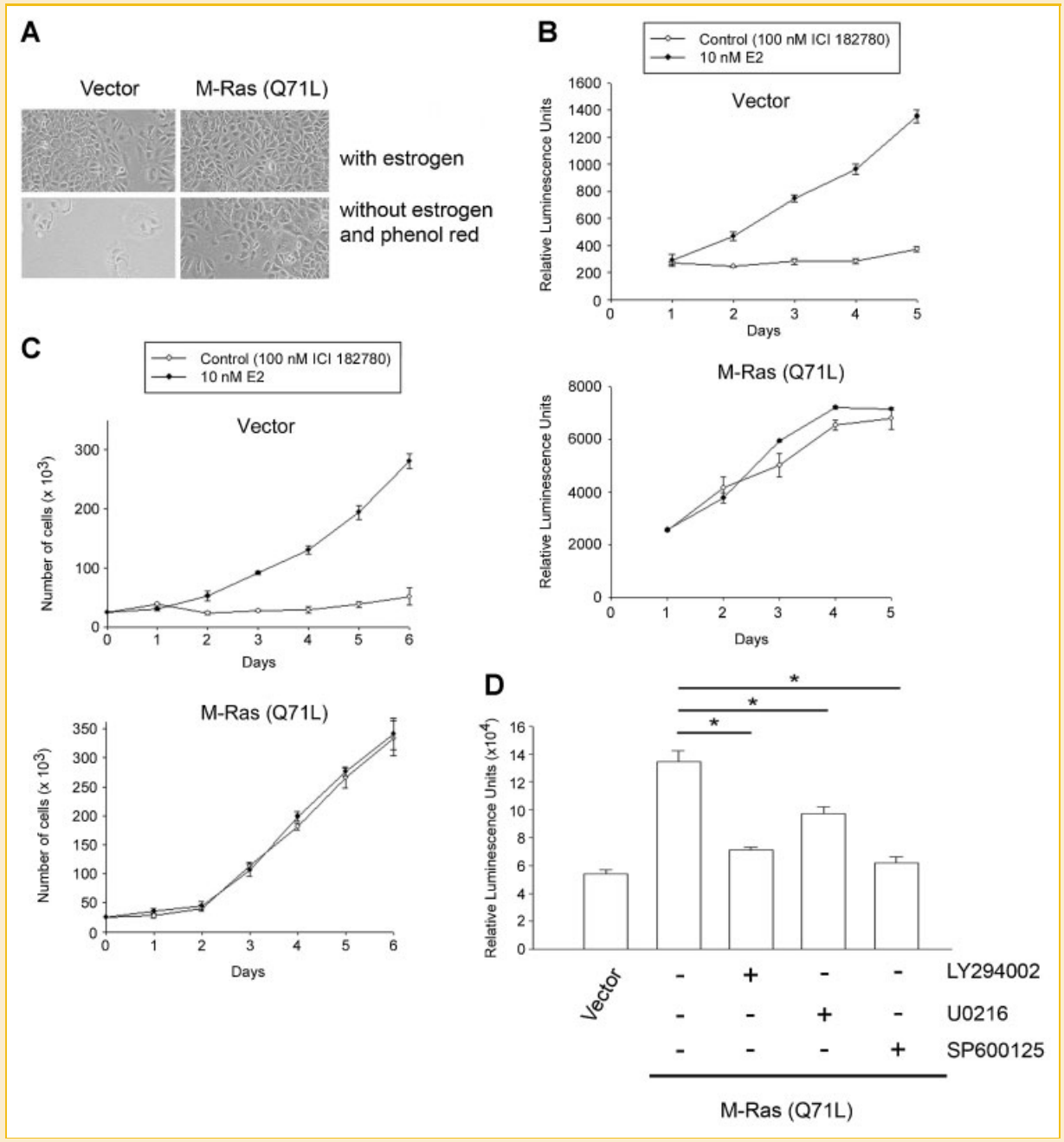

Fig. 7. M-Ras induces estrogen-independent growth in MCF-7 cells. A: MCF-7 cells stably expressing empty pCGN vector or vector encoding M-Ras (71L) were grown in the absence of estrogen (phenol red-free EMEM with 5% charcoal-stripped FBS plus 100 nM ICI 182780) for approximately 7 days. M-Ras (Q71L) expression overcame the estrogen-dependency of MCF-7 cells. Representative images were photographed at 4× magnification. Data are representative of three independent experiments performed in duplicate. B: Same as A, but cell survival of pCGN (vector) or M-Ras (71L) expressing cells on estrogen-free media plus ICI 182780 was compared with that in the presence of 10 nM E2. Cell survival was evaluated at the indicated times by bioluminescent measurement of ATP levels as described in Materials and Methods Section. C: Same as B, but quantified by cell counting. Experiments in (B) and (C) were performed in triplicate and are representative of two independent experiments. D: MCF-7 cells stably expressing empty pCGN vector or vector encoding M-Ras (71L) were grown in the absence of estrogen (phenol red-free EMEM with 5% charcoal-stripped FBS plus 100 nM ICI 182780), +/− indicated kinase inhibitors for approximately 7 days. Cell survival was evaluated by using a CytoTox-Glo cytotoxicity assay kit that measures dead cell protease activity. Experiments were performed in triplicate and are representative of two independent experiments. *Student's *t*-test ($P < 0.05$).

supporting estrogen-independent signaling. The importance of defining Ras protein signaling within each cell type, especially when designing treatments for Ras-induced cancer, is highlighted here.

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