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The putative tumor suppressor RASSF1A homodimerizes and heterodimerizes with the Ras-GTP binding protein Nore1

Sara Ortiz-Vega[1,2,3,5], Andrei Khokhlatchev[1,2,3,5], Maria Nedwidek[1], Xian-feng Zhang[1], Reinhard Dammann[4], Gerd P Pfeifer[4] and Joseph Avruch*[,1]

[1]*Diabetes Unit and Medical Services, Massachusetts General Hospital, Boston, Massachusetts, MA 02114, USA;* [2]*Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts, MA 02114, USA;* [3]*Department of Medicine, Harvard Medical School, Boston, MA 02114, USA;* [4]*Beckman Research Institute, City of Hope, 1500 E. Duarte Rd, Duarte, California, CA 91010, USA*

Nore and RASSF1A are noncatalytic proteins that share 50% identity over their carboxyterminal 300 AA, a segment that encompasses a putative Ras-Rap association (RA) domain. RASSF1 is expressed as several splice variants, each of which contain an RA domain, however the 340 AA RASSF1A, but not the shorter RASSF1C variant, is a putative tumor suppressor. Nore binds to Ras and several Ras-like GTPases in a GTP dependent fashion however neither RASSF1 (A or C) or the *C. elegans* Nore/RASSF1 homolog, T24F1.3 exhibit any interaction with Ras or six other Ras-like GTPases in a yeast two-hybrid expression assay. A low recovery of RASSF1A (but not RASSF1C) in association with RasG12V is observed however on transient expression in COS cells. Nore and RASSF1A can each efficiently homodimerize and heterodimerize with each other through their nonhomologous aminoterminal segments. Recombinant RASSF1C exhibits a much weaker ability to homodimerize or heterodimerize; thus the binding of RASSF1C to Nore is very much less than the binding of RASSF1A to Nore. The association of RASSF1A with RasG12V in COS cells appears to reflect the heterodimerization of RASSF1A with Nore, inasmuch the recovery of RASSF1A with RasG12V is increased by concurrent expression of full length Nore, and abolished by expression of Nore deleted of its RA domain. The preferential ability of RASSF1A to heterodimerize with Nore and thereby associate with Ras-like GTPases may be relevant to its putative tumor suppressor function.
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Introduction

Ras is a small GTP-binding protein involved in many cellular processes including proliferation, differentiation and apoptosis (Shields *et al*., 2000). This small GTPase exists in two conformations, a GTP-bound active state and a GDP-bound inactive state. This conformational change renders Ras able to interact with its downstream effectors, which are defined as proteins that specifically bind to GTP-bound form of Ras, and mediate some component of Ras' cellular actions. The best studied effectors are Raf (Avruch *et al*., 1994; Morrison and Cutler, 1997; Keikhoft and Rapp, 1998; Avruch *et al*., 2001), a serine-threonine kinase that controls the MEK-ERK pathway; PI-3 kinase (Rodriquez-Viciana *et al*., 1994, 1996, 1997) whose activity is required for activation of PKB; and the members of the Ral GEF family (White *et al*., 1996; Feig *et al*., 1996; Wolthuis and Bos, 1999) that control the activation state of the Ras-related protein Ral. Several noncatalytic proteins also interact with active Ras. These include AF-6 (Kuriyama *et al*., 1996), Rin-1 (Han *et al*., 1997), and Nore1 (Vavvas *et al*., 1998).

Nore1 was discovered as a protein that interacts specifically with active Ras in a two-hybrid screen, and was subsequently shown to bind Ras *in vivo* in response to EGF or serum-stimulation. Nore1 is a 413 amino acid protein that contains several PXXP motifs in its aminoterminal segment (AA 17–108), a central C1 zinc finger (AA118–165) and a Ras-Rap association domain (AA267–358) homologous to those in AF-6 and the Ral GDS family (Ponting and Benjamin, 1996). The closest mammalian homolog to Nore is the putative tumor suppressor RASSF1A, one of the longer (340AA) splice variants of the RASSF1 gene located on Chr 3p21 (Dammann *et al*., 2000; Lerman and Minna, 2000).

Recently, several additional Nore homologs have been reported, namely RASSF2, RASSF3 and AD037; the biologic functions of these polypeptides is not known.

The carboxyterminal 300 amino acids of Nore and RASSF1A, encompassing the zinc finger and RA domains, are approximately 50% identical (70%

*Correspondence: J Avruch, Diabetes Research Laboratory, Department of Molecular Biology, Massachusetts General Hospital, 50 Blossom Street, Boston, MA 02114, USA;
E-mail: avruch@helix.mgh.harvard.edu
[5]These authors contributed equally to this work
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similar) to each other in amino acid sequence; moreover this segment of each protein is about 40% identical to a central region of the 615AA *C. elegans* polypeptide T24F1.3. The considerable phylogenetic conservation of the amino acid sequence points to the likelihood that one or more conserved functions common to all three polypeptides is mediated by this segment. Inasmuch as all three polypeptides contain within this conserved segment a domain corresponding to that in the Ral GDS/AF-6 polypeptides that binds selectively to Ras GTP, and Nore has been shown to bind directly to Ras GTP, we inferred that the GTP-dependent binding of a Ras-like GTPase was probably the common conserved function. This view was reinforced by a recent report describing the binding of RASSF1C to Ras GTP *in vivo* and *in vitro* (Vos *et al*., 2000). In seeking to confirm this hypothesis however, we were surprised to find that neither RASSF1 or T24F1.3 exhibited significant binding to active mutants of Ras in a yeast two-hybrid assay. We therefore set out to compare the ability of Nore and RASSF1 to bind Ras-like GTPases, and to each other.

Results

Employing the yeast two-hybrid expression assay as described by Durfee *et al*. (1993) Nore, RASSF1A, RASSF1C and a fragment of T24 F1.3 encompassing amino acids 247 to 601 were coexpressed in *S. Cerevisiae* with each of seven Ras-like GTPases. The latter were modified by replacement of a Glycine in the GTP-binding domain so as to inhibit intrinsic GTPase activity, and by deletion of the carboxyterminal prenylation motifs, so as to facilitate nuclear transfer and avoid growth suppressive artifacts. The relative binding, estimated by assay of the activity of the induced beta galactosidase, is shown in Table 1. Nore associates most strongly with M-Ras/Rras3, Ki-Ras, R-Ras and Rap 2A, followed by TC21/R-Ras2. Ha-Ras and Rap1b exhibit comparable binding. No significant association with RalA was observed. Inasmuch as we have previously shown that endogenous Nore can be recruited to endogenous Ras in response to EGF (Vavvas *et al*., 1998), it is possible that the binding of Nore to other GTPases, e.g. M-Ras/R-Ras3 or R-Ras also occurs *in vivo*. We have confirmed that Nore binds to an active mutant of M-Ras/R-Ras3 during transient expression in mammalian cells (not shown).

In marked contrast to the ability of Nore to interact with several Ras-like GTPases, the Nore homologs RASSF1A and C, as well as T24F1.3 showed no induction of beta galactosidase that was significantly different than that seen in the negative controls, e.g. Ras vs. p53 (Table 1). The expression of the other Nore homologs was verified by HA-immunoblot of yeast extracts; RASSF1C and T24F1.3 are expressed at levels similar to the small GTPases, whereas the expression of Nore and RASSF1A is substantially lower (data not shown). Nevertheless, RASSF1A shows significant interaction with cloneA. Moreover, inadequate expression does not account for the inability of RASSF1A to interact with small GTPases as RASSF1A and RASSF1C share an identical RA domain, and RASSF1C, although well expressed, also lacks detectable binding to the Ras-like GTPases. Thus it appears that RASSF1 and T24F1.3 do not interact directly with the Ras-like GTPases examined.

In view of the prior detection of a RASSF1C/RasG12V interaction during transient expression in mammalian cells (Vos *et al*., 2000), we compared the ability of RasG12V to associate with Nore, RASSF1A, RASSF1C and T24F1.3 during transient expression in COS cells. As is evident in Figure 1, Nore associates strongly with RasG12V, and conversion to Ala of a set of amino acids (301–304 LKKF) in the Nore RA domain, that is well conserved in RASSF1 (LRKF) and T24F1.3 (LKKF) nearly eliminates the ability of Nore to bind RasG12V (Figure 1a); thus the binding of Nore to Ras-GTP is mediated through the Nore RA domain, as expected. In contrast to Nore, neither RASSF1 (A or C) or T24F1.3 exhibit detectable association with RasG12V (Figure 1a). In other experiments however, detectable recovery of RASSF1A, but never RASSF1C, with RasG12V could be observed (Figure 1b,c); thus whereas approximately 20% of coexpressed Nore is recovered in the Ha

Table 1 Interaction of Nore1 and its homologs with Ras-like GTPases in a yeast two-hybrid assay (values represent the mean ± s.d. of four experiments)

	Ha Ras	*Ki Ras*	*R-Ras*	*RRas2/TC21*	*MRas/RRas3*	*Rap1B*	*Rap2A*	*RalA*	*CloneA*
Nore	4.0 (±1.88)	7.8 (±3.12)	7.3 (±2.59)	5.8 (±2.1)	9.6 (±4.17)	3.0 (±1.15)	8.0 (±1.65)	1.3 (±0.58)	2.4 (±0.43)
RASSF1A	1.3 (±0.30)	1.0 (±0.54)	1.0 (±0.71)	1.4 (±0.35)	0.8 (±0.37)	1.1 (±0.46)	2.0 (±0.16)	0.8 (±0.70)	4.3 (±1.12)
RASSF1C	0.9 (±0.34)	1.1 (±0.59)	1.1 (±0.46)	1.0 (±0.19)	1.0 (±0.4)	1.2 (±0.68)	1.3 (±0.22)	1.4 (±0.16)	1.9 (±0.45)
T24F1.3	0.6 (±0.16)	1.0 (±0.6)	1.3 (±1.14)	1.0 (±0.11)	1.0 (±0.32)	0.7 (±0.56)	1.2 (±0.11)	1.0 (±0.47)	17.4 (±2.61)
Raf	19.5 (±5.3)	–	–	–	–	–	–	–	–
P53	0.8 (±0.11)	1.1 (±0.2)	1.2 (±0.59)	1.5 (±0.2)	0.9 (±0.55)	1.1 (±0.14)	1.5 (±0.17)	1.0 (±0.37)	1.3 (±0.24)

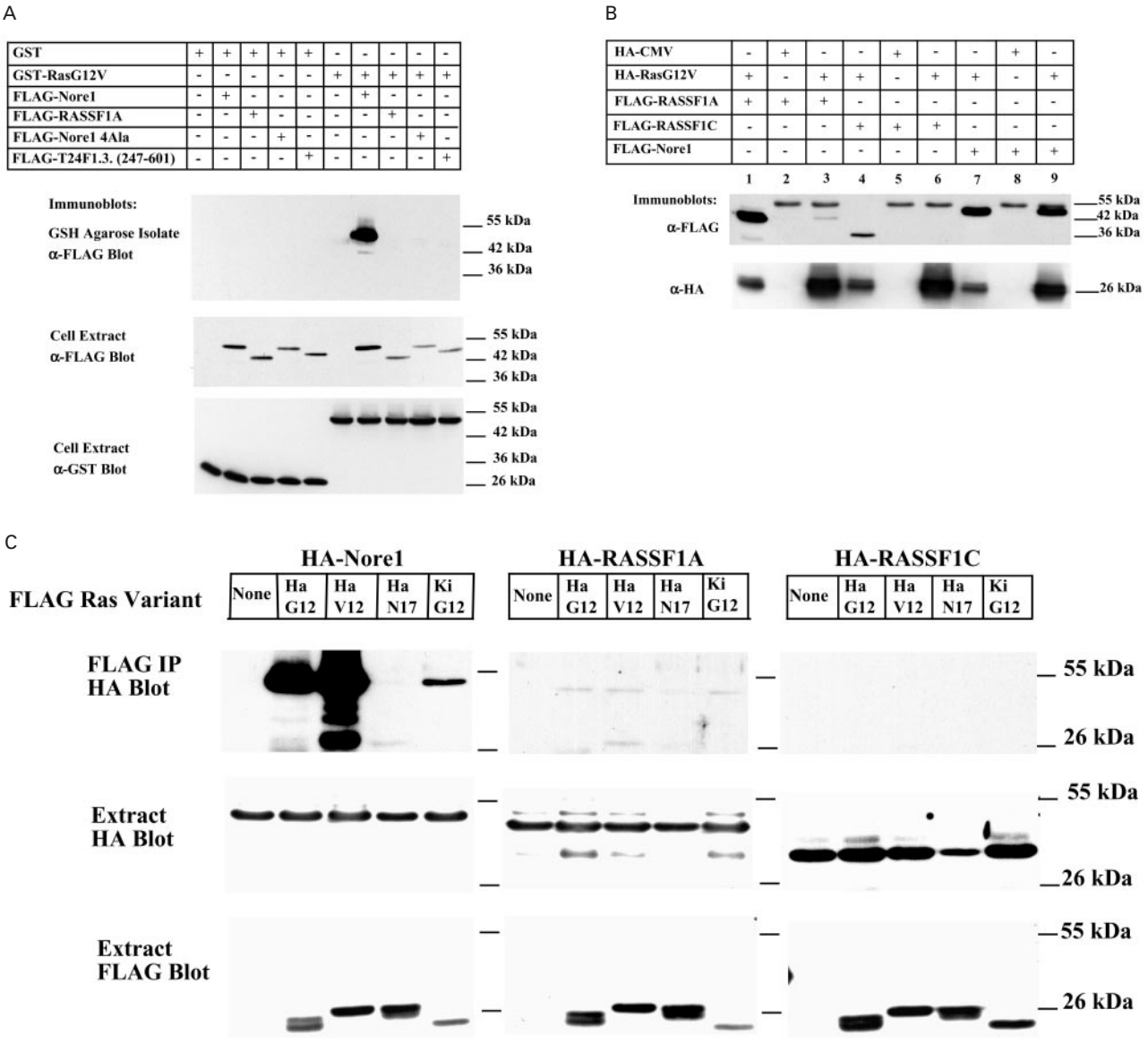


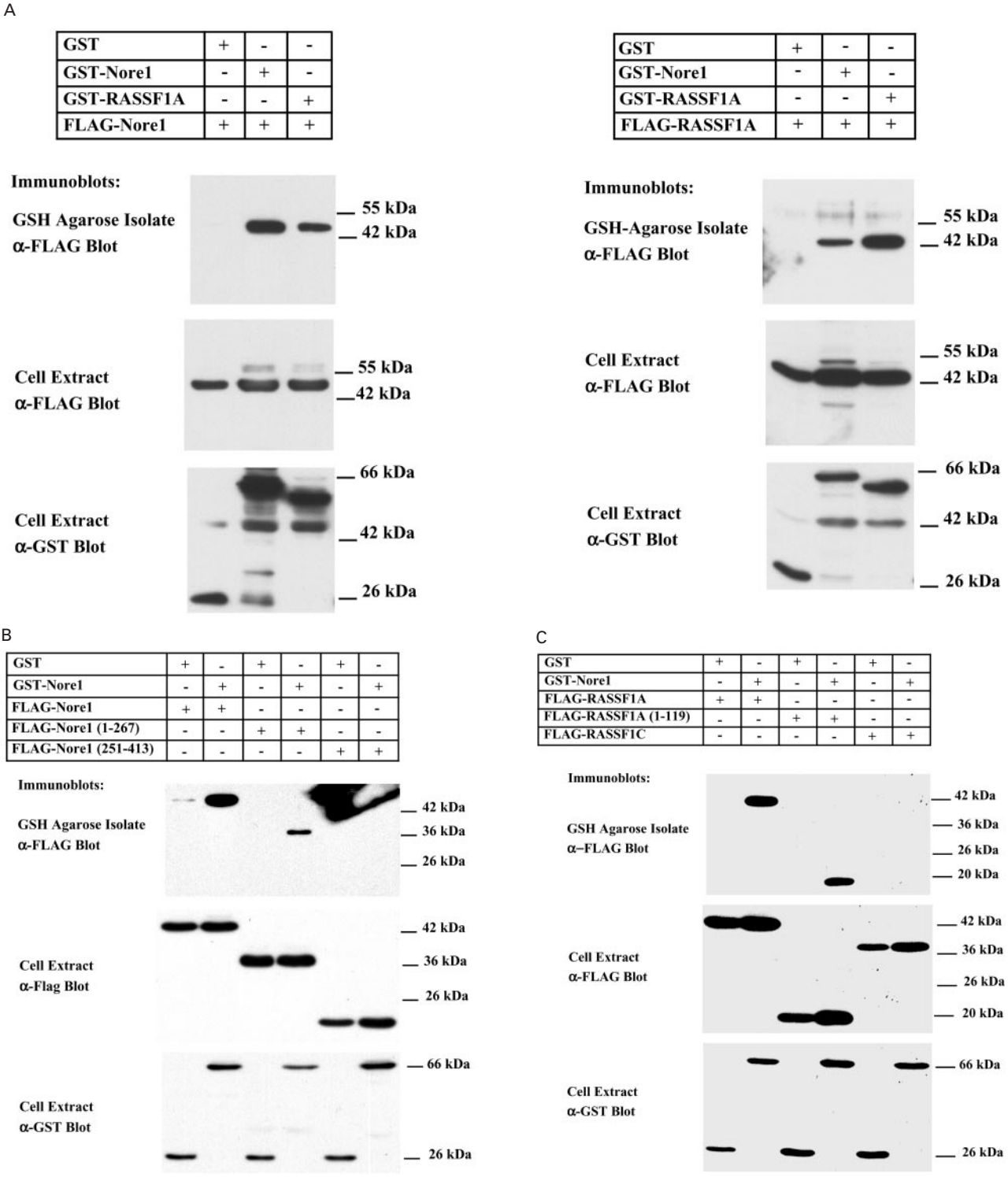
Figure 1 Association of Nore1 and its homologs to RasG12V *in vivo*. (**a**) Cos-7 cells, grown on 10 cm plates, were transfected with 7 μg of either pEBG (lanes 1–5) or pEBG-RasG12V (lanes 6–10) and 12 μg of FLAG-vector or FLAG-RA domain proteins, as indicated. Cells were lysed, GST-tagged proteins were isolated from cell extract and subjected to Western blot analysis as described in experimental procedures. Upper panel: proteins retained on GSH agarose beads were probed with anti-FLAG antibody. Middle panel: extract of lysed cells was probed for protein expression with anti-FLAG antibodies. Lower panel: extract of lysed cells was probed for protein expression with anti-GST antibodies. Nore1 4Ala, Nore1 mutant L301A, K302A, K303A, F304A. (**b**) Cos-7 cells grown in six-well dishes were transfected with either CMV-HA vector or CMV-HA -Ha RasG12V, FLAG-RASSF1 (A or C) (0.3 μg) or FLAG-Nore (0.3 μg). Cells were extracted 40 h after transfection and aliquots containing equal protein were subjected to anti HA immunoprecipitation. The FLAG blot of the HA immunoprecipitates shown in the upper panel (lanes 2,3,5,6,8 and 9); and the HA blot in the lower panel (lanes 1,4 and 7) contain 1% of the extract used in (lanes 3,6 and 9) respectively. (**c**) COS-7 cells grown in 6-well dishes were transfected with vectors encoding Ha-Nore (0.3 μg) or HA RASSF1A (0.3 μg) or Ha-RASSF1C (0.3 μg), each with 0.3 μg of CMV5-FLAG vector, or CMV5 FLAG encoding cHa-Ras, Ha-Ras G12V, Ha-Ras S17N or cKi-Ras. Cells were extracted 40 h after transfection. Aliquots of the extracts, matched for protein, were subjected to anti-FLAG immunoprecipitation. The washed FLAG IPs and aliquots of the extracts were subjected to immunoblot as indicated. The blot transfers of the FLAG IP were incubated with anti HA antibody and processed together, so that the relative recovery of the Ras variants with Nore, RASSF1A and RASSF1C shown in the upper panels is directly comparable

RasG12V immunoprecipitate, the recovery of RASSF1A is well under 2% (Figure 1b).

Zhang *et al*. (2001) recently presented compelling evidence that the wildtype allele of Ki Ras2 acts as tumor suppressor. Consistent with this idea, Ras S17N, a constitutively GDP-liganded mutant, has been shown to suppress the transforming activity of RasG12V (Stewart and Guan, 2000). Such an action of Ki-Ras might be explained if Ras-GDP were able to recruit an effector with tumor suppressor activity (e.g., RASS-

F1A). We therefore examined the ability of RASSF1A, RASSF1C and Nore to bind wildtype Ha-and Ki Ras, as well as the mutants G12V and S17N (Figure 1c). Nore binds strongly to the wildtype Ras polypeptides and especially to Ras G12V, but not at all to Ras S17N. RASSF1A exhibits barely detectable binding to wildtype Ha-and Ki-Ras and to RasG12V, but no binding Ras S17N. RASSF1C shows no detectable binding to any of the Ras variants (Figure 1c). Thus, RASSF1A exhibits no ability to bind Ras-GDP and is therefore unlikely to serve as a Ras-GDP effector.

In considering the mechanisms that might account for slight binding of RasG12V to RASSF1A but not RASSF1C in mammalian transfection experiments,



whereas neither RASSF1 isoform exhibits significant association with Ras G12V in a yeast two-hybrid assay, the hypothesis arose that RASSF1A might heterodimerize with a Ras-GTP binding protein, such as Nore1. We therefore examined whether the RASSF1 and Nore polypeptides are capable of homo- and heterodimerization. As shown in Figure 2 left, GST Nore (but not GST) binds FLAG Nore as well as FLAG RASSF1A; reciprocally GST RASSF1A (but not GST) binds both FLAG RASSF1A and FLAG

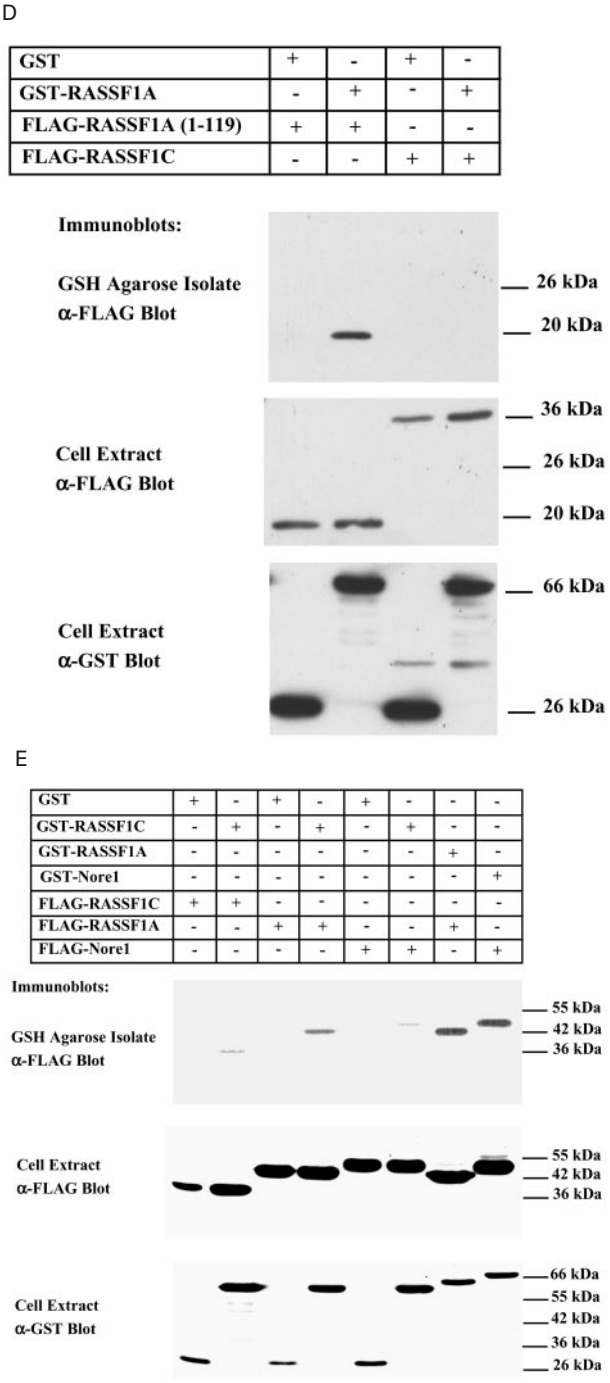


Figure 2 Dimerization of Nore1 and RASSF1A. cDNAs (0.3 μg) encoding Nore1 and RASSF1 variants in either CMV5-FLAG or pEBG-GST were transiently expressed in Cos-7 cells. GSH agarose was used to immunopurify GST-containing proteins. GSH-Agarose isolates were probed with FLAG-M2 monoclonal antibodies. (**a**) Association of FLAG-Nore (left) or FLAG-RASSF1A (right) with GST-Nore or GST-RASSF1A. (**b**) Nore homodimerization occurs through the Nore amino terminal segment. (**c**) RASSF1A heterodimerizes with Nore through its unique amino terminal segment. (**d**) RASSF1A homodimerizes through aminoterminal sequences not present in RASSF1C. (**e**) RASSF1C homodimerizes weakly in comparison to RASSF1A and Nore, and heterodimerizes weakly with Nore

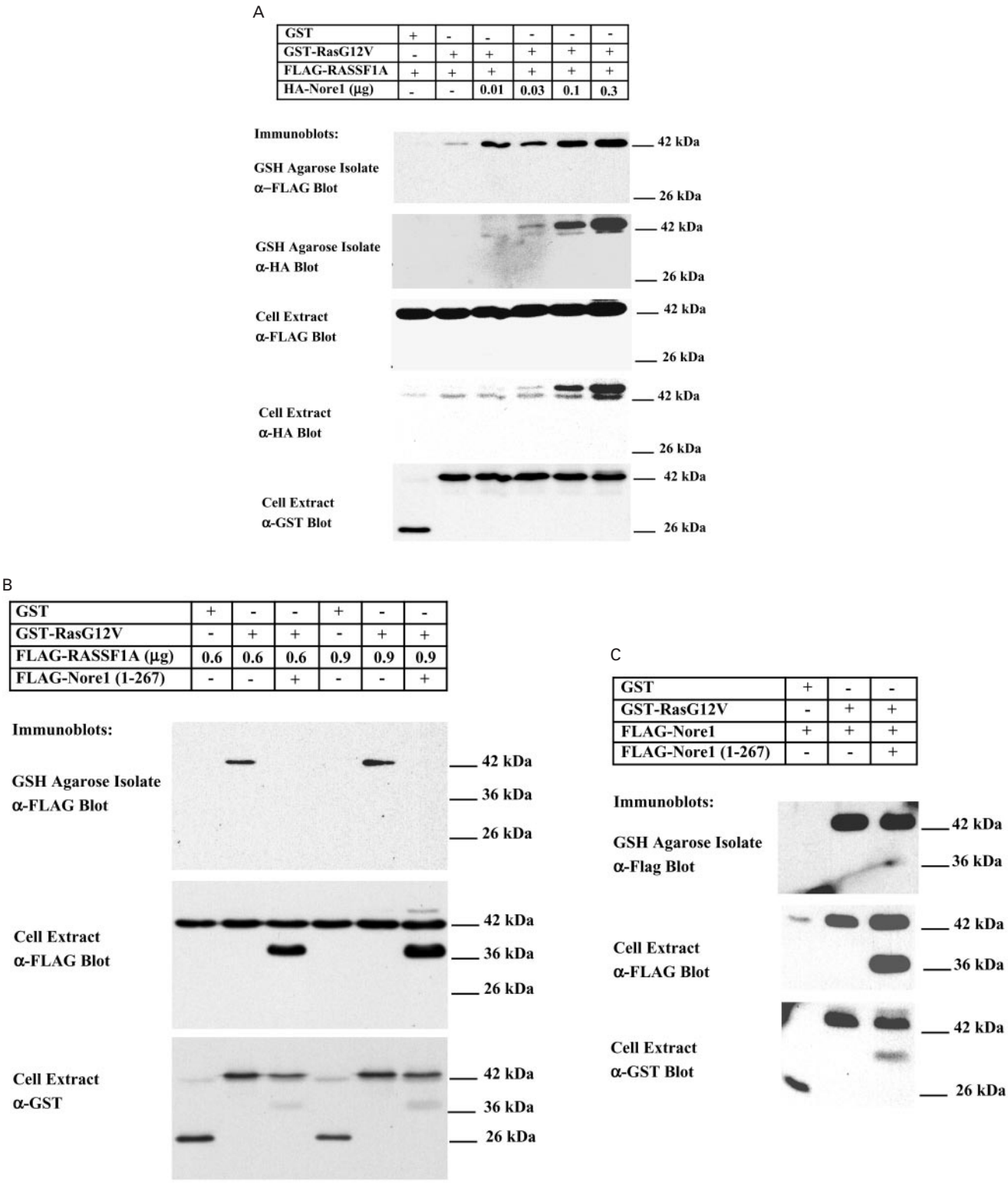


Figure 3 RASSF1A association with RasG12V in COS cells occurs through a Nore-RASSF1A heterodimer. (**a**) GST-RasG12V (0.3 μg) and FLAG-RASSF1A (0.3 μg) were expressed together with different amounts of HA-Nore1 cDNA in COS cells, as indicated. The recombinant GST-RasG12V was purified using GSH agarose, the GSH-agarose isolate was resolved by SDS–PAGE and subjected to immunoblot with anti-FLAG (top panel) and anti-HA (second panel) antibodies. The third, fourth, and the fifth panels are the blots of the cell extract to verify protein expression. (**b**) Different amounts of FLAG-RASSF1A cDNA were co-expressed with either RasG12V (0.3 μg) alone or RasG12V (0.3 μg) or/and FLAG-Nore1 (1–267) (0.4 μg). (**c**) cDNA encoding FLAG-Nore1 was transfected together with RasG12V alone or RasG12V and FLAG-Nore (1–267)

Nore (Figure 2a, right). The ability of Nore to dimerize resides in the Nore aminoterminal segment, amino acids 1–267; the carboxyterminal Nore segment (amino acids 251–413) does not associate with full length Nore (Figure 2b). The ability of Nore to bind RASSF1A occurs through the RASSF1A aminoterminal domain (amino acids 1–119) (Figure 2c). This segment is expressed exclusively in the RASSF1A isoform, and is not present in the RASSF1B or RASSF1C splice variants. The ability of RASSF1A to form homodimers also resides in this aminoterminal domain; thus full length RASSF1A binds RASSF1A (1–119), but not to RASSF1C in most experiments (Figure 2d). RASSF1C exhibits a much lesser ability to homodimerize as compared with RASSF1A or Nore (Figure 2e) and little (Figure 2e) or no (Figure 2c) ability to heterodimerize with Nore, especially as compared with RASSF1A (Figure 2c). Thus RASSF1A but not RASSF1C can readily form a dimer with the Ras-GTP binding protein, Nore.

If the ability of RasG12V to coprecipitate RASSF1A depends on the ability of RASSF1A to form heterodimers with an endogenous Ras-GTP binding protein such as Nore, the expression of additional amounts of recombinant Nore should promote the recovery of recombinant RASSF1A with RasG12V. The occurrence of such a facilitation is shown in Figure 3a; coexpression of increasing amounts of Ha-Nore with FLAG RASSF1A causes a progressive and substantial increase in the recovery of FLAG RASSF1A with RasG12V.

If the association of recombinant RASSF1A with RasG12V is dependent on the ability of RASSF1A to form heterodimers with endogenous Nore then overexpression of a truncated Nore fragment, that can compete with endogenous Nore for RASSF1A, but is unable to convey RASSF1A to RasG12V, should interfere with the ability of RASSF1A to associate with RasG12V. As shown in Figure 3b, coexpression of the Nore aminoterminal fragment (1–267) lacking the RA domain, abolishes completely the association of RASSF1A with RasG12V. By contrast, this Nore aminoterminal fragment does not interfere with the binding of full length Nore to RasG12V (Figure 3c). Based on the results shown in Figures 2 and 3, we conclude that RASSF1A associates with RasG12V in mammalian cells indirectly, through its ability to heterodimerize with a Ras-GTP binding protein, either Nore itself, or another Nore homolog capable of binding both RASSF1A and Ras-GTP.

Discussion

This report describes three novel findings: (1) Both Nore and RASSF1A are able to homodimerize as well as heterodimerize with each other whereas RASSF1C has very much weaker ability to homo- or heterodimerize. The dimerization of RASSF1A appears to require the RASSF1A aminoterminal 119AA which are not found in the B and C isoforms, and thus significant RASSF1 dimerization is probably restricted to splice variants that contain the segment encoded in exon 1a. (2) Neither RASSF1 (A or C) or its *C. elegans* homolog T24F1.3 is able to bind directly to any of seven Ras-like GTPases, although the ability of RASSF1A to heterodimerize with Nore may confer an indirect association with Ras/GTP *in vivo*. (3) Nore binds to several Ras-like GTPases in addition to Ha-Ras, especially members of the R-Ras branch of the subfamily. Moreover, although Nore binds a non-prenylated Ki RasG12V slightly better than Ha-RasG12V in a two-hybrid assay, wildtype cHa-Ras binds Nore better than cKi-Ras during transient expression in COS cells.

The function of Nore is not yet known, and consequently the functional significance of Nore dimerization is also unknown. Nevertheless, the ability of Nore to associate with Ras *in vivo* after serum stimulation indicates that Nore does mediate some aspect of Ras action. Moreover, inasmuch as Raf, a bona fide Ras effector, has been shown to occur as dimers (Luo *et al*., 1996), and to be activated by forced dimerization, and Ras-GTP activation of Raf *in vitro* appears to require Ras dimerization (Inouye *et al*., 2000), it is likely that Nore dimerization will also prove to be relevant to its function.

The RASSF1 gene is located on Chr 3p21 in a region that shows loss-of-heterozygosity (LOH) in nearly all small cell lung cancer (SCLC)-derived cell lines, a large fraction of NSCLC and several other human tumors (Dammann *et al*., 2000; Lerman and Minna, 2000). Damman *et al*. recently reported that the expression of the RASSF1A splice variant is selectively extinguished in nearly all SCLC derived cell lines examined, whereas the expression of RASSF1C isoform is unimpaired. Moreover, reintroduction of RASSF1A expression in SCLC lines reduces colony formation, growth in soft agar and tumorigenesis in nude mice, leading to the proposal that the RASSF1A isoform is (at least one of) the major tumor suppressor gene products encoded in this region.

Our inability to detect any direct interaction between RASSF1C and RasG12V contrasts with a recent report (Vos *et al*., 2000) describing the binding of RASSF1C to Ras-GTP in cell extracts and during transient expression in 293T cells. The latter experiments employed much higher amounts of RASSF1 cDNA and 10–30-fold higher ratios of RASSF1 to Ras DNA than used in the present experiments. Our results establish that the relative affinity for Ras of RASSF1 as compared to Nore must be very low, and we consider it unlikely that RASSF1 will interact directly with Ras-GTP *in vivo*. Conversely, the indirect and selective association of the RASSF1A isoform with Ras-GTP through RASSF1A heterodimerization with Nore may be relevant to the putative tumor suppressor action of RASSF1A. As yet however, the understanding of the biochemical actions of RASSF1A is at an early stage. Vos *et al*. reported that overexpression of RASSF1C could promote apoptosis of 293T cells in a RasG12V-dependent manner. Thus the

roles of Ras, other Ras-like GTPases, Nore and apoptosis in the putative tumor suppressive function of RASSF1 will require much further investigation.

The family of proteins homologous to Nore, i.e. RASSF1-3, AD037 and the *C. elegans* polypeptide T24F1.3 each contains a putative Ras-Rap association (RA) domain (Ponting and Benjamin, 1996) in their carboxy-terminal segment, and Nore was isolated by its affinity for Ras-GTP. Consequently the finding that two members of this polypeptide family exhibit no ability to bind to any of seven Ras-related GTPases was unexpected, but not unprecedented. Thus Kalhammer *et al*. (1997) reported that the predicted RA domain in myr5 showed no binding to Ras in a two-hybrid assay or *in vitro*. The features that define an RA domain were compiled using the sequences of the known Ras-Rap binding domains or AF-6, Canoe (Dros AF6) and the Ral-GDS family (Ponting and Benjamin, 1996). The motif thereby developed predicted the presence of the previously identified RA domain in the Rin polypeptides and predicted the presence of previously unknown RA domains in a number of polypeptides (including T24F1.3) several of which subsequently have been verified experimentally. Thus the *C. elegans* phospholipase C, PLC210 (F31B12.1) (Shibatohge *et al*., 1998) and its human homolog PLCε do contain two functional RA domains, as predicted. Although both appear to be required for Ras-GTP activation of PLCε (Song *et al*., 2001; Kelley *et al*., 2001) *in vivo*, only RA-2 binds Ras-GTP > Ras-GDP; RA-1 binds Ras specifically but with a much lower affinity than RA-2 and independently of GTP (Kelley *et al*., 2001). Thus among the RA domains examined thus far, several bind to a Ras-like GTPase in a GTP specific manner, at least one binds to Ras specifically, but in a GTP independent manner (PLCε RA-1), and RASSF1 and T24F1.3 do not bind at all to any of seven Ras-like GTPase. We have not yet examined several Ras-like GTPases (e.g. Rheb, Rin, Rit) for their ability to bind to RASSF1, however those RA domains that do bind a Ras-like GTPase actually bind to several members with varying affinity; the lack of any detectable binding of RASSF1/T24F1.3 to any of seven Ras-like Smgs makes it much less likely that another member of the Ras- like subfamily will prove to be a physiologic ligand for the RASSF1 RA domain.

The RA domain in T24F1.3 is about 40% identical in amino acid sequence to those in both Nore and RASSF1. This degree of sequence conservation implies a conserved function yet Nore binds several GTPases and RASSF1 binds none of those examined. The Interpro project has identified two *S. Cerevisiae* polypeptides that contain RA domains, namely Ste50 and Adenyl Cyclase (Rubin *et al*., 2000). The ability of Ste50 to bind Ras has not been reported, but the RA domain of *S. Pombe* homolog Ste4 does not bind Ras in a two-hybrid assay (Barr *et al*., 1996). *S. Cerevisiae* Adenyl Cyclase is a Ras-GTP binding protein, however this interaction is not mediated by the RA domain (Akasaka *et al*., 1996; Shima *et al*., 1997), but by the leucine rich repeats that are situated carboxyterminal to the RA domain. Thus we infer that the primary 'founding' ligand partner for the RA domain is not a small GTPase, but another partner yet to be identified. The ability of RA domains to bind Ras-like small GTPases appears to have been acquired subsequent to the emergence of this domain, and it is possible that those RA domains that have acquired the ability to bind Ras-like GTPases may y*et al*so retain the ability to bind another class of partners, presumably related to those that bind the RASSF1 RA domain. It is important to note that the primary sequence of the Ras-binding domain of the Raf kinase does not conform to the RA motif, suggesting that the ability to bind Ras-like Smgs evolved independently, but in a convergent manner; in spite of their lack of sequence similarity, the tertiary structure of the Raf RBD (Nassar *et al*., 1995) and the Ral GDS RA domain (Huang *et al*., 1997) exhibit a similar ubiquitin superfold. The diversity in Ras-binding motifs is greater yet, as the aminoterminus of the Byr2 kinase binds Ras-GTP specifically (Akasaka *et al*., 1996) but shares no resemblance in primary sequence to either the Raf RBD or RA motifs.

Nore, although isolated as a putative Ras effector, binds to several other Ras-like GTPases, in particular M-Ras/Ras3 and R-Ras. We have confirmed the ability of Nore to bind R-Ras or R-Ras3 on coexpression in mammalian cells (data not shown). Inasmuch as endogenous Nore has been shown to associate with endogenous Ras in response to EGF in intact cells (Vavvas *et al*., 1998), it is likely that activation of R-Ras or R-Ras3 will recruit Nore, i.e., based on avidity of interaction, Nore is as likely an effector of both R-Ras and R-Ras3 as of Ras. Little is known of the physiologic programs controlled by R-Ras3 (Reuther and Der, 2000). Although M-Ras/R-Ras3 is most abundant in brain, skeletal and cardiac muscle, it is widely expressed, and in NIH3T3 cells, e.g. is more abundant than Ras (Gotz *et al*., 1999). This GTPase, like Ras can be activated *in vitro* by both mSOS and Ras GRF, and is therefore likely to be recruited by many of the same stimuli that activate Ras (Ohba *et al*., 2000). GTPase deficient forms of M-Ras can weakly transform NIH3T3 cells, and induce IL-3 independent growth in BaF3 cells (Gotz *et al*., 1999). R-Ras3 interacts weakly with Rafs, PI-3Kδ and Ral-GDS, but more avidly with Nore1, AF6, Rin and the novel Ral-GDS homolog RPM/RGL3 (Quilliam *et al*., 1999; Ehrhardt *et al*., 2001). The present studies support the idea that Nore functions as an M-Ras/Ras3 effector, and introduces the possibility that RASSF1A, through its ability to dimerize with Nore, may also be recruited by M-Ras/R-Ras-3.

Materials and methods

DNA constructs

cDNAs encoding the different proteins were subcloned in either CMV5-FLAG, CMV5-HA or pEBG as described in the figure legends. Nore1 full length coding region,

RASSF1A and C isoform coding sequences and a fragment of *C. elegans* T24F1.3 encompassing amino acids 247–601 were subcloned into the yeast two-hybrid bait vector, pAS1-CYH2. The small GTPase proteins were mutated to a GTPase deficient form, deleted of their carboxyterminal prenylation motif and inserted into the vector pACT2. The small GTPases examined were: Ha-Ras (G12V), Rap 1b (G12V), RalA (G12V), Rap2A (G12V), MRas (G22V) and RRas (G38V). Clone A encoded in pACT2 was found to interact with pAS1-CYH2 encoded-Nore, RASSF1A and C and T24F1.3 but not pAS1-CYH2 alone or encoding p53, and was therefore employed to verify expression of the Nore-related sequences.

Quantitative two-hybrid assays

The yeast strain Y190 yeast was transformed with the desired plasmid pairs using standard methods (Clontech manual yeast protocols handbook PT 3024-1). Following selection on CSM plates supplemented with appropriate amino acids (-TRP-LEU and other dropouts mixes from BIO101), double transformant clones were isolated. Liquid medium selective for the TRP and LEU plasmids was used to grow up Y190 cells harboring the indicated plasmid pairs overnight; aliquots were sub-inoculated into YPD medium, grown to an OD of 0.5 to 0.8, and harvested. Pellets were processed using the Pierce Yeast Beta Galactosidase assay kit (#75768) according to manufacturers instructions. Beta galactosidase activity was measured at 2 min intervals, on a Molecular devices Spectramax 384 instrument interfaced with PC-based Softmax 3.1 software, by subtracting the OD 660 from the OD420 of cell suspensions plus substrate for each sample. Initial rates were estimated by extrapolation of the linear component of the progress curve. Controls used to calibrate the progress of both assays were as follows: full length GAL4 alone (plasmid CL1 Clontech); plasmid pair p53/SV40 Large T antigen (VA3-1/TD1, Clontech), productive of a positive protein-protein interaction. Candidate pACT2 clones plus p53 in pAS1-CYH2 (VA3-1), and Candidate pAS1-CYH2 constructs plus SV40 large T in pACT2 (TD1-1) did not produce Beta-galactosidase and served as negative controls.

Transfection and binding in COS cells

Cos-7 cells were maintained in DMEM supplemented with 10% FBS. Cells in 6-well tissue culture plates were transfected using Lipofectamine (6 μl per well) according to the manufacturer's instructions. Extractions were performed 40 h after transfection. Cells were lysed in a buffer containing 30 mm HEPES, pH 7.4, 1% Triton X-100, 20 mm β-glycerophosphate, 1 mM orthovanadate, 20 mm NaF, 20 mm KCl, 2 mm EGTA, 7.5 mm MgCl2, 14 mm β-mercaptoethanol, and a mixture of protease inhibitors. Lysates were centrifuged at 15000 r.p.m. $\times$ 10 min. Supernatants were incubated with GSH-agarose beads for 3 h at 40°C, the beads were washed four times with lysis buffer. The bound proteins were eluted in SDS sample buffer and subjected to SDS–PAGE, transferred to PVDF membranes, and probed using the antibodies indicated. Bound antibodies were detected using ECL.

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References

Akasaka K, Tamada M, Wang F, Kariya K-i, Kikuchi A, Yamamoto M, Shirouzu M, Yokoyama S and Kataoka T. (1996). *J. Biol. Chem.,* **271,** 5353–5360.
Avruch J, Zhang X-f and Kyriakis JM. (1994). *TIBS.,* **19,** 279–283.
Avruch J, Khokhlatchev A, Kyriakis JM, Luo Z, Tzivion G, Vavvas D and Zhang X-f. (2001). *Recent Prog. Horm. Res.,* **56,** 127–155.
Barr MM, Tu H, Van Aelst L and Wigler M. (1996). *Mol. Cell. Biol.,* **16,** 5597–5603.
Dammann R, Li C, Yoon J-H, Chin PL, Bates S and Pfeifer DP. (2000). *Nat. Genet.,* **25,** 315–319.
Durfee T, Becherer K, Chen P, Yeh SH, Yan Y, Kilurn AE, Lee WH and Elledge SJ. (1993). *Genes Dev.,* **7,** 555–569.
Ehrhardt GRA, Korherr C, Wieler JS, Knaus M and Schrader JW. (2001). *Oncogene,* **20,** 188–197.
Feig LA, Cantor T and Cantor S. (1996). *Trends Biochem. Sci.,* **11,** 438–431.
Gotz RA, Ehrhardt LKB, Lee F, Wieler JS and Schrader JW. (1999). *Blood,* **94,** 2433–2444.
Han L, Wong D, Dhaka A, Afar D, White M, Xie W, Herschman H, Witte O and Colicelli J. (1997). *Proc. Natl. Acad. Sci. USA,* **94,** 494–4959.
Huang L, Weng X, Hofer F, Martin GS and Kim SH. (1997). *Nat. Struct. Biol.,* **4,** 609–615.
Inouye K, Mizutani S, Koide H and Kaziro Y. (2000). *J. Biol. Chem.,* **275,** 3738–3740.
Kalhammer G, Bahler M, Schmits M, Schmitz F, Jockel J and Block C. (1997). *FEBS.,* **414,** 599–602.
Keikhoft E and Rapp UR. (1998). *Oncogene,* **17,** 1457–1462.
Kelley GG, Reks SE, Ondrako JM and Smrcka AV. (2001). *EMBO J.,* **20,** 743–754.
Kuriyama M, Harada N, Kuroda S, Yamamoto T, Nakafuku M, Iwamatsu A, Yamoto D, Prasad R, Croce C and Canaani E. (1996). *J. Biol. Chem.,* **271,** 607–610.
Lerman MI and Minna JD. (2000). *Cancer Res.,* **60,** 6116–6133.
Luo Z, Tzivion G, Belshaw PJ, Marshall M and Avruch J. (1996). *Nature,* **383,** 181–185.
Morrison DK and Cutler Jr RE. (1997). *Curr. Opin. Cell Biol.,* **274,** 174–179.
Nassar N, Horn G, Herrmann C, Scherer A, McCormick F and Wittinghofer, A. (1995). *Nature,* **375,** 554–560.
Ohba Y, Mochizuki N, Yamashita S, Chan AM, Schrader JW, Hattori S, Nagashima K and Matsuda M. (2000). *J. Biol. Chem.,* **275,** 20020–20026.
Ponting CP and Benjamin DR. (1996). *TIBS.,* **21,** 422–425.
Quilliam LA, Castro AF, Rogers-Graham KS, Martin CB, Der CJ and Bi C. (1999). *J. Biol. Chem.,* **274,** 23850–23857.
Reuther GW and Der CJ. (2000). *Curr. Biol.,* **12,** 157–165.
Rodriquez-Viciana P, Warne PH, Dhand R, Vanhaesebroeck B, Gout I, Fry MJ, Waterfield MD and Downward J. (1994). *Nature,* **370,** 527–532.

Rodriquez-Viciana P, Warne PH, Khwaja A, Marte BM, Pappin D, Das P, Waterfield MD, Ridley A and Downward J. (1997). *Cell,* **89,** 457–467.

Rodriquez-Viciana P, Warne PH, Vanhaesebroeck B, Waterfield MD and Downward J. (1996). *EMBO J.,* **15,** 2442–2451.

Rubin GM, Yandell MD, Wortman JR, Gabor Miklos GL, Nelson CR, Hariharan IK, Fortini ME, Li PW, Apweiler R, Fleischmann W, Cherry JM, Henikoff S, Skupski MP, Misra S, Ashurner M, Birney W, Boguski MS, Brody T, Brokstein P, Celniker SE, Chervitz SA, Coates D, Cravchik A, Gabrielian A, Galle RF, Gelbart WM, George RA, Goldstein LSB, Gong F, Guan P, Harris NL, Hay BA, Hoskins RA, Li J, Li Z, Hynes RO, Jones SJM, Kuehl PM, Lamaitre B, Littleton JT, Morrison DK, Mungall C, O'Farrell PH, Pickeral OK, Shue C, Vosshall LB, Zhang J, Zhao Q, Zheng ZH, Zhong F, Zhong W, Gibbs R, Vener JC, Adams MD and Lewis S. (2000). *Science,* **287,** 2204–2215.

Shibatohge M, Kariya K, Liao Y, Hu C-D, Watari Y, Goshima M, Shima F and Kataoka T. (1998). *J. Biol. Chem.,* **273,** 6218–6222.

Shields JM, Pruitt K, McFall A, Shaub A and Der CJ. (2000). *Cell. Biol.,* **4,** 147–154.

Shima F, Yamawaki-Kataoka Y, Yanagihara C, Tamada M, Okada T, Kariya K-i and Kataoka T. (1997). *Mol. Cell. Biol.,* **17,** 1057–1064.

Stewart S and Guan KL. (2000). *J. Biol. Chem.,* **275,** 8854–8862.

Song C, Hu C-D, Masago M, Kariya K-i, Yamawaki-Kataoka Y, Shibatohge M, Wu D, Satoh T and Kataoka T. (2001). *J. Biol. Chem.,* **276,** 2752–2757.

Vavvas D, Li X, Avruch J and Zhang X-f. (1998). *J. Biol. Chem.,* **273,** 5439–5442.

Vos MD, Ellis CA, Bell A, Birrer MJ and Clark GJ. (2000). *J. Biol. Chem.,* **275,** 35669–35672.

White MA, Vale T, Chamonis JH, Schaefer E and Wigler MHL. (1996). *J. Biol. Chem.,* **271,** 16439–16442.

Wolthuis RM and Bos JL. (1999). *Curr. Opin. Genet. Dev.,* **1,** 112–117.

Zhang Z, Wang Y, Vikis HG, Johnson L, Liu G, Li J, Anderson MW, Sills RC, Hong HL, Devereux TR, Jacks T, Guan K-L and You M. (2001). *Nat. Genet.,* **29,** 25–33.