

Mxi2 promotes stimulus-independent ERK nuclear translocation

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Spatial regulation of ERK1/2 MAP kinases is an essential yet largely unveiled mechanism for ensuring the fidelity and specificity of their signals. Mxi2 is a p38 α isoform with the ability to bind ERK1/2. Herein we show that Mxi2 has profound effects on ERK1/2 nucleocytoplasmic distribution, promoting their accumulation in the nucleus. Downregulation of endogenous Mxi2 by RNAi causes a marked reduction of ERK1/2 in the nucleus, accompanied by a pronounced decline in cellular proliferation. We demonstrate that Mxi2 functions in nuclear shuttling of ERK1/2 by enhancing the nuclear accumulation of both phosphorylated and unphosphorylated forms in the absence of stimulation. This process requires the direct interaction of both proteins and a high-affinity binding of Mxi2 to ERK-binding sites in nucleoporins. In this respect, Mxi2 acts antagonistically to PEA15, displacing it from ERK1/2 complexes. These results point to Mxi2 as a key spatial regulator for ERK1/2 and disclose an unprecedented stimulus-independent mechanism for ERK nuclear import.

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Introduction

ERK1 and ERK2 mitogen-activated protein kinases (MAPKs) are cytoplasmic kinases that become activated in response to a wide array of stimuli, including those that regulate cell proliferation, differentiation and survival. ERK1/2 are key elements in the transduction of signals from the surface to the interior of the cell. They lie downstream of a signaling

module that includes sequentially Raf family MAPKKs and MEK 1 and 2 dual-specificity MAPKKs. This cascade is regulated at its origin by Ras GTPases. Once activated, ERK1/2 phosphorylate a broad spectrum of substrates distributed throughout different subcellular compartments (Pearson *et al*, 2001). The balance among these site-specific components of ERK signals is the determinant for the biological outcomes resulting from ERK activation (Robinson *et al*, 1998; Ajenjo *et al*, 2004).

Under resting conditions, unphosphorylated ERK1/2 are primarily located in the cytoplasm, largely as a consequence of their interaction with cytoplasmic anchors, including MEK1, certain protein phosphatases and the cytoskeleton (Reszka *et al*, 1995; Fukuda *et al*, 1997b; Blanco-Aparicio *et al*, 1999). Upon stimulation, ERK1/2 lose affinity for these partners and redistribute throughout the cell, including the nucleus. Phosphorylation may be sufficient to promote ERK1/2 transit to the nucleus, but their kinase activity is not necessary (Lenormand *et al*, 1993). Several models explaining ERK1/2 nuclear import have been suggested: ERK1/2 enter the nucleus, in both phosphorylated and unphosphorylated forms, by a carrier- and energy-independent mechanism that involves direct interaction with the nuclear pore complex (NPC) (Matsubayashi *et al*, 2001; Whitehurst *et al*, 2002). The phosphorylated proteins may also enter the nucleus, perhaps in a dimeric form, by active transport (Khokhlatchev *et al*, 1998; Adachi *et al*, 1999; Ranganathan *et al*, 2006). Some proteins, like PEA15, prevent ERK1/2 translocation by interfering with their binding to the nuclear pore (Formstecher *et al*, 2001; Whitehurst *et al*, 2004). ERK1/2 nucleocytoplasmic distribution is also dependent on its nuclear export. ERK1/2 nuclear efflux can be mediated by MEK, which has a nuclear export sequence (NES) that complexes with the nuclear export receptor CRM1 (Fukuda *et al*, 1997a). ERK1/2, once dephosphorylated in the nucleus, bind to MEK and are exported to the cytoplasm (Adachi *et al*, 2000). ERK1/2 nucleocytoplasmic distribution can also be affected by the number and affinity of ERK1/2 binding partners in each compartment (Pouyssegur and Lenormand, 2003; Burack and Shaw, 2005). ERK1/2 nuclear translocation and retention requires neosynthesis of nuclear anchoring proteins (Lenormand *et al*, 1993). Topoisomerase II and kinetochores have been identified as nuclear anchors for ERK1/2 (Shapiro *et al*, 1998), but the identity of the majority of these remains elusive.

Mxi2 is a splice isoform of p38 α . It is identical to p38 from amino acids 1 to 280 and harbors a unique, 17-amino-acid C-terminus (Zervos *et al*, 1995). We have recently reported that some Mxi2 biochemical properties make it unique among p38 proteins (Sanz *et al*, 2000). As such, Mxi2 directly binds to ERK1/2 and in so doing it prolongs their activation. This interaction has profound effects on ERK1/2 nuclear but not cytoplasmic signaling (Sanz-Moreno *et al*, 2003). Herein, we have studied the mechanism of Mxi2 action on ERK1/2 functions. We report that Mxi2 is a nuclear protein with the

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ability to alter ERK1/2 nucleocytoplasmic distribution by facilitating ERK1/2 nuclear entry. In the absence of additional stimulation, Mxi2 expression is sufficient to promote ERK1/2 nuclear translocation. Our results disclose for the first time a stimulus-independent nuclear shuttle for ERK1/2 capable of modifying ERK1/2 nucleocytoplasmic distribution.

Results

Mxi2 is a nuclear protein

To gain an initial insight into how Mxi2 regulates ERK1/2 functions, we investigated its subcellular distribution. Mxi2 was expressed in COS7 cells and its localization was visualized by immunofluorescence. Mxi2 was predominantly nuclear irrespective of the stimulatory conditions, although particularly when cells were growing or under acute mitogenic stimulation (Figure 1A). Quantification of Mxi2 nuclear and cytoplasmic fractions confirmed that, under starvation, over 60% of Mxi2 was nuclear and that this proportion was greater than 80% when growing or upon stimulation with EGF (Figure 1B). The possibility existed that Mxi2 nuclear localization was a consequence of its inability to interact with cytoplasmic MAPKKs. Thus, cells were cotransfected with

HA-tagged Mxi2 or p38 together with MKK3 or MKK6 and the presence of these MAPKKs in the anti-HA immunoprecipitates was analyzed. It was found that both MAPKKs efficiently associated with Mxi2, although in the case of MKK3 not as intensely as with p38 (Figure 1C). Furthermore, overexpression of these MAPKKs resulted in a pronounced retrieval of Mxi2 from the nucleus (Supplementary Figure 1).

Mxi2 alters ERK1/2 subcellular distribution

Next, we investigated how Mxi2 affected ERK1/2 distribution between the nucleus and cytoplasm. In parental COS7 cells, under resting conditions, nearly 20% of the total ERK1/2 pool was nuclear. This proportion increased to ~50% under mitogenic stimulation, upon which phosphorylated ERK1/2 also segregated evenly between the nucleus and the cytoplasm (Figure 2A). Conversely, in cells transfected with Mxi2, about 80% of ERK1/2 was nuclear under basal conditions, a proportion that increased to over 90% under EGF stimulation. In line with our previous results (Sanz-Moreno *et al*, 2003), the presence of Mxi2 was sufficient to increase the levels of phosphorylated ERK1/2 even under starved conditions, the majority of which was located in the nucleus. On

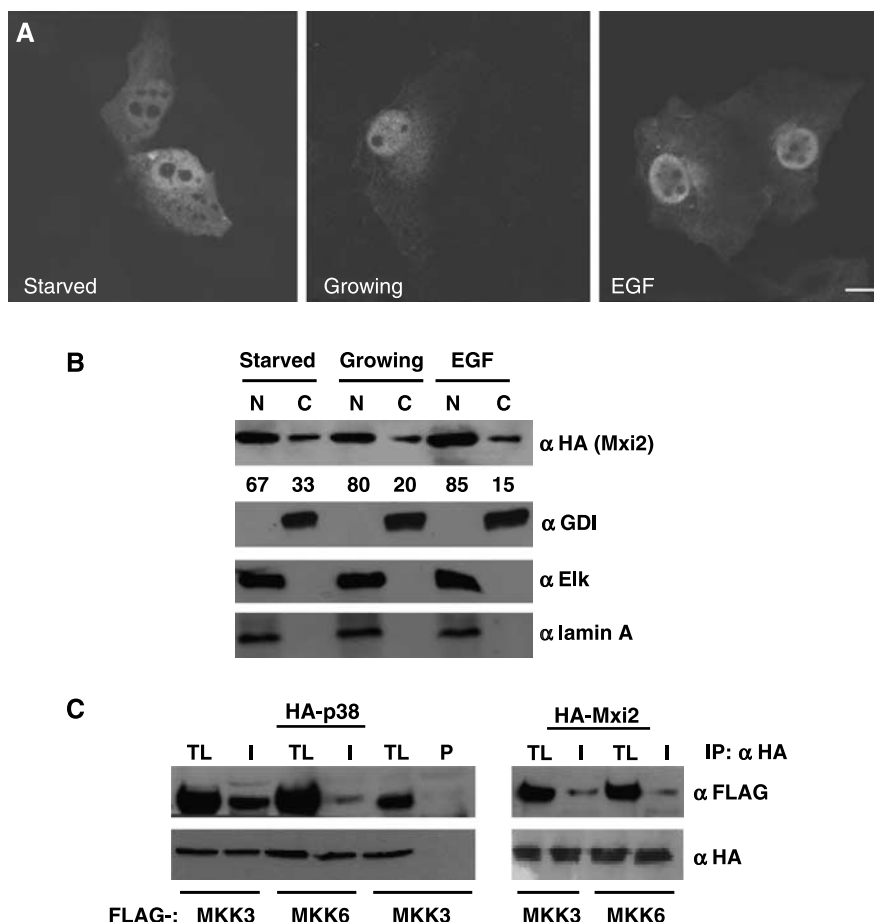


Figure 1 Subcellular localization of Mxi2. COS7 cells transfected with HA-Mxi2 (1 µg), serum-starved, growing or stimulated with EGF (100 ng/ml, 5 min) were analyzed for Mxi2 distribution as follows: (A) Anti-HA immunofluorescence and confocal microscopy. Scale bar: 10 µm. (B) Nucleocytoplasmic fractionation: Determined by anti-HA immunoblotting in nuclear (N) and cytoplasmic (C) fractions. Figures show the percentage of Mxi2 present in each compartment. The absence of cross-contamination in N and C extracts was ascertained using nuclear (Elk, Lamin A) and cytoplasmic (Rho-GDI) markers. (C) Interaction of Mxi2 with MAPKKs. Cells were transfected with HA-p38 or HA-Mxi2, plus FLAG-tagged MKK3 or MKK6 (1 µg each). Lysates were immunoprecipitated with anti-HA antibody (I) or preimmune serum (P) and probed for associated MKK 3 or 6. Protein levels were determined by anti-HA immunoblotting. TL = total lysate.

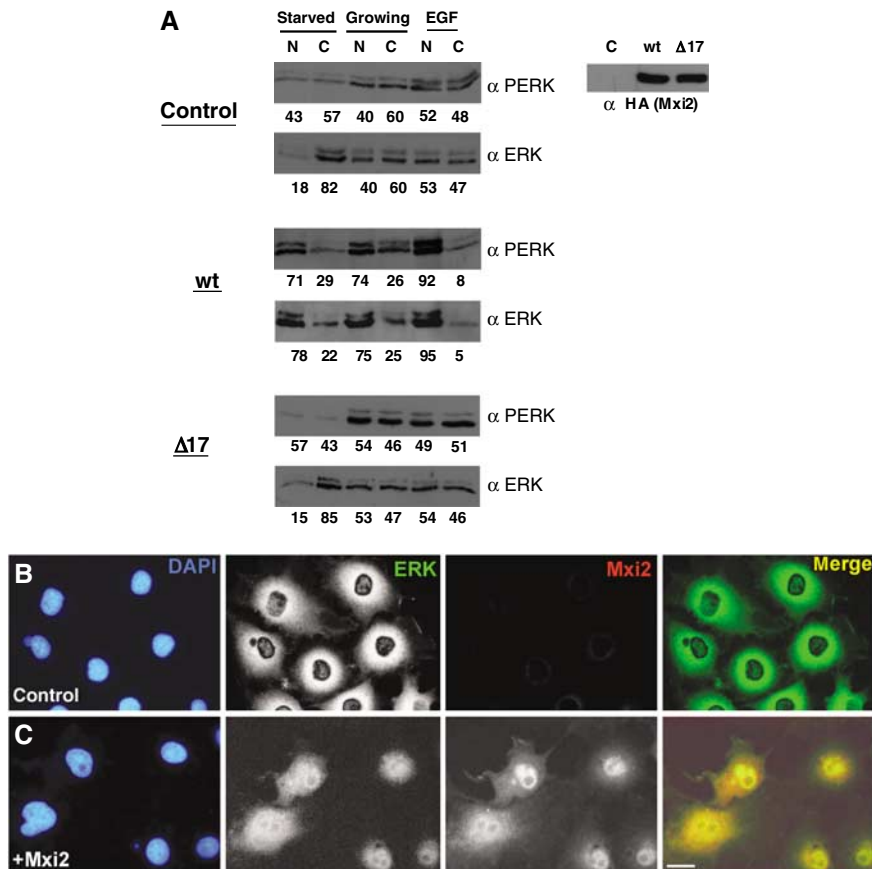


Figure 2 Mxi2 alters ERK1/2 nucleocytoplasmic distribution. (A) Lysates from COS7 cells transfected with vector (control) or HA-Mxi2, wild type and Δ17 (1 μg); starved, growing or EGF-stimulated (100 ng/ml, 5 min) were separated into nuclear (N) and cytoplasmic (C) fractions. Protein levels were analyzed by immunoblotting. Figures show the percentage of ERK1/2 present in each compartment. Right panel: expression levels of Mxi2 wild type and Δ17. (B, C) Mxi2 tethers ERK1/2 to the nucleus. Mxi2 and ERK1/2 distributions were determined by anti HA-Texas Red/ERK-FITC Red immunofluorescence in serum-starved (control) and HA-Mxi2-transfected cells. DAPI staining revealed nuclei. Scale bar: 10 μm.

the other hand, in cells transfected with Mxi2 Δ17, a mutant form that cannot bind ERK1/2 (Sanz-Moreno *et al*, 2003), the localization of total and phosphorylated ERK1/2 closely resembled that detected in control cells. This suggested that direct binding to Mxi2 was required for ERK1/2 redistribution. These observations were confirmed by immunofluorescence. In serum-starved control cells, ERK1/2 were pronouncedly cytoplasmic (Figure 2B). By contrast, in cells expressing Mxi2, ERK1/2 underwent a dramatic nuclear translocation where they markedly colocalized with Mxi2 (Figure 2C).

In MDCK cells, endogenous Mxi2 acts as a nuclear tether for ERK1/2

The above results had the limitations inherent to every ectopic expression system. Thus, it was essential to ascertain whether the same phenomena applied under physiological conditions. We generated an antibody that specifically recognized Mxi2 and, upon screening some cell lines, we found that the canine renal epithelium cell line MDCK endogenously expressed Mxi2 at high levels (Supplementary Figure 2). When we examined the nucleocytoplasmic distribution of endogenous Mxi2 in MDCK cells, it was found primarily at the nucleus, under starved and stimulated

conditions (Figure 3A), mirroring the results obtained by ectopic expression in COS7 cells.

We wished to know whether endogenous Mxi2 influenced ERK1/2 distribution. To do so, we downregulated its expression in MDCK cells by means of RNA interference. Because the canine sequence for the region encoding Mxi2 C-terminus, exon 11', had not been described, we sequenced it using DNA from MDCK cells. The resultant sequence (GenBank accession number DQ388883) had a 98% identity with the human one. Next, oligonucleotides encoding for an siRNA targeting Mxi2 at the region encoding amino acids 282–287 were cloned into a short-hairpin plasmid and stably expressed in MDCK cells. We obtained a pool of 10 clones named MDMx–, in which Mxi2 expression was undetectable and p38 were unaffected (Figure 3B). In cells expressing random control siRNAs, neither Mxi2 nor p38 expression were affected (data not shown). Next, we investigated the impact of Mxi2 downregulation on ERK1/2 distribution. In MDCK cells whether starved, growing or stimulated with EGF, both total and phosphorylated ERK1/2 were prominently nuclear, a pattern almost identical to that found in COS7 cells transfected with Mxi2. Conversely, in MDMx– cells, ERK1/2 were distributed more evenly between the nucleus and cytoplasm, and the amounts of nuclear, phosphorylated ERK1/2 were lower (Figure 3C), resembling

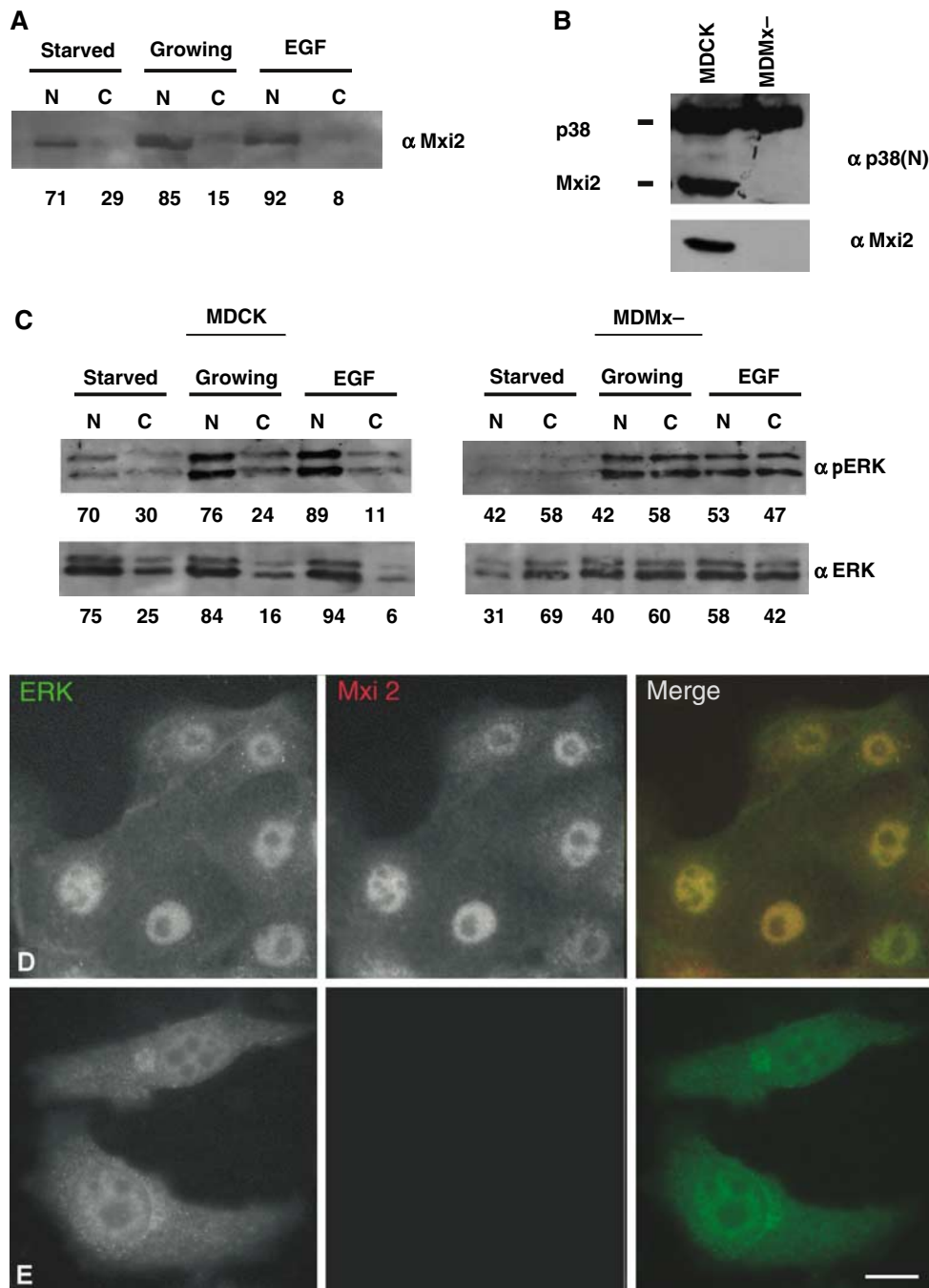


Figure 3 Mxi2 is expressed endogenously in MDCK cells. (A) Nucleocytoplasmic distribution of Mxi2: Lysates from cells starved, growing or stimulated with EGF (100 ng/ml, 5 min) were separated in nuclear (N) and cytoplasmic (C) fractions. Mxi2 distribution was determined by immunoblotting. Figures show the percentage of Mxi2 present in each compartment. (B) Down-regulation of Mxi2 expression in MDCK cells by RNAsi. Mxi2 protein levels in parental MDCK and in MDMx- cells, in which Mxi2 expression was suppressed, were determined by anti-Mxi2 and anti-p38 (N-terminal) immunoblotting. (C) Downregulation of Mxi2 alters ERK1/2 nucleocytoplasmic distribution. Lysates from MDCK and MDMx- cells, starved, growing or stimulated with EGF, were separated into nuclear (N) and cytoplasmic (C) fractions. Figures show the percentage of ERK1/2 present in each compartment. Distribution of endogenous Mxi2 and ERK1/2 in (D) MDCK cells and (E) MDMx- cells, as determined by anti ERK-FITC/Mxi2-Texas Red immunofluorescence. Scale bar: (G) 15 μ m; (H) 10 μ m.

parental COS7 cells. These results were confirmed by immunofluorescence. In parental MDCK cells, Mxi2 was markedly nuclear, excluded from nucleoli and mainly found at the nucleoplasm, where it profusely colocalized with ERK1/2 (Figure 3D). On the other hand, in MDMx- cells, suppression of Mxi2 expression resulted in a decrease in the nuclear ERK1/2, which became more uniformly distributed throughout the cell (Figure 3E). These results indicated that Mxi2,

whether ectopic or endogenous, was capable of profoundly altering ERK1/2 distribution by promoting its localization to the nucleus.

It was important to gain a notion of the physiological consequences of ERK1/2 nucleocytoplasmic redistribution resulting from Mxi2 downregulation. To this end, we compared the growth kinetics of MDCK and MDMx- cells. In MDCK cells, the MEK inhibitor UO126 abrogated

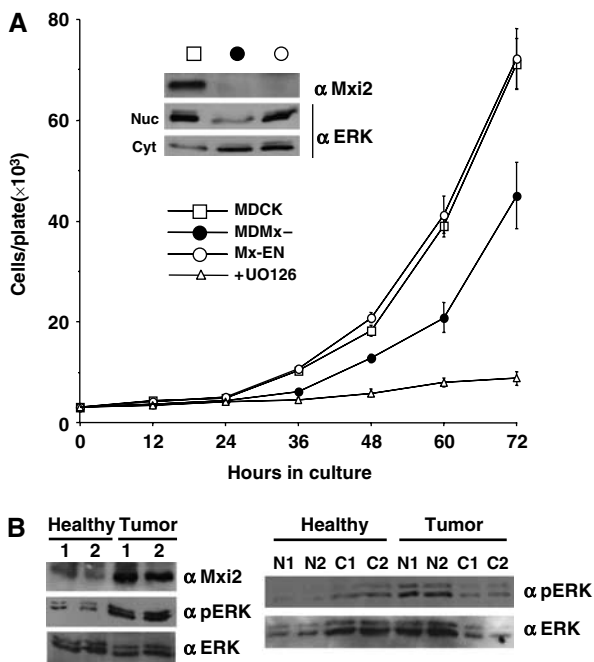


Figure 4 Mxi2 expression associated to cellular proliferation and transformation. (A) Downregulation of Mxi2 expression slows cellular proliferation. Proliferation rates of the cells lines indicated in the legend, growing in 10% calf serum. Where indicated, MDCK cells were grown in the presence of 2 μ M UO126. Results show average \pm s.e.m. of at least three independent experiments. Inset: Mxi2 expression and ERK1/2 nucleocytoplasmic distribution in the starved cell lines indicated by symbols. (B) Mxi2 expression and ERK1/2 distribution in renal tumors. Left panel: Lysates from two adenocarcinomas (tumor 1 and 2) and from healthy renal tissue of the same individuals (healthy 1 and 2) were analyzed by immunoblotting. Right panel: Lysates from the tumor and healthy samples were fractionated into nuclear (N) and cytoplasmic (C) fractions and analyzed by immunoblotting.

proliferation, indicating that this process was dependent on ERK activation (Figure 4A). Noticeably, the proliferation rate in MDMx- cells was reduced by nearly 40%. To verify that such a decrease on proliferative capacity was due to the drop on ERK1/2 nuclear levels and not to some unrelated Mxi2-mediated effect, we generated a cell line derived from MDMx- cells (Mx-EN) expressing an ERK2 harboring SV40 NLS at its C-terminus, to bolster its accumulation in the nucleus (Figure 3A, inset). It was found that in Mx-EN cells, the restoration of ERK nuclear levels rescued cellular proliferation up to levels similar to those exhibited by parental MDCK cells.

We then took an insight into how Mxi2 affected ERK distribution under pathological conditions. To this aim, we looked at several samples of renal adenocarcinomas. Interestingly, in the tumor samples, Mxi2 was markedly overexpressed and they possessed much higher phospho-ERK levels, as compared with healthy renal tissue from the same individuals (Figure 4B, left panel). When evaluating ERK1/2 distribution, it was found that in the tumors both total and phosphorylated ERK1/2 accumulated mainly at the nucleus, as opposed to healthy tissue in which they were mainly cytoplasmic (Figure 4B, right panel). These results verified that under pathological conditions, Mxi2 also acted as a nuclear tether for ERK1/2.

Mxi2 potentiates ERK1/2 binding to nuclear pore proteins

The mechanism whereby Mxi2 made possible the accumulation of ERK1/2 at the nucleus was explored. One possibility was that Mxi2 could be interfering with ERK1/2 nuclear export, thereby prolonging its residence therein. To test this hypothesis, as it has been suggested that ERK1/2 leave the nucleus associated to MEK (Adachi *et al*, 2000), we analyzed whether Mxi2 had an effect on MEK nucleocytoplasmic shuttling. We found that the expression of Mxi2 was incapable of altering MEK nucleocytoplasmic distribution in COS7 cells, neither under basal conditions (Figure 5A) nor under EGF stimulation (Supplementary Figure 3). In MDCK cells, in which, unlike COS7 cells, MEK is mainly located at the cytoplasm, downregulation of endogenous Mxi2 did not have any noticeable effect on MEK distribution either (Supplementary Figure 3).

Another possibility was that Mxi2 potentiated ERK1/2 nuclear entry. As it has been shown that ERK1/2 interact with the NPC to gain access to the nucleus (Matsubayashi *et al*, 2001; Whitehurst *et al*, 2002), we investigated if Mxi2 could impact this interaction. We expressed the C-terminus of nucleoporin 153 (Whitehurst *et al*, 2002) as a GST fusion and tested *in vitro* its binding to ERK1/2 in lysates from cells transfected with increasing concentrations of Mxi2. As shown in Figure 5B, ERK1/2 were found to bind to GST-Nup153c *per se*, as previously shown (Whitehurst *et al*, 2002). Interestingly, Mxi2 markedly augmented the amount of ERK1/2 bound to the nucleoporin. To gain a quantitative notion of this effect, increasing concentrations of purified ERK2 were allowed to bind *in vitro* to GST-Nup153c in the presence or absence of Mxi2 and bound ERK2 was recovered by affinity pull-down. As shown in Figure 5C, the affinity of ERK2 towards the nucleoporin ($K_d \sim 90$ nM) increased nearly two-fold ($K_d \sim 50$ nM) in the presence of Mxi2. Mxi2-mediated enhancement of ERK1/2 affinity for Nup 153 was dependent on the ability of Mxi2 to bind ERK1/2, because only wild-type Mxi2 could exert this effect. Contrarily, Mxi2 Δ 17 and E293A, two mutants defective for binding to ERK1/2 (Sanz-Moreno *et al*, 2003; our unpublished results), were unable to potentiate the interaction of ERK1/2 with the NPC protein, even though these mutants could themselves bind to Nup153c as effectively as wild-type Mxi2 (Figure 5D). These results demonstrate that Mxi2 itself can directly bind to the NPC, and that the presence of Mxi2 in Nup153 affinity pull-downs (Figure 5B) was not a consequence of its interaction with ERK but rather with the nucleoporin itself. To further substantiate this point, we evaluated Mxi2 *in vitro* binding affinity towards Nup153, following the same methodology described above. As shown in Figure 5E, Mxi2 displayed a remarkable affinity ($K_d = 58$ nM) towards this nuclear pore component.

Mxi2 antagonizes PEA15

It has been shown that the cytoplasmic protein PEA15 (Formstecher *et al*, 2001) interferes with ERK1/2 binding to the nuclear pore, thereby preventing their nuclear import (Whitehurst *et al*, 2004). As such, it was of interest to explore if Mxi2 could counteract PEA15 effects. As expected, expression of PEA15 in COS7 cells markedly prevented the accumulation of total and phosphorylated ERK1/2 in the nucleus upon stimulation with EGF. Conversely, when expressed together with Mxi2, PEA15 no longer interfered

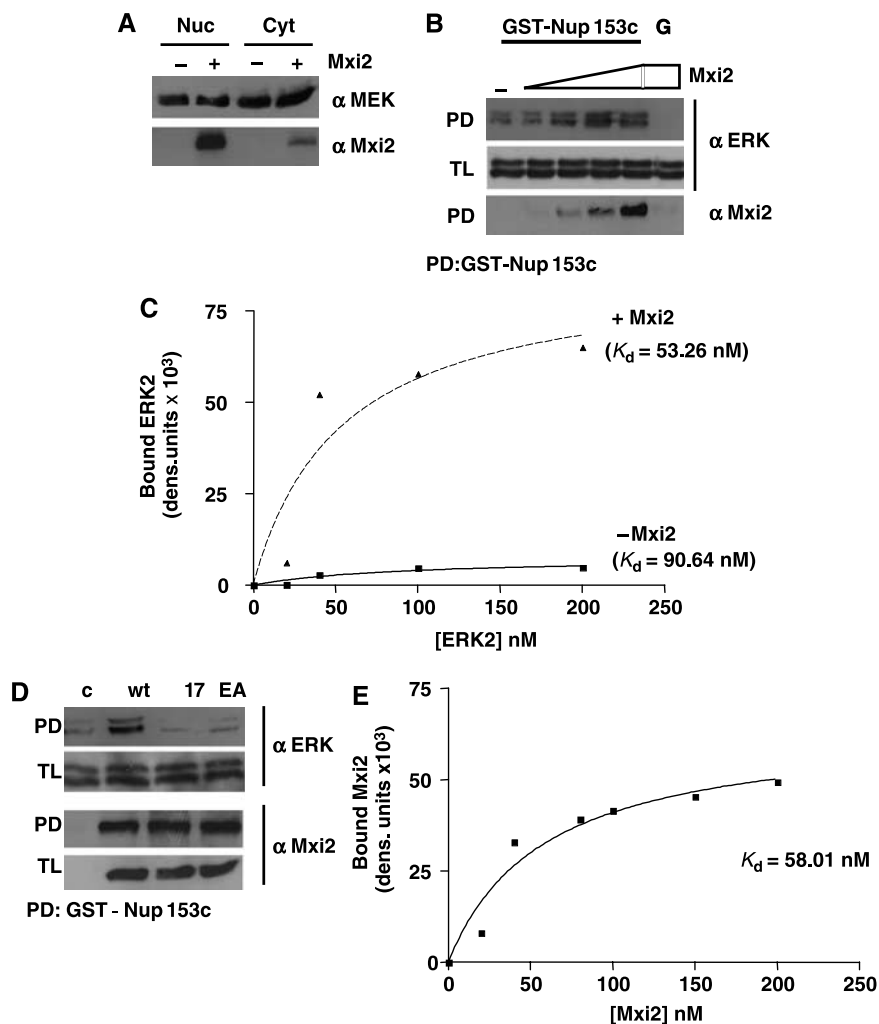


Figure 5 Mxi2 affects ERK1/2 nuclear import. (A) The expression of Mxi2 does not affect MEK distribution. Lysates from control (–) and Mxi2-transfected (+) COS7 cells were separated in nuclear and cytoplasmic fractions and MEK levels were analyzed by immunoblotting. (B) Mxi2 potentiates ERK1/2 interaction with nucleoporins. Lysates from COS7 cells transfected with increasing amounts (0.25–2 µg) of Mxi2 were incubated with GST Nup153c or GST (G) beads. ERK1/2 and Mxi2 levels in pull-downs were analyzed by immunoblotting. (C) Mxi2 increases ERK1/2 *in vitro* affinity for Nup 153c. Increasing concentrations of purified ERK2 were incubated with 200 nM GST-Nup153c, with or without 100 nM Mxi2 as shown. ERK2 bound to Nup153c was affinity pulled-down and quantified. Results show average of three independent experiments. (D) Mxi2 mutants defective for binding ERK1/2 do not promote ERK association to the NPC. Lysates from cells transfected with HA-Mxi2 wild-type (wt) and mutants Δ 17 and E293 (1 µg), were incubated with GST Nup153c beads. Proteins in the pull-downs were analyzed by immunoblotting. (E) Mxi2 binds Nup 153c *in vitro*. Increasing concentrations of purified Mxi2 were incubated with 200 nM GST-Nup153c. Bound Mxi2 was affinity pulled-down and quantitated. Results show average of three independent experiments. PD, affinity precipitates; TL, total lysates.

with ERK1/2 nuclear transit, neither under starvation nor after EGF stimulation (Figure 6A), suggesting that Mxi2 and PEA15 had antagonistic effects on ERK1/2 nuclear import, and that Mxi2 could overcome the inhibitory effects of PEA15. To extend this observation, we investigated whether Mxi2 and PEA15 would compete for binding to ERK1/2. PEA15 was expressed with AU5-tagged ERK2 in addition to increasing concentrations of Mxi2 and its presence in anti-AU5 immunoprecipitates was analyzed. It was found that the amount of PEA15 detected in association with ERK2 decreased concomitantly with the increasing appearance of Mxi2 in ERK2 complexes (Figure 6B), thus supporting the conclusion that Mxi2 competes with PEA15 to associate with ERK1/2.

It has been shown that PEA15 interacts with ERK2 through the Insert region (Whitehurst *et al*, 2004); so we asked if PEA15 and Mxi2 shared this common binding site for binding to ERK1/2. To this aim, we coexpressed Mxi2 with an ERK2 Insert region deletion mutant (Δ 241–272) (Whitehurst *et al*,

2004) and assayed their ability to complex. It was found that ERK2 Δ 241–272 associated with Mxi2 to a much lesser extent than wild-type ERK2 (Figure 6C), suggesting that the Insert region was an essential site for Mxi2 binding to ERK2. The possibility existed that the deletion of the whole Insert region introduced gross conformational changes on ERK2 so as to perturb Mxi2 binding. To rule out this possibility, we tested the ability of Mxi2 to bind to an ERK2 point mutant within this region, Y261N, previously shown to be defective for binding to PEA15 (Whitehurst *et al*, 2004). As shown in Figure 6C, Mxi2 association to this ERK2 mutant form was remarkably impaired. These experiments demonstrated that Mxi2 and PEA15 shared a common ERK2-binding site.

Mxi2 is a stimulus-independent nuclear shuttle for ERK1/2

As Mxi2 was able to facilitate ERK1/2 binding to the NPC, we asked if, by this mechanism, Mxi2 would be sufficient to

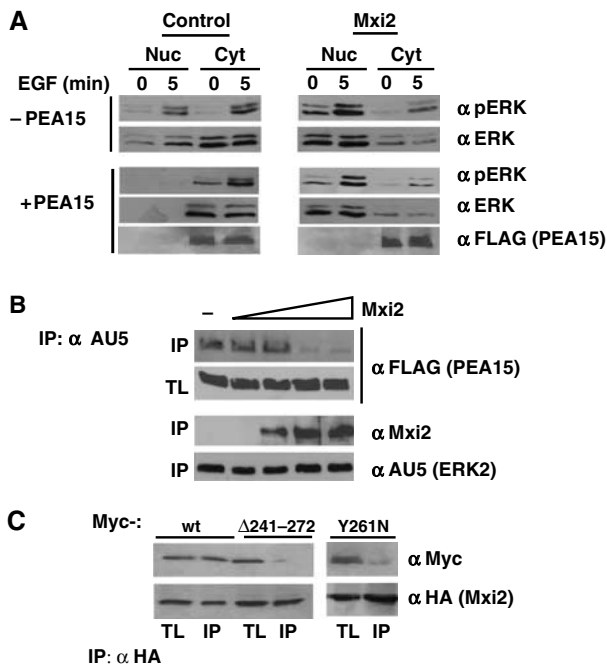


Figure 6 Mxi2 antagonizes PEA15. (A) Mxi2 counteracts PEA15-mediated blockade of ERK1/2 nuclear translocation. Vector (control) and HA-Mxi2 were transfected, with or without FLAG-PEA15, into COS7 cells. After starvation and stimulation with EGF where indicated, lysates were separated into nuclear and cytoplasmic fractions. Protein levels were analyzed by immunoblotting. (B) Mxi2 outcompetes PEA15 for binding to ERK1/2. COS7 were transfected with AU5-ERK2 and FLAG-PEA15 (1 μg each) along with increasing concentrations of Mxi2 (0.25–2 μg). PEA15 and Mxi2 in anti-AU5 immunoprecipitates were determined by immunoblotting. (C) Mxi2 requires the Insert region for binding to ERK2. HA-Mxi2 was cotransfected into COS7 cells with Myc-tagged ERK2 wild type, the Insert region-defective mutant Δ241–272 or the point mutant Y261A (1 μg each). ERK2 in anti-HA immunoprecipitates was determined by immunoblotting. TL, total lysates; IP, immunoprecipitates.

drive ERK1/2 into the nucleus in the absence of additional stimuli known to promote ERK1/2 nuclear translocation. To this end, we utilized a conditional vector in which Mxi2 expression was regulated by the zinc-inducible metallothionein promoter. Once transfected into COS7 cells and after serum starvation, ZnSO₄ was added to induce the expression of Mxi2, which was apparent after 12 h. As expected, the majority of the newly expressed Mxi2 accumulated in the nucleus (Figure 7A). Noticeably, concomitant with the appearance of Mxi2, ERK1/2 underwent a dramatic redistribution, translocating from the cytoplasm to the nucleus. On the other hand, the expression of Mxi2 E293A did not affect ERK1/2 distribution, in spite of itself being able to enter the nucleus. The possibility existed that Mxi2 could promote ERK1/2 nuclear entry by inducing the expression of some growth factor and thereby, an autocrine loop that would induce ERK nuclear import. However, conditioned media from Mxi2-transfected COS7 cells and from MDCK cells failed to stimulate ERK1/2 nuclear translocation (data not shown), suggesting that this was not the case. These results clearly supported the notion that Mxi2 could suffice to promote the transport ERK1/2 to the nucleus in the absence of further stimulation, and that a direct interaction between Mxi2 and ERK1/2 was essential for this process.

To confirm that Mxi2-induced ERK1/2 nuclear translocation proceeded through the nuclear pore, we utilized wheat

germ agglutinin (WGA), which binds to *N*-acetylglucosamine residues on nucleoporins, thus preventing all transit that requires interactions with the nuclear pore (Finlay *et al*, 1987). Treatment with WGA completely abrogated nuclear entrance of both Mxi2 and ERK1/2 (Figure 7B). To implicate an active transport mechanism in the import of the Mxi2-ERK complex, we utilized the GTPase-deficient mutant Ran Q69L that inhibits active nuclear transport in a dominant-negative manner (Adachi *et al*, 1999). The presence of Ran Q69L completely inhibited the nuclear accumulation of ERK1/2 triggered by Mxi2 expression (Figure 7C). Neither treatment with WGA nor expression of Ran Q69L affected Mxi2 binding to ERK1/2 (Supplementary Figure 4). These results are consistent with the idea that the Mxi2-ERK complex entered the nucleus by a mechanism that required Ran-mediated active transport. The nuclear entry of Mxi2 itself was also blocked, suggesting that its nuclear influx also occurs by an active import mechanism.

The fact that Mxi2 could induce ERK1/2 nuclear translocation in the absence of stimulation implied that ERK1/2 transported into the nucleus was an unphosphorylated state. To verify that Mxi2 was indeed capable of promoting nuclear import of unphosphorylated ERK1/2, we utilized an ERK2 mutant (AEF) in which the phosphoacceptor threonine and tyrosine had been mutated to alanine and phenylalanine respectively, rendering it unphosphorylatable (Wolf *et al*, 2001). Upon cotransfection into COS7 with the Zn-inducible Mxi2 construct, it was found that Mxi2 could evoke the nuclear translocation of ERK2-AEF just as efficiently as that of wild-type ERK2 (Figure 7D), demonstrating that Mxi2 was capable of inducing the nuclear translocation of unphosphorylated ERK1/2.

It has been shown that phosphorylated ERK1/2 forms dimers, and it is likely that dimerization plays a role in their nuclear translocation (Khokhlatchev *et al*, 1998). Thus, it was interesting to test whether Mxi2-dependent nuclear translocation required ERK1/2 dimerization. For this, we utilized the ERK2 mutant H176 L₄A (HL), defective for dimerization (Khokhlatchev *et al*, 1998). It was found that Mxi2 co-immunoprecipitated with ERK2-HL (Supplementary Figure 5), indicating that Mxi2 could form a complex with monomeric ERK2. Moreover, upon cotransfecting ERK2-HL with the Zn-inducible Mxi2 construct in COS7 cells, we found that Mxi2 could efficiently promote nuclear translocation of ERK2-HL (Figure 7E), thus demonstrating that dimerization was not a requirement for Mxi2-mediated ERK1/2 nuclear translocation.

As it has been suggested that in the absence of stimulation, ERK1/2 are retained in the cytoplasm largely as a result of their interaction with cytoplasmic anchors such as MEK1 (Fukuda *et al*, 1997b), it was conceivable that a mechanism whereby Mxi2 could induce stimulation-independent ERK1/2 nuclear translocation could be by liberating ERK1/2 from their cytoplasmic partners. To test this possibility, we analyzed if Mxi2 could release ERK1/2 from their complex with MEK. Increasing concentrations of Mxi2 were expressed in COS7 cells and the amounts of ERK1/2 associated with MEK were evaluated. As shown in Figure 7F, in spite of the gradual increase on Mxi2 cellular concentration, the levels of ERK1/2 in association with MEK were unaffected. This result supported the notion that Mxi2 did not affect ERK-MEK complex stability.

Reconstitution in a permeabilized cell system was then used to examine the effect of Mxi2 on individual steps in

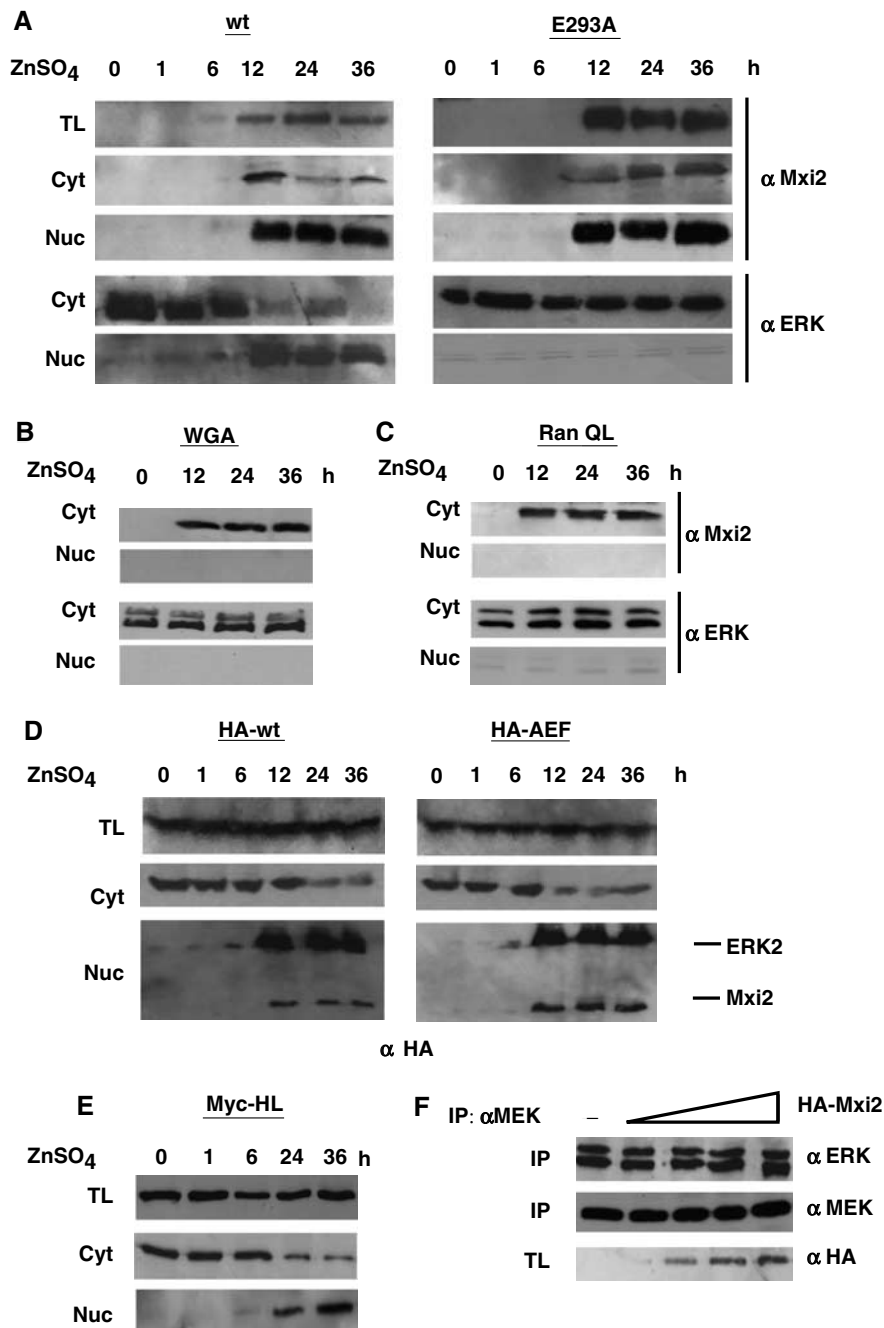


Figure 7 Mxi2 is a nuclear shuttle for ERK1/2. **(A)** Mxi2 expression is sufficient to promote ERK1/2 nuclear translocation. COS7 cells were transfected with Zn-inducible vectors expressing Mxi2 wild type (wt) and E293A. After 18 h of serum starvation, 75 μ M ZnSO₄ was added and cells were incubated for the indicated periods. Lysates were separated into nuclear and cytoplasmic fractions and protein levels were analyzed by immunoblotting. **(B)** Effects of WGA on the nuclear import of the ERK-Mxi2 complex. Cells were transfected with the Zn-inducible Mxi2. After starvation, ZnSO₄ was added. WGA (40 μ g/ml) was added after 6 h. Lysates were fractionated and protein levels were analyzed by immunoblotting. **(C)** Effects of a Ran inhibitory mutant on the nuclear import of the ERK-Mxi2 complex. Cells were transfected with the Zn-inducible Mxi2 and a vector expressing Ran Q69L (1 μ g). After starvation, ZnSO₄ was added. Lysates were fractionated and protein levels were analyzed. **(D)** Mxi2 promotes nuclear entry of unphosphorylated ERK1/2. COS7 cells were transfected with the HA-Mxi2 Zn-inducible vector in addition to plasmids encoding for HA-ERK2 or HA-ERK2 AEF (1 μ g each), and MEK (1.5 μ g) to serve as a cytoplasmic anchor. After starvation, ZnSO₄ was added. Lysates were fractionated and protein levels were analyzed by anti-HA immunoblotting. **(E)** Mxi2 translocates monomeric ERK2 into the nucleus. Cells were cotransfected with the Mxi2 Zn-inducible vector plus Myc-tagged ERK2 H176 L₄A dimerization-defective mutant (HL) (1 μ g). After starvation, ZnSO₄ was added. Lysates were fractionated and analyzed by anti-Myc immunoblotting. **(F)** Mxi2 cannot displace ERK1/2 from ERK-MEK complexes. Cells were transfected with increasing concentrations of HA-Mxi2 (0.25–2 μ g). Endogenous ERK1/2 present in anti-MEK immunoprecipitates was determined by immunoblotting. IP, immunoprecipitates; TL, total lysates.

the nucleocytoplasmic shuttling of unphosphorylated ERK2. Permeabilized cells lose most transport factors and Ran; thus, a complementation assay with cytosol as the source of transport factors was used to investigate ERK2 nuclear

uptake and export. In the absence of cytosol and energy, GFP-ERK2 was imported in the *in vitro* import assay as previously shown (Matsubayashi *et al*, 2001; Whitehurst *et al*, 2002). Under these same conditions, addition of in-

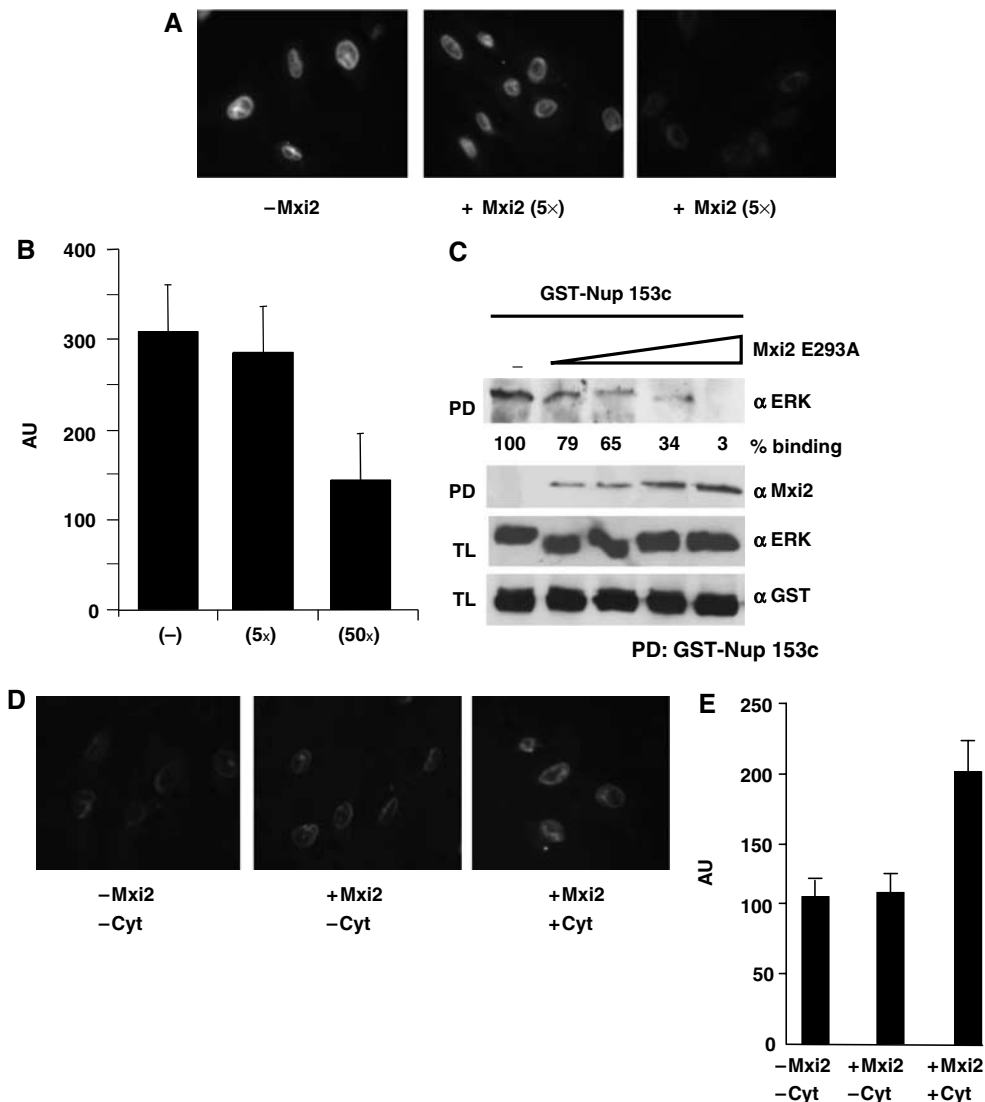


Figure 8 Effects of Mxi2 on ERK2 nuclear import/export in the digitonin permeabilization *in vitro* nuclear import assay, in BJ fibroblasts. (A) Import of GFP-ERK2 is not increased in the absence of cytosol for the import of Mxi2. Nuclear import of GFP-ERK2 (1.5 μ g) was performed in transport buffer without cytosol or energy, plus or minus two different concentrations of Mxi2 as shown. (B) Quantification of two independent experiments (average \pm s.d.). (C) Mxi2 outcompetes ERK1/2 for binding to nucleoporins. Lysates from COS7 cells transfected with Mxi2 E293A (0.25–2 μ g) were incubated with GST Nup153c. Affinity precipitates (PD) and total lysates (TL) were analyzed by immunoblotting. Figures show percentage ERK binding relative to the value in control cells. (D) Nuclear ERK2 levels increase when Mxi2 is imported into the nucleus in the presence of cytosol and energy. Where indicated, Mxi2 (1.5 μ g) was first incubated under import conditions for 15 min, either with or without cytosol and energy. After washing, import of GFP-ERK2 was carried out for 15 min in the absence of cytosol and energy, followed by a 15-min export assay. (E) Quantification of three independent experiments (average \pm s.d.). AU, arbitrary units.

creasing concentrations of Mxi2 reduced the import of GFP-ERK2 (Figure 8A and B), suggesting that Mxi2 did not enter the nucleus without energy and transport factors. ERK2 import may have been reduced either due to binding to Mxi2 under circumstances in which Mxi2 could not translocate to the nucleus or due to Mxi2 competing with ERK2 for binding to nucleoporins. In order to clarify this point, we tested whether Mxi2 E293A could outcompete ERK1/2 from binding to nucleoporins, by analyzing the amounts of ERK1/2 that associated to GST-Nup153 in the presence of increasing concentrations of this ERK binding-defective Mxi2 mutant. It was found that the quantity of ERK1/2 bound to the nucleoporin markedly diminished, in parallel to the increase of Mxi2 E293A in the Nup153 affinity pull-downs (Figure 8C), thus demonstrating that Mxi2 and ERK1/2 competed for the same binding sites in Nup153.

Finally, to test the idea that ERK2 binding to Mxi2 was influencing the distribution of ERK2, GFP-ERK2 was imported into the nucleus with or without Mxi2, plus or minus cytosol and energy. The import mixture was removed and ERK2 was allowed to be exported for 15 min. It was found that more GFP-ERK2 remained in the nucleus of the cells that have had Mxi2 imported with cytosol (Figure 8D and E), supporting the idea that Mxi2 binds tightly enough to ERK2 to cause its retention in the compartment to which it is localized.

Discussion

In this study, we explored the regulation of ERK1/2 by Mxi2. We have found that Mxi2, both ectopically expressed and endogenous, localizes predominantly to the nucleus. Mxi2 accumulation in the nucleus is not due to its inability to interact with putative

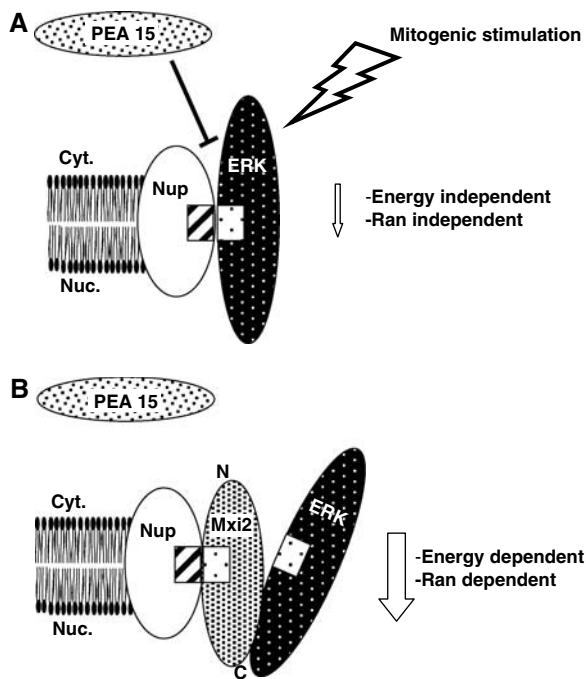


Figure 9 Model for Mxi2-mediated ERK1/2 nuclear translocation. As described in Discussion, (A) ERK1/2 nuclear translocation under mitogenic stimulation in the absence of Mxi2. (B) ERK1/2 nuclear translocation in the presence of Mxi2, without stimulation. Hatched boxes: ERK-binding sites in nucleoporins. Dotted boxes: homologous hydrophobic regions in Mxi2 and ERK1/2.

cytoplasmic anchors, as Mxi2 is capable of interacting with MKK 3 and 6. We have observed that upon stimulation, Mxi2 is enriched further in the nucleus, unlike p38 that exits the nucleus once activated (Ben-Levy *et al*, 1998). This difference may be due to Mxi2 being unable to interact with MAPKAP K2 (our unpublished results), which acts as a p38 nuclear exit shuttle (Ben-Levy *et al*, 1998). Maybe the inability of Mxi2 to couple to this or similar nuclear export mechanisms could contribute to its persistent nuclear localization.

Our results demonstrate that Mxi2 expression evokes a dramatic redistribution of ERK1/2 from the cytoplasm to the nucleus. This is highlighted when examining the endogenous proteins in MDCK cells, in which downregulation of Mxi2 by RNAi results in a marked drop in ERK1/2 nuclear localization. Furthermore, Mxi2 enhances the accumulation of total and phosphorylated ERK1/2 in the nucleus, even under starvation. Our findings suggest that Mxi2 binding to ERK1/2 hinders their interaction with deactivating phosphatases (Sanz *et al*, 2000; Sanz-Moreno *et al*, 2003; unpublished results). Our results herein hint that Mxi2 could be inhibiting ERK1/2 nuclear but not cytoplasmic phosphatases, since, even though about 20% of Mxi2 is localized at the cytoplasm, this fraction seems unable to affect the levels of phosphorylated ERK1/2 therein. One possible explanation could be that Mxi2 affinity for ERK1/2 is higher than that of nuclear phosphatases, but lower than the affinity exhibited by cytoplasmic phosphatases. Thus, only a high concentration of Mxi2 could bring about changes in ERK1/2 cytoplasmic signals.

Searching for the mechanisms underlying in Mxi2 effects on ERK1/2 nucleocytoplasmic distribution, we have found that Mxi2 markedly potentiates ERK1/2 binding to nucleo-

porin 153. This requires a direct interaction between Mxi2 and ERK1/2, as Mxi2 mutants defective for binding to ERK1/2 are unable to enhance either their association with nucleoporins or their translocation into the nucleus. It seems possible that this effect of Mxi2 contributes to enhance the nuclear entry of ERK1/2. However, *in vitro* import assays suggest that this effect is not sufficient in the absence of nuclear entry of Mxi2 itself. As such, the possibility of Mxi2 also serving as some sort of nuclear anchor for ERK1/2 should be considered. Another implication is that the interaction of Mxi2 with ERK2 may cause conformational changes in ERK2 that affect its capacity to interact with other proteins. Changes in interactions with phosphatases, for example, may be one manifestation of this effect.

It has been proposed that ERK1/2 bind to nucleoporins at the FFX motifs (Jacobs *et al*, 1999) present in many of these, through a hydrophobic region proximal to the activation lip (Lee *et al*, 2004; Whitehurst *et al*, 2004). We show that Mxi2 itself can directly bind to nucleoporin 153. The ERK1/2 hydrophobic residues thought to mediate the NPC interaction are conserved in p38 α /Mxi2. Thus, it is likely that Mxi2 binds to nucleoporins through this same region, although with a higher affinity than ERK1/2, ~60 vs ~200 nM (Whitehurst *et al*, 2002). In agreement, we demonstrate that Mxi2 can displace ERK1/2 from binding to nucleoporins. In support of this notion, using the *in vitro* nuclear import assay, we have observed that excess Mxi2 can prevent ERK2 nuclear import. Even though p38 α also possesses an FFX-binding region, it cannot promote ERKs nuclear translocation, probably owing to its low affinity towards ERK1/2, compared with Mxi2 (Sanz-Moreno *et al*, 2003).

The relevance of the influence of Mxi2 on ERK1/2 binding to the NPC is also highlighted by its antagonism with PEA15, which prevents ERK1/2 nuclear entry by interfering with ERK1/2 binding to nucleoporins (Whitehurst *et al*, 2004). Mxi2 can overcome this inhibition and promote ERK1/2 nuclear translocation even in the presence of PEA15. Mxi2 antagonistic effects on PEA15 can be explained by its ability to outcompete this protein for binding to ERK1/2, as expected from the reported *in vitro* binding affinities that Mxi2 and PEA15 display for ERK1/2: 20 nM (Sanz-Moreno *et al*, 2003) and 200 nM (Callaway *et al*, 2005), respectively. Along these lines, our results indicate that the association between Mxi2 and ERK2 is mediated through the Insert region, similar to PEA15 (Whitehurst *et al*, 2004), thus demonstrating that Mxi2 and PEA15 share a common binding site on ERK1/2. Interestingly, it has been proposed that PEA15 C-terminal 121–127 amino acids, whereby it binds to ERK1/2, constitute a reverse D domain, with a consensus sequence: R/K- ϕ_a -X_{3/4}- ϕ_b (where ϕ are Leu, Ile or Val) (Callaway *et al*, 2005). Noticeably, the Mxi2 C-terminus, which mediates its interaction with ERK1/2 (Sanz-Moreno *et al*, 2003), displays a very similar sequence: K- ϕ_a -X₅- ϕ_b .

ERK1/2 lack a canonical NLS and have not been found associated with importins (Adachi *et al*, 1999). Even though canonical ERK1/2 nuclear translocation can take place independently of NPC functionality and Ran (Adachi *et al*, 1999), our results show that Mxi2-mediated ERK1/2 nuclear transport is dependent on NPC integrity and this GTPase, as it is inhibited by WGA and a Ran inhibitory mutant. The requirement for energy for Mxi2-mediated transport is further

supported by our results showing that Mxi2 effects on ERK2 nuclear accumulation require cytosolic factors. Even though Mxi2 does not have an NLS either, at this point, it cannot be discarded that the Mxi2-ERK complex could somehow bind to other proteins that possess that motif, thereby facilitating further its entry to the nucleus.

An enhanced interaction with the NPC by the ERK-Mxi2 complex is likely to be the mechanism whereby Mxi2 promotes ERK1/2 nuclear accumulation. Another possibility worth considering is the blockade of ERK1/2 nuclear export. We have previously shown that Mxi2 sustains the levels of nuclear, phosphorylated ERK1/2 (Sanz-Moreno *et al*, 2003). As a consequence, the formation of ERK export complexes would be delayed and ERK1/2 would be retained for longer periods in the nucleus. However, our results clearly demonstrate that Mxi2 can induce the nuclear accumulation of ERK1/2 irrespective of their phosphorylation state.

Herein, we demonstrate that Mxi2 can promote ERK1/2 massive translocation into the nucleus, under starvation conditions, in the absence of any additional stimulation. Moreover, we have found that Mxi2 is capable of inducing the nuclear translocation of unphosphorylatable ERK2 mutants as efficiently as that of wild-type ERK2. Our data clearly show that ERK1/2 can be transported into the nucleus in an unphosphorylated form. This notion is further supported by our results showing that Mxi2 can promote the nuclear entry of monomeric ERK2. Thus, even though ERK1/2 phosphorylation can be sufficient to trigger their nuclear import in transformed cells (Lenormand *et al*, 1993), such an event does not appear not be an essential requirement and, under the right circumstances, unphosphorylated ERK1/2 can also be translocated into the nucleus. Our results point to Mxi2 as a unique protein in retaining unphosphorylated ERK1/2 in the nucleus.

Even though the biological rationale that underlies the stimulus-independent nucleocytoplasmic distribution of ERK1/2, as undertaken by Mxi2, is largely unknown, we demonstrate that altering such an event can have profound physiological implications, as evidenced by its effects on cellular proliferation and by the association of Mxi2 overexpression and ERK1/2 redistribution to renal tumors.

The currently accepted model for ERK1/2 distribution postulates that in the absence of stimuli, unphosphorylated ERK1/2 are retained at the cytoplasm through their interaction with MEK and other cytoplasmic anchors (Fukuda *et al*, 1997b; Blanco-Aparicio *et al*, 1999). It would be expected that in order to promote ERK1/2 nuclear entry without stimulation, Mxi2 should be able to withdraw ERK1/2 from their complexes with MEK. However, our results indicate otherwise. This would seem at odds with the weak affinity that MEK displays for ERK1/2 *in vitro* ($K_d = 10\text{--}15\ \mu\text{M}$) (Bardwell *et al*, 2001) compared with Mxi2. One likely explanation for this discrepancy could be that in physiological contexts MEK-ERK affinity is much greater, probably as a consequence of the stabilizing effect of other proteins, such as scaffolds. Another possibility could be that the presence of MEK-ERK scaffolds could pose some degree of structural hindrance for the interaction of ERK with Mxi2. On the other hand, a recent model suggests that ERK1/2 nucleocytoplasmic distribution would be primarily controlled by changes in the affinity of ERK1/2 towards its nuclear and cytoplasmic anchors and hints for the existence of significant amounts of uncomplexed

ERK1/2 in both compartments (Burack and Shaw, 2005; Callaway *et al*, 2005). Under such premises, Mxi2 could bring about ERK1/2 nuclear translocation by the recruitment of the uncomplexed cytoplasmic fraction, without significantly affecting the fraction bound to MEK.

In summary, we propose a model for Mxi2-mediated ERK1/2 nuclear translocation, as depicted in Figure 9: (a) In the absence of Mxi2 and under mitogenic stimulation, ERK1/2 translocate to the nucleus through the interaction with NPC, taking place between ERK-binding sites at nucleoporins and ERK1/2 hydrophobic residues. Such a process would occur independently of energy and without the participation of Ran and subject to inhibition by PEA15. (b) The presence of Mxi2 is sufficient to drive ERK1/2 into the nucleus, even without stimulation. Mxi2 constitutively binds to ERK1/2, this interaction taking place through the Mxi2 C-terminus and ERK1/2 Insert region. Hydrophobic residues are conserved in Mxi2, whereby it binds ERK-binding sites in nucleoporins, although with much higher affinity. Mxi2 transports ERK1/2 into the nucleus by a high-efficiency mechanism that is dependent on energy and Ran and is unaffected by PEA15. In this respect, we have unveiled Mxi2 as a unique protein in its role of promoting stimulus-independent ERK1/2 nuclear import.

Materials and methods

Plasmids

We have previously described the vectors encoding for HA-Mxi2, HA-Mxi2 Δ 17, FLAG-MKK3 and FLAG-MKK6 and GST-Mxi2, GST-p38 and GST-ERK2 (Sanz-Moreno *et al*, 2003); ERK2 H176 L₄A (Khokhlatchev *et al*, 1998); ERK2 Δ 241-272 and Y261N (Whitehurst *et al*, 2004). HA-ERK2 AEF (Wolf *et al*, 2001) was provided by R Seger, FLAG-PEA15 by H Chneiweiss and Ran Q69L by A Wittinghofer. Mxi2 E293A was generated by PCR site-directed mutagenesis. Zinc-inducible Mxi2 constructs were subcloned in MT CB6, driven by the sheep metallothionein promoter. To generate ERK2-NLS, SV40T antigen nuclear localization signal (PKKKRKV) was introduced by PCR directly downstream of ERK2 last codon.

Cell culture and transfection

COS7 and MDCK cells were grown in DMEM-10% FCS. Cells were transfected by the calcium phosphate technique (Sanz-Moreno *et al*, 2003). Wheat germ agglutinin and UO126 were from Sigma.

Clinical samples

Samples from renal tumors and corresponding healthy tissue were obtained directly from surgeries performed at the Department of Urology, Hospital Universitario Marqués de Valdecilla (Santander, Spain), in accordance with the center and the EEC Ethical Regulations.

Immunoblotting and immunoprecipitations

These were performed as described (Sanz-Moreno *et al*, 2003). Mouse monoclonals anti-HA, anti-Myc and anti-FLAG were from Babco. Mouse monoclonals anti-ERK1/2 and anti-phospho-ERK from Santa Cruz and Cell Signaling. Rabbit polyclonals anti-Rho-GDI, anti-MEK, anti-Lamin A and anti-p38 (C terminus) were from Santa Cruz. Rabbit polyclonal anti-Ran was from Dr JL Rosa (Barcelona). The anti-Mxi2 polyclonal antibody was generated as described (Esparis-Ogando *et al*, 2002), immunizing rabbits with a peptide for Mxi2 unique 17-amino-acid C-terminus.

Cellular proliferation assays

These assays were performed as described (Ajenjo *et al*, 2004).

Subcellular fractionations

These were performed as described (Ajenjo *et al*, 2004).

Nuclear Import assays

These assays were performed as described (Whitehurst *et al*, 2002).

Immunofluorescences

These were performed as described (Ajenjo *et al*, 2004).

In vitro protein binding assays

These assays were performed basically as described previously (Sanz-Moreno *et al*, 2003), using 200 ng of GST-Nup 153c. Bound ERK was quantitated by densitometry, using the program NIH Image 1.61. Analyses of the results were performed and plotted using the program GraphPad Prism 3 (GraphPad Software Inc.).

Mxi2 knockdown

The region comprising exon 11' of canine p38 was sequenced by PCR using DNA from MDCK cells. Flanking primers annealing to exons 11 and 12 were designed based on the p38 canine sequence (GenBank #AF003597). A sense-antisense 19-base oligonucleotide, targeting Mxi2 at nucleotides 888–907 (amino acids 282–287), separated by a hairpin, was cloned into pSUPER retro and

transfected into MDCK. After selection in puromycin for 3 weeks, clones were selected by virtue of their decreased Mxi2 expression levels. The sequences of the inhibitory and control oligonucleotides are available upon request.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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